Unisexual Reproduction in *Huntiella moniliformis*

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ABSTRACT

Sexual reproduction in fungi is controlled by genes present at the mating type (*MAT*) locus, which typically harbours transcription factors that influence the expression of many sex-related genes. The *MAT* locus exists as two alternative idiomorphs in ascomycetous fungi and sexual reproduction is initiated when genes from both idiomorphs are expressed. Thus, the gene content of this locus determines whether a fungus is heterothallic (self-sterile) or homothallic (self-fertile). Recently, a unique sub-class of homothallism has been described in fungi, where individuals possessing a single *MAT* idiomorph can reproduce sexually in the absence of a partner. Using various mycological, molecular and bioinformatic techniques, we investigated the sexual strategies and characterized the *MAT* loci in two tree wound-infecting fungi, *Huntiella moniliformis* and *Huntiella omanensis*. *H. omanensis* was shown to exhibit a typically heterothallic sexual reproductive cycle, with isolates possessing either the *MAT1-1* or *MAT1-2* idiomorph. This was in contrast to the homothallism via unisexual reproduction that was shown in *H. moniliformis,* where only the *MAT1-2-1* gene was present in sexually reproducing cultures. While the evolutionary benefit and mechanisms underpinning a unisexual mating strategy remain unknown, it could

have evolved to minimize the costs, while retaining the benefits, of normal sexual reproduction.

KEYWORDS

- *Ceratocystis*
- *Huntiella*
- Unisexual reproduction
- Mating type (*MAT*) locus
- Heterothallic

1. INTRODUCTION

Despite being a costly process, sexual reproduction allows for the generation of genetic diversity and can act to purge deleterious alleles from a population (BARTON 2009; NIELSEN 2006). These benefits likely explain the ubiquitous nature of sex in the Eukaryota (NIELSEN 2006). While many fungi are able to reproduce asexually, sexual reproduction is found in all four major fungal phyla (LEE *et al.* 2010). The numerous and diverse strategies employed by these organisms to retain sexual reproduction, demonstrates the benefits of preserving this type of reproduction, whilst also providing numerous opportunities to minimize the high costs associated with this process (ROACH *et al.* 2014).

Sexual reproduction in fungi is controlled by genes present at the mating type (*MAT*) locus, which are typically involved in the transcriptional regulation of other sex-related genes, such as those involved in mate recognition and meiosis (KRONSTAD and STABEN 1997). In the ascomycetes, this locus has two alternative forms, known as the *MAT1-1* and *MAT1-2* idiomorphs, which possess non-allelic gene combinations (METZENBERG and GLASS 1990). The *MAT1-1* idiomorph characteristically possesses, at minimum, the *MAT1-1-1* gene, which encodes a protein with an alpha box DNA-binding domain, and is homologous to *MATα1* of *Saccharomyces cerevisiae* (TURGEON and YODER 2000). Other genes, including *MAT1-1-2* and *MAT1-1-3* can also be present in the idiomorph (FERREIRA *et al.* 1996;

WILKEN *et al.* 2014). Similarly, the *MAT1-2* idiomorph typically harbours the *MAT1-2-1* gene, encoding an HMG box protein (TURGEON and YODER 2000). Although this is generally the only gene present at this locus, other genes including the *MAT1-2-2*, *MAT1-2-3, MAT1-2-4* and *MAT1-2-5* genes have also been described and are named in the order of their discovery (BIHON *et al.* 2014; MANDEL *et al.* 2007; MARTIN *et al.* 2011; PÖGGELER and KÜCK 2000). The expression of genes from both these idiomorphs is typically required for the completion of a sexual cycle (COPPIN *et al.* 1997) and thus, fungal mating systems can be classified based on the structure and gene content of the *MAT* locus.

Sexual reproduction has classically been divided into two mating systems. Of these, heterothallism is characterized by the requirement of two compatible mating partners for sexual reproduction (KRONSTAD and STABEN 1997). In this case, each self-sterile partner possesses genes from a different *MAT* idiomorph and the combined expression of both idiomorphs results in a successful mating interaction. Species exhibiting heterothallism are wide-spread (BILLIARD *et al.* 2012) and include commonly studied fungi such as *Neurospora crassa* and *Podospora anserina* (GLASS *et al.* 1990a; PICARD *et al.* 1991). In contrast, homothallism represents a situation where a single individual is able to progress through a full sexual cycle without an opposite mating partner (KRONSTAD and STABEN 1997). Homothallic fungi are thus self-fertile and typically possess genes from both *MAT* idiomorphs in a single genome (COPPIN *et al.* 1997).

Homothallism encompasses a heterogeneous assemblage of mechanisms and it has thus been further sub-divided into discrete categories. These include primary homothallism (LIN and HEITMAN 2007), as exhibited by *Sodaria macrospora* where both *MAT* idiomorph genes are present in a single genome (PÖGGELER *et al.* 1997); mating type switching, found in *S. cerevisiae* where the *MAT* locus identity can change via gene conversion (HABER 1998) and pseudohomothallism, such as that occurring in *N. tetrasperma* where nuclei of opposite mating types are packaged in a single ascospore (MERINO *et al.* 1996). A unique sub-class of homothallism known as unisexual reproduction has also been discovered in fungi, including four *Neurospora* spp., *Cryptococcus neoformans* and *Candida albicans* (ALBY and BENNETT 2011; GLASS and SMITH 1994; LIN *et al.* 2005). In unisexual reproduction, also known as same-sex mating, isolates with only a single mating type have the ability to initiate

and proceed through an entire sexual cycle. This can occur within a single cell or between two cells of different lineage, but identical mating type (ROACH *et al.* 2014).

Fungal species in the Ceratocystidaceae (DE BEER *et al.* 2014) include tree-infecting species and important tree pathogens in genera such as *Ceratocystis* (WINGFIELD *et al.* 2012). Genera in this family typically exhibit a wide variety of sexual reproductive strategies, including heterothallism as employed by *Davidsoniella eucalypti* (KILE *et al.* 1996), primary homothallism as seen in *Thielaviopsis cerberus (MBENOUN et al. 2014)* and homothallism via unidirectional mating type switching as observed in *Ce. fimbriata* (HARRINGTON and MCNEW 1997; WILKEN et al. 2014; WITTHUHN et al. 2000).

Species in the genus *Huntiella*, previously accommodated in the *Ce. moniliformis* complex (DE BEER *et al.* 2014), have been described as homothallic (AL-SUBHI *et al.* 2006; HARRINGTON 2007). However, these species are cosmopolitan saprobes occurring on freshly made tree wounds (VAN WYK *et al.* 2006) and their mating strategies have not been intensively studied. The aim of this study was to investigate the mating strategy and characterize the *MAT* loci of two *Huntiella* species, *H. omanensis* and *H. moniliformis*.

2. METHODS AND MATERIALS

2.1 Sexual Reproduction in *Huntiella omanensis and H. moniliformis* Cultures

Two isolates of *H. omanensis* and eight isolates *H. moniliformis* were used in this study (Table 1). All cultures are preserved in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. The isolates were grown and maintained on 2% malt extract agar plates (20 $g.L^{-1}$ malt extract (Biolab, Merck), 20 $g.L^{-1}$ agar (Biolab, Merck), supplemented with thiamine (100mg/l, SIGMA, Steinheim, Germany) and streptomycin (150mg/l, SIGMA, Steinheim, Germany), forthwith referred to as MEA-ST plates. The cultures were kept at 22°C for the duration of the study.

H. omanensis and *H. moniliformis* isolates that produced mature sexual structures (ascomata from which ascospore masses exuded) were used as parental cultures from which single ascospore progeny were generated. These isolates were

Table 1: *Huntiella moniliformis* and *H. omanensis* Isolates Used in This Study

Species	CMW Number [a]	Details of Isolation (Host, Country, Locality, Year)	Sporulating
H. moniliformis	10134	*Eucalyptus grandis*, South Africa, Mpumalanga, 2002	Yes
H. moniliformis	36895	*Theobroma cacao*, Cameroon, Ebolowa, 2009	Yes
H. moniliformis	36896	*Theobroma cacao*, Cameroon, Ebolowa, 2009	Yes
H. moniliformis	36897	*Theobroma cacao*, Cameroon, Ebolowa, 2009	Yes
H. moniliformis	36908	*Theobroma cacao*, Cameroon, Bokito, 2009	Yes
H. moniliformis	36919	*Theobroma cacao*, Cameroon, Ngomedzap, 2009	Yes
H. moniliformis	36923	*Theobroma cacao*, Cameroon, Ngomedzap, 2009	No
H. moniliformis	37105	*Terminalia superbra,* Cameroon, Ngomedzap, 2009	No
H. omanensis	11046	*Mangifera indica*, Oman, 2003	Yes
H. omanensis	11046.1	Single spore progeny of CMW 11046 (*MAT1-2*) [b]	No
H. omanensis	11046.2	Single spore progeny of CMW 11046 (*MAT1-1*) [b]	No
H. omanensis	11046.3	Single spore progeny of CMW 11046 (*MAT1-2*) [b]	No
H. omanensis	11046.4	Single spore progeny of CMW 11046 (*MAT1-2*) [b]	No
H. omanensis	11046.5	Single spore progeny of CMW 11046 (*MAT1-1*) [b]	No
H. omanensis	11056	*Mangifera indica*, Oman, 2003	Yes
H. omanensis	11056.1	Single spore progeny of CMW 11056 (*MAT1-1*) [b]	No
H. omanensis	11056.2	Single spore progeny of CMW 11056 (*MAT1-2*) [b]	No
H. omanensis	11056.3	Single spore progeny of CMW 11056 (*MAT1-1*) [b]	No
H. omanensis	11056.4	Single spore progeny of CMW 11056 (*MAT1-1*) [b]	No
H. omanensis	11056.5	Single spore progeny of CMW 11056 (*MAT1-2*) [b]	No

[a] CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria

[b] *MAT1-1* and *MAT1-2: MAT* genes amplified from the *H. omanensis* single spore progeny

generated by soaking ascospore masses in 20 µl Soltrol 130 oil (Chemfit, Gauteng, SA) and streaking them out on fresh MEA plates. After approximately 12 hours, individual germinating ascospores were transferred to fresh MEA-ST plates and allowed to grow for 10 to 15 days. These single ascospore progeny were then visually screened for the presence of ascomata exuding ascospore masses. The

production of ascomata and ascospore masses was used as an indication of successful sexual reproduction.

Cultures that had not shown signs of ascomatal production were then paired in all possible combinations with other non-sporulating cultures. This involved placing a mycelium-covered agar block from each partner approximately 2cm apart on a single 2% MEA-ST plate. The paired cultures were allowed to grow for 10 days, and were then screened daily for the presence of ascomata. In order to test the viability of the ascospore masses produced by these cultures, two to three masses per culture were transferred to fresh 2% MEA plates and allowed 10 to 15 days to grow.

In addition to the intra-species crosses performed between non-sporulating isolates, inter-species crosses between *H. omanensis* and *H. moniliformis* isolates were also conducted. The culturing method used was the same as that for the intra-species pairings. In this case, MAT1 single ascospore isolates produced from *H. omanensis* (CMW 11056) were co-incubated with all eight isolates of *H. moniliformis*. Furthermore, MAT2 single ascospore isolates produced from *H. omanensis* (CMW 11056) were also co-incubated with the *H. moniliformis* isolates.

Micrographs of the ascomata and ascospores were captured using an AxioCAm MRc mounted on a Zeiss Axioskop 2 Plus compound microscope. The ascomata at different stages of development were prepared and arranged for microscopy on 2% MEA medium, whereas the hyphae and ascomatal bases were prepared on glass slides and mounted in 85% lactic acid.

2.2 *MAT* Gene Discovery and *MAT* Locus Structure

The genes present at and associated with the *MAT* locus in *Ce. fimbriata* (accession number KF033902, WILKEN *et al.* 2014) were used in local tBLASTn searches against the draft genome assembly of *H. omanensis* (accession number JMSH00000000, VAN DER NEST *et al.* 2014b) as well as the draft genome assembly of *H. moniliformis* (accession number JSUI00000000, VAN DER NEST *et al.* 2014a) using CLC Genomics Workbench 7.5 (CLC Bio, Denmark). These included the *MAT* genes: *MAT1-1-1*, *MAT1-1-2* and *MAT1-2-1* as well as the *MAT* locus-associated genes: *Cytoskeleton assembly protein* (*SLA*), *Anaphase promoting complex* (*APC*)

and *DNA lyase* (*APN*). Furthermore, a number of other *MAT1-1* gene sequences from various ascomycetous fungi were used in local tBLASTn searches against the two genome assemblies (Table 3).

Contigs showing hits with an E-value of 10^{-5} or less for the *MAT* and *MAT* locus-associated genes were subjected to gene prediction using the web-based AUGUSTUS gene prediction tool (STANKE *et al.* 2004). These contigs were annotated using the AUGUSTUS GFF output file and the "annotate with GFF/GTF/GVF file" function on CLC Main Workbench 6.8 (CLC Bio, Denmark). Gene identity was confirmed using these sequences as queries in an NCBI BLASTn search within the non-redundant nucleotide database (ALTSCHUL *et al.* 1990). Any *MAT* genes identified in *H. omanensis* were used as queries in local tBLASTn searches against the *H. moniliformis* genome.

The corresponding nucleotide sequences of the *MAT* and *MAT* locus-associated genes in *H. omanensis* were compared with those in *H. moniliformis.* This involved aligning the homologs with a gap cost of 10.0 and a gap extension cost of 1.0, before calculating percentage identity using the "create pairwise comparison" option in CLC Main Workbench 6.8. The coding sequence (CDS) of each gene was also translated to its corresponding amino acid sequence, aligned and compared using the technique described above. The translated amino acid sequence of the *MAT* and *MAT* locus-associated genes were also subjected to conserved domain prediction. This was completed using NCBI Conserved Domain Search (MARCHLER-BAUER *et al.* 2011), InterPro Protein Sequence Analysis and Prediction (HUNTER *et al.* 2011) as well as PROSITE (SIGRIST *et al.* 2012), using the default settings for each tool.

In addition, the raw sequence data generated during the sequencing of both genomes was mapped to the *MAT* regions of the sister species. To do this, the raw data for each species was obtained from the authors (VAN DER NEST *et al.* 2014a; VAN DER NEST *et al.* 2014b). The *H. moniliformis MAT* locus was used as a reference sequence to which the raw reads from the *H. omanensis* genome were mapped. Similarly, the raw reads from the *H. moniliformis* genome were mapped to the *H. omanensis MAT* loci. Both of the analyses were carried out in CLC Genomics Workbench 7.5.

2.3 Screening for *MAT* Genes in *Huntiella* Isolates

A PCR approach was used to determine which of the *MAT* genes were present in *H. omanensis* and *H. moniliformis* cultures for which genome sequence was not available. This included cultures preserved in the CMW culture collection as well as at least six single spore isolates generated from each sporulating culture. To do this, PCR primers were designed using CLC Main Workbench 6.8 (Table 2). Primers for *MAT1-1-1* (targeting the conserved alpha box) and *MAT1-1-2* were designed based on the *H. omanensis* gene sequences. Primers for *MAT1-2-1* were designed based on an alignment of the *H. omanensis* and *H. moniliformis* gene sequences, using areas conserved between the two species as target sites.

Table 2: *MAT* Gene Primer Sequences

Primer Name	Sequence (5' → 3')	Target Gene	Amplicon Size (bp)
Oman_111_F	CGGCTCATCCCCAAATCT	*MAT1-1-1*	335
Oman_111_R	AGCTCCCCTACTTCGTTAC		
Oman_112_F	GGGATTGAGACCGGCAAA	*MAT1-1-2*	298
Oman_112_R	CAGTTCGTGTTTTCTCTGT		
Om_Mo_121_F	ATTGCTGGCTGATTTCACG	*MAT1-2-1*	572
Om_Mo_121_R	TAGTCTGGGTGGGTGTTC		

DNA was extracted for PCR from 6 to 8-day-old cultures that were grown on 2% MEA-TS plates. Mycelium was harvested by scraping the surface of cultures before using a standard phenol/chloroform extraction method (PŁAZA *et al.* 2004). Standard 25 µl PCR reactions were subsequently conducted using the protocol supplied with KAPA Taq (KapaBiosystems, Massachusetts, USA). The protocol required 1X Buffer A, $MgCl_2$ at 1.5 mM, total dNTPs at 0.8 mM, forward and reverse primers at 0.5 µM each, 1 unit of KAPA Taq and approximately 25 ng of DNA. Amplification was carried out on a BioRad C1000 Touch^{TM} thermocycler (BioRad, California, USA) using the following protocol: initial denaturation at 95°C for 3 min, 35 cycles of denaturation (95°C for 30 sec), annealing (53°C for 30 sec) and extension (72°C for 1 min) and a final extension at 72°C for 1 min. The products were kept at 4°C until they were electrophoresed through 2% (w/v) agarose gels at 80V for 45 min.

PCR products were purified using the Zymo Research DNA Clean and ConcentratorTM-5 Kit (Zymo Research Corporation, Irvine, USA) following the manufacturer's protocol for dsDNA products. The purified products were then cycle-sequenced using a sequencing reaction with the BigDye® Terminator Cycle Sequencing Kit v3.1 (Life Technologies, Carlsbad, USA), also per the manufacturer's instructions. The sequencing protocol was carried out on a Bio-Rad C1000 TouchTM Thermal Cycler. Finally, the sequencing products were precipitated using a standard ethanol precipitation protocol (SAMBROOK *et al.* 1989) and submitted to the Bioinformatics and Computational Biology Unit at the University of Pretoria for Sanger sequencing.

3. RESULTS

3.1 Sexual Reproduction in *Huntiella omanensis* and *H. moniliformis* Cultures

Visual inspection revealed that the *H. omanensis* cultures used in this study were able to produce ascomata and viable ascospores after 10 to 15 days of growth (Figure 1A). The single ascospore isolates generated from these parental cultures were unable to reproduce sexually in isolation. However, when these isolates were paired in various combinations, ascomata and ascospores were once again produced in a number of the crosses (Figure 2, A & D).

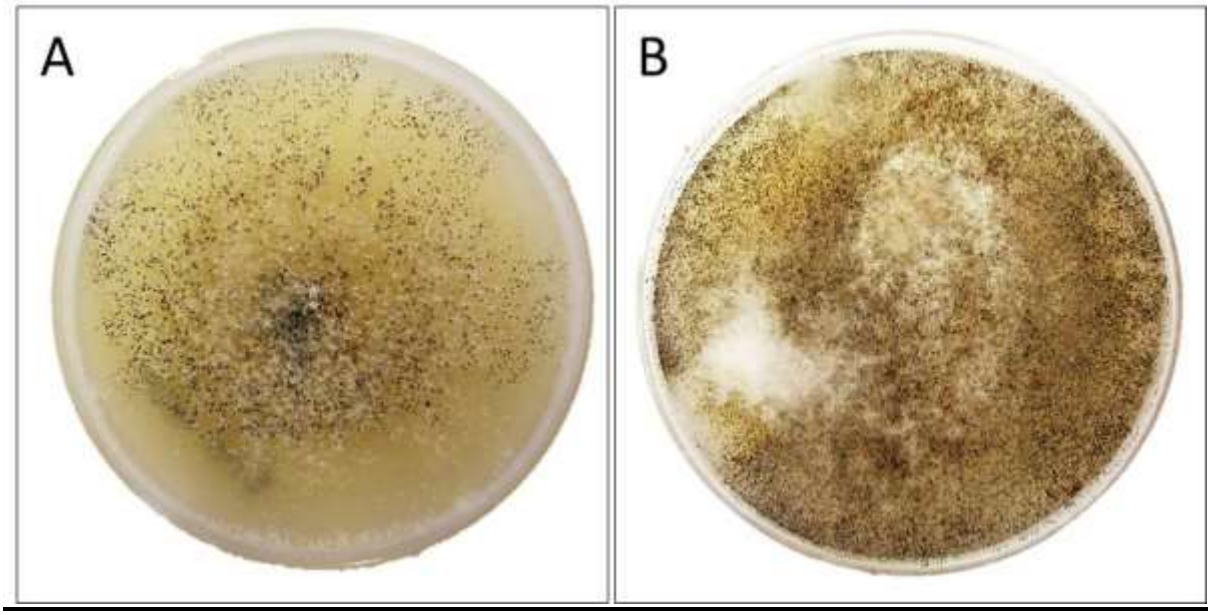

Figure 1: Sporulating cultures of the two *Huntiella* species. **A:** *H. omanensis* undergoes sexual reproduction when two opposite mating types isolates are cultured together. **B:** *H. moniliformis* is able to undergo sexual reproduction from single isolated ascospores.

Of the eight *H. moniliformis* cultures used in this study, six were able to produce ascomata and viable ascospores after 10 to 15 days of growth (Figure 1B). An average of five of the six single ascospore progeny generated from each of the parental *H. moniliformis* cultures retained their ability to reproduce sexually (Figure 2, e & h). When paired in various combinations, however, none of the sterile *H. moniliformis* isolates produced ascomata.

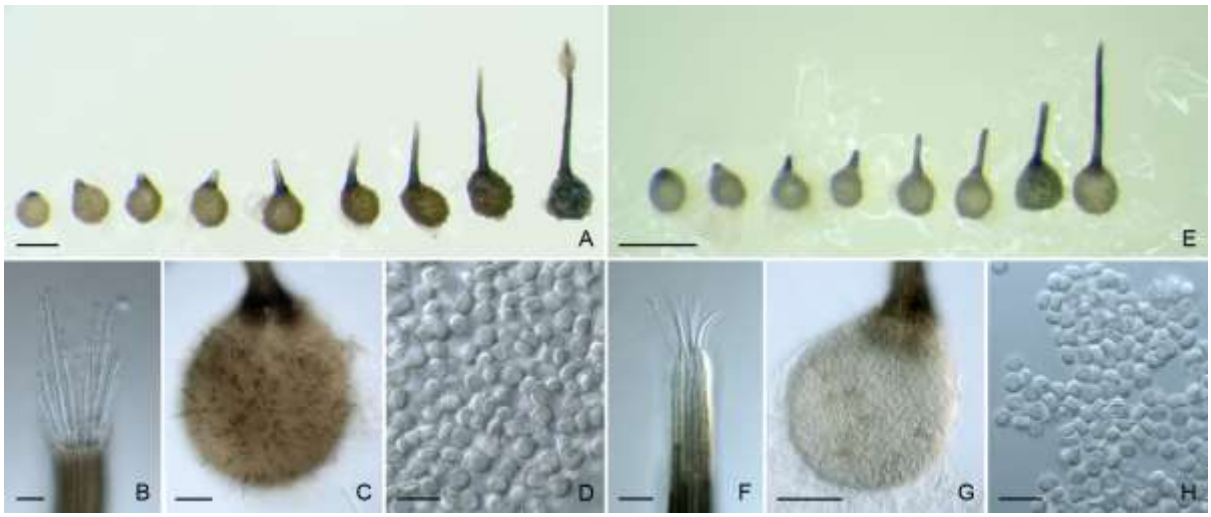

Figure 2: Microscope images of characteristics representing the sexual structures of *H. omanensis* (**A**-**D**) and *H. moniliformis* (**E**-**H**). **A** & **E**: Ascomata at different stages of development, from young (left) to mature (right). **B** & **F**: Divergent ostiolar hyphae. **C** & **G**: Ascomatal bases. **D** & **H**: Hat-shaped ascospores. Scale bars: **A** & **E** = 200 µm; **B**, **F**, **D** & **H** = 10 µm; **C** & **G** = 50 µm.

No successful mating was observed in the inter-species crosses performed between isolates of *H. omanensis* and *H. moniliformis*. The various MAT1 and MAT2 *H. omanensis* isolates were unable to reproduce sexually and form ascomata and ascospores when cultured alongside any of the eight *H. moniliformis* isolates. Thus, there was no evidence of hybridization between the two species.

3.2 *MAT* Gene Discovery and *MAT* Locus Structure

The tBLASTn searches revealed the presence of *MAT1-1-1*, *MAT1-1-2*, *MAT1-2-1*, *SLA*, *APC* and *APN* gene homologs in the *H. omanensis* assembly. The *MAT1-1-1* and *MAT1-1-2* genes were found together on a single contig, while the *MAT1-2-1* gene was found on a separate contig. The *MAT1-1* idiomorph (possessing the *MAT1-1-1* and *MAT1-1-2* genes) as well as the *MAT1-2* idiomorph (possessing the *MAT1-2-1* gene) were both found to be associated with the *SLA* and *APC* genes (Figure 3A). AUGUSTUS predicted an additional two genes flanking the other side of

Table 3: *MAT1-1* Gene Sequences used as queries for Local tBLASTn Searches against the *H. moniliformis* Genome Assembly

Gene	Species	NCBI Gene ID/ Accession No.	Reference
MAT1-1-1	*Aspergillus flavus*	7920875	(YU *et al.* 2005)
MAT1-1-1	*Aspergillus niger*	4985235	(PEL *et al.* 2007)
MAT1-1-1	*Aspergillus nidulans*	BK001307	(DYER *et al.* 2003)
MAT1-1-1	*Aspergillus oryzae*	5996547	(MACHIDA *et al.* 2005)
MAT1-1-1	*Beauveria bassiana*	19890745	(XIAO *et al.* 2012)
MAT1-1-1	*Fusarium pseudograminearum*	20366840	(GARDINER *et al.* 2012)
MAT1-1-2	*Fusarium pseudograminearum*	20366841	(GARDINER *et al.* 2012)
MAT1-1-3	*Fusarium pseudograminearum*	20366842	(GARDINER *et al.* 2012)
MAT1-1-1	*Magnaporthe oryzae*	AB080668	(KANAMORI *et al.* 2007)
MAT1-1-2	*Magnaporthe oryzae*	AB080668	(KANAMORI *et al.* 2007)
MAT1-1-1	*Mycosphaerella graminicola*	13401352	(GOODWIN *et al.* 2011)
MAT1-1-1	*Neofusicoccum parvum*	19020696	(BLANCO-ULATE *et al.* 2013)
MAT A1	*Neurospora crassa*	3880391	(GALAGAN *et al.* 2003)
MAT A2	*Neurospora crassa*	3880488	(GALAGAN *et al.* 2003)
MAT A3	*Neurospora crassa*	3880489	(GALAGAN *et al.* 2003)
MAT-	*Podospora anserina*	X64194	(DEBUCHY and COPPIN 1992)
MAT α	*Saccharomyces cerevisiae*	850407	(OLIVER *et al.* 1992)
MAT1-1-1	*Trichoderma reesei*	FJ599756	(SEIDL *et al.* 2009)
MAT1-1-2	*Trichoderma reesei*	FJ599756	(SEIDL *et al.* 2009)
MAT1-1-3	*Trichoderma reesei*	FJ599756	(SEIDL *et al.* 2009)

the *MAT* locus. Neither of these genes showed similarity to any proteins present in the NCBI database.

In contrast to *H. omanensis,* the tBLASTn searches revealed the presence of the *MAT1-2-1*, *SLA*, *APC* and *APN* genes in the *H. moniliformis* genome, while neither the *MAT1-1-1* nor the *MAT1-1-2* gene was identified. Despite using *MAT1-1* gene sequences from many other ascomycetous species (Table 3) as search queries, no *MAT1-1* gene homologs were ever identified in the *H. moniliformis* genome. The *SLA* and *APC* genes as well as two unknown genes flanking the *MAT* locus in *H. omanensis* were present linked to the locus in *H. moniliformis* (Figure 2B). In both species, the *APN* homolog was found elsewhere in the genome and is not associated with any known *MAT* genes. This is in contrast to the *MAT* locus of *Ce. fimbriata*, where *APN* is found downstream of the *MAT1-1-1* gene, adjacent to *APC* (WILKEN *et al.* 2014).

The structure of the *MAT1-2* idiomorph was highly conserved between *H. moniliformis* and *H. omanensis*. In both cases, the *SLA* and *APC* genes were found upstream of the *MAT1-2-1* gene while two unknown proteins were found downstream. The only notable difference between the two *MAT1-2* idiomorphs of these species was the presence of an unknown gene directly upstream of the *MAT1-2-1* gene in *H. omanensis*. Due to this gene's association with the *MAT1-2* idiomorph, its absence in the flanking region of the *MAT1-1* idiomorph and its lack of homology to any other gene previously observed at the *MAT* locus, we have designated it as *MAT1-2-7.* This reflects the fact that it is the seventh gene to have been identified as part of the *MAT1-2* locus in ascomycetous fungi. When this gene was used in a local BLASTn search against the *H. moniliformis* genome, the corresponding sequence in *H. moniliformis* showed a 90% similarity between the two species. However, the region does not encode the full *MAT1-2-7* gene present in *H. omanensis* and instead, what is apparently a pseudogene was present. This pseudogene would encode a truncated protein in *H. moniliformis* if it were transcribed and subsequently translated because it possesses a nonsense mutation in the reading frame that produces a premature stop codon. There is no evidence to suggest that there is an intron present which would potentially allow for this region to encode the full gene as observed in *H. omanensis*. This explains why it was not predicted using the default parameters of AUGUSTUS.

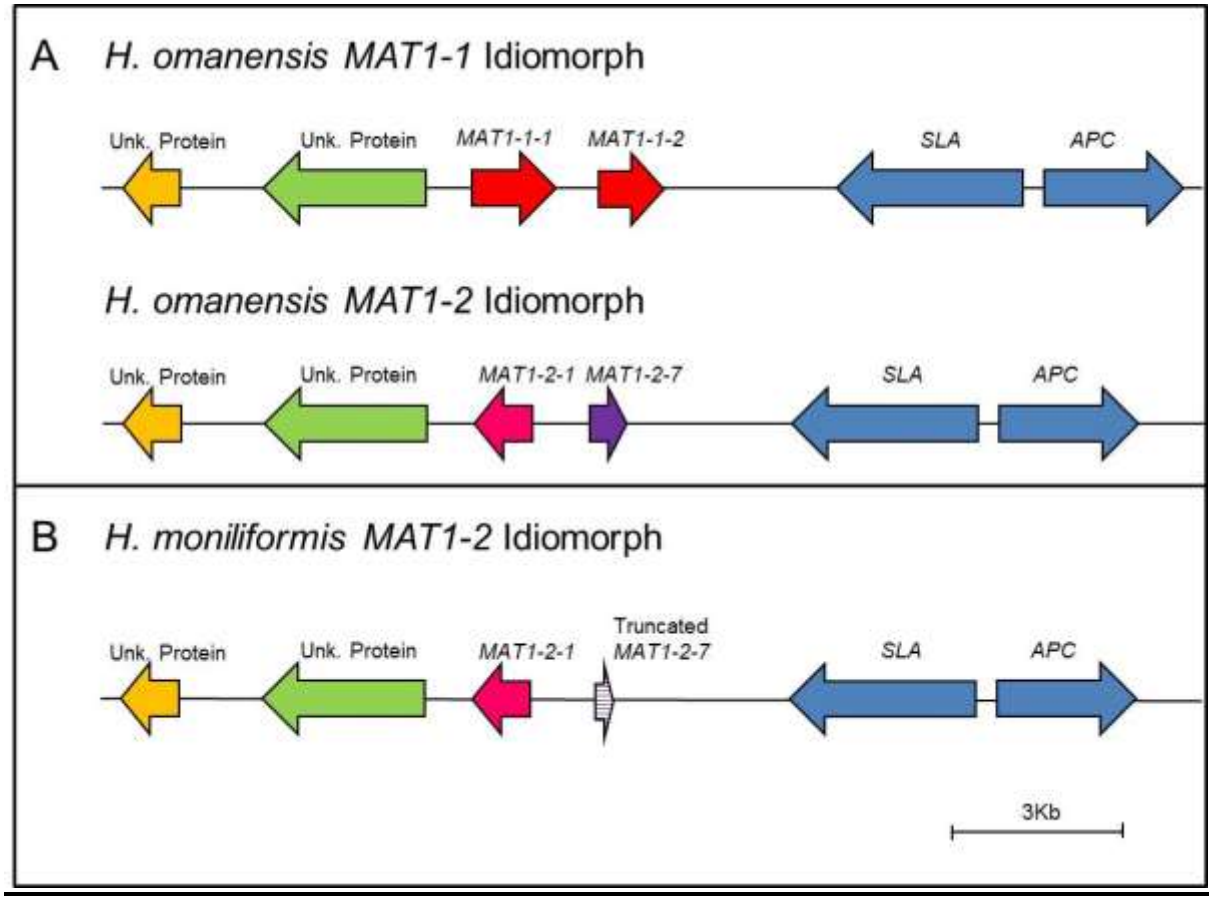

Figure 3: A schematic representation of the *H. moniliformis* and *H. omanensis MAT* loci. **A:** The *MAT1-1* and *MAT1-2* idiomorphs of *H. omanensis*. **B:** The *MAT1-2* idiomorph of *H. moniliformis*. **Unk. Protein:** Unknown Protein.

Significant sequence and structure similarity was seen between the *MAT* locus and flanking regions of the two *Huntiella* species. The *MAT1-1* idiomorph in *H. omanensis* possesses the *MAT1-1-1* and *MAT1-1-2* genes. The predicted *MAT1-1-1* gene was 1188bp long, comprised of an 1134bp CDS and a single intron (54bp) and encoded a 377aa protein. The predicted protein harboured the characteristic alpha box that spans the intron. The predicted *MAT1-1-2* gene was 1527bp long and was composed of a 1299bp CDS and four introns (65, 48, 54 and 61bp). The gene encoded a 432aa protein, possessing no conserved motifs. The *MAT1-2* idiomorph of *H. omanensis* possessed the *MAT1-2-1* and *MAT1-2-7* genes (Figure 3A). The predicted *H. omanensis MAT1-2-1* gene was 938bp long and consisted of an 825bp CDS and two introns (53, 60bp), encoding a 274aa protein harbouring the characteristic HMG box domain. The predicted *MAT1-2-7* gene was 467bp long, did not possess any introns and encodes a putative protein of 155aa with no conserved

domains. As in *H. omanensis*, the *H. moniliformis MAT1-2-1* gene was also 938bp long with an 85% nucleic acid identity to that of *H. omanensis*. It was comprised of an 825bp CDS region and two introns (53, 60bp), encoding a 274aa protein with a 91% identity to the corresponding protein of *H. omanensis*. The predicted protein also possessed the characteristic HMG box motif, which has a 97% identity to the same motif in *H. omanensis* (Figure 4). The truncated *MAT1-2-7* gene in *H. moniliformis* was 147bp long, encoding a protein of only 48aa and is likely non-functional.

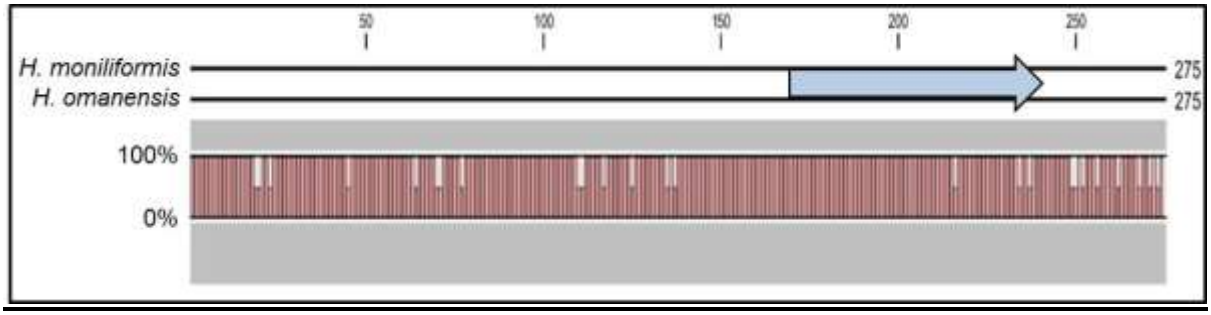

Figure 4: Amino acid alignment of the *H. moniliformis* and *H. omanensis* MAT1-2-1 protein. The proteins show a 91% similarity across the species. The arrow indicates the position of the conserved HMG box domain, which shows a 97% similarity.

When the *H. omanensis MAT1-1* idiomorph was used as a reference, the raw reads from *H. moniliformis* aligned to most of the sequence flanking the *MAT* locus. No reads aligned to either the *MAT1-1-1* or *MAT1-1-2* genes, despite an average coverage of 25X across other genes in the vicinity (Figure 5A). In contrast, the raw genome sequence reads of *H. moniliformis* aligned to all genes (including *MAT1-2-1* and *MAT1-2-7*) and most non-genic sequence present at the *MAT1-2* idiomorph of *H. omanensis* (Figure 5B). Although few raw reads from the *H. moniliformis* genome aligned to the unknown protein flanking the *H. omanensis MAT* loci, the gene sequence of this unknown protein shares a 67% similarity between the two species and can be identified via reciprocal local BLASTn searches.

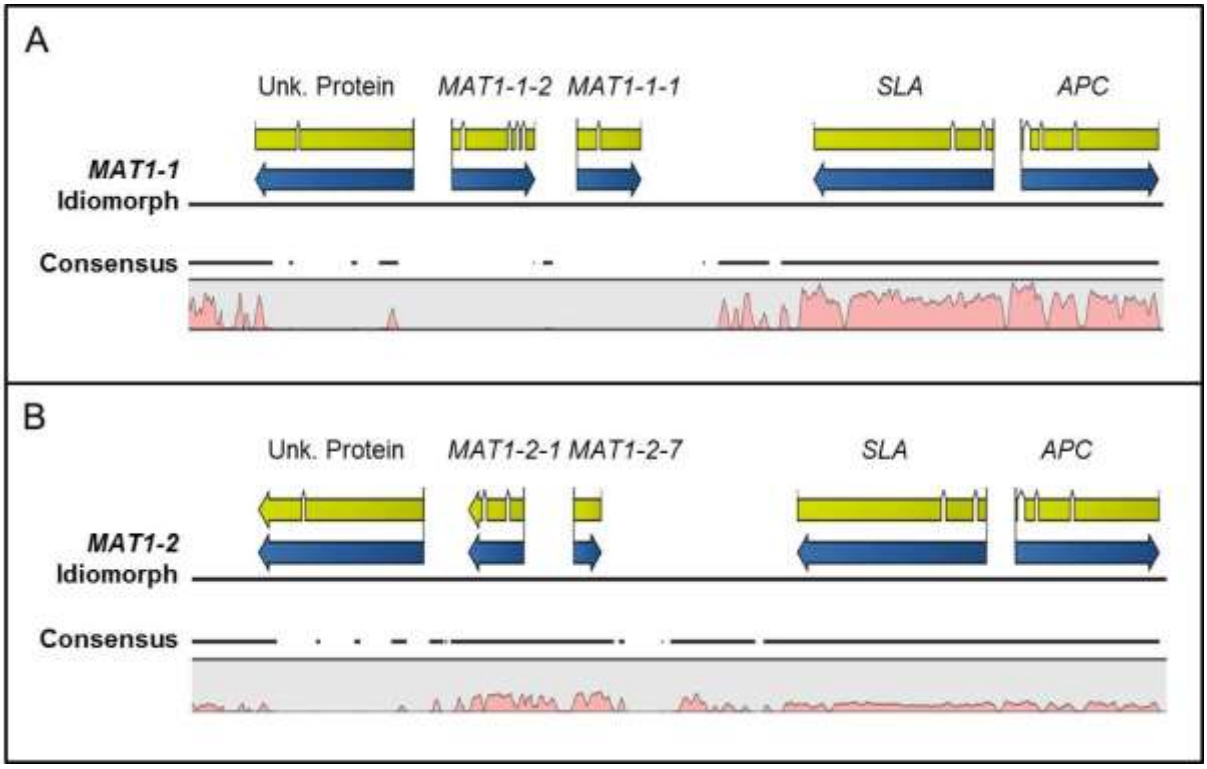

Figure 5: Raw genomic sequence reads of *H. moniliformis* mapped to the two *MAT* idiomorphs from *H. omanensis*. The consensus sequence indicated by the dotted black line underneath the idiomorphs represents an average coverage of these reads to the reference. This coverage was between 26 and 46X. **A)** *MAT1-1* idiomorph as reference: raw reads map to all *MAT* locus-flanking genes, but do not map to *MAT1-1-1 n*or *MAT1-1-2*. **B)** *MAT1-2* idiomorph as reference: raw reads map to the *MAT1-2-1* gene as well as its flanking regions. **Unk. Protein**: Unknown protein

3.3 Screening for *MAT* Genes in *Huntiella* Isolates

Genes from both the *MAT1-1* and *MAT1-2* idiomorphs were successfully amplified from the parental *H. omanensis* cultures. This was expected because the genome assembly also contained both idiomorphs and the presence of both idiomorphs resulted in the production of ascomata. However, only genes from a single idiomorph (either *MAT1-1* or *MAT1-2)* were amplified from the *H. omanensis* non-sporulating, single ascospore isolates. In contrast, *MAT1-2-1* was the only gene region to be successfully amplified in all eight *H. moniliformis* cultures used in this study. This gene region was also found in all single ascospore progeny generated from each of the six parental cultures. Once sequenced, these *MAT1-2-1* amplicons were shown to be identical for all the parental and single spore progeny. Thus, in both sporulating

and non-sporulating cultures, the only *MAT* gene present was *MAT1-2-1* and no amplicons corresponding to either of the *MAT1-1* genes were present.

4. DISCUSSION

The availability of sporulating cultures as well as genome sequences for *H. omanensis* and *H. moniliformis* allowed us to investigate the modes of sexual reproduction and to characterize the *MAT* loci in these species for the first time. It was intriguing that these species, thought to be closely related (DE BEER *et al.* 2014), had distinct mating strategies. *H. omanensis* was shown to be heterothallic, with individuals in the natural population possessing either the *MAT1-1* idiomorph, harbouring the *MAT1-1-1* and *MAT1-1-2* genes or the *MAT1-2* idiomorph, harbouring the *MAT1-2-1* and *MAT1-2-7* genes. This was in contrast to *H. moniliformis*, which was found to be self-fertile, with all sporulating and non-sporulating isolates possessing only the *MAT1-2-1* gene. This provides evidence for a unisexual reproductive cycle in *H. moniliformis* and is the first record of this strategy in a species of the important fungal family Ceratocystidaceae. In addition to the discovery of this novel mating system, results of this study also showed that the *H. omanensis MAT1-2* idiomorph harbours an undescribed *MAT* gene, which we have designated as *MAT1-2-7*. Remnants of this gene were found in *H. moniliformis*; however a premature in-frame stop codon in the sequence produces a truncated predicted protein.

Results of this study suggested that *H. omanensis* has a typical bipolar heterothallic mating system. Heterothallism involves the presence of a single *MAT* locus, possessing one of two distinct idiomorphs that confer mating type (COPPIN *et al.* 1997). Thus, sexual reproduction requires an interaction between two individuals of opposite mating type. This is identical to what was observed in *H. omanensis*, where single ascospore isolates failed to produce sexual structures in culture, unless they were paired with individuals of the opposite mating type. This mating system is similar to that of *D. eucalypti* (KILE *et al.* 1996), another species in the Ceratocystidaceae that was previously accommodated in *Ceratocystis* (DE BEER *et al.* 2014).

Both the *MAT1-1* and *MAT1-2* idiomorphs of *H. omanensis* were characterized from the single genome assembly that is publicly available (VAN DER NEST *et al.* 2014b). Given that the culture-based approach used in this study showed that the species is heterothallic, the presence of both idiomorphs within a single genome sequence seems implausible. The isolate (CMW 11056) from which the genome sequence was generated was obtained from the CMW culture collection and was able to produce abundant ascomata as well as viable ascospores. However, through the production of single ascospore isolates, approximately equal ratios of MAT1 to MAT2 individuals were recovered, neither of which were able to produce ascomata in isolation. PCR analysis also showed that each of these isolates harboured either the *MAT1-1* or the *MAT1-2* idiomorph, but never both. Taken collectively, these results showed that the observed fertility in isolate CMW11056 was the result of the presence of two or more individuals of both mating types in a single culture.

The standard procedure for maintaining cultures of *Ceratocystidaceae* spp. is to transfer ascospore masses from the necks of ascomata of sporulating cultures to fresh plates. This is common mycological practise to ensure that ascomata and ascospore production is maintained after successive rounds of sub-culturing (HANLIN 1985). The practise is particularly important in mycological collections where dimensions of the ascomata and ascospores are essential for taxonomic descriptions of filamentous fungi. While this is not an ideal technique for culturing fungi for which the genomes are to be sequenced, in the case of the present study, it allowed for the elucidation of both *MAT* idiomorphs of *H. omanensis*, aiding in the identification of the two distinct mating types.

The results of this study show that *H. moniliformis* represents an example of self-fertility conferred by unisexual reproduction. Previously, *H. moniliformis* has been treated as homothallic because single ascospore cultures were shown to produce ascomata and viable ascospores in culture (HARRINGTON 2007), an observation that was confirmed in the current study. Because ascospore production can be observed in a pure culture arising from a single ascospore, this reproductive strategy is a selfing event. Because we lack informative segregating markers, this form of reproduction would make it very difficult to trace recombination and the segregation of genes.

Based on the gene content of the *MAT* locus in *Ce. fimbriata* (WILKEN *et al.* 2014), another homothallic fungus in the Ceratocystidaceae, our hypothesis was that *H. moniliformis* would harbour genes of both idiomorphs at its *MAT* locus. However, the bioinformatic analyses employed identified only the *MAT1-2-1* gene and no genes associated with the *MAT1-1* locus. Molecular analyses including dot blot analysis (data not shown), PCR amplification and sequencing were also unable to identify any *MAT1-1* genes. These observations, combined with the ability of single ascospore cultures to produce ascomata with viable ascospores, suggest that the fungus represents an example of homothallism via unisexual reproduction. This would be the first time that a unisexual reproductive strategy has been found in a species of the Ceratocystidaceae, but it is known in other Sordariomycete species (GLASS and SMITH 1994).

The Sordariomycete species, *N. africana,* was the first fungal species in which unisexual reproduction was described (GLASS and SMITH 1994). To date, only the *mat A* idiomorph (homologous to *MAT1-1*) has been identified in sexually reproducing isolates of *N. africana.* This fungus provides an excellent example of homothallic mating despite the absence of essential *MAT* genes (GLASS and SMITH 1994). It is thus possible that the genetic mechanisms of unisexual reproduction in *H. moniliformis* are analogous to those of *N. africana*.

A second species in which unisexual reproduction has been found is the basidiomycetous yeast, *C. neoformans*. This fungus has a well-described heterothallic mating system, where mating types have been classified as **α** and **a** (reviewed in HEITMAN *et al.* 2013). Naturally occurring and clinical populations of *C. neoformans* show evidence of clonality and an extreme excess of the **α** mating type (KWON-CHUNG and BENNETT 1978). While this was originally attributed to a predominance of asexual reproduction in the species, the discovery of a unisexual cycle also explained the observed clonality. This cycle involves a tissue differentiation process by cells of the **α** mating type that is similar to that in classical sexual reproduction (LIN *et al.* 2005). Though distantly related to species in the Ceratocystidaceae, the presence of a unisexual pathway in *C. neoformans* may provide insights into the genetic mechanisms employed by *H. moniliformis* to

reproduce sexually in conditions where locating a suitable opposite mating partner may be highly improbable.

The *MAT* locus of *H. omanensis* is comparable to that of many heterothallic fungi. The presence of both *MAT* idiomorphs in the natural population of *H. omanensis*, each harbouring either *MAT1-1* or *MAT1-2* genes is typical of most heterothallic species, including the model species *N. crassa* as well as *Fusarium fujikuroi* and *Mycosphaerella graminicola* (CONDE-FERRAEZ *et al.* 2007; GLASS *et al.* 1990a; STABEN and YANOFSKY 1990; YUN *et al.* 2000). However, it is not only the gene content of the locus that is conserved, the genes present just outside the *H. omanensis MAT* locus also feature as *MAT* locus-associated genes in many other ascomycetous fungi. *SLA* (AMSELEM *et al.* 2011; ARONSTEIN *et al.* 2007; WADA *et al.* 2012; WILKEN *et al.* 2014) and *APC* (CONDE-FERRAEZ et al. 2007; COZIJNSEN and HOWLETT 2003; WAALWIJK et al. 2002; WILKEN et al. 2014), in particular, have been described as *MAT* flanking genes in various species.

The fact that this locus seems so conserved across a number of heterothallic species raises the question as to whether *H. moniliformis,* also exhibiting the *MAT1-2* locus structure seen in *H. omanensis*, is able to undergo heterothallic reproduction in the presence of a partner possessing the *MAT1-1* genes. In this study, only eight *H. moniliformis* isolates were available for screening and while these all harboured only the *MAT1-2-1* gene, this does not exclude the possibility of finding the opposite mating type in the future. Furthermore, the similarity of the *MAT1-2* idiomorphs of the two *Huntiella* species as well as the presence of the *MAT1-1* idiomorph in *H. omanensis* lends credence to the view that this idiomorph exists in *H. moniliformis*. However, it is important to consider that we were unable to perform successful interspecies crosses between MAT1 isolates of *H. omanensis* and MAT2 isolates of *H. moniliformis* and thus, in order to test this hypothesis, MAT1 isolates of H. moniliformis need to be identified. Under the assumption that *H. moniliformis* is able to employ the same mating strategy as *H. omanensis* under favourable conditions, unisexual reproduction could represent an alternative reproductive strategy that isolates possessing the *MAT1-2* idiomorph undergo in the absence of a possibly rare suitable partner. This would then represent a situation very similar to that seen in *C.*

neoformans, rather than *N. africana* where the opposite mating type has never been found (GLASS and SMITH 1994).

The fact that *N. africana* possesses *MAT1-1* sequence while *H. moniliformis* possesses only *MAT1-2* sequence could indicate that the genetic mechanisms of unisexual reproduction do not rely on the unique function of the *MAT* genes. This might rather be due to differences in the downstream gene targets of the *MAT* transcription factors. This has been shown to be the case in *C. neoformans*, where a number of experiments have identified genes that are essential for heterothallic mating yet dispensable for unisexual reproduction. For example, the homeodomain Sxi1α/Sci2**a** complex is required for cell identity during heterothallic mating, but is not required for unisexual mating, where cell identity is not a prerequisite (FERETZAKI and HEITMAN 2013; HULL *et al.* 2002). In contrast, genes that play a large role in unisexual reproduction but contribute only modestly to heterothallic mating have also been identified. The *Znf3* gene product is the dominant regulator of hyphal development in unisexual reproduction but is far less important for the same process in heterothallic mating (FERETZAKI and HEITMAN 2013). This supports the hypothesis that unisexual reproduction is not the result of a unique *MAT* locus function. However, it also suggests the presence of parallel pathways that can allow for sexual reproduction in species of only a single mating type (FERETZAKI and HEITMAN 2013). It may also explain why *H. omanensis* is unable to reproduce unisexually, despite its highly similar *MAT1-2* idiomorph.

Support for the view that unisexual reproduction is not the result of genes at a single *MAT* idiomorph replacing the functions of the other typically essential genes has been provided by transformation studies conducted in sterile *Neurospora* species. Transformation of a self-sterile relative with the *N. africana mat A* sequence does not confer self-fertility seen in *N. africana* (GLASS and SMITH 1994). While transformation experiments have not been conducted using the *MAT1-2* idiomorph of *H. moniliformis*, the presence of the highly similar *MAT1-2-1* gene in *H. omanensis* provides a framework for comparison. The MAT1-2-1 protein shares a 91% similarity between the two *Huntiella* species, with the conserved DNA binding domain sharing a 97% similarity. However, this gene does not confer self-fertility in *H. omanensis*.

Unisexual reproduction, evidence for which has been found in *H. moniliformis* and various other fungi (ALBY and BENNETT 2011; GLASS and SMITH 1994; LIN *et al.* 2005), may have evolved as a mechanism allowing species to preserve sexual reproduction and its benefits while at the same time minimising the costs (ROACH *et al.* 2014). The cost of locating an opposite mating partner is a major barrier to efficient sexual reproduction in heterothallic fungi. This is particularly evident in natural populations of *C. neoformans*, where the **a** mating type makes up less than 1% of the population, making it incredibly costly for an **α** individual to locate a suitable, opposite mating partner (LIN *et al.* 2005). By permitting **α** individuals to mate with one another, this particular cost is minimized. Even though energy is required to locate a partner, all potential partners are compatible (NI *et al.* 2013). At present, the *H. moniliformis* system appears to mirror that seen in *N. africana* (GLASS *et al.* 1990b), where only a single mating type is present in the natural population. It is possible that the unisexual mating system has evolved in a mating type-biased population, thereby preserving sexual reproduction despite the difficulty of locating an opposite mating partner. This would reduce the need for the rare mating type to be located and subsequently lead to a population composed entirely of a single mating type. In this situation, *C. neoformans* would represent an intermediate state of this process, with *N. africana* and possibly *H. moniliformis* representing the final state.

5. CONCLUSION

This study describes the first case of unisexual reproduction in the Ceratocystidaceae, a family known for its diverse sexual strategies. Unisexual reproduction was recognised relatively recently as a unique form of homothallism, allowing individuals to reproduce sexually, in the absence of a partner of opposite mating type. While this is apparently not a common sexual strategy in the fungi, it may have evolved in order to diminish the costs associated with typical sexual reproduction, while still maintaining its benefits. This study provides a foundation for further investigations into the evolution of sexual strategies across a variety of fungal species. It also provides a model organism in which the evolutionary benefits and molecular mechanisms of unisexual reproduction can be further studied.

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