Prevalence of inversion positive and inversion negative mating type (MAT) alleles and MAT heterokaryons in Sclerotinia sclerotiorum in the United States.

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<td>Manuscript ID:</td>
<td>cjb-2015-0035.R2</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>05-May-2015</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Chitrampalam, Periasamy; North Dakota State University, Plant Pathology Qiu, Chengxiang; North Dakota State University, Plant Pathology Aldrich-Wolfe, Laura; Concordia College, Leng, Yueqiang; North Dakota State University, Plant Pathology Zhong, Shaobin; North Dakota State University, Plant Pathology Nelson Jr., Berlin; North Dakota State University, Plant Pathology</td>
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<td>Keyword:</td>
<td>Fungi, Sclerotinia sclerotiorum, Mating type (MAT) alleles, MAT heterokaryon</td>
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Prevalence of inversion positive and inversion negative mating type (MAT) alleles and MAT heterokaryons in Sclerotinia sclerotiorum in the United States.

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Abstract

*Sclerotinia sclerotiorum* is a yield limiting pathogen of several economically important crops, and it reproduces sexually by self-fertilization. Based on the presence of an inversion in the mating type locus, *S. sclerotiorum* can be grouped as inversion negative (Inv- *MAT*) and inversion positive (Inv+ *MAT*) isolates. This study was conducted to determine the prevalence of Inv- and Inv+ *MAT* *S. sclerotiorum* isolates across the United States. In total, 164 isolates from 16 hosts and 22 states were evaluated, including 87 isolates from North Dakota-northern Minnesota and 47 isolates from soybean. PCR screening was performed separately for Inv- and Inv+ *MAT* with specific primers. Of the two kinds of *MAT* homokaryons, Inv- *MAT* isolates were the most frequent (31.7%) and were identified in 15 states. Another 12.8% of isolates were Inv+ *MAT*, and were identified in 8 states. The majority (55.5%) of isolates screened were *MAT* heterokaryons, and these were identified from 18 states and 11 hosts. The implications of *MAT* heterokaryons for the biology and management of *S. sclerotiorum* are discussed.

Keywords: Fungi, *Sclerotinia sclerotiorum*, Mating type (*MAT*) alleles, *MAT* heterokaryon
Introduction

*Sclerotinia sclerotiorum* is an ascomycete phytopathogen which attacks more than 400 plant species, including numerous economically important crops worldwide (Boland and Hall 1994). Sunflower, soybean, oilseed rape, dry bean, chick pea, peanut, dry pea, lentils and numerous vegetables are some of the agriculturally important crops susceptible to this pathogen. The losses from this pathogen are significant worldwide (Saharan and Mehta 2008) with annual losses in the United States alone exceeding $200 million (Bolton et al. 2006).

This soil-borne fungus produces large numbers of black sclerotia (asexual hyphal structures covered by a melanin layer) on infected plants. Sclerotia can survive in soil for several years (Adams and Ayers 1979). Depending on environmental conditions and the host, sclerotia serve either as primary inoculum and directly initiate infection through mycelial germination or undergo sexual reproduction and initiate infection through ascospores. Sexual reproduction in *S. sclerotiorum* requires specific conditions, such as low soil temperature and high soil moisture for an extended period of time (Wu and Subbarao 2008). In Arizona and other states with a warm and dry climate, such conditions rarely occur; therefore, under these conditions infection is mainly initiated through mycelial germination of sclerotia and rarely through ascospores (Matheron and Porchas 2004). However, prevailing climatic conditions in other states, such as California, North Dakota, and Washington are favorable for sexual reproduction in *S. sclerotiorum* and hence infection of most susceptible crops is mainly initiated through ascospores in these states (Wu et al. 2011; del Rio et al. 2004; Atallah et al. 2004).

Ascomycete fungi are sexually either homothallic (self-compatible) or heterothallic (self-incompatible) and reproduce generally by self-fertilization or cross-fertilization, respectively.
Sexual compatibility is controlled by two idiomorphs, *MAT1-1* and *MAT1-2*, which are dissimilar in sequence and occupy the same locus on the chromosome, called the *MAT* locus (Yun et al. 1999). *MAT1-1* is identified by a gene encoding a protein with alpha domain (*MAT1-1-1*), and likewise *MAT1-2* is identified by a gene encoding a protein with high mobility group (HMG) DNA binding domain (*MAT1-2-1*) (Coppin et al. 1997). In addition to the above two candidate genes, additional *MAT* gene(s) can also be associated with each idiomorph depending on the fungal species (Turgeon and Yoder 2000). In homothallic fungi, both idiomorphs are located within an individual mostly at the same locus (Lu et al. 2011), with few exceptions (Yun et al. 1999; Paoletti et al. 2007). However, in heterothallic fungi each individual carries a single idiomorph, and therefore, sexual reproduction occurs only between *MAT1-1* and *MAT1-2* individuals (Lee et al. 2010). Moreover, several unusual modes of sexual reproduction such as pseudohomothallism (Coppin et al. 1997; Raju and Perkins 1994; Raju and Perkins 2000), mating type switching (Harrington and McNew 1997; Uhm and Fujii 1983a, 1983b), and dual mating (Amselem et al. 2011; Faretra et al. 1996) have also been reported in ascomycetes. Furthermore, two unusual mating type alleles have also recently been reported within *S. sclerotiorum* (Chitrampalam et al. 2013).

*Sclerotinia sclerotiorum* is homothallic and contains both *MAT1-1* and *MAT1-2* idiomorphs at the same locus between the conserved flanking genes *APN2* and *SLA2* at upstream and downstream, respectively, as in other ascomycetes (Amselem et al. 2011). Each idiomorph contains two genes, and *MAT1-1-1* and *MAT1-1-5* are specific for idiomorph *MAT1-1* and *MAT1-2-1* and *MAT1-2-4* are specific for idiomorph *MAT1-2*. They are arranged in the following order *MAT1-1-5, MAT1-1-1, MAT1-2-4* and *MAT1-2-1* (Amselem et al. 2011).
However, recently a new mating type allele was described in *S. sclerotiorum* which contained a 3.6 kb inversion relative to the previously described MAT allele, and hence it was called an inversion positive MAT allele (Inv+ MAT). Conversely a MAT allele without an inversion was termed an inversion negative MAT allele (Inv- MAT; Chitrampalam et al. 2013). Although the inversion affected three of the four MAT genes in the Inv+ MAT allele, the Inv+ MAT *S. sclerotiorum* isolates successfully undergo sexual reproduction. In addition, the evidence suggests that the MAT inversion occurs during sexual reproduction in homokaryotic *S. sclerotiorum* in every meiotic generation. The MAT inversion is reversible so that Inv- MAT allele converts into an Inv+ MAT allele, and vice versa. This MAT conversion occurs only during sexual reproduction and is not associated with the asexual vegetative phase (Chitrampalam et al. 2013).

Both the Inv+ MAT allele and the MAT conversion are newly described phenomena in *S. sclerotiorum*, and the distribution of Inv- and Inv+ MAT *S. sclerotiorum* isolates has not been studied in detail across the United States. Therefore, this study was conducted to determine the prevalence of Inv- and Inv+ MAT *S. sclerotiorum* isolates from across a broad portion of the United States with an emphasis on the fungus from the North Dakota-northern Minnesota region where there is a large acreage of susceptible crops.

**Materials and methods**

**Isolate collection**

A total of 164 isolates from 22 states, ranging from FL to WA, and 16 hosts were used in this study (Table 1, S1). Of these, 87 isolates were from North Dakota and northern Minnesota.
and 47 were from soybean. These isolates were obtained as sclerotia between 2008 and 2011 from plant scientists in individual states as part of a project on genetic variation in *S. sclerotiorum* in the United States (Aldrich-Wolfe et al. 2010; Qiu and Nelson 2013). Isolates from North Dakota and northwestern Minnesota were collected by the authors. A single sclerotium from each sample was surface sterilized for 30 s in 10% bleach, then 30 s in 70% ethanol, washed twice in sterilized water, dried on a sterilized paper towel, and plated on 2% water agar. A hyphal tip from the emerging colony was transferred onto potato dextrose agar (PDA) and incubated at room temperature. Sclerotia from pure cultures were dried, placed in vials with Drierite (Hammond Drierite Co., Xenia, OH) and stored at room temperature.

**DNA extraction**

Each isolate was grown separately on an autoclaved polycarbonate membrane filter (0.4 µm pore size; GE Healthcare, Cleveland, OH) layered on top of PDA in a 100 x 15 mm petri dish and incubated in the dark at 23°C for a week. Mycelia were harvested, ground in liquid nitrogen with pestle and mortar and immediately used for DNA extraction. DNA was extracted using Qiagen DNeasy plant mini kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. DNA was quantified using a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE) and the concentration was adjusted to 10 ng/µL in sterilized water for polymerase chain reaction (PCR).

**Polymerase chain reaction**

For each isolate, two PCR were performed separately for both Inv- and Inv+ *MAT* alleles using allelic specific primers (Chitrampalam et al. 2013). PCR was performed in a 25 µL
reaction mixture which included 2 µL of *S. sclerotiorum* DNA (10 ng/µL), 12.5 µL of GoTaq PCR mix (Promega, Madison, WI), 1 µL each of forward and reverse primer and 8.5 µL of sterilized water. The genomic DNA replaced with an equal volume of autoclaved water in the PCR mixture served as the negative control. For positive control for Inv- and Inv+ *MAT*, DNA from previously characterized homokaryotic Inv- and Inv+ *MAT* *S. minor* isolates SM1 and SM2, respectively were used (Chitrampalam and Pryor 2014). Amplification was performed in MJ Research DNA Engine thermocyclers (MJ Research, Inc., Waltham, MA) following the PCR conditions specified by Chitrampalam et al. (2013). PCR conditions were: initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C, 60°C, and 72°C for 30, 30 and 90 sec respectively, with 5 min final extension at 72°C. Aliquots of PCR products (6 µL) were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized under a UV trans-illuminator (Ultra-Violet Products Ltd, Cambridge, UK). Representative PCR products from Inv-, Inv+ and *MAT* heterokaryotic categories were cleaned with Exo-I and rAPid (Roche, Inc., Indianapolis, IN) following the manufacturer’s protocol and sequenced at MCLAB Sequencing facility (MCLAB, San Francisco, CA).

**Sequencing**

For three, representative *MAT* heterokaryotic isolates both Inv- and Inv+ *MAT* were amplified and sequenced with allelic specific primers for each *MAT* heterokaryon. Inv- and Inv+ *MAT* PCR products from respective *MAT* homokaryon isolates were also sequenced for three representative isolates in each category. PCR products were cleaned with Exo-I and rAPid (Roche, Inc., Indianapolis, IN) following the manufacturer’s protocol and sequenced at MCLAB Sequencing facility (MCLAB, San Francisco, CA). Sequencing was performed at both directions using PCR
primers. Sequences were submitted to Genbank, and the accession numbers were listed in Table S2. Inv- and Inv+ MAT sequences from MAT heterokaryon isolates were aligned and compared with sequences from respective MAT homokaryon isolates using MEGA5 (Tamura et al. 2011).

Southern hybridization

To further confirm MAT heterokaryons in S. sclerotiorum Southern hybridization was performed. One representative isolate each from Inv-, Inv+ and MAT heterokaryon identified by PCR screening were arbitrarily selected and included in the Southern hybridization. These were isolates 303 MAT heterokaryon, 320 Inv+ MAT and 250 Inv- MAT (see Table S1 for descriptions of isolates). For a MAT heterokaryon positive control, genomic DNA from putative Inv- and Inv+ MAT isolates were mixed manually at equal concentrations. For DNA extraction each isolate was cultured in 50 mL of potato dextrose broth (Becton, Dickinson and Company, MD) in 250 mL flask for 5 days at room temperature. Mycelia were harvested and washed with sterilized water. DNA was extracted using Qiagen DNeasy plant mini kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. About 5 µg of DNA was digested with BsaHI (Chitrampalam et al. 2013) by following manufacturer’s procedure (New England BioLabs, MA). Digested DNAs were run on 0.8% agarose gel by electrophoresis overnight at 22 V and transferred to Hybond N+ membrane (Amersham Biosciences, Piscataway, NJ, USA). A probe specific to MAT1-2-1 was prepared in PCR using primers MAT1-2-F and MAT1-2-R (Malvarez et al. 2007) and labelled with [α-32P]-dCTP (MP Biomedicals, Santa Ana, CA, USA). The hybridization and detection procedures were performed according to the method described previously by Zhong et al. (2002).

Results
PCR screening of S. sclerotiorum for MAT alleles

Polymerase chain reaction amplification was successful with all isolates tested, and PCR products specific for Inv- and Inv+ MAT were obtained with the allele specific primer (Fig. 1). MAT homokaryon isolates yielded a PCR product either for Inv- or Inv+ MAT, while MAT heterokaryon isolates yielded two PCR products, one for Inv- and one for Inv+ MAT (Fig. 1). A single discrete band corresponding to Inv- and Inv+ MAT was obtained with S. minor isolates SM1 and SM24, respectively which were used as positive controls for homokaryotic Inv- and Inv+ MAT, respectively (Chitrampalam and Pryor 2014).

Among 164 isolates from 22 states, 52 isolates (31.7%) were Inv- and 21 isolates (12.8%) were Inv+ MAT (Table 1, Fig. 1). The remaining 91 isolates (55.5%) were MAT heterokaryons (Table 1). MAT heterokaryons were observed in isolates from 18 states. All three MAT genotypes were also observed in isolates from North Dakota-northern Minnesota, and of 87 isolates screened 22 (25.3%), 11 (12.6%) and 64 (62.1%) isolates were Inv-, Inv+ and MAT heterokaryon, respectively (Table 1). Twelve of 16 host species represented contained more than two isolates for screening (Table 1), and both Inv- and Inv+ MAT alleles were found either in MAT homokaryons or heterokaryons in those isolates. MAT heterokaryons were observed in 11 of 14 host species screened. The greatest number of isolates were from soybean, of which 9, 6, and 32 isolates were Inv-, Inv+ and MAT heterokaryon, respectively (Table 1).

MAT sequences comparison between MAT heterokaryon and MAT homokaryon isolates

Sequencing Inv- and Inv+ MAT PCR products from the MAT heterokaryon isolate and the respective MAT homokaryon isolates generated 591 and 1227 nucleotides for Inv- and Inv+ MAT, respectively. Comparison of the partial sequences of Inv- and Inv+ MAT regions from the
MAT heterokaryon isolate with the corresponding MAT homokaryon isolates revealed that they were > 99% identical for both MAT alleles at the sequenced MAT regions.

**Southern hybridization**

Southern hybridization was performed to confirm MAT heterokaryon in *S. sclerotiorum* using MAT1-2-1 as a probe as suggested by Chitrampalam et al. (2013). The expected single band with a 1.16 kb size specific for Inv- MAT was obtained only for putative Inv- MAT isolate (250) as predicted by PCR screening (Fig. 2). Likewise, the expected single band (1.5 kb) specific for Inv+ MAT allele was obtained exclusively for Inv+ isolate (320). Both Inv- and Inv+ MAT specific bands were obtained with a putative MAT heterokaryon isolate (303). These two bands were also observed with MAT heterokaryon positive control created manually by mixing equal quantity of DNA from both putative Inv- (250) and Inv+ MAT (320) isolates (Fig. 2).

**Discussion**

Two kinds of mating type alleles, Inv- MAT and Inv+ MAT, were recently described in homothallic *S. sclerotiorum* (Amselem *et al.*, 2011; Chitrampalam *et al.* 2013). Both *S. sclerotiorum* isolates with Inv- MAT allele and isolates with Inv+ MAT allele are self-fertile despite the inversion affecting three of four MAT genes in Inv+ MAT isolates (Chitrampalam *et al.* 2013). Prevalence of Inv- and Inv+ MAT *S. sclerotiorum* isolates were previously determined for twelve states on eight hosts, but of the 12 states screened, eight states including North Dakota contained only one isolate for screening (Chitrampalam *et al.* 2013). In this study we determined the distribution of Inv- and Inv+ MAT *S. sclerotiorum* isolates in a large collection from North Dakota and northern Minnesota along with samples from 20 other states in the United States. The results expand the distribution of these alleles to 12 additional states and 11 additional hosts.
In addition these data expand the known geographic distribution of Inv- and Inv+ MAT alleles from coast to coast in the United States.

Overall, of the Inv- and Inv+ MAT S. sclerotiorum isolates, Inv- MAT isolates were the most predominant. A previous study reporting the prevalence of both MAT S. sclerotiorum isolates in lettuce fields of California found a roughly equal frequency for the two types (Chitrampalam et al. 2013). In the current study, MAT heterokaryons are reported for the first time in S. sclerotiorum. They appear to be prevalent across the United States, as 55.5 % of the isolates were MAT heterokaryons. Both sequencing of MAT PCR products and Southern hybridization confirmed the presence of MAT heterokaryon in S. sclerotiorum. However, in most of the MAT heterokaryon isolates usually only one of the MAT alleles is amplified strongly in PCR with a more intense band on the electrophoresis gel compared to the other MAT allele which shows a weaker band on gel (Fig 1). This differential amplification of MAT alleles in MAT heterokaryon isolates could possibly be due to unequal distribution of MAT nuclei in heterokaryon isolates (Liberti et al. 2012). Therefore, the absence of MAT heterokaryons in a previous study at California (Chitrampalam et al. 2013) could possibly be due to the inadvertent omission of a weaker band for one of the two MAT alleles. For example, weaker bands observed in the previous MAT screening study with S. sclerotiorum were omitted as false positive (refer to figure 10 in Chitrampalam et al. 2013 study), and which could potentially have been MAT heterokaryons. Further detailed screening of isolates from CA may identify MAT heterokaryons in that area.

In the current study Southern hybridization was performed with a single representative isolate from each of the MAT categories, and the results confirmed the identification of heterokaryotic MAT isolates based on the PCR results. However, in future studies on MAT
heterokaryons in this pathogen, it would be advantageous to conduct Southern hybridization assay with every putative *MAT* heterokaryotic isolate predicated by PCR to determine the relative abundance of these two nuclei in the heterokaryotic isolates, and also to precisely estimate the *MAT* heterokaryon population in nature. In addition, the stability of the observed *MAT* heterokaryons in *S. sclerotiorum* over several mitotic generations has not been tested in the current study. Information on these various aspects of this pathogen may give us new insight into heterokaryosis in *S. sclerotiorum*.

*Sclerotinia sclerotiorum* has multinucleate hyphal cells with an average of > 3.5 nuclei per cell (Ford et al. 1995). When nuclei produced from mitosis in *MAT* heterokaryotic isolates move into the newly formed hyphal tip cells or branches, there is a possibility that the new hyphal cells may receive fewer or no nuclei of one of the two kinds of *MAT* nuclei, influencing the proportion of *MAT* nuclei in *MAT* heterokaryon isolates. This type of unequal distribution of nuclei has also been previously reported in *S. sclerotiorum* in which the GFP-transformed nuclei were low compared to non-transformed nuclei in GFP-transformed *S. sclerotiorum* strains (de Silva et al. 2009).

While heterokaryosis, the existence of more than one genetically distinct nucleus within a mycelium, is the basis for sexual mating and somatic or parasexual recombination, the occurrence of heterokaryosis in vegetative cells independent of mating is believed by some researchers to be rare in filamentous fungi in nature (Glass and Kaneko 2003). However, a recent study revealed that the occurrence of heterokaryosis in the vegetative phase of *Cryphonectria parasitica* is not uncommon, as a significant percentage of isolates in the field were *MAT* heterokaryons (McGuire et al. 2004, 2005). *MAT* heterokaryons have also been
reported in *Botrytis cinerea*, *S. minor*, and *S. homoeocarpa* (Faretra and Pollastro 1996; Chitrampalam and Pryor 2014; Liberti et al. 2012).

Population structure is substantially influenced by the reproductive biology of an organism (Anderson and Kohn 1995). As *S. sclerotiorum* is a homothallic fungus and reproduces sexually by self-fertilization, the resulting population is expected to be clonal with limited variability in population. In support of this, several previous reports documented clonal structure in *S. sclerotiorum* populations (Cubeta et al. 1997; Kohli and Kohn 1998; Kohli et al. 1995). However, high genetic variability has also been reported in *S. sclerotiorum*, and outcrossing is hypothesized to be one reason for the observed high variability (Atallah et al. 2004; Attanayake et al. 2012, 2014; Hemmati et al. 2009; Sexton and Howlett 2004). The mechanism for outcrossing is not clearly understood in *S. sclerotiorum*. The observance of a wide distribution of MAT heterokaryons in *S. sclerotiorum* in agricultural fields suggests that the formation of heterokaryons during the vegetative phase could be the basis for outcrossing. Ford et al. 1995 found that under controlled conditions, heterokaryotic sclerotia generated through crossings in the laboratory, successfully outcross, form apothecia and segregate both parent genotypes in the subsequent ascospore progenies in equal proportion. Although pairing between hyphae of the same colony and between hyphae of the same strain are a universal phenomenon in filamentous fungi, pairing between strains followed by heterokaryon formation is generally regulated by one or more vegetative compatibility loci in ascomycetes (Saupe, 2000). The observance of many mycelial compatibility groupings (MCG) in *S. sclerotiorum* (Wu and Subbarao 2006) suggested that heterokaryon formation in *S. sclerotiorum* is highly regulated and probably occurs only within the same MCG groups.
There is a possibility that the observed \( \text{MAT} \) heterokaryons in \( S. \) sclerotiorum could also result from \( \text{MAT} \) conversion during mitosis. However, the observance of homokaryotic \( S. \) sclerotiorum isolates from field in the current study and also in the previous study (Chitrampalam et al., 2013) suggests that \( \text{MAT} \) conversion during mitosis is not common in \( S. \) sclerotiorum isolates. If \( \text{MAT} \) conversion occurred commonly during mitosis, one would expect that \( \text{MAT} \) heterokaryons would be more common than indicated by the results of this study, and more likely, most if not all isolates, would be \( \text{MAT} \) heterokaryons if the conversion was a common event during mitosis. The \( \text{MAT} \) conversion during mitosis might also be conditionally regulated, thus only occur under specific conditions. A more detailed study of this phenomenon will be needed to determine if conversion occurs during mitosis and what factors are associated with the event.

The phenomenon of \( \text{MAT} \) conversion, which according to the current evidence is associated exclusively with sexual reproduction, has recently been observed in \( S. \) sclerotiorum in which Inv- \( \text{MAT} \) allele converts into Inv+ \( \text{MAT} \) allele or vice versa resulting in 4 Inv- and 4 Inv+ \( \text{MAT} \) ascospores (1:1) at the end of every meiotic cycle regardless of whether the parent genotype is Inv- or Inv+ \( \text{MAT} \) (Chitrampalam et al. 2013). In addition, the pattern of segregation of \( \text{MAT} \) genotypes among sibling ascospores within an ascus (2:2:2:2 or 4:4) revealed that the \( \text{MAT} \) conversion precedes meiosis and could possibly be associated with the nuclear recognition event during diploidization in \( S. \) sclerotiorum (Chitrampalam et al. 2013). In heterothallic ascomycete fungi sexual fusion occurs only between \( \text{MAT1-1} \) and \( \text{MAT1-2} \) individuals (Lee et al. 2010). In homothallic \( S. \) sclerotiorum, nuclear fusion is hypothesized to occur only between Inv- and Inv+ \( \text{MAT} \) nuclei. Consequently, nuclear fusion in a homokaryotic isolate may be mediated by a preceding \( \text{MAT} \) conversion event, which would ensure the presence of both \( \text{MAT} \) nuclei at
the fusion site (Chitrampalam et al. 2013). However, the widespread observance of \textit{MAT} heterokaryons in \textit{S. sclerotiorum}, and the fact that \textit{MAT} conversion can occur in every sexual cycle raises questions on the possible impact of heterokaryons on \textit{MAT} conversion in \textit{S. sclerotiorum}. Are there \textit{MAT} conversions in \textit{MAT} heterokaryotic isolates similar to those in \textit{MAT} homokaryotic \textit{S. sclerotiorum}? Or is \textit{MAT} conversion exclusively associated with homokaryotic \textit{S. sclerotiorum} isolates? To address this question, the proper approach would be examining apothecia from heterokaryotic \textit{S. sclerotiorum} isolates containing the Inv- and Inv+ \textit{MAT} alleles tagged with green and red fluorescent protein (GFP and RFP), respectively.

Three possible segregation patterns (Fig. 3) would be expected to occur in heterokaryotic \textit{S. sclerotiorum} isolates tagged with GFP Inv- \textit{MAT} and RFP Inv+ \textit{MAT}. (I) If there is no \textit{MAT} conversion in a tagged \textit{MAT} heterokaryotic isolate, then the nuclear fusion expected to occur between GFP tagged Inv- \textit{MAT} and RFP tagged Inv+ \textit{MAT} nucleus, and the successive meiotic ascospore progeny in an ascus would be 4 GFP Inv- \textit{MAT} and 4 RFP Inv+ \textit{MAT} (Fig. 3). (IIa) If there is a \textit{MAT} conversion from GFP Inv- \textit{MAT} to GFP Inv+ \textit{MAT} allele followed by nuclear fusion and meiosis then the expected outcome would again be 4 Inv- and 4 Inv+ \textit{MAT} ascospores; however, all should be green. (IIb) Conversely if there is \textit{MAT} conversion from RFP Inv+ to RFP Inv- \textit{MAT} followed by nuclear fusion and meiosis then the expected outcome would again be 4 Inv- and 4 Inv+ \textit{MAT} ascospores, all red (Fig. 3). Furthermore, it is also possible that both GFP Inv-\textit{MAT} and RFP Inv+ \textit{MAT} alleles could simultaneously convert into GFP Inv+ \textit{MAT} and RFP Inv- \textit{MAT}, respectively, and which results into segregation of either 4 GFP Inv+ \textit{MAT} and 4 RFP Inv- \textit{MAT} among ascospore progenies or segregation as observed with IIa and IIb.
Considering the number of $MAT$ heterokaryons of $S. sclerotiorum$ documented in this study, heterokaryosis in $S. sclerotiorum$ may be more common and widespread than previously thought. The level of heterokaryons of $S. sclerotiorum$ in nature would be expected to be greater than the level of $MAT$ heterokaryons found in this study. Heterokaryons in fungal populations are considered more responsive to changing environmental conditions than homokaryons (Kessler et al. 2013). Part of the difficulty in managing diseases caused by $S. sclerotiorum$ could be due to the pathogen readily forming heterokaryons and thereby increasing genetic variability within the population. Developing technologies that can assess the heterokaryotic nature of a population might improve our understanding of how to manage this pathogen.

In conclusion, this study revealed for the first time the presence of $MAT$ heterokaryons in isolates of $S. sclerotiorum$ collected from across the United States and representing a broad range of hosts. The effect of $MAT$ heterokaryons on the genetics and diversity of $S. sclerotiorum$ and their implications for the currently available management strategies are still unknown and warrant detailed study.

Acknowledgements

This research was funded by the USDA Sclerotinia Initiative Research Project, ARS Specific Cooperative Agreement #58-5442-8-233. This work also is/was supported by the USDA National Institute of Food and Agriculture, Hatch project ND02223. The authors thank the numerous plant scientists that sent isolates of this fungus from the various states. The authors also thank anonymous reviewers for their insightful reviews.

References


Table 1. Distribution of Inv-, Inv+, and MAT heterokaryotic isolates of *Sclerotinia sclerotiorum* across the United States.

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<td>Minnesota</td>
<td>12</td>
<td><em>Phaseolus vulgaris</em> (Dry bean), <em>Glycine max, Helianthus annus, Daucus carota, Brassica napus</em></td>
<td>2</td>
</tr>
<tr>
<td>Missouri</td>
<td>2</td>
<td><em>Glycine max.</em></td>
<td>0</td>
</tr>
<tr>
<td>Montana</td>
<td>1</td>
<td><em>Carthamus tinctorius</em></td>
<td>0</td>
</tr>
<tr>
<td>Nebraska</td>
<td>4</td>
<td><em>Phaseolus vulgaris</em> (Dry bean), <em>Glycine max</em></td>
<td>2</td>
</tr>
<tr>
<td>North Carolina</td>
<td>4</td>
<td><em>Brassica oleracea</em> var. <em>capitata, Lactuca sativa, Petroselinum crispum, Phaseolus vulgaris</em> (Snap bean)</td>
<td>2</td>
</tr>
<tr>
<td>North Dakota</td>
<td>75</td>
<td><em>Phaseolus vulgaris</em> (Dry bean), <em>Brassica napus, Glycine max, Helianthus annus</em></td>
<td>20</td>
</tr>
<tr>
<td>Oklahoma</td>
<td>1</td>
<td><em>Brassica oleracea</em> var. <em>capitata</em></td>
<td>0</td>
</tr>
<tr>
<td>State</td>
<td>Plants Count</td>
<td>Species</td>
<td>HW</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
<td>----------------------------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>Ohio</td>
<td>1</td>
<td><em>Glycine max</em></td>
<td>0</td>
</tr>
<tr>
<td>Oregon</td>
<td>2</td>
<td><em>Solanum tuberosum, Phaseolus vulgaris</em> (Snap bean)</td>
<td>1</td>
</tr>
<tr>
<td>South Carolina</td>
<td>2</td>
<td><em>Capsicum annuum, Phaseolus vulgaris</em> (Snap bean)</td>
<td>1</td>
</tr>
<tr>
<td>South Dakota</td>
<td>1</td>
<td><em>Glycine max</em></td>
<td>0</td>
</tr>
<tr>
<td>Wisconsin</td>
<td>12</td>
<td><em>Glycine max, Nicotiana tabacum</em></td>
<td>3</td>
</tr>
<tr>
<td>Washington</td>
<td>13</td>
<td><em>Lens culinaris, Piscum sativum, Cucurbita pepo, Solanum tuberosum,</em></td>
<td>9</td>
</tr>
<tr>
<td>Wyoming</td>
<td>2</td>
<td><em>Phaseolus vulgaris</em> (Dry bean)</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>164</td>
<td>16</td>
<td>52</td>
</tr>
</tbody>
</table>

(31.7%) (12.8%) (55.5%)

-H- MAT heterokaryon. Common name for *Phaseolus vulgaris* has been noted in the parenthesis.
Figure 1. Representative electrophoresis gel images for PCR based screening of *S. sclerotiorum* for *MAT* alleles. PCR was performed separately for Inv- *MAT* allele and Inv+ *MAT* allele using allelic specific primers. Lane 1-19 are *S. sclerotiorum* isolates UND187c, UND188c, UND189c, DCO196, PCO198, RMN205, SIN206, SIN207c, SIA214c, SIA215c, SWI223, SWI224c, SWI225, TWI232, TWI233, SMO234, SMO243, CND700c, and CND800. SM1 and SM24 are Inv- and Inv+ *MAT* *S. minor* isolates (Chitrampalam et al. 2014), and they were used as positive control for Inv- and Inv+ *MAT* homokaryon, respectively. M and N are 100 bp DNA marker and negative control, respectively. Isolates with positive amplification for both Inv- and Inv+ *MAT* are *MAT* heterokaryons.

Figure 2. Confirmation of *MAT* heterokaryon in *Sclerotinia sclerotiorum* by Southern hybridization. Genomic DNA was digested with *Bsa*HI, separated on 0.8% agarose gel, transferred to a nylon membrane and hybridized with radiolabelled 0.53 kb fragment of *MAT1-2-1*. Lanes 1-3 are *S. sclerotiorum* isolates 303, 320, and 250 (see Table S1 for information on isolates). Lane 1 shows the *MAT* heterokaryon. Lane 4 is the artificial mixture of DNA from isolates 320 and 250 for *MAT* heterokaryon positive control. 1.5 kb and 1.16 kb bands are specific for Inv+ and Inv- *MAT* allele, respectively.

Figure 3. Pictorial depiction of theoretical segregation of *MAT* genotypes at first and second division of meiosis in *MAT* heterokaryotic *S. sclerotiorum*. A. Heterokaryotic sclerotia of *S. sclerotiorum* with GFP tagged Inv- *MAT* (denoted as white circle with -) and RFP tagged Inv+ *MAT* (denoted as grey circle with +) nuclei. (I) Segregation pattern when there is no *MAT* conversion, and diploidization between GFP tagged Inv- and RFP tagged Inv+ *MAT* followed by
meiosis. (II) segregation pattern with $MAT$ conversion, (a) when $MAT$ conversion occurs from GFP tagged Inv- to GFP tagged Inv+ $MAT$ followed by diploidization and meiosis or (b) when $MAT$ conversion occurs from RFP tagged Inv+ to RFP tagged Inv- $MAT$ followed by diploidization and meiosis. On the figure, - and + indicate Inv- and Inv+ $MAT$ genotype, respectively.
76x27mm (300 x 300 DPI)