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Mating system complexity and cryptic speciation in the seed bank pathogen *Pyrenophora semeniperda*

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Mating strategies contributing to a balance between inbreeding and outcrossing are understudied in all but a few model fungal pathogens. This study examined factors that influence the occurrence of the sexual state of *Pyrenophora semeniperda*. It was consistently found to have functional copies of the *MAT1-1* and *MAT1-2* idiomorphs essential for sexual reproduction, despite considerable polymorphism in both single nucleotide polymorphisms (SNPs) and number of 18-base minisatellite repeats. The two idiomorphs occurred at approximately equal frequencies across 25 populations on *Bromus tectorum* seeds in the western United States, suggesting maintenance of sexual reproduction. The putative mating system is described as facultative pseudohomothallism, with only one of the two *MAT1* idiomorphs found in a nucleus. Unikaryotic strains of opposite mating type can potentially mate, as can nuclei of opposite mating type in a thallus that results from anastomosis between vegetatively compatible unikaryotic strains. Strains shown to be dikaryotic using simple sequence repeat (SSR) markers may contain either or both *MAT1* idiomorphs. Most populations consist of a mixture of *MAT1-1*, *MAT1-2* and *MAT1-1/MAT1-2* genotypes. A possible constraint on recombination is the presence of multiple strain groups characterized by ITS haplotype. These are apparently vegetatively incompatible, as even dikaryotic strains are invariably composed of a single ITS haplotype. Different ITS haplotypes also have unique combinations of *MAT1-1* and *MAT1-2* allelic variants, suggesting that perhaps these strain groups are also sexually incompatible. Phylogenetic analysis using both genome-wide SNP/indel polymorphisms and SSR markers demonstrated genetic divergence among ITS haplotypes, supporting the hypothesis that these strain groups may represent cryptic species.

Keywords: biological species concept, heterothallism, phylogenetic species concept, pseudohomothallism, vegetative incompatibility

Introduction

In ascomycete fungi, the mating-type (*MAT1*) locus is the primary genetic factor controlling sexual reproduction. The *MAT1* locus is occupied by the complementary idiomorphs *MAT1-1* and *MAT1-2*, and both must normally be present in order for successful mating to occur (Metzenberg & Glass, 1990; Coppin *et al.*, 1997; Kronstad & Staben, 1997; Fraser & Heitman, 2004). *MAT1-1* and *MAT1-2* are referred to as idiomorphs rather than alleles because they show high sequence divergence, even though they usually occupy the same chromosomal locus (Metzenberg & Glass, 1990; Turgeon & Yoder, 2000; Martin *et al.*, 2010).

MAT1-1 and *MAT1-2* idiomorphs contain genes encoding transcription factors that initiate interaction between individuals of complementary mating type through the production of specific pheromones and pheromone receptors and that also promote internuclear recognition as the sexual cycle progresses (Debuchy *et al.*, 2010; Martin *et al.*, 2010). The *MAT1-1* idiomorph may include multiple genes but is characterized by the presence of a gene encoding an α-box DNA-binding domain protein, whereas the *MAT1-2* idiomorph is characterized by a single gene encoding a high mobility group (HMG) DNA-binding domain protein (Coppin *et al.*, 1997; Turgeon & Yoder, 2000). These genes are the master regulators of sexual reproduction.

The apparent simplicity of the ascomycete bipolar mating system is belied by the complex suite of alternative mating strategies presented by this group of fungi (Ni *et al.*, 2011; Bennett & Turgeon, 2017). The fundamental distinction is between heterothallism, which ensures outcrossing, and homothallism, which presumably provides sexual reproductive assurance in the absence of a mate. In heterothallic species, each individual contains a single nuclear type, and each nuclear type contains only one of the two *MAT1* idiomorphs, so that mating requires the presence of two individuals with nuclei of opposite mating type. In strictly homothallic species, a single nuclear type contains both *MAT1-1* and *MAT1-2* idiomorphs, so that sexual reproduction does not require a mate (Kronstad & Staben, 1997).

As pointed out by Wilson *et al.* (2015), the term homothallism is an umbrella concept that can include

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several different mating strategies. Strictly homothallic species have the two *MAT1* idiomorphs within a single haploid genome, but these may be fused or in close proximity at a common locus (e.g. Lepoint *et al.*, 2010), or they may occupy unlinked positions on different chromosomes (e.g. Hutchinson *et al.*, 2016). Many ascomycete fungi have mating systems that represent further modifications of the homothallic scheme, including mating-type switching in yeasts (Butler *et al.*, 2004) and unisexuality (sexual reproduction through a single *MAT1* idiomorph; Debuchy & Turgeon, 2006). Another mating strategy is pseudohomothallism, in which the two *MAT1* idiomorphs are in separate nuclear types that cohabit in a dikaryotic mycelium (Grognet & Silar, 2015). In several well-described species with this mating system, the products of meiosis are packaged into spores that contain nuclei of both mating types, ensuring that the pseudohomothallic condition will persist across generations. The system is sometimes somewhat 'leaky', with a small fraction of uninucleate spores produced, potentially preserving some outcrossing opportunity (Grognet & Silar, 2015).

Many ascomycete fungi have no known sexual state, although most of those examined have been found to possess apparently intact and functional *MAT1* loci (Debuchy & Turgeon, 2006) and may have a population genetic structure that suggests past or current recombination (e.g. Groenewald *et al.*, 2006). The conditions that induce mating can be very specific and hard to ascertain, so that 'cryptic sex' under field conditions is often assumed to occur even when mating cannot be obtained in culture. Another possible source of recombination for truly asexual species arises when two vegetatively compatible strains anastomose to form a dikaryotic mycelium. Parasexuality, a mitotic process that results in recombination, can then potentially occur (Grubisha & Cotty, 2010).

Vegetative incompatibility represents a system of non-self recognition in fungi that generally operates independently of the mating type locus, but it can also have major implications for population genetic structure (Grubisha & Cotty, 2010). It is regulated by a series of loci referred to as *het* or *vic* loci, which vary in number and location in different fungi (Glass *et al.*, 2000). In many vegetative compatibility systems, two strains must have matching alleles at every *het* locus to be vegetatively compatible, that is, capable of anastomosis to form a dikaryotic mycelium (heterokaryosis). If this condition is not met when two strains attempt anastomosis, compartmentalization and cell lysis prevent hyphal fusion. For strictly asexually reproducing fungi, this means that members of different vegetative compatibility groups (VCGs) are genetically isolated from each other, in effect forming 'cryptic species' according to the biological species concept (Giraud *et al.*, 2008). Although readily obtained in culture, heterokaryosis is thought to be 'virtually excluded in nature' because of the low probability of encounter between two vegetatively compatible strains (Glass *et al.*, 2000).

In species with sexual recombination, members of different VCGs may be sexually compatible even though they cannot anastomose vegetatively. In fact, in strictly heterothallic species such as *Neurospora crassa*, strains of the two mating types are not vegetatively compatible, thus preventing the formation of dikaryons with the potential for pseudohomothallic mating (Glass *et al.*, 2000), so that only strains belonging to different VCGs can mate.

A common generalist seed bank pathogen, *Pyrenophora semeniperda*, was used here to address the question of the relative importance of sexual reproduction, mating system structure, and vegetative incompatibility in mediating potential levels of outcrossing and patterns of genetic variation. This organism was described originally and is known primarily in its mitosporic or asexually reproducing form, but the formation of sexual structures was described from field collections by Shoemaker (1966). It has been reported to reproduce sexually in culture only twice, and cultural conditions for reliable production of the sexual state have not been determined (Brittlebank & Adam, 1924; Paul, 1969). The efforts here to obtain crosses of this fungus for the purpose of generating recombinant strains with superior biocontrol potential against seeds of the annual grass weed *Bromus tectorum* have been unsuccessful, prompting this research to better understand its mating system and outcrossing potential.

Pyrenophora semeniperda can infect dispersed seeds of many grass species but is most abundant in field seed banks of weedy annual grasses, especially *B. tectorum* (Beckstead *et al.*, 2014, 2016). It penetrates through the caryopsis wall, sends haustoria directly into the endosperm tissue, and produces macroscopic stromata that protrude from the infected seed (Finch-Boekweg *et al.*, 2016). Slow-germinating seeds are killed, while rapidly germinating seeds may escape mortality even though conidial production occurs (Beckstead *et al.*, 2007). A seed may be infected by multiple strains, as evidenced by simple sequence repeat (SSR) fingerprints of cultures derived from individual stromata on a single seed (Meyer *et al.*, 2015; authors' unpublished data). This potentially provides an opportunity for direct interactions between strains, either through vegetative anastomosis or sexual reproduction.

This study had the following objectives: (i) to sequence the *MAT1* locus in multiple strains of *P. semeniperda* from *B. tectorum* to identify and characterize genetic variation in the *MAT1-1* and *MAT1-2* idiomorphs; (ii) to determine mating type frequency distributions within a large *P. semeniperda* strain collection from 25 populations from *B. tectorum* seeds across the western United States to estimate the potential for sexual reproduction; (iii) to describe the mating system and life cycle of *P. semeniperda* in the context of existing information about its genetic composition and life history; and (iv) to determine whether the known high level of polymorphism at the ITS (internal transcribed spacer of the ribosomal RNA gene) locus in this species has any bearing on potential patterns of outcrossing.

Based on the fact that the sexual state in *P. semeniperda* is known, it was expected to possess intact,

functional *MAT1* idiomorphs, and that the two idiomorphs would occur at approximately equal frequencies across and within populations, supporting the hypothesis of regular sexual reproduction in the field. It was expected that its life cycle would present a balance between clonal reproduction through asexually produced conidia and a pattern of sexual reproduction via a mating system that includes at least some potential for outcrossing. Previous work has shown that polymorphism at the ITS locus in this species is extreme, with 13 known haplotypes and wide sequence divergence (Boose *et al.*, 2011). It was hypothesized that this ITS polymorphism could be indirectly associated with some degree of reproductive isolation among ITS haplotypes, so that ITS haplotypes represent markers of genome-wide genetic differentiation.

Materials and methods

MAT1 locus sequencing and *MAT1* haplotype identification

To characterize the *P. semeniperda* mating-type locus, the *MAT1* idiomorphs were first identified in assembled genomes from strains CCB06 (Soliai *et al.*, 2014) and STR15 (authors' unpublished data) based on homology with the *MAT1* sequences from the related species *Pyrenophora teres* (Rau *et al.*, 2007). Single-spore cultures of 58 randomly selected *P. semeniperda* strains were obtained by isolating germinated conidial spores on water agar (Meyer *et al.*, 2010). DNA was extracted from these single-spore cultures as described by Boose *et al.* (2011), or with a DNeasy Plant Mini kit (QIAGEN) using freeze-dried tissue pulverized in a 2 mL screwtop container with 3 mm bashing beads. Much of the DNA used for *MAT1* locus sequencing and genotyping was extracted as part of an earlier ITS study described by Boose *et al.* (2011). To obtain DNA sequence from the coding region of the single genes comprising each of the two *MAT1* idiomorphs, three overlapping fragments from each idiomorph were amplified by PCR using primers designed with PRIMER3 in the GENEIOUS software package (Biomatters) and GoTaq HotStart Master Mix (Promega). The primer sets are listed in Table 1 as MAT1-1A, B, C and MAT1-2A, B, C. The PCR products were cleaned using the QIAquick PCR Purification kit (QIAGEN) and submitted for Sanger sequencing at the Brigham Young University DNA Sequencing Center. Sequenced fragments from each strain were assembled and consensus sequences were aligned to identify polymorphisms using GENEIOUS (cost matrix = 65% similarity, gap open penalty = 12, gap extension penalty = 3, global alignment with free end gaps). Multiple sequence alignment and tree construction were performed using CLUSTALW within GENEIOUS to identify haplogroups and haplotypes within haplogroups for each idiomorph. The complete sequence of each *MAT1* idiomorph was also obtained, as well as flanking regions, from the draft genome assemblies (described below) of representative *MAT1-1* and *MAT1-2* strains. Lastly, a dN/dS analysis was performed on the polymorphism exhibited by each idiomorph to determine whether these were under positive, purifying, or neutral selection, but as predicted by Kryazhimskiy & Plotkin (2008), there was insufficient within-idiomorph sequence variation to use this analytical approach.

Polymorphism in the *MAT1-1* and *MAT1-2* idiomorphs was related to ITS haplotype by examining the frequency distribution of MAT haplogroups for each idiomorph in each of the six common ITS haplotypes. ITS haplotypes are designated here by the abbreviation HT followed by the haplotype letter designation from Boose *et al.* (2011). This dataset produced clear-cut results with no exceptions; it was not subjected to further statistical analysis.

MAT1 genotyping

Pyrenophora semeniperda strains were isolated from killed *B. tectorum* seeds collected from the soil seed bank at 25 sites in Colorado, Idaho, Nevada, Utah and Washington, USA (Boose *et al.*, 2011). DNA was extracted from 505 strains as described above. Using the program PRIMER3 within the GENEIOUS software package, PCR primers specific to the *MAT1-1* and *MAT1-2* idiomorphs were designed (MAT1gt and MAT2gt, respectively; Table 1). These primer sets were used, together with GoTaq HotStart Master Mix, to amplify DNA from each of the 505 strains in separate PCRs. The amplification products from each reaction were analysed by gel electrophoresis and each isolate was scored as *MAT1-1* only, *MAT1-2* only or *MAT1-1/MAT1-2*, depending on the presence or absence of amplification products from the two PCRs.

The *MAT1* genotyping dataset was analysed in two ways. First, the relative frequencies of *MAT1* idiomorphs and *MAT1* genotypes (*MAT1-1*, *MAT1-2* and *MAT1-1/MAT1-2*) were calculated in each sample population and across all populations. Next, these frequencies were calculated for each of the six common ITS haplotypes. Differences in *MAT1* haplotype and genotype relative frequencies were examined among ITS haplotypes using a Fisher's exact test (SAS PROC FREQ) of the raw count data against the null hypothesis that all ITS haplotypes would have the same *MAT1* haplotype or *MAT1* genotype frequency distribution.

SSR fragment length analysis

Five polymorphic SSR loci (BFOFF9, BFOFF19, BFOFF25, BFOFF37 and BFOFF41) were used to genotype 126 strains selected to include representative proportions of each *MAT1* genotype, as well as an additional 18 strains from a concurrent study (Beckstead *et al.*, 2016; not MAT-genotyped) for use in the ITS phylogenetic analysis (Table S1). Although these SSRs were described earlier (Meyer *et al.*, 2015), new primers were designed using PRIMER3 within the GENEIOUS software package to better match the cycling parameters of the Type-it Microsatellite PCR kit (QIAGEN) used to amplify the markers (Table 1). Cycling parameters for all SSR markers used in the analysis were set according to the manufacturer's instructions. Fragments were analysed at the Brigham Young University DNA Sequencing Center using an ABI PRISM 3700 Genetic Analyzer and peak calls were made using GENEIOUS.

The SSR data were used to test the relative frequency of polymorphic and monomorphic strains in each of the three observed *MAT1* genotype groups (*MAT1-1*, *MAT1-2* and *MAT1-1/MAT1-2*) against the null hypothesis of equal representation of polymorphic and monomorphic strains. The objective was to determine whether the *MAT1-1/MAT1-2* genotype was associated with SSR polymorphism in dikaryotic strains. Fisher's exact test (SAS PROC FREQ) using raw count data was used for this analysis.

ITS phylogenetic analysis

To test the hypothesis that ITS haplotypes would be genetically differentiated, the study took advantage of draft genome

Table 1 Primer sequences used in *Pyrenophora semeniperda* mating-type (*MAT1*) locus and SSR genotyping.

Name	Forward primer (5′–3′)	Reverse primer (5′–3′)
MAT		
MAT1-1A	CCCACTCTCTTCTCACTTTC	GTAACGCTAGTAGGGGCATAG
MAT1-1B	TTCGTGACCAACTCACCAAG	TGAATCCAGTTCCCGCATTG
MAT1-1C	ATGATCTCATTGCCGCTGTC	ATAGGCCTGCAGGTACAACTC
MAT1-2A	CTTCTGCTCAATTTCTCACTAC	CTATCGGTCGATGAGGAAAC
MAT1-2B	CTATTGAGGCTGCTCCCAC	GGCAAGCATACCAACTGAAG
MAT1-2C	CAAGTATAGCCCTAGGAAGC	TTCATGGGACGTGGAGTTC
MAT1gt	TGCAAAGTGCTGACCCCCTACTT	AGCGCCAGGAACGAAAGCGT
MAT2gt	TGTCCCGCAGACGGACCTGA	AGCTCGGGTCGTGGAAGGCT
SSR		
BFOFF9	TATTGTTGAGGGATTGGTTGTG	TCCGCAGTTGGAATAAACGTCAAAAC
BFOFF19	ACGTCTCTCCCACATGGCTCCCC	GGCTTAGAGAACCCTGTCCGCCATGG
BFOFF25	CCGATCAGTCGTGCTCCTTCTAC	GCAGACTCCTTGCATGAGAATCGTCCA
BFOFF37	CATGCCAAAGGGTGTCTTGGG	GCAACAAGCAAAAGAGCAGCGAGAAC
BFOFF41	CCAGCCACCACGTCTGCCCTGA	GCCAGTCTGCCAGTCTCGGTCTTGG

assemblies obtained via Illumina sequencing for 12 strains belonging to four ITS haplotypes. DNA was extracted from air-dried cultures using a ZR Fungal/Bacterial DNA Miniprep kit (Zymo Research). Libraries for sequencing were constructed using Illumina TruSeq DNA Sample Prep with custom size selection by the Huntsman Cancer Institute, and 1 × 50 DNA sequencing was performed on an Illumina HiSeq 2000 machine. DNA sequencing resulted in *c.* 25 million reads per library. Sequence data from each of these 12 strains are deposited in the Small Read Archive at NCBI as accessions SRX3242201-SRX324212. Reads from each sample library were assembled separately using the *P. semeniperda* genome sequence for strain CCB06 deposited in GenBank (accession SRP007005; Soliai *et al.*, 2014) as a reference and the GENEIOUS software package. Coverage of each assembled genome ranged between 20× and 30×.

Each assembled genome produced 54 scaffolds, corresponding to the 54 scaffolds found in the original *P. semeniperda* genome assembly (Soliai *et al.*, 2014). A subset of 30 randomly selected scaffolds was included in the analysis. Selected scaffolds from the 12 genome assemblies that matched each reference scaffold were compared to one another using the sequence aligner CLUSTALW (Larkin *et al.*, 2007). This produced separate alignments for each included scaffold. These alignments were curated to remove any regions that did not have at least 1× coverage in all the genomes. Furthermore, any region that had no variability between genomes was removed. Thus, only single nucleotide polymorphisms (SNPs) and indels among the 12 genomes were kept. This resulted in inclusion of 141 786 polymorphic markers. A table was generated for each of the selected alignments, with columns representing each genome. Each row represented a SNP/indel location. Genomes that had identical nucleotides at that SNP or indel were given identical numbers on the row for that polymorphic locus. The tables were then concatenated together, resulting in a table representing SNP haplotypes across a large subset of the entire genome for each of the 12 genome assemblies, along with the original reference genome. Using the PVCLUST package in R (Suzuki & Shimodaira, 2006), cluster analysis that included bootstrapping was performed on the data to produce a dendrogram that approximates phylogeny. PVCLUST uses hierarchical clustering based on a distance matrix generated via correlation and constructs the clusters based on average distances (http://stat.sys.i.kyoto-u.ac.jp/prog/pvclust/). This generates a dendrogram that has branch tips of equal length. The bootstrap analysis was performed with 1000 replications.

As a second test of genetic differentiation among ITS haplotypes, the SSR dataset discussed earlier was used to construct a phylogeny for the six common ITS haplotypes for which sufficient data were available. As the strains had been selected without reference to their ITS haplotype, each haplotype was represented at approximately the same abundance found range-wide in the field. The analysis included 129 strains, of which 51 were dikaryotic. A dikaryotic strain was treated as two haplotypes for the allele frequency analysis, increasing the sample size to 180. ARLEQUIN (Excoffier & Lischer, 2010) was used to calculate SSR allele frequencies for each ITS haplotype and the resulting F_{ST} distance matrix was used as input to the program NEIGHBOR in the PHYLIP software package (Felsenstein, 1989). The UPGMA clustering protocol with default settings was used to generate a dendrogram showing degrees of genetic similarity among the ITS haplotypes. It was not possible to perform bootstrap analysis on this dataset because of the small number of SSR markers included.

Results

MAT1 locus sequencing and haplotype identification

MAT1-1 was sequenced from 43 strains and *MAT1-2* from 40 strains, including strains that contained both *MAT1* idiomorphs. The identification of gene features reported here is deduced from DNA sequence homology with *MAT1* genes from related fungal species, namely *P. teres* and *Pyrenophora tritici-repentis*, the only other *Pyrenophora* species for which sequence information is available (Lepoint *et al.*, 2010; Lu *et al.*, 2010). The organization of these features is described using nucleotide positions relative to the putative start codon of the gene.

MAT1-1 characterization

The α-box DNA-binding motif typical of the MAT1-1 protein was encoded by nucleotides 63–639 and was interrupted by a single 53 bp intron (Fig. 1a). An 18 bp minisatellite repeat unique to the *MAT1-1* idiomorph was identified inserted after base 1065, and was repeated

from three to 10 times. The most common number of repeats was seven, in 17 of the 43 strains. Although no sequences with eight or nine repeats were detected, one of the strains had 10 repeats. The length of the *MAT1-1* coding sequence plus intron varied from 1306 to 1430 bp depending on the number of minisatellite repeats present. Length variation of the minisatellite repeat and SNPs at 17 variant positions defined nine *MAT1-1* haplotypes among the strains sequenced (GenBank accession numbers KP718750–KP718758). The nine haplotypes were grouped into three haplogroups designated 1A, 1B and 1C, according to sequence similarity (Fig. 1b). Haplogroup 1A was predominant, identified in 22 of the 43 strains. Of the four haplotypes comprising haplogroup 1A, only one, haplotype 5, varied from the others in its nucleotide sequence at two positions. The main distinguishing feature that separated haplogroup 1A haplotypes was the number of minisatellite repeats, which ranged from four to 10. The two haplotypes in haplogroup 1B differed only at a single nucleotide, whereas the three haplogroup 1C haplotypes differed only in the number of minisatellite repeats (Fig. 1c).

Sixteen of the 17 SNPs were in the coding region of the gene, with six of those located in the sequences encoding the α-box domain of the MAT1-1 protein (nucleotides 66, 75, 87, 103, 504 and 560; Fig. 1c). Of the 16 coding SNPs, eight were nonsynonymous (shown in bold in Fig. 1c) with two of the nonsynonymous variants located in the α-box coding region (nucleotides 103 and 504). The nonsynonymous SNP at nucleotide 504 occurred only in haplotype 1–5, resulting in an alanine substitution for proline in the encoded protein. The nonsynonymous SNP at nucleotide 103 distinguished haplogroup 1C from 1A and 1B and resulted in a valine substitution for leucine. The 18 bp minisatellite repeats were not perfectly conserved, as variation was detected between repeats (Fig. 1d). Additionally, minisatellite

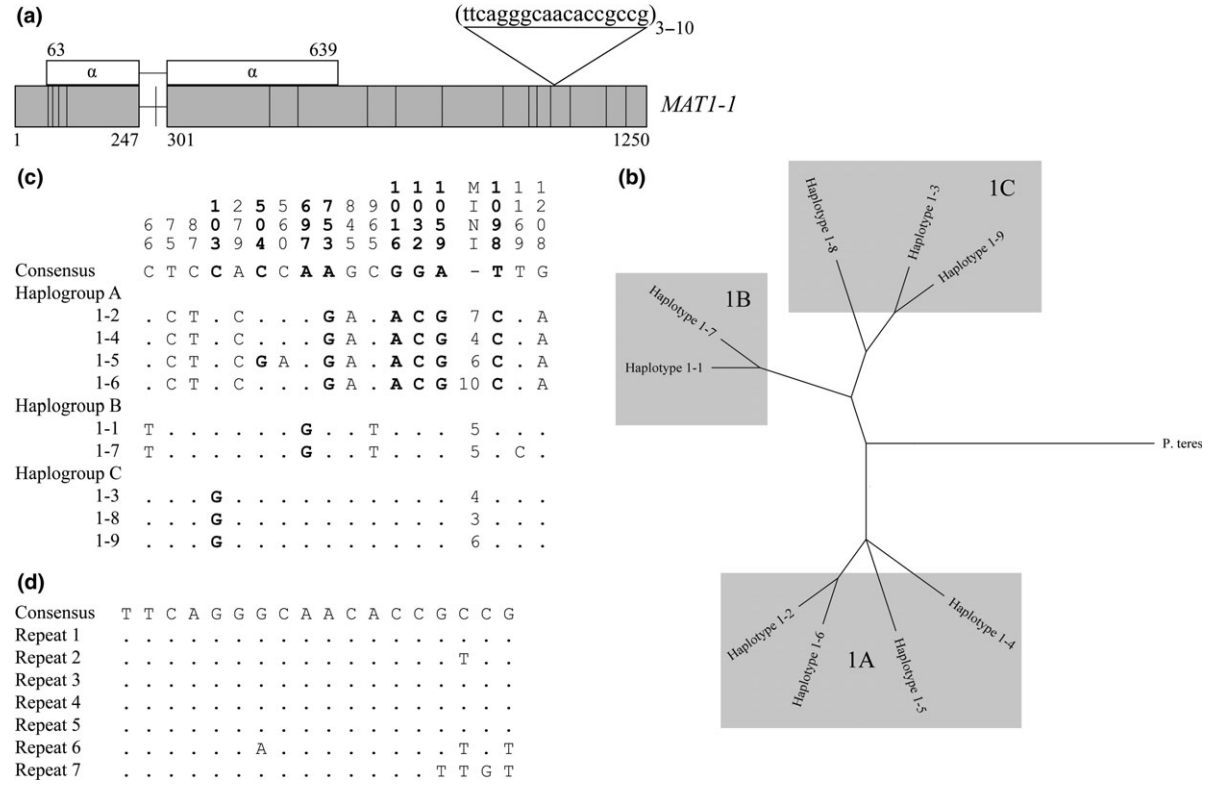

Figure 1 Characterization of the *MAT1-1* idiomorph from *Pyrenophora semeniperda*. (a) Map of *MAT1-1* coding sequences and intron showing two exons (grey boxes) and a single 53 bp intron (horizontal line). The numbers below the exons indicate the base positions for the beginning and end of the coding sequence within the exons relative to the first base of the start codon. The α-box DNA-binding domain is represented by the open box above the exons with the beginning and ending bases of the domain shown above the box. The consensus sequence of an 18 bp minisatellite is shown in parentheses above the second exon with the range of repeat numbers identified in the nine haplotypes. Variant base positions between the nine haplotypes are shown as black vertical lines within the gene exons. (b) Neighbour-joining tree of the nine *P. semeniperda MAT1-1* haplotypes with the *Pyrenophora teres* idiomorph included as an out-group. Three haplogroups, A, B and C, are shown as shaded rectangles. (c) Nine *P. semeniperda MAT1-1* haplotypes vary at 17 positions within the gene and in the number of minisatellite tandem repeats. The base number of the variant position relative to the first base of the start codon is shown above a consensus sequence and bases are shown for each haplotype when it varies from the consensus. The numbers below MINI indicate the number of minisatellite tandem repeats found in each haplotype. (d) The consensus sequence of the seven repeats in the haplotype 1–2 minisatellite is shown with variant nucleotides in repeats 2, 6 and 7.

sequence variation was found between the different haplotypes, with detected polymorphisms generally shared within haplogroups (data not shown).

MAT1-2 characterization

The HMG DNA-binding motif typical of the MAT1-2 protein was encoded by nucleotides 367–652 and was interrupted by a single 55 bp intron (Fig. 2a). An 18 bp minisatellite was also identified in the *MAT1-2* idiomorph that was inserted after base 322, but its sequence was not the same as the *MAT1-1* minisatellite. It repeated from three to five times, resulting in a variable length of the coding sequence plus intron of 1120–1156 bp. Seven *MAT1-2* haplotypes were identified, defined by 17 SNPs and length variation of the minisatellite (GenBank accession numbers KP718741–KP718747). The seven haplotypes could be grouped into two distinct haplogroups, designated 2A and 2B (Fig. 2b). Haplogroup 2A was widely distributed across the entire range as it was found at every site sampled, whereas the much less common haplogroup 2B was only identified at six of the 20 sampled locations. The four haplotypes that comprised haplogroup 2A varied at only three nucleotide positions but they included the full range of minisatellite repeats. Haplogroup 2B haplotypes all had four minisatellite repeats and showed sequence variation at four nucleotide positions (Fig. 2c).

Fifteen of the 17 SNPs were in the coding region of the gene, with only two of those SNPs located in the sequences encoding the HMG DNA-binding domain of the MAT1-2 protein (nucleotides 525 and 580). Nine of the 15 coding SNPs were nonsynonymous (shown in bold in Fig. 2c), although the adjacent SNPs at nucleotides 984 and 985 were in the same codon. One of the two SNPs in the HMG domain encoding region of the gene was nonsynonymous (nucleotide 525) resulting in an arginine in the haplogroup 2A protein and histidine in the haplogroup 2B protein. As with the minisatellite repeat found in the *MAT1-1* gene, the *MAT1-2* gene minisatellite also had some sequence variation between the repeats (Fig. 2d) and variation between haplotypes that helped define the two haplogroups (data not shown).

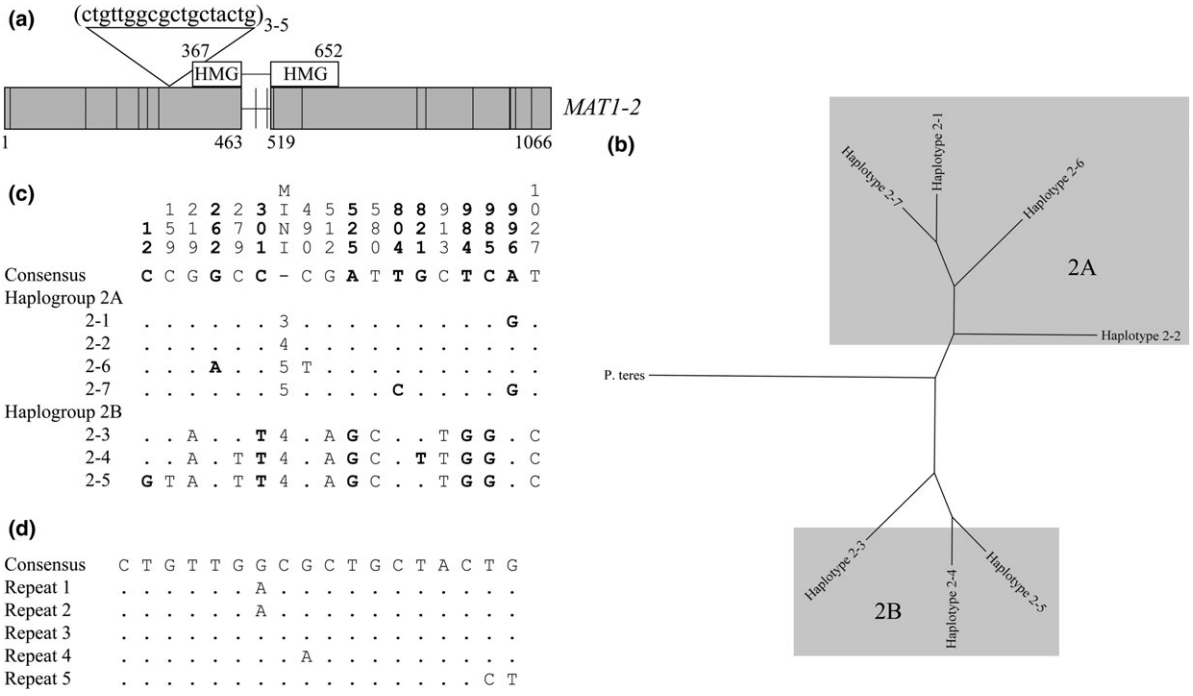

Figure 2 Characterization of the *MAT1-2* idiomorph from *Pyrenophora semeniperda*. (a) Map of *MAT1-2* coding sequences and intron showing two exons (grey boxes) and a single 55 bp intron (horizontal line). The numbers below the exons indicate the base positions for the beginning and end of the coding sequence within the exons relative to the first base of the start codon. The HMG-box DNA-binding domain is represented by the open box above the exons with the beginning and ending bases of the domain shown above the box. The consensus sequence of an 18 bp minisatellite is shown in parentheses above the first exon with the range of repeat numbers identified in the seven haplotypes. Variant base positions between the seven haplotypes are shown as black vertical lines within the gene exons. (b) Neighbour-joining tree of the seven *P. semeniperda MAT1-2* haplotypes with the *P. teres* idiomorph included as an out-group. Two haplogroups, A and B, are shown as shaded rectangles. (c) Seven *P. semeniperda MAT1-2* haplotypes vary at 17 base positions within the coding region of the gene and in the number of minisatellite tandem repeats. The base number of the variant position relative to the first base of the start codon is shown above a consensus sequence and bases are shown for each haplotype when it varies from the consensus. The numbers below MINI indicate the number of minisatellite tandem repeats found in each haplotype. (d) The consensus sequence of the five repeats in the haplotype 2-6 minisatellite is shown with variant nucleotides in repeats 1, 2, 4 and 5.

MAT locus similarity to other Pyrenophora *species*

To determine how similar the MAT1 proteins from *P. semeniperda* were to proteins from related species, the deduced amino acid sequences were aligned and compared to homologous proteins from *P. teres* and *P. tritici-repentis*. Although the *MAT1* sequences from *P. graminea* are published, they are nearly identical to the *MAT1* sequences from *P. teres* (Rau *et al.*, 2007). The deduced amino acid sequence from the *P. semeniperda* MAT1-1 protein was 45.4% and 43.5% identical to the *P. teres* and *P. tritici-repentis* proteins, respectively. Higher sequence conservation was observed in the α-box DNA-binding domain where the identities were 61.7% and 59.9%, respectively. The *P. semeniperda* MAT1-2 protein was 55.0% identical to the homologous protein from *P. teres* and 51.1% identical to the *P. tritici-repentis* protein. Again, the HMG DNA-binding domain of the protein was more conserved than the protein as a whole, with 71.4% and 67.5% identity of *P. semeniperda* when compared to *P. teres* and *P. tritici-repentis*, respectively.

In examining sequence for the *MAT1* locus and flanking regions based on assembled genomes, it was found that both the *MAT1-1* and *MAT1-2* idiomorphs contained only genes for the DNA-binding proteins. The characteristic open reading frame (ORF) was found immediately upstream from the sequenced locus in both mating types and a putative DNA lyase gene unrelated to sexual reproduction was found immediately downstream. The presence of a single gene in each idiomorph is similar to the locus structure in *P. teres* (Rau *et al.*, 2007) and throughout the dothidiomycetes (Bennett & Turgeon, 2017).

MAT1 polymorphism and ITS haplotype

When the *MAT1-1* and *MAT1-2* haplogroup identities of strains in each of the six common ITS haplotypes included in the *MAT1* sequencing dataset were tabulated, a striking pattern emerged, with each ITS haplotype characterized by a unique combination of *MAT1-1* and *MAT1-2* haplogroups (Table 2). The only exception was for ITS haplotypes HTH and HTI, which were closely similar at the ITS locus, differing only by a single base pair (Boose *et al.*, 2011). The strongly non-random distribution of *MAT1* haplotypes among ITS haplotypes suggests that the *MAT1* polymorphisms observed may have some functional significance.

MAT1 genotyping

Across all populations, 34% of the strains assayed contained only *MAT1-1*, 28% contained only *MAT1-2*, and 38% contained both mating-type idiomorphs (genotype *MAT1-1/MAT1-2*; Table 3). Within the 505 strains assayed, 263 (52%) possessed *MAT1-1* idiomorphs and 242 (48%) possessed *MAT1-2* idiomorphs, a ratio that does not depart significantly from 1:1. Both idiomorphs were identified in all populations assayed and all three *MAT1* genotypes (*MAT1-1* only, *MAT1-2* only and *MAT1-1/MAT1-2*) were identified in each of the 25 populations except Peavine Mountain, where none of the strains assayed had the *MAT1-2* idiomorph occurring singly (Fig. 3). Due perhaps to small sample size, no population had a *MAT1-1*:*MAT1-2* haplotype ratio that differed significantly from a 1:1 ratio. However, there were often large differences in the proportions of the three *MAT1* genotypes present in a population, with the *MAT1-1/MAT1-2* genotype dominant in some populations (e.g. Cricket Mountain, Fishtrap) and rare in others (e.g. Gusher, Kahlotus; Fig. 3).

The most common ITS haplotype (HTA) accounted for 62% of all strains, with remaining common haplotypes each accounting for 4.2–11.6% of the total (Fig. 4a). The ratio of *MAT1-1* to *MAT1-2* idiomorphs within ITS haplotypes was generally not significantly different from a 1:1 ratio, suggesting that sexual

Table 2 Distribution of *MAT1-1* and *MAT1-2* haplogroups among 54 strains of the six most common ITS haplotypes.

ITS haplotype	No. of strains	No. of populations	*MAT1-1* haplogroup	*MAT1-1* haplotypes	*MAT1-2* haplogroup	*MAT1-2* haplotypes
HTA	24	15	1A	1A-2 (14)	2A	2A-2 (5)
				1A-4 (2)		2A-6 (8)
				1A-5 (1)		
				1A-6 (1)		
HTB	8	8	1C	1C-3 (8)	2A	2A-6 (7)
HTD	4	4	1A	1A-2 (4)	2B	2B-3 (4)
HTH	10	8	1B	1B-1 (4)	2A	2A-1 (3)
				1B-7 (1)		2A-2 (2)
						2A-6 (3)
HTI	5	5	1B	1B-1 (3)	2A	2A-1 (4)
HTK	3	3	1C	1C-8 (1)*	2B	2B-4 (1)
				1C-9 (1)		

Strains for *MAT1* locus sequencing were chosen at random with respect to ITS haplotype. Within each ITS haplotype–*MAT1* haplogroup combination, the number of strains identified in each *MAT1* haplotype is listed. *MAT1* haplogroup and haplotype designations are from Figure 1 (*MAT1-1*) and Figure 2 (*MAT1-2*). Single strains of four rare ITS haplotypes were also sequenced: HTL (1C-8)*, HTM (2B-5), HTN (2A-7) and HTO (2A-2). Shaded cells indicate *MAT1* haplotypes shared among ITS haplotypes.

Table 3 *Pyrenophora semeniperda* idiomorph frequencies at 25 collection sites in the western USA

Name[a]	Collection year	Collection site	Idiomorph frequency[b]		
			N	*MAT1-1*	*MAT1-2*
DIN	2005	Dinosaur, CO	25	0.62	0.38
CCB	2005	Cindercone Butte, ID	25	0.48	0.52
CCF	2008	Cindercone Flat, ID	19	0.50	0.50
BFL	2008	Bedell Flat, NV	24	0.67	0.33
LSC	2008	Lower Smoke Creek, NV	22	0.50	0.50
PVM	2008	Peavine Mountain, NV	18	0.70	0.30
SWR	2008	Stillwater, NV	17	0.61	0.39
CKT	2005	Cricket Mountains, UT	17	0.45	0.55
CNF	2005	Confusion Range, UT	16	0.44	0.56
DOG	2005	Dog Valley, UT	20	0.58	0.42
DUJ	2005	Dutch John, UT	17	0.44	0.56
GUS	2005	Gusher, UT	16	0.38	0.62
HRN	2005	House Range, UT	19	0.26	0.74
MIL	2005	Milk Ranch, UT	18	0.28	0.72
SQC	2008	Santaquin Canyon, UT	23	0.63	0.37
STR	2005	Strawberry, UT	19	0.53	0.47
TMC	2005	Ten Mile Creek, UT	22	0.66	0.34
WHV	2005	White's Valley, UT	22	0.53	0.47
WRK	2005	Whiterocks, UT	26	0.57	0.43
DRL	2006	Dr Lefcourt, WA	27	0.56	0.44
FSH	2006	Fishtrap, WA	25	0.56	0.44
KAH	2006	Kahlotus, WA	15	0.43	0.57
MAR	2006	Marcellus, WA	20	0.55	0.45
PKC	2006	Packer Creek, WA	18	0.56	0.44
SDM	2006	Saddle Mountain, WA	15	0.30	0.70

A total of 505 strains were included in the *MAT1* genotyping. See Figure 3 for locations.

[a]The three-letter name abbreviations correspond to those shown on the map in Figure 3.

[b]*MAT1* idiomorph frequencies were determined at each location from both unikaryotic and dikaryotic strains. Because of relatively small sample sizes, none of the population *MAT1-1*:*MAT1-2* ratios depart significantly from a 1:1 ratio according to χ^2 tests.

recombination would be likely within ITS haplotypes even if they were reproductively isolated from each other (Fig. 4b). The one significant exception was haplotype HTH, in which *MAT1-2* was significantly over-represented. Haplotype HTI showed the reverse pattern but the difference was not significant due to smaller sample size. *MAT1* genotype proportions, on the other hand, varied significantly among ITS haplotypes (Fisher's exact test $P < 0.0001$; Fig. 4c). Haplotype HTA had a *MAT1* genotype distribution very similar to the overall average, with an approximately even distribution of strains among the three genotypes, as did haplotype HTK. Haplotype HTI had approximately the same proportion of *MAT1-1*/*MAT1-2* strains as HTH, but was deficient in the *MAT1-2* genotype, whereas HTH was deficient in the *MAT1-1* genotype. Haplotype HTD was deficient in the *MAT1-1* genotype, while the *MAT1-1*/*MAT1-2* genotype was over-represented, whereas in HTB, the *MAT1-2* genotype was under-represented and *MAT1-1*/*MAT1-2* was over-represented.

MAT1 genotype and SSR polymorphism

The apparently common occurrence of both structurally heterothallic and homothallic strains within populations of *P. semeniperda* led to the hypothesis that each nucleus contains a single *MAT1* idiomorph, but that anastomosis between members of opposite mating type within a VCG could result in dikaryotic strains that are structurally pseudohomothallic. As a test of this hypothesis, polymorphic SSR markers were used to detect the dikaryotic condition independent of *MAT1* genotype and predicted that the *MAT1-1*/*MAT1-2* genotype would be associated with SSR polymorphism and therefore with dikaryotic strains. It was found that, of 25 strains with the *MAT1-1*/*MAT1-2* genotype, 24 also exhibited SSR polymorphism indicative of the dikaryotic condition, providing strong evidence that this genotype represents structural pseudohomothallism (Table 4). Strains with the *MAT1-1* genotype were mostly monomorphic for SSRs, suggesting that they represent unikaryotic strains, and the same trend was seen for the *MAT1-2* genotype, though in this case the association was not significant. Dikaryotic strains detected by SSR polymorphism can show either *MAT1-1* or *MAT1-2* genotypes; these strains must contain two copies of a single *MAT1* idiomorph, one in each nuclear type. This shows that vegetative compatibility and mating type are not associated, as anastomosis can occur both between strains of the same mating type and also of opposite mating types, so that dikaryotic strains can show any of the three *MAT1* genotypes. In contrast, unikaryotic strains exhibit both SSR monomorphism and either *MAT1-1* or *MAT1-2* genotypes.

ITS phylogenetic analysis

In ITS genotyping of over 500 strains of *P. semeniperda* using the standard fungal barcoding primers ITS4 and ITS5 (White *et al.*, 1990), not a single strain was found that was polymorphic for ITS haplotype (Boose *et al.*, 2011). This meant that the presence of dikaryotic strains in this fungus was overlooked, but the *MAT1* and SSR genotyping data presented here show conclusively that the dikaryotic condition that presumably arises from anastomosis within a VCG is a common occurrence. The logical conclusion from this set of observations is that strains of different ITS haplotypes must belong to different VCGs, as they apparently never occur together in dikaryotic strains. The distribution of *MAT1* idiomorph allelic variation presented earlier in this paper suggests that ITS haplotypes may also be reproductively isolated. If this is the case, it is reasoned that there should be evidence for genetic differentiation among ITS haplotypes.

When genome assemblies of 12 strains of *P. semeniperda* from Illumina sequencing were compared

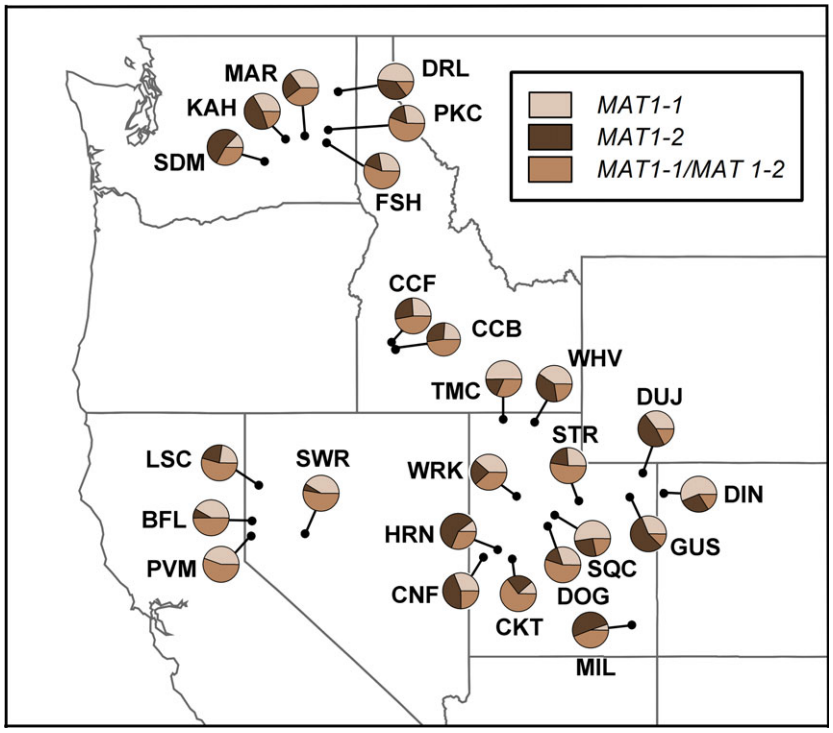

Figure 3 Locations of 25 *Pyrenophora semeniperda* populations included in the *MAT1* genotyping, with pie charts showing the relative frequency of *MAT1-1*, *MAT1-2* and *MAT1-1*/*MAT1-2* genotypes in each population. See Table 3 for population names corresponding to three-letter codes. [Colour figure can be viewed at wileyonlinelibrary.com].

using SNP/indel markers from throughout the genome, a pattern of genetic differentiation among ITS haplotypes was clearly demonstrated (Fig. 5a). The seven strains belonging to the common haplotype HTA clustered very closely with one another, as did two strains belonging to the common haplotype HTH. These two haplotype groups were joined at intermediate genetic distance in the dendrogram. Two strains of haplotype HTK also clustered closely together, along with the HTK reference strain that was used as an assembly template. The single strain of haplotype HTD was separated from the HTK group, but HTD and HTK strains were more similar to each other than to the larger group containing HTA and HTH strains. Each node of the dendrogram was supported at 100% in the bootstrap analysis, which means that all bootstrap replicates resulted in trees with identical topology. This was probably because of the very large number of markers included in the analysis, so that taking random subsets of markers did not affect the result.

The dendrogram prepared from SSR allele frequencies for strains belonging to the six common ITS haplotypes generally supported results of the genome-wide SNP/indel analysis for a much larger number of strains (Fig. 5b). ITS haplotypes were clearly differentiated, and the pattern was similar to that observed with genome-wide SNP/indel markers. Haplotypes HTA and HTH were more similar to each other than to haplotypes HTK and HTD. One haplotype not in the Illumina dataset (HTI) clustered most closely with HTH, as expected from their nearly identical ITS sequences mentioned earlier. ITS haplotype HTB clustered more closely with haplotype HTD.

Discussion

Nucleotide sequencing of *P. semeniperda MAT1* idiomorphs showed that both idiomorphs possess intact sequence and share homology with reported sequences from the related fungal species *P. teres* and *P. tritici-repentis*, both of which are known to have functional sexual phases. Remarkably, both *P. semeniperda MAT1* idiomorphs contain separate minisatellite repeats that increase the size of the mating type proteins considerably relative to proteins from other ascomycete fungi. Nevertheless, the inserted minisatellite sequences do not appear to disrupt the reading frame of the coding sequence, including sequence encoding the α-box and HMG DNA-binding domains. The DNA-binding domains of the two idiomorphs are highly conserved across *P. semeniperda* and related *Pyrenophora* species, suggesting these domains are under selection pressure to retain protein function.

MAT1 sequencing revealed substantial variation within each of the idiomorphs in terms of both single nucleotide substitutions and number of minisatellite repeats, allowing identification of three distinct *MAT1-1* haplogroups and two distinct *MAT1-2* haplogroups. The strong

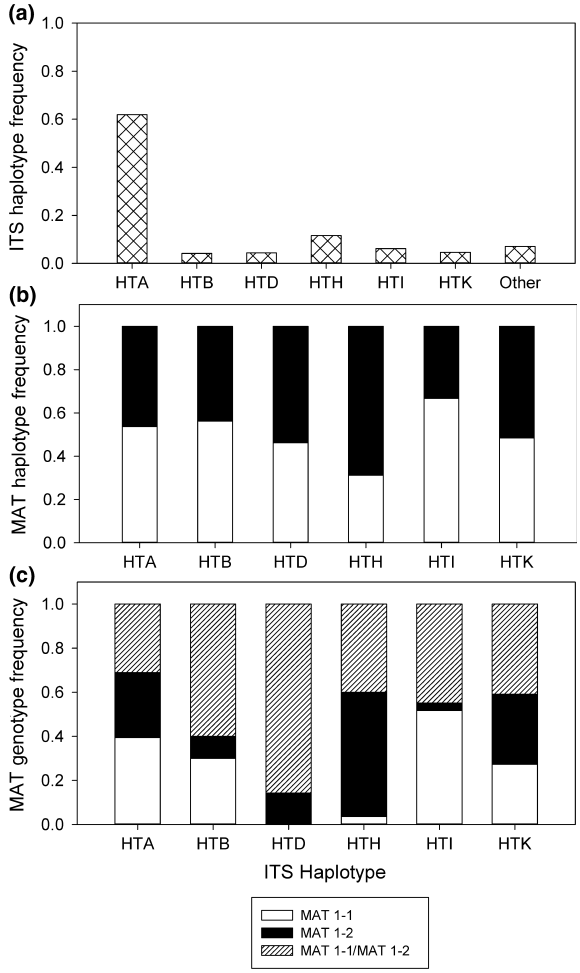

Figure 4 (a) Relative frequencies of the six most common *Pyrenophora semeniperda* ITS haplotypes in a collection of 505 strains from 25 populations in six western US states; (b) relative frequencies of the *MAT1-1* and *MAT1-2* idiomorphs within each of the six most common ITS haplotypes, regardless of whether they occur alone or in combination within dikaryotic strains; (c) relative frequencies of *MAT1-1*, *MAT1-2* and *MAT1-1*/*MAT1-2* genotypes within each of the six most common ITS haplotypes.

Table 4 The relationship between *MAT1* genotype and simple sequence repeat (SSR) genotype based on fragment analysis for 126 *Pyrenophora semeniperda* isolates belonging to three *MAT1* genotypes.

		SSR genotype		Fisher's exact test
MAT1 genotype	Sample number	All monomorphic	Some polymorphic	independence probability
MAT1-1 only	58	46 (0.793)	12 (0.207)	0.0017
MAT1-2 only	43	27 (0.628)	16 (0.372)	0.2820
MAT1-1/1-2	25	1 (0.040)	24 (0.960)	0.0003

Five polymorphic SSRs were included in the genotyping. The probability (Fisher's exact test) reports the likelihood of obtaining the observed ratio of SSR-polymorphic to SSR-monomorphic isolates if the population contained isolates with equal representation in the two SSR groups.

association between *MAT1* haplogroup identity and ITS haplotype identity provided a first indication that these *MAT1* polymorphisms might function to reproductively isolate groups of strains with different *MAT1-1* and *MAT1-2* haplogroup combinations.

A similar *MAT1* locus allelic diversity analysis was conducted for the related species *P. teres*, resulting in identification of two haplotypes for both *MAT1-1* and *MAT1-2* (Lu *et al.*, 2010). Divergence at the *MAT1* locus in *P. teres* distinguishes the net form of barley net blotch (*P. teres* f. *teres*) from the spot form (*P. teres* f. *maculata*), and these two forms are not known to hybridize under field conditions, indicating that they are probably reproductively isolated (Rau *et al.*, 2007). Differentiation between ITS haplotypes that is correlated with *MAT1* polymorphism is also reported for Chinese black truffle (*Tuber indicum*; Belfiori *et al.*, 2013). These reports make reproductive isolation among ITS haplotypes of *P. semeniperda* due to *MAT1* locus polymorphisms a plausible hypothesis. How polymorphisms associated with large changes in the size of the coding regions caused by minisatellite repeats might change mating compatibility is not known.

From evidence presented earlier, it is clear that each haploid nuclear type in *P. semeniperda* possesses only one of the two possible *MAT1* idiomorphs, and that an encounter between nuclei possessing opposite mating types would be necessary for mating to occur. Although there are no data to address the functional significance of these results, it is proposed that this species has a mixed mating system that combines heterothallism (mating between two strains with nuclei of opposite mating types) and a form of pseudohomothallism (mating between nuclei of opposite mating type contained within the mycelium of a dikaryotic strain) (Fig. 6). While this mating system seems to result as a natural consequence of the ability of vegetatively compatible strains of opposite mating type to form heterokaryons that then have the potential to sexually reproduce, it is remarkably hard to find reports of such a mating system. On the one hand, obligately heterothallic species have mechanisms to prevent heterokaryosis that includes both mating types, such as the link between mating type and vegetative compatibility in *N. crassa* mentioned earlier (Glass *et al.*, 2000). On the other hand, pseudohomothallism as classically described includes the ability to generate meiotic products that generally retain the dikaryotic configuration of the parent mycelium (e.g. *Podospora anserina*; Grognet & Silar, 2015). In *P. semeniperda*, the presence of both unikaryotic and dikaryotic strains as well as *MAT1-1*, *MAT1-2* and *MAT1-1*/*MAT1-2* mating types at high frequency in many populations could indicate an alternation between creation of the dikaryotic condition through anastomosis and restoration of the unikaryotic

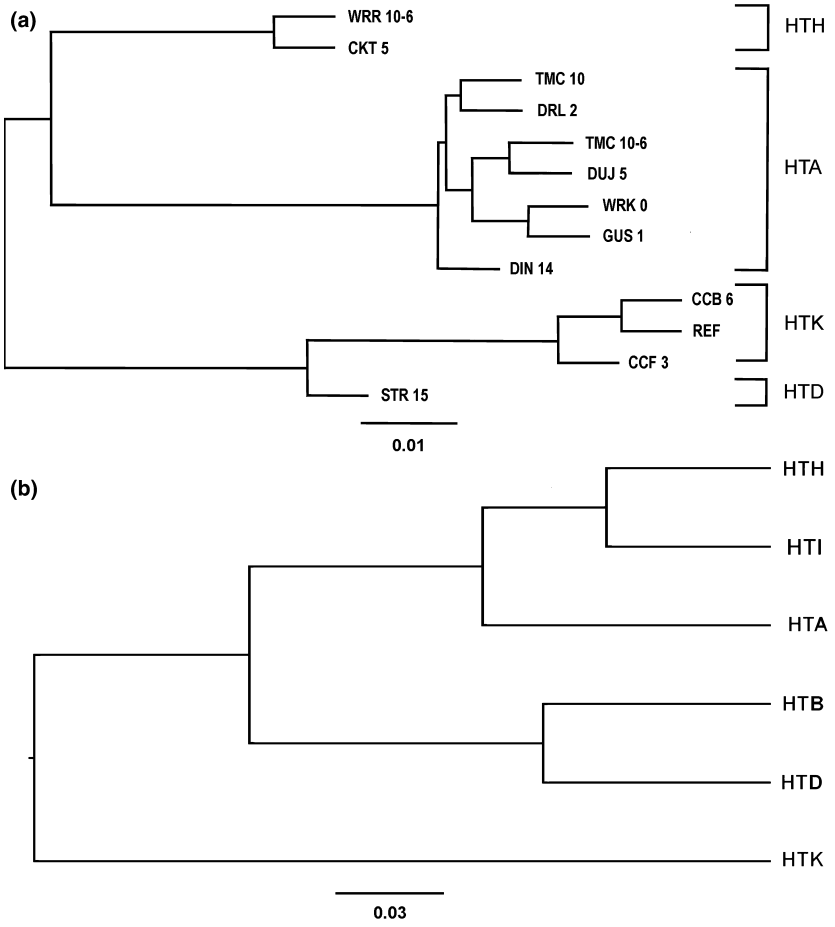

Figure 5 (a) Hierarchical cluster dendrogram showing relationships among 12 strains of *Pyrenophora semeniperda* belonging to four common ITS haplotypes, based on genome-wide SNP/indel polymorphisms obtained from draft Illumina genome assemblies for each strain using the originally sequenced genome of this organism (CCB6; Soliai *et al.*, 2014) as a reference genome (marked REF); (b) UPGMA dendrogram showing relationships among six common *P. semeniperda* ITS haplotypes, based on an F_{ST} distance matrix calculated from allele frequencies at five polymorphic SSR loci (n = 129 strains). Scale bars indicate genetic distance. Strains were selected for Illumina sequencing and for SSR genotyping without reference to their ITS haplotypes.

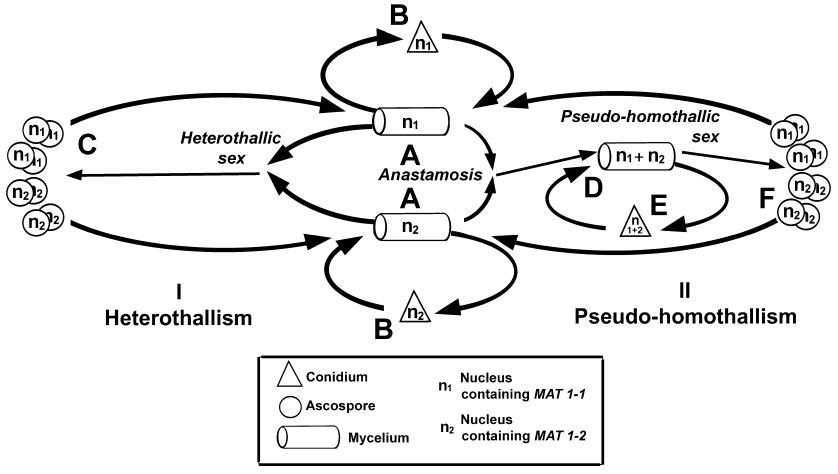

Figure 6 Provisional schematic diagram proposing a facultatively pseudohomothallic life cycle for *Pyrenophora semeniperda*. Part I: Heterothallism. Haploid mycelia of each mating type (A) produce conidial spores (B) that germinate to form mycelium identical to the parental type, OR haploid mycelia of opposite mating type engage in sexual reproduction to produce haploid recombinant ascospores (C) that germinate to produce haploid mycelia of opposite mating type (A). Part II. Pseudohomothallism. Haploid mycelia (A) that are vegetatively compatible undergo anastomosis to form a dikaryotic mycelium (D) that produces conidial spores (E) that are also dikaryotic and identical to the parental type OR dikaryotic mycelium that contains nuclei of opposite mating types engages in sexual self-reproduction to produce haploid recombinant ascospores (F). These ascospores germinate to produce haploid mycelia of opposite mating type (A).

condition through a sexual process that results in uninucleate ascospores, in other words, facultative pseudohomothallism (Fig. 6).

It has been observed that transitions between homothallism and heterothallism are common, even within species (Lin & Heitman, 2007), but these authors do not discuss facultative pseudohomothallism as proposed here based on evidence for *P. semeniperda*. This type of mixed mating system may be so common that it is not noteworthy, but if heterokaryosis is truly rare in natural systems (Glass *et al.*, 2000), then the case presented by *P. semeniperda* may be exceptional. The fact that multiple strains of the pathogen can share the same infection court within a single seed may make heterokaryosis more likely in this pathosystem than is generally the case.

Not every transition in the provisional life cycle diagram illustrated in Figure 6 has been documented, but there is considerable evidence to support it. It is known that both unikaryotic and dikaryotic strains can occur at high frequency within a population. SSR studies have confirmed that the nuclear condition of the parent mycelium is preserved through asexual production of conidia, so that conidial reproduction does not provide an avenue of return to the unikaryotic condition for dikaryotic strains (authors' unpublished data). There is no proof that dikaryotic strains produce unikaryotic ascospores through the normal process of meiosis, as ascospores have not yet been recovered for genetic analysis, but it is difficult to envision another process whereby the observed balance between unikaryotic and dikaryotic strains could be maintained.

It appears that, rather than representing a transition between functional homothallism and heterothallism, the life cycle of *P. semeniperda* may represent a more generalized system that lacks both specialization for control over anastomosis of opposite mating types seen in obligately heterothallic species and specialization for the maintenance of the dikaryotic condition across generations seen in true pseudohomothallic species. Such a system would preserve options for outcrossing with other strains, while increasing the probability of sexual reproduction by placing nuclei of opposite mating type in close proximity in dikaryotic strains. If dikaryotic strains are repeatedly generated from recombined unikaryotic strains, this sexual reproductive assurance would not even come at the cost of greatly reduced outbreeding potential. Asexual conidial production and the clonal structure that it engenders are still probably the primary mode of reproduction in this fungus, so that the balance between preserving adapted gene complexes and providing outcrossing opportunities would be maintained.

The discovery of strain groups within *P. semeniperda* that are vegetatively incompatible and possibly also reproductively isolated adds another layer of complexity to *P. semeniperda* population biology. The fact that strain groups characterized by different ITS marker haplotypes also show genetic divergence across the genome strengthens the case for reproductive isolation mediated through both vegetative incompatibility and *MAT1* locus allelic variation. These strain groups could probably qualify as cryptic species in terms of phylogenetic species recognition criteria (Giraud *et al.*, 2008). Species boundaries in *Fusarium* species complexes have been defined in these terms (O'Donnell *et al.*, 2004).

If further experimental work confirms both vegetative incompatibility and sexual incompatibility among ITS haplotypes in *P. semeniperda*, then they would also qualify as cryptic species by a biological species definition (Giraud *et al.*, 2008). There is no evidence to suggest that these strain groups are phenotypically distinguishable, but work on phenotypic variation in this fungus (e.g. Meyer *et al.*, 2010, 2015) has focused mostly on strains belonging to haplotype HTA, the common haplotype in Utah deserts (Boose *et al.*, 2011), with no systematic effort to detect among-ITS haplotype differences.

It is possible that cryptic speciation in *P. semeniperda* is somehow associated with host specialization, but the experimental evidence indicates a remarkable lack of physiological host specialization in this fungus (Beckstead *et al.*, 2014, 2016). The host *B. tectorum* is genetically diverse but highly selfing, with populations made up of strongly differentiated maternal lineages (Meyer *et al.*, 2013, 2016). Reciprocal inoculation studies found no evidence that different strains of *P. semeniperda* were physiologically specialized to attack particular host genotypes (Beckstead *et al.*, 2016). Weak genetic structure among populations on different hosts based on ITS and SSR allelic composition appeared to be more related to founder effects, probably as a result of seedborne conidial dispersal (Meyer *et al.*, 2008).

The presence of multiple cryptic biological species would lessen the chances for outcrossing in this fungus, particularly for the less common ITS haplotypes. It would increase the advantage of pseudohomothallism for these rare haplotypes, as compatible mating types could remain paired through clonal increase in this system and thereby increase their chances of producing sexual progeny without the necessity of encountering a mate.

This investigation of the mating system of *P. semeniperda* emphasizes the need for mating system studies across a broad phylogenetic range of non-model pathogens in the Ascomycota, to determine what is truly common and what is rare in terms of mating strategies. It also sets the stage for further experimental work that could lead to the achievement of the original goal of breeding strains of this pathogen with superior potential for biocontrol of invasive annual brome seed banks.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. This file contains ITS haplotypes, MAT genotypes and SSR genotypes at five loci for 143 strains of *Pyrenophora semeniperda* included in the genetic analyses.