Fungi in a hot, dry, changing world

Miriam I. Hutchinson

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FUNGI IN A HOT, DRY, CHANGING WORLD

BY

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B.S., Biology, University of New Mexico, 2011
M.S., Biology, University of New Mexico, 2017

Ph.D. DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Biology

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With gratitude to my family and friends for their unending support, especially to my grandfathers Charles and Karl-Theodor for paving the way for me in the sciences. This also could not have been possible without my mentors, first Dr. Andrea Porras-Alfaro while I was an undergraduate and Dr. Don Natvig during my time as a laboratory technician and graduate student. I would also like to thank my fellow graduate students: Aaron Robinson, Renée Brown, Noelle Martinez, Rae DeVan and Nicole Caimi for their advice with all things computer— I could not have done this without you.
FUNGI IN A HOT, DRY, CHANGING WORLD

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ABSTRACT

My doctoral work focused on understanding the reciprocal relationship between fungi and their environment, namely how fungi respond to environmental flux, as well as how fungi can modify and structure their habitats, especially in the context of climate change. As such, I aimed my research on fungi with distinct adaptations to their environmental niches: endophytic fungi that inhabit plant tissue and thermophilic fungi that are capable of growing at the upper temperature limit for eukaryotic life. My research consisted of three studies. First, I investigated the thermophilic species *Myceliophthora heterothallica* to demonstrate its use as a model organism for efficient cellulose decomposition by identifying its optimal growth and reproductive conditions, as well as the genes involved in mating. In experiments, *M. heterothallica* proved to be a tractable organism for genetic manipulation as it was easily grown and successfully mutagenized, and strains could be readily crossed. Sequencing of genomes for several strains of *M. heterothallica* as well as related species led to the discovery that the structure of the mating-type region of heterothallic (outcrossing) species is atypical when compared to related groups in the same order. Although thermophilic fungi are well understood in applied science, their ecology is still unclear. Therefore, in a second study I surveyed a transect in western North America, spanning from Mexico in Canada, in attempts to define the biogeography of fungal thermophiles. Using culture-based methods, I found that thermophilic fungi can be isolated from a variety of substrates and from diverse habitats. Similar to results from previous studies, there was little specificity to their distribution, but the frequency of recovery showed a reciprocal relationship with latitude. For my final study, I conducted a survey on a more local scale
at the Sevilleta National Wildlife Refuge. I collected roots from creosote (*Larrea tridentata*) and black grama (*Bouteloua eriopoda*) in order to assess the composition of root endophyte populations in these dominant desert plants and to understand what impact shrub encroachment may have on the abundance of different fungi. Using next-generation sequencing, I characterized the communities of root-associated fungi of these plants within a shrub encroachment zone where creosote is expanding into black grama grassland. Fungal root communities were shaped by the host plant as well as the year of collection. The most abundant members of the community included unclassified fungi related to common pathogens, dark-septate endophytes and, notably, a thermophilic species.
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INTRODUCTION

The fungal kingdom is a diverse lineage of organisms that occupy a wide variety of ecological niches, although all are heterotrophic. Some fungi are saprotrophs and decompose organic material for nutrition. Others are parasitic and acquire nutrition from a host. Finally, some fungi are known to form mutualisms with other organisms, most notably as lichen mycobionts, as the fungal associates in mycorrhizae and as endophytes. Moreover, fungi also possess adaptations to a spectrum of ecological factors such as salinity, pH and temperature. The aim of my doctoral research is to investigate the responses of fungi to fluctuating environments, and in turn, how fungi can structure and alter their environments. In addition, I am interested in the interplay between fungi and climate change. To address these objectives, I have studied fungal taxa that fit into two different functional categories: those that are adapted to thrive at high temperatures as thermophiles, those that form endophytic relationships with plants, as well as fungi at the intersection of these groups.

Endophytic and thermophilic fungi are of interest for several ecological and evolutionary reasons. For instance, fungal endophytes are well-known to aide their host plants in the acquisition of resources and to confer tolerance to environmental stresses such as drought, ultraviolet radiation, herbivory and temperature (Porras-Alfarò and Bayman 2011). Moreover, the presence of endophytic fungi in plant tissue may alter the decomposition of plant matter, competition among host plants, and the succession of plant species (Lemons et al. 2005; Clay and Hollah 1999). Every plant species examined to date appears to harbor fungal endophytes and these fungi represent several diverse lineages in the Ascomycota, Basidiomycota and Zygomycota (Porras-Alfarò and Bayman 2011).

Thermophilic fungi, on the other hand, are restricted to the orders Sordariales, Eurotiales, and Onygenales in the Ascomycota and the Mucorales in the Zygomycota (Morgenstern et al.
These fungi are the only eukaryotes demonstrated to grow at temperatures up to 60°C (Tansey and Brock 1978). Previous studies report thermophiles from such diverse materials as animal nests, mushroom compost and hay bales, all of which are insulated and therefore self-heating, and which exhibit ideal temperature ranges for thermophile growth (Fergus and Sinden 1969; Tansey 1971, 1973, 1977). To date, however, no thermophilic fungi have been isolated from within living plant tissue.

The aim of my graduate research is to investigate the links between these fungal groups and climate change. To this end, I have carried out three studies for my doctorate dissertation research. One, I have evaluated the species Myceliophthora heterothallica for its potential as a model organism for thermophilic fungi by elucidating its reproductive genetics and growth characteristics. Next, I have conducted a survey for thermophilic fungi to clarify their biogeography in Western North America. Finally, I surveyed the roots of both creosote (Larrea tridentata) and black grama (Bouteloua eriopoda) for endophytes to determine how the populations of root fungi can vary by host plant and how they may shape the distributions of these plants.

References


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 (MAT1-1,2,3) region in addition to an unlinked mat a-1 (MAT1-2-1) gene, reflecting one type of homothallism commonly observed in diverse Ascomycota.

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 (e.g. US Patents 4081328, 5602004 and 569598; Canadian Patent Application 1075181 A1, and PCT Patent Applications WO2014060379 A1, WO2013029170 A1 and WO2014059541 A1), and the sequenc- ing 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 prom- ise 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 con- tamination in large-scale fermentation (Beckner et al., 2011; Rubin, 2008; Visser et al., 2011).

Although members of the Chaetomiaceae have long been known for the ability to degrade celluloxic 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 Thie- lavia 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 analy- sis (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 ther- mophiles in general. We confirmed sexual recombination by demonstrating the independent assortment of specific genetic markers, and we have characterized the growth conditions neces- sary 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 compati- bility, 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 sys- tems in other members of the Sordariales and those of homothal- lic members of the family.

2. Materials and methods

2.1. Mating gene nomenclature

While we have a preference for the mating-type gene nomen- clature employed for species of Neurospora (Perkins, 1999), we acknowledge the wide use of the nomenclature recommended by Turgon 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-I-1 using the Turgon and Yoder system. In part, our use of the Neurospora nomen- clature 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 devel- oped 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 Schimmelm- cultures, 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 Gen- Bank 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-fun- galgenomics.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, 1.4 M 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) amplifi- cations were conducted in 13.5 µL reactions with 6.5 µL Premix ExTaq polymerase (Takara, Mountain View, California), 1 µL of each (5 µM) primer, 2 µL of 2% bovine serum albumin, 2 µL of milliQ water and 1 µL of template DNA. PCR products were purified enzymi- cally with ExoSAP-IT (Affymetrix, Santa Clara, California) according to the manufacturer’s recommendations. Sequences were obtained by Sanger sequencing using 10 µL reactions with 0.5 µL BigDye Terminator v3.1, 2 µL of 5× Sequencing Buffer (Life Technologies/Applied Biosystems, Carlsbad, California), 1 µL of 3 µM concentrations of the same primers used for PCR (1 primer per reaction), 5.5 µL of milliQ water and 1 µL of template DNA.

2.4. Genetic analysis

Genetic crosses were performed by inoculating strains of differ- ent 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 dis- necing needle, then rinsed three times with sterile water to remove adhering conidia and mycelial fragments. To release ascosp- ores, 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 incu- bated at 45–50 °C for 12–18 h. Using light microscopy, germlings that were confirmed to derive from ascospores (Fig. 1) were indi- vidually 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(sh)) 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, \(5\text{C-88F}^+\) (5'-CAGGTSATCACCATYGGMAAYGARCG-3') and \(5\text{C-88F}^-\) (5’-CCTSCTTSGASRTCCA CATCTGCTG-3’), originally used to identify the \(\text{Act88F}\) actin gene in \(Drosophila\) melanogaster (Lovato et al., 2001). The resulting sequences were then used to design internal primers specific to \(M.\) heterothallica: \(\text{ACTFWD}\) (5’-TATCCACGTCACCACCTTCA-3’) and \(\text{ACTREV}\) (5’-GATCCAGAGACCGAGTACTTGC-3’).

Benomyl (DuPont, Wilmington, Delaware) resistant strains, presumably carrying a mutation in the \(\beta\)-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 s, 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 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 µL of spore suspension and were incubated at 25, 37, 45, 50, and 55 °C.

To determine optimal crossing temperature, crossing plates were incubated at 25, 29, 33, 37 and 41 °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 1 h, placed overnight at −80 °C, plated and incubated at 45 °C. Subsequent experiments showed that the −80 °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 °C. None of
these treatments resulted in a substantial increase in germination rates, which at 45 °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 PRJNA327775) and N. tetrasperma (mat a; FGSC 2509; Genbank AF010000000) were queried by BLAST analysis (Joint Genome Institute) to identify homologs of slat2 (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 (Giotti 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 slat2 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) (Zerbino and Birney, 2008).

Mating gene annotations were performed using alignments with related genera 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 Locus12875v1rpkm1.66 (mat A-1), Locus12875v1rpkm1.82 (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. hinnulae 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 10⁷ spores/ml were inoculated in 10× 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 21–26 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 45480) was grown on three additional carbon sources (all at 2%): avicel, beechnut xylan and citrus pectin (all from Sigma-Aldrich). RNA was sequenced using the mRNA-seq method of Illumina’s Solexa IG at the McGill University-Génôme 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), KR19055 (CBS202.75, complete mating region), KR19057 (ThNM146, complete mating region), KR19058 (ThNM053, complete mating region), KR632512 (CBS 663.74, truncated mat A-1), KR632510 (CBS 131.65, mat A-1), KR632511 (CBS 375.69, mat A-1). Entries for other species are KR261945 (M. fergusii CBS 405.69, truncated mat A-1), KR261946 (M. hinnulae ATCC 52474, truncated mat A-1), KR632513 (H. hyalothermophila CBS 454.80, truncated mat A-1), M33876 (N. crassa OR74A) and JTEW00000000 (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 dnaps 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 non-parental genotypes, indicated by the presence of a mating type idiomorph of one parent and an actin-like polymorphism of 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) 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).

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

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mat A (MAT1-1)</th>
<th>Mat a (MAT1-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progeny scored for actin polymorphism (from CBS 203.75 x CBS 202.75)</td>
<td>4</td>
<td>4*</td>
</tr>
<tr>
<td>Actin morph 203.75</td>
<td>8*</td>
<td>8</td>
</tr>
<tr>
<td>Actin morph 202.75</td>
<td>8*</td>
<td>8</td>
</tr>
<tr>
<td>Progeny scored for benomyl resistance (from CBS 203.75 x CBS 202.75)</td>
<td>3</td>
<td>8*</td>
</tr>
<tr>
<td>Benomyl sensitive</td>
<td>3</td>
<td>8*</td>
</tr>
<tr>
<td>Benomyl resistant</td>
<td>3*</td>
<td>8</td>
</tr>
</tbody>
</table>

* Progeny with non-parental genotypes.

---

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 (drapers, 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.
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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 slas2 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 (MAT1-1-2) and the flanking genes slas2 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 homothallicism (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 HMGI 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.
Table 2
Reproductive modes among Chaetomiaceae and Sordariaceae and the status of homologs of *M. heterothallica* mat a-1 (MAT1-2-1).

<table>
<thead>
<tr>
<th>Species (strain)</th>
<th>Reproduction</th>
<th>Best mat a-1 BLASTp hit</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chaetomiaceae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. heterothallica</em> (CBS 203.75)</td>
<td>Heterothallic mat A</td>
<td>e-value = 6e−13 JGI Myche1(^a)</td>
<td>HMG homolog not in mat A region. No true mat a-1 ortholog. Intact mat A-1,2,3 region</td>
</tr>
<tr>
<td><em>M. heterothallica</em> (CBS 202.75)</td>
<td>Heterothallic mat a</td>
<td>e-value = 0.0 Genbank: KR119055 mat a-1</td>
<td>mat a-1 ortholog adjacent to 5' truncated mat A-1 with no mat A-2 or mat A-3</td>
</tr>
<tr>
<td><em>M. fergusii</em> (CBS 405.69)</td>
<td>Reported to be heterothallic(^b) presumed mat a</td>
<td>e-value = 4e−136 Genozymes: Corth2p4_004280</td>
<td>mat a-1 ortholog (Genbank: KR261943) adjacent to 5' truncated mat A-1 with no mat A-2 or mat A-3</td>
</tr>
<tr>
<td><em>M. himmulae</em> (ATCC 52474)</td>
<td>Unknown, but has <em>M. heterothallica</em> mat a gene arrangement</td>
<td>e-value = 0.0 Genbank: KR261944 mat a-1</td>
<td>mat a-1 ortholog adjacent to 5' truncated mat A-1 with no mat A-2 or mat A-3</td>
</tr>
<tr>
<td><em>M. thermophila</em> (ATCC 42464)</td>
<td>Sexual reproduction not observed</td>
<td>e-value = 2e−13 JGI SpotH2_2130558</td>
<td>Intact mat A-1,2,3 region with no true mat a-1 ortholog</td>
</tr>
<tr>
<td><em>M. sepedonium</em> (ATCC 9787)</td>
<td>Reported to be homothallic(^a)</td>
<td>e-value = 5e−44 Genozymes: Corse1p7_009534</td>
<td>Intact mat A-1,2,3 region with mat a-1 ortholog not in mat A region</td>
</tr>
<tr>
<td><em>Chaetomium globosum</em> (CBS 148.51)</td>
<td>Homothallic</td>
<td>e-value = 4e−172 Genbank: XP_001230096 Hypothetical protein</td>
<td>Intact mat A-1,2,3 region with mat a-1 ortholog not in mat A region</td>
</tr>
<tr>
<td><em>C. thermophilum</em> (DSM 1495)</td>
<td>Homothallic</td>
<td>e-value = 3e−100 Genbank: XP_006690660 Hypothetical protein CTHT_0001070</td>
<td>Intact mat A-1,2,3 region with mat a-1 ortholog not in mat A region</td>
</tr>
<tr>
<td><strong>Sordariaceae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thielavia terrestris</em> (NRRL 8126)</td>
<td>Unknown(^c)</td>
<td>e-value = 2e−08 Genbank: XP_0013651350 Hypothetical protein</td>
<td>Intact mat A-1,2,3 region. No true mat a-1 ortholog</td>
</tr>
<tr>
<td><em>T. hyrcanae</em> (CBS 757.83)</td>
<td>Homothallic</td>
<td>e-value = 2.36e−132 JGI Thyl1(^d)</td>
<td>489377</td>
</tr>
<tr>
<td><em>T. appendiculata</em> (CBS 731.68)</td>
<td>Homothallic</td>
<td>e-value = 9.2e−137 JGI Thiap1(^d)</td>
<td>591717</td>
</tr>
<tr>
<td><em>T. australiensis</em> (ATCC 28236)</td>
<td>Homothallic</td>
<td>e-value = 1e−107 Genozymes: Thiau2p7_024287</td>
<td>Intact mat A-1,2,3 region with mat a-1 ortholog not in mat A region</td>
</tr>
<tr>
<td><em>Mycothermus thermophilus</em> (CBS 625.91)</td>
<td>Sexual reproduction not observed</td>
<td>e-value = 9e−14 Genozymes: Scyth2p4_008521</td>
<td>Intact mat A-1,2,3 region with no true mat a-1 ortholog</td>
</tr>
<tr>
<td><strong>Neurospora crassa</strong> (FGSC 4200)</td>
<td>Heterothallic mat a</td>
<td>e-value = 2e−44 Genbank: P36981 mat a-1 (defining record)</td>
<td>mat a-1 with no mat A-1,2,3</td>
</tr>
<tr>
<td><em>N. crassa</em> (FGSC 2489)</td>
<td>Heterothallic mat A</td>
<td>e-value = 8e−12 Genbank: XP_0305370 Hypothetical protein</td>
<td>HMG homolog not in mat A region. No true mat a-1 ortholog</td>
</tr>
<tr>
<td><em>N. pannonica</em> (FGSC 7221)</td>
<td>Homothallic</td>
<td>e-value = 4e−46 Genbank: CCD57792 mat a-1</td>
<td>mat a-1 and mat A adjacent on same chromosome(^b)</td>
</tr>
<tr>
<td><em>N. sublineolata</em> (FGSC 5508)</td>
<td>Homothallic</td>
<td>e-value = 2e−47 Genbank: CCD57795 mat a-1</td>
<td>mat a-1 not linked to mat A region(^b)</td>
</tr>
</tbody>
</table>

\(^a\) We 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; Navog et al., 2015).

\(^b\) BLASTp 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.

\(^c\) Conclusions 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.

\(^d\) von Klopotek (1974).

\(^e\) Emmons (1932).

\(^f\) Also reported by Debuchy and Turgeon (2006) based on comparison with *Podospora ameraena*.

\(^g\) Strain whose genome sequence was reported in Berk 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.

\(^h\) Gioti et al. (2012).
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 seasonal 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 homothalic 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 (*Grosmaniella 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-1 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 (x1) domain (Martin et al., 2010) or alpha box (Arie et al., 2000), present in all previously reported mating-type A (MAT1-1) 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 undefined 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 et al., 2012), it would appear that the reproductively 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 being included in the same genome with mat a-1 (Glass and Kuldau, 1992). 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. thermophilotum*, *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. terrestis, 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 Hunsillla moniliformis; Wilson et al., 2015). Alternatively, it is possible that the isolate of T. terrestis examined here is in fact heterothallic.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.11.007.

References


Acknowledgments

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We thank George Rosenberg of the UNM Department of Biology’s Molecular Biology Facility for help with sequence assembly, and we acknowledge additional technical support from this facility under National Institutes of Health grant P30GM110907.


Supplemental Table S1. Percent identities for mat A-1 homologous regions from *M. heterothallica* mating-type A (CBS203.75, ThNM146) and a (CBS202.75, ThNM053) strains. Pairwise genetic distance estimates were obtained using ClustalW2. Letters in parentheses indicate the mating types of the strains from which the mat A-1 genes were derived.

<table>
<thead>
<tr>
<th></th>
<th>203.75A1(A)</th>
<th>NM146A1(A)</th>
<th>202.75A1(a)</th>
<th>NM053A1(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBS203.75A1(A)</td>
<td>100</td>
<td>99.7</td>
<td>99.6</td>
<td>99.6</td>
</tr>
<tr>
<td>ThNM146A1(A)</td>
<td>100</td>
<td>99.3</td>
<td>99.3</td>
<td></td>
</tr>
<tr>
<td>CBS202.75A1(a)</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ThNM053A1(a)</td>
<td></td>
<td>100</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>
Supplemental Table S2. Scaffold locations for mat a-1 (MAT1-2-1), mat A-1, 2, 3 (MAT1-1-2,3, sla2 and apn2 in homothallic members of the Chaetomiaceae. BLAST searches were performed at Genbank, genome portals at the US DOE Joint Genome Institute (http://jgi.doe.gov/fungi/), or at the Genozymes website supported by Genome Canada and Génome Québec (www.fungalgenomics.ca/) as indicated.

<table>
<thead>
<tr>
<th>Species (strain)</th>
<th>Genome search location</th>
<th>Locations of mat A-1,2,3; sla2 and apn2</th>
<th>Location of mat a-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myceliophthora sepedonium (ATCC 9787)</td>
<td>Genome Canada</td>
<td>Scaffold_0065</td>
<td>Scaffold_0111</td>
</tr>
<tr>
<td>Chaetomium globosum (CBS 148.51)</td>
<td>Genbank: XP_001230096</td>
<td>Scaffold_3</td>
<td>Scaffold_2(^a)</td>
</tr>
<tr>
<td>C. thermophilum (DSM 1495)</td>
<td>Genbank: EGS23418</td>
<td>scaffold scf718000011814</td>
<td>scaffold scf718000011806</td>
</tr>
<tr>
<td>Thielavia hyrcaniae (CBS 757.83)</td>
<td>Joint Genome Institute</td>
<td>Scaffold_10</td>
<td>Scaffold_58(^b)</td>
</tr>
<tr>
<td>T. appendiculata (CBS 731.68)</td>
<td>Joint Genome Institute</td>
<td>Scaffold_4</td>
<td>Scaffold_3(^c)</td>
</tr>
<tr>
<td>T. australiensis (ATCC 28236)</td>
<td>Genome Canada</td>
<td>Scaffold_0067(^d)</td>
<td>Scaffold_0349(^d)</td>
</tr>
</tbody>
</table>

\(^a\)This scaffold possesses a sequence with homology to mat A-2 (adjacent to mat a-1) in addition to the mat A-2 gene in the mat A-1 region of Scaffold_3.

\(^b\)This scaffold possesses a copy of the apn2 gene (adjacent to mat a-1) in addition to the copy that is in the mat A-1 region of Scaffold 10.

\(^c\)This scaffold possesses a copy of the mat A-3 gene in addition to the copy in the mat A-1 region of Scaffold_4.

\(^d\)The sla2 gene is located on Scaffold_0349 along with mat a-1. In addition, the mat a-1 gene is near one end of Scaffold_0349, whereas mat A-1 is near one end of Scaffold_0067. This leaves open the possibility that the two scaffolds represent adjacent regions of the same chromosome.
Supplemental Table S3. Numbers of RNA-seq reads for mating-type genes (truncated \textit{mat A-1}, \textit{mat a-1}) and flanking genes (\textit{sla2}, \textit{apn2}) in mating-type \textit{A} (\textit{Mat1-1}) strains (see Materials and Methods for growth conditions and RNA sequencing methods). Gene sizes are shown in kilobase pairs (kb).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon Source</th>
<th>Total reads</th>
<th>\textit{sla2} \hspace{1em} (3.4 kb)</th>
<th>\textit{mat A-1}\textsuperscript{a} \hspace{1em} (MATI-1-1) \hspace{1em} (0.76 kb)</th>
<th>\textit{mat a-1}\textsuperscript{a} \hspace{1em} (MATI-2-1) \hspace{1em} (1.1 kb)</th>
<th>\textit{apn2} \hspace{1em} (2.2 kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Myceliophthora} \hspace{1em} \textit{heterothallica} \hspace{1em} CBS 202.75</td>
<td>barley/alfalfa</td>
<td>27.2 x 10^6</td>
<td>2478</td>
<td>0</td>
<td>74</td>
<td>1307</td>
</tr>
<tr>
<td>\textit{M. fergusii} \hspace{1em} CBS 454.80</td>
<td>barley/alfalfa</td>
<td>16.5 x 10^6</td>
<td>2675</td>
<td>27</td>
<td>1256</td>
<td>432</td>
</tr>
<tr>
<td>\textit{M. fergusii} \hspace{1em} CBS 454.80</td>
<td>avicel</td>
<td>6.6 x 10^6</td>
<td>1531</td>
<td>16</td>
<td>741</td>
<td>114</td>
</tr>
<tr>
<td>\textit{M. fergusii} \hspace{1em} CBS 454.80</td>
<td>xylan</td>
<td>4.8 x 10^6</td>
<td>917</td>
<td>6</td>
<td>292</td>
<td>56</td>
</tr>
<tr>
<td>\textit{M. fergusii} \hspace{1em} CBS 454.80</td>
<td>pectin</td>
<td>2.5 x 10^6</td>
<td>689</td>
<td>19</td>
<td>113</td>
<td>22</td>
</tr>
<tr>
<td>\textit{M. fergusii} \hspace{1em} CBS 454.80</td>
<td>glucose</td>
<td>16.4 x 10^6</td>
<td>5625</td>
<td>7</td>
<td>2637</td>
<td>382</td>
</tr>
<tr>
<td>\textit{M. hinnulea} \hspace{1em} ATCC 52474</td>
<td>barley/alfalfa</td>
<td>86.8 x 10^6</td>
<td>38304</td>
<td>3</td>
<td>212</td>
<td>4272</td>
</tr>
<tr>
<td>\textit{Humicola} \hspace{1em} \textit{hyalothermophila} \hspace{1em} CBS 454.80</td>
<td>barley/alfalfa</td>
<td>47.1 x 10^6</td>
<td>20640</td>
<td>4</td>
<td>1153</td>
<td>1336</td>
</tr>
</tbody>
</table>

\textsuperscript{a}RNA-seq reads obtained for \textit{mat a-1} lacked intron sequences present in the gene, indicating that the reads were derived from mRNA. The results for truncated \textit{mat A-1} genes suggest low levels of transcription but were less definitive. Unlike the other species examined, the \textit{M. fergusii} \textit{mat A-1} lacks an intron at the 3' end (the sole intron in truncated versions of the gene). \textit{M. hinnulea} possesses the intron, but the RNA-seq reads observed did not span this region of the gene. The RNA-seq reads obtained for \textit{H. hyalothermophila} included sequences present in the putative intron. However, although this putative intron in \textit{H. hyalothermophila} possesses potential 5' and 3' splice sites, it is substantially shorter than the intron observed for strains of \textit{M. heterothallica} (an intron confirmed by RNA-seq for \textit{mat A} strain CBS 203.75) and possibly is no longer functional. Moreover, this putative intron from \textit{H. hyalothermophila} contains an in-frame stop codon that would shorten the protein by only two amino acids in a region that is not evolutionarily conserved.
Supplemental Figure S1. CLUSTAL 2.1 multiple sequence alignment of mat A-1 genes from *M. heterothallica* mating-type A and a strains. The two A mating-type strains (CBS203.75 and ThNM146)
possess a 5’ region corresponding to the first 83 amino acids of the encoded protein that is not shared with mating-type \( a \) strains (CBS202.75 and ThNM053). This 5’ region is present in \( mat \ A-1 \) homologs across diverse members of the Ascomycota. Start and stop codons, introns and an 18-base-pair indel present in strain ThNM146 are coded in red.
S_cerevisiae                  MFTSKPAFKIKNKASKSYRNTAVSKKLKEKRLAEEHRPSCFNIIRPLKDK
G_clavigera_SS274          ----------------------------------
G_clavigera_SS278          ----------------------------------
N_crassa                   MSGV------------------------DGIVKTF--ADLAEDDREAAMRASRMR
M_heterothallica_202.75a   MAGI-----------------------NEILKTF--EGLAEGRTRFMALSAIMRS
M_grisea                   MNAS-----------------------LSPDIARL--P------QETLT----SLLRA
C_heterostrophus           MAHA-----------------------RDPTEAIARFI--A-------TRTGQVMQLMRC
P_europaea_122.3           -------
P_europaea_136.1           -------

S_cerevisiae                  IQIPVPSSRFLNKIQIHRIASGSQNTQFRQFKTSIKSSKKYLNSFMAFR
G_clavigera_SS274          ----------------------------------
G_clavigera_SS278          ----------------------------------
N_crassa                   GTEP------------------------RQPAKKKKNFGFR
M_heterothallica_202.75a   ENQ------------------------AVAAASKKKNFGFR
M_grisea                   NDEK------------------------AVAAASKKKNFGFR
C_heterostrophus           IEKPAQAFTAALKLLVVPAVS----------------GRPATPEKALNAFVGFR
P_europaea_122.3           -------
P_europaea_136.1           -------

S_cerevisiae                  AYYSQF--GGVKQKNVLSSLLAAEWHADKMQHGHIWDYFAQYNFIPNGF
G_clavigera_SS274          ----------------------------------
G_clavigera_SS278          ----------------------------------
N_crassa                   SFYNRM------------------------VPQPAKKKKNFGFR
M_heterothallica_202.75a   AYYSSR------------------------VPQPAKKKKNFGFR
M_heterothallica_203.75A   SYYAGI------------------------VPQPAKKKKNFGFR
M_grisea                   SYYAGI------------------------AVAAASKKKNFGFR
C_heterostrophus           CYVCTIPMFSDKLSLIGNLNWADPN---KSLWSLMAKAWSTIRDQIG
P_europaea_122.3           -------
P_europaea_136.1           -------

S_cerevisiae                  FVEWLTNNYAEVRGDDGQYWEDVQVHLAL-----------------
G_clavigera_SS274          ----------------------------------
G_clavigera_SS278          ----------------------------------
N_crassa                   FVEWLTNNYAEVRGDDGQYWEDVQVHLAL-----------------
M_heterothallica_202.75a   VCPKIGIVDDERYLNLQNWTCQILTDGIAA
M_heterothallica_203.75A   KQN------------------------APLDRFLSIVCPKIGIVDDERYLQNLNWTCQLTGDIAA
M_grisea                   VCPKIGIVDDERYLQNLNWTCQLTGDIAA
C_heterostrophus           KQN------------------------APLDRFLSIVCPKIGIVDDERYLQNLNWTCQLTGDIAA
P_europaea_122.3           -------
P_europaea_136.1           -------

S_cerevisiae                  LKEYDEAMAPTVSSSIPE--------------------------LAERDGSIGERKRN
G_clavigera_SS274          ----------------------------------
G_clavigera_SS278          ----------------------------------
N_crassa                   LKEYDEAMAPTVSSSIPE--------------------------LAERDGSIGERKRN
M_heterothallica_202.75a   LQKIMR--------------------------LAERDGSIGERKRN
M_heterothallica_203.75A   LQKIMR--------------------------LAERDGSIGERKRN
M_grisea                   LQKIMR--------------------------LAERDGSIGERKRN
C_heterostrophus           LQKIMR--------------------------LAERDGSIGERKRN
P_europaea_122.3           -------
P_europaea_136.1           -------

S_cerevisiae                  LQKIMR--------------------------LAERDGSIGERKRN
G_clavigera_SS274          ----------------------------------
G_clavigera_SS278          ----------------------------------
N_crassa                   LQKIMR--------------------------LAERDGSIGERKRN
M_heterothallica_202.75a   LQKIMR--------------------------LAERDGSIGERKRN
M_heterothallica_203.75A   LQKIMR--------------------------LAERDGSIGERKRN
M_grisea                   LQKIMR--------------------------LAERDGSIGERKRN
C_heterostrophus           LQKIMR--------------------------LAERDGSIGERKRN
P_europaea_122.3           -------
P_europaea_136.1           -------
G_clavigera_SS278             AQARAVAGPLIVA---PKTKNGSGSIRKTPYTQMQAYQTSDLAGQFHV
N_crassa                     --PYNMALAIDVP--MMGFGNGA--------------------------
M_heterothallica_202.75a     KERLMMSTSMGGMGGMGGMGNTTDFN-------------------
M_heterothallica_203.75A     KERLMMSTSMGGMGGMGGMGNTTDF--DILERFRC---------
M_grisea                     --STVV----------------SFPGEHGHP-----ETQYSYP-----
C_heterostrophus             --TTLSD-----QIPTGQDAGHLNDA--HLNFDYSLP---
P_europaea_122.3             --ERFLSA-----SGAVGWKAGEGVD----------FP------
P_europaea_136.1             --ERFLSA-----SGAVGWKAGEGVD----------FP------

S_cerevisiae
G_clavigera_SS274             FSSQPSMQSSSRNDEAAAAPTDSIITMESDMGWHLHEMDMAQRAAALQP
G_clavigera_SS278             FSSQPSMQSSSRNDEAAAAPTDSIITMESDMGWHLHEMDMAQRAAALQP
N_crassa                     M_heterothallica_202.75a
M_heterothallica_202.75a     M_heterothallica_203.75A
M_grisea                     C_heterostrophus
P_europaea_122.3             P_europaea_136.1

S_cerevisiae
G_clavigera_SS274             QSHSEAGESSGNVSEATTVLAAAGEVDAMWHGHQAEDADAERVYLDHEHD
G_clavigera_SS278             QSHSEAGESSGNVSEATTVLAAAGEVDAMWHGHQAEDADAERVYLDHEHD
N_crassa                     M_heterothallica_202.75a
M_heterothallica_202.75a     M_heterothallica_203.75A
M_grisea                     C_heterostrophus
P_europaea_122.3             P_europaea_136.1

S_cerevisiae
G_clavigera_SS274             SSVGLSAPGLHFVDDLSEQSASSLGKHGRDQIEQIEVNTAGDEPS
G_clavigera_SS278             SSVGLSAPGLHFVDDLSEQSASSLGKHGRDQIEQIEVNTAGDEPS
N_crassa                     M_heterothallica_202.75a
M_heterothallica_202.75a     M_heterothallica_203.75A
M_grisea                     C_heterostrophus
P_europaea_122.3             P_europaea_136.1

S_cerevisiae
G_clavigera_SS274             KRCRLYDDLHLMGAMQQDEVSLEQLARGQRPAPFADYRSQAAAAMEG
G_clavigera_SS278             KRCRLYDDLHLMGAMQQDEVSLEQLARGQRPAPFADYRSQAAAAMEG
N_crassa                     M_heterothallica_202.75a
M_heterothallica_202.75a     M_heterothallica_203.75A
M_grisea                     C_heterostrophus
P_europaea_122.3             P_europaea_136.1
Supplemental Fig. S2. Multiple sequence alignment of mat A-1 (MAT1-1-1) predicted proteins from *M. heterothallica* mating-type A and a strains and homologs from other Ascomycota. The alignment was created using Clustal 2.1 followed by manual adjustment informed by similar alignments presented in Fig. 5 of Saupe et al. (1996) and Fig. 2 of Arie et al. (2000). Accession numbers for *M. heterothallica* strains are presented in the text. Accession numbers for other species are AAC37478 (*N. crassa*), CAA48465 (*Cochliobolus heterostrophus*), BAC65087 (*Magnaporthe grisea*), JX402934 (*Grosmannia clavigera* SS274), JX402945 (*G. clavigera* SS278), HM347274 (*Phialocephala europaea* 122.3), HM347275 (*P. europaea* 136.1) and NP_009867 (*Saccharomyces cerevisiae*).
New Perspectives on the Distribution and Roles of Thermophilic Fungi

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Abstract

Defined as fungi that grow better at 25°C than at 45°C, thermophilic fungi were discovered more than a century ago. Nevertheless, little is known about the natural roles and distribution of these organisms. Although common in “sun-heated soils” and other natural substrates they have most often been recovered from manmade composts, and one hypothesis suggests that they evolved as decomposers in natural compost. This hypothesis suggests that propagules found outside compost have been dispersed by wind, an idea that seems nearly impossible to reconcile with their high frequency and broad distribution. In this chapter we briefly review the biology, history and evolution of thermophilic fungi. We also present new results from ongoing efforts to map the range of habitats from which thermophilic fungi can be obtained. We have isolated thermophilic fungi over small and large spatial scales. Our surveys have focused on soil, litter and herbivore droppings sampled from diverse ecosystems (deserts, grasslands and forests) across eight western states, Mexico and Canada--from southern deserts to alpine ecosystems in Colorado and Montana. Our results show that thermophiles can be isolated readily from all of these substrates at nearly every latitude and elevation. We observed that the success of recovering thermophilic fungi from soil decreases with increasing latitude. During this survey we also discovered that several species of thermophilic fungi can survive storage in soil samples for several years at -80°C.

Keywords: Thermophile, Ecology, Chaetomiaceae, Eurotiales, Biogeography

1. Introduction

The goal of this chapter is two-fold. First, we briefly review the history, basic biology, evolution, and industrial relevance of thermophilic fungi. Second, we address ongoing questions concerning the ecology of these organisms. In the past two decades, several excellent reviews have
considered one or more of these topics (Maheshwari et al. 2000; Mouchacca 2000 a, b; Salar and Aneja 2007; Salar 2018). Here, we give particular attention to topics for which there has been some difference of opinion. These include a discussion of the definition of thermophilic as it pertains to fungi and an evaluation of the types of microhabitats that are most relevant to the growth and distribution of these organisms. We argue that the microenvironments capable of supporting the growth of thermophilic fungi are widespread and often transient. In the latter context, we present the results of a recent previously unpublished survey of thermophilic fungi in diverse ecosystems of the western United States, Mexico and Canada.

**Definition.** While thermophilic fungi do not grow at the extreme temperatures that are optimal for many thermophilic bacteria and archaea, they are the only eukaryotes demonstrated to grow at temperatures up to 60°C (Tansey and Brock 1978). In practice, the term thermophilic, when applied to fungi, has sometimes been used quite loosely, and there is no universally-accepted definition. Cooney and Emerson (1964), who wrote the first monograph for thermophilic fungi, considered such fungi to be those that have “a maximum temperature for growth at or above 50°C and a minimum temperature for growth at or above 20°C.” We have adopted a simpler working definition (Powell et al. 2012, Hutchinson et al. 2015). Namely, we consider a thermophilic fungus to be one that grows better at 45°C than at 25°C. One practical advantage of this latter definition is that it permits easy evaluation of fungal isolates.

Less consistent in the literature is the distinction between thermotolerance and thermophily. Cooney and Emerson considered thermotolerant fungi to be those with a maximum growth temperature near 50°C while having a minimum growth temperature “well below” 20°C. This definition is quite restrictive on the high end. Although it permits inclusion of the ubiquitous
Aspergillus fumigatus, it excludes many fungi, for example the model organism Neurospora crassa, that can grow at temperatures near or above 45°C while having temperature optima below 50°C. From a practical point of view, 45°C is a temperature that is lethal or stress-inducing for most organisms, and we consider fungi that can grow at 45°C to be thermotolerant.

History. The first reported thermophilic fungus, Rhizomucor pusillus, was isolated from bread by Lindt in the 1880s (Lindt 1886). Later, Tiklinskaya (1899) identified another thermophile, Thermomyces lanuginosus, growing on potatoes. In the early 1900s, Hugo Miehe (1907, 1930a, b) published a series of papers derived from his investigations regarding the role of living organisms in the self-heating of stored hay. One result was the description of two new thermophiles, Thermoidium sulfureum (Malbranchea cinnamomea), and Thermoascus aurantiacus.

The study of these organisms languished for several decades before they were discovered to be part of the composting process associated with the production of rubber from the desert shrub Guayule (Parthenium argentatum). During World War II, the United States and allies lost access to rubber-plant plantations in the Pacific, which hindered the manufacture of rubber badly-needed for the war effort. The US Department of Agriculture had a large-scale program aimed at developing the Guayule latex as an alternative source of rubber. One of the experimental approaches involved chopping the shrub into pieces and composting it in piles. These “rets” were strongly thermogenic as a result of microbial activity, and the characterization of the organisms involved led to the identification of new and previously-recognized thermophilic fungi (Cooney and Emerson 1964).

The single publication by Allen and Emerson (1949) that resulted from the study of the effects of microbial activity on rubber quality did not detail the organisms involved in the process. The importance of the guayule project in the “rediscovery” of thermophilic fungi as the basis for the
studies that led to the Cooney and Emerson (1964) monograph of thermophilic fungi was recounted in the latter.

*Industry.* In recent decades much of the attention given to thermophilic fungi has been in industry. This interest stems in large part from the ability of these fungi to yield thermostable enzymes, especially those that are cellulose-active. These enzymes function at temperatures high enough to exclude contaminants, and they accelerate reactions that convert cellulose into fermentable sugars for bio-ethanol (Beckner et al. 2011; Rubin 2008; van den Brink et al. 2013). To understand the genetic mechanisms of thermophily and thermostability, the genomes of several fungal thermophiles have been sequenced (Berka et al. 2012).

2. Evolution

Of the more than 100 thousand described species of fungi, only approximately 50 species are thermophilic, representing a small fraction of the 2.2 to 3.8 million estimated fungal species (Salar and Aneja 2007; Hawksworth and Lücking 2017). Thermophilic fungi are known from two phyla, the Ascomycota and the Mucoromycota. In the Ascomycota, thermophiles are restricted to the orders Sordariales, Eurotiales, and Onygenales. Thermophiles in the Mucoromycota occur in the Mucorales (Salar 2018) and a recently created order, the Calcarisporiellales (Hirose et al. 2012; Morgenstern et al. 2012; Tedersoo et al. 2018). The order Mucorales contains two families with thermophiles, the Rhizopodaceae and the Lichtheimiaceae (Hoffman et al. 2013). The Calcarisporiellales contains the thermophilic species *Calcarisporiella thermophile*. In the Sordariales, all known thermophilic species belong to the family Chaetomiaceae, which contains the greatest diversity of thermophilic fungi (Morgenstern et al. 2012). Among the Eurotiales, two families are
considered to possess thermophilic members, the Trichocomaceae and the Thermoascaceae (Houbraken et al. 2014; Houbraken et al. 2016). A sole species of thermophilic fungus, Malbranchea cinnamomea, is found in the Onygenales (Morgenstern et al. 2012). Thermophilic Basidiomycota have been described by Straatsma et al (1994) and Fergus (1971) but these species have either not been confirmed to be thermophilic or, as in the case of Myriococcum thermophilum, have been found to belong in the Ascomycota instead (Morgenstern et al. 2012; Koukol 2016).

Taxonomy for thermophilic fungi is in a state of considerable flux (Mouchacca 2000b; Oliveira et al. 2015; Natvig et al. 2015). This results in part from the fact that under the “One Fungus = One Name” convention recently adopted by the International Code of Nomenclature for Algae, Fungi, and Plants the names for many thermophiles in the fungal kingdom need to be revised (Oliveira et al. 2015). This convention requires that the asexual and sexual nomenclature be unified and that a single name be assigned to a single species. In addition to name changes that have been required by changes in nomenclatural codes, in many cases, thermophilic fungi have simply been misclassified because of the failure to identify correct taxonomic affinities. The genus Myceliophthora provides examples of name changes required by new nomenclatural rules and by molecular phylogenetic studies that reveal true relationships (van den Brink et al. 2012). For example, the species recently recognized as Myceliophthora heterothallica was previously known under the teleomorphic names Theilavia heterothallica and Corynascus heterothallicus. To add to the confusion, as T. heterothallica, this species was once thought to be the teleomorph of Chrysosporium thermophilum, now recognized as M. thermophila (von Klopotek 1976; Hutchinson et al. 2015; van den Brink et al. 2012). A similar case exists for Rasamsonia, a genus erected to accommodate teleomorphs of Geosmithia and Talaromyces species, which were improperly identified (Houbraken et
al. 2012). As a final example, the genus *Mycothermus* was recently erected to accommodate fungal strains previously known as *Scytalidium thermophilum*, placed in a genus (*Scytalidium*) that is appropriate for organisms in a different fungal class (Natvig et al. 2015).

3. Ecology

Despite advances in industry and genetics, comparatively little is known about the natural role and distribution of thermophilic fungi. Although commonly isolated from compost, these fungi are known to exhibit a variety of lifestyles, including as animal and plant associates, and as saprotrophs (Salar 2018). For example, the thermophilic species *Myceliophthora thermophila* was identified as an endophyte of foliar tissue from a desert tree, *Parkinsonia microphylla* (Massimo et al. 2015). Another thermophile, *Rhizomucor pusillus*, has been reported to cause human infections, especially in immune-compromised individuals (St-Germain et al. 1993; Andrey et al. 2017). Cooney and Emerson (1964) noted that thermophilic fungi often remain unrecognized in culture when moderate incubation temperatures are used. As such, it may be that many thermophilic fungi remain undescribed.

A debate exists regarding how broadly distributed are the habitats in which thermophilic fungi can thrive. One hypothesis suggests that most thermophilic fungi are specialists of insulated compost-like substrates and that the presence of these fungi in soil and other non-compost substrates represents dispersal of aerial propagules (Maheshwari et al. 2000). Support for this idea has been presented for *Thermomyces lanuginosus*, which though common in soil was not competitive with mesophilic and thermotolerant fungi in soil microcosm experiments performed under fluctuating temperature regimes, unless temperatures were maintained above 40°C. In addition, spores of *T.*
lanuginosus failed to germinate in soil under conditions favorable for growth (Rajasekaran and Maheshwari 1993).

On the other hand, it possible to wonder if understanding the role of thermophilic fungi in soil requires consideration of specific microhabitats and substrates suitable for growth. The proportion of physiologically active microorganisms in soil can be small compared to the total microbial biomass, and the level of activity for a microorganism or microbial group is dependent on substrate availability (Blagodatskaya and Kuzyakov 2013). Moreover, microcosm experiments performed with only mesophilic “soil” fungi demonstrate that the performance of one species relative to another is substrate dependent (e.g. Deacon et al. 2006). Therefore, while previous studies have reported thermophiles from diverse compost or pseudo-compost materials such as animal nests, mushroom compost and self-heating hay bales (Fergus and Sinden 1969; Tansey 1971, 1973, 1975, 1977), it is likely that even a small 5-cm mass of leaf litter can be sufficiently insulated, moist and solar-heated to encourage growth of thermophilic fungi (Subrahmanyam 1999). Indeed, recent studies of arid ecosystems (where sizeable composts are rare, if not absent), including the Sevilleta Long Term Ecological Research (LTER) site in New Mexico, have demonstrated that thermophilic fungi are common in certain microhabitats (Powell et al. 2012). We recovered isolates from a variety of substrates including soil, biological soil crusts, leaf litter and herbivore droppings. While these and other previous studies have shed light on microhabitats and distributions, the extent to which thermophilic fungi exhibit habitat specificity is unclear, as is the prevalence of thermophilic fungi on a regional scale.

Microhabitats suitable for the growth of thermophilic fungi are common in diverse ecosystems. Although the early studies of thermophilic fungi examined substrates that were self-heating as a
result of microbial activity (Miehe 1907; Cooney and Emerson 1964), soil and other substrates can achieve temperature and moisture conditions suitable for thermophiles as a result of solar gain (Tansey and Jack 1976; Powell et al 2012). In reality, soil, litter and herbivore droppings in temperate ecosystems often reach temperatures at or above those suitable for thermophilic fungi. In an experiment designed to follow the succession of thermophiles in a natural setting, we monitored temperatures in the droppings of three herbivores (elk, oryx and rabbit) over a period of approximately one year (Fig. 1) at the Sevilleta National Wildlife Refuge. Even during winter months, daytime temperatures were often near or above 40°C. In warmer months, daytime temperatures often reached 60-75°C, temperatures at which fungal growth has ceased. In a single 24-hr period temperatures could swing from 15°C to above 60°C (Fig. 1). Droppings in this environment therefore represent an extreme microhabitat with dramatic and rapid changes in temperature and moisture. Thermophilic fungi are common in this microenvironment, and they participate in decomposition along with a complex community of bacteria, non-thermophilic fungi and microfauna.

4. A survey of thermophilic fungi from across the western United States

In a previously unpublished study, we surveyed thermophilic fungi in soils, plant litter and herbivore droppings from a wide range of latitudes, elevations, and distinct climatic regions across sites from central Mexico to southern Canada. One goal was to evaluate the extent to which these fungi are common in locations where the opportunities for natural compost are rare. A second goal was to evaluate whether there exists geographic, latitudinal or substrate differences in the distributions of major thermophile groups. Our sampling focused on soil, litter and herbivore
droppings. In addition, deep-frozen (-80°C) rhizosphere soil samples collected from under blue grama grass (*Bouteloua gracilis*) were tested for the presence of thermophilic fungi.

**Experimental approach.** Samples were collected in two phases. From May through June of 2008, 10 samples of rhizosphere soil were collected from each of five stands of *Bouteloua gracilis* in western North America as part of a separate study of root-associated fungi (Herrera et al. 2010). Soils were transported from the field on ice within 48 hours and ultimately stored at -80°C. These samples were plated in January of 2013. In a second effort, soil, herbivore droppings and leaf litter samples were collected from each of 10 locations in the western United States between March 2012 and May 2013 (Fig. 2, Table 1). These samples were stored at 4-5°C for no more than 4 days before plating.

All samples were plated onto Malt Extract Agar (MEA) with 50 µg/mL ampicillin (to exclude bacteria) and incubated up to 10 days at 50°C (see Bustamante 2006). Approximately 0.5-1.0 g of substrate was used for each plate. Rhizosphere soils from the Herrera et al. (2010) study were plated in replicates of 3. Resulting colonies from all cultures were then sub-cultured to obtain axenic isolates.

A CTAB (cetyl trimethylammonium bromide) DNA extraction procedure modified from Winnepenninckx et al. (1993) was used to isolate DNA from cultures, using methods previously described (Hutchinson et al. 2016). DNA was amplified by PCR of the ribosomal internal transcribed spacer (ITS) region using the fungal-specific primers ITS4 and ITS1F (White et al. 1990; Gardes and Bruns 1993). Each reaction consisted of 6.5 µL ExTaq polymerase (Takara, Mountain View, CA), 1 µL of each (5 µM) primers, 2 µL of 2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 2 µL milliQ purified water and 1 µL of template DNA, for a total of 13.5
μL. The following thermocycler settings were used: 95°C for 5 min, 30 cycles at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 45 sec, followed by a final extension of 72°C for 7 min. After PCR, reactions were purified by an enzyme procedure using the ExoSAP-IT kit (Affymetrix, Santa Clara, CA) and manufacturer’s specifications.

Amplicons were Sanger sequenced with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) in 10 μL reactions containing 0.5 μL BigDye Terminator v3.1, 2 μL of 5X Sequencing Buffer (Life Technologies/Applied Biosystems, Carlsbad, CA) 1 μL of 3 μM primer and 5.5 μL of milliQ water. A Big Dye STeP protocol was used with the following parameters: 96°C for 60 seconds followed by 15 cycles: of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 1 minute 15 seconds, then 5 cycles: of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 1 minute 30 seconds and a final 5 cycles: of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 2 minutes seconds (Platt et al. 2007).

Chromatogram files for the forward and reverse reads were edited and assembled into contigs using Sequencher v5.1 (Gene Codes, Ann Arbor MI). To determine the overall species richness among the isolates, ITS sequences were assembled into Operational Taxonomic Units (OTUs) using UPARSE 9.0 (Edgar 2013). OTU cutoffs were set to 97% identity. To obtain taxonomic information, the resulting OTUs were then queried at NCBI (National Center for Biotechnology Information) GenBank with BLASTN (Basic Local Alignment Search Tool Nucleotide) searches using the option to exclude uncultured and environmental samples.

Phylogenetic Analyses. ITS sequences were aligned in MUSCLE implemented through the European Bioinformatics Institute web interface (Edgar 2004; Li et al. 2015). Alignments were then visualized and trimmed in AliView v1.2.1 (Larsson 2014). Reference sequences from GenBank were
included as a comparison to the newly acquired sequences and type strains were selected as references when possible (Table 2, Table 3). Trees were constructed with the RaxML (Randomized Axelerated Maximum Likelihood) program v7.3.2 using 1000 bootstrap replicates (Stamatakis 2006). Because ITS sequences align poorly across distant phylogenetic groups, we built separate trees for each of the three orders to which the sequences were classified. Trees were visualized and edited with Mesquite v2.75 (Maddison and Maddison 2011).

**Results.** Thermophilic and thermotolerant fungi were recovered from every substrate type and nearly every location. Notably, propagules of thermophilic fungi from the rhizosphere soil were also able to survive storage at -80°C for nearly 5 years. Sixty-two total isolates were recovered. After excluding duplicates from the same sample, 55 isolates were characterized at the sequence level, resulting in 14 putative OTUs, 10 genera and 13 known species. The identity of each of the OTUs is summarized in Table 2. Most isolates fell into the fungal orders Eurotiales (34 isolates) and Sordariales (17 isolates). Only 4 isolates belonged to the Mucorales, and no isolates from the Onygenales were identified. The lack of isolates from the Onygenales may owe to the types of substrates and media used, as this group of fungi is known to be keratinophilic (Sharpton et al. 2009). The most common species was *Thermomyces lanuginosis*, represented by 16 isolates, followed by *Aspergillus fumigatus*, represented by 8 isolates, and *Chaetomium thermophilum var. dissitum*, represented by 6 isolates.

Several of the isolates were from species viewed as thermotolerant rather than thermophilic. Mouchacca (2000a) suggests that *A. fumigatus, A. nidulans*, and *C. jodhpurense* have been erroneously reported as thermophiles when they actually possess lower temperature optima than true thermophiles. Additionally, *Thielavia gigaspora* is a thermotolerant species previously isolated in
Egypt (Moustafa and Abdel-Azeem 2008). Mouchacca (2000a) also reported *Rhizopus microsporus* as a misattributed thermophile, but Peixoto-Nogueira demonstrated that isolates grow optimally at 45°C. Overall, thermostolerant species represented 29% of all of our isolates. Excluding the thermostolerant species, there were 25 isolates from the Eurotiales and 14 from the Sordariales.

Independent-samples Welch’s t-tests were employed to compare elevation and latitude specificity for thermophilic isolates in the Eurotiales and Sordariales. Because the Mucorales were comparatively rare, they were not included in statistical analyses. For elevation, there was no significant difference between the distributions of Eurotiales and Sordariales ($M_{EUROTIALES}$ = 2038.28m, SD=900.51; $M_{SORDARIALES}$ = 1765m, SD=823.66; $t(29)=0.96$, $p=0.05$). For latitude, again, there was no significant difference between the distributions of Eurotiales and Sordariales ($M_{EUROTIALES}$ = 38.79°, SD= 16.35; $M_{SORDARIALES}$ = 41.44°, SD= 28.92; $t(18)=-0.32$, $p=0.05$).

In terms of substrate preference, thermophilic samples in Eurotiales were most frequently isolated from litter (44%), while for samples in the Sordariales, the top sources were droppings (35.71%) and top soil (35.71%). Overall, the most thermophilic isolates originated from litter substrates (35.9%), followed by droppings (30.7%), soil (20%) and finally rhizosphere, which represented 12.8% of the samples.

For the soils collected in 2008 and stored at -80°C, there appeared to be a latitudinal gradient in terms of the success of platings. Just over half (62.5%) of soils collected in Saskatchewan, Canada were positive for thermophiles, compared to 80% of soils from Custer, South Dakota, 86.7% from Socorro, New Mexico, 93.9% from Janos (Chihuahua), Mexico and 89.7% from Ojuelos (Jalisco), Mexico. With the exception of the soils from Janos (which showed a higher percentage than Ojuelos to the south), plating success declined with increasing latitude. In pairwise
comparisons, plating success for Saskatchewan was an outlier, and significantly different from all other locations except South Dakota according to a Pearson’s N-1 chi-square test $\chi^2_{\text{SOUTHDAKOTA}}(1, N = 54) = 2.00, p = 0.16; \chi^2_{\text{NEWMEXICO}}(1, N = 54) = 4.20, p = 0.04; \chi^2_{\text{JALISCO}}(1, N = 63) = 6.60, p = 0.003; \chi^2_{\text{CHIHUAHUA}}(1, N = 57) = 8.63, p = 0.01$. No other pair-wise comparisons were significantly different.

**Discussion.** Our results indicate that thermophilic fungi are readily isolated from various substrates, from elevations as low as 40m above sea level to as high as 3951m and from a great range of latitudes between Mexico and Canada. We observed no correlation between phylogeny and environment. Specifically, isolates from the Eurotiales and Sordariales did not differ significantly for substrate preference, elevation or latitude. Even within a single OTU cluster, constituent sequences were derived from diverse locations and substrates. For example, OTU1 (*Thermomyces lanuginosus*) represents isolates from as far south as Ojuelos, Jalisco to as far north as the Beartooth Highway in Wyoming. This cluster also consisted of multiple isolates from every substrate type and of elevations from 315m to above timberline at 3951m. Indeed, at the resolution of OTUs at the 97% level, there appears to be no specificity of thermophilic fungi to a particular habitat. It is possible, however, that the 97% cutoff is too generous and blurs the finer distinctions among the isolates. To develop a better sense of the phylogenetic relationships between the isolates, one might also collect data for functional DNA regions that are less variable and more reliable at predicting deeper levels of taxonomy.

Studies show that members of the Chaetomiaceae (Sordariales) are proficient in decomposing cellulosic biomass, so they are thought to associate with plant-based substrates in nature (Ames 1963; Mehrotra and Aneja 1990). They have been previously isolated from herbivore
droppings, leaf litter and even from live plants (Kerekes et al. 2013; Richardson 2001; Alhamed and Shebany 2012). Chaetomiaceae are also prevalent in composts (Cooney and Emerson 1964; Kane and Mullins 1973; Straatsma 1994). For example, using an ITS barcoding approach Neher et al. (2013) showed *Chaetomium* species to be dominant members of the fungal OTUs across all of the compost recipes they tested, especially in the earlier stages of composting. As discussed previously, composts have been proposed as the primary habitats for thermophilic fungi, with the suggestion that specimens found on other substrates are likely inactive propagules dispersed from compost (Rajasekaran and Maheshwari 1993). However, soil is also sufficiently rich in cellulose as it is one of the top sources of complex carbon polymers (Kögel-Knabner 2002; López-Mondéjar 2016). Thus, it is perhaps unsurprising that many thermophilic species in the Chaetomiaceae have been identified from soil (Tansey and Jack 1976; Pan et al. 2010; Powell et al. 2012). Mesophilic Chaetomiaceae have been demonstrated to be both present and active in the soil. Using Stable Isotope Probing with $^{13}$C cellulose substrate, Eichorst and Kuske (2012) showed that species of *Chaetomium* actively decay cellulose added to soil. It is reasonable to believe that thermophilic members of the Chaetomiaceae do the same.

Species in the order Eurotiales are also commonly associated with decaying plant material. For example, the well-known fungus *Aspergillus fumigatus* is cited as one of the most frequent species recovered from composts and other plant debris (Taylor et al. 2015). *A. fumigatus* also shows a pan-global distribution, which Pringle et al. (2005) have suggested may be due to the role of humans in expanding composting processes. Another member of the Eurotiales, the thermophilic *Thermomyces lanuginosus* also shows seemingly ubiquitous distribution. In our present study, it was the most frequently isolated taxon and derived from a variety of substrates and locations. Langarica-Fuentes et
al. (2014) also found that along with *Talaromyces thermophilus* (another species in the Eurotiales), *T. lanuginosus* accounted for 65% of sequences obtained via 454’ barcoding of the fungal community in the middle and center of an in-vessel compost system. Similarly, it was the top isolate in studies of thermophilic fungi from soils in India (Maheshwari et al. 1987; Rajasekaran and Maheshwari 1993). Still, Rajasekaran and Maheshwari (1993) were unable to detect actively growing *T. lanuginosus* in soil with immunofluorescence assays. However, Hedger and Hudson (1974) reported that *T. lanuginosus* shows commensal interactions with cellulolytic fungal thermophiles (*Chaetomium thermophile* and *Humicola insolens*) and subsists on the sugar byproducts from cellulose decomposition. Thus, it may be that this species performs best in a consortium with cellulolytic thermophiles and requires other fungal partners to grow. If there is adequate cellulose in a given substrate, cellulose degrading fungi can likely support commensal fungi thus provide a niche in soil for species such as *T. lanuginosus*.

Soils undergo diurnal temperature fluctuations to upwards of 70°C, so soil is a suitably hot substrate for thermophilic fungi (Powell et al. 2012). Leaf litter and herbivore droppings also experience similar swings in temperature (Fig. 1). In addition, thermophilic fungi are more readily isolated from soil after precipitation events, indicating that they are responsive to changes in the soil environment (Powell et al. 2012). Taken together, these factors suggest that thermophilic fungi can inhabit many microhabitats, including soil, provided that they have access to moisture and appropriate temperatures.
5. Conclusions

Much remains to be learned about the ecology of thermophilic fungi. Although it has long been known that these fungi can be isolated from soil, herbivore droppings and other substrates, most studies have focused on composted plant materials in either natural or anthropogenic settings. In contrast, our surveys have shown that nearly all ecosystems provide thermophilic fungi with at least transient access to decomposing plant material, and sufficiently high temperature and moisture (see Fig. 1). Our results suggest that such transient microenvironments might be the primary habitats. At the level of resolution provided by ribosomal ITS sequences, there is little evidence for habitat specialization or geographical restrictions among thermophiles. Thermophiles in the Ascomycota are distributed across three orders, with several phylogenetic lineages within each order. We found members of most lineages across wide latitudes, elevations, substrate and ecosystem types, ranging from desert shrublands and grasslands to montane forests to northern grasslands.

6. Future perspectives

Thermophilic fungi have provided many contributions to science, both in their utility to industry and in the advancement of basic understanding in biology. Information on the distribution of thermophilic fungi, and a better grasp on their natural diversity and roles in the environment, will help further the field of microbial ecology and will aid in bioprospecting new, potentially useful organisms for biotechnology. Although next-generation sequencing methods can detect thermophilic fungi in environmental samples, many thermophiles have close mesophilic relatives, and as a result, the assessment of thermophily often requires evaluation based on growth in the laboratory rather than on sequence analysis alone. Accordingly, it is likely that fungal thermophiles are overlooked in
environmental sequencing data. Similarly, culture-based methods of community analysis often employ only temperatures suitable for mesophiles, and temperatures optimal for the growth of thermophiles or psychrophiles are not considered. Moreover, it is possible that certain fungal thermophiles are unculturable and are only detected as DNA in environmental surveys. These circumstances thereby result in a need for a unified, comprehensive approach to appraising and understanding not only the biology of thermophilic fungi, but also the ecology of non-thermophilic microbes that share environments with thermophiles.

Acknowledgements

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References


Figure legends

Fig. 1. Extreme microenvironments are common in temperate ecosystems. A. Variation in soil temperature for a typical 23-hour period (1:00 AM to midnight) in July at the Sevilleta National Wildlife Refuge in central New Mexico (adapted from Fig. 1 in Powell et al. 2012, used with permission of the copyright holder, Taylor and Francis). B. Dramatic swings in internal temperatures for herbivore droppings and litter in the foothills of the Los Pinos mountains in central New Mexico over 19 days surrounding the transition to the monsoon season in 2013. The temperature swings were frequently from 12-15°C in early morning to over 70°C at midday. The high temperatures were driven by solar gain. Air temperatures did not exceed 35°C. Temperatures were measured with a small thermocouple and recorded on a Campbell Scientific CR1000 datalogger.

Fig. 2. Locations of soil, litter and herbivore dropping samples employed for the thermophile survey presented here. Details of the samples are given in Table 1.

Fig. 3. Ribosomal RNA ITS gene trees for three orders of thermophilic fungi recovered from a recent survey (collection sites are presented in Fig. 2 and Table 1): Eurotiales (A), Sordariales (B), Mucorales (C). Trees were rooted with *Coccidioides immitis*, *Neurospora crassa* and *Pilobolus crystallinus*, respectively. New isolates are color coded by substrate type, while reference strains are colored by temperature optimum. Bootstrap values (1000 replicates) are displayed for all nodes receiving 65% or greater support. All new isolates form well-supported clades with previously identified species and represent diverse substrate types and locations.
Fig. 1

A. McKenzie Flats Soil Temperature

B. Start of rainy season

Temperatures and Precipitation (mm)

- Oryx Droppings (°C)
- Litter (°C)
- Elk Droppings (°C)
- Rabbit Droppings (°C)
- Precipitation (mm)
Fig. 3B

Neurospora crassa strain CBS 709.71
  WBg9_2 Wind Cave, SD
  Thielavia gigaspora CBS 112062
  Thielavia subthermophila CBS 125981
    Thielavia arenaria CBS 507.74
    GBg10_1 Grasslands, SK, Canada
    18E_L1 Thermopolis, WY
    Th044-2 Gila National Forest, AZ
    15C_L1 Cody, WY
  Chaetomium globosum CBS 160.62
    WBg10_2 Wind Cave, SD
    Th044 Gila National Forest, AZ
    Th022 Gila National Forest, AZ
    Th041 Central Valley, CA
    Myceliophthora heterothallica CBS 202.75
    Th021 Val Verde, CA
  13C Lake Butte, WY
    Mycothermus thermophilus CBS 629.91
    10B_II Lake Butte, WY
    12_II Lake Butte, WY
    Chaetomium thermophilum var. dissitum LC4128
    31 Grey Cliff Prairie Dog State Park, MT
    WBg1_MH1 Wind Cave, SD
    Chaetomium thermophilum var. dissitum NBRC 31807
    16_I Thermopolis, WY
    32A_II Maxwell Wildlike Refuge, NM

Color Key:
- Thermophile reference strain
- Mesophile reference strain
- Rhizosphere soil
- Soil
- Droppings
- Litter
Fig. 3C

Pilobolus crystallinus ATCC 36186

Rhizomucor miehei CBS 182.67

Tho40 Near Phoenix, AZ

SBg6_3 Sevilleta, NM

SBg10_2 Sevilleta, NM

Rhizopus microsporus CBS 130158

JBg17_2 Janos, CHH, Mexico
<table>
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<th>OTU (Putative Species)</th>
<th>Isolate(s) Represented</th>
<th>Substrate Type</th>
<th>Collection Date</th>
<th>GPS Coordinates</th>
<th>Location</th>
<th>Elevation (meters)</th>
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<td>6II</td>
<td>Litter</td>
<td>May 2013</td>
<td>44°56.593’N 110°38.397’W</td>
<td>Undine Falls, WY</td>
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<td>34°24.094’N 106°40.662’W</td>
<td>Sevilleta, NM</td>
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<td>Th002</td>
<td></td>
<td>Soil</td>
<td>March 2012</td>
<td>34°15.267’N 109°24.267’W</td>
<td>Apache County, AZ</td>
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*Type Strain*
CHAPTER 3

Plant-endophyte interactions across a Creosote (*Larrea tridentata*) shrub encroachment zone

Abstract

Desertification is a significant global phenomenon and shrub encroachment is considered one of the primary causes. Shrub encroachment, the conversion of grasslands into shrublands, is a well-documented process but the mechanisms by which encroachment occurs are debated. Here we investigate how interactions between grasses and their root associated fungi (endophytes) may be influenced by shrub expansion into an arid grassland by examining the fungal colonization of roots from two Chihuahuan desert plants, the C3 shrub *Larrea tridentata* (creosote) and the C4 grass *Bouteloua eriopoda* (black grama). Our sampling spanned two years (2012 and 2015) and a narrow spatial gradient at the Sevilleta National Wildlife Refuge, NM, where there is a distinct ecotone between shrubland and grassland. To assess fungal colonization, we sequenced the fungal ribosomal internal transcribed spacer (ITS) from the total DNA of harvested roots via high-throughput sequencing. The community composition of root fungi was distinct across years and also showed significant shifts among the fungal communities of the three sample types (although grama samples were more similar to each other than to creosote). While previous studies have demonstrated an aridland fungal endophyte community shared across diverse plant species at the Sevilleta, our results support the fact that endophyte communities are also shaped by host species. This in turn suggests the possibility that encroachment can influence plant distribution by altering endophyte communities.
INTRODUCTION

Shrub encroachment is a global phenomenon and has been well-documented for many diverse ecosystems, although arid and semi-arid regions appear to be especially susceptible (Eldridge et al. 2011). This process is marked by the replacement of grasslands, savannahs and woodlands by indigenous woody and shrub-like plants (Eldridge et al. 2011; van Auken 2009). In the desert southwest of North America, mesquite (Prosopis spp.) and creosote (Larrea tridentata) are the primary species responsible for woody plant encroachment (van Auken 2000). Although these shrubs have existed in the southwest for at least 4000 years, they were likely only dominant at local scales (Grover and Musik 1990). For example, historical records of vegetation in New Mexico suggest that perennial grasses were the dominant plant form in the state until the early twentieth century, while shrubs were confined to gravelly, well-drained areas (Grover and Musik 1990; Gross and Dick-Peddie 1979). However, as of the year 2000, creosote and mesquite had replaced 19 and 38 million hectares, respectively, of land that was once dominated by grasses (van Auken 2000).

This unprecedented increase in shrubland cover may have several environmental and economic consequences. For instance, the loss of viable grass-covered rangeland can threaten pastoral practices (Eldridge and Soliveres 2015). From an environmental perspective, shrub encroachment can also result in the loss of species diversity, as seen for example by declines in mammal and reptile diversity, documented in a 2017 meta-analysis (Stanton et al. 2017). Encroachment of woody plants is also cited as a cause of desertification, which is defined by the United Nations (under the Convention to Combat Desertification) as “land degradation in arid, semi-arid, and dry subhumid areas” (UNCCD 1994; Reynolds et al. 2007).
Various factors are thought to contribute to shrub encroachment. Some potential causes are directly anthropogenic, such as overgrazing and fire suppression, but even indirect causes likely relate to human influences on climate (Eldridge et al. 2011). For example, the increase of CO\textsubscript{2} and the deposition of N are both implicated in driving shrub encroachment (Archer 2010). Creosote encroachment, in particular, is linked with changes in microclimate because as creosote becomes the dominant vegetation type, more soil becomes exposed, thus increasing solar gain and ambient nighttime temperatures (D’Odorico et al. 2010). Indeed, stands of creosote have been reported to exhibit mean and mean minimum temperatures that are approximately 2°C higher than in the adjacent grassland (D’Odorico et al. 2010). Even this minor shift in temperature is thought to tip the scales in favor of creosote and promote its establishment.

Creosote is also likely able to compete with other plant species and increase bare ground via allelopathy. Indeed, creosote bushes have been recorded to inhibit not only neighboring \textit{Ambrosia dumosa} but also conspecifics via allelopathic chemicals (Mahall and Callaway 1990). For example, nordihydroguaiaretic acid isolated from creosote has been shown to suppress the seedling root growth of various plants, including rye grass, barnyard grass, red millet, lettuce and alfalfa (Elakovich and Stevens 1985). In addition, creosote is known to produce a host of volatile organic compounds (VOCs) such as terpenes, ketones, benzene derivatives and alcohols, as well as other hydrocarbons and their derivatives (Strobel et al. 2011). Chemicals in these classes have been proposed as allelopathic but also demonstrate ability to suppress fungal pathogens (Reigosa et al. 2006). Creosote leaf extract and resin have been shown to inhibit fungal genera such as \textit{Aspergillus}, \textit{Penicillium} and \textit{Fusarium} as well as the fungus-like pathogens \textit{Phytophthora capsica} and \textit{Pythium sp.} (Tequida-Meneses et al. 2002; Mojica-Marin et al. 2011; Lira Saldívar et al. 2003).
Further, it has been suggested that plant-associated fungi may be responsible for producing many of these allelopathic compounds. For example, the fungal endophyte *Alternaria alternata* (phylotype CID 120) isolated from an invasive forb *Centaurea stoebe* doubled the allelopathic effect of its host, compared to an endophyte-free host (Aschehoug et al. 2014). In creosote, a foliar endophyte (*Phoma sp.*) was reported to be responsible for producing many of the allelopathic VOCs known from creosote and these compounds inhibited the growth of several pathogenic fungi (Strobel et al. 2011). These findings indicate that creosote may be capable of modifying the soil environment via its endophytic fungi and thereby affect the growth of neighboring plants and microbes.

Indeed, plant-microbial feedbacks are well known drivers of dynamics between plant species, such as invasion and competition (Klironomos 2002). For example, studies have suggested that mutualistic microbes can enable alien plant invasions, as in the case of symbioses with nitrogen-fixing bacteria and mycorrhizal fungi which both serve their host plants by bringing in nutrients that may otherwise be unavailable in new habitats (van Kleunen et al. 2018). Mutualisms with mycorrhizal fungi may be vital to the establishment of invasive plants, seen for instance in pine tree invasions in South America that were facilitated by the co-introduction of ectomycorrhizal fungi (Hayward et al. 2015). Previous studies have also shown that exotic plants can even disrupt existing mutualisms between native plants and their mycorrhizal fungi (Stinson et al. 2006; Meinhardt and Gehring 2012).

Creosote, although endemic to the lands it is encroaching, has itself been termed a “native invasive” as shrub expansion shares many of the same characteristics as alien plant invasion (Nackley et al. 2017). As such, it is relevant to assess the presence of fungal mutualists in creosote to determine
if such associations may contribute to shrub encroachment. Furthermore, there is indication that invasive plants may be flexible in their mutualisms with fungi and can co-opt mutualists associated with native species (Marler et al. 1999). Knapp et al. found that native and exotic plants in the arid Great Hungarian Plain can share species of dark septate endophytes (DSE), melanized root colonizing fungi that are thought to mitigate plant stress in dry and nutrient poor environments (Knapp et al. 2012). Notably, fungi belonging to the same clades of DSE found in Hungary have also been recovered from blue grama grass (*Bouteloua gracilis*) in the southwestern state of New Mexico, USA (Porras-Alfaró et al. 2008). Here, at the southern edge of its range, blue grama also overlaps with a congeneric black grama grass (*B. eriopoda*), which in turn overlaps with and is being encroached upon by creosote. Given the close phylogenetic relationship of the grama species, the two can be predicted to share root associated fungi. In fact, *B. gracilis*, *B. eriopoda* and co-occurring native dropseed grass (*Sporobolus nealleyi*) have all been shown to share a similar root fungal consortium (Khidir et al. 2010). Knapp et al. have hypothesized that plants in arid environments can share root-associated fungi, particularly DSE, on a global scale (2012). This suggests that root associated fungi in these environments are often generalists in terms of host preference and can possibly be leveraged by invading and encroaching plants.

Although there has been no focus on the root fungal consortium and creosote encroachment, previous studies have addressed other components of the fungal community that associate with creosote, specifically foliar endophytes, fungi from soil under the plant canopy, and rhizosphere fungi. In a study of foliar endophytes, Massimo et al. isolated fungi in culture and determined that isolation frequency was low in that only 2.0% of tissue segments produced a successful culture (2015). Furthermore, isolates from creosote were dominated by members of the *Preussia* species
complex to the which belong to the order Pleosporales, a group among which many species are considered dark-septate endophytes (Massimo et al. 2015; Porras-Alfaro and Bayman 2011). *P. viridicata* is also known to be a coprophilous species, and is closely related to *Sporormiella*, a dung fungus that has been used as a proxy for the presence of megafauna in the fossil record (Cain 1961; Burney et al. 2003).

When examining soil near creosote, Ewing et al. (2007) found that microbial communities were larger in soil at the shrub base than in soil between shrubs, and that the communities differed in composition between these two locations. Measures for polar lipid fatty acids inferred to be from fungi also decreased with distance from the main shrub stem. In contrast, Steven et al. found no significant difference in mean fungal abundance and a 60% increase in bacterial abundance when comparing biocrust soil and the rhizosphere of creosote (2014). Furthermore, the biocrusts and root zones shared many of the most abundant bacteria and fungi, but the relative proportions of taxa were different. Although most fungal OTUs mapped to the Ascomycota for both sample types, the classes Dothideomycetes, Leotiomycetes and Eurotiomycetes were more abundant in the root zone and fungi in the Pezizomycetes were more abundant in the biocrusts.

In the desert southwest United States, creosote is an attractive system for testing whether fungal mutualisms aide encroachment in arid conditions. Since desert ecosystems pose extreme constraints on organisms, symbiotic relationships with DSE and other fungal communities are thought to support the establishment and survival of their host plants (Porras-Alfaro and Bayman 2011). Moreover, since shrub encroachment occurs in concert with desertification, the potential role that endophytes may play is especially relevant to high temperature aridlands. This feedback loop and the resulting temperature increase could alter microbial populations on the encroachment front.
Despite the knowledge of microbe-host interactions in creosote, there has been no study of the role that microbes may play in creosote shrub encroachment. To investigate the potential that creosote may impact microbe communities and their plant hosts, in this study we have the targeted root-associated fungi of creosote and the co-occurring grass species, black grama. We have used next-generation sequence-based techniques to assess the composition of these fungal communities, in order to understand the common and distinguishing taxa associated with the two plant types. To address the impact of creosote establishment on the root fungal microbiota of black grama, we also sampled from black grama at the transition zone into creosote shrubland.

**MATERIALS AND METHODS**

**Field Site**

This study was conducted at the Sevilleta National Wildlife Refuge in south-central New Mexico which spans 100,000 hectares and includes a mosaic of ecotypes such as the Rio Grande riparian corridor, the Chihuahuan Desert, and the Great Plains Short-Grass Steppe. Notably, the refuge also contains a transition zone or “ecotone” between two biomes: shortgrass prairie and desert grassland-shrubland (Buxbaum and Vanderbilt 2007). Creosote (*Larrea tridentata*), blue grama (*Bouteloua gracilis*) and black grama (*Bouteloua eriopoda*), are three of the dominant plant species across at the Sevilleta but are distributed along the distinct north to south gradient (Kröel-Dulay et al. 2004). The focus of this study was the transition zone between the creosote-dominated shrubland and the black grama-dominated grassland.
**Sampling**

Our experimental design consisted of two variables, host plant and year. To understand the impact of creosote on the microbial community of black grama, we sampled roots from both grama living among creosote plants in the transition zone and black grama from grassland approximately 1.5 km north of the transition zone. The grass samples are referred to as “grama-in” and “grama-out” hereafter, respectively. To detect any temporal variation in the root fungal communities, sampling was first conducted in 2012 and repeated in 2015. Samples were collected from two locations on the refuge, near the Five Points Creosote Bush site (34°20’18”N, 106°44’15”W) and McKenzie flats (34°20’17”N, 106°41’55”W) in both June 2012 and July 2015. In 2012, roots were sampled from 10 creosote plants, 5 adjacent black grama plants within the same creosote stand, as well as from five black grama plants from the McKenzie flats. In 2015, roots were sampled from nine creosote plants and four adjacent black grama plants from the Five Points site, as well as from four black grama plants from the McKenzie flats. Roots were selected if they were shallow, at least 5 cm long (Khidir et al. 2010) and met the following criteria established by Porras-Alfaro and Bayman, 2007: (1) connected to green leaves, (2) have fine root hairs, and (3) free of lesions. A total of 10 root segments were collected per plant.

**Laboratory Methods**

Harvested root tissue was surface-sterilized with a 70% ethanol wash, followed by a 1% sodium hypochlorite wash and a rinse with sterilized ultra-pure water as previously described by Porras-Alfaro and Bayman (2007). The roots were briefly pressed into 2% malt extract agar (MEA) plates which were in turn incubated at 25°C to confirm adequate removal of surface propagules (Khidir et al. 2010)
DNA was isolated directly from roots with a CTAB (Cetyl trimethylammonium bromide) extraction protocol as previously described (Hutchinson et al. 2016). Lysis buffer contained 2% CTAB, 1.4M NaCl, 20 mM EDTA, 100 mM Tris-HCl, and 0.2% 2-mercaptoethanol. Prior to DNA extraction, roots were ground in liquid nitrogen with a sterile mortar and pestle. Samples were amplified for the internal transcribed spacer (ITS) region using the fungal specific primers ITS1F and ITS4 (White et al. 1990, Gardes and Bruns 1993). Total root DNA was provided to MR DNA (Molecular Research LP, Shallowater, TX) for Roche 454 sequencing.

Roche 454 sequencing was conducted by MR DNA LP as previously described by Dowd et al. (2008). Samples were processed using a trademarked procedure (bTEFAP®). PCR of the ITS region with primers ITS4 and ITS1F, HotStarTaq polymerase and the Plus Master Mix Kit (Qiagen, Valencia, CA) was conducted in a single-step 30 cycle procedure with the following steps: 94°C for 60 seconds, 53° for 40 seconds, 72° for 60 seconds, and a final extension of 72° for 5 minutes. PCR products were combined in equal concentrations, then purified with the Agencourt AMPure bead kit (Beckman Coulter, Brea CA) before sequencing with the ITS1F primer on the Roche 454 FLX Titanium instrument as specified by the manufacturer.

Data Processing and Statistical Analyses

Reads were processed using a combination of scripts and pipelines. The Standard Flowgram File (SFF) was converted to FASTA format with a quality file using the SFF converter at GALAXY (Afgan et al. 2016). The resulting FASTA file and the quality file were merged using the ‘faqual2fastq.py’ script available from drive5 Bioinformatics. Barcodes and primers were removed using the ‘fastq_strip_barcode_relabel2.py’ script also available from drive5 Bioinformatics. Quality filtering, global trimming and Operation Taxonomic Unit (OTU) assembly were performed with
the USEARCH v9 algorithm (Edgar 2010). Prior to OTU picking, raw reads were filtered such that sequences with greater than 1.0 expected error were removed and passing sequences were then trimmed to 200 nucleotides. OTUs were clustered at the 97% identity threshold, chimeras were filtered denovo and singleton reads were retained. To build the OTU table, all raw reads including previously filtered sequences were trimmed to 200 nucleotides or padded with n’s to a length of 200 nucleotides, then matched to OTUs. This step greatly increased the number of reads that could be assigned to an OTU. Taxonomy was assigned using CONSTAX, a Python tool designed to determine the consensus classification for OTUs using three methods, the Ribosomal Database (RDP) Naïve Bayesian classifier, UTAX and SINTAX (Gdanetz et al. 2017, Wang et al. 2007, Edgar 2013, Edgar 2016). The UNITE v7.2 (01.12.2017) general FASTA release database served as the reference (Kõljalg et al. 2013). CONSTAX was utilized with default settings (a confidence threshold of 0.8) and the following versions of classifiers: the RDP classifier v11.5, the UTAX algorithm from UPARSE v8.1.1861, the SINTAX algorithm from UPARSE v10.0.240. Taxonomy for members of the Mortierellomycota and Glomeromycota was amended to reflect their current designation as subphyla the Mucoromycota (Stajich 2017; Spatafora et al. 2018).

Following classification with CONSTAX, attempts were made to better assign the top 25 OTUs with incomplete taxonomy using BLAST (Basic Local Alignment Search Tool) nucleotide queries of the NCBI database (Altschul et al. 1990). Searches were modified to exclude uncultured/environmental sample sequences, but all other options were set to default. To assign these OTUs to functional categories of fungi, the top 25 OTU table was also analyzed with FUNguild (Nguyen et al. 2016).
Statistical analyses were conducted in R v3.5.0, primarily with packages phyloseq v3.8 and vegan v2.5-3, and with the use of other packages as noted (R Core Team 2018; McMurdie and Holmes 2013; Oksanen et al. 2013). Figures were created primarily in ggplot2 v3.0 (Wickham 2016).

Prior to ordination, counts in the OTU table were converted to relative abundance with the ‘transform_sample_counts’ command in phyloseq. This approach was adopted rather than rarefaction because rarefaction is reported to perform poorly for differential abundance testing (McMurdie and Holmes 2014). However, for alpha diversity measures, samples were rarefied without replacement to the minimum sample size of 720 using the ‘rarefy_even_depth’ command in phyloseq (random seed 123). Alpha diversity indices (Shannon, Simpson, Chao and Observed) were computed with the ‘estimate_richness’ command in phyloseq on both the rarefied and the raw data. The distribution of measures was visualized with a histogram and tested for normality with a Shapiro-Wilks test (‘shapiro.test’) in the R base package. Indices with normal distributions were tested for significant differences between host type using an ANOVA (‘aov’), followed by a Tukey honest significant differences test for pairwise comparisons. These indices were also tested for differences in sampling year (2012 vs. 2015) with a Welch’s Two Sample t-test (‘t.test’). For non-normally distributed diversity indices, a Kruskal Wallace rank sum test (‘kruskal.test’) was used for the comparisons by host, followed by a Wilcoxon rank sum test (‘pairwise.wilcox.test’) with a false discovery rate (FDR) p-value adjustment method, while a two-sample Wilcoxon test (‘wilcox.test’) was used for comparisons by year.

Ordinations were computed using an NMDS (Non Metric Multidimensional Scaling) technique with the phyloseq command ‘ordinate’ and Bray-Curtis as the distance metric. Three
dimensions adequately reduced stress, so a value of $k = 3$ was used for the ordination. The NMDS plot was visualized with the ‘plot ordination’ command, an implementation of ggplot2 in phyloseq and ellipses were added with the ‘stat_ellipse’ command using default parameters. Ordinations were conducted for the relativized OTU table and an OTU table that was limited to only the top 25 most abundant OTUs, in order to determine the impact of these OTUs in driving the overall relationships among samples. A PERMANOVA (Permutational Analysis of Variance) with the ‘adonis’ command (in vegan) was used to estimate the relative impact of the variables host type, year of collection and the interaction of both variables. For variables with significant effect, a post-hoc adonis (‘pairwise.adonis’) with Bonferroni p-value corrections was used to assess pairwise between-group differences (Martinez Arbizu 2019).

Samples were also tested for differences in relative abundance of representative phyla, classes, orders and families by host plant and year. Only taxa with overall relative abundances of more than 1% were tested. Histograms and Shapiro-Wilks tests were performed to test for normality, as described for alpha diversity measures. Because none of the distributions for the percentages of each taxon were normal, Kruskal Wallace rank sum tests were conducted for host type and a two-sample Wilcoxon test were used for yearly comparisons, again, as previously described for alpha diversity measures. Similar statistical procedures were conducted to detect differences in relative abundance for the top 25 OTUs, relative to collection year and host type.

Additionally, the RAM package and function ‘core.OTU’ was used to determine which OTUs, if any were shared between all host types or years (Chen et al. 2018). A Venn diagram (with r package VennDiagram 1.6.20) was also constructed to visualize the overlaps in OTUs between variables (Chen and Boutros 2011).
RESULTS

Taxonomic Representation and Functional Classifications

The OTU table contained 359 OTUs which were represented by 88.4% Ascomycota, 7.1% Basidiomycota, 3.0% unidentified at the phylum level, and 1.5% Mucoromycota. Overall, the three plant types shared 65 OTUs, while 134 OTUs were unique to creosote, and 15 were associated with black grama regardless of location (Figure 2B). There were 41 OTUs unique to only grama-in and 25 specific to grama-out. The two years shared 138 OTUs, while 160 OTUs were unique to 2012 and 61 were unique to 2015. When tested for differences in phylum composition by host plant or by year, no phyla showed a significant change by year or by host type at the p<0.05 level. At the class level, only the Glomeromycetes showed significant differences by year (W = 96, p = 0.005275). Trends for the Glomeromycetes were recapitulated at lower levels of taxonomy within that phylum because all of the constituent OTUs fell into the same family (Glomeraceae). In other words, the Glomeromycetes (W = 96, p = 0.005275), Glomerales (p = W = 89, p-value = 0.002651) and Glomeraceae (W = 90, p = 0.002561) all increased from 0.07% in 2012 to 5.2% in 2015. Aside from Glomeraceae, the Tricholomataceae was the only other family to show significant differences by year of collection with p = 0.0035. Members of Tricholomataceae (Agaricales) accounted for 2.9% of all sequences in 2012 but were absent in 2015. No other taxa, at any level of classification showed significant differences by year.

The taxonomy at the class level consisted of 44.3% Sordariomycetes, 26.7% Dothideomycetes, 13.0% Unidentified, 7.0% Eurotiomycetes, 6.0% Agaricomycetes and 1.5% Glomeromycetes, while the rest made up fewer than 1%. In statistical comparisons of the relative abundance of Sordariomycetes by host plant type, grama-in (29.3%) and grama-out (8.3%) were
both significantly different from creosote (65.2%) ($p_{\text{Grama}_{\text{in}}/\text{Creosote}} = 0.031$; $p_{\text{Grama}_{\text{out}}/\text{Creosote}} = 0.031$), but were not significantly different from one another ($p_{\text{Grama}_{\text{in}}/\text{Grama}_{\text{out}}} = 0.859$). The Agaricomycetes were significantly less abundant in creosote compared to either of the grass types ($p_{\text{Grama}_{\text{in}}/\text{Creosote}} = 0.013$; $p_{\text{Grama}_{\text{out}}/\text{Creosote}} = 0.014$), but there was no significant difference among the grass samples ($p_{\text{Grama}_{\text{in}}/\text{Grama}_{\text{out}}} = 0.478$). This class accounted for 0.5% of all creosote sequences, 16.9% of all grama-in sequences and 11.1% of all grama-out sequences. There were no other significant correlations between the remaining classes and host type.

The most abundant order was the Xylariales (39.6%), followed by Pleosporales (25.2%), 15.2% unidentified, 6.8% Onygenales, 5.8% Agaricales, 4.0% Sordariales, 2.5% Glomerales and 1.3% Capnodiales, while the remainder of orders made up less than 1%. When comparing relative abundance of Xylariales between different plant hosts, differences between grama-in samples and creosote samples ($p=0.015$) as well as differences between grama-out and creosote ($p=0.031$) were both significant but the differences between grama-in and grama-out samples were not significant at level ($p=0.964$). The Xylariales accounted for 61.2% of all creosote sequences but comparatively fewer sequences in the grama-in (17.2%) and grama-out (6.7%) samples. Similar results were found for the Agaricales, with significant differences between creosote and both grass types, but again no significant differences when comparing grass types ($p_{\text{Grama}_{\text{in}}/\text{Creosote}} = 0.0046$; $p_{\text{Grama}_{\text{out}}/\text{Creosote}} = 0.0132$; $p_{\text{Grama}_{\text{in}}/\text{Grama}_{\text{out}}} = 0.6555$). The Agaricales were extremely rare in the creosote samples at 0.03% but made up 16.9% of the grama-in samples and 11.1% of the grama-out samples. None of the other orders showed significant differences by host type.

At the family level, 64.6% were unidentified, Lentitheciaceae represented 17.7%, followed by Sporormiaceae at 4.5%, Marasmiaceae at 2.9%, Tricholomataceae at 2.1%, Chaetomiaceae at
1.6%, Glomeraceae at 1.5% and Cladosporiaceae at 1.0%. All of the remaining families represented fewer than 1% of the sequences. Statistically, only the Lentitheciaceae were shown to be responsive to host type, and both grama-in (8.4%) and grama-out (61%) showed significantly greater relative abundance than creosote (0.7%) ($p_{\text{Grama\_in}/\text{Creosote}} = 0.010$; $p_{\text{Grama\_in}/\text{Creosote}} = 0.014$), but not from one another ($p_{\text{Grama\_in}/\text{Grama\_out}} = 0.965$).

The top 25 OTUs accounted for a majority of the sequences (83.1%). Initially, many of these OTUs were unclassified, but after revising taxonomy based on blastn searches, most of these OTUs were found to belong to the Ascomycota (94.1%), and the rest (5.8%) were identified as Basidiomycota. In the Ascomycota, over half were in the class Sordariomycetes (56.2%), 42.7% were Dothideomycetes, while the rest belonged to the Pezizomycetes (1.0%). All of the Basidiomycota (100%) were classified to the class Agaricomycetes and the order Agaricales. The most abundant genus (45.5%) among the top 25 OTUs was classified as *Monosporascus* (Sordariomycetes, Xylariales, Xylariales insertae sedis), followed by *Darksidea* (Dothideomycetes, Pleosporales, Lentitheciaceae) at 25.3% and *Paraconiothyrium* (Dothideomycetes, Pleosporales, Didymosphaeriaceae) at 8.2%. The rest of the genera accounted for less than 5% of sequences among the top 25 OTUs.

After Kruskal-Wallace statistical tests at the p<0.05 level, 7 of the 25 top OTUs showed significant differences in relative abundance by plant host and 5 showed significant differences by year. In comparisons by host type, most of the responsive OTUs fell into the genus *Darksidea* (OTU2, OTU177, OTU20 and OTU111). OTU2 showed statistically higher abundance in grama-out compared to creosote (48.7% vs. 0.7%). OTU177 was nearly absent in creosote (0.06%) and the abundances in grama-in and grama-out were significantly higher (6.9% and 14.0%, respectively). Similarly, OTU20 was absent in creosote samples but abundant in grama-in samples.
and although relatively rare in grama-out samples (1.2%), there were still statistically significant differences between each of the grass types and creosote. OTU111 was low in overall abundance but showed significant differences in abundance between both grama types (grama-in = 0.08%, grama-out = 2.4%) and creosote (0.5%). OTU6 and OTU170 which classified to the genus *Monosporascus* both showed differences between grama-out and creosote, although OTU170 also showed differences between grama-in and creosote. Both were abundant in creosote (OTU6 = 9.9%, OTU170 = 4.5%) but rare or absent in the grama samples. OTU10 (*Fusarium*) and OTU18 (*Delastria*) both were statistically different in abundance when comparing grama-out and creosote. Both were moderately abundant in grama-out samples but rare or absent in the grama-in samples and creosote. Additionally, none of the 7 OTUs that were responsive to host type showed significant differences between grama-in and grama-out.

Two of the OTUs that were responsive to year of collection belonged to the genus *Monosporascus*, and both showed declines from 2012 to 2015. OTU1 decreased from 14.1% abundance to 1.7%, while OTU3 declined from 9.3% in 2012 to complete absence in 2015. OTU13, identified as *Mycena olida* (Agaricales), and OTU16 identified to the genus *Cladosporium* (Capnodiales) decreased from 3.2% and 1.5%, respectively in 2012, to absence in 2015. OTU177, a species of *Darksidea* (Pleosporales) showed the opposite trend and increased from 0.2% in 2012 to 20.5% in 2015.

Based on analyses using FUNGuild, the top 25 OTUs were found to represent a variety of trophic modes and guilds. Trophic modes were broadly categorized as follows: “(1) pathotroph = receiving nutrients by harming host cells (including phagotrophs); (2) symbiotroph = receiving nutrients by exchanging resources with host cells; and (3) saprotroph = receiving nutrients by
breaking down dead host cells” (Nguyen et al. 2016). Under these broad trophic modes were 12 possible guild categories to reflect lifestyle: animal pathogens, arbuscular mycorrhizal fungi, ectomycorrhizal fungi, ericoid mycorrhizal fungi, foliar endophytes, lichenicolous fungi, lichenized fungi, mycoparasites, plant pathogens, undefined root endophytes, undefined saprotrophs, and wood saprotrophs. FUNGuild designations can also be combined, as many fungi may change categories during their lifecycle or based on environmental conditions.

Creosote samples were dominated by OTUs considered to be pathotrophs (71.0%) but had some representation of saprotrophs (21.2%) and very low representation of other guild types. The pathotrophs fell exclusively in the plant pathogen guild designation, while the saprotrophs were either undefined in terms of guild or considered dung/plant saprotrophs. Grama-in and grama-out samples contained comparatively fewer OTUs associated with pathotrophy, with 19.7% and 7.0% respectively. In addition, grama samples contained OTUs on the pathotroph-saprotroph continuum, with 22.9% in grama-in and 12.0% in grama-out as well as OTUs on the pathotroph-saprotroph-symbiotroph continuum, with 9.7% in grama-in and 11.3% in grama-out. OTUs designated pathotroph-saprotrophs represented guilds of plant pathogens, wood and leaf saprotrophs, as well as undefined saprotrophs, while the pathotroph-saprotroph-symbiotroph trophic mode consisted a wide range of guilds: plant and animal pathogens, lichen parasites, soil and wood saprotrophs, epiphytes and endophytes. In the symbiotroph trophic level, the constituent guilds were either endophytes or ectomycorrhizal. These were almost absent among creosote OTUs (0.8%) but dominant in grama-in (41.5%) OTUs and most abundant in grama-out OTUs (69.6%).

Samples in 2012 were dominated by pathotrophs (48.0%), with less than half as many symbiotrophs (21.9%) while 2015 showed relatively equal representation of pathotrophs (37.9%)
and symbiotrophs (40.4%). Proportions of saprotrophs were fairly consistent across years (11.9% in 2012 and 16.9% in 2015) and pathotroph-saprotrophs showed also similar abundance (7.3% in 2012 and 4.6% in 2015). More OTUs fell into the pathotroph-saprotroph-symbiotroph continuum in 2012 (5.9%) than in 2012 (0.3%).

**Diversity Measures and Ordinations**

For both the raw and rarefied datasets, only the Shannon diversity measures showed a normal distribution. However, this measure did not vary significantly by host type or year of collection when tested by ANOVA at the p<0.05 level. For the diversity measures on the raw dataset, the Chao index showed significant differences for year of collection (2012 vs. 2015), as demonstrated by a Wilcoxon rank sum test (W = 299.5, p<0.001513). Observed diversity also showed significant differences by collection year (W = 293.5, p<0.002649).

NMDS ordinations showed distinct groupings by year, and by host type. For host type, (Figure 1B.) there was some overlap between the grama-in samples and the creosote samples, while grama-out samples were nested within the grama-in samples and did not show any overlap with the creosote samples. For year, (Figure 1A.) there was slight overlap between year 2012 and 2015, but overall these samples were clearly separated.

Per adonis PERMANOVA, both year and host type were shown to be highly significant in terms of structuring the fungal community (p<year = 0.001, R² = 0.052; p<host = 0.001, R² = 0.081). Moreover, there was also a significant effect of the interaction between year and host type (p = 0.013, R² = 0.065). According to a post-hoc, pairwise adonis PERMANOVA, the difference between creosote fungal community and the grama-out fungal community was highly significant (p = 0.003, R² = 0.065), while the creosote samples were also different from the grama-in fungal
community but slightly less so (p = 0.024, R² = 0.050). Differences between the grama-in and grama-out samples were not significant (p = 0.190, R² = 0.071).

DISCUSSION

As variables, year of collection and host type were clear drivers of differences in the root-associated fungal communities of both black grama and creosote. This conclusion is borne out in the NMDS ordinations and adonis PERMANOVA results, which both show that grama-in, grama-out and creosote all host varying fungal consortia, although the differences between the two grama types were not statistically significant at the p<0.05 level. Moreover, the grama-in samples collected from the creosote-grassland transition zone were less dissimilar to creosote than the grama-out samples. Additionally, there appears to be an effect of year of collection in structuring these fungal communities, as well as interactive effect of both year and host type.

Comparisons between grama and creosote by host location and by year at different taxonomic levels also revealed significant differences at every level. However, in no case were the grama-in and grama-out samples statistically dissimilar. On the whole, the grama-out samples were more different from the creosote samples than the grama-in samples were from creosote. This trend can be seen visually in the NMDS ordination in which there is some overlap between the scatter of creosote and grama-out samples but no overlap between grama-in and creosote. On the other hand, differences in taxonomic composition are evident for the two grama types suggesting that shrub encroachment may contribute to shifts in the relative abundances of certain taxa.

In comparisons by year, only the families Glomeraceae (Glomerales) and Tricholomataceae (Agaricales) were found to be responsive and showed opposite trends in terms of abundance at each
time point. The Tricholomataceae decreased in abundance from 2012 to 2015, a trend which also held for one of the top OTUs, OTU13 (*Mycena olida*), because it was the only OTU classified to the family in the dataset. According to FUNGuild, *Mycena olida* has been previously identified as a leaf and wood saprotroph, plant pathogen, as well as an unidentified saprotroph. It is unclear what factors may account for the decreased abundance of *Mycena* from 2012 to 2015.

On the other hand, the Glomeraceae significantly increased in abundance from 2012 to 2015. This group belongs to the arbuscular mycorrhizal fungi (AMF), a lineage that forms symbioses with nearly 80% of vascular plants and can function to increase nutrient uptake from the soil (Schüßler et al. 2001). In other studies, the abundance of AMF appears to correlate with meteorological factors such as sunlight and precipitation (Lingfei et al. 2005) as well as show temporal variation (Husband et al. 2002). In 2012, annual precipitation for our study site at the Sevilleta National Wildlife Refuge was lower than the average in previous years and in fact, the monsoon for that year was 22% below the average for 2001 through 2010 (Petrie et al. 2015). In January through May of 2012 the area received approximately 25% less precipitation, while 2015 received average rainfall in the months preceding the date of sample collection (Moore 2019). Previous studies at the Sevilleta indicate that AMF abundance decreases with lower annual rainfall. This trend was demonstrated for *Bouteloua gracilis* for which Johnson et al. (2003) reported up to 80% AMF root colonization in a high precipitation year, whereas Barrow (2003) and Porras-Alfaro et al. (2007) reported appreciably less colonization in low precipitation years. Declines in AMF were accompanied by concomitant increases in dark septate endophytes (Porras-Alfaro et al. 2007). In our study, however, OTU177, a species of *Darksideo* (Pleosporales) and dark septate endophyte also significantly increased in abundance alongside species in the Glomeraceae. Additionally, AMF fungi
in the phylum Glomeromycota were quite rare overall, reaching a maximum abundance of 5.2% in 2015. Their rarity in the dataset may also owe to primer biases, as the ITS1F and ITS4 are not optimal for AMF taxa and have shown poor amplification in previous studies (Porras-Alfaro et al. 2007).

Other OTUs among the top 25 that showed temporal responses were classified to *Monosporascus* (OTU1 and OTU3) and *Cladosporium* (OTU16). These OTUs all significantly decreased in abundance from 2013 to 2015, in some cases becoming completely absent in the 2015 dataset. According to FUNGuild, these taxa represent a range of guilds. *Cladosporium*, for example, is cited as an animal and plant pathogen, lichen parasite, endophyte and wood saprotroph. Some isolates of *Cladosporium* have been shown to have mutualistic effects on dominant plants in an alpine tundra, by both increasing germination rates and host growth (Tobias et al. 2017). *Monosporascus*, on the other hand, is exclusively designated as a plant pathogen in the FUNGuild database. However, the role that this genus may play in the arid environment of the Sevilleta is ambiguous because it is pervasive in surveys of endophytes and has been isolated from a variety of plants that do not show any obvious signs of pathology (Dean et al. 2015; Porras-Alfaro et al. 2008; Porras-Alfaro et al. 2014). There is also evidence of *Monosporascus* as an endophyte of plants inhabiting saline soils in Spain (Collado et al. 2002). On the other hand, some agricultural strains of *Monosporascus* have been documented to cause root rot and vine decline of cucurbits (Edelstein et al. 1999; Salem et al. 2013). In terms of responses to environmental factors, mycelial growth and reproduction of the species *Monosporascus cannonballus*, a known melon rot pathogen, has been shown to be hindered by reduced water potential (Ferrin and Stanghellini 2006). As such, drought might reduce the root colonization of *M. cannonballus* and other members of the genus. Conversely, sporulation in
*Cladosporium* is shown to be accelerated during warm, dry weather (Kasprzyk et al. 2016). As mentioned, 2012 at the Sevilleta was recorded to be drier year than 2015 so the decreased abundance of *Monosporascus* OTUs in 2015 does not align with previous findings in the literature, while the possibility that *Cladosporium* may have shown increased sporulation in 2012 relative to 2015 is consistent with the literature.

In comparisons by plant for the top 25 OTUs, OTU6 and OTU170 which also classified to *Monosporascus* both showed differences between grama-out and creosote, although OTU170 also showed differences between grama-in and creosote. Both were abundant in creosote (OTU6 = 9.9%, OTU170 = 4.5%) but rare or absent in the grama samples, suggesting that these particular taxa may show specificity to creosote. Although *Monosporascus* was the most abundant genus among the top 25 OTUs, no other OTUs classified to *Monosporascus* showed significant fidelity to creosote samples, nor did any of the other OTUs. At higher levels of classification, the order Xylariales, to which *Monosporascus* belongs, accounted for 61.2% of all creosote sequences and significantly fewer sequences in the grama-in (17.2%) and grama-out (6.7%) samples. Indeed, the Xylariales appear to be associated with creosote, and the fact that grama-in samples contained more than twice the abundance of Xylariales compared to grama-out may relate to their close proximity to creosote. This trend puts forth the possibility that creosote is bringing *Monosporascus* and related endophytes with it during the encroachment process. The transmission mode, however, is unclear as none of the *Monosporascus* species previously isolated from the Sevilleta have been demonstrated to sporulate (Robinson and Natvig 2019). Furthermore, *Monosporascus* may represent a latent pathogen although currently no symptoms of disease are present in any of the plants. Indeed, among the top 25 OTUs, creosote showed the highest representation of pathotrophs (71.0%), whereas grama-in and grama-
out samples showed much lower abundance of pathotrophs, with 19.7% and 7.0% respectively. Instead grama-out and grama-in samples were dominated by symbiotrophs that were either endophytes or ectomycorrhizal at 69.9% and 41.5% respectively, a stark comparison to the 0.8% seen in creosote. In this case, the percentage of symbiotrophs declines with proximity to creosote, suggesting that creosote may be capable of disrupting mutualisms in black grama. Indeed, the introduction of new pathogens and the inference with existing microbial interactions are two known mechanisms by which invasive plants move into new territory (Desprez-Loustau et al. 2007; Pringle et al. 2009).

However, in comparison to creosote, both grama-in and grama-out samples showed higher representation of pathotroph-saprotroph and pathotroph-saprotroph-symbiotroph taxa among the top 25 OTUs, so black grama is not completely devoid of potential pathogens. It is clear, however, that pathogenicity is context dependent and endophytes are known to produce disease upon external stress to the plant (Porras-Alfaro and Bayman 2011). Among these potential pathogens was OTU9 which corresponded to the genus Moniliophthora and was detected exclusively in grama-out samples. Although this genus is acts as a destructive pathogen on cacao, it has appeared previously in surveys of root-associated fungi and was among the dominant taxa in a survey of blue grama in western North America, as well as one of the two most common OTUs in studies of a variety of plants conducted at the Sevilleta NWR (Herrera et al. 2010; Khidir et al. 2010). Again, none of the North American plants infected with Moniliophthora were symptomatic, as seen with Monosporascus, indicating that either its pathogenicity has not been triggered or it in fact acts in a truly endophytic manner.
Another potential pathotroph that was present in black grama but nearly absent in creosote (0.007%) was *Fusarium*. In addition, the genus was quite rare in grama-in samples (0.047%) but the fourth most abundant taxon for grama-out (7.2%) among the top 25 OTUs. Here we see, again, a gradient effect relating to proximity of creosote and the abundance of *Fusarium*, similar to what was seen for symbiotrophs. The nearer a black grama plant is to creosote, the lower representation of *Fusarium* it has. *Fusarium* is also common in plants at the Sevilleta and seems to belong to the core root fungal microbiome as it has been previously isolated from three abundant native plant species: blue grama (*B. gracilis*), sand dropseed grass (*Sporobolus cryptandrus*) and yucca (*Yucca glauca*) (Khidir et al. 2010). As noted by Tequida-Meneses et al. (2002), however, creosote extract can have a potent antifungal effect on *Fusarium*, which might explain its near absence in samples from the creosote stand and the grassland transition zone.

In terms of known symbiotrophs, a gradient effect was also visible for OTUs in the top 25 classified to the genus *Darksidea*. In general, abundance of *Darksidea* was greatest for grama-out samples, with intermediate abundance for grama-in samples and low abundance or absence in creosote. Such was the case for all but two OTUs, OTU11 and OTU20, which both peaked in abundance for grama-in samples (19.6% and 10.2%, respectively) but were rare in both grama-out and creosote samples. At NCBI, these two OTUs received a best BLAST hit to the species *Darksidea zeta*, whereas the other 3 *Darksidea* OTUs received a best hit of *Darksidea alpha*. In molecular studies, *D. zeta* was found to differ from *D. alpha* (CBS 135650) for several genetic markers (Knapp et al 2015). Both species were first classified from isolates derived from plant roots in Hungary, although other members of the genus have also been collected from plants on two other continents, namely in the southwest of North America and from a semi-arid Mongolian steppe in Asia (Knapp
et al. 2012; Porras-Alfaro et al. 2008; Su et al. 2010). One strain of *Darksidea* from the Mongolian steppe, most closely related to *D. zeta*, was found to enhance uptake of potassium, and increase root and shoot biomass of its host plant compared to an un-inoculated host (Li et al. 2018). The authors of this study suggest that this strain may enable its host to withstand drought and nutrient poor conditions. Indeed, as its name suggests, *Darksidea* is a member of the dark septate endophytes (DSE), a group frequently associated with plants in aridlands and generally thought to provide benefits to their hosts (Knapp et al. 2015; Newsham 2011). As Knapp et al. (2012) suggested, DSE in regions of high abiotic stress may show low affinity to a particular host, and instead may act as generalists, even colonizing invasive plants. In our study, however, this appears not to be the case. Sequences matching *Darksidea* among the top 25 OTUs showed high fidelity to black grama, with only miniscule abundance in creosote. The fact that *Darksidea* abundance was generally lower in grama-in samples suggests that creosote may be capable of disrupting this mutualism.

Other OTUs of interest among the top 25, include a match to a thermophilic fungus, *Myceliphthora heterothallica*, and two matches to the saprotroph genus *Preussia*. Both of these fungi have been previously isolated from herbivore dung and belong to families noted for their ability to decompose plant material (Ames 1963, Cain 1961). Saprotrophic fungi, and coprophilous (dung-decomposing) fungi have been previously recovered from plants at the Sevilleta (Porras-Alfaro et al. 2009; Herrera et al. 2011). One hypothesis suggests that these fungi may represent early colonizers that act as latent saprotrophs in plant tissue and only become active either after tissue senescence or after ingestion and excretion by herbivores (Porras-Alfaro and Bayman 2011; Herrera et al. 2011). Indeed, some fungal spores have been shown to require passage through the digestive tract to induce germination (Webster 1970). The presence of a *M. heterothallica* is also particularly notable because
no previous reports of thermophilic root associated fungi have been published. However, there is a record of an unclassified fungal clone sequence from foliar tissue of a desert tree *Parkinsonia microphylla*, that hits to a sister species, *M. thermophila* (Massimo et al. 2015). Here, we find that *M. heterothallica* is virtually exclusive to grama-in samples, being absent in creosote, and low in abundance (1.1%) in “grama out.” *Preussia*, on the other hand was nearly absent in grama-out and one OTU showed specificity to creosote, while the other showed specificity to grama-in samples. Overall, it is unclear how these saprotrophic fungi may impact their hosts and why some OTUs may show differential abundance between hosts.

**CONCLUSIONS**

Here we report for the first time a comprehensive survey of the root associated fungi in creosote and co-occurring black grama, within and outside a creosote stand, in attempts to understand the microbial implications of shrub encroachment. Our results revealed a significant effect of both host type and year of collection on the fungal communities colonizing the roots of these plants. Specifically, the root-fungal community of creosote was found to be distinct from both the black grama plants within the creosote stand and black grama plants in the main grassland. Although the communities of fungi from both black grama types were not significantly different, there were some notable trends. Among the top 25 OTUs in the entire dataset, those classified as symbiotrophs were most common in black grama outside the creosote stand, intermediate for black grama within the creosote stand and virtually absent for creosote samples. These OTUs matched to species in the genus *Darksidea*, a taxon classified as a dark-septate endophyte and known for mutualistic interactions with plants (Li et al. 2018). Top OTUs classified as pathotrophs show the opposite trend, with peak abundance in creosote samples, intermediate abundance for grama within
the creosote stand and lowest abundance for grama outside the creosote stand. Most of these pathotrophs were best classified to the genus Monosporascus, a nebulous yet abundant group of fungi associated with plants growing in environments with abiotic stress (Dean et al. 2015; Porras-Alfaro et al. 2008; Porras-Alfaro et al. 2014; Collado et al. 2002).

Black grama plants did however harbor some putative pathogens such as Fusarium and Moniliophthora that were rare and absent in creosote, respectively. However, both of these genera are commonly recovered from plants at the Sevilleta and show no apparent ill effect to their hosts (Herrera et al. 2010; Khidir et al. 2010). The decreased abundance of Fusarium for grama-in samples compared to grama-out samples may relate to the demonstrated antifungal effect of creosote on this fungus. Furthermore, this antifungal activity may disrupt the existing association between Fusarium and its black grama hosts. There is precedent for this mechanism in other invasive plants, and it may act in concert with the introduction of new pathogenic fungi.

The findings for creosote parallel other studies on shrub encroachment and its effect on microbial communities has been examined for other systems. For example, in an Inner Mongolian grassland, when compared to control grassland sites, sites experiencing encroachment displayed greater bacterial alpha diversity in soils and with changes in functional traits of the community (Xiang et al. 2018). Based on phospholipid fatty acid assays, soils in these shrub sites also showed higher biomass and abundance of gram-negative bacteria, arbuscular mycorrhizal fungi and actinomycetes (Li et al. 2017). Results from a study of mesquite encroachment in the Great plains echo the findings for Mongolian grasslands, and higher bacterial and fungal diversity were observed for shrub sites than for grassland sites and mesquite soil contained a unique fungal community.
compared to other plant types (Hollister et al. 2010). Together, these studies suggest that as shrubs encroach, they can alter the communities of microbes in their new territory.

References


Fig 1. Taxonomic composition by sample types. A and B: Phylum representation by year and plant type. C and D: Representation by taxonomic orders in the Basidiomycota. E and F: Abundance for orders in the Ascomycota.
Fig 2. Overlap in OTU composition. A. Number of shared and unique OTUs between years. B. Number of shared and unique OTUs among host plant types.
Fig 3. Figures A and B depict identical NMDS Ordinations, but 3A is colored by year while 3B is colored by plant type. Ellipses are fitted by variable. Distinct groupings are visible for both variables.
Table 1: Results for adonis PERMANOVA.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Df</th>
<th>Sums of Sqs</th>
<th>Mean Sqs</th>
<th>F Model</th>
<th>R²</th>
<th>p-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td>1</td>
<td>0.9273</td>
<td>0.92725</td>
<td>2.1342</td>
<td>0.05186</td>
<td>0.001</td>
<td>***</td>
</tr>
<tr>
<td>Plant</td>
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<td>1.4496</td>
<td>0.72480</td>
<td>1.6682</td>
<td>0.08108</td>
<td>0.001</td>
<td>***</td>
</tr>
<tr>
<td>Year x Plant</td>
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<td>1.1640</td>
<td>0.58200</td>
<td>1.3396</td>
<td>0.06511</td>
<td>0.013</td>
<td>*</td>
</tr>
</tbody>
</table>

Table 2: Results for post-hoc adonis PERMANOVA.

<table>
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<th>Pairs</th>
<th>Df</th>
<th>Sums of Sqs</th>
<th>F Model</th>
<th>R²</th>
<th>p-value</th>
<th>p-adjusted</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creosote vs Grama Out</td>
<td>1</td>
<td>0.8815771</td>
<td>1.941513</td>
<td>0.0648435 0</td>
<td>0.001</td>
<td>0.003</td>
<td>*</td>
</tr>
<tr>
<td>Creosote vs Grama In</td>
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<td>0.6835587</td>
<td>1.473665</td>
<td>0.0499993 8</td>
<td>0.008</td>
<td>0.024</td>
<td>.</td>
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<tr>
<td>Grama Out vs Grama In</td>
<td>1</td>
<td>0.5459648</td>
<td>1.222898</td>
<td>0.0710042 1</td>
<td>0.190</td>
<td>0.570</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 contains adonis PERMANOVA findings. As variables, both year and plant are highly significant while the interaction of both is slightly less significant. Table 2 contains post-hoc adonis results. Creosote vs. Grama-Out differences were highly significant, while Creosote vs. Grama-In differences were somewhat less significant.
Summary

Fungi possess remarkable adaptations to their given environmental niches. Within this dissertation, I have presented research on fungi that thrive under extreme heat and those that form endosymbioses with plants. The primary findings of the three chapters of my dissertation are summarized below.

Studies with thermophilic fungi

1. The fungus *Myceliophthora heterothallica* shows promise as a model organism for the production of thermally stable enzymes, especially those that are cellulose-active. Also, as implied by its name, it was thought to be a heterothallic fungus that must outcross to reproduce sexually. Heterothallism is a valuable trait because it enables researchers to cross strains to select for traits and to understand the genetics that underlie phenotypes. Crosses performed with wild-type strains of *M. heterothallica* revealed the independent assortment of traits for mating compatibility and an actin-like gene. Crosses between a UV mutagenized, fungicide resistant strain and a wild-type parent also produced progeny with non-parental phenotypes. These results confirmed that heterothallism is in fact the reproductive mode used by *M. heterothallica*. Furthermore, *M. heterothallica* is a good fit under the definition of a thermophilic or “heat-loving” fungus, with a growth optimum of ~45°C. On the other hand, sexual reproduction was observed to occur most readily at 29°C. Together, the traits of thermophily and heterothallism make *M. heterothallica* a good candidate for strain-engineering in industrial applications. *M. heterothallica* can also serve as a model for understanding the reproductive modes of other members of its family, the Chaetomiaceae. Strains containing the mat A idiomorph region have the canonical mating gene arrangements...
seen in other model fungi such as *Neurospora crassa*. However, strains with the mat a idiomorph contain not only the typical mat a-1 gene but also a fragment of the A-1 gene that is characteristic of strains with the mat A idiomorph region. This fragment is truncated at the 5’ end and lacks most of an Alpha 1 domain and all of the HMG domain which are thought to be required for fertility. Typically, the introduction of the Alpha 1 domain from one strain into a strain of opposite mating type is known to cause a lethal incompatibility reaction, but this outcome may be avoided in *M. heterothallica* because only a fragment of this domain is present. After obtaining genome sequences for several strains of *M. heterothallica* and other relatives in the Chaetomiaceae, the trend seen in mat A strains was found to be consistent for other out-crossing members of the group. Self-fertile members of the Chaetomiaceae contained an intact mat A region with the addition of a mat a-1 ortholog elsewhere in the genome. On the other hand, several species with unknown reproductive modes were found to contain an intact mat A region without a known mat a-1 ortholog in the genome, leaving open the possibility that these may be obligately out-crossing species.

2. While thermophilic fungi are well understood in industrial applications, their natural biology and ecology have received far less attention. To address this gap in knowledge, I expanded upon a survey conducted at the Sevilleta National Wildlife Refuge and collected thermophilic fungi from a variety of substrates on a transect of western North America. Thermophilic fungi could be recovered from every substrate type and nearly every location, from latitudes of 21° N to 49° N and elevations of 2574 feet to 9950 feet. Isolates represented 14 putative OTUs and were derived from three known orders of thermophilic
fungi: the Eurotiales, the Sordariales and Mucorales. Species in the Mucorales were isolated exclusively from soil, either in the form of topsoil or rhizosphere soil. The isolates in other two orders derived from all substrate types, including both soil types as well as herbivore droppings and leaf litter. Overall, there was no specificity observed for the Eurotiales or the Sordariales, in terms of substrate preference or geographic location. However, comparatively more isolates were recovered from lower latitudes that higher latitudes, suggesting that thermophilic fungi may be adapted to the warmer climate in these regions.

**Studies with endophytic fungi**

1. Root-associated fungi are known to drive interactions between plant species. For this reason, I chose to investigate the fungi associated with an encroaching shrub species, *Larrea tridentata* (creosote), as well as fungi associated with the grass species *Bouteloua gracilis* (black grama), which is currently losing territory to creosote. I harvested roots from creosote, black grama growing among creosote in the encroachment area, and black grama growing in the unencroached grassland. Using next generation sequencing techniques, I was able to characterize the root fungal communities of these three plant types and found that creosote root samples were significantly different from roots of either of the two black grama types but that the black grama types were not significantly different from one another. However, there appeared to be shifts in the black grama root fungal community based on the proximity to creosote. Specifically, dark-septate endophytes in the Pleosporales, primarily in the genus *Darksidea*, were most abundant in black grama in the main grassland, intermediate in abundance for black grama in the creosote stand and least abundant in creosote. Conversely,
fungi in the Xylariales, primarily in the genus *Monosporascus*, were most abundant in creosote, intermediate for black grama in the creosote stand and least abundant in black grama from the main grassland. Pleosporales are known to provide benefit to their host plant in terms of drought resistant, ultra-violet protection and defense from herbivory. On the other hand, the genus *Monosporascus* contains species that are known pathogens of cucurbits although it has also been frequently isolated from a variety of plants at the Sevilleta which show no signs of pathology. Currently, the effect of *Monosporascus* species on black grama and creosote is unknown. Although creosote is not technically an invasive species, the mechanisms of shrub encroachment are thought to parallel invasion of exotic plants. Invasive plants are known to disrupt existing mutualisms between microbes and native plants, as well as to introduce foreign microbes. It is therefore possible that creosote is capable of disrupting the association between black grama and members of the Pleosporales, while introducing fungi related to the pathogenic fungus *Monosporascus*. 