Genetics of mating in members of the Chaetomiaceae as revealed by experimental and genomic characterization of reproduction in *Myceliophthora heterothallica*

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Abstract

Members of the Chaetomiaceae are among the most studied fungi in industry and among the most reported in investigations of biomass degradation in both natural and laboratory settings. The family is recognized for production of carbohydrate-active enzymes and antibiotics. Thermophilic species are of special interest for their abilities to produce thermally stable enzymes and to be grown under conditions that are unsuitable for potential contaminant microorganisms. Such interests led to the recent acquisition of genome sequences from several members of the family, including thermophilic species, several of which are reported here for the first time. To date, however, thermophilic fungi in industry have served primarily as parts reservoirs and there has been no good genetic model for species in the family Chaetomiaceae or

for thermophiles in general. We report here on the reproductive biology of the thermophile *Myceliophthora heterothallica*, which is heterothallic, unlike most described species in the family. We confirmed heterothallism genetically by following the segregation of mating type idiomorphs and other markers. We have expanded the number of known sexually-compatible individuals from the original isolates from Indiana and Germany to include several isolates from New Mexico. An interesting aspect of development in *M. heterothallica* is that ascocarp formation is optimal at approximately 30 °C, whereas vegetative growth is optimal at 45 °C. Genome sequences obtained from several strains, including isolates of each mating type, revealed mating-type regions whose genes are organized similarly to those of other members of the Sordariales, except for the presence of a truncated version of the *mat A-1* (*MAT1-1-1*) gene in mating-type *a* (*MAT1-2*) strains. In *M. heterothallica* and other Chaetomiaceae, mating-type *A* (*MAT1-1*) strains have the full-length version of *mat A-1* that is typical of mating-type *A* strains of diverse Ascomycota, whereas *a* strains have only the truncated version. This truncated *mat A-1* has an intact open reading frame and a derived start codon that is not present in *mat A-1* from *A* strains. The predicted protein contains a region that is conserved across diverse *mat A-1* genes, but it lacks the major alpha1 domain, which characterizes proteins in this family and is known to be required for fertility in *A* strains from other Ascomycota. Finally, we have used genes from *M. heterothallica* to probe for mating genes in other homothallic and heterothallic members of the Chaetomiaceae. The majority of homothallic species examined have a typical *mat A-1,2,3* (*MAT1-1-1,2,3*) region in addition to an unlinked *mat a-1* (*MAT1-2-1*) gene, reflecting one type of homothallism commonly observed in diverse Ascomycota.

Keywords: *Myceliophthora heterothallica*; thermophile; Chaetomiaceae; heterothallism; mating; genomics

1. Introduction

Thermophily, defined as better vegetative growth at 45 °C than at 25 °C, has been reported for fungi in three orders of the Ascomycota, the Eurotiales, Onygenales and Sordariales (Morgenstern et al., 2012). Among the Sordariales, thermophily is restricted to members of the Chaetomiaceae, a family whose species are well known for their abilities to degrade cellulose (Acharya and Chaudhary, 2012). Considerable attention has been given to thermophilic members of the family for their thermally stable cellulose-active enzymes. This has fostered substantial interest in industry, patents (eg. US Patents 4081328, 5602004 and 569598; Canadian Patent Application 1075181 A1, and PCT Patent Applications WO2014060379 A1, WO2013029170 A1 and WO2014059541 A1), and the sequencing of the genomes for several species, including *Myceliophthora thermophila* and *Thielavia terrestris*, in part to explore the value of fungal systems in the development of biofuels (Berka et al., 2011). Thermophilic members of the Chaetomiaceae show substantial promise in the context of the need to produce fermentable sugars from complex polysaccharides, the need for stable enzymes and the advantages high temperatures have in reducing the threat of contamination in large-scale fermentation (Beckner et al. 2011, Rubin 2008).

Although members of the Chaetomiaceae have long been known for the ability to degrade cellulosic materials (Ames, 1963), there has been little research focusing on the genetics of species in the family. In fact, there is neither a genetic model for any member of the family nor a thermophilic fungus from any group with a history in genetic research, a circumstance that limits the tractability of these organisms in research. Here we focus on *M. heterothallica*, which is a truly heterothallic species. The production of fruiting bodies had been observed originally for this species by von Klopotek (1976), who described it as *Thielavia heterothallica*. The species

was placed in *Myceliophthora* by van den Brink et al. (2012), who confirmed crossing behavior and later presented evidence for recombination using AFLP analysis (van den Brink et al., 2013). We have sought to characterize further the reproductive biology of this species, toward the goal of providing a genetic model for both Chaetomiaceae and thermophiles in general. We confirmed sexual recombination by demonstrating the independent assortment of specific genetic markers, and we have characterized the growth conditions necessary for optimal vegetative growth and ascocarp production. Using a combination of PCR-based sequence analysis and genome sequencing, we identified the genes involved in mating compatibility, providing the first such analysis for a heterothallic member of the Chaetomiaceae. In addition, we compared the genetic mechanisms of mating within *M. heterothallica* with mating systems in other members of the Sordariales and those of homothallic members of the family.

2. Materials and Methods

2.1. Mating gene nomenclature

While we have a preference for the mating-type gene nomenclature employed for species of *Neurospora* (Perkins 1999), we acknowledge the wide use of the nomenclature recommended by Turgeon and Yoder (2000). Accordingly, in this paper we have included designations using both conventions. For example, the *mat A-1* of *Neurospora* is also referenced as *MAT1-1-1* using the Turgeon and Yoder system. In part, our use of the *Neurospora* nomenclature reflects the fact that species of *Neurospora* have served as our primary references for comparative analyses of mating-type gene organization. The genus is the logical choice as a reference because it is in the same taxonomic order as *Myceliophthora*, its species display a diversity of reproductive modes that have been characterized at the molecular level (for example, Gioti et al.,

2012), and it is the genus in the Sordariales with the best developed phylogenetic framework (for example, Dettman et al., 2003).

2.2. *Fungal strains*

Crossing studies employed *M. heterothallica* strains CBS 202.75 and CBS 203.75, obtained from the Centraalbureau voor Schimmelcultures, and isolates ThNM053 and ThNM146, from a previous study of thermophilic fungi from the Sevilleta National Wildlife Refuge in central New Mexico (Powell et al., 2012). Strains ThNM053 and ThNM146 were concluded to belong to *M. heterothallica* during the course of this study (discussed below) and have been deposited at the Fungal Genetics Stock Center (FGSC 25265 and FGSC 26266, respectively).

Our comparative analyses of mating-type genes employed sequences derived from genome sequencing efforts encompassing more than twenty strains representing diverse species across the Chaetomiaceae, including the four *M. heterothallica* strains listed above. These strains are listed in the sections that follow. Sequences relevant to our analyses have been deposited at GenBank as described below. The genome projects that generated these sequences were publicly funded through the US DOE Joint Genome Institute or Genome Canada. Genome sequences and annotations are available at <http://jgi.doe.gov/fungi> and www.fungalgenomics.ca.

2.3. *Molecular methods*

DNA from strains employed in genetic analyses was typically isolated from tissues ground in liquid nitrogen, resuspended in cetyltrimethylammonium bromide (CTAB) lysis buffer (2% CTAB, 1.4M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 0.2% 2-mercaptoethanol), followed by a phenol and chloroform extraction, and ethanol precipitation. Polymerase chain reaction (PCR) amplifications were conducted in 13.5 μ L reactions with 6.5 μ L Premix ExTaq polymerase

(Takara, Mountain View, California), 1 μ L of each (5 μ M) primer, 2 μ L of 2% bovine serum albumin, 2 μ L of milliQ water and 1 μ L of template DNA. PCR products were purified enzymatically with ExoSAP-IT (Affymetrix, Santa Clara, California) according to the manufacturer's recommendations. Sequences were obtained by Sanger sequencing using 10 μ L reactions with 0.5 μ L BigDye Terminator v3.1, 2 μ L of 5X Sequencing Buffer (Life Technologies/Applied Biosystems, Carlsbad, California), 1 μ L of 3 μ M concentrations of the same primers used for PCR (1 primer per reaction), 5.5 μ L of milliQ water and 1 μ L of template DNA.

2.4. Genetic analysis

Genetic crosses were performed by inoculating strains of different mating type opposite one another on plates containing malt extract medium [MEA, 2% malt extract (w/v), 1% agar (w/v)]. Progeny were derived from ascospores dissected from mature ascocarps, which lack ostioles and forcible ascospore ejection. Ascocarps were removed from crossing plates with a sterile dissecting needle, then rinsed three times with sterile water to remove adhering conidia and mycelial fragments. To release ascospores, the ascocarps were transferred to a microscope slide and broken with sterile forceps. The ascospores were then suspended in sterile water and pipetted onto MEA plates, which were incubated at 45-50 °C for 12-18 hours. Using light microscopy, germlings that were confirmed to derive from ascospores (Fig. 1) were individually transferred to fresh MEA plates, by removing a germling and the surrounding agar with a sterile dissecting needle.

Progeny from crosses employing CBS strains 203.75 and 202.75 along with a benomyl-resistant mutant strain derived from CBS 202.75 (designated 202.75^{ben}) were analyzed for independent assortment of specific markers: the mating type idiomorph, a molecular

polymorphism between strains 202.75 and 203.75 in an actin-like gene, and benomyl resistance. The polymorphism in the actin-like gene was scored by sequencing. The sequences of both the CBS 202.75 and 203.75 actin-like genes were first obtained using two degenerate primers, 5C-88F+ (5'-CAGGTSATCACCATYGGMAAYGARCG-3') and 5C-88F- (5'-CCTSCTTSGASRTCCACATCTGCTG-3'), originally used to identify the *Act88F* actin gene in *Drosophila melanogaster* (Lovato et al., 2001). The resulting sequences were then used to design internal primers specific to *M. heterothallica*: ACTFWD (5'-TATCCACGTCACCACCTTCA-3') and ACTREV (5'-GATCCAGAGACCGAGTACTTGC-3').

Benomyl (DuPont, Wilmington, Delaware) resistant strains, presumably carrying a mutation in the β -tubulin gene (Orbach et al., 1986), were obtained by UV mutagenesis. Conidia were harvested from culture flasks of CBS 202.75 and suspended in sterile water. This conidial suspension was poured into a 40 mm petri dish and stirred with a mini stir bar and stir plate. The conidia were exposed to UV radiation for 105 sec, using a Panasonic (G15 T8) germicidal lamp placed at a distance of 13 cm. Irradiated conidia (0.5 ml suspension) were spread onto benomyl agar (MEA with 1.5 μ g/mL benomyl), and plates were incubated in the dark at 45 °C. Resulting colonies were subcultured on benomyl agar plates to confirm resistance. Resistant isolates were then crossed with CBS 203.75 on MEA agar to obtain progeny. These progeny were screened on benomyl agar plates to determine benomyl resistance. Mating type was first determined by crossing each progeny strain to both parental strains and was confirmed by amplifying the mating type region with PCR. Non-parental genotypes were identified as those with a mating-type idiomorph from one parent and an actin-like or benomyl resistance marker allele from the other parent.

2.5. Effects of temperature on growth and reproduction

The optimal temperature for vegetative growth was determined by measuring the maximum radial growth per day in millimeters. Ten centimeter MEA plates were inoculated at the center with 5 μ L of spore suspension and were incubated at 25, 37, 45, 50, and 55 $^{\circ}$ C.

To determine optimal crossing temperature, crossing plates were incubated at 25, 29, 33, 37 and 41 $^{\circ}$ C. Parental strains, CBS 202.75 and 203.75, were inoculated opposite one another on 10-cm MEA plates using conidia or a plug of mycelium from a vigorously growing culture. Crossing plates were incubated in the dark for up to a month. The optimal temperature for ascocarp development was determined by measuring the total area of the plate covered by ascocarps. Ascocarp density was determined using scans of the undersides of crossing plates. Images from scans were adjusted for optimal contrast between dark regions (ascocarps) and light areas (mycelia), and the area occupied by ascocarps was estimated using ImageJ software (Rasband, 1997-2012).

2.6. *Ascospore germination*

Our preliminary studies resulted in several failed attempts to achieve ascospore germination. Our first success came with ascospores that had been dried in air for approximately one hour, placed overnight at -80 $^{\circ}$ C, plated and incubated at 45 $^{\circ}$ C. Subsequent experiments showed that the -80 $^{\circ}$ C treatment is not required, although it may help increase germination frequency. We explored a range of pH, incubation temperatures and media types. We also tested a range of pretreatment temperatures up to 60 $^{\circ}$ C. None of these treatments resulted in a substantial increase in germination rates, which at 45 $^{\circ}$ C varied from a few percent to greater than 50%.

2.7. *Genomic analysis, characterization and expression of mating-type genes*

Preliminary comparative analyses of the *M. heterothallica* mating-type regions were performed prior to the availability of genome sequences for mating-type *a* strain *N. crassa* FGSC 73 and *M. heterothallica* strains 202.75 and 203.75 (see below). Genome sequences of *M. thermophila* (*mat A*; ATCC 42464; Genbank PRJNA32775) and *N. tetrasperma* (*mat a*; FGSC 2509; Genbank AFCY00000000) were queried by BLAST analysis (Joint Genome Institute) to identify homologs of *sla2* (NCU11202) and *apn2* (NCU01961), two genes that flank the mating-type idiomorphs of *mat A* (*MAT1-1*) and *mat a* (*MAT1-2*) regions in diverse Ascomycota (Gioti et al., 2012). The mating-type regions of *M. heterothallica* strains were obtained by long-range PCR using primers designed by referencing conserved sequences in *sla2* and *apn2*, followed by Sanger sequencing using “primer walking” (results not presented). While the results presented below are derived largely from the availability of genome sequences, these preliminary experiments, together with the results presented below, indicated conservation of mating-gene arrangements across diverse species of *Myceliophthora* and *Neurospora*.

Subsequently, we obtained genome sequences for four *M. heterothallica* strains using Illumina technology in conjunction with a Community Sequencing Project award from the Joint Genome Institute (JGI). This project resulted in draft sequences for CBS strains 203.75 and 202.75 and genomic sequences at lower coverage for ThNM146 and ThNM053. Genome sequences for strains 203.75 and 202.75 were obtained and assembled using the standard JGI pipeline for draft fungal sequences. The ThNM146 and ThNM053 genomes were assembled using Velvet version 1.2.10 (31 kmer with exp_cov set to auto).

Mating gene annotations were performed using alignments with related genes and proteins, and comparisons with transcripts. Transcripts were identified for *mat A-1* (*MAT1-1-1*), *mat A-2* (*MAT1-1-2*) and *mat A-3* (*MAT1-1-3*) in BLASTn searches of EST assemblies for *M.*

heterothallica CBS strain 203.75 (mating type A), available at the DOE Joint Genome Institute (<http://jgi.doe.gov/fungi>). These assemblies were used to confirm both expression and intron annotations. The JGI designations are Locus807v1rpkm214.84 (*mat A-1*), Locus12481v1rpkm1.82 (*mat A-2*) and Locus12875v1rpkm1.66 (*mat A-3*).

Expression of the truncated *mat A-1* (*MAT1-1-1*) and *mat a-1* (*MAT1-1-3*) genes in strains of *M. heterothallica*, *M. fergusii*, *M. hennulea* and *H. hyalothermophila* was assessed using the Illumina sequencing platform to determine the sequences of expressed RNAs. Fungi were grown in liquid suspensions. Conidia at 10⁶ spores/mL were inoculated in 10x TDM (Roy and Archibald 1993) containing 2% carbon source, and cultures were incubated at 45 °C with shaking at 150 rpm. A carbon source used for all fungi was a mixture (1:1 ratio) of alfalfa and barley straws (gifts from Lethbridge Research Centre of Agriculture and Agri-Food Canada). The straws were ground to 0.5 mm before use. Following growth for 21 h to 26 h, mycelia were harvested by filtering through Miracloth (Calbiochem, San Diego, CA, USA), ground in liquid nitrogen, and total RNA was extracted with Trizol (Invitrogen) as described previously (Semova et al. 2006). *M. fergusii* (CBS 454.80) was grown on three additional carbon sources (all at 2%): avicel, beechwood xylan and citris pectin (all from Sigma-Aldrich). RNA was sequenced using the mRNA-seq method of Illumina's Solexa IG at the McGill University-Génome Québec Innovation Centre. The RNA-seq reads, 100 nucleotides in length, were mapped and analyzed as described by Berka et al. (2011).

2.8. Crossing experiments

M. heterothallica was named by von Klopotek (1976) based on sexual crossing experiments carried out with CBS strains 203.75 and 202.75. We performed additional crosses

to examine the compatibility of these strains with other closely related isolates. Both *M. heterothallica* strains were also crossed with isolates ThNM146 and ThNM053, which were obtained from biological soil crusts at the Sevilleta National Wildlife Refuge, Socorro, New Mexico. Previously, these strains were shown to be closely related to *M. heterothallica* (Powell et al., 2012), and so our ultimate assignment of these strains to *M. heterothallica* was based on both genetic relationships and crossing behavior as discussed below.

2.9. Genbank accessions

ITS sequences for *M. heterothallica* strains employed for phylogenetic tree building have the following Genbank accession numbers: JN659509 (ThNM006), JN659502 (ThNM053), JN659490 (ThNM109), JN659493 (ThNM140), JN659494 (ThNM142), JN659495 (ThNM146), JN659496 (ThNM147), JN659479 (CBS 203.75), JN659478 (CBS 202.75), JN659509 (ThNM006). Entries for other species are JF412005 (*M. fergusii* CBS 405.79), JF412003 (*M. thermophila* ATCC 42464) and AY681193 (*N. crassa*).

Mating-type genes for *M. heterothallica* strains examined have been deposited in Genbank under the following accession numbers: KR119056 (CBS203.75, complete mating region), KR119055 (CBS202.75, complete mating region), KR119057 (ThNM146, complete mating region), KR119058 (ThNM053, complete mating region), KR632512 (CBS 663.74, truncated *mat A-I*), KR632510 (CBS 131.65, *mat A-I*), KR632511 (CBS 375.69, *mat A-I*). Entries for other species are KR261945 (*M. fergusii* CBS 405.69, truncated *mat A-I*), KR261946 (*M. hinnulea* ATCC 52474, truncated *mat A-I*), KR632513 (*Humicola hyalothermophila* CBS 454.80, truncated *mat A-I*), M33876 (*N. crassa* OR74A) and JTEW000000000 (*N. crassa* FGSC 73, mating-type *a* region; Baker et al., 2015).

2.10. Phylogenetic tree building and WebLogo analysis

Phylogenetic analyses were performed with the PHYLIP version 3.67 *dnapars* program (Felsenstein, 1989) employing 1000 bootstrap replicates. Alignments have been deposited at TreeBASE (study number 17141). Pairwise genetic distance estimates were obtained using ClustalW2 (Larkin et al., 2007).

WebLogo-assisted analyses (Crooks et al. 2004; <http://weblogo.berkeley.edu>) employed *mat A-1* (*MAT1-1-1*) predicted proteins from *M. heterothallica* and homologs from diverse Ascomycota.

3. Results

3.1. Developmental stages have distinct temperature optima

Growth experiments indicated that *M. heterothallica* reaches optimal vegetative growth at 45 °C, while ascocarp development is greatest at 29 °C (Fig. 2). Vegetative growth was enhanced by increases in temperature from 25 °C to 45 °C but after peaking, growth dropped sharply between 45 °C and 55 °C. At 45 °C, the maximum growth rate was as much as 50 mm/day.

3.2. Progeny genotypes demonstrate independent assortment

A type of mating behavior termed “homothallism with cross-feeding” has been reported for the related species *T. terrestris* (Samson et al., 1977). The term refers to the hypothesis that one strain, whether of opposite mating type or not, can induce homothallic sexual reproduction in another without true mating. Given that report, it was necessary to determine whether *M. heterothallica* exhibits true heterothallism, as reported by von Klopotek (1976). Recombination was confirmed by the independent assortment of the mating-type with actin-like and benomyl-

resistance loci in crosses of CBS strains 203.75 and 202.75. Of 24 progeny obtained from wild-type crosses, 12 had non-parental genotypes, indicated by the presence of a mating type idiomorph of one parent and an actin-like polymorphism of the other parent. Twenty-two progeny were obtained from the cross between the benomyl-resistant CBS 202.75 strain and the wild-type 203.75 strain. Eleven of these progeny represented non-parental genotypes, with benomyl sensitivity or resistance conferred from one parent and a mating-type idiomorph from the other parent. These results are summarized in Table 1.

Neither CBS 203.75 nor CBS 202.75 was capable of crossing with *M. thermophila* (ATCC 42464), as no fruiting bodies were successfully produced. However, the *M. heterothallica* strains did cross with the ThNM isolates. Specifically, ThNM053 crossed with CBS 203.75 and ThNM146 crossed with CBS 202.75. These results are consistent with the phylogenetic analysis of the rRNA ITS region (Fig. 3).

3.3. Mating-type regions of *M. heterothallica* possess both conserved and unique features

Mating-type A (*MAT1-1*) strains of *M. heterothallica* possess a gene arrangement that is conserved across diverse heterothallic species in the Sordariales (Fig. 4). Both gene order and directionality are conserved. In contrast, mating-type *a* (*MAT1-2*) strains of *M. heterothallica* possess a truncated version of the *mat A-1* (*MAT1-1-1*) gene, a gene typically viewed as the hallmark of mating type A. The predicted *mat A-1* proteins of *M. heterothallica* strains CBS 202.75 and ThNM053 are lacking the first 83 amino acids relative to mating-type A strains CBS 203.75 and ThNM146 (Supplemental Fig. S1).

The portion of *mat A-1* present in mating-type *a* strains is highly conserved in terms of nucleotide sequence, and its reading frame is preserved. The percent identity between *mat A-1* genes is 99.5% for mating-type A versus *a*, 99.7% for the two mating-type A strains, and 100%

for the two mating-type *a* strains (Supplemental Table S1, Fig. 5). Moreover, one of the few positions of divergence between *A* and *a* strains includes an in-frame start codon at the beginning of the *mat A-1* homolog in mating-type *a*. This methionine codon is not present in the corresponding position in *mat A-1* genes from mating-type *A* strains, suggesting selection toward a functional start codon in mating-type *a* (Supplemental Fig. S1).

We also observed the close linkage of a truncated *mat A-1* and *mat a-1* in searches conducted against genomic sequences of *M. fergusii* strain CBS 405.69, *M. hinnulea* strain ATCC 52474 and *Humicola hyalothermophila* strain CBS 454.80 (Fig. 5, Table 2). This strain of *M. fergusii*, which was reported to be heterothallic (von Klopotek, 1974), lacks *mat A-2* (*MAT1-1-2*) and *mat A-3* (*MAT1-1-3*), and accordingly appears to be mating type *a* (*MAT1-2*). To our knowledge, *M. hinnulea* and *H. hyalothermophila* have not been observed to reproduce sexually, but the mating gene arrangement in the strains examined is like that of *M. heterothallica* mating-type *a*.

The truncated *mat A-1* sequences from *M. fergusii*, *M. hinnulea*, *H. hyalothermophila* and *M. heterothallica* are substantially divergent from one another, in contrast with the small amount of divergence between the sequences from *a* and *A* strains of *M. heterothallica* when compared over the region of homology (Fig. 5). Given that the truncated version of *mat A-1* quite clearly existed in mating-type *a* strains prior to the divergence of species within the family, it appears that crossing over in this region has served to maintain substantial sequence similarity between the full-length and truncated *mat A-1* genes within *M. heterothallica*. This inference is supported by the fact that mating-type *A* and *a* strains possess substantial sequence identity that spans from the flanking *sla2* homolog through the portion of *mat A-1* that is shared between mating types (Fig. 4). In contrast, the intergenic region between the truncated *mat A-1* and *mat a-1* is not

conserved between mating types. This observation suggests that homology-dependent crossing over occurs in the region that includes a portion of *mat A-1* without extending past the truncated (5') end of the *mat A-1* gene (Fig. 4).

RNA-sequence analyses of *M. fergusii* (CBS 405.79), *M. hinnulea* (ATCC 52474) and *H. hyalothermophila* (CBS 454.80) revealed apparent low expression of the truncated *mat A-1* (*MAT1-1-1*) relative to *mat a-1* (*MAT1-2-1*) and the flanking genes *sla2* and *apn2* (Supplemental Table S2). We did not observe expression of the truncated *mat A-1* gene in mRNA pools from *M. heterothallica* CBS 202.75. We assume that this lack of detection derives from either low expression or transient expression during development, or both.

3.4. Mating genes and reproductive mode in the Chaetomiaceae

We employed the *mat a-1* (*MAT1-2-1*) gene from *M. heterothallica* CBS 202.75 in BLAST searches against selected homothallic members of the Chaetomiaceae and Sordariaceae to determine the value of this gene for probing the mating genetics of homothallism (Table 2, Supplemental Table S2). The identification of *mat a-1* orthologs is complicated by the fact that fungal genomes typically have paralogous genes from the HMG family, as well as by the fact that among homothallic species true *mat a-1* orthologs can occur in different chromosomal locations. Nevertheless, comparisons among results obtained from BLAST searches demonstrated that *mat a-1* orthologs can be inferred across substantial phylogenetic distances. This was observed in BLAST searches employing the *M. heterothallica mat a-1* gene to probe homothallic and heterothallic members of the genus *Neurospora*, where, as expected, much better matches were observed with mating-type *a* and homothallic strains than with mating-type *A* strains. In addition, conclusions regarding orthology were supported by bidirectional BLAST searches with putative *mat a-1* orthologs and paralogs from *M. heterothallica* and other species

(Table 2). In most instances, homothallic members of the Chaetomiaceae possessed unlinked *mat a-1* and *mat A-1* orthologs. As discussed below, we failed to find a *mat a-1* ortholog in *T. terrestris*, reported to be homothallic with cross feeding.

4. Discussion

4.1. Heterothallism in *M. heterothallica*

Given that another member of the Chaetomiaceae, *T. terrestris*, is reported to exhibit homothallism with cross-feeding (Samson et al., 1977), which at the gross level looks like heterothallism, it was important to confirm independent assortment of segregating markers for *M. heterothallica*. Recombinant progeny were successfully obtained from crosses of wild-type parental strains and from crosses where the UV mutagenized strain, CBS 202.75, was crossed to the wild-type strain CBS 203.75. Genetic markers for mating type, an actin-like gene and benomyl resistance were recombined among progeny genotypes. Reports of heterothallism are rare for the Chaetomiaceae, and *M. heterothallica* is the first heterothallic species for which sexual recombination of marker genes has been confirmed.

4.2. Temperature requirements for sexual development versus vegetative growth

Sexual development and vegetative growth in *M. heterothallica* reach optima at different temperatures, with ascocarp formation occurring optimally at a temperature 15 °C below that of the optimum vegetative growth temperature. This disconnect between the temperature optima for different stages of development has been observed previously for species of *Aspergillus* and other Ascomycota (Dyer and O’Gorman, 2012). In the case of *M. heterothallica*, one possibility is that the pathway for sexual development is evolutionarily constrained to mesophilic temperatures, for example by regulatory proteins that have not evolved tolerance to high

temperature. Alternatively, the difference in temperature optima for vegetative growth and sexual development could represent an adaptation to promote sexual reproduction under conditions suboptimal for vegetative growth, as microhabitats undergo successional or seasonal cooling and reduction in carbon substrates and nutrients. Perhaps arguing against the notion of evolutionary constraint is the fact that other members of the Chaetomiaceae that are both homothallic and thermophilic, for example *Chaetomium thermophilum*, can form ascocarps at or above 40 °C (Cooney and Emerson, 1964). Nevertheless, it is possible that an evolutionary constraint could exist for biochemical processes that are required for heterothallism but not homothallism.

4.3. Mating-type genes

Comparative analysis of the mating gene architecture in *M. heterothallica* and other closely related species in the Sordariales enabled an examination of mating systems in the group. The *mat A* strains of *M. heterothallica* show a gene arrangement that is conserved in terms of gene order and directionality with respect to other heterothallic *mat A* members of the Sordariales. In contrast, mating-type *a* strains of *M. heterothallica* and *M. fergusii* contain a portion of a *mat A-1* homolog in addition to the canonical *mat a* genes. The presence of a truncated *mat A-1* homolog in mating-type *a* (*MAT1-2*) strains has been observed previously in heterothallic Sordariomycetes belonging to the Ophiostomatales (*Grosmannia clavigera*) and the Helotiales (*Phialocephala europaea*) (Tsui et al., 2013; Duong et al., 2013; Zaffarano et al., 2010), but the origin and function of the truncated *mat A-1* gene remain unknown (discussed below).

With the possible exception of *T. terrestris* (discussed below), the homothallic Chaetomiaceae species examined here contain genes from both mating idiomorphs, *mat a*-

I and *mat A-1,2,3*. In most cases, the mating-type *a* and *A* regions appeared to be unlinked, based in part on the fact that they occur on different scaffolds associated with genome assemblies (Table 2, Supplemental Table S2). In addition, in cases where a presumed homothallic strain appeared to possess unlinked *mat A-1* and *mat a-1* genes, the *mat A-1* region contains homologs of the flanking genes *apn2* and *sla2*, whereas the *mat a-1* region does not. The one exception to this pattern, *T. australiensis*, possesses *mat a-1* and *sla2* on one genomic scaffold, with *apn2* and the *mat A-1* region on another scaffold. This leaves open the possibility that the two scaffolds represent adjacent regions and therefore that *mat a-1* is linked to the *mat A-1* region (Supplemental Table S2).

4.4. Significance of *mat A-1* genes in mating type *a* strains

Homologs of *mat A-1* (*MAT1-1-1*) genes define one mating type among diverse heterothallic members of the Ascomycota, in addition to being present in homothallic species. They are important for fertilization and sexual development (Debuchy and Turgeon, 2006; Martin et al., 2010; Saupe et al., 1996). These genes are characterized by a conserved domain, designated the alpha1 ($\alpha 1$) domain (Martin et al., 2010) or alpha box (Arie et al., 2000), present in all previously reported mating-type *A* (*MAT 1-1*) strains. In contrast, the predicted protein of the *mat A-1* homolog in mating-type *a* strains of *M. heterothallica* lacks most of this alpha1 domain region, which is known to be required for fertility in mating type *A* members of the Sordariales (Fig. 6). The alpha1 domain region is also missing from the truncated *mat A-1/MAT1-1-1* genes of mating-type *a* (*MAT1-2*) strains of *P. europaea* (Helotiales) and *G. clavigera* (Ophiostomatales) (Fig. 6, Supplemental Fig. S2). The truncated *mat A-1* predicted protein in mating-type *a* strains does, however, possess regions of unknown function that are conserved in *mat A-1/MAT1-1-1* encoded proteins but are downstream of the alpha1 domain and

downstream of the conserved region previously correlated with fertility (Fig. 6). The gene structures and sequence divergence among the *mat A-1* genes in the Chaetomiaceae suggest that the divergence between the mating type *a* and *A* forms of this gene occurred in the ancestors of extant lineages (Fig. 5, Supplemental Fig. S1).

Although it is difficult to speculate on the role of this *mat A-1* gene in *a* mating-type strains, taken together, the conserved gene organization, the high level of sequence conservation across all *a* and *A* *M. heterothallica* strains, the conserved reading frame, and the derived start codon (Supplemental Fig. S1 and Supplemental Table S1) strongly suggest that the truncated *mat A-1* gene in mating type *a* has some as yet undetermined function. The presence of the truncated version of *mat A-1* in the presumed mating-type *a* strains of other species and other sordariomycete orders provides additional support for the ancestral nature of the arrangement in the Chaetomiaceae (Fig. 5). In addition, because phylogenetic analysis of the elongation factor gene *EF1A* further suggests that *M. fergusii* is outside the clade that contains *M. heterothallica* and the homothallic species *M. sepedonium*, as well as other species of uncertain life cycle (van den Brink, 2012), it would appear that the reproductive mode possessed by *M. heterothallica* and *M. fergusii* is not a recent derivation of heterothallism from a homothallic ancestor.

Although there is substantial divergence between the truncated *mat A-1* homologs identified from the Chaetomiaceae, Ophiostomatales and Helotiales, all are truncated for a majority of the alpha1 domain. In *Neurospora crassa*, sequences in the region of the alpha1 domain are responsible for both heterokaryon incompatibility and mating. The presence of this region in mating-type *a* strains has been shown to produce an incompatibility reaction (Saupe et al. 1996). In addition, physiological and developmental processes associated with mating, for example reciprocal mating pheromone production and recognition, require complementary

controls by *mat A-1* and *mat a-1* genes (Kim et al. 2012). As a result, the presence of the *mat A-1* alpha1 domain in mating-type *a* would have the potential to disrupt both mating and development.

4.5. Mating genes in homothallic *Chaetomiaceae* species

The *M. heterothallica* mating genes, and in particular the *mat a-1* gene, together with genomic sequences from members of the *Chaetomiaceae*, provide a mechanism for probing reproductive systems. In the genus *Neurospora* and other Ascomycota, the evolution of homothallism from heterothallism has typically included genome rearrangements that result in the *mat A-1* region (*mat A-1*, *A-2* and *A-3*) being included in the same genome with *mat a-1*. In some instances, *mat a-1* is adjacent to the *mat A-1* region, while in others *mat a-1* is located elsewhere in the genome (Yun et al., 1999; Gioti et al., 2012). Our analyses demonstrated that the homothallic species *Chaetomium globosum*, *C. thermophilum*, *T. hyrcaniae* and *T. appendiculata* possess *mat a-1* genes not linked to the *mat A-1* region; and as expected, heterothallic mating-type *A* strains do not possess *mat a-1* (Table 2). Interestingly, the isolate assigned to *T. terrestris*, a thermophilic member of the *Chaetomiaceae* for which a complete genome sequence has been reported (Berka et al., 2011), does not contain a *mat a-1* ortholog. This species is reported to be “homothallic with cross feeding” (Samson et al., 1977), although sexual reproduction has apparently not been observed for the isolate in question (NRRL 8126). If this isolate is indeed homothallic, the lack of a *mat a-1* gene suggests that “cross feeding” replaces the need for *mat a-1*. There is precedent for homothallic strains possessing a single mating-type gene (for example *Huntiella moniliformis*; Wilson et al., 2015). Alternatively, it is possible that the isolate of *T. terrestris* examined here is in fact heterothallic.

4.6. Potential of *M. heterothallica* as a model organism

M. heterothallica possesses several traits that make it a viable candidate as a model organism. Cultures can be grown rapidly and maintained with little effort. All strains investigated here produce abundant conidia, facilitating mutagenesis, transformation and other experimental procedures where large numbers of conidia are desirable. Crosses result in mature ascocarps within a month and these are easily dissected from crossing plates. On the negative side, ascocarps lack a mechanism to forcibly eject ascospores, which complicates the separation of ascospores from conidia toward the goal of obtaining progeny. In addition, the germination of ascospores to date has been quite variable, and further investigation will be needed to determine the conditions needed to reliably induce ascospore germination.

Crossing experiments revealed that *M. heterothallica* isolates are sexually incompatible with isolates of *M. thermophila*. Combined with phylogenetic studies (van den Brink et al., 2012) this result supports the conclusion that isolates of *M. heterothallica* represent a distinct species, not the teleomorphic form of *M. thermophila* as suggested by von Klopotek (1976). The ThNM strains from New Mexico are sexually compatible with the CBS 202.75 and 203.75 *M. heterothallica*, are very closely related (>99% similarity for sequences examined to date), and in all likelihood are the same species. Moreover, *M. heterothallica* has also been identified from other geographically distant locations, including Senegal, Canada and Sweden, and this broad distribution of isolates provides a potential reservoir for ecologically diverse genes that may be of industrial interest. “Reverse ecology” approaches as have been used in *Neurospora crassa* (Ellison et al., 2011) may be useful to identify functional genes such as those that regulate thermophily.

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Appendix A. Supplementary material

Supplementary data for this article can be found at: [add]

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Figure legends

Fig. 1. Colony appearance, ascocarp morphology, and spore germination in *M. heterothallica*.

A. Most strains of *M. heterothallica* produce abundant conidia on agar medium, as shown here for ThNM146. B. Ascocarps (arrow) on agar media are non-ostiolate and are frequently embedded in the medium. On crossing plates (insert), ascocarp development typically begins where strains meet at the center (note darkly pigmented areas), followed by ascocarps forming more broadly across the plate. C. Ascospore (arrow) germination. D. Conidium (arrow) germination.

Fig. 2. Different developmental stages of *M. heterothallica* have distinct temperature optima.

Ascocarp development is optimal near 30 °C, whereas mycelial growth is optimal near 45 °C. Results are shown for New Mexico strains ThNM053 and ThNM146. Experiments with CBS strains 203.75 and 202.75 produced results similar to those presented here and as first reported by von Klopotek (1976). Vertical bars represent the standard error of the mean for three replicates.

Fig. 3. Relationships among *M. heterothallica* isolates and related fungi determined by rRNA internal transcribed spacer (ITS) regions. CBS strains 203.75 and 202.75 were isolated from soils in Indiana and Germany, respectively. The other *M. heterothallica* (interfertile strains) were isolated from biological soil crusts from the Sevilleta National Wildlife Refuge in central New Mexico. Crossing behavior and relationships support the conclusion that the interfertile strains belong to a distinct species with a broad distribution. Parsimony analysis (*dnapars*, Felsenstein 1989) produced the single tree shown. Branch lengths indicate relative genetic distances. The percentage of trees supported in bootstrap analyses (1000 replicates) is shown for each node supported in 70% or more replicates.

Fig. 4. Mating-type regions in *M. heterothallica*. Mating gene arrangements are conserved across *M. heterothallica* and *Neurospora crassa* with the exception that mating-type *a* strains of *M. heterothallica* strains possess a truncated version of *mat A-1* not present in *a* strains in other heterothallic Sordariales. The genes coded in blue, *sla2* and *apn2*, are non-mating-type genes that are present in both mating types and conserved across the order. Gene spacings are to scale (bar = 1000 base pairs). For CBS strains 203.75 and 202.75, the two flanking intergenic regions, *sla2* to *mat A-1* and *mat A-3/a-1* to *apn2*, shared high levels of identity (99% and 98%,

respectively). This high level of identity included the full length of the left flank (*sla2* through the truncated *mat A-1* gene, but not the intergenic sequence 5' of the latter) and the right flank beginning approximately 50 base pairs downstream (3') of the *mat A-3* and *mat a-1* genes and continuing through *apn2*. None of the intergenic regions between the mating-type genes exhibited clear identity within or across strains. We note that mating-type *a* (*MAT1-2*) strains from the other Chaetomiaceae species examined here had identical gene arrangements and nearly identical gene spacings to those shown here for *M. heterothallica* CBS 202.75. These included *M. fergusii* (CBS 405.69), *M. hinnulea* (ATCC 52474) and *H. hyalothermophila* (CBS 454.80). Pöggeler, S. and U. Kück identified an open reading frame in the *mat a-1* region of *N. crassa* and designated the gene *mat a-2*. We have not detected a region with homology to the *mat a-2* gene in members of the Chaetomiaceae.

Fig. 5. Maximum parsimony tree depicting relationships among homologous regions from *mat A-1* genes from mating-type *A* and *a* strains. The tree was created using an alignment that excluded the 5' region found only in mating type *A* strains (see Supplemental Fig. S1). There was only one most parsimonious tree, and this topology was supported in 95% of the trees generated in a 1000-replicate bootstrap analysis. The letters following strain numbers indicate *A* and *a* mating types. The presence of the truncated *mat A-1* gene in *H. hyalothermophila* indicates that the presence of this gene in mating-type *a* strains is ancestral to *M. heterothallica*. The close relationships among *mat A-1* genes from certain *A* and *a* strains of *M. heterothallica*, therefore suggests concerted evolution in *mat A-1* from *A* and *a* mating-type lineages. The tree was constructed using *dnaphars* as described in the Fig. 3 legend.

713 Fig. 6. Conserved regions in *mat A-1* (*MAT1-1-1*) homologs relative to the truncated *mat A-1*
 714 gene of the mating *a* strain *M. heterothallica* CBS 202.75. The *mat A-1* genes from CBS 202.75
 715 (putative start shown with red arrow, *Mh*) and other *a* strains in the Chaetomiaceae lack the
 716 alpha1 domain (Arie et al. 2000), which includes a proposed HMG domain (Martin et al., 2010)
 717 and the region tentatively reported to be necessary and sufficient for female fertility in *N. crassa*
 718 (Saupe et al., 1996). The truncated *mat A-1* in mating *a* strains does, however, possess a
 719 previously recognized conserved region downstream of the alpha1 domain, which is present in
 720 diverse filamentous Ascomycota (Martin et al., 2010) and possesses residues required for male
 721 fertility (Saupe et al., 1996). Similar truncations observed in *mat A-1* homologs present in
 722 mating type *a* (*MAT1-2*) strains of *P. europaea* and *G. clavigera* are also indicated with red
 723 arrows (*Pe* and *Gc*). The WebLogo figure was drawn using an alignment that included predicted
 724 *mat A-1* proteins from *M. heterothallica* strains and other Ascomycota chosen to represent
 725 diversity across the phylum, which included *N. crassa*, *Cochliobolus heterostrophus*,
 726 *Magnaporthe grisea*, *Phialocephala europaea*, *Grosmannia clavigera* and *Saccharomyces*
 727 *cerevisiae*. Alignment details and GenBank accession numbers are presented in Supplemental
 728 Fig. S2.

Table 1. Segregation of markers in *M. heterothallica* crosses

Marker	Mat A (<i>MAT1-1</i>)	Mat a (<i>MAT1-2</i>)
Progeny scored for actin polymorphism (from CBS 203.75 x CBS 202.75)		
Actin morph 203.75	4	4*
Actin morph 202.75	8*	8
Progeny scored for benomyl resistance (from CBS 203.75 x 202.75 ^{ben})		
Benomyl sensitive	3	8*
Benomyl resistant	3*	8

*Progeny with non-parental genotypes

Table 2. Reproductive modes among Chaetomiaceae and Sordariaceae and the status of homologs of *M. heterothallica* *mat a-1* (*MAT1-2-1*)

Species (strain) ^a	Reproduction	Best <i>mat a-1</i> BLASTp hit ^b	Comments ^c
Chaetomiaceae			
<i>M. heterothallica</i> (CBS 203.75)	Heterothallic <i>mat A</i>	e-value=6e-13 JGI Myche1 744144	HMG homolog not in <i>mat A</i> region. No true <i>mat a-1</i> ortholog. Intact <i>mat A-1,2,3</i> region
<i>M. heterothallica</i> (CBS 202.75)	Heterothallic <i>mat a</i>	e-value=0.0 Genbank: KR119055 <i>mat a-1</i>	<i>mat a-1</i> ortholog adjacent to 5' truncated <i>mat A-1</i> with no <i>mat A-2</i> or <i>mat A-3</i>
<i>M. fergusii</i> (CBS 405.69)	Reported to be heterothallic ^d presumed <i>mat a</i>	e-value=4e-136 Genozymes: Corth2p4_004280	<i>mat a-1</i> ortholog (Genbank: KR261943) adjacent to 5' truncated <i>mat A-1</i> with no <i>mat A-2</i> or <i>mat A-3</i>
<i>M. hinnulea</i> (ATCC 52474)	Unknown, but has <i>M. heterothallica</i> <i>mat a</i> gene arrangement	e-value=0.0 Genbank: KR261944 <i>mat a-1</i>	<i>mat a-1</i> ortholog adjacent to 5' truncated <i>mat A-1</i> with no <i>mat A-2</i> or <i>mat A-3</i>
<i>M. thermophila</i> (ATCC 42464)	Sexual reproduction not observed	e-value=2e-13 JGI Spoth2 2130558	Intact <i>mat A-1,2,3</i> region with no true <i>mat a-1</i> ortholog
<i>M. sepedonium</i> (ATCC 9787)	Reported to be homothallic ^e	e-value=5e-44 Genozymes: Corse1p7_009534	Intact <i>mat A-1,2,3</i> region with <i>mat a-1</i> ortholog not in <i>mat A</i> region
<i>Chaetomium globosum</i> (CBS 148.51)	Homothallic	e-value=4e-172 Genbank: XP_001230096 Hypothetical protein	Intact <i>mat A-1,2,3</i> region with <i>mat a-1</i> ortholog not in <i>mat A</i> region ^f
<i>C. thermophilum</i> (DSM 1495)	Homothallic	e-value=3e-100 Genbank: XP_006690660 Hypothetical protein CTHT_0001070	Intact <i>mat A-1,2,3</i> region with <i>mat a-1</i> ortholog not in <i>mat A</i> region
<i>Thielavia terrestris</i> (NRRL 8126)	Unknown ^g	e-value=2e-08 Genbank: XP_003651350 Hypothetical protein	Intact <i>mat A-1,2,3</i> region. No true <i>mat a-1</i> ortholog
<i>T. hyrcaniae</i> (CBS 757.83)	Homothallic	e-value=2.36e-132 JGI Thihy1 489377	Intact <i>mat A-1,2,3</i> region with <i>mat a-1</i> ortholog not in <i>mat A</i> region
<i>T. appendiculata</i> (CBS 731.68)	Homothallic	e-value=9.2e-137 JGI Thiap1 591717	Intact <i>mat A-1,2,3</i> region with <i>mat a-1</i> ortholog not in <i>mat A</i> region
<i>T. australiensis</i> (ATCC 28236)	Homothallic	e-value=1e-107 Genozymes: Thiau2p7_024287	Intact <i>mat A-1,2,3</i> region with <i>mat a-1</i> ortholog not in <i>mat A</i> region

<i>Mycothermus thermophilus</i> (CBS 625.91)	Sexual reproduction not observed	e-value=9e-14 Genozymes: Scyth2p4_008521	Intact <i>mat A-1,2,3</i> region with no true <i>mat a-1</i> ortholog
Sordariaceae			
<i>Neurospora crassa</i> (FGSC 4200)	Heterothallic <i>mat a</i>	e-value=2e-44 Genbank: P36981 <i>mat a-1</i> (defining record)	<i>mat a-1</i> with no <i>mat A-1, 2, 3</i>
<i>N. crassa</i> (FGSC 2489)	Heterothallic <i>mat A</i>	e-value=8e-12 Genbank: XP_956370 Hypothetical protein	HMG homolog not in <i>mat A</i> region. No true <i>mat a-1</i> ortholog
<i>N. pannonica</i> (FGSC 7221)	Homothallic	e-value=4e-46 Genbank: CCD57792 <i>mat a-1</i>	<i>mat a-1</i> and <i>mat A</i> adjacent on same chromosome ^h
<i>N. sublineolata</i> (FGSC 5508)	Homothallic	e-value=2e-47 Genbank: CCD57795 <i>mat a-1</i>	<i>mat a-1</i> not linked to <i>mat A</i> region ^h

^aWe note that phylogenetic frameworks for most of the species and strains referenced here have been reported previously (Morgenstern et al., 2012; van den Brink et al., 2012; Natvig et al., 2015).

^bBLASTp searches were performed at Genbank, the US DOE Joint Genome Institute (<http://jgi.doe.gov/fungi>), or at the Genozymes website supported by Genome Canada (<http://blast.fungalgenomics.ca>) as indicated. Designations in this column for genomes available at JGI and Genozymes websites, for example Myche1 and Corth2, indicate the names assigned to these genomes and the assembly versions.

^cConclusions regarding *mat a-1* orthology and non-orthology were further supported by bidirectional BLAST searches employing the current assembly of the *M. heterothallica* CBS 202.75 genome (<http://blast.fungalgenomics.ca>). In each instance, the best BLAST hit for a putative *mat a-1* ortholog from another species was to the *mat a-1* gene located on scaffold_21 of the *M. heterothallica* CBS 202.75 genome, regardless of whether this gene was contained within

the canonical mating region. On the other hand, all of the HMG homologs referenced in this table that were deemed not to be *mat a-1* produced best hits to a gene on scaffold_146.

^dvon Klopotek (1974)

^eEmmons (1932)

^fAlso reported by Debuchy and Turgeon (2006) based on comparison with *Podospora anserina*.

^gStrain whose genome sequence was reported in Berka et al. (2011). Although *T. terrestris* is reported to be homothallic (Samson et al. 1977), this strain appears not to make ascocarps and possibly is heterothallic.

^hGioti et al. (2012)

Figure 1

FIGURES

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6