Meiosis-Specific Gene Expression in the Arbuscular Mycorrhizal Fungus *Rhizophagus irregularis*

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are a group of root obligate symbionts that are part of the fungal sub-phylum Glomeromycotina, which provide water, nutrients, and pathogen protection to about 80% of land plants in exchange for their photosynthetic products. AMF thus act as "biofertilizers", have a profound effect and influence on the biodiversity of plants, and play a major role in life on land. From an evolutionary point of view, AMF are a puzzling group of organisms, thought to have propagated for over 400 million years without sexual reproduction, a rarity among eukaryotes. However, this assumption is largely based on the absence of definitive observations of sexual reproduction through microscopic tools. One clue into the sexual activity of AMF is evidence of a dikaryotic-like genome organization in their multi-nucleated mycelium. The recent identification of multi-allelic mating-type loci (*MAT* locus) potentially places AMF among other heterothallic or bipolar species, who's mating compatibility is determined by their *MAT* locus. The presence of a hidden sexual cycle in AMF is still a possibility, and recent findings on the meiotic gene content of AMF suggests an alternative narrative to how these fungi have escaped extinction for so long. Seven meiosis-specific genes (MSG) were found to exist in AMF, indicating that these fungi are likely undergoing a cryptic sexual cycle. The main goal of this research is to determine if/when MSG are expressed in an in-vitro model of AMF. To build onto this research, we established crossings between isolates with hypothetically compatible mating types, in order to determine if fusion of their hyphae can trigger the expression of MSG. Together, these experiments will assess expression at varying stages of the putative cycle of sexual reproduction and give further insight into the elusive sexual life of AMF.

RÉSUMÉ

Les champignons mycorhiziens à arbuscules (AMF) sont un groupe de symbiotes racinaires obligatoires qui font partie du sous-embranchement fongique Glomeromycotina, qui fournissent de l'eau, des nutriments et une protection contre les agents pathogènes à environ 80% des plantes terrestres en échange de leurs produits photosynthétiques. L'AMF agit ainsi comme biofertilisant, a un effet et une influence profonds sur la biodiversité des plantes, et joue un rôle majeur dans la vie terrestre. D'un point de vue évolutif, les AMF sont un groupe déroutant d'organismes qui se seraient propagés pendant plus de 400 millions d'années sans reproduction sexuelle, une rareté chez les eucaryotes. Cependant, cette hypothèse est largement basée sur l'absence d'observations définitives de la reproduction sexuelle à l'aide d'outils microscopiques. Un indice de l'activité sexuelle de l'AMF est la preuve d'une hétérocaryose dans leur mycélium multinucléé. L'identification récente de loci de type d'accouplement multi-alléliques (loci *MAT*) place potentiellement l'AMF parmi d'autres espèces hétérothalliques ou bipolaires, dont la compatibilité d'accouplement est déterminée par leurs loci *MAT*. La présence d'un cycle sexuel caché dans l'AMF est toujours une possibilité, et des découvertes récentes sur la teneur en gènes méiotiques de l'AMF suggèrent une narration alternative à la façon dont ces champignons ont échappé à l'extinction pendant si longtemps. Sept gènes spécifiques à la méiose (MSG) se sont avérés exister dans AMF, indiquant que ces champignons subissent probablement un cycle sexuel cryptique. L'objectif principal de la recherche est de déterminer si / quand les MSG sont exprimés dans un modèle in vitro d'AMF. Pour approfondir cette recherche, nous avons établi des croisements entre des isolats avec des types d'accouplement hypothétiquement compatibles, afin de déterminer si la fusion de leurs hyphes peut déclencher l'expression de MSG. Ensemble,

ces expériences évalueront l'expression à différents stades du cycle putatif de la reproduction sexuelle et donneront un aperçu supplémentaire de la vie sexuelle insaisissable de l'AMF.

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ABBREVIATIONS

AMF – Arbuscular mycorrhizal fungi

P – Phosphorus

qRT-PCR – Real-time quantitative polymerase chain reaction

ddPCR – Digital droplet polymerase chain reaction

RT-PCR – Reverse-transcriptase polymerase chain reaction

MSG – Meiosis-specific genes

cDNA – Complementary DNA

HMG – High mobility group

CCL – Concerted chromosome loss

BIR – Break-induced replication

MSR – Modified Strullu-Romand

Kb – Kilobase

Bp – Base pairs

TE – Transposable elements

CHAPTER ONE

Introduction

1.1 Overview of Arbuscular Mycorrhizal Fungi

1.1.1 Unique Symbiosis

The arbuscular mycorrhizal (AM) symbiosis has been around for over 400 million years, with the earliest evidence discovered in the Devonian land plant, *Aglaophyton major* (Remy et al. 1994). This mutualistic symbiosis is found in over 80% of land plants in nearly every corner of the world (Savary et al. 2018; S. E. Smith and Read 1997). AM fungi (sub-phylum Glomeromycotina), or AMF, have co-evolved with these host plants, and potentially aided in the transition of plants from water to land (Paszkowski 2006; Pirozynski and Malloch 1975). To say this relationship is integral in shaping the planet as we know it today is an understatement. AMF are vital in the survival of many biologically important plant groups, including the crops that we grow for food (Bagyaraj, Sharma, and Maiti 2015; Gianinazzi-pearson 1996). In many ways, this unique symbiosis increases the fitness of both the fungus and its host (Bonfante and Genre 2008). It all begins with a germinated hypha from a soilborne fungal spore that comes into contact with a host's root. At this point, the hypha penetrates the cortex and forms arbuscules (intracellular haustoria) deep in the root cortical cells, which is the site of nutrient exchange (Gadkar et al. 2001; Wang et al. 2017). Arbuscules contain immense surface area for the transfer of nutrients to occur and mature over the course of several days (Harrison 2005). While the fungus grows, many more arbuscules continuously form and degenerate in an unsynchronized fashion throughout the roots (Gianinazzi-pearson 1996). The fungal mycelium acts as an auxiliary root system that provides the host with enhanced water use efficiency (Antunes et al. 2011; Augé 2001), nutrient aquisition (mainly phosphorous and nitrogen (Marschner and Dell 1994)), and pathogen protection (Borowicz 2001; Wehner et al. 2010), while the plant exchanges carbon (primarily in the form of lipids and carbohydrates (Douds, Pfeffer, and Shachar-Hill

2000)) with which the fungus uses to grow its mycelium. AMF are obligate symbionts, which means that they require a host to survive because they cannot create their own source of carbon (Schwarzott and Walker 2001). Current knowledge in this symbiosis postulates that it is ultimately up to the plant's defence mechanisms (Song et al. 2011) and genes involved with plant-microbe interactions to decide whether or not to allow a fungus to colonize its roots (Gianinazzi-pearson 1984).

1.1.2 AMF as "Biofertilizers"

Because of their unique and long-standing beneficial relationship with plants, research has been invested into the commercial potential of AMF as a "biofertilizer" in agriculture, or an auxiliary root system of sorts which aids the plant at siphoning more fertilizer already in the soil. Because of this, they represent a potential alternative to harmful chemical fertilizers that are currently widely being used globally for agriculture. Not only are conventional fertilizers damaging to marine ecosystems (runoff that cause harmful algal blooms (Beman, Arrigo, and Matson 2005; Fried, Mackie, and Nothwehr 2003)), but there is also a finite availability of P-rock fertilizer globally (Li et al. 2017). Increasing populations and an increasing global food demand means that fertilizer reserves will face shortages in the near future, which prompts the search for alternate ways to keep up with the growing market (Roy-bolduc and Hijri 2011). AMF have already been used in many agricultural sectors as "biofertilizers" for decades (Menge 1983; Ndung et al. 2013), but their effectiveness as an inoculant is often debated. Many studies show inconsistent results (Carpio, Davies, and Arnold 1962) anywhere from increased crop growth (Cozzolino, Meo, and Piccolo 2013) to no noticeable effect, with the expectation of increasing yields from commercial inoculants (Kokkoris et al. 2019). To further complicate things, studies shows that isolates within species, specifically *R. irregularis*, which vary in phenotype and

genotype, are demonstrated to affect plant growth in different ways (Koch et al. 2004). The plant responses observed from genetically diverse isolates suggests that AMF and their host's fitness is underscored by this inter-isolate diversity (Chen, Morin, Beaudet, Noel, Yildirir, Ndikumana, Charron, St-onge, et al. 2018). There is still much vital research needed in this field to harness the full potential of AMF as a viable challenge to the agricultural status quo.

1.2 Sex in Fungi

1.2.1 Mating in Related Clades

Sex in fungi is incredibly rich and diverse, with even sub-phyla evolving their own unique ways of regulating sex (Zeyl 2009). In general, sex occurs in three main steps in the fungal kingdom. At first, two compatible mating partners undergo plasmogamy, or fusion of their cell membranes. Then, the parental nuclei fuse together in the second step, karyogamy. This is instantaneous for chytridiomycetes, most zygomycetes, and some ascomycetes. For other fungal lineages like the basidiomycetes, this process is delayed in the formation of a stable dikaryotic sexual stage, where the fungus lives in a state with two haploid nuclei. The final step is meiosis, where the nuclei fuse to form a brief diploid stage that recombines and produces haploid progeny. Fungal sex is directed by the *MAT* locus, or mating type, which is a highly evolved and diverse genetic locus that regulates sex (Fraser et al. 2007). In the simplest of forms, two alleles of this *MAT* locus, usually denoted as *MAT***a** and *MAT*α, produce transcription factors that, upon contact, signals both compatible mating partners to fuse and undergo meiosis (Lee et al. 2010). However, for many fungi, this process is highly diversified and complex, involving thousands of mating types in one species alone (Kues and Casselton 1993).

1.2.2 Ancient Asexuals

AMF are an interesting group of filamentous fungi due to the complete absence of evidence that these fungi have ever sexually reproduced. This places them among a select group of organisms, dubbed "ancient asexuals", that have survived (and sometimes thrived) for hundreds of millions of years in the absence of sexual reproduction (Judson and Normark 1996). Examples of these asexuals include some Bdelloid rotifers (Gladyshev and Arkhipova 2010), Microsporidia, and some related fungi. Most of these organisms, including Bdelloid rotifers and Microsporidia, have meiotic proteins (Spo11, Rec8, Hop1, Msh4, Msh5 in the rotifers; Spo11 and Dmc1 in Microsporidia) which would usually indicate some form of sexuality, although the lack of evidence suggests otherwise (Hofstatter and Lahr 2019b). For many taxa, their asexual label relies on circumstantial evidence such as fossil records, the lack of males in a population, or specifically in AMF, the lack of an observable sexual stage. Additionally, many of these organisms prove difficult to study due to their small size or the difficulty in establishing populations in a laboratory setting (Gandolfi et al. 2002). This quickly raises an important question: how have AMF and other ancient asexuals staved off the accumulation of deleterious mutations for so long? Evolutionary theory predicts that asexual lineages will eventually go extinct due to their lack of ability to adapt to environmental change (M. Smith 1978). Clearly, there is more going on in these organisms that has let them defy these odds for hundreds of millions of years. Genetic sequencing has allowed for more questions to be answered in these organisms, particularly in AMF. Evidence for recombination events has been seen in these fungi, which at the very least demonstrates that there are mechanisms in place for creating genetic diversity (den Bakker et al. 2010; Croll and Sanders 2009; Vandenkoornhuyse, Leyval, and Bonnin 2001). Whether these events involve sexual processes such as meiosis or some asexual

alternatives (transposable elements) is still up in the air (Riley and Corradi 2013; Yildirir et al. 2020).

1.2.3 Heterokaryosis in AMF

A single mycelium can have hundreds of thousands of identical individual haploid nuclei free-floating in their cytoplasm, a feature unique to AMF. Their genetic organisation had been hotly debated for years before a breakthrough study (Ropars et al. 2016) saw evidence for dikaryotic-like life stages in the model AMF species, *Rhizophagus irregularis*. Based on allelic frequency data, three isolates of this species (A4, A5, SL1) seemingly harbored two genetically distinct populations of nuclei and were thus dubbed dikaryon-like isolates. The data showed a 50% drop in coverage at certain genomic regions compared to their homokaryotic counterparts. A drop in coverage results from mismatched reads in the assembly, which is explained by the highly diverged allelic regions. The coexistence of two distinct genotypes in some AMF strains, each linked with a specific *MAT* locus has been confirmed using single nucleus sequences (Chen, Mathieu, Hoffrichter, Sedzielewska-Toro, Peart, Pelin, Ndikumana, Ropars, et al. 2018). These are not true dikaryons, however, because of their multinucleated nature, but functionally could represent a stage of sexual reproduction in AMF where the two nuclei could fuse and recombine to form a new homokaryon isolate with a unique genetic makeup.

1.2.4 Multi-Allelic Mating Locus

Along with the discovery evidence for heterokaryosis in *R. irregularis*, was the breakthrough discovery of a *MAT* locus or mating-type region in the genome. As previously mentioned, this locus is vital for sexual reproduction in fungi and gives an organism its sexual identity with which it uses to determine compatibility with another individual. The genes located

in this region, notably orthologues of *sex*M and *sex*P, encode for high mobility group proteins (HMG) which give sexual identity to other close AMF relatives in the phylum previously named Zygomycota, now Mucoromycota (Riley and Corradi 2013). This region also hosts genes that encode for pheromones and pheromone receptors, which are important for the detection of a compatible mating partner (Ropars et al. 2016). The *MAT* locus represents one of the first key steps in a putative cycle of sexual reproduction in AMF. In addition to the discovery of this locus is the identification of six alleles, *MAT* 1-6, which postulates that AMF are heterothallic species where an organism can only mate with a partner that has the complimentary mating type. Current evidence shows that each homokaryotic isolate of *R. irregularis* harbors one of these six mating types within each of their nuclei while dikaryotic isolates have a mix of two nuclei with different mating types. These dikaryotic isolates likely originated from two compatible homokaryon isolates, but whether compatibility is driven by the mating type or other regions of the genome is unclear. Table 1.2 lists identified isolates of *R. irregularis* with their corresponding mating types. These mating types may give unique sexual identity to different isolates of *R. irregularis*, which would potentially allow them to anastomose and form a dikaryotic stage.

1.2.5 Putative Cycle of Sexual Reproduction in AMF

The theory of heterokaryosis in conjunction with the multinucleated nature of AMF places this group in a unique category of sexual reproduction in the fungal kingdom. Assuming that AMF indeed have hidden sexual lives, their putative cycle of sexual reproduction would look most like their fungal relatives, the basidiomycetes. The existence of a prolonged dikaryotic stage is a hallmark of these two groups and would represent the second step of sex in fungi. Modified from Ropars et al. 2016, the Figure 1.1 depicts the hypothetical cycle of sexual reproduction in AMF if meiosis was undergoing in their mycelium. This, however, differs from

the basidiomycetes as AMF do not have an observable sexual stage; there is no evidence that AMF produce sexual spores which contain only two nuclei with different mating types. In theory, two compatible homokaryon isolates would come into contact, at which point their hypha would anastomose and exchange nuclei. This process, which we now confirmed exist in AMF (Mathieu, Unpublished), would lead to the formation of a dikaryotic stage where two genetically different nuclei would be present in the same mycelium. Eventually, certain factors would trigger the fusion of these nuclei to form a diploid stage where the chromosomes would cross over and meiosis would occur, producing haploid progeny with new genetic composition. The resulting homokaryotic spores containing identical nuclei would germinate and undergo clonal propagation until the mycelium grew enough to start the cycle anew. To date, AM dikaryotic spores have been shown to have hundreds of nuclei within them. It remains unclear how such a seemingly chaotic soup of nuclei could possibly coordinate to undergo meiosis in synchrony. It is possible that select regions of the mycelium could host sexual events, however, there is no evidence to date of this occurring either (Yildirir et al. 2020). Although this putative cycle of sexual reproduction remains unconfirmed, it is a useful blueprint with which hypotheses can be created and tested. AMF may very well be true asexual organisms, or they could even be undergoing processes far different than their fungal relatives. However, mounting research increases the weight of evidence against their supposed asexuality, and more towards a cryptic sexual identity.

1.3 Evidence for a Cryptic Sexual Cycle

1.3.1 Core Meiotic Machinery

Research into the genetics of AMF has loosened the asexual identity that has for many decades been labelled onto this strange group of organisms. Asexuality often results from the

loss of sexual ability that originated from a sexual predecessor. Many times, asexual organisms do harbour remnants of the core meiotic machinery in their genomes (Hofstatter and Lahr 2019a) but are incapable of utilizing the genes due to deleterious mutations that may have accumulated over time or massive gene losses. In 2011, AMF were discovered to have a conserved repertoire of meiotic genes within their genomes (Halary et al. 2011). Figure 1.2 details the genes present in the genus formerly known as *Glomus*, now *Rhizophagus*. 51 meiosis-related genes were identified in this genus, which represents roughly 85% of the core meiotic machinery. The only AMF core genes that were not detected in this study were homologs of *Pch2*, *Hop1*, *Mei4*, and *Mer3*, all genes whose loss does not affect successful completion of meiosis in many fungi (Kumar, Bourbon, and Massy 2010). These genes are notably absent in a meiosis-capable fungal relative of AMF, *R. oryzae* (Malik et al. 2008). Some eukaryotic organisms known to reproduce sexually have even less core meiotic genes than AMF; *N. crassa*, *G. zeae*, *D. melanogaster* are all missing *Hop2* and *Mer3*, and are known sexual organisms (Malik et al. 2008). Among the gene repertoire in *Rhizophagus*, are seven meiosis-specific genes which are only known to be involved in meiotic processes (*Spo*11, *Mnd*1, *Dmc*1, *Hop*2, *Msh*4, *Msh*5, and *Rec*8). These genes are near universally essential for eukaryotic organisms' ability to undergo sexual reproduction through meiosis. The functions of each of these proteins is listed in Table 1.1 and range anywhere from facilitating chromosome homolog crossovers to the creation of double stranded breaks. With this genetic catalogue, AMF could theoretically be more than capable of undergoing meiosis. This breakthrough has laid the foundation for many experiments to dive deeper into the sexual lives of AMF and is the premise for the work in this thesis. Further analyses into the genome of *R. irregularis* have uncovered even more evidence to support the argument against asexuality.

1.3.2 Preliminary work on meiotic gene expression

With the discovery of a multi-allelic mating locus and a core meiotic gene repertoire *in R. irregularis*, the focus of current research tilted towards the identification of more concrete evidence to point towards an underlying sexual mechanism. Preliminary unpublished research by Timea Marton in the Corradi laboratory (Figure 1.3) analyzed the expression of the seven meiosis-specific genes in three *R. irregularis* isolates (A4, C2, and B3). A4 is a dikaryotic isolate with the mating types 1 and 2, while C2 and B3 are homokaryotic isolates with the mating types 6 and 3, respectively. RNA was extracted from cultures of these isolates and the expression of their meiotic genes were analyzed using qRT-PCR in standalone conditions (Figure 1.3a) and in plantae conditions (Figure 1.3b). The results showed a lack of ubiquitous expression of all meiosis-specific genes in these isolates, which prompted the deeper investigation of these genes in this thesis. Since C2 and B3 are homokaryotic isolates, the results were unsurprising since one would not expect meiotic genes to be constitutively expressed in a non-sexual stage of the putative cycle of reproduction for AMF. However, when looking at the dikaryon A4, which holds potential for meiosis to occur as a putative sexual stage, it is interesting to see the same lack of expression. These results, although they appear negative, may simply mean that the meiotic genes are only expressed in highly specific times and circumstances – i.e. meiosis.

1.3.3 Anastomosis and isolate compatibility

The first step of sexual reproduction in related fungi is the fusion of cell membranes. In the case of the putative cycle of sexual reproduction in AMF, this step is called anastomosis (the fusion of hyphae). This mechanism has multiple uses in the fungal kingdom: sexual fusion, exploration of the environment with the formation of complex lattices, and the exchange of

protoplasmic and genetic material (De La Providencia et al. 2005). Most often, this occurs in vegetative hyphae where spores of the same organism germinate and fuse with one another to expand the mycelial network. These hyphal bridges see the bidirectional transfer of protoplasmic material, including nuclear migration (Giovannetti, Azzolini, and Citernesi 1999). This mechanism has also been observed to be involved in healing, by isolating damaged areas of the mycelium (Bago et al. 1999) or reconnecting broken or blocked hyphae (de Souza and Declerck 2003). Much is still unknown about what controls diverse anastomosis behaviours in different AMF species, which display a wide range of successful fusion events (Pepe, Giovannetti, and Sbrana 2016). Unpublished preliminary data from collaborators at the University of Pisa shows that, on rare occurrences, hyphae of genetically distinct but potentially compatible isolates of the same species can fuse together and possibly exchange nuclei (Figure 1.4). These results indicate that under a small percentage of encounters (3.8%), hyphae from different isolates of *R. irregularis* (A1 and C2) are capable of forming complete hyphal fusions. In the majority of encounters, however, this pairing saw no contacts (77.8%) with occasional incompatible contacts or fusions (0.9% and 17.5%, respectively). Incompatible contacts describe hyphae that approach one another and then divert, while incompatible fusions describes the fusion of hyphae with the formation of septal walls so that protoplasmic material cannot be exchanged (Pepe, Giovannetti, and Sbrana 2016). The assay tested the anastomosis rates specifically in isolates with the mating types 3 and 6, which co-exist in the dikaryon, A5. However, as shown in the figure, different pairings of isolates with the same mating types did not see successful fusion of hyphae. Although these results indicate that AMF compatibility may not be entirely based on mating types, the rates of anastomosis between genetically distinct isolates of *R. irregularis* prompted further investigation into the relationship between A1 and C2 in this thesis.

1.4 Research Justification and Goals

Increasing interest has been underway in the role that AMF can have in the agricultural industry as a "biofertilizer" and potential replacement for harmful commercial phosphate fertilizers. Evolving for hundreds of millions of years alongside land plants, AMF are a promising eco-friendly alternative way to alleviate the stress that crops face in the worsening climate crisis. Steady progress has been made on elucidating the intricate mechanisms that direct functional specificity within certain hosts, but so far studies have only scratched the surface. An important step in harnessing the benefits of AMF as a commercial inoculum involves investigating their hotly debated sexuality, or lack-thereof. After the discovery a near-complete core meiotic repertoire, questions arose about whether these were ancient genetic artefacts or lasting functional tools used by AMF to survive for so long. This study seeks to assess the expression of six meiosis-specific genes in various homokaryotic and dikaryotic isolates of the model AMF, *R. irregularis*. Using quantitative techniques, we seek to establish a baseline level of expression, compare to those levels to hypothetically sexual stages (dikaryon, active homokaryon crossings), and try to answer the age-old question: are AMF undergoing a cryptic sexual cycle?

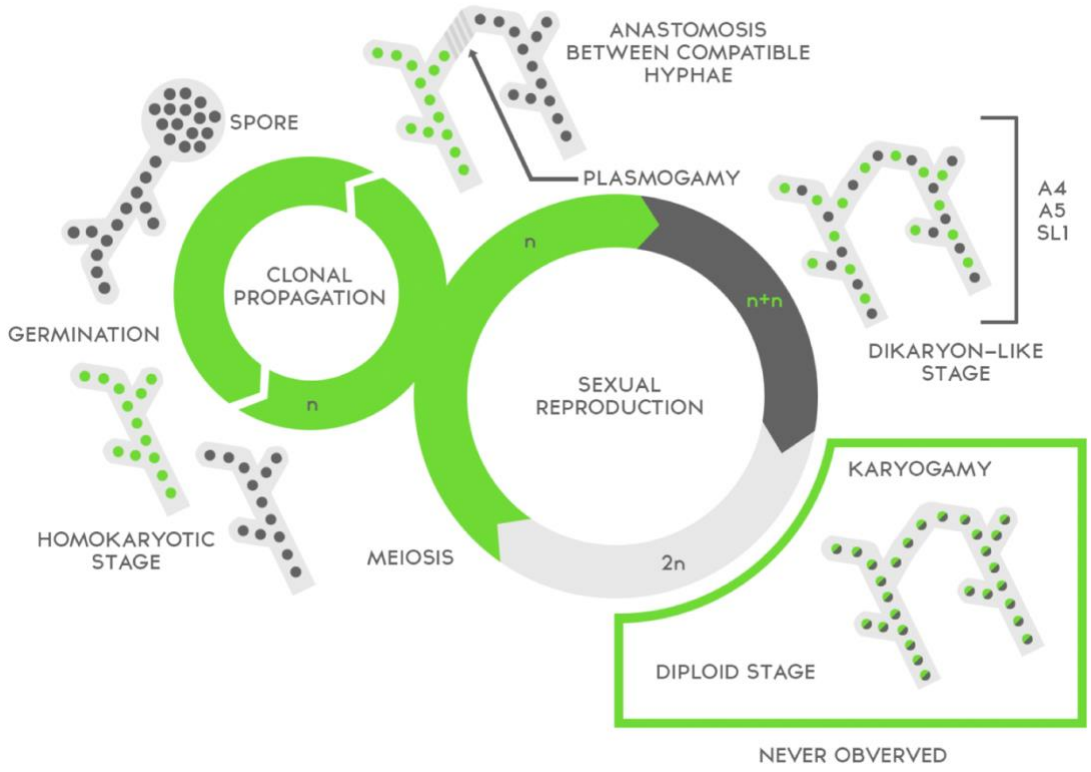


Figure 1.1. Putative cycle of sexual reproduction in AMF. AMF spores germinate and grow asexually in clonal propagation. Eventually, compatible homokaryon isolates anastomose, exchange nuclei, and create a dikaryon-like stage. The nuclei within this stage fuse and form a diploid stage. Meiosis occurs and the diploid nuclei forms new haploid nuclei that goes on to form a new homokaryon isolate, which can then grow asexually. Figure modified from Ropars et al. (2016).

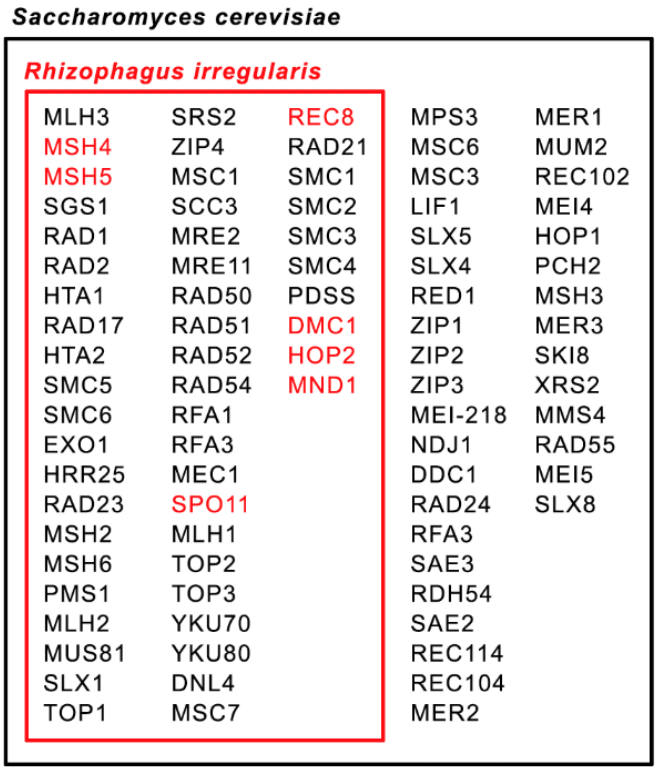


Figure 1.2. Core meiotic gene repertoire present in *Rhizophagus irregularis*. Total meiotic gene repertoire shown by *S. cerevisiae*. *R. irregularis* meiotic genes shown in red box. Meiosis-specific genes highlighted in red font. List of genes obtained from Halary et al. (2011).

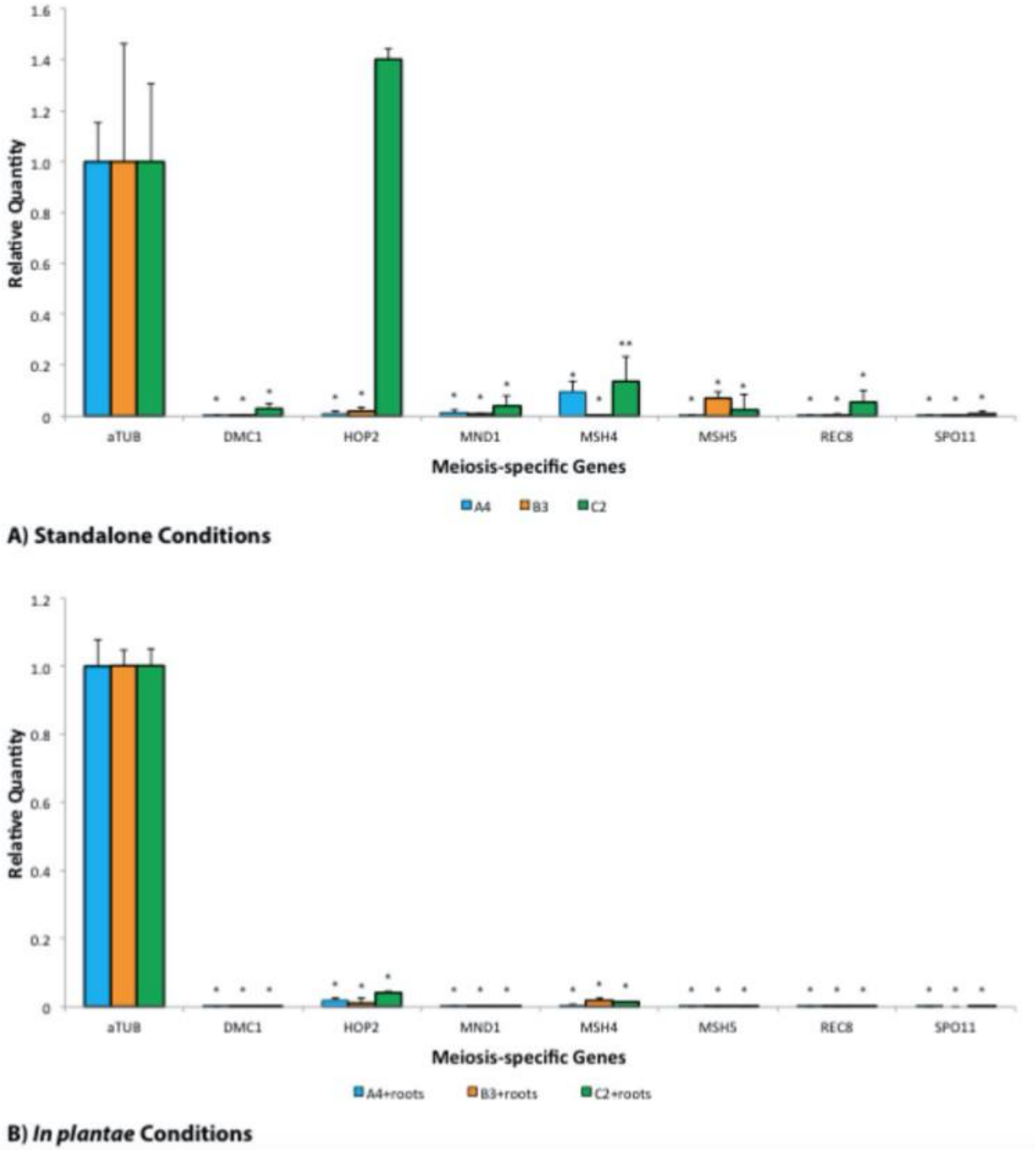


Figure 1.3. Relative quantity of meiosis-specific genes in *R. irregularis* isolates. Seven meiosis-specific genes analyzed using qRT-PCR in three isolates (A4, B3, and C2). A4 is a dikaryon-like isolate while B3 and C2 are homokaryotic isolates. a) displays relative quantity in standalone conditions (MSR media in 25˚C incubator). b) displays relative quantity *in plantae*.

Table 1.1. Anastomosis rates between homokaryon crossings. Unpublished results from Cristiana Sbrana & Manuela Giovannetti at the University of Pisa. Homokaryon crossing studied in this thesis highlighted by red box. Perfect fusions denote complete hyphal fusions with no septal wall formation. Incompatible fusions denote hyphal fusions with septal wall formation. Incompatible contacts denote close encounter but no hyphal fusion. No interactions denote no close encounters nor hyphal fusions. Data listed as percentage of tracked interactions.

MAT types		**Pairings**		**Perfect Fusions**	**Incompatible fusions**	**Incompatible contacts**	**No interactions**
3	3	A1	A1	47.0±1.8	1.8±1.2	0.7±0.7	50.5±2.8
6	6	C2	C2	56.3±2.4	1.0±1.0	1.4±0.9	12.8±2.8
3	6	A1	C2	3.8±2.2	17.5±2.2	0.9±0.6	77.8±4.2
3	6	B3	C2	0.0	3.2±2.5	12.9±2.2	83.8±3.8
3	6	A1	4401	0.0	26.1±0.4	0.0	73.9±0.4

Table 1.2. Meiosis-specific genes and their corresponding function in meiotic processes. List of meiosis specific genes present in *R. irregularis* obtained from Halary et al. (2011).

Protein	Function
Msh4	Protein involved in meiotic recombination, required for normal levels of crossing over, colocalizes with Zip2p to discrete foci on meiotic chromosomes, has homology to bacterial MutS protein (Novak, Ross-macdonald, and Roeder 2001).
Msh5	Protein of the MutS family, forms a dimer with Msh4p that facilitates crossovers between homologs during meiosis (Halsey et al. 1995).
Mnd1	Protein required for recombination and meiotic nuclear division; forms a complex with Hop2p, which is involved in chromosome pairing and repair of meiotic double-strand breaks (Pezza et al. 2007).
Rec8	Regulator of chromatin structure and function during meiosis. Component of sister chromatid cohesion complex; maintains cohesion between sister chromatids and between centromeres of sister chromatids (Golubovskaya et al. 2005).
Dmc1	Meiosis-specific protein required for repair of double-strand breaks and pairing between homologous chromosomes; homolog of Rad51p and the bacterial RecA protein (Shinohara and Shinohara 2004).
Spo11	Type II topoisomerase; Meiosis-specific protein that initiates meiotic recombination by catalyzing the formation of double-strand breaks in DNA via a transesterification reaction; required for homologous chromosome pairing (Celerin et al. 2000).
Hop2	Protein involved in homologous pairing of chromosomes and proper intragenic recombination during meiosis. Works with Dmc1 for the resolution of meiotic double-stranded breaks (Leu, Chua, and Roeder 1998).

Table 1.3. List of *R. irregularis* isolates with their corresponding mating type allele. Dikaryotic isolates have an Asterix (*); Isolates used in these studies highlighted in red. Information derived from phylogenetic analysis conducted in Ropars et al. (2016).

***MAT* locus allele**	***R. irregularis* isolate**
1	SL1*
	A4*
2	A4*
	DAOM-229455 (330)
3	A5*
	A1
	B3
	DAOM-240270 (66)
	DAOM-194475 (327)
	DAOM-240721 (143)
4	DAOM-241558 (309)
	DAOM-220723 (73)
	DAOM-240159 (163)
	DAOM-197198
5	SL1*
	DAOM-229264 (56)
	DAOM-240448 (101)
	DAOM-664340 (96)
	DAOM-212349 (445)
6	A5*
	C2
	DAOM-229457 (414)
	DAOM-211734 (289)
	DAOM-240425 (57)
	DAOM-234180 (454)
	DAOM-240446 (98)
	DAOM-240442 (80)
	DAOM-240434 (79)
	DAOM-240424 (55)
	DAOM-240201 (178)

CHAPTER TWO

Meiosis-specific gene expression in the arbuscular mycorrhizal fungus *Rhizophagus irregularis*

2.1 Abstract

Arbuscular mycorrhizal fungi (AMF) are a group of root obligate symbionts that are part of the fungal sub-phylum Glomeromycotina, which provide water, nutrients, and pathogen protection to about 80% of land plants in exchange for their photosynthetic products. AMF thus act as "biofertilizers", have a profound effect and influence on the biodiversity of plants, and play a major role in life on land. From an evolutionary point of view, AMF are a puzzling group of organisms, thought to have propagated for over 400 million years without sexual reproduction, a rarity among eukaryotes. However, this assumption is largely based on the absence of definitive observations of sexual reproduction through microscopic tools. One clue into the sexual activity of AMF is evidence of heterokaryosis in their multi-nucleated mycelium. The recent identification of multi-allelic mating-type loci (*MAT* locus) potentially places AMF among other heterothallic or bipolar species, who's mating compatibility is determined by their *MAT* locus. The presence of a hidden sexual cycle in AMF is still a possibility, and recent findings on the meiotic gene content of AMF suggests an alternative narrative to how these fungi have escaped extinction for so long. Seven meiosis-specific genes (MSG) were found to exist in AMF, indicating that these fungi are likely undergoing a cryptic sexual cycle. The main goal of this research is to determine if/when MSG are expressed in an *in-vitro* model of AMF.

2.2 Keywords

Arbuscular mycorrhizal fungi, *Rhizophagus irregularis*, homokaryon, dikaryon, meiosis-specific gene, digital droplet PCR, ancient asexual, gene expression

2.3 Introduction

Meiosis is near universal among eukaryotes and is essential for long-term persistence and the adaptation of species (Goddard, Godfray, and Burt 2005; Goodenough and Heitman 2014). Not only does it lead to the production of offspring, but in doing so, drives recombination of genes, and results in the shuffling of genetic material which, through natural selection, purges deleterious mutations that may accumulate over time (Glémin 2003). Some rare eukaryotic organisms, however, have seemingly defied this conserved and ancient mechanism and appear to have persisted for millions of years exclusively through asexual means. These "ancient asexual" lineages have not only defied the basic principles of evolutionary theory (Gandolfi et al. 2002; Scho 2003), but in some cases, have thrived ecologically (Little and Hebert 1996). Examples of these anomalous organisms include arbuscular mycorrhizal fungi (AMF), bdelloid rotifers, and a number of protist lineages (Halary et al. 2011; Normark, Judson, and Moran 2003). Notably, the classification of these select individuals has often been based on the absence of evidence for sexual reproduction (Judson and Normark 1996) as opposed to the evidence for asexuality (genetic structure, mutation rates, gene decay).

It is possible that asexual means to generate genetic diversity such as unisexual and parasexual reproduction (Joseph Heitman 2010), or horizontal gene transfers (Nocturnal and Schwander 2016), could offset the deleterious attributes common to asexuality in these lineages. Still, many asexual species whose genomes have been sequenced harbor most of the genetic repertoire needed for proper completion of meiotic recombination, derived from their sexual ancestors (Halary et al. 2011; Pöggeler 2002), suggesting that these organisms may undergo cryptic sex.

AMF are a textbook example of an ancient asexual, as they are thought to have propagated for over 400 million years without meiosis. They are a group of root obligate, biotrophic symbionts that are part of the fungal sub-phylum Glomeromycotina, which provide water, nutrients (Govindarajulu et al. 2005), and pathogen protection (Wehner et al. 2010) to more than 80% of land plants in exchange for their photosynthetic products (Berruti et al. 2016). AMF thus act as "biofertilizers", have a profound effect and influence on the biodiversity of plants, and play a major role on life on land (Igiehon and Babalola 2017a; Sch, Schwarzott, and Walker 2001). How they have persisted for so long and had such a strong impact on our ecosystems has been an ever-increasing topic of research.

Evidence for a cryptic sexual cycle in AMF began with the identification of 51 meiosis related genes present in the genus formerly known as *Glomus*, now *Rhizophagus* (Halary et al. 2011). This repertoire accounts for approximately 85% of the core-meiotic machinery and would theoretically encode enough genes to allow AMF to undergo sexual reproduction. The AMF core genes that were not detected in this study do not affect successful completion of meiosis in many fungi (Kumar, Bourbon, and Massy 2010). For example, these genes are notably absent in fungal models of mating and meiosis-capable fungal relatives (Malik et al. 2008). Some eukaryotic organisms known to reproduce sexually have even less core meiotic genes than AMF; *N. crassa*, *G. zeae*, *D. melanogaster* are all missing important proteins (*Hop2* and *Mer3*), and are known sexual organisms (Malik et al. 2008). In summary, although no direct observations of sexual structures in AMF have ever been observed, their genome is more than equipped to undergo a

potential cryptic sexual cycle, which has shifted the focus away from morphological observations of sexual stages and into deeper analyses of their transcriptomes.

An additional clue into the sexual activity of AMF is the evidence of a dikaryote-like life cycle in some isolates, where by thousands of nuclei originating from 2 distinct strains co-exist in one large syncytium (Boon et al. 2015; Ropars et al. 2016). Furthermore, the recent identification of multi-allelic putative mating-type loci (*MAT* locus) in AMF suggests that they may be heterothallic or bipolar species, whose mating compatibility is determined by their *MAT* locus (Hsueh and Heitman 2008). The *MAT* locus is a specialized genomic region that controls fungal compatibility by coding for pheromones that govern the fusion of cells from two different individuals (Casselton and Olesnicky 1998; Coppin et al. 1997; J Heitman et al. 2007). In theory, two homokaryotic (containing one *MAT* locus allele) isolates could undergo anastomosis (hyphal fusions) to exchange nuclei (Giovannetti et al. 2001) and form a new dikaryon-like (containing two distinct *MAT* locus alleles) isolate, of which three (A4, A5, SL1) have been identified thus far (Ropars et al. 2016).

Mounting signs point toward AMF being capable of meiosis, however, akin to many of its fungal relatives, a number of other processes might be undergoing that parallels sexual reproduction in the absence of meiosis. Recent evidence in *C. albicans*, an asexual fungal parasite, shows that a parameiotic mechanism exists in this species, which allows for genetic recombination in the absence of sex. Core meiotic genes which are also found in AMF, *Spo11* and *Rec8*, play a central role in concerted chromosome loss (CCL) and parameiosis in *C. albicans*, which mimics the haploidization of meiosis by hijacking genes usually involved in chromosome stability and

interhomolog recombination (Anderson et al. 2019). Although AMF may not be undergoing parameiosis like *C. albicans* (Yildirir et al. 2020), the discovery of this process suggests that AMF could use their repertoire of core meiotic genes to undergo analogous pseudo-meiotic processes such as parasexual reproduction.

Among the 51 core meiotic genes present in AMF, seven of them (*Spo11*, *Rec8*, *Mnd1*, *Dmc1*, *Msh4*, *Msh5*, and *Hop*2) are meiosis-specific genes (MSG), which means, as far as we know, they are not generally linked to any other processes other than meiosis in model organisms (Halary et al. 2011). In particular, these genes are specifically involved in meiotic recombination, sister chromatid cohesion, homolog crossover, nuclear division, and the repair of double-strand breaks (Bishop et al. 1992; Keeney, Giroux, and Kleckner 1997; Nishant et al. 2010; Watanabe and Nurse 1999; Zierhut et al. 2004). Still, the formal absence of meiosis observations in AMF raises the possibility that MSG may be implicated in frequent cellular processes, such as mitosis or DNA replication in these organisms, highlighting the need for expression profiling of these genes.

Here, we examine the expression of MSG in a variety of isolates in the model AMF species *R. irregularis*. The differences in their expression will be assessed in three homokaryon isolates (56, C2, and 454) and three dikaryon isolates (A4, A5, SL1) and is analyzed using the quantitative techniques, qRT-PCR and digital droplet PCR (ddPCR). The present study allowed us to determine a baseline level of MSG expression in six *R. irregularis* isolates while highlighting differences and similarities between the two known genome organizations known in AMF. After determining the optimal technique for the analyses of these genes, we analyzed the

expression of MSG in an environment where two genetically different homokaryon isolates of *R. irregularis* (A1 and C2) coexist, anastomose, and have the potential to exchange nuclei, a vital stage of the putative sexual cycle of AMF (Ropars et al. 2016). These analyses open the door for future studies on the conditions involved in activating meiotic pathways within these fungi and represent vital steps in elucidating the complex sexual lives of AMF.

2.4 Materials and Methods

2.4.1 Primer design and In silico identification of meiosis-specific genes

Primers for MSG were designed based on the *R. irregularis* isolate A4 genome acquired from JGI Mycocosm. Gene regions where primers are located were aligned against fungal relatives to confirm homologue identity and to show that the primers did not lie within introns (Supplemental Fig S1a-f) Primer design was completed in SnapGene and ordered from IDT. Primers were rehydrated with autoclaved water and diluted ten-fold to a concentration of 10 ng/uL. Amplicon lengths were approximately 150 bp and were tested on stock DNA of *R. irregularis* isolate A4 to ensure they worked properly.

2.4.2 Fungal isolates and mycelium extraction

Fungal isolates (homokaryon and dikaryon) were plated on circular split-plates (Supplemental Figure S2a) with MSR media. One semi-circle housed a sucrose-nutrients mix and the other semi-circle contained the same nutrient mix without the sucrose. The fungal isolates were acquired from pre-existing cultures in our inventory and were plated on the sucrose positive side with pieces of transformed carrot roots to facilitate symbiosis and allow the fungus to grow into the other compartment of the plate. For the crossings experiment, compatible homokaryon isolates

were plated in a 3-well split plate system (Supplemental Figure S2b) with identical media to the other experiments. An empty well was in between both homokaryon compartments to allow them to grow together, anastomose, and be isolated from the carrot roots for extraction. After around 3-4 months of growth in a 25˚C incubator and weekly redirections of the transformed carrot roots, the fungal side (sucrose negative) of the plate was extracted using a sterilized scalpel and placed in a sodium citrate/citric acid buffer which solubilizes the MSR media. A stir-bar was added to spin and allowed the fungal mycelium to lump together. After one hour, the fungal tissue was removed from the buffer, strained using a sieve, and dried using air suction. The fungal tissue was then collected using sterilized tweezers and placed in a 1.5 mL Eppendorf tube on ice.

2.4.3 RNA Extraction

The fungal tissue was placed in a 2.5-inch diameter mortar and frozen with liquid nitrogen. Once frozen, the fungal tissue was crushed to a powder with a pestle and collected with a thin metal spatula and placed in a 2 mL tube. A modified Qaigen Plant mini RNA Extraction protocol was used: 450 uL of Buffer RLC is added to the tube with the fungal tissue and is vortexed at maximum speed for 5-10 seconds until the tissue is well mixed with the buffer. The tube is then incubated at 56˚C for three minutes and shaken at 300 rpm to promote destruction of the tissue. The liquid formed is transferred to a Qaigen shredder column and centrifuged at 15000 rpm for two minutes. The flow-through is then taken from the collection tube and transferred to a 1.5 mL Eppendorf tube where 250 uL of 99% ethanol is added and mixed by pipetting. The liquid is then immediately transferred to a second Qaigen column and centrifuged at 15000 rpm for 15 seconds. The flow-through is then discarded and 700 uL of buffer RW1 is added to the column and centrifuged at 15000 rpm for 15 seconds with a new 2 mL collection tube below it. The flow-through is discarded and 500 uL of buffer RLP is added to the column with a new collection tube

and centrifuged at 15000 rpm for 15 seconds. The flow-through is discarded again and 500 uL of buffer RLP is added a second time to the column with a new collection tube underneath. The column is then centrifuged for two minutes at 15000 rpm and the flow-through is discarded. A 1.5 mL Eppendorf tube replaces the collection tube below the column and 40 uL of RNAse free water is added to the centre of the column. It is then centrifuged at 15000 rpm for one minute and the column is then discarded, leaving the liquid in the Eppendorf tube.

2.4.4 DNA Removal

RNA RapidOut DNA removal kit was used and modified for the purification of the RNA sample. 5 uL of MgCl buffer and 5 uL of DNAse solution is added to the sample. The solution is mixed by pipetting and placed in a shaker-incubator at 37˚C for 30 minutes at 300 rpm. After the incubation, 10 uL of DNAse removal reagent is added to the solution and it is mixed by pipetting once every minute for 3 minutes, at which point it is placed in a centrifuge at 15000 rpm for one minute. The DNAse removal regent is pelleted and the supernatant is then transferred to a new 1.5 mL Eppendorf tube. This process is repeated a second time to ensure the complete removal of DNA within the sample.

2.4.5 cDNA synthesis

After the DNA removal is complete, 30 uL of the sample was transferred to a PCR tube and the iScript cDNA synthesis kit was used. 8 uL of the iScript buffer is added to the sample along with 2 uL of reverse transcriptase enzyme. The tube was vortexed and centrifuged and placed in a PCR machine with the following parameters: 5 min at 25˚C, 20 min at 46˚C, 1 min at 95˚C, followed by a hold at 4˚C. The remaining RNA not converted to cDNA was stored on ice, used for Qubit RNA concentration analysis, and subsequently stored at -80˚C indefinitely.

2.4.6 PCR confirmation of successful extraction

After cDNA synthesis, 1 uL of the sample was used for PCR amplification with the reference gene *bTub*. The primers for this gene were designed around an intron to ensure cDNA confirmation and no genomic DNA contamination. 7.5 uL of Econotaq is added to 4.5 uL of autoclaved water along with 1.0 uL of each forward and reverse primer and 1.0 uL of the sample. Once the PCR amplification is complete, the sample was run through (0.6%) agarose gel electrophoresis to determine the size of the fragments. A positive control was used with genomic DNA from the same isolate extracted. This is used to ensure that there is no more genomic DNA in the sample and that the RNA extraction/cDNA synthesis worked. An example of this is shown in the supplemental figure S3.

2.4.7 qRT-PCR procedure and analysis

Meiosis-specific gene expression was analyzed using qRT-PCR. Since the concentration of RNA/cDNA of the samples are so low (3-40 ng/uL), the samples were diluted 1:2 for qRT-PCR analysis of MSG expression and diluted 1:10 for qRT-PCR analysis of the reference gene expression. 7.0 uL of SsoFast Taq polymerase is added to 6.0 uL of the diluted cDNA sample and 1.0 uL of each forward and reverse primer. There are 6 primer sets for MSG (*Msh*4, *Msh*5, *Spo*11, *Rec*8, *Mnd*1, and *Dmc*1) and 3 reference genes (*bTub*, *aTub*, *Ef*1*a*). Technical triplicates were included for each sample and ran in the qRT-PCR machine. The data was analysed using CFX Maestro.

2.4.8 ddPCR procedure and analysis

Meiosis-specific gene expression was also analyzed using ddPCR. Since the concentration of RNA/cDNA of the samples are so low (3-40 ng/uL), the samples are diluted 1:2 for ddPCR analysis of the MSG expression and diluted 1:10 for ddPCR analysis of the reference gene expression. 11.5 uL of QX200 ddPCR EvaGreen Supermix, 1 uL of each forward and reverse primer, 6 uL of nuclease-free H2O, and 5 uL of template was used to prepare the mastermix. Three technical triplicates were done for each of the three biological samples and ran in the ddPCR machine, then was subsequently placed in the digital droplet reader for analysis. The data was recorded using QuantaSoft.

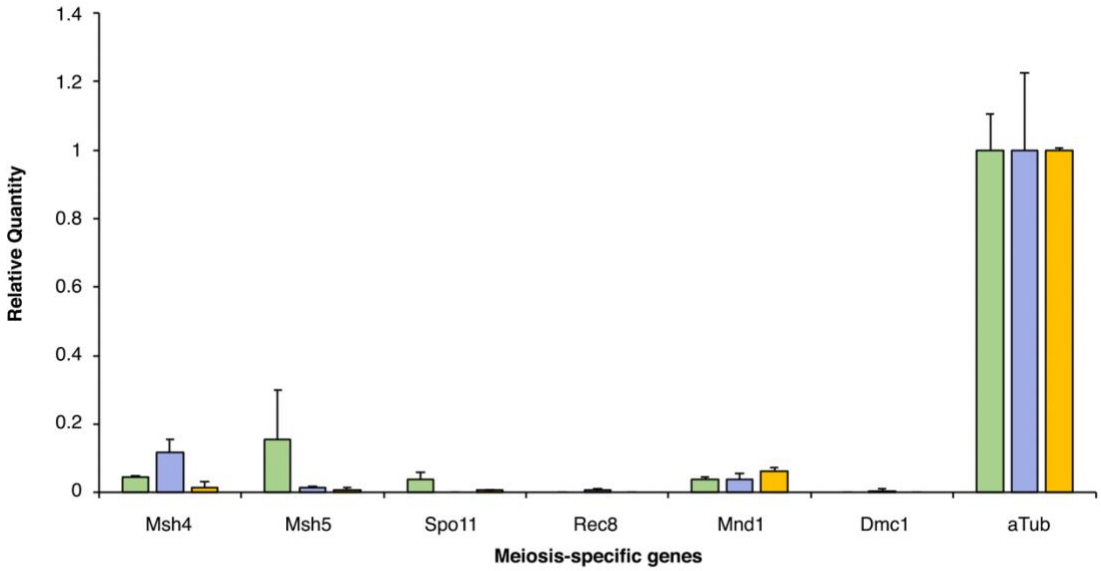
2.5 Results

2.5.1 Meiosis-specific gene expression in homokaryon and dikaryon AMF in-vitro cultures

The isolates studied in this paper are all of the model AMF species *Rhizophagus irregularis* and contain the complete set of core-meiotic genes previously identified in Glomeromycotina (Halary et al. 2011). Among these, six meiosis-specific genes (MSG) were selected for expression analysis due to their almost exclusive role in meiotic processes. *Hop*2 was removed from the analysis due to non-specific overexpression, shown in previous unpublished data (Figure 1.3). The remaining genes selected encode proteins involved in meiotic recombination (Msh4, Spo11, and Mnd1), formation of the sister chromatic cohesion complex (Rec8), pairing between homologous chromosomes (Dmc1), and crossover events during meiosis (Msh5). Initial analysis of homokaryon and dikaryon isolates using qRT-PCR revealed that the MSG are expressed at extremely low levels compared to reference genes (Supplementary Fig 4). However, RNA extractions yielded, on average, low concentrations (3-40 ng/µL) and

amplification curves lied beyond the limit of quantification for this technique. To address this, MSG profiles for the same strains were analyzed with the high-resolution ddPCR technique. Specifically, ddPCR allows for quantification of transcripts down to single copy numbers to be identified and is thus ideal for analyzing MSG expression profiles. The ddPCR results corroborated those obtained using qRT-PCR, as they all indicate that these genes are all weakly expressed compared to the reference genes (*aTub*, *bTub*, *Ef1a*) and display some variability of expression across homokaryon and dikaryon isolates. Among MSG, highest levels of expression are seen in *Msh*4, *Msh*5, and *Mnd*1, with remarkably little to no expression shown for *Spo*11, and especially *Rec*8 and *Dmc*1. No notable differences in levels of expression are seen between the homokaryon and dikaryon isolates (Figure 4ab).

a



b

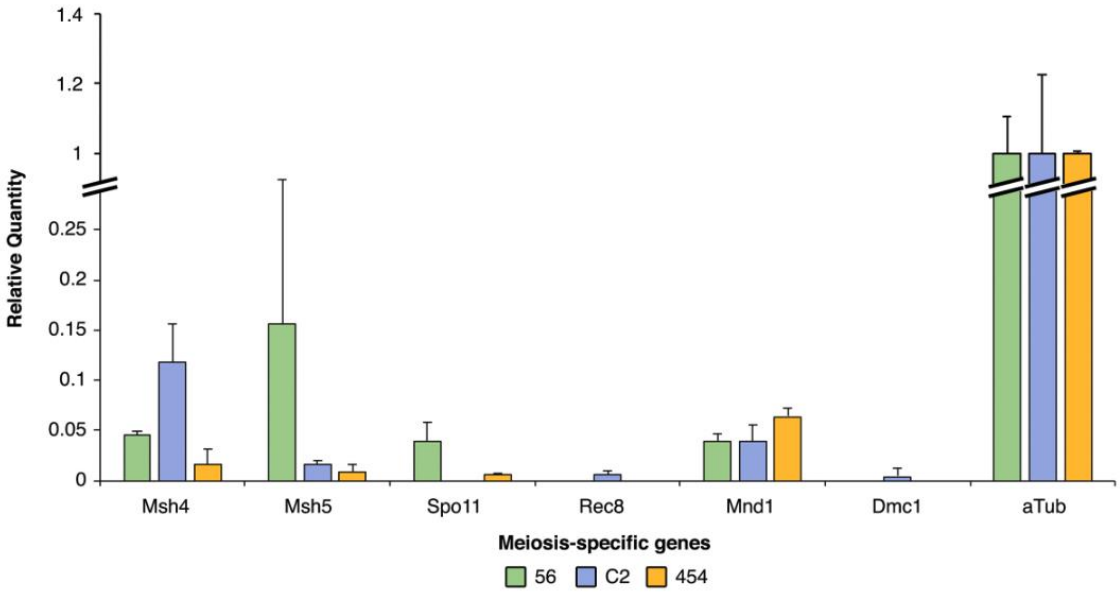
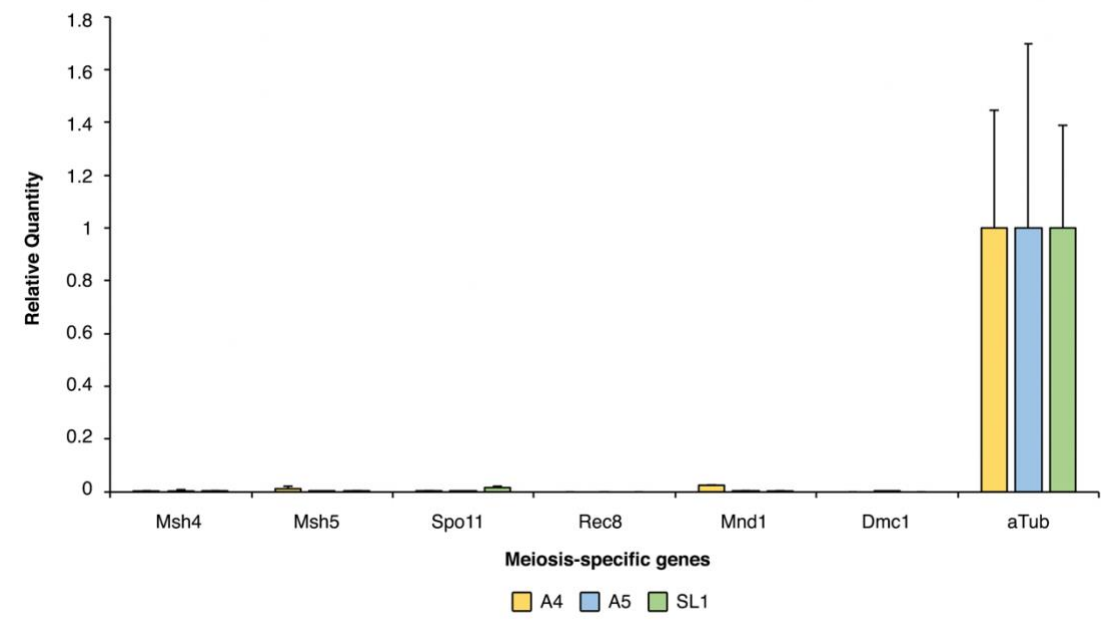


Figure 2.1. Gene expression levels of meiosis-specific genes (MSG) in *R. irregularis* homokaryon isolates measured using ddPCR. Biological replicates (n =3) for every isolate and technical replicates (n = 3) for every biological replicate included in analysis. a) displays the relative quantity of MSG normalized to the reference gene (*aTub*) of the homokaryon isolates C2, 454, and 56 in standard in-vitro culture extracted after 4 months of growth. b) displays the same homokaryon isolates with a break in the scale to better view individual differences in expression between meiosis-specific genes.

a



b

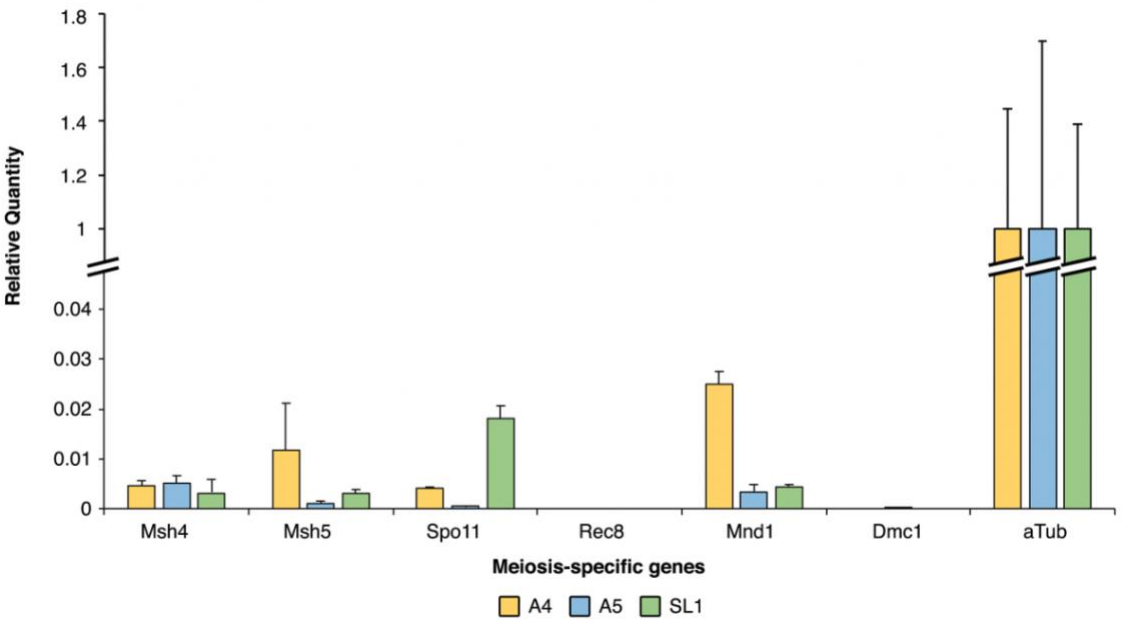
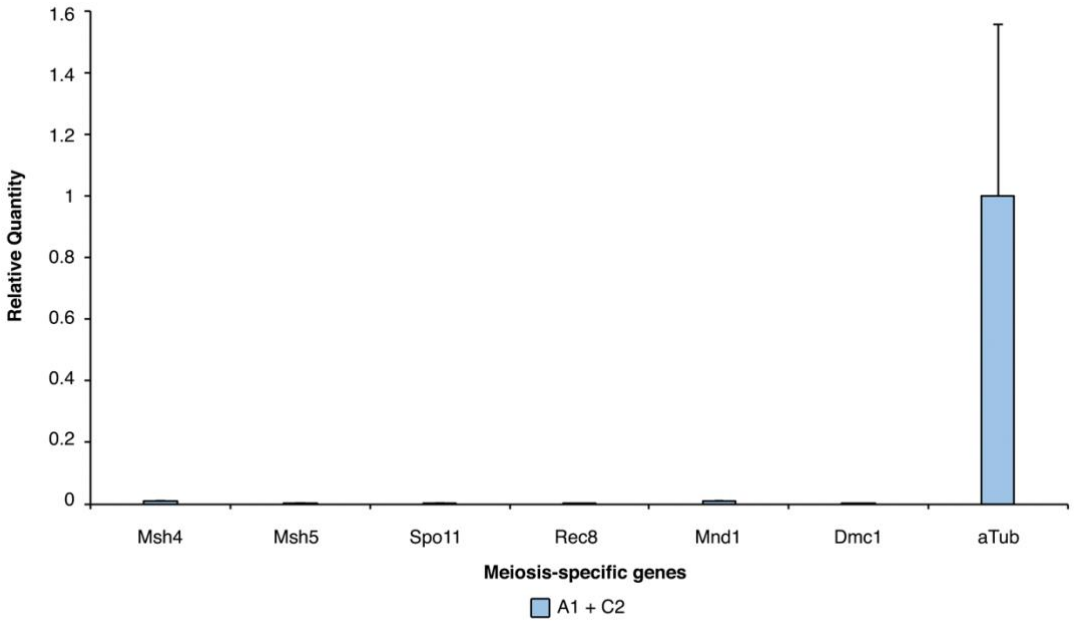


Figure 2.2. Gene expression levels of meiosis-specific genes (MSG) in *R. irregularis* homokaryon isolates measured using ddPCR. Biological replicates (n =3) for isolate A4, SL1, and (n =2) A5 as well as technical replicates (n = 3) for every biological replicate included in analysis. a) displays the relative quantity of MSG normalized to the reference gene (*aTub*) of the dikaryon isolates in standard in-vitro culture extracted after 4 months of growth. b) displays the same dikaryon isolates with a break in the scale to better view individual differences in expression between meiosis-specific genes.

2.5.2 Meiosis-specific gene expression in Homokaryon crossing AMF in-vitro cultures

Two homokaryon isolates, A1 and C2, represent isolates with potentially compatible mating types (*MAT*3 and *MAT*6, respectively). In theory, these two isolates would anastomose, exchange nuclei, and undergo sexual reproduction given the right circumstances. These isolates contain the complete set of core-meiotic genes previously identified in Glomeromycotina (Halary et al. 2011), including the six MSG of interest. Gene expression was analysed using ddPCR. The results indicate weak expression of all MSG normalized to the reference gene, *aTub* (Figure 3ab). Among the six genes, the greatest level of expression among the MSG is seen from *Msh*4 and *Mnd*1, however when normalized to the reference genes, display similar expression to both the homokaryon and dikaryon isolates (Figure 4ab). The absence of ubiquitous expression in these genes suggests that the active interaction between A1 and C2 in standard culture conditions is not sufficient in activating MSG. Crossings may need to be analyzed under induced stress to demonstrate an upregulation in expression.

a



b

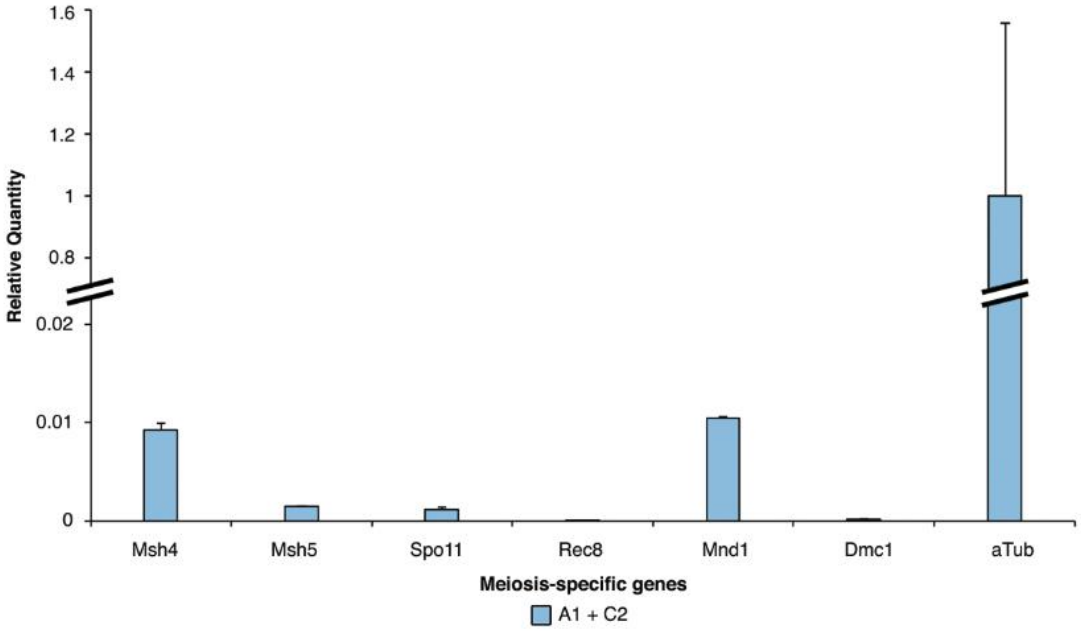
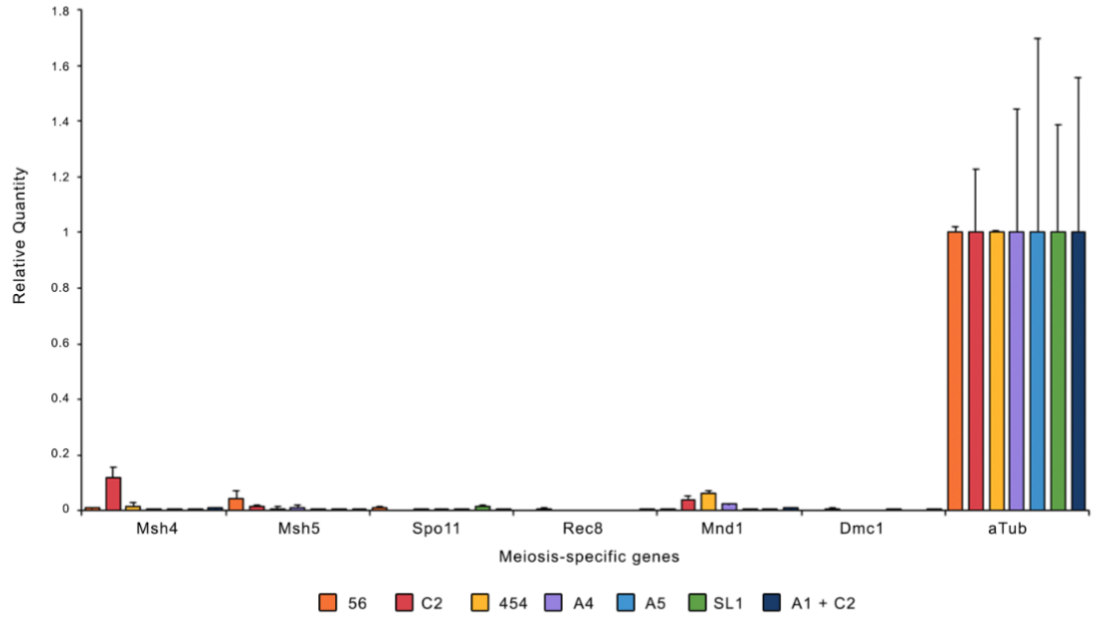


Figure 2.3. Gene expression levels of meiosis-specific genes (MSG) in *R. irregularis* homokaryon isolate crossings measured using ddPCR. Biological replicates (n =3) and technical replicates (n = 3) for every biological replicate included in analysis. a) displays the relative quantity of MSG normalized to the reference gene (*aTub*) of the homokaryon crossing between A1 and C2 in 3-well split plate in-vitro culture extracted after 4 months of growth. b) displays the same homokaryon crossing with a break in the scale to better view individual differences in expression between meiosis-specific genes.

a



b

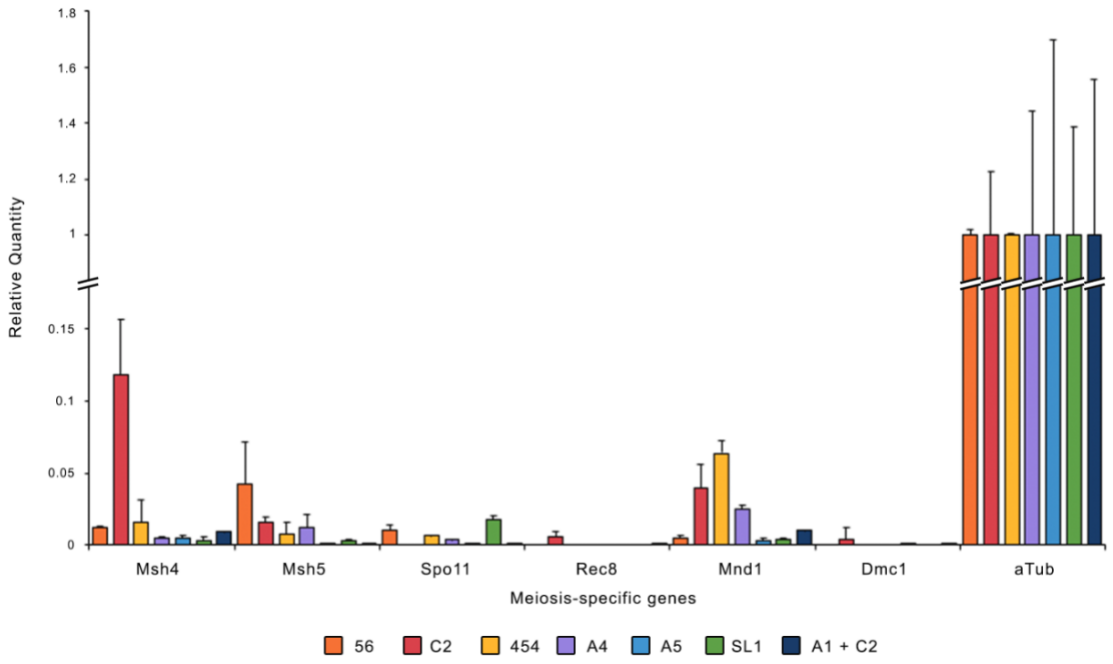


Figure 2.4. Comparing meiosis-specific gene expression levels in all *R. irregularis* isolates analyzed using ddPCR. Biological replicates (n =2-3) and technical replicates (n = 3) for every biological replicate included in analysis. a) displays the relative quantity of MSG normalized to the reference gene (*aTub*) of all isolates in 2-well and 3-well split plate in-vitro culture extracted after 4 months of growth. b) displays the same isolates with a break in the scale to better view individual differences in expression between meiosis-specific genes.

2.6 Discussion

Eukaryotes and fungi by and large undergo sexual reproduction in nature (Bernstein and Bernstein 2013). The generation of genetic diversity has expunged mutations and deleterious traits from accumulating in populations for over a billion years, almost exclusively through meiotic processes (Egel and Penny 2008; Muller 1964). However, exceptions exist, and this very select group of eukaryotes (dubbed "ancient asexuals") have seemingly thrived in the absence of sexual reproduction for hundreds of millions of years. However, with the advent of advances in molecular and microbiological technology, the list of ancient asexuals continues to diminish in numbers. Recent findings in the genome of *R. irregularis*, a model AMF species and member of this exclusive list, presents an interesting argument against their supposed lack of sexual reproduction. The improved genomes of these fungi, coupled with the discovery of a core meiotic gene repertoire along with a *MAT* locus, prompted this study's investigation into the expression of meiosis-specific genes.

Overall, our analyses revealed a generally very weak expression for all six MSG in the three assays performed, particularly compared to known housekeeping genes. At a minimum, this finding indicates that many MSG are constitutively expressed to fulfill continued cellular requirements in AMF such as mitosis or DNA replication. This result is also supported by the similar expression levels found in AMF homokaryons and AMF dikaryons. Specifically, our analyses do not indicate that MSG are expressed at high levels in AMF dikaryons, which is the stage that represents the first step in the putative cycle of AMF sexual reproduction. The expression levels in the homokaryon assay were used to compare to the other two assays that we performed (Figure 2.4ab), which represent later stages of the putative cycle of sexual

reproduction where one might expect an upregulation of these genes, if meiosis were to occur. In AMF dikaryons, which contain two sets of nuclei, each with genetically distinct *MAT* loci, the analysis showed a notable lack of ubiquitous expression. When looking at the homokaryon crossing assay between A1 and C2, similar results can be seen. Although these two isolates do show evidence of plasmogamy (Table 1.1), other homokaryon pairings may be more suited for genetic meiotic recombination to occur, or meiosis may undergo under very unique environmental conditions or stress. It is certainly within the realm of possibility that AMF require unfavourable environmental conditions to induce a stress response which promotes the need for sexual reproduction. Conditions such as an imbalance in soil (or in this case media) pH, drought, nutrient deficiency, or limited carbon allocation are all factors that could all potentially induce sexual reproduction in AMF. Indeed, it has been demonstrated in numerous organisms, including filamentous fungi, that environmental stress can induce the upregulation of genes and processes involved in sexual reproduction (Bell 1982; Schoustra, Rundle, and Dali 2010), and different plant hosts may also play an important role in the sexual lives of AMF.

Our fungi are cultured alongside transformed carrot roots, largely for the ease of culturing and quick production of mycelium, which may not be optimal for sexual reproduction. However, previous unpublished preliminary work in our lab has evaluated the differences in MSG expression in plantae versus in vitro, but no significant differences in expression were seen (Figure 1.3) Furthermore, the conditions in our cultures do not mimic the natural environment perfectly and have largely stayed stagnant for dozens of generations. Our isolates may have grown accustomed to the conditions in which they are cultured in and are thus more likely to continue propagating clonally rather than sexually reproducing. As these fungi take months to

grow, it was outside of the scope of this study to test the effects of different environmental conditions on the expression of MSG. However, future studies can certainly address this limitation and use the baseline levels of expression we observed in our isolates to compare with their findings.

Secondly, although AMF possess most of the core meiotic machinery needed for sexual reproduction, these fungi may also have evolved a novel, undefined, cryptic process used to generate genetic diversity which has allowed them to thrive for over 400 million years. This would not be the first time that a fungus which was previously thought to lack sexuality has turned out to undergo alternate processes. Interestingly, *R. irregularis* isolates did not show substantial expression of *Spo*11 and *Rec*8, which were notably upregulated in the discovery of parameiosis in *C. albicans* (Anderson et al. 2019). Although it is possible that a cryptic sexual mechanism may be undergoing in AMF, another scenario is a series of parasexual events, which are widespread in the fungal kingdom. Due to the close proximity of haploid nuclei within the cytoplasm of AMF, certain events such as break-induced replication (BIR) or TE-mediated gene exchange could be occurring (Yildirir et al. 2020). BIR specifically could utilize *Spo*11 and *Rec*8 for double-strand break repairs, but their lack of expression in our analyses continues to suggest that this would occur under highly specific circumstances or at levels rare enough to avoid detection using total RNA extracts. Clearly, sexual reproduction or some novel parasexual process for creating genetic recombination occur in AMF, yet catching it happening in the right situation, at the right time, is one of the greater challenges in attempting to understand how these organisms have thrived for so long.

There is certainly much more to be discovered about the hidden sexual life of AMF. Although this study does not by any means allude to sexual reproduction occurring in these fungi, it provides evidence against the notion that they are constitutively involved in common cellular processes, and represents a clear baseline of expression for these genes that one would expect to be upregulated if meiosis were to occur. Future studies must now build on this data and assess the effects of stressful environmental conditions on dikaryotic isolates as well as crossings of homokaryotic isolates to see if upregulation of meiosis-specific genes occurs. Sexual reproduction is very situation-based and most likely would occur under time-dependent environmental circumstances (Nieuwenhuis and James 2016). As such, there is still much to be done and much to be learned as we chip away at the mystery of how AMF have survived for so long without sex. This study provides essential framework for future experiments on the sexuality of AMF and unveils that a lack of ubiquitous expression of MSG occurs under standalone culture conditions.

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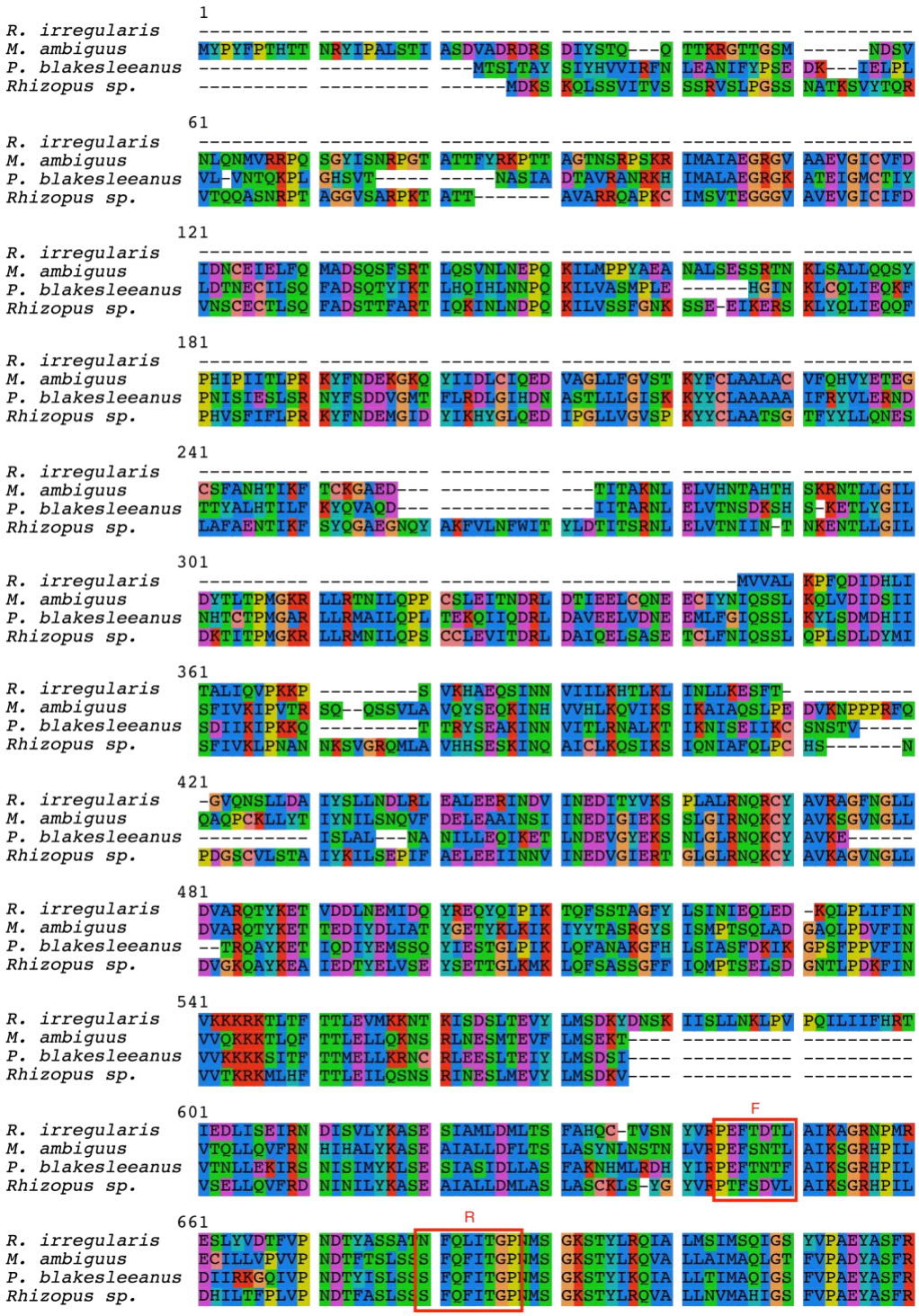
2.7 Supplementary Data

Figure S1a. Msh4 amino acid conservation with fungal relatives from phylum Mucoromycota.



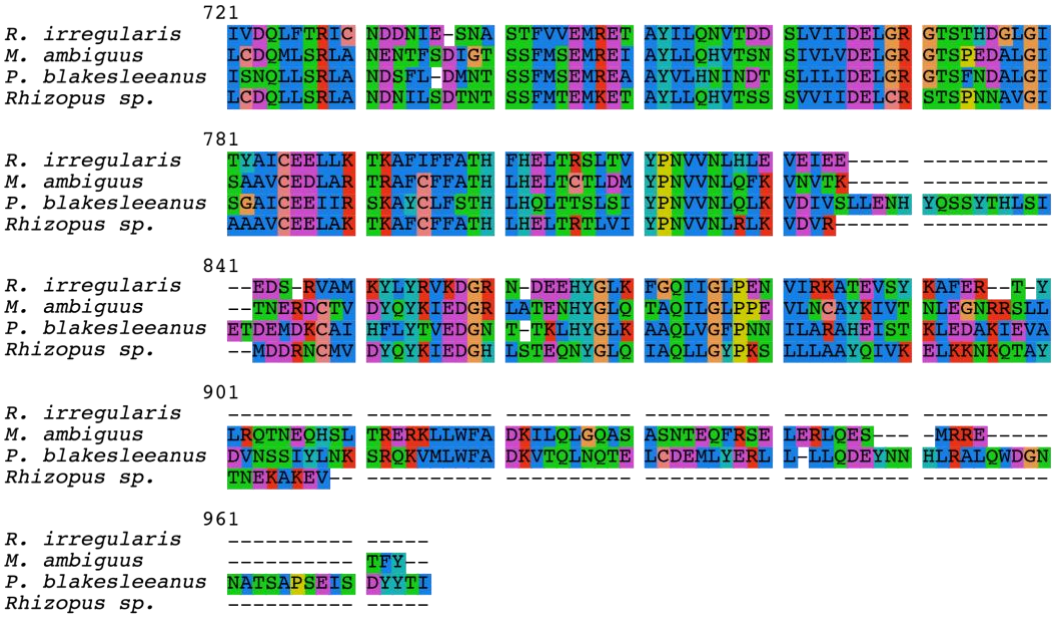
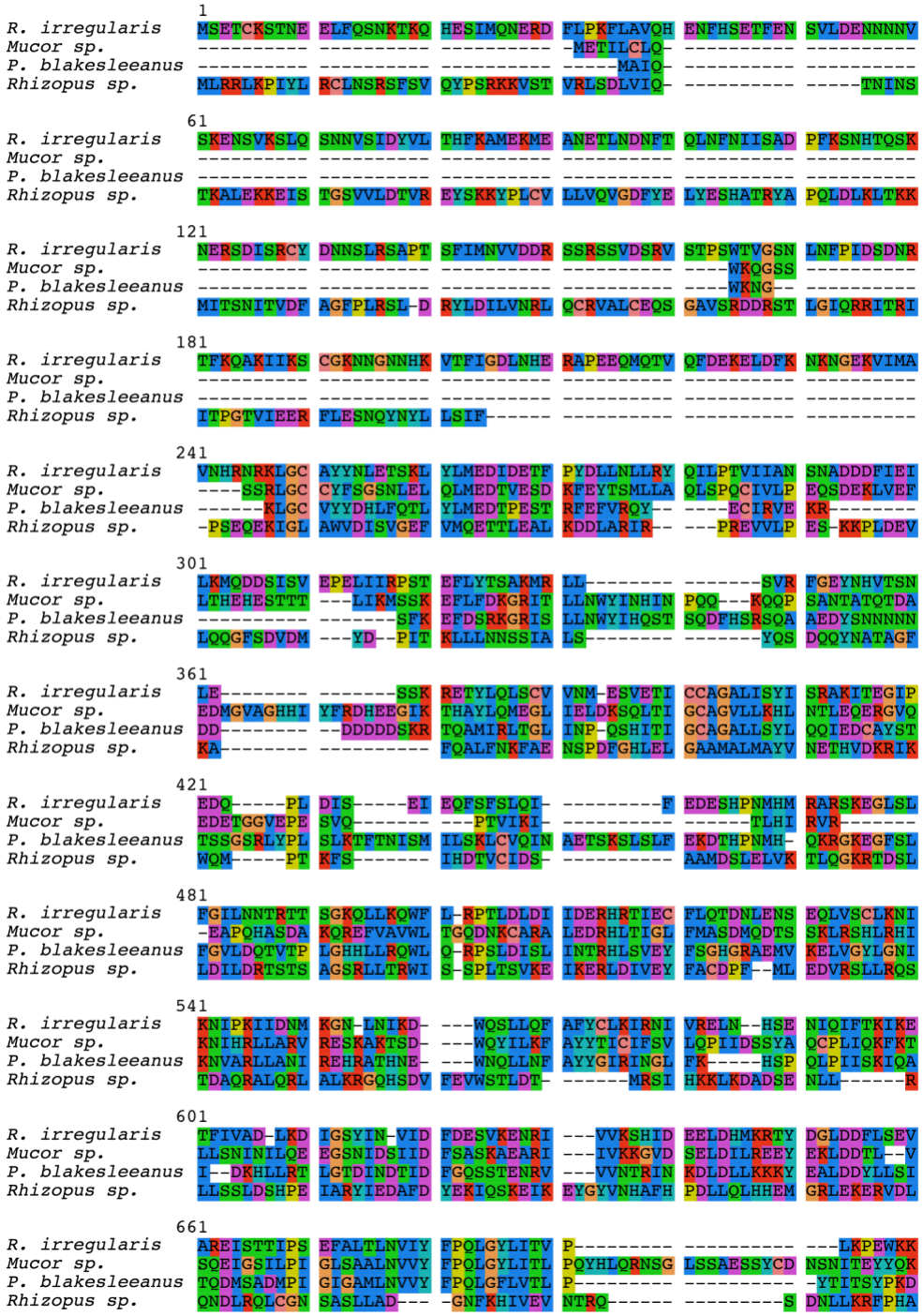

721
R. irregularis IVDQLFTRIC NDDNIE-SNA STFVVEMRET AYILQNVTDD SLVIIDELGR GTSTHDGLGI
M. ambiguus LCDQMLSRLA NENTFSDIGT SSFMSEMREI AYLLQHVTSN SIVLVDELGR GTSPEDALGI
P. blakesleeanus ISNQLLSRLA NDSFL-DMNT SSFMSEMREA AYVLHNINDT SLILIDELGR GTSFNDALGI
Rhizopus sp. LCDQLLSRLA NDNILSDTNT SSFMTEMKET AYLLQHVTSS SVVIIDELCR STSPNNAVGI
781
R. irregularis TYAICEELLK TKAFIFFATH FHELTRSLTV YPNVVNLHLE VEIEE----- ----------
M. ambiguus SAAVCEDLAR TRAFCFFATH LHELTCTLDM YPNVVNLQFK VNVTK----- ----------
P. blakesleeanus SGAICEEIIR SKAYCLFSTH LHQLTTSLSI YPNVVNLQLK VDIVSLLENH YQSSYTHLSI
Rhizopus sp. AAAVCEELAK TKAFCFFATH LHELTRTLVI YPNVVNLRLK VDVR------ ----------
841
R. irregularis --EDS-RVAM KYLYRVKDGR N-DEEHYGLK FGQIIGLPEN VIRKATEVSY KAFER--T-Y
M. ambiguus --TNERDCTV DYQYKIEDGR LATENHYGLQ TAQILGLPPE VLNCAYKIVT NLEGNRRSLL
P. blakesleeanus ETDEMDKCAI HFLYTVEDGN T-TKLHYGLK AAQLVGFPNN ILARAHEIST KLEDAKIEVA
Rhizopus sp. --MDDRNCMV DYQYKIEDGH LSTEQNYGLQ IAQLLGYPKS LLLAAYQIVK ELKKNKQTAY
901
R. irregularis ---------- ---------- ---------- ---------- ---------- ----------
M. ambiguus LRQTNEQHSL TRERKLLWFA DKILQLGQAS ASNTEQFRSE LERLQES--- -MRRE-----
P. blakesleeanus DVNSSIYLNK SRQKVMLWFA DKVTQLNQTE LCDEMLYERL L-LLQDEYNN HLRALQWDGN
Rhizopus sp. TNEKAKEV-- ---------- ---------- ---------- ---------- ----------
961
R. irregularis ---------- -----
M. ambiguus ---------- TFY--
P. blakesleeanus NATSAPSEIS DYYTI
Rhizopus sp. ---------- -----

Figure S1b. Msh5 amino acid conservation with fungal relatives from phylum Mucoromycota.



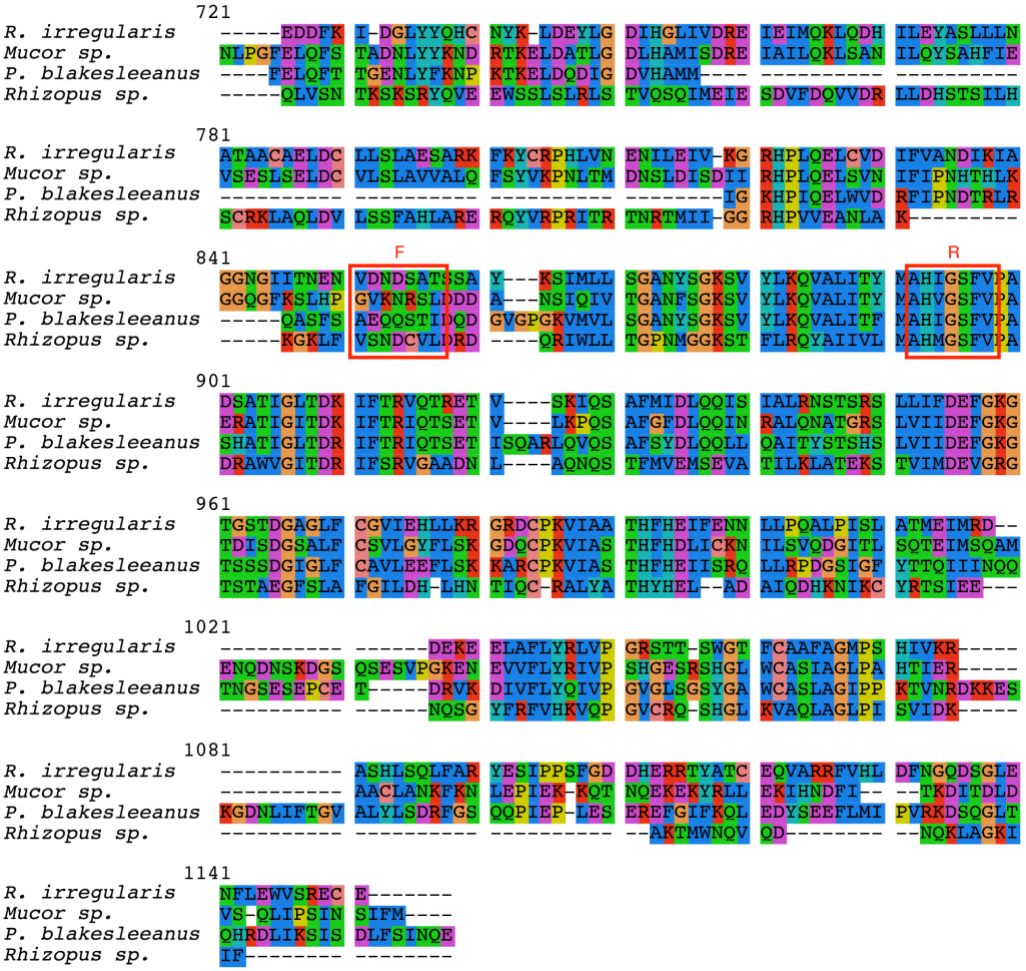

721
R. irregularis -----EDDFK I-DGLYYQHC NYK-LDEYLG DIHGLIVDRE IEIMQKLQDH ILEYASLLLN
Mucor sp. NLPGFELQFS TADNLYYKND RTKELDATLG DLHAMISDRE IAILQKLSAN ILQYSAHFIE
P. blakesleeanus ----FELQFT TGENLYFKNP KTKELDQDIG DVHAMM---- ---------- ----------
Rhizopus sp. -----QLVSN TKSKSRYQVE EWSSLSLRLS TVQSQIMEIE SDVFDQVVDR LLDHSTSILH
781
R. irregularis ATAACAELDC LLSLAESARK FKYCRPHLVN ENILEIV-KG RHPLQELCVD IFVANDIKIA
Mucor sp. VSESLSELDC VLSLAVVALQ FSYVKPNLTM DNSLDISDII RHPLQELSVN IFIPNHTHLK
P. blakesleeanus ---------- ---------- ---------- --------IG KHPIQELWVD RFIPNDTRLR
Rhizopus sp. SCRKLAQLDV LSSFAHLARE RQYVRPRITR TNRTMII-GG RHPVVEANLA K---------
841
F
R
R. irregularis GGNGIITNEN VDNDSATSSA Y---KSIMLL SGANYSGKSV YLKQVALITY MAHIGSFVPA
Mucor sp. GGQGFKSLHP GVKNRSLDDD A---NSIQIV TGANFSGKSV YLKQVALITY MAHVGSFVPA
P. blakesleeanus -----QASFS AEQQSTIDQD GVGPGKVMVL SGANYSGKSV YLKQVALITF MAHIGSFVPA
Rhizopus sp. -----KGKLF VSNDCVLDRD ----QRIWLL TGPNMGGKST FLRQYAIIVL MAHMGSFVPA
901
R. irregularis DSATIGLTDK IFTRVQTRET V----SKIQS AFMIDLQQIS IALRNSTSRS LLIFDEFGKG
Mucor sp. ERATIGITDK IFTRIQTSET V----LKPQS AFGFDLQQIN RALQNATGRS LVIIDEFGKG
P. blakesleeanus SHATIGLTDR IFTRIQTSET ISQARLQVQS AFSYDLQQLL QAITYSTSHS LVIIDEFGKG
Rhizopus sp. DRAWVGITDR IFSRVGAADN L----AQNQS TFMVEMSEVA TILKLATEKS TVIMDEVGRG
961
R. irregularis TGSTDGAGLF CGVIEHLLKR GRDCPKVIAA THFHEIFENN LLPQALPISL ATMEIMRD--
Mucor sp. TDISDGSALF CSVLGYFLSK GDQCPKVIAS THFHDLICKN ILSVQDGITL SQTEIMSQAM
P. blakesleeanus TSSSDGIGLF CAVLEEFLSK KARCPKVIAS THFHEIISRQ LLRPDGSIGF YTTQIIINQQ
Rhizopus sp. TSTAEGFSLA FGILDH-LHN TIQC-RALYA THYHEL--AD AIQDHKNIKC YRTSIEE---
1021
R. irregularis ---------- ------DEKE ELAFLYRLVP GRSTT-SWGT FCAAFAGMPS HIVKR-----
Mucor sp. ENQDNSKDGS QSESVPGKEN EVVFLYRIVP SHGESRSHGL WCASIAGLPA HTIER-----
P. blakesleeanus TNGSESEPCE T-----DRVK DIVFLYQIVP GVGLSGSYGA WCASLAGIPP KTVNRDKKES
Rhizopus sp. ---------- ------NQSG YFRFVHKVQP GVCRQ-SHGL KVAQLAGLPI SVIDK-----
1081
R. irregularis ---------- ASHLSQLFAR YESIPPSFGD DHERRTYATC EQVARRFVHL DFNGQDSGLE
Mucor sp. ---------- AACLANKFKN LEPIEK-KQT NQEKEKYRLL EKIHNDFI-- --TKDITDLD
P. blakesleeanus KGDNLIFTGV ALYLSDRFGS QQPIEP-LES EREFGIFKQL EDYSEEFLMI PVRKDSQGLT
Rhizopus sp. ---------- ---------- ---------- --AKTMWNQV QD-------- --NQKLAGKI
1141
R. irregularis NFLEWVSREC E-------
Mucor sp. VS-QLIPSIN SIFM----
P. blakesleeanus QHRDLIKSIS DLFSINQE
Rhizopus sp. IF-------- --------

Figure S1c. Mnd1 amino acid conservation with fungal relatives from phylum Mucoromycota.

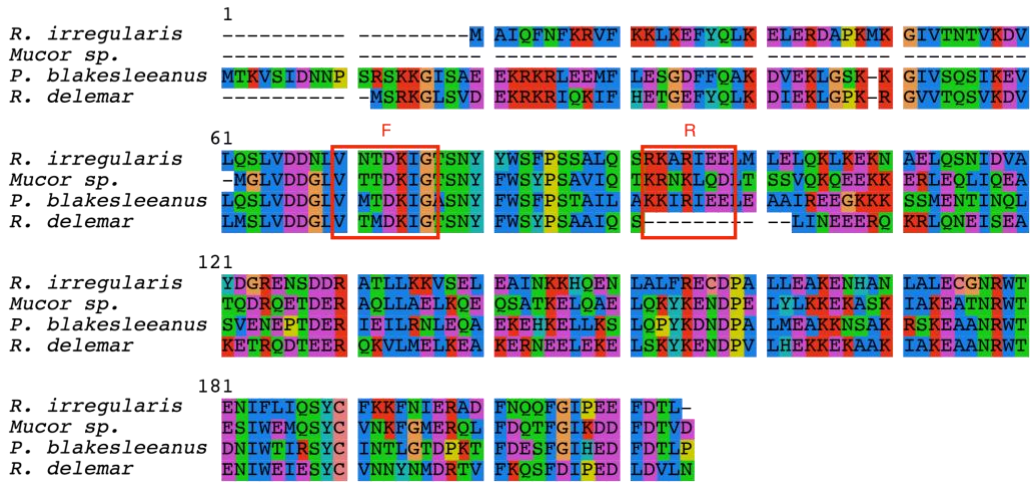


Figure S1d. Rec8 amino acid conservation with fungal relatives from phylum Mucoromycota.

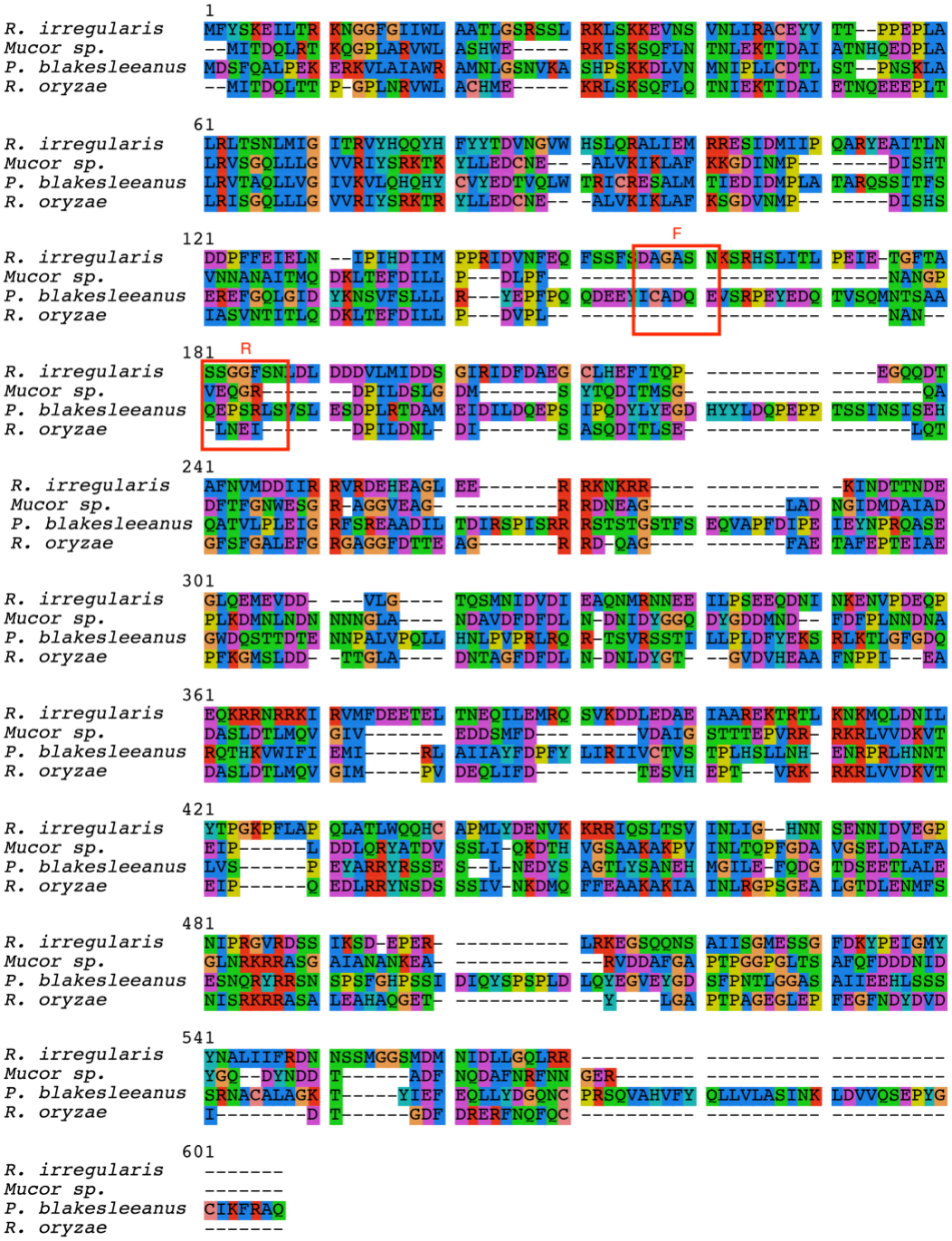


Figure S1e. Dmc1 amino acid conservation with fungal relatives from phylum Mucoromycota.

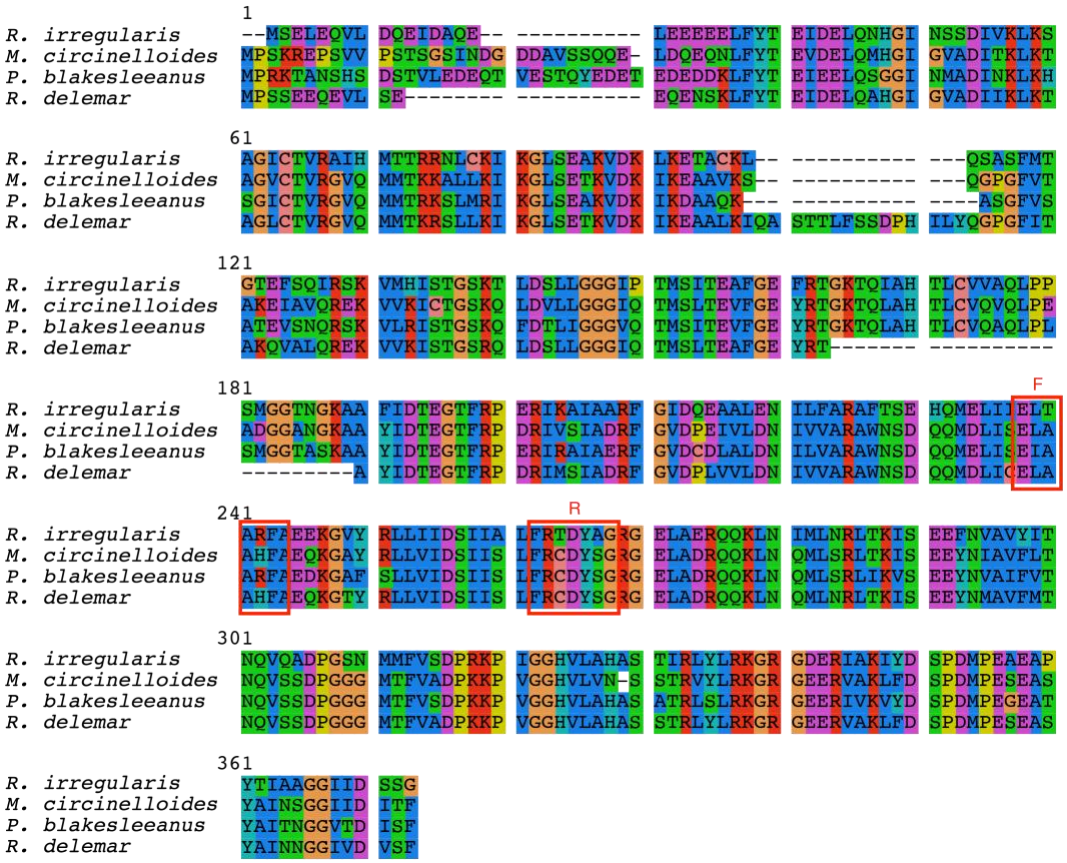
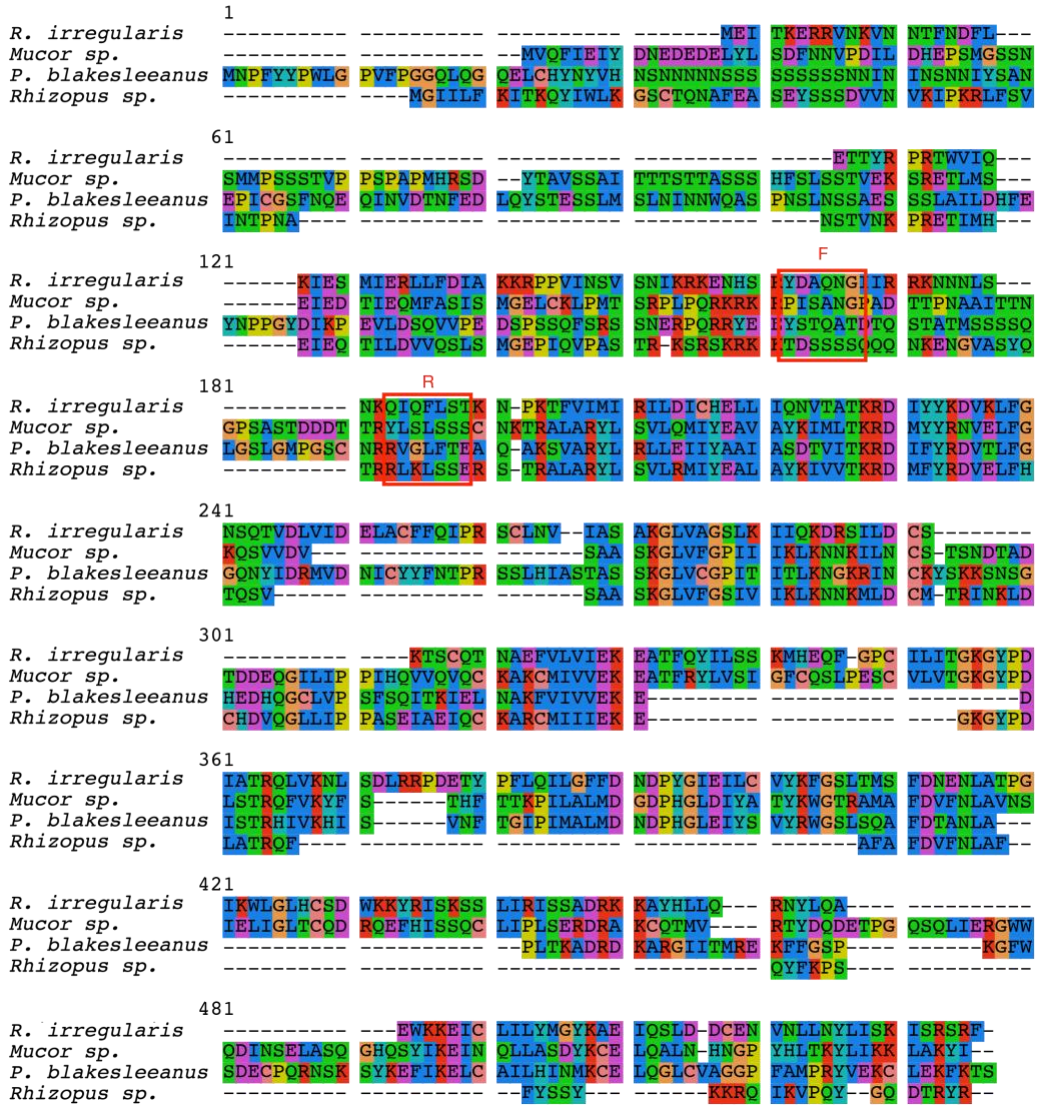


Figure S1f. Spo11 amino acid conservation with fungal relatives from phylum Mucoromycota.



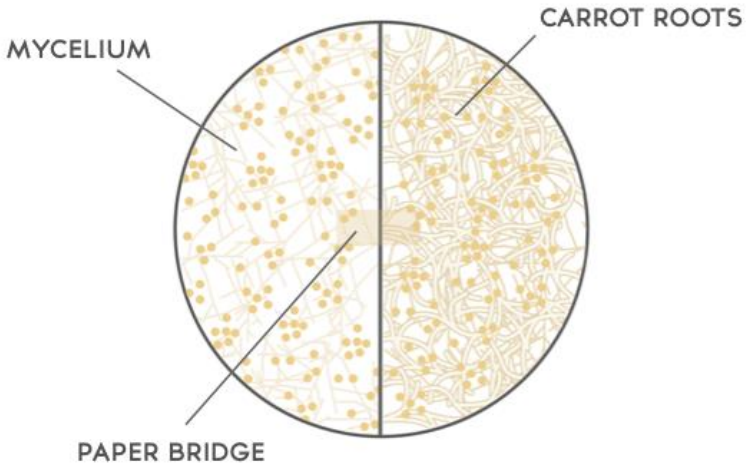


Figure S2a. Homokaryon and dikaryon culture semi-circular compartment plate set-up. Fungal mycelium plated with transformed carrot roots on a sucrose (+) MSR medium on the right side and allowed to grow into the sucrose (-) left side over 4 months at which point it can be extracted.

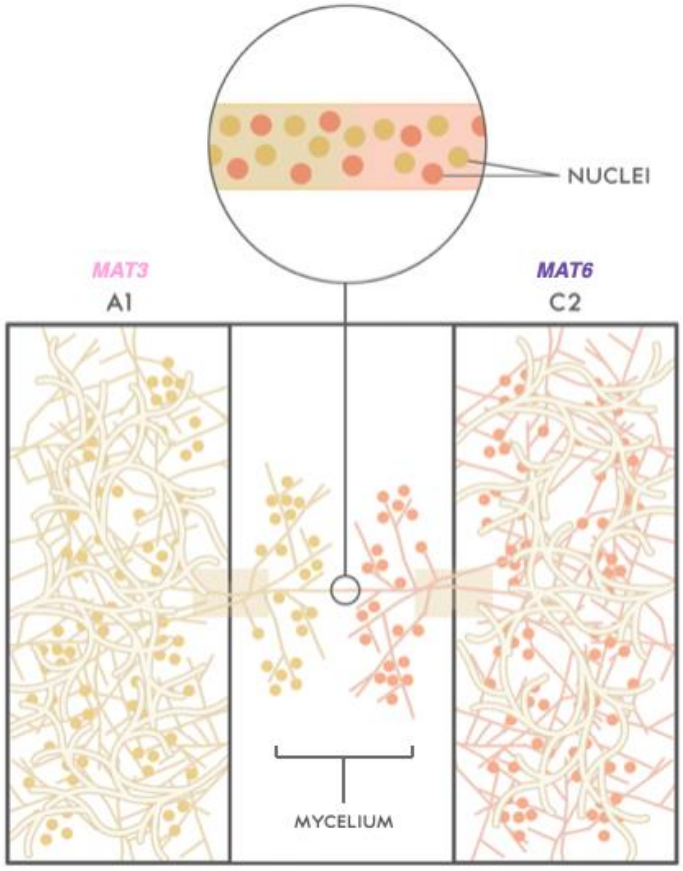


Figure S2b. Homokaryon crossing culture 3-well compartment plate set-up. Fungal mycelium plated with transformed carrot roots on each out compartment on a sucrose (+) MSR medium. Fungal tissue grows into center sucrose (-) compartment for 4 months at which point it can be extracted. Homokaryon crossings A1 and C2 depicted along with their corresponding mating type (*MAT*3 and *MAT*6 respectively). Magnified circle depicts the hypothetical anastomosis of hyphae and exchange of nuclei to form potential dikaryotic stage.

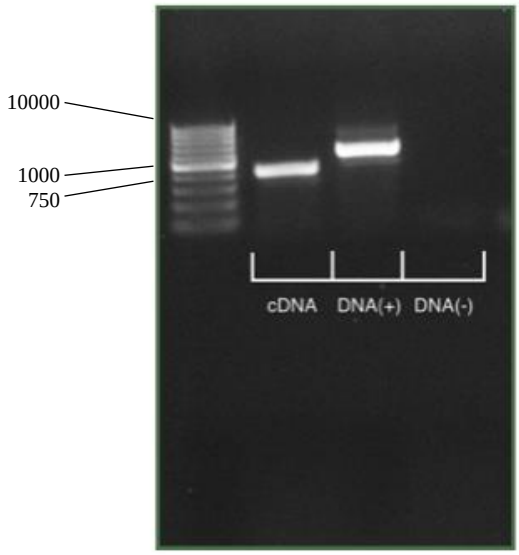


Figure S3. Example of PCR confirmation of cDNA synthesis from extracted fungal RNA. First lane is a 1kb DNA ladder; second lane is the cDNA of *bTub* synthesized from extracted fungal RNA (fragment size is smaller than positive control because the intron has been spliced out in the transcription process); third lane is positive control of DNA from the *R. irregularis* isolate A4 also showing *bTub*; fourth lane is a negative control with no template DNA.

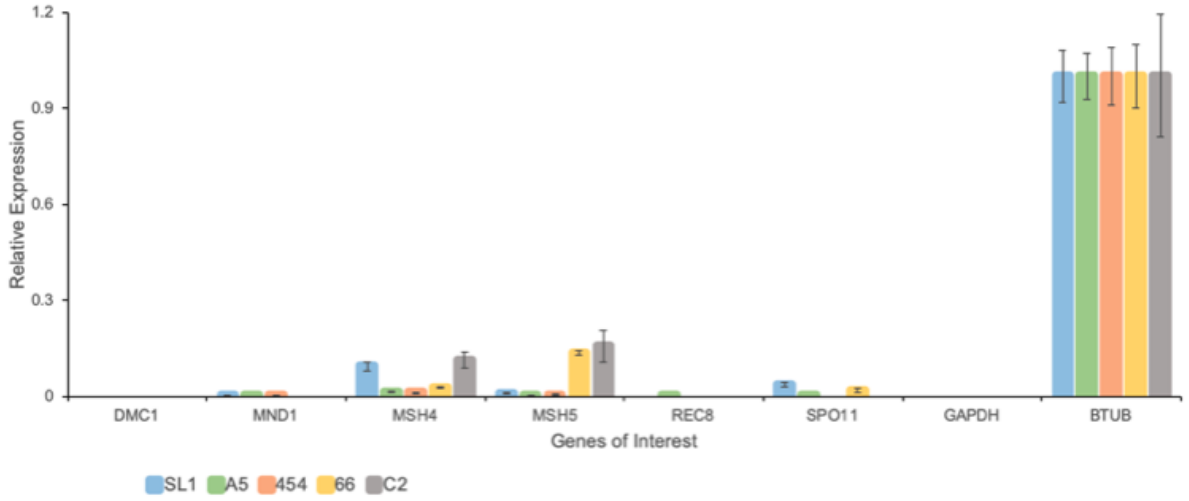


Figure S4. Preliminary qRT-PCR analysis of meiosis-specific gene expression in various isolates of *R. irregularis*. Reference gene is *bTub* and negative control is *GAPDH*. Biological replicates for each isolate is n=3; technical replicates for each biological replicate is n=3.Two dikaryon (SL1 and A5) and three homokaryon (454, 66, and C2) isolates were analyzed.

Table S1. Average raw transcript copy numbers of meiosis-specific genes across all analyses per 20 µL sample. Average calculated using three biological replicates each with three technical replicates for all isolates of *R. irregularis*. Transcript copy numbers obtained from ddPCR and adjusted for initial sample concentration. Copy numbers rounded to whole numbers. Homokaryotic isolates are in red; dikaryotic isolates are in blue; homokaryon crossing is in purple.

Isolate	bTub	aTub	Ef1a	Msh4	Msh5	Mnd1	Spo11	Dmc1	Rec8
C2	1931	6665	1790	228	31	76	0	8	11
454	7606	10756	6164	24	12	97	10	0	0
56	5439	19909	6622	249	847	105	209	0	0
A4	9458	7900	12520	36	93	198	32	0	0
A5	12356	85526	12022	437	87	294	44	9	0
SL1	4261	13045	4050	42	39	58	236	0	0
A1 & C2	10635	55150	11310	521	83	576	65	8	1

Table S2. Fungal representatives used to show conservation in amino acid sequences of meiosis-specific genes. Accession numbers and amino acid sequences obtained from the National Center for Biotechnology Information (NCBI).

Protein	Organism	Accession
Msh4	*Rhizophagus irregularis*	AOP03995.1
	Mucor ambiguus	GAN05554.1
	Phycomyces blakesleeanus	XP_018291952.1
	Rhizopus azygosporus	RCH93530.1
Msh5	*Rhizophagus irregularis*	AOP03990.1
	Mucor ambiguus	GAN10577.1
	Phycomyces blakesleeanus	XP_018296026.1
	Rhizopus azygosporus	RCH88492.1
Mnd1	*Rhizophagus irregularis*	AOP04000.1
	Mucor ambiguus	GAN01903.1
	Phycomyces blakesleeanus	XP_018286555.1
	Rhizopus delemar	EIE82560.1
Rec8	*Rhizophagus irregularis*	AOP03985.1
	Mucor ambiguus	GAN01903.1
	Phycomyces blakesleeanus	XP_018286555.1
	Rhizopus delemar	EIE77841.1
Dmc1	*Rhizophagus irregularis*	AOP04025.1
	Mucor circinelloides	EPB88075.1
	Phycomyces blakesleeanus	XP_018296192.1
	Rhizopus delemar	EIE79168.1
Spo11	*Rhizophagus irregularis*	AOP03980.1
	Mucor ambiguus	GAN07020.1
	Phycomyces blakesleeanus	XP_018289141.1
	Rhizopus delemar	EIE81169.1

Table S3. Primers used for qRT-PCR and ddPCR reactions for meiosis-specific genes and reference genes. Primer sequences obtained from *R. irregularis* isolate A4. Designed using SnapGene program and ordered using IDT.

Primer Name	**Sequence (5' – 3')**
MSG_Msh4_F	CGA CCT GAA TTC ACG GAT AC
MSG_Msh4_R	GGA CCA GTG ATT AAT TGG AAG TT
MSG_Msh5_F	GAG CTT ATT ACC TTC CGA TAT GTG
MSG_Msh5_R	TGT AGA TAA TGA TAG CGC GAC AA
MSG_Spo11_F	CTA TGA CCA AGA CGA ATT CAG C
MSG_Spo11_R	GAA CGC TTA TAC CAA CTA TGG ATC
MSG_Rec8_F	GAT GCA GGT ACT TAC AAT ATC GC
MSG_Rec8_R	ACT AAA TCC ACC AGA TGA TGC A
MSG_Mnd1_F	GTA AAC ACG GAC AAG ATT GGT
MSG_Mnd1_R	CTT CGA TTC TAG CTT TAC GCT
DMC1_F_2	GAG TTG ACT GCT CGA TTT GCA
DMC1_R_2	ACC AGC ATA ATC CGT TCG GA
BTUB_F	GTC AAG TAA CGA CCG TGT CGA
BTUB_R	CTT CTT CAT GGT CGG ATT TGC
aTUB_F_1	AAA CCA GAT CCG GTACCA CC
aTUB_R_1	AAT CGT CGA CCT CGT CCT TG
EF1a_F	GCC CTT AAC TTT GGC ACC AC
EF1a_R	CAG GCT TTA TTA AAC GTG TTG G

CHAPTER THREE

Discussion, Future Directions, and Concluding Remarks

3.1 Importance of Research

Humankind stands at a pivotal point in its history and is seeing a transition into a new world. With the advent of technological booms, massive leaps in healthcare, infrastructure, and transportation, our global population is growing at an extraordinarily rapid pace. An increasing population in the midst of a climate crisis (Archer and Rahmstorf 2010) is putting enormous strain on our agriculture and our ability to maintain food supplies in sustainable ways (Faisal and Parveen 2004; Molyneux et al. 2012; Rosenzweig et al. 2001). Regions around the world are experiencing the effects of global warming every year with longer, harsher summers (Christidis, Jones, and Stott 2015; Seneviratne et al. 2014) and more frequent rare violent weather events and natural disasters (Moore et al. 2015; Stott 2016), which can be detrimental to the survival of sensitive agricultural sectors (Cheeseman 2016; Mendelsohn 2008). P-rock fertilizer is widely used to nourish crops in nutrient-poor soil around the world (Jasinski 1999), but at the current pace that it is being used, global stores are set to be depleted in mere decades (Cordell, Drangert, and White 2009; Herring and Fantel 1993; Neset and Cordell 2012). Regardless of its finite availability, commercial fertilizer can be extremely damaging to local aquatic ecosystems (Sharpley, McDowell, and Kleinman 2001) and contribute to the formation of massive algal blooms (Garrett et al. 2011).This not only contributes to the climate crisis, it creates a negative feedback loop that depletes the farmable fertile soil and solidifies the need for even more fertilizer use.

Although we live in uncertain times and the future may seem bleak, human ingenuity and our resourcefulness has allowed us to overcome many challenges in the recent past and over thousands of years. An unprecedented amount of research is being conducted in the agricultural

and ecological sector due to the strain of climate change being experienced around the world. The search for alternative ways to grow, nourish, and sustain our food supplies is vital to the prosperity of our species, especially in developing countries with climbing population growth. AMF, with their unique symbiotic relationship with plants, are a promising alternative to the harmful fertilizers currently being used (Igiehon and Babalola 2017b; Suhag 2016). Not only do AMF provide plants with phosphorous and other important nutrients (George, Marschner, and Jakobsen 1995), but they have been shown to provide protection from pathogens (Al-Askar and Rashad 2010; Sikes 2010) and increase efficiency of water regulation to avoid drought (Al-Karaki 1998; Farahani et al. 2013). They are, in a way, nature's "biofertilizer" and have been for over 400 million years. They operate in a wide gamut of hosts, approximately 300,000 plant species (Klironomos 2000), and have substantial potential to impact the agricultural sector as continuous research on this topic is conducted.

This study sought to understand how for so long this group of organisms has survived and thrived in the absence of conventional sex. This information is essential in understanding the biology of these fungi and how they have evolved alongside terrestrial plants in their intricate mutualist symbiosis. Certain species and even isolates within species of AMF may confer specific advantages to particular plant hosts but much more research must be conducted on the functional diversity in this group of fungi. Harnessing the ability to cross isolates of AMF that could support agricultural crops in highly individual ways while simultaneously being sustainable and harmless to the local environment is the overarching goal of why we study these strange fungi. The research in this thesis is a steppingstone in our attempts to understand the sexual lives of AMF. The expression of meiotic genes is a clear sign that an organism is

undergoing sexual reproduction. The discovery of a core meiotic machinery in *R. irregularis* coupled with the identification of a multi-allelic mating locus was more than enough to warrant an investigation into whether meiosis-specific genes were being expressed in AMF.

3.2 Summary of Novel Findings

3.2.1 Baseline MSG expression in homokaryon isolates

Preliminary work investigating the expression of meiosis-specific genes in various isolates of *R. irregularis* concluded low overall expression across the board. This study sought to confirm and reproduce these findings as well as analyze different isolates of *R. irregularis* using similar and more sensitive quantitative techniques (qRT-PCR as used in Figure 1.3, Figure S4; ddPCR Figure 2.1ab, Figure 2.2ab, Figure 2.3ab, and Figure 2.4ab). The low expression of these genes required the use of ddPCR in order to quantify the transcription levels down to single copies. The results of our findings show that all six meiosis-specific genes analyzed (*Msh4*, *Msh5*, *Dmc1*, *Spo11*, *Rec8*, and *Mnd1*) are not expressed in any significant levels of homokaryotic isolates (C2, 56, and 454) of *R. irregularis* when normalized to a reference gene (*aTub*). When taking into account the putative cycle of sexual reproduction (Figure 1.1), the expression of these genes would not be expected in these homokaryotic fungal isolates, as they only contain one mating type within their multi-nucleated mycelium. Thus, the results are viewed as baseline levels of expression when compared to the other experiments in this study.

3.2.2 MSG expression in dikaryon isolates

As in the homokaryotic isolates, previous work had investigated the expression of meiosis-specific genes in one dikaryotic isolate of *R. irregularis* (A4) using qRT-PCR (Figure 1.1). Those results indicated very low levels of expression compared to the reference gene and

showed no significant differences to the homokaryotic isolates tested. This experiment was recreated and tested in preliminary work also using qRT-PCR and confirmed the low expression of A4 (Figure S4). Further analyses on dikaryotic isolates were conducted on A5 and SL1, the two other dikaryotic isolates studied in our laboratory, using ddPCR. The results showed that all three dikaryotic isolates exhibited similar very low levels of expression of MSG as seen in their homokaryotic counterparts, suggesting a few explanations. The putative cycle of sexual reproduction (Figure 1.1) hypothesizes that if AMF were to undergo meiotic processes, this would occur in a dikaryotic stage where two populations of compatible nuclei coexist within one mycelium. Although many fungal groups see the near-instantaneous fusion of nuclei after the fusion of mating partner cells, AMF may appear to partake in similar sexual cycles to that of their fungal relatives, the basidiomycetes. In this group, dikaryotic stages are often prolonged and karyogamy is not instantaneous (Lee et al. 2010). Although MSG are indeed present in *R. irregularis*, our results do not confirm that they are currently being used to undergo some form of sexual reproduction. ddPCR has the advantage that it can detect miniscule levels of expression, but it only captures a snapshot of time in a potentially long sexual stage. This does leave the possibility that certain conditions may need to be met in order for the final step of fungal sex to occur, which prompted the final analysis in this study, which observed the expression of MSG in an active crossing between two hypothetically compatible homokaryotic isolates (A1 and C2).

3.2.3 MSG expression in homokaryon crossings

The final analysis in this study sought to assess the expression of meiosis-specific genes in an active environment between two homokaryotic isolates (A1 and C2) of *R. irregularis* that were hypothetically compatible based on their mating types (*MAT*3 and *MAT*6, respectively).

Their compatibility is modeled around the mating type of the dikaryon A5 which also contains nuclei with *MAT*3 and *MAT*6. Furthermore, unpublished analyses from the University of Pisa (Figure 1.4) concluded that isolates A1 and C2 were indeed capable of forming perfect hyphal fusions with one another, albeit in 3.8% of the interactions observed. Out of all the anastomosis assays conducted, this pairing appeared as the most promising candidate to see if anastomosis and the exchange of nuclei could occur. Surprisingly, an analysis by Stephanie Mathieu in our laboratory concluded that dikaryotic spores were indeed created from this pairing. With this information in mind, ddPCR was conducted on the crossing to see if meiosis-specific genes were being expressed. As seen in Figure 1.3, meiosis-specific genes remained expressed in very low quantities compared to the reference gene (*aTub*) and showed no notable differences to the dikaryotic isolates assessed in Figure 1.2.

3.3 Further Implications and Future Directions

3.3.1 Implications on AMF sexuality

The results compiled in this study, particularly that of the notable lack of ubiquitous expression of meiosis-specific genes in active homokaryon crossings, have multiple implications. Firstly, given the confirmation of the formation of a dikaryon from these cultures coupled with the low expression of these genes, one could hypothesize that AMF may have a prolonged dikaryotic stage similar to many basidiomycetes and certain environmental triggers may be necessary to move past the second stage and into meiosis. Secondly, parasexual mechanisms may be at play in AMF like somatic recombination or transposable element-induced genetic transfers (Yildirir et al. 2020). In these processes, random recombination may be occurring in the dikaryotic isolates that could lead to higher genetic diversity. AMF do have the advantage of having thousands of nuclei at their disposal and could make use of transposable elements, which

are abundant in AMF (Chen, Morin, Beaudet, Noel, Yildirir, Ndikumana, Charron, St-Onge, et al. 2018), to shuffle around genetic material from one parental nuclei to the other. Lastly, the almost complete lack of expression of some MSG (*Dmc*1, *Rec*8) may be explained by AMF evolving a completely novel way to generate genetic diversity and have simply carried the remnants of a sexual ancestor in their genomes for millions of years. All in all, the notion that AMF are ancient asexual organisms is continually under question as more data and information provides evidence against it. The main enigma remains: how have AMF survived for hundreds of millions of years in the absence of sex? The answer to this question might lie in a radically slow sexual cycle that may only take place under highly specific circumstances. Whether AMF can stave off the effects of asexuality through sexual processes or other means remains a mystery. However, what is abundantly clear is that AMF possess all the capable tools to undergo meiosis, and it may simply come down to catching them in the act.

3.3.2 Future Directions

If meiosis is indeed happening in AMF, future work must focus on what conditions must be met in order for it to occur. Stress is the defining factor that forces populations to adapt to their environments to survive and drives evolution at its core (Nevo 2011). The laboratory conditions that the dikaryotic cultures used in this study were grown in have been stagnant for generations of clonal propagation. These fungi are given optimal nutrients to grow which could stunt steps for meiosis to even begin. Experimental procedures should be developed to test the effects of environmental stressors like drought, pH imbalance, nutrient deficiency, carbon shortage, and suboptimal hosts on the expression of meiosis-specific genes in both existing dikaryotic isolates and homokaryon crossings.

In addition to this, many more homokaryon crossing assays must be analyzed for compatibility, creation of dikaryotic spores, and expression of meiosis-specific genes. Unpublished data shows evidence that other homokaryon crossings (such as A1 and 4401) are capable of producing dikaryotic spores. The investigation of homokaryon combinations coupled with environmental stressors may be the perfect storm for meiosis (or another process of genetic recombination) to occur. As previously stated, the challenge will inevitably be catching these fungi at the right time and under the right circumstances, as it is very possible that recombination events occur infrequently over large periods of time.

Looking far into the future, if there is concrete evidence of meiotic recombination observed in AMF, experimental procedures must be established to assess how the functional diversity in various isolates and species of AMF can affect the growth of plants. If certain genetic differences can be attributed to symbiotic advantages for specific species or groups of host plants, then the door can open for highly specialized commercial inoculants to jump on the market and replace the existing finite and harmful P fertilizers currently being used. These experiments must also extend beyond the laboratory and into the field to assess the real-world efficacy when external variables are not mitigated, as nature is not a sterile environment like the laboratory cultures that our species are currently being grown in.

3.4 Concluding Remarks

Much work is needed to peel away at the hidden secrets that lie within the intricate networks that these unique fungi form with terrestrial plants. The agriculture sector is feeling continual pressure from the effects of climate change and will gradually require a solution to the global food crisis on top of dealing with the depleting availability of phosphate rock fertilizer

reserves. There are only decades remaining to develop alternative ways to fuel our world's food supply and feed our growing populations. This research serves as one step in uncovering the sexual lives of an evolutionary scandal. In agreement with previous research done on the expression of meiosis-specific genes, this study concludes definitively that there is a complete lack of ubiquitous expression across homokaryotic and dikaryotic isolates of *R. irregularis* using high-resolution quantitative techniques. For the very first time, the expression of these genes was also assessed in homokaryotic crossings, which represent an important step in the putative cycle of sexual reproduction for AMF. A baseline level of expression has thus been identified in standalone laboratory conditions, which can now be used in a multitude of future studies to compare the expression levels of these genes. All in all, this study sheds a light on a promising group of organisms that have puzzled researchers for decades and highlights the urgent need for continued research into their cryptic sexual lives.

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