The genetics of mating in arbuscular mycorrhizal fungi

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Abstract

Sexual reproduction is an important process amongst eukaryotic organisms, with one function being to maintain genetic variation. The idea that complex eukaryotic species can persist for millions of years in the absence of sex defies fundamental evolutionary dogma, yet a group of organisms known as ancient asexuals were thought to have evolved clonally under deep evolutionary time. Prominent among these are the arbuscular mycorrhizal fungi (AMF), which are obligate plant symbionts that colonize the root cells of plants and extend their hyphae into the soil assisting the plant in acquiring key nutrients. Unlike most eukaryotes, AMF cells are multinucleate with thousands of nuclei moving through a continuous cytoplasm. Genomic analyses have identified a putative mating-type (MAT) locus within the nuclear genomes of model AMF *Rhizophagus irregularis*, a region that in other fungi dictates the process of sexual reproduction. Additional findings demonstrated that AMF strains carry one of two nuclear organizations. They can be either homokaryotic (AMF homokaryons), where all nuclei within the cytoplasm are virtually identical, or heterokaryotic (AMF dikaryons), where two MAT-locus variants co-exist within the cytoplasm. Despite a lack of observable traits indicative of sex, this homo/heterokaryotic dichotomy is reminiscent of the nuclear organization of sexual fungi.

My research aims to build on these findings to investigate the actual role of the MAT-locus in driving AMF reproduction. To address this, I build my thesis into three main chapters. The first chapter reviews our current understanding of AMF genetics and what drives genome evolution in these organisms. The second chapter establishes a relatively easy, inexpensive, and reproducible approach to genotype known MAT variants of *R. irregularis* in natural and experimental conditions. The last chapter uses experimental crossings between strains to assess cytoplasmic compatibility and nuclear exchange. I demonstrate that dikaryotic spore progenies

can be formed after co-culturing two distinct AMF homokaryotic strains. Further analyses of various genomic regions also reveal possible recombination in homokaryotic spore progenies from co-cultures. Overall, this research provides new experimental insights into the origin of genetic diversity in AMF. These findings open avenues to produce genetically new AMF strains in the lab using conventional crossing procedures and provide a glimpse of the mechanisms that generate AMF genetic diversity in the field.

Résumé

La reproduction sexuée est un processus important pour le maintien de la variation génétique chez les organismes eucaryotes. L'idée que des espèces eucaryotes complexes puissent persister pendant des millions d'années en l'absence de sexe défie les tendances fondamentales de l'évolution. Pourtant, on pense qu'un groupe d'organismes connus sous le nom d'anciens asexués a évolué de manière clonale au cours d'une évolution profonde. Les champignons mycorhiziens à arbuscules (AMF) sont des symbiotes obligatoires des plantes qui colonisent les cellules racinaires des plantes et étendent leurs hyphes dans le sol pour aider la plante à acquérir des nutriments essentiels. Les cellules des AMF sont multinucléées, avec des milliers de noyaux présents dans un cytoplasme continu, contrairement à la plupart des eucaryotes. Les analyses génomiques ont permis d'identifier un locus putatif de type d'accouplement (MAT) dans les génomes nucléaires du modèle AMF *Rhizophagus irregularis*, une région qui, chez d'autres champignons, dicte le processus de reproduction sexuelle. D'autres résultats ont démontré que les souches d'AMF portent l'une de deux organisations nucléaires. Elles peuvent être soit homocaryotes (homocaryons AMF), où tous les noyaux du cytoplasme sont pratiquement identiques, soit hétérocaryotes (dikaryons AMF), où deux variantes de locus MAT coexistent dans le cytoplasme. Malgré l'absence de traits observables indiquant le sexe, cette dichotomie homo/hétérocaryote ressemble à l'organisation nucléaire des champignons sexués.

Ma recherche vise à appuyer ces résultats pour étudier le rôle réel du locus MAT dans la reproduction de l'AMF. Pour ce faire, j'ai construit ma thèse en trois chapitres principaux. Le premier chapitre fait le point sur notre compréhension actuelle de la génétique des AMF et sur les facteurs d'évolution du génome de ces organismes. Le deuxième chapitre établit une approche relativement facile, peu coûteuse et fiable pour génotyper les variants MAT connus de *R.*

irregularis dans des conditions naturelles et expérimentales. Le dernier chapitre utilise des croisements expérimentaux pour évaluer la compatibilité cytoplasmique et l'échange nucléaire entre souches. Je démontre que des descendances de spores dikaryotiques peuvent être formées après la co-culture de deux souches homocaryotiques distinctes d'AMF. Des analyses plus approfondies de diverses régions génomiques révèlent une recombinaison possible dans les descendants de spores résultants. Dans l'ensemble, cette recherche fournit de nouvelles perspectives expérimentales sur l'origine de la diversité génétique des AMF. Ces résultats ouvrent la voie à la production de nouvelles souches de AMF en laboratoire à l'aide de procédures de croisement conventionnelles et donnent un aperçu des mécanismes qui génèrent la diversité génétique des AMF sur le terrain.

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Introduction

Keystone species, such as arbuscular mycorrhizal fungi (AMF), play a crucial role in shaping the ecosystems around them. AMF are an important component of the underground fungal network that provides structure to the soil and reallocates nutrients to maintain ecosystem stability (van der Heijden et al. 2015; Chen et al. 2018c; Whiteside et al. 2019). Despite the essential functions that AMF provide, much of their biology has remained elusive. In particular, the mechanisms that drive their nuclear exchange and reproduction are poorly understood.

Modes of reproduction

Reproduction is the process that passes genetic material on to the next generation. This process can be broadly divided into two contrasting modes: asexual and sexual reproduction. In asexual reproduction offspring are the product of a single parent, of which they are essentially clones (Schurko et al. 2008). In eukaryotes, asexual reproduction generally takes the form of a mitotic cell division which produces two daughter cells identical to the mother (Kondrashov 1993; Schurko et al. 2008). In sexual reproduction homologous chromosomes undergo recombination during meiosis and haploid gametes from two different individuals fuse to produce offspring (Barton and Charlesworth 1998; Schurko et al. 2008; Lee et al. 2010).

It is generally accepted that sexual reproduction is more prevalent than asexual reproduction in eukaryotic organisms (Barton and Charlesworth 1998; Otto and Lenormand 2002; Neiman et al. 2005, 2009). The main arguments supporting the ubiquity of sexual reproduction are that it facilitates adaptation by either strengthening the purging of deleterious mutations, accelerating the fixation of beneficial mutations, or by increasing genotypic diversity in the

population (Maynard Smith 1971, 1978; Kondrashov 1993; Barton and Charlesworth 1998; Agrawal 2001; Siller 2001; Lee et al. 2010; Ni et al. 2011). However, there are also many costs associated with sexual reproduction. For example, this process may break up favourable gene combinations following recombination (Kondrashov 1993; Otto 2009). It can also lead to an increased cost of resource allocation in co-sexes as well as introduce genetic conflict, where different sexes have different fitness optima (Otto and Lenormand 2002; Otto 2009; Lee et al. 2010). Despite this, the evolutionary advantages conferred by sexual reproduction seem to outweigh its costs, as the majority of eukaryotic species engage in either obligate or facultative sexual reproduction (Otto 2009). A prominent example is the New Zealand freshwater snail, *Potamopyrgus antipodarum*. In this species, sexually reproducing and asexually reproducing populations co-exist. However, under the influence of parasitism, snails undergoing sexual reproduction were able to outcompete their asexual counterparts (Lively 1987; King et al. 2009, 2011). Additional experiments using the facultatively sexual rotifer *Brachionus calciflorus* demonstrates that sexual reproduction facilitates adaptation in novel environments, where rates of sex are seen to increase until a fitness plateau is reached (Becks and Agrawal 2012; Luijckx et al. 2017).

Nonetheless many asexual lineages appear throughout history, however, completely asexual lineages are considered short-lived on an evolutionary time scale. In particular, clonal organisms are perceived as being unable to adapt to natural environmental fluctuations (Barton and Charlesworth 1998; Otto and Lenormand 2002; Normark et al. 2003; Neiman et al. 2005, 2009). In contrast to this view, a handful of organisms termed ancient asexuals have been said to thrive for millions of years in the absence of sex (Otto and Lenormand 2002; Schurko et al. 2008). These ancient asexuals are viewed as evolutionary scandals since they defy our understanding of

reproduction and bring into question the fundamental theories that have been developed to explain the widespread evolution of sexual reproduction (Neiman et al. 2005, 2009; Schurko et al. 2008). Some of the organisms that fall into this category include the Bdelloid rotifers, Darwinulid ostracods and the arbuscular mycorrhizal fungi (Law and Crespi 2002; Normark et al. 2003; Neiman et al. 2009).

Overview of arbuscular mycorrhizal fungi (AMF)

Mycorrhizae are symbiotic relationships that arise between fungi and many terrestrial plants. The most frequent (and understood) mycorrhizae can be grouped into two categories based on how they interact with the plant: ectomycorrhizae and endomycorrhizae (Basu et al. 2018; Strullu-Derrien et al. 2018). Ectomycorrhizae develop primarily outside of the plant root, as their name suggests, and are composed of three main structures defined by the locality of hyphal growth: the mantle or hyphal sheath that envelops the roots, the extraradical hyphae that grow into the soil and the internal structure of the Hartig net, which extends its growth within the intercellular space of the plant root (**Figure I.1**; Bonfante and Genre 2010; Strullu-Derrien et al. 2018). These associations are primarily formed by fungi of the phyla Ascomycota and Basidiomycota in association with small shrubs and trees from temperate, boreal and Mediterranean regions (Strullu-Derrien et al. 2018). In contrast, the primary structures of endomycorrhizae are found mostly within the plant root cells. As with their ectomycorrhizal counterparts, endomycorrhiza form extraradical hyphae that extend into the soil. However, in endomycorrhiza, hyphae invade the plant root, producing storage vesicles and complex nutrient exchange centres that reside within the cortical cells of the roots of vascular plants (**Figure I.1**). Endomycorrhizae are recognized as the

most widespread plant symbiont associations and include the arbuscular mycorrhizae (AM) (Strullu-Derrien et al. 2018).

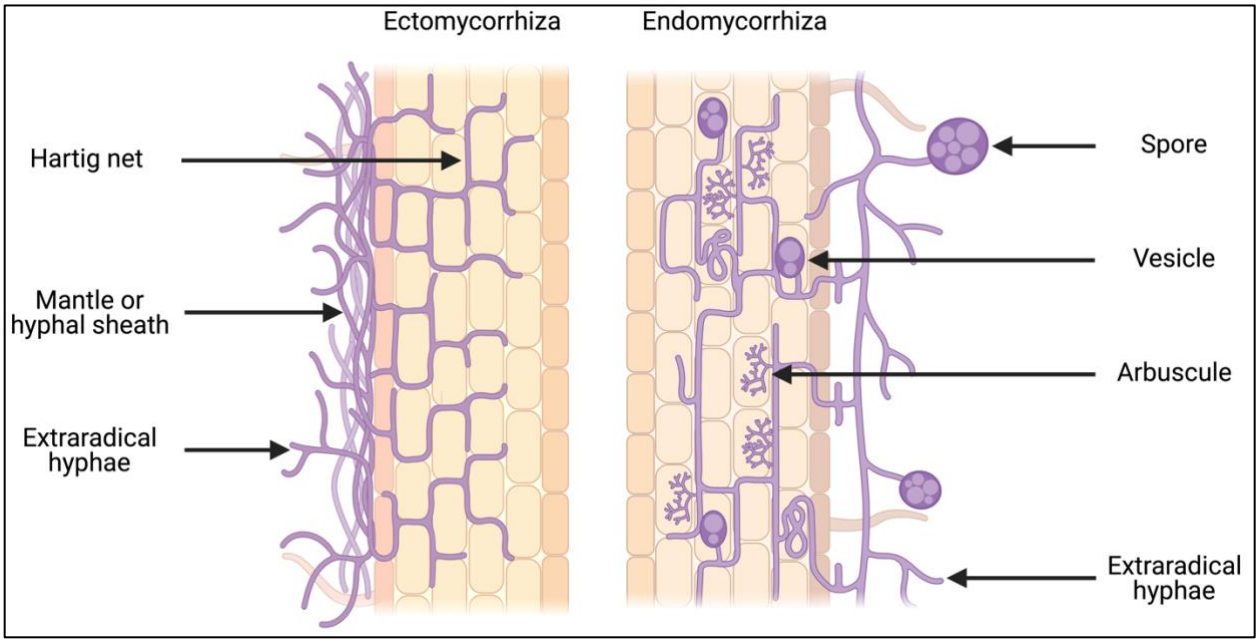

Figure I.1. Depiction of the primary structures formed by ectomycorrhizae (left) and endomycorrhizae (right) both within and outside of the plant root. Image generated using BioRender.

AMF form the most common type of mycorrhizal symbiosis, interacting with approximately 72% of land plants and occurring in the majority of natural habitats and virtually all ecozones, spanning arctic regions, tropical forests and deserts (Basu et al. 2018; Chen et al. 2018c; Cosme et al. 2018; Bonfante and Venice 2020). Interactions with AMF are initiated by strigolactone, a root-borne signal that is detected by the fungi and promotes the growth of their hyphae towards the plant's roots and the response secretion of lipochito-oligosaccharides, which activate the common symbiosis signaling pathway in the plant (Maclean et al. 2017; Basu et al. 2018; Chen et al. 2018c; Cosme et al. 2018). The fungal hyphae then enter the apoplastic space

between the plant cells in addition to penetrating the cells, where they form arbuscules, the highly branched structures from which they derive their name (Strullu-Derrien et al. 2018; Bonfante and Venice 2020). The arbuscules serve as the nutrient exchange site between the plant and the fungus. They are surrounded by a host-derived peri-arbuscular membrane which further increases the contact surface area. The main nutrient provided by the fungus is phosphate in addition to other nutrients such as: ammonium, potassium, calcium, sulphur, copper and zinc (Pellegrino and Bedini 2014; Berruti et al. 2016). In return, the plant provides the fungus with carbohydrates as well as fatty acids, which the fungus cannot synthesize on its own.

This symbiosis also provides a variety of benefits to the plants and the environments in which they occur, offering important ecological services such as: allowing plants to receive nutrients beyond the depletion zones of their rhizosphere, providing abiotic and biotic stress resistance and increasing soil structure and fertility (der Heijden et al. 1998; Lanfranco et al. 2017; Chen et al. 2018c). The hyphae of AMF can grow to be kilometres long, far exceeding the length of the plant roots. This increases the contact surface area and allows a greater abundance of nutrients to be accessed and ultimately absorbed by the plant host. By producing an extended hyphal network, AMF can also interact with multiple host plants simultaneously, and host plants can undergo symbiotic interactions with multiple AMF species. This interconnected community plays an important role in providing ecosystem stability. For example, plants experiencing environmental stress can benefit from the common mycorrhizal network to boost their nutrient intake. Furthermore, stress signals generated by some plants due to pest invasion can be transmitted across the mycelial network to neighboring plants as a warning system (Babikova et al. 2013; Basu et al. 2018; Chen et al. 2018c). These dense hyphal networks also create a three-dimensional matrix underground that produce the glycoprotein glomalin and crosslinks the soil

particles creating stable soil aggregates that can protect against erosion (Singh et al. 2013; Chen et al. 2018c).

In addition, the majority of crops that feed the global human population are excellent hosts for AMF, from cereals to vegetables to fruiting trees (Berruti et al. 2016; Chen et al. 2018c; Bonfante and Venice 2020). AMF show the potential to increase soil nutrient retention, by preventing nutrient run-off and generating closed nutrient cycles. These fungi can also promote long-term soil fertility, thereby decreasing the amount of fertilization required by agricultural practices without causing a reduction in yield for farmers (Hijri 2016; Chen et al. 2018c). AMF also have the potential to benefit large-scale reforestation, as they have been shown to store about 2-5% of soil carbon, thus compounding the beneficial carbon sink that trees provide and rendering efforts more sustainable. Bioremediation projects have also proposed using AMF to sequester toxic compounds from the soil and transfer them to resistant plants in order to reduce the environmental footprint of industrialization (Wang et al. 2005; Basu et al. 2018).

However, all such applications have been limited by a lack of knowledge of the reproductive biology of these organisms. Understanding the reproductive strategy of these fungi may lead to better selective breeding programs by increasing the efficiency of AMF inoculum (Corradi and Brachmann 2017; Chen et al. 2018c).

Genetics and mating in AMF

As with most organisms, fungi were originally classified based on morphological traits. Sex was first characterized by the appearance of specialized sexual structures known as fruiting bodies (such as basidioma and ascoma in basidiomycetes and ascomycetes, respectively). AMF,

however, lacked any of these structures as well as any other obvious signs of sexual reproduction. As a result, these organisms were long considered to be completely asexual.

An interesting aspect of AMF biology was the discovery that they are coenocytic, such that cells contain many nuclei and are not separated by septa. For the multinucleate AMF, this means hundreds of thousands of nuclei move between spore and hyphae in a continuous cytoplasm (Angelard and Sanders 2011; Kokkoris et al. 2020). The co-existence of thousands of nuclei in the same cell, combined with the formal absence of sexual reproduction gave rise to the AMF heterokaryosis hypothesis (Sanders 1999; Kuhn et al. 2001). This hypothesis stated that nuclei co-existing in AMF cells are very different from one another, and this rich inter-nuclear diversity was thought to compensate for the lack of sexual reproduction, thus explaining the longevity and broad host range of these ancient asexuals. Specifically, this hypothesis proposed that any deleterious mutations found in some nuclei could be compensated by beneficial mutations present in other co-existing nuclei, eliminating the need for sexual reproduction.

This hypothesis gained support from a study by Kuhn et al (2001) that used DNA-DNA *in-situ* hybridization (FISH) to show the co-existence of genetically different nuclei within a single spore of the arbuscular mycorrhizal fungus *Scutellospora castenea*, based on two divergent sequences of the rDNA ITS2 region. Furthermore, this work as well as a follow-up study by Hijri and Sanders (2005) concluded that spores must harbour many unique nuclei, as multiple variants of presumed single copy genes were found within a single spore. However, later studies showed that the genes once thought to be single copy exist in multiple paralogs throughout the genome, suggesting that these studies confounded intra-nuclear variation for inter-nuclear variation (Lin et al. 2014; Corradi and Brachmann 2017). In addition, the rDNA sequences (such as ITS2) were shown to harbour a large number of single nucleotide polymorphisms (SNP), making them

unreliable for genotyping AMF nuclei (Simon and Weiß 2008; Schoch et al. 2012; Corradi and Brachmann 2017).

Overall, these new findings brought into question the plausibility of the heterokaryosis hypothesis, while increased availability of AMF genome data allowed novel approaches and hypotheses to be explored. For instance, the AMF genome was found to contain many homologs of genes known to be involved in sexual reproduction in eukaryotes as well as a large abundance of transposable elements (TE) (Hickey 1982; Normark et al. 2003; Halary et al. 2011, 2013; Riley et al. 2014; Ropars et al. 2016; Bonfante and Venice 2020). TE are mobile DNA sequences that can change position and duplicate within the genome (Dolgin and Charlesworth 2006; Feschotte and Pritham 2007). The spread of TE between lineages within a population is greatly aided by sexual reproduction, specifically meiosis (Wright and Finnegan 2001; Normark et al. 2003). However, since most asexual lineages arise from sexual ancestors, TE are present in the genomes of nearly all living species (Dolgin and Charlesworth 2006; Bast et al. 2016). Upon abandoning sexual reproduction in favour of an asexual lifestyle individuals with lower TE loads would be expected to be favoured, as a high TE load can lead to an accumulation of deleterious genomic content which can exacerbate the process of Muller's ratchet (Wright and Finnegan 2001; Dolgin and Charlesworth 2006; Bast et al. 2016). Furthermore, both the TE and genes involved in sexual reproduction should become inactive in asexual organisms, thereby undergoing relaxed selection, leading to degeneration. If, however, AMF were not exclusively clonal, this could explain the presence of these genetic elements and we could expect to find additional strong signatures of sexual reproduction in their genome, as has been the case for several other formerly presumed ancient asexuals such as Colpodean ciliates, *Trichomonas vaginalis*, and *Candida albicans* (Fraser and Heitman 2003; Malik et al. 2008; Dunthorn et al. 2017).

In addition to the presence of abundant TE, other signatures of sexual reproduction have also been found in AMF. Halary et al. (2011) identified 51 meiosis related genes including seven that are meiosis-specific within the AMF genome, representing more than 85% of core meiotic genes. A follow up study by Riley et al. (2014) showed that the genome of model AMF *Rhizophagus irregularis* carries at least 76 homologues of MATA-HMGs, which are broadly related to mate recognition in fungi, which led to the search for a mating-type locus in *R. irregularis*.

What is a mating-type (MAT) locus?

The MAT-locus is a region of the fungal genome that establishes cell identity and governs the sexual cycle (Casselton 2002; Fraser and Heitman 2003; David-Palma et al. 2016). It does this through transcriptional factors, such as homeodomains, that bind to specific DNA regions and regulate the expression of genes involved in sexual developmental cascades (Hsueh and Heitman 2008; Vonk and Ohm 2018). There are two main mating systems based on MAT-loci arrangements, bipolar and tetrapolar (Fraser and Heitman 2003; Billiard et al. 2011).

In a bipolar system a single MAT-locus is encoded in the genome but is present as two alternate allelic versions within one population. Each version codes for a distinct mating type, typically denoted a and α or + (plus) and – (minus) (Fraser and Heitman 2003; Fraser et al. 2007). The two alternate, and generally non-homologous mating types are referred to as idiomorphs. Despite being on homologous chromosomes, the sequences of the MAT-locus are completely dissimilar between mating types and code for different proteins (Casselton 2002). In order for partners to mate, they must be of the opposite mating type (Fraser et al. 2007). This can occur in two different ways: either two individuals with opposite mating types come in contact with each

other (heterothallic; **Figure I.2b**) or a single individual is able to self-fertilize (homothallic; **Figure I.2a**; Fraser and Heitman 2003). Homothallism is less common but is seen in species such as *Saccharomyces cerevisiae*, *S. pombe* and *Kluyveromyces lactis* (Fraser and Heitman 2003; Ni et al. 2011). Bipolar mating systems are considered to be ancestral in fungi and are found in basal fungal lineages as well as throughout the ascomycete phylum (Hsueh and Heitman 2008; Raudaskoski and Kothe 2010).

In contrast, a tetrapolar system has two unlinked MAT-loci within the genome, typically denoted A and B (**Figure I.2c**, Casselton and Olesnicky 1998). Each MAT-locus is highly polymorphic and can have anywhere from a handful of alleles to extreme cases where hundreds of MAT alleles exist within a population (Casselton 2002; Fraser and Heitman 2003). The A locus contains divergently transcribed homeodomain protein-encoding genes, HD1 and HD2 (Raudaskoski and Kothe 2010; David-Palma et al. 2016). Both domains bind to DNA, but HD1 has been shown to carry nuclear localization signals and an activation domain (Raudaskoski and Kothe 2010). Furthermore, the N-terminal of both the HD1 and HD2 domain have dimerization motifs that allow heterodimerization within the cytoplasm of the two domains (Raudaskoski and Kothe 2010). This heterodimer can only be formed between genetically different partners and allows the HD2 protein to be transported to the nucleus and bind to sex related genes (Kues et al. 1994; Spit et al. 1998; Raudaskoski and Kothe 2010). The B locus contains pheromones and pheromone receptors that direct the initial stages of mate recognition and cell fusion (David-Palma et al. 2016). In order for mating to occur in tetrapolar fungi both A and B regions must differ (Fraser and Heitman 2003). This mating system is found in basidiomycete fungi and is considered ancestral to this phylum.

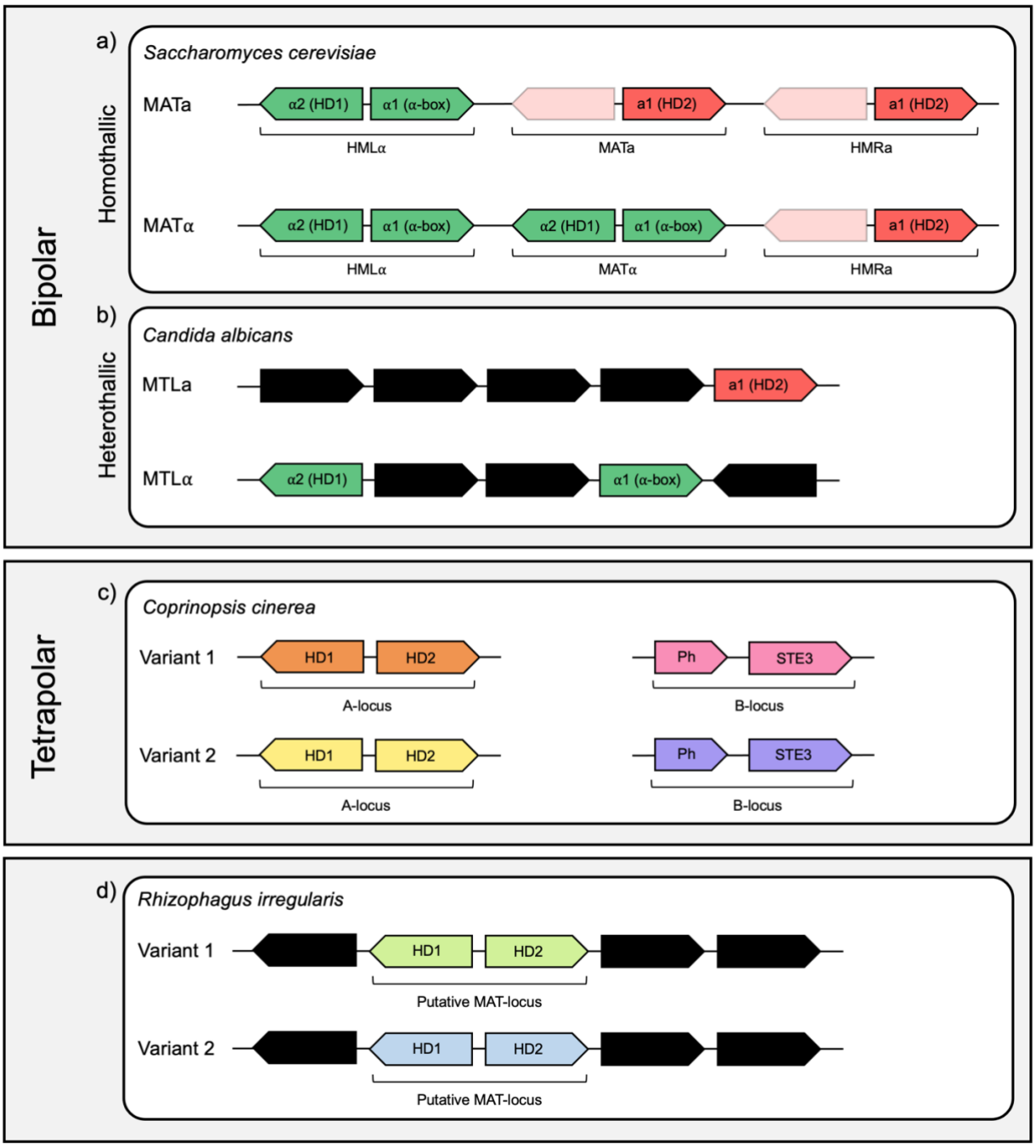

Figure I.2. Representations of the MAT-locus in different species. a) Bipolar homothallic mating system represented by the ascomycete *Saccharomyces cerevisiae*, with the MATa and MATα loci as well as the HMRa and HMLα domains responsible for mating type switching. b) Bipolar heterothallic mating system represented by the ascomycete *Candida albicans* with the

MTLa and MTLα loci. c) Tetrapolar mating system represented by the basidiomycete *Coprinopsis cinerea* with the A-locus containing homeodomains (HD1 and HD2) and the B-locus containing example pheromone and pheromone receptor genes (Ph and STE3). The two variants are representative of the many versions of the alleles that exist. d) The putative mating-type locus identified in model AMF species *Rhizophagus irregularis* with homeodomains HD1 and HD2. The two variants are representative of the multiple different versions of the locus that have been identified to date. Arrows represent the direction of transcription and black arrows represent additional genes present on the loci. Species chosen for the bipolar and tetrapolar mating systems are representative and do not comprise the only versions of the MAT-loci present in these different mating systems. Drawings of the MAT-loci have also been simplified for clarity.

A new hypothesis for AMF reproduction

A putative MAT-locus, with several variants, has been identified in *in vitro* samples of model AMF *R. irregularis* (**Figure I.2d**, Ropars et al. 2016; Kokkoris et al. 2021). This MAT-locus shares the gene order with the MAT-locus of basidiomycetes as it encodes for two divergently transcribed homeodomains, HD1-like and HD2. Despite its similarities to the A locus of basidiomycetes, there has yet to be a B locus identified, suggesting that AMF may use a bipolar mating type system.

Using genome and single nucleus data AMF isolates analysed to date were found to be either homokaryotic, with one nuclear genotype present in the cell, or dikaryon-like, with two nuclear genotypes co-existing in one cell. The term dikaryon (or AMF dikaryon) will be used throughout this thesis to define "a genetic organization where thousands of nuclei originating from two parental strains co-exist in one cell". Such organization differs from the previously proposed

AMF heterokaryosis hypothesis, where more than two genotypes were thought to co-exist within one cell, independent of nuclear exchange.

The identification of one putative mating-type locus per nucleus in AMF, and the presence of homokaryon/dikaryon genome organization led to a new hypothesized life cycle for AMF species (**Figure I.3**). In this model, it is proposed that AMF reproduce clonally most of the time. However, under conditions that remain to be determined, two homokaryotic hyphae of different mating types may undergo plasmogamy (fusion of hyphae and exchange of cytoplasmic contents) to form an AMF dikaryon. This model is based on current knowledge of Dikarya fungi and proposes that AMF can stay in a dikaryotic state for prolonged periods of time until an additional signal would initiate karyogamy (fusion of nuclei) and meiosis to return to the homokaryotic stage.

Research justification and goals

Though the current homokaryotic/dikaryotic view of the AMF life cycle is now widely accepted in the field, it is still based solely on genomic data and comparative genetic analyses. In particular, the hypothesis lacks corroboration and support from either *in vitro* or *in vivo* experiments. My thesis aims to determine whether the MAT-locus is a potential mating regulator in the AMF species *R. irregularis* and determine if isolates of this species can undergo plasmogamy to produce a dikaryotic spore progeny, providing evidence of nuclear exchange driven by mating in a laboratory environment.

My thesis first focuses on a review that provides the reader with up-to-date knowledge of AMF genomes laying the framework for my experiments. This is followed by the development of a genotyping technique that can be widely used to identify karyogamy of single spores based on the MAT-locus. This technique is then used to test the hypothesis of plasmogamy, nuclear

exchange and mating under laboratory conditions in my third chapter. Here, I examine actual crosses between isolates to determine whether a dikaryon isolate can be created from two homokaryotic parental lineages, and whether recombination occurs following plasmogamy. Altogether, this work will add fundamental understanding of the life cycle of these fungi and will help further elucidate how they reproduce. Ultimately, this will build a repertoire of knowledge that may one day be used to enhance the roles of AMF in the various applications previously discussed.

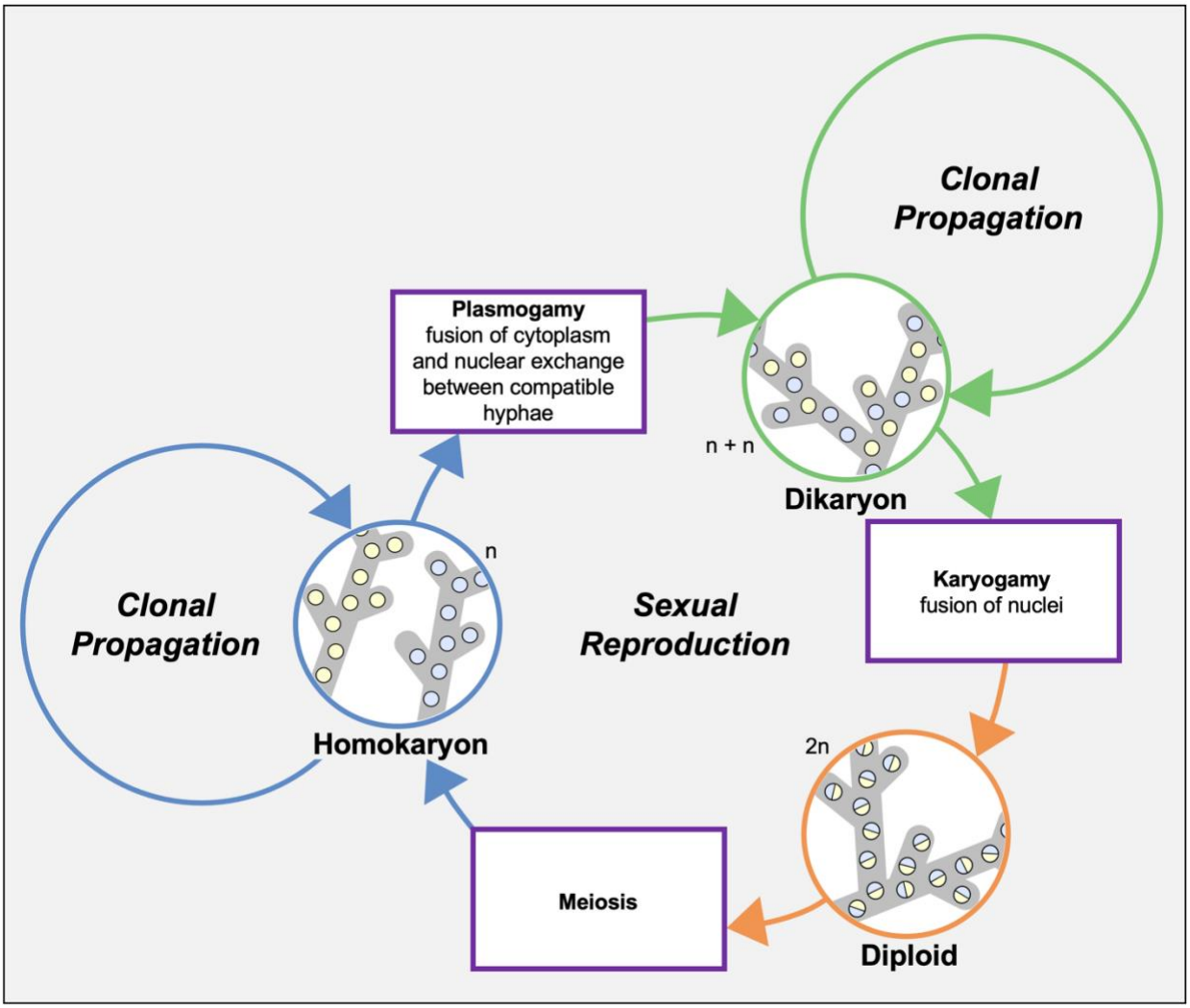

Figure I.3. Depiction of the hypothesized life cycle of AMF. Isolates spend most of their time in the homokaryon state (ploidy = n) undergoing clonal propagation (blue), until a compatible partner is located, which would initiate plasmogamy and the formation of a dikaryon (ploidy = n + n; green). The dikaryon stage can persist for a yet undetermined amount of time before karyogamy occurs and a diploid (ploidy = 2n) is formed (orange). The diploid then undergoes meiosis to return to the homokaryotic (ploidy = n) stage. Blue and yellow circles represent nuclei of two different MAT variants. Figure modified from Ropars *et al.* 2016.

Chapter 1: Arbuscular mycorrhizal fungi: intraspecific diversity and pangenomes

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Summary

Arbuscular mycorrhizal fungi (AMF) are ubiquitous plant symbionts with an intriguing population biology. Conspecific AMF strains can vary substantially at the genetic and phenotypic levels, leading to direct and quantifiable variation in plant growth. Recent studies have shown that high intraspecific diversity is very common in AMF, and not only found in model species. Studies have also revealed how the phenotype of conspecific isolates varies depending on the plant host, highlighting the functional relevance of intraspecific phenotypic plasticity for the AMF ecology and mycorrhizal symbiosis. Recent work has also demonstrated that conspecific isolates of the model AMF *Rhizophagus irregularis* harbor large and highly variable pangenomes, highlighting the potential role of intraspecific genome diversity for the ecological adaptation of these symbionts.

Keywords

arbuscular mycorrhizal fungi (AMF), intraspecific diversity, pangenomes, *Rhizophagus irregularis*, transposable elements

Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate plant symbionts that associate with over 80% of vascular plants; an association believed to date back nearly 400 Myr to the colonization of land by plants (Martin et al. 2017; Strullu-Derrien et al. 2018). These fungi participate in underground nutrient exchanges by associating with plant roots, providing them with increased access to soil nutrients, such as nitrates and phosphates, in exchange for carbon-based

photosynthetic by-products (Jakobsen & Rosendahl, 1990; Parniske, 2008), particularly sugars (Trépanier et al. 2005; Helber et al. 2011) and lipids (Bravo et al. 2017; Jiang et al. 2017; Keymer et al. 2017; Luginbuehl et al. 2017). In addition to trophic advantages, these fungi enhance plant protection against parasites and increase drought tolerance in their host, resulting in higher plant fitness (der Heijden et al. 1998; Corradi and Bonfante 2012; Chitarra et al. 2016).

To date, c. 300 species of Glomeromycotina have been described based on spore morphology, but environmental ribosomal DNA sequences suggest that up to 1600 operational taxonomic units (OTUs) may exist (Öpik and Davison 2016). Furthermore, AMF have been shown to interact with c. 200 000 plant species, which led for some time to the confounding question of how few taxa with restricted morphological diversity can interact with such a broad host range (Brundrett 2009; Öpik et al. 2013; Rodríguez-Echeverría et al. 2017). Technological advances in genomics are finally providing answers to this question and are revealing new aspects of AMF molecular diversity that could lead to a better understanding of their evolution and ecology.

Intraspecific phenotypic variation and the plant host

Seminal work by Koch and colleagues has shown that hyphal length, as well as spore counts and density, can vary significantly among conspecific AMF isolates harvested from the same field, and that this variation is correlated with differences in plant growth (Koch et al. 2006). It is now generally accepted that AMF intraspecific diversity has wide-ranging effects on terrestrial ecosystems, a finding with many potential applications in agricultural and environmental procedures. In particular, recent data has shown that the plasticity observed among isolates correlates with downstream functional effects on the level of host interactions, whereby some

isolates provide greater benefits to the plant while others can be detrimental (Hart and Reader 2002; Munkvold et al. 2004; Koch et al. 2006).

In a recent study based on a large taxonomical sampling of AMF species, Koch and colleagues (Koch et al. 2017) have further confirmed that within-species variation is an important determinant of plant growth, regardless of the AM species investigated. The latter point is particularly relevant as it shows that intraspecific functional variation is not restricted to model species. In this study, authors found that most variation in plant fitness occurs as a result of using different isolates of the same species, rather than different species. In the future, it will be interesting to see if the isolates of all AMF families show similar functional benefits to the plant, and whether some lineages or species produce isolates with a particularly high functional diversity.

Koch *et al.* (2017) also found evidence that the AMF phenotype varies depending on the plant host. Specifically, conspecific isolates were found to grow at various rates and to produce different structures depending on the identity of their host. For example, some isolates produced either more or larger spores when forming symbioses with the plant *Achillea millefolium* but would otherwise extend larger extraradical hyphal networks when in symbiosis with *Medicago trunculata*. Future studies should now aim to identify the molecular mechanisms underlying this intraspecific phenotypic plasticity.

High inter-isolate genetic diversity in model AMF

Cultures produced from single spores (*i.e.* isolates) of the same AMF species can also differ substantially at the genetic level. The existence of high genetic variability among conspecific AMF strains has been known to exist for over a decade through studies of the model species

Rhizophagus irregularis (Koch et al. 2004). Originally, this intraspecific diversity was proposed to result from a combination of heterokaryosis, and additional polymorphism due to copy number variation (CNV), as based on data obtained using amplified fragment length polymorphism (AFLP) and single gene comparisons between strains (Koch et al. 2004, 2006; Corradi and Sanders 2006; Corradi et al. 2007; Croll et al. 2009).

More recent attempts to explore intraspecific diversity in these plant symbionts have focused on genome-wide diversity scans of *R. irregularis* isolates harvested from different geographical locations using a 'restriction site associated DNA sequencing' approach (RAD-Seq) (Wyss et al. 2016). The RAD-seq method allows sequencing of short DNA genome fragments generated through digestion of genomic DNA with the *Mse*I and *Eco*RI restriction enzymes using the Illumina technology. By mapping reads onto a reference genome (*e.g. R. irregularis* DAOM 197198), Wyss and colleagues uncovered large levels of inter-isolate diversity, as indicated by a 20-fold variation in the number of single nucleotide substitutions (SNP). These findings revealed that some of the isolates analysed diverge substantially from the reference, and this wide-ranging inter-isolate variability was proposed to result from varying genomic conditions such as heterokaryosis, CNV, aneuploidy or a combination of these.

Genome diversity within the model AM fungus *Rhizophagus irregularis*

For some time, studies have shown that within-species diversity in AMF is high and important for their interaction with their surrounding environment. What has been unclear, however, is how this elevated diversity originates within their genomes and which genomic regions are most affected by such variation. This lack of knowledge stemmed from the methods previously used to detect AMF genome variability, such as AFLP, microsatellite analysis, single-gene

sequencing, or RAD-seq. In particular, these techniques are unable to extract meaningful functional information from genome data, as they provide a mainly quantitative overview of genetic diversity present between individuals.

An improved view of intraspecific diversity in AMF was recently obtained through genome analyses of six model isolates. Specifically, Chen and colleagues (Chen et al. 2018b) have discovered that conspecific *R. irregularis* isolates share as little as 50% of their gene repertoire. This genome diversity is remarkable, as similar analyses performed on conspecific strains of distant fungi show variation in gene count reaching at most 3% (Lysøe et al. 2014; Walkowiak et al. 2015; Plissonneau et al. 2016). In *R. irregularis*, most molecular functions are affected by CNV, with inter-isolate variability occurring in crucial pathways such as those predicted to be involved in the dialogue between the host and appears to affect almost all Pfam domains. These findings indicate substantial variation in the molecular function of each isolate, with many implications for our understanding of AMF population and genome dynamics.

Comparing *R. irregularis* genomes also revealed the potential role of transposable elements (TE) in creating genome diversity. TE abundance influences the extent of genome reorganization in many taxa by increasing adaptability to environmental change – for example, a host switch – particularly in organisms where sex is seemingly absent (Seidl and Thomma 2014; Faino et al. 2016). Most recent analyses indicate that TE compose at least 23% of the *R. irregularis* genome (Chen et al. 2018b). These TE are also extremely variable among conspecific isolates, with certain TE families being abundant in some *R. irregularis* isolates and absent in others. To our knowledge, the degree of TE variability discovered in *R. irregularis* is unprecedented, and it is possible that AMF genome evolution follows a 'two-speed genome' model, which posits that genomic regions containing a high density of TE evolve more rapidly (Raffaele and Kamoun 2012; Dong et al.

2015). These mobile elements are also widely expressed in *R. irregularis*, as measured by RNA-seq read mapping (Chen et al. 2018b). Evidence for their expression indicates that they are active and may play a driving role in reshaping genome diversity within AMF species (Faino et al. 2016). Since the evolution of effector genes is tightly linked to the activity of transposable elements (Plissonneau et al. 2017), future analyses should aim to understand whether the observed variation in TE abundance and activity correlates with effector diversity and abundance within AM species.

Pangenomes and the future of AMF ecological genomics

The most recent genome studies have also introduced the notion of 'pangenome' in AMF research. The term generally indicates that the total number of genes available to one microbial species can far exceed those encoded by one single individual (Plissonneau et al. 2018). In *R. irregularis*, the pangenome already exceeds 150 000 genes (Chen *et al.*, 2018), which means that model AMF (and probably all lineages in the group) carry vast arrays of 'accessory genes'. Accessory genes are thought to represent genomic regions providing evolutionary and ecological advantages to some isolates under changing environments – for example, the ability to colonize certain plant hosts, or some environments more efficiently (**Figure 1**). Within this context, the molecular diversity that exists in just six laboratory-grown *R. irregularis* isolates offers only a small view of the genome diversity present in the natural populations of these symbionts.

In order to facilitate future work linking AMF genome diversity with the ecosystem – that is, ecological genomics – studies should now aim to acquire optimal assemblies from as many AMF lineages as possible. Indeed, all genome data currently available from this group comes from only one model species, and the genome content and diversity of most AM families is virtually unknown. It will also be essential to improve both current and future AMF genome

assemblies because, with the exception of DAOM 197198, all available genome data are fragmented into tens of thousands of contigs. This fragmentation hampers the detection of synteny blocks and structural variants, and thus the identification of mechanisms that create variability within and among species. The identification of single-copy regions in these highly paralogous genomes also requires clean assemblies, which is particularly relevant to distinguish between intra- and inter-isolate polymorphism in genetic studies based on fingerprinting methods such as RAD-seq or low-coverage sequencing (Sanders and Rodriguez 2016). Improving assemblies can be done by combining long-read sequencing technologies (Rhoads and Au 2015; Jain et al. 2018), with Illumina sequencing data obtained from paired end and mate paired libraries. In return, optimal assemblies will allow us to obtain a precise view of the changes that occur among homologous regions of closely related individuals, for example, by revealing which TE or genes are more prone to move among or change within isolates and along the genome.

Because long-read technologies are based on single DNA molecule sequencing, their use will also expose the extent and location of variations that can exist among nuclei coexisting within the AMF mycelium or within single spores. When applied to DNA from conspecific isolates, this method could help us to determine how frequently isolates can exchange genetic material in the field (Chen et al. 2018b), and whether such events result from mating processes – that is, recombination events occurring between closely related isolates (Riley and Corradi 2013) – or from horizontal gene transfers (Fitzpatrick 2012; Gluck-Thaler and Slot 2015).

These analyses will also benefit greatly from recent breakthroughs in the area of single-cell (and single nucleus) genomics and transcriptomics (de Bekker *et al.*, 2011; Shapiro *et al.*, 2013; Kolisko *et al.*, 2014; Macaulay & Voet, 2014). Questions to be addressed using these techniques include: are certain mutations/genes/genomic locations particularly abundant under

certain environmental conditions, and are these specific genomic conditions always linked with a unique culture phenotype, or with the AMF association with a specific plant species?

Ultimately, understanding intraspecific fungal phenotypic variation and its effect on plant and ecosystems will also require a better understanding of AMF gene expression regulatory networks, through transcriptomics and epigenetics studies. Single cell transcriptomics (SCT) procedures are now available (de Bekker *et al.*, 2011; Kolisko *et al.*, 2014), and could be used to understand where or when conspecific AMF single spores are transcriptionally active in the field, or whether changes in transcriptional patterns are linked to variability in host-specificity or soil parameters (*e.g.* pH, salinity), stratification and plant abundance. Recently, SCT procedures were successfully applied to obtain transcript datasets from several AMF taxa, including genetically obscure genera such as *Paraglomus*, *Ambispora* and *Diversispora* (Beaudet *et al.*, 2018) isolated from pot-cultures. As discussed earlier, optimal reference genomes from most AMF families and genera will be crucial to investigate intraspecific variation in transcriptional patterns across the Glomeromycotina phylogeny. These data will also be key to identifying intraspecific variability in the epigenome using ChIP-seq (chromatin immunoprecipitation sequencing) or whole-genome bisulfite sequencing approaches (Chung et al. 2014; Jeon et al. 2015; So et al. 2018). Such techniques respectively allow the detection of binding sites for transcription factors and methylation patterns that have direct effects on the fungal phenotype (Chujo and Scott 2014; Jeon et al. 2015; Kronholm et al. 2016). In AMF, such epimutations could modulate intraspecific phenotypic diversity, for example, in response to symbiosis with different plant species or environmental variations.

Better taxonomical definitions would also benefit our understanding of AMF ecological genomics. Critical issues surrounding AMF taxonomy have been raised recently by others (Bruns

et al. 2017), as species delineation is at the core of how we define populations. Conspecific isolates are expected to undergo anastomosis and exchange nuclei (Croll et al. 2009; Sbrana et al. 2018), thus, in addition to morphology and phylogenetics, species designation could also be supported by anastomosis experiments (Giovannetti et al. 2003). Genome-driven approaches to AM taxonomy may also resolve recent challenges in taxonomy, for example, by exploring TE exchange across isolates with sequenced genomes. Indeed, the presence of TE is linked with sexual reproduction, and compatible isolates of the same species are expected to share more TE. The recent genome study by Chen and colleagues (Chen et al. 2018b) has revealed that not all *R. irregularis* isolates with sequenced genomes share the same amount of TE. Lastly, isolates of the same species should also be able to exchange nuclei, undergo karyogamy, and eventually recombine through meiosis or parasexuality. Past genome explorations have proven useful in detecting the presence of such events in AMF (Croll and Sanders 2009; Riley et al. 2014; Ropars et al. 2016).

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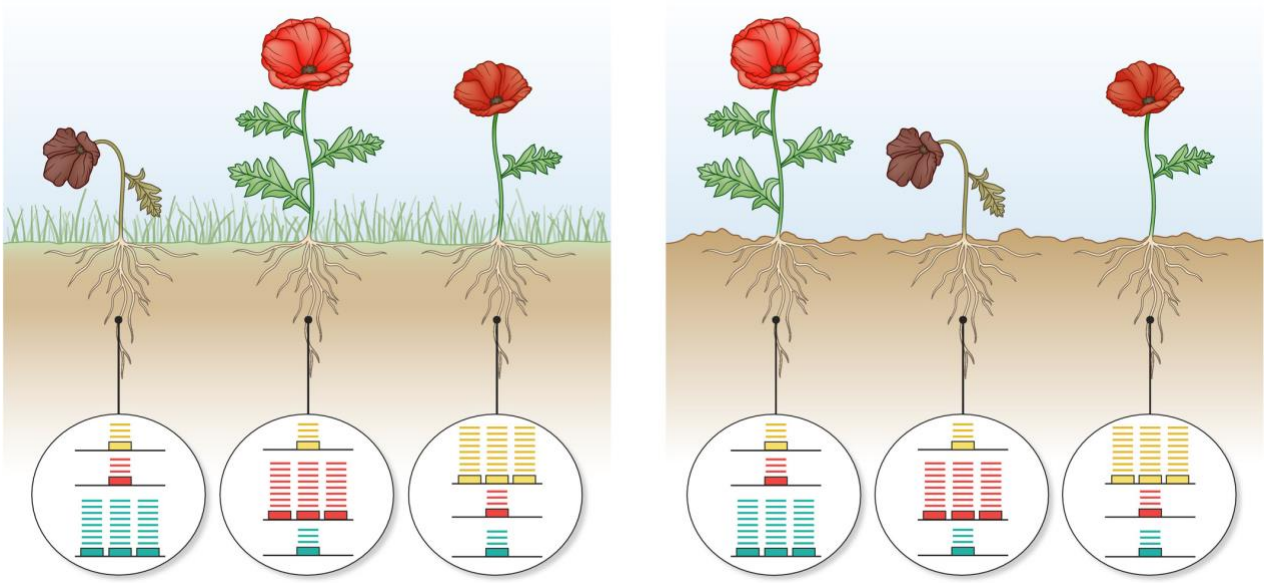

Figure 1.1. Hypothesis on the effect of copy number variation (CNV) on plants located in natural (left) and disturbed (right) environments. Conspecific arbuscular mycorrhizal fungal (AMF) spores are represented by circles and different gene families are shown as small rectangles with different colours (green, red and yellow). Variation in the number of rectangles indicates intraspecific CNV. In this example, high copy number of red genes is proposed to be beneficial in the first environment (left) but detrimental in the second (right). The opposite is true for green genes, whereas the yellow gene family confers equal growth in both environments. Horizontal lines represent transcripts produced by these genes, which should theoretically affect the AMF and plant phenotype.

Chapter 2: Simple and cost-effective method of genotyping model arbuscular mycorrhizal fungus *Rhizophagus irregularis*

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SM designed the experiment, performed data analyses, and wrote the initial draft of the manuscript. NC was the principal investigator and provided insight throughout data collection and important edits to the manuscript.

Abstract

Arbuscular mycorrhizal (AM) fungi are soil microorganisms that form beneficial associations with most vascular plants. Despite having been characterized as ancient asexuals for many years, it was recently found that the model AM fungus *Rhizophagus irregularis* carries a putative mating-type (MAT) locus and a nuclear organization that mirrors that of sexual fungi. All *R. irregularis* isolates analysed to date harbour either one or two genotypes linked to the MAT-locus. The ability to determine the identity of the MAT-locus amongst different AM isolates is essential to characterize the nuclear organization of isolates across culture collections and within natural populations. Here, we report a method based on restriction fragment length polymorphism (RFLP) which allows for an accurate and cost-effective discrimination of MAT-locus identity and genome organization in *R. irregularis*.

Keywords

arbuscular mycorrhiza, MAT-locus, RFLP, genotyping, genome organization

Introduction

Arbuscular mycorrhizal (AM) fungi are obligate symbionts that engage in nutrient exchange with most terrestrial plants and facilitate inter-plant communication (Babikova et al. 2013; Johnson and Gilbert 2015; Keymer et al. 2017; Luginbuehl et al. 2017; Brundrett and Tedersoo 2018). Having appeared nearly 400 Mya, AM fungi were long considered to be evolutionary outliers as they lacked any observable sexual traits, an unexpected missing feature among most eukaryotic organisms (Remy et al. 1994; Normark et al. 2003; Martin et al. 2017). However, the idea that AM fungi are asexual has now been challenged, as many genome analyses

showed that these organisms carry intact mating-related genes (Halary et al. 2011, 2013; Riley and Corradi 2013; Riley et al. 2014), including a putative mating-type (MAT) locus (Ropars et al. 2016), and a nuclear organization similar to that of sexual fungi (Ropars et al. 2016; Chen et al. 2018a).

In AM fungi, genome organization is currently defined by the number of MAT-locus variants found within an "individual" mycelium (Corradi and Brachmann 2017). Specifically, AM isolates that carry one MAT-locus variant are referred to as AMF homokaryons, while isolates that carry two distinct MAT-locus variants are referred to as AMF dikaryons (**Figure S2.1**; Ropars et al. 2016). The two distinct co-existing nucleotypes of dikaryotic isolates were proposed to have emerged from plasmogamy (hyphal fusion and nuclear exchange) between two sexually compatible parental strains.

The notion that AM fungi can have sex, as seen in Dikarya fungi of the phyla Ascomycota and Basidiomycota, represents a paradigm shift from a decades long assumption that these organisms are ancient asexuals (Sanders 1999; Hijri and Sanders 2005). Practically, the finding of homokaryotic and dikaryotic life-stages offer novel opportunities to further our understanding of these fascinating plant symbionts. From this perspective, important questions that are yet to be answered include: how frequently is dikaryosis found in AM fungi culture collections versus field populations, and is this genetic condition associated with specific environmental factors, such as stress or host plant identity?

The ability to rapidly genotype isolates and single spores based on their MAT-locus is essential to address outstanding questions about the life cycle of AM fungi. Here, we report a genotyping method that allows for easy and cost-effective identification of six MAT-locus

variants, and the underlying strain specific nuclear organization, using total DNA extracts from either mycelium or single spores without the need for DNA sequencing.

Materials and methods

For this study DNA of the 27 isolates examined in Ropars *et al* (2016) was used since their MAT identity had already been verified by Sanger sequencing. When single spores were analyzed, individual spores were collected from *in vitro* cultures by selecting a small piece of MSR medium containing spores and dissolving it in a 0.1M citric acid and 0.1M sodium citrate buffer. Spores were then manually isolated and selected using a pipette under a dissection microscope. They were then crushed with a pestle in 20 µL of TE (10 mM Tris and 0.1 mM EDTA) buffer, which was heated at 56 °C for 30min to inactivate DNases.

Primer design and PCR amplification

Primers, universal to all variants, were designed on the cDNA of the homeodomain 2 (HD2) region of the mating-type (MAT) locus in *R. irregularis* because of the higher levels of sequence variability between the six MAT variants. PCR was then performed on either whole mycelium DNA or on single spores. PCRs on whole mycelium DNA were done using a reaction volume of 40 µL with 20 µL of 2x Econotaq (Lucigen) mastermix, 1 µL (10 mM) of each primer (F: 5'-CRCGTTYCACTGRCTCAAC-3', R: 5'-KTGAKTCTTYGACGGAAATTTGA-3') and 20 ng of DNA. Amplifications were performed in a Biometra DNA Engine Thermal cycler with 3 min at 96 °C followed by 35 cycles of 30 s at 96 °C, 30 s at 51 °C and 60 s at 60 °C and completed with a final 10 min extension at 60 °C. Semi-nested PCRs were performed on the single spores. The first PCR was performed using a 20 µL reaction volume with 10 µL 2x Econotaq (Lucigen)

mastermix, 0.5µL (10 mM) of each primer pair (F: 5'-CRCGTTYCACTGRCTCAAC-3', R: 5'-TTCACAATTGCGTCGGTGG-3') and 2 µL of DNA extract of a single spore. Amplifications were performed in a Biometra DNA Engine Thermal cycler with a 3 min cycle at 96 °C followed by 35 cycles of 30 s at 96 °C, 30 s at 52 °C and 60 s at 60 °C, followed by a final 10 min extension at 60 °C. The second reaction used a 1:10 dilution of products from the first PCR as the template and followed the same thermal cycling program as for whole mycelium DNA. Success of amplification was then verified on 1.5% agarose gels run in 1 X TBE buffer and compared to a GeneRuler 100 bp DNA ladder (Thermo Scientific). All PCR amplicons had a final length ranging from 522 bp to 552 bp (**Table S2.1**).

Choice of discriminatory restriction endonucleases for the MAT variants

Within the amplified region of the MAT-locus sites unique to each MAT variant were identified using SnapGene® software (GSL Biotech; available at https://www.snapgene.com). Three enzymes; AlwI BbvI and NspI (New England BioLabs Inc.) cut the PCR amplicons and produced distinct fragment sizes. The enzyme AlwI recognizes the sequence 5'…GGATC$(N)_4$▼…3' / 3'…CCTAG$(N)_5$▲…5', BbvI recognizes the sequence 5'…GCAGC$(N)_8$▼…3' / 3'…CGTCG$(N)_{12}$▲…5', and NspI recognizes the sequence 5'…RCATG▼Y…3' / 3'…Y▲GTACR…5'. These enzymes were used to individually digest the PCR amplicons in a total reaction volume of 50 µL with 1X NEB CutSmart buffer, 10 µL of PCR product and either 12.5 U of enzyme AlwI, 4 U of enzyme BbvI or 2.5 U of enzyme NspI. Digestions were performed in a Biometra DNA Engine Thermal cycler for 120 min at 37 °C followed by a denaturation step for 20 min at 65 °C. Digestion products were visualized on 3% agarose gels run at 45 V for 3 hours in 1 X TBE buffer and compared to a GeneRuler 100 bp DNA

ladder (Thermo Scientific). The fragment lengths can be found in **Table S2.1** and viewed on *in silico* generated gels in **Figure S2.2**.

Sensitivity test for dikaryon detection

To confirm that RFLP can be used to detect the MAT-locus type and the genome organization even at low and skewed nucleotype abundance, genomic DNA from whole mycelia of the isolates A1 (MAT-3) and C2 (MAT-6) were diluted 1:8 to mirror the concentration of DNA in a single spore. These DNA templates were mixed in various ratios, ranging from 99:1 to 1:99 and were PCR amplified, following the protocol for single spores. These amplicons were digested with the restriction enzyme AlwI and the fragments resolved on 3 % agarose gels (as outlined above).

Results

Initial RFLP analyses were carried out on whole mycelium DNA of the 24 homokaryotic isolates characterised by Ropars *et al*. (2016) to verify that the MAT-locus could be correctly distinguished (**Figure 2.1a**). We found that the restriction enzyme (RE) AlwI clearly distinguished the MAT-5 locus, producing a MAT-5 specific band of 346 bp. The enzyme AlwI also cut the MAT-2, MAT-3, and MAT-4 locus variants, but in these cases all restriction fragments are approximately the same length of 408 ± 10 bp. However, this enzyme did not digest the MAT-6 amplicons, leaving a band of 552 bp. The BbvI enzyme cuts Mat-4 to produce bands of 269 bp and 172 bp. The restriction fragments of MAT-3 are 271 bp and 269 bp and for MAT-6, fragments of 283 bp and 269 bp are generated. The PCR amplicon of MAT-2 and MAT-5 are not digested by BbvI leaving a band of 522 bp and 531 bp respectively. Finally, RE NspI specifically cuts the

PCR amplicons of MAT-4 producing distinct bands at 362 bp and 181 bp. All other MAT-loci were not digested, leaving a band of the PCR amplicons at approximately 538 ± 15 bp. The three RE AlwI, BbvI and NspI thus resolve all currently known MAT-loci as predicted from the information in Ropars *et al*. (2016). Furthermore, these results also show that MAT-5 can be identified with only RE AlwI, MAT-3 and MAT-6 with only RE BbvI, and MAT-4 with only RE NspI.

Once the method was verified on whole mycelium DNA, identical analyses were carried out on DNA of homokaryon single spores. Spores of representative cultures of each MAT-locus variant were genotyped (**Figure 2.1b**). In all instances, bands produced were consistent with the isolate's MAT and karyotype, demonstrating the ability of RFLP analysis to screen populations of single spores.

One important goal of the study was to construct a method that is of use to identify AMF dikaryons. In all cases, use of RE confirmed the dikaryotic status of *R. irregularis* isolates A4, A5 and DAOM 240409, using both whole mycelium and single spores (**Figure 2.2a,b**; Ropars et al. 2016; Chen et al. 2018). The two nucleotypes of each dikaryon could be distinguished with a single RE. Specifically, the isolate A5 had restriction fragments of 522 bp and 419 bp with RE AlwI as expected for an isolate carrying MAT-3 and MAT-6 nuclei. Similarly, A4 had restriction fragments of 522 bp, 269 bp and 172 bp and DAOM 240409 had restriction fragments of 531 bp, 269 bp and 172 bp with RE BbvI attributable, respectively, to MAT-1 and MAT-2, and MAT-1 and MAT-5. Mixing whole mycelium DNA of homokaryotic strains in different ratios showed that dikaryosis can be reliably detected from a range of 80:20 to 30:70 MAT-3 and MAT-6 at a template concentration corresponding to that of a single spore (**Figure 2.2c**). Sensitivity of

dikaryon detection was only tested on the A5-like dikaryon (MAT-3 and MAT-6) as no MAT-1 homokaryon samples were available at the time.

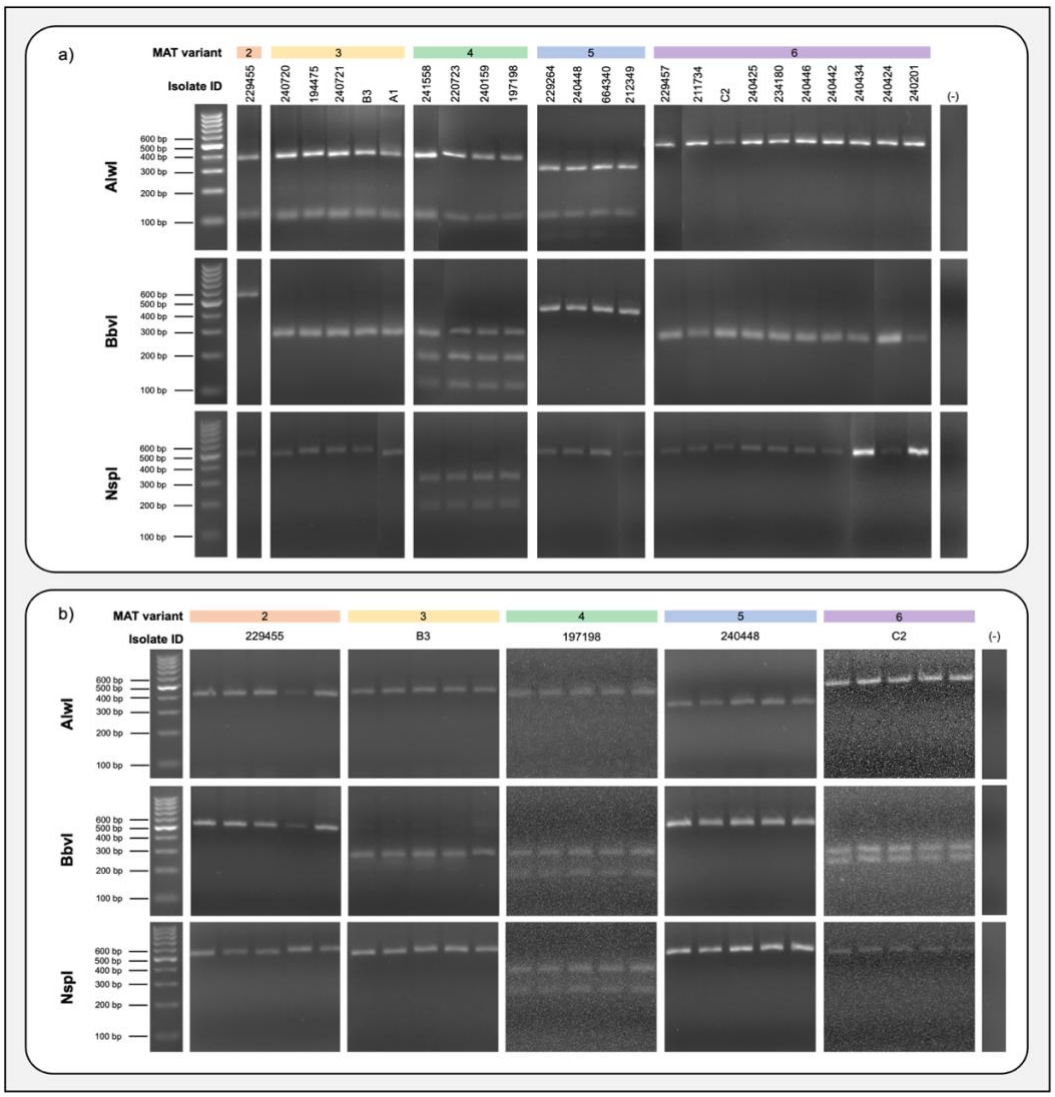

Figure 2.1. Restriction enzyme digestion of the homeodomain 2 (HD2) region of homokaryotic *Rhizophagus irregularis* isolates of different MAT variants. Isolate ID refers to the DAOM number assigned to each isolate. All samples are visualized on 3% agarose gels. Exact sizes of fragments generated can be found in **Table S2.1**. a) Digestion of amplicons from whole mycelium for the 24 identified homokaryotic isolates in Ropars *et al.* (2016) with restriction enzymes AlwI, BbvI, and NspI. b) Digestion of amplicons from single spores for representative isolates of each MAT variant with restriction enzymes AlwI, BbvI, and NspI.

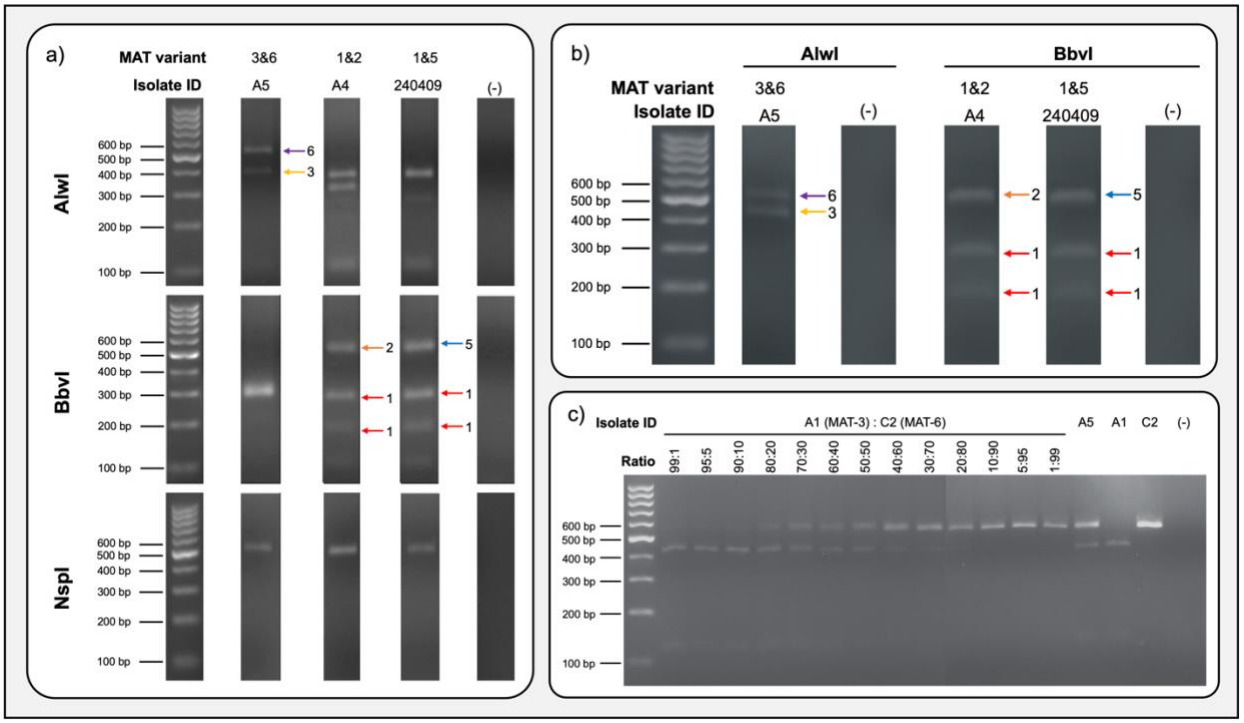

Figure 2.2. Restriction enzyme digestion of the homeodomain 2 (HD2) region of dikaryotic *Rhizophagus irregularis* isolates. Isolate ID refers to the DAOM number assigned to each isolate. All samples are visualized on 3% agarose gels. Exact sizes of bands generated can be found in **Table S2.1**. a) Digestion of whole mycelium amplicons for the three identified dikaryotic isolates in Ropars *et al.* (2016) with restriction enzymes AlwI, BbvI, and NspI. Arrows indicate bands that clearly distinguish one of the two MAT variants present within the sample. b) Digestion of single spore amplicons of each dikaryotic isolate with the restriction enzyme capable of distinguishing the two MAT variants present within the sample. Arrows indicate bands that are unique to one of the two MAT variants present within the sample. c) Sensitivity test for dikaryon detection with samples containing A1 (MAT-3) and C2 (MAT-6) diluted DNA. All samples were digested with RE AlwI with the ratios written as MAT-3 : MAT-6. Positive controls of a dikaryon, MAT-3 and MAT-6 are represented by A5, A1, and C2 respectively.

Discussion

Overall, this study shows that PCR-RFLP can be used to rapidly determine the MAT-locus identity and state of karyosis of *R. irregularis* strains, using DNA extracted from whole mycelium as well as individual spores (**Figure 2.1 and Figure2.2a,b**). Furthermore, the method is inexpensive compared to identical analyses involving direct Sanger sequencing of PCR products. Specifically, Sanger sequencing all PCR products shown in this study would have increased the cost by at least 50 % (based on direct Sanger sequencing costs at the McGill Genome Québec sequencing centre in 2021). Further inflation of the costs would be encountered for samples with co-existing mating-type variants, as they must undergo bacterial cloning and additional sequencing.

However, as with any method there are limitations with RFLP. For instance, all restriction enzymes have a measure of their endonuclease activity, as defined by the amount of DNA that they can efficiently digest (Davis et al. 1986). As a result, samples with higher DNA concentrations could be subjected to incomplete digestion by the restriction enzymes. Something else to keep in mind when performing RFLP analysis is that results can at times be subject to interpretation, specifically if the bands visualized on the agarose gel after restriction enzyme digestion are faint. These constraints can be resolved by using a more sensitive or higher throughput technology, such as capillary electrophoresis, qPCR or ddPCR (Cohen et al. 1988; Zhou et al. 2013; Kokkoris et al. 2021a). Though these technologies may acquire more detailed information on the samples, they will also result in a large increase in cost.

In conclusion, the RFLP procedures we established allow for simple and cost-effective genotyping of *Rhizophagus* isolates and can thus assist in furthering our understanding of genome organization in these organisms. This protocol could be applied to verify the MAT identity of

individual spore progenies generated from crossings between homokaryotic isolates, or of spores produced under different environmental conditions, including different plant hosts (Corradi and Brachmann 2017). Furthermore, this technique could easily be expanded to genotype environmental samples, given its ability to generate accurate results on single spores, allowing, for example, to assess whether dikaryosis is more likely to emerge under environmental stress.

More generally, it has been proposed that the AMF research community should shift away from using traditional markers, such as morphology and ribosomal sequence markers, and focus instead on protein-encoding genes with biologically or ecologically important functions to better classify AM fungi (Gamper et al. 2010). From this point of view, the putative AMF MAT-locus represents an optimal genetic marker: it is single copy, highly traceable, and is potentially highly relevant for the biology of AMF.

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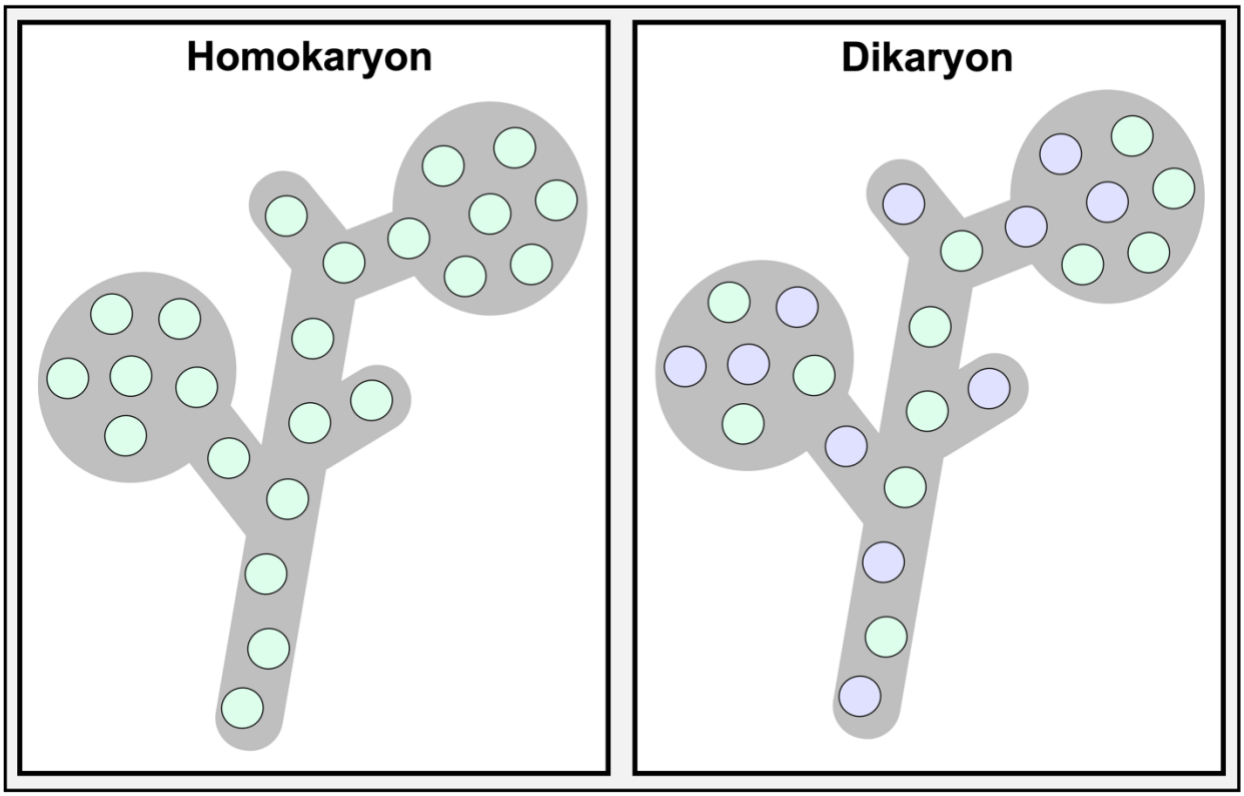

Figure S2.1. Visual comparison of the nuclear organization of homokaryotic and dikaryotic isolates. In the AMF homokaryon, all nuclei possess the same mating-type (MAT) locus and overall similar genotypes (nuclei represented by green circles). In the AMF dikaryon, two distinct populations of nuclei co-exist, each with a different variant of the MAT-locus (nuclei represented by green and purple circles).

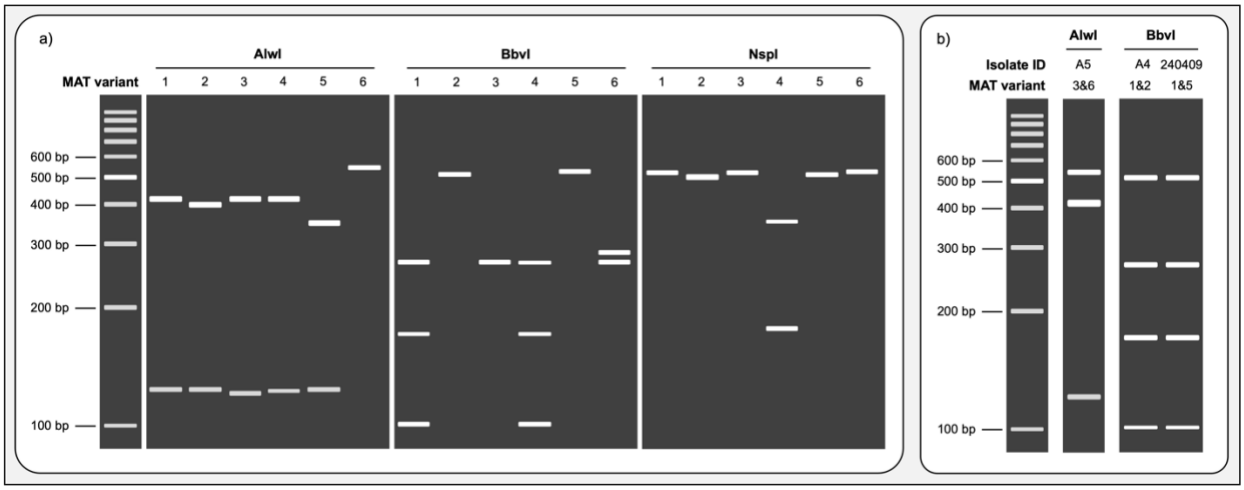

Figure S2.2. ***In silico*** **gels generated to show the expected RFLP fragment lengths for each of the mating-type (MAT) locus variants with different restriction enzymes based on cDNA sequences of the homeodomain 2 (HD2) region.** a) Expected fragment lengths of homokaryotic isolates of each of the MAT-locus variants being individually digested with the three restriction enzymes: AlwI, BbvI and NspI. b) Expected fragment lengths of dikaryotic isolates digested with the endonuclease that allows the two nucleotypes of each isolate to be identified. Both *in silico* gels use a GeneRuler 100bp DNA ladder.

Table S2.1. Information used in restriction fragment length polymorphism (RFLP) analysis. Strain identifier (DAOM), mating-type (MAT) locus variant identity, homeodomain 2 (HD2) accession number, PCR amplicon lengths and predicted lengths of the resulting restriction fragments produced from the three enzymes: AlwI, BbvI and NspI based on *in silico* analyses of the cDNA sequences.

DAOM	MAT identity	Accession number	Length of PCR amplicon (in bp)	Length of restriction fragments produced (in bp)		
				AlwI	BbvI	NspI
229455	2	KT946653	522	398 124	NA°	NA
240720	3	KT946673	540	419 121	271 269	NA
194475	3	KT946675	540	419 121	271 269	NA
240721	3	KT946662	540	419 121	271 269	NA
B3	3	KT946660	540	419 121	271 269	NA
A1	3	KT946661	540	419 121	271 269	NA
241558	4	KT946666	543	419 124	269 172 102	362 181
220723	4	KT946665	543	419 124	269 172 102	362 181
240159	4	KT946664	543	419 124	269 172 102	362 181
197198	4	KT946663	543	419 124	269 172 102	362 181
229264	5	KT946671	531	346 124 61	NA	NA
240448	5	KT946670	531	346 124 61	NA	NA
664340	5	KT946668	531	346 124 61	NA	NA

DAOM	MAT identity	Accession number	Length of PCR amplicon (in bp)	Length of restriction fragments produced (in bp)		
				AlwI	BbvI	NspI
212349	5	KT946667	531	346 124 61	NA	NA
229457	6	KT946658	543	NA	274 269	NA
211734	6	KT946680	552	NA	283 269	NA
C2	6	KT946677	552	NA	283 269	NA
240425	6	KT946678	552	NA	283 269	NA
234180	6	KT946679	552	NA	283 269	NA
240446	6	KT946682	552	NA	283 269	NA
240442	6	KT946683	552	NA	283 269	NA
240434	6	KT946684	552	NA	283 269	NA
240424	6	KT946686	552	NA	283 269	NA
240201	6	KT946685	552	NA	283 269	NA
A4*	1	KT946659 (scaffold511)	543	419 124	269 172 102	NA
	2	KT946654 (scaffold5308)	522	398 124	NA	NA
A5*	3	KT946676 (scaffold4735)	540	419 121	271 269	NA
	6	KT946681 (scaffold2225)	552	NA	283 269	NA
240409*	1	KT946674 (allele1)	543	419 124	269 172 102	NA
	5	KT946672 (allele2)	531	346 124 61	NA	NA

° Enzyme does not cut the sequence

* Dikaryons

Chapter 3: Co-existence of homokaryotic arbuscular mycorrhizal fungi generates heterokaryotic spore progenies

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SM designed the crossing and recombination experiment, performed data analyses, and wrote the initial draft of the manuscript. CS and MG designed the hyphal compatibility assay and performed data analyses. NC was the principal investigator and provided insight throughout data collection and important edits to the manuscript.

Abstract

Arbuscular mycorrhizal fungi (AMF) form mutualistic associations with 72% of land plants, establishing important subterranean nutrient exchange networks that can benefit entire terrestrial ecosystems. AMF strains are either homokaryotic (AMF homokaryons), carrying thousands of highly similar nuclei, or heterokaryotic (AMF dikaryons) carrying distinct co-existing nucleotypes. It was proposed that AMF dikaryons originate through nuclear exchange between compatible homokaryotic strains as seen in sexual fungi, but this hypothesis has not been tested experimentally. Here, we show that co-culturing theoretically compatible AMF homokaryons can indeed produce spore progeny that carry two parental genotypes. Furthermore, we found that some homokaryotic spores present in co-cultures carry signatures of inter-strain recombination, indicating that following nuclear exchange genetic diversity may be redistributed either through meiotic or somatic recombination. Overall, this work supports the model that AMF can switch from a clonal to a sexual life stage when compatible strains co-exist and provides a glance into the mechanisms that generate and maintain AMF genetic diversity in terrestrial ecosystems.

Keywords

arbuscular mycorrhiza, plasmogamy, recombination, genome organisation, mating-type locus

Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate plant symbionts that are thought to have played a key role in the emergence of terrestrial plant species (Bucking et al. 2012; Field et al. 2015; Bonfante and Venice 2020). These fungi have a peculiar biology, as they produce large

coenocytic cells that carry thousands of nuclei that flow bi-directionally along interconnected hyphae and within spores (Cooke et al. 1987; Marleau et al. 2011; Kokkoris et al. 2020). For some time, these organisms have been characterized as ancient asexuals; a group of evolutionarily unrelated eukaryotes thought to have propagated clonally for many millions of years (Law and Crespi 2002; Normark et al. 2003). However, growing research into the biology of these supposed ancient asexuals has shown that genetic exchange is nearly ubiquitous amongst these organisms (Fraser and Heitman 2003; Malik et al. 2008; Dunthorn et al. 2017). For example, the presumed asexual protozoan parasite *Giardia duodenalis* was found to undergo cell division twice without DNA replication during the process of excystation, a process reminiscent of meiosis (Bernander et al. 2001), and a multi-locus sequence analysis of *G. duodenalis* revealed evidence of meiotic recombination in this species (Cooper et al. 2007). Similarly, the opportunistic fungal pathogen *Candida albicans* was previously considered asexual until a mating-type locus was identified in the genome (Hull and Johnson 1999), and subsequent observations of sexual stages (from schmooing through to nuclear migration, septation and daughter cell budding) were reported for this species (Lockhart et al. 2003).

In the case of AMF, genome data has uncovered many signatures linked to sexual reproduction, including an abundance of transposable elements, a core set of meiotic genes and a putative mating-type (MAT) locus (Halary et al. 2011, 2013; Riley et al. 2014; Ropars et al. 2016). In fungi, the MAT locus is responsible for sexual identity and dictates compatibility between partners, such that fungal strains with compatible MAT loci can fuse their hyphae and exchange nuclei that then undergo meiotic recombination (Casselton 2002; Fraser and Heitman 2003). As such, the identification of this MAT locus resulted in a paradigm shift for AMF by demonstrating the stark similarities in their genetics with that of sexual fungi.

In AMF the MAT-locus mirrors the genetic architecture of the fungal phyla Basidiomycota, which contains inversely transcribed homeodomains (Ropars et al. 2016; Chen et al. 2018a). In Basidiomycota nuclear exchange results in the formation of dikaryotic cells, where each cell carries two distinct nuclei, one from each compatible parent. The cell may persist in this dikaryotic stage for a prolonged period of time until karyogamy and meiosis (sexual reproduction) occurs (Brown and Casselton 2001), resulting in recombined homokaryotic offspring (Middleton 1964; Clark and Anderson 2004).

Remarkably, some strains of the model AMF *Rhizophagus irregularis* show a dikaryote-like genetic pattern that is similar to that of basidiomycetes (Ropars et al. 2016; Corradi and Brachmann 2017), but in this case thousands of nuclei, presumably originating from two parental strains, co-exist in one continuous cytoplasm (Ropars et al. 2016; Chen et al. 2018a; Kokkoris et al. 2021a). This discovery supported the view that AMF follow similar life-stages to those of sexual fungi. Specifically, it was proposed that two homokaryotic AMF strains that carry compatible genomes could fuse hypha to generate AMF dikaryons (Ropars et al. 2016; Corradi and Brachmann 2017; Kokkoris et al. 2021a). This view of AMF genetics is presently built on knowledge from sexually reproducing fungi, but actual experimental evidence that homokaryotic relatives can produce spores with two parental genotypes is currently unavailable. It is also unknown if crossing AMF homokaryotic relatives can produce a recombined homokaryotic spore progeny.

Here, we hypothesize that spores containing nuclei originating from two parental isolates can emerge from co-culturing theoretically compatible homokaryotic strains. To test this, we co-cultivated two homokaryotic strains that carry seemingly compatible MAT-loci in a Root Organ

Culture system (ROCs), and genotyped individual spores to detect evidence of inter-strain nuclear exchange (spores carrying two genotypes) and putative inter-strain recombination.

Materials and Methods

Hyphal compatibility assay

Chicory seeds (*Cichorium intybus* L.) were surface-sterilized, germinated, and grown for 15 days in sterile plastic boxes filled with steam-sterilized quartz grit (mean diameter size 2 mm) before AMF inoculation. *R. irregularis* inoculum consisting of spores, mycelium and chopped colonised roots, was obtained from ROCs of the different isolates, after Modified Strullu and Romand (MSR) medium solubilization in citrate buffer (Doner and Bécard 1991) and sieving through a 30-µm-mesh size sieve. One gram (fresh weight) of the prepared inoculum was laid on the root system of each plant, then chicory seedlings were grown in disinfected 10-cm diameter pots, filled with the steam-sterilized quartz grit. Plants were placed into sun-transparent bags (Sigma-Aldrich), maintained in a growth chamber at 25 °C, with 16 h of light per day and watered weekly with sterile distilled water. After 3 weeks' growth, root colonisation was checked by detecting intraradical fungal autofluorescence at 450-490 nm excitation wavelengths (Jabaji-Hare et al. 1984) under a Leica DM IRB inverted microscope (Leica, Wien).

Extraradical hyphae adhering to colonised plant roots were carefully removed with forceps and each root system was then wrapped in a 41 µm mesh nylon net (Millipore) to obtain a flat mesh pocket (Pepe et al. 2017). Couples of mycorrhizal plants with nylon-enclosed root systems, hosting either the same or different *R. irregularis* isolates, were paired on 13-cm diameter membranes (0.45-µm pore diameter size, MF-Millipore™). Each membrane was laid on moist sterile quartz grit placed on the bottom of a sterile 14-cm diameter plastic Petri dish with a hole

allowing shoot growth. After sealing, plates were wrapped in aluminium foil and the whole-plant systems were placed into sun-transparent bags. Plants were maintained in the growth chamber and grit was moistened every three days with 2 mL of sterile distilled water. Eight replicate plates for each pairing were prepared.

After 20 days, the extraradical mycelia (ERMs) growing from the nylon nets of the paired plants were treated to detect hyphal contacts between the two ERMs and assess their viability, using a succinate dehydrogenase (SDH) activity test and Trypan blue staining, as described in Pepe *et al.* (2016). The membrane area containing the paired ERMs contact zone was cut into 25 x 50 mm pieces, to be mounted on microscope slides and observed under a Reichert Jung Polyvar microscope (Leica, Wien). All hyphal contacts occurring among hyphae belonging to ERMs produced by the paired plants were enumerated at magnifications of ×320 – ×1250 and classified into four categories. The outcome of hyphal interactions based on the deposition of formazan salts due to SDH activity allowed the assessment of hyphal viability and protoplasmic continuity in fusing hyphae. Anastomosed hyphae showing viable protoplasm continuity were recorded as perfect fusions, while anastomosed hyphae showing protoplasm withdrawal and septa isolating fused hyphal compartments in one or both hyphae were scored as post-fusion incompatible interactions. When septa and protoplasm retraction were detected before hyphal fusion but hyphal tip swelling or hyphal homing occurred, the contact was scored as pre-fusion incompatible interaction (Sbrana et al. 2011). All hyphal contacts where hyphae crossed with no interactions were scored as non-interacting. For each hyphal contact category, frequencies were calculated as the ratio between contact event number and the total hyphal contacts detected. Images were acquired under the Reichert Jung Polyvar microscope with Reichert 40x plan Iris (NA 0.75) or plan 100 oil iris (NA 1.25) lenses.

Establishing crossing cultures and collecting single spores

Cultures for the crossings between isolates were established in three-compartment rectangular Petri dishes, where isolates A1 and C2 were placed in the outer compartments with transformed carrot roots and could only interact in the middle compartment (**Figure S3.1**). Cultures were grown in MSR medium containing 1 %W/V sucrose and were allowed to grow to maturity for approximately six months – i.e., when hyphae and spores from both isolates could be seen to overlap in the middle compartment and sporulate. Once spores had grown to maturity, single spores were isolated from the cultures by dissolving a small piece of the MSR medium in a 17 % 0.1 M citric acid and 83 % 0.1 M sodium citrate solution. The isolated spores were then crushed in TE buffer (10 mM Tris and 0.1 mM EDTA) and heated at 56 °C for 30 min to break down the spore membranes, burst the nuclei and inactivate DNases.

Determining the genome organization of single spores

The extracted DNA from each single spore was analyzed to determine its karyosis (homokaryotic: containing only one of the parental mating types, or dikaryotic: containing both parental mating types).

RFLP was used to genotype the spores, based on a segment of the homeodomain 2 (HD2) region of the MAT-locus. This region was first amplified by semi-nested PCR with 2 X Econotaq (Lucigen) mastermix, 10 mM of each primer pair (first PCR, F: 5'-CRCGTTYCACTGRCTCAAC-3', R: 5'-TTCACAATTGCGTCGGTGG-3' and second PCR, 5'-CRCGTTYCACTGRCTCAAC-3', R: 5'-KTGAKTCTTYGACGGAAATTTGA-3') and 2 µL of DNA. Amplifications were performed in a Biometra DNA Engine Thermal cycler with 3 min at 96 °C followed by 35 cycles of 30 s at 96 °C, 30 s at 52 °C and 60 s at 60 °C and completed with

a final 10 min extension at 60 °C. Success of amplification was then verified on a 1.5 % agarose gel run in 1 X TBE buffer and compared to a GeneRuler 100 bp DNA ladder (Thermo Scientific). The amplified PCR products were then digested with the restriction enzyme AlwI (New England BioLabs Inc.) in a 50 µL reaction with 1 X NEB CutSmart buffer, 10 µL of PCR product and 4 U of enzyme. The enzyme was shown to generate unique patterns when used to digest MAT-3 and MAT-6 variants isolated from homokaryotic relatives (as seen in chapter 2).

Digestions were performed in a Biometra DNA Engine Thermal cycler for 120 min at 37 °C followed by a denaturation step for 20 min at 65 °C. Digestion products were visualized on a 3 % agarose gel run at 45 V for 3 hours in 1 X TBE buffer and compared to a GeneRuler 100 bp DNA ladder (Thermo Scientific). Several PCR products were also sent for Sanger sequencing (McGill Genome Québec sequencing centre) to verify the findings of the RFLP (data not shown).

Additional single spores were then analyzed using droplet digital PCR (ddPCR) to validate the RFLP genotyping of spores isolated from the crossings. ddPCR was performed with the MAT-3 and MAT-6 primers and following the protocol from Kokkoris *et al.* (2021). In brief, these single spores were collected and placed directly into a PCR tube in a 2.4 µL volume of distilled water. The spores were then crushed using a needle and the PCR reaction mixture was added to the tube. The mixture consisted of 1 X Supermix for probes (Bio-Rad), 500 nM primer/250 nM probe mixture for both MAT-3 and MAT-6. The PCR reaction was performed in a C100 Touch Thermal Cycler (Bio-Rad) with a 10 min cycle at 95 °C followed by 45 cycles at 94 °C for 1 min and 52.5 °C for 2 min and completing with a 1 min cycle at 98 °C. All steps had a ramp rate of 1 °C/sec applied. Amplified droplets were then read with the QX200 Droplet Reader (Bio-Rad) and analyzed using the QuantaSoft Analysis Pro Software (Bio-Rad). Thresholds were set at points where positive and negative droplets were clearly distinguishable.

Growing single spore cultures from spore progenies produced during crossings

Once crossing cultures had reached maturity (approximately six months) additional spores were collected, plated (10 spores spaced out on a 100 x 15 mm Petri dish) on MSR medium containing 1 %W/V sucrose and grown under 25 °C. Spores were then observed daily for germination and once observed, the section of medium containing the germinated spore was cut from the Petri dish using a scalpel under a laminar flow hood and placed spore side down into a new 100 x 15 mm Petri dish containing MSR medium with 1 %W/V sucrose. Two sections of ROCs were then added on either side of the spore and the new culture was grown under 25 °C. Cultures were checked regularly for hyphal growth and hyphal/root contact. Once sporulation occurred, sections of the culture were collected by dissolving a small piece of the MSR media in a 17 % 0.1 M citric acid and 83 % 0.1 M sodium citrate solution. These cultures were then genotyped as explained above.

Checking for evidence of recombination

A MUMmer plot was generated for the A1 genome against the C2 genome. The data from this plot was then extracted and homologous scaffolds were arranged by length, where the pairs of scaffolds with the longest lengths were extracted, to maximize the chances of finding variations within the sequence. PCR primers were designed on single copy regions showing strain-specific genotypes as denoted by specific insertions and deletions, thus allowing for rapid distinction of strain-specific amplicons on an agarose gel based on size (**Table S3.1**). These regions, as well as the primers used to amplify them, were analysed using Blast (Altschul et al. 1990) against their respective publicly available genomes (Chen et al. 2018b) to further ensure that the sequence was single copy and that the primers were specific to that region. Similar analyses performed on

unpublished chromosome-level data from A1 and C2 confirmed their single-copy and strain-specific nature.

DNA from single spores collected in the middle portion of the co-cultures (**Figure S3.1**) was extracted and genotyped as previously described, and 84 spores identified as homokaryotic (43 of MAT-3 and 41 of MAT-6) were further investigated for recombination using the strain-specific primers (**Table S3.1**). The PCR amplification was carried out in a Biometra DNA Engine Thermal cycler for 3 min at 96 °C followed by 40 cycles of 30 s at 96 °C, 30 s at 54.1 °C and 60 s at 60 °C and completed with a final 10 min extension at 60 °C. Success of amplification and identification of the identity of each region was then verified on a 2 % agarose gel run in 1 X TBE buffer and compared to a GeneRuler 100 bp DNA ladder (Thermo Scientific). Negative and positive controls were run on the parental isolates A1 (known MAT-3) and C2 (known MAT-6) following the same protocol.

Results

In vivo hyphal interaction assay to identify candidate homokaryotic isolates for crossing

To date, a total of five AMF dikaryotic strains have been identified (Ropars et al. 2016; Masclaux et al. 2018; Kokkoris et al. 2021a) in culture collections, and these carry the following MAT-loci combinations: MAT-1/2 (*R. irregularis* strains A4 and C3), MAT-1/5 (*R. irregularis* strains DAOM 240409 and G1) and MAT-3/6 (*R. irregularis* strain A5). At the time of this experiment, only *R. irregularis* homokaryotic relatives containing MAT-3 and MAT-6 were available in our lab and were thus readily testable for hyphal compatibility.

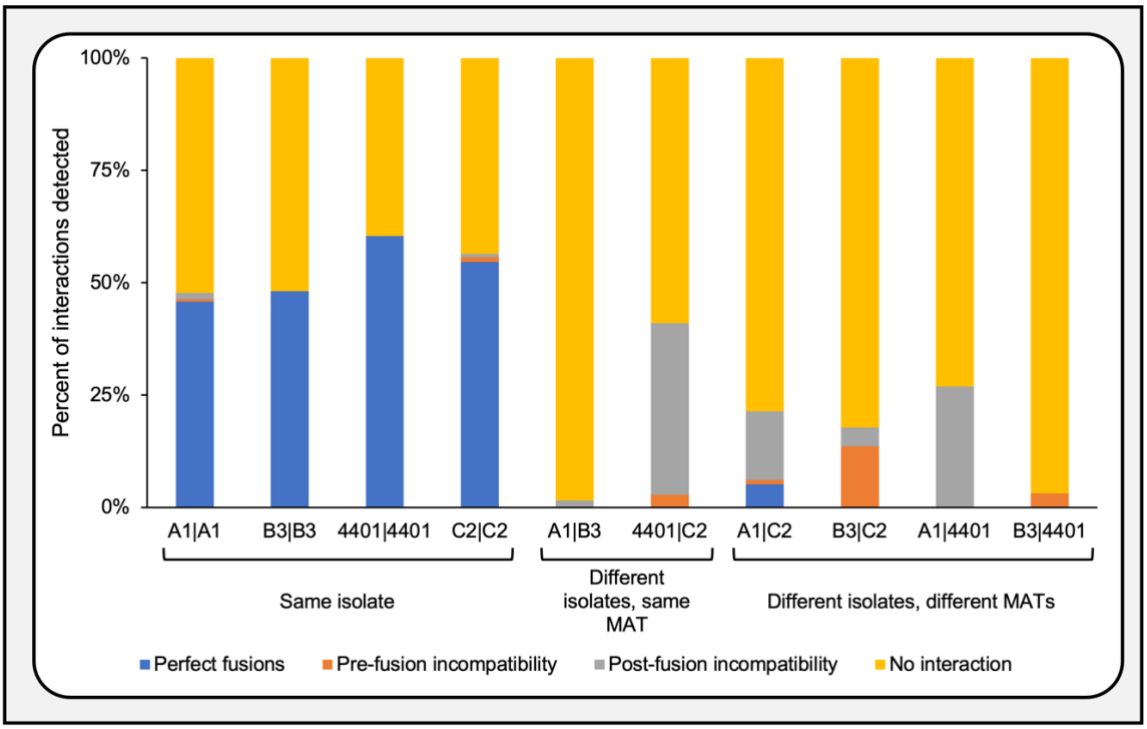

Figure 3.1. *In vivo* interactions between different isolate pairings of *R. irregularis* in symbiosis with chicory. Isolates selected were all of MAT-3 (A1, B3) or MAT-6 (C2, 4401) based on the previously identified dikaryon (A5) of this combination. Three different types of pairings were made between: spores from the same isolate (n = 203 for A1|A1; 293 for B3|B3; 505 for 4401|4401; 171 for C2|C2), spores from two different isolates that shared the same MAT type (n = 418 for A1|B3; 475 for 4401|C2) and different isolates that had different MAT types (n = 265 for A1|C2; 224 for B3|C2; 561 for A1|4401; 326 for B3|4401). Interactions observed are perfect fusions (blue), pre-fusion incompatibility (orange), post-fusion incompatibility (grey) and no interaction (yellow).

To identify the isolate combination that is most likely to undergo gene exchange, we measured the frequency of hyphal fusion and protoplasmic continuity between pairs of *R. irregularis* MAT-3 and MAT-6 isolates (either same isolate or different isolate pairings) using an *in vivo* symbiotic assay with chicory (**Figure 3.1**, **Table S3.2**). Phylogenies based on protein-

encoding genes were used to distinguish *R. irregularis* isolates with distinct phylogenetic backgrounds (Sokolski et al. 2011; Ropars et al. 2016).

These assays showed that perfect fusions ranged from 45.8 % to 60.4 % between hyphae from the same isolate, while perfect fusions were rare or not observed among genetically distinct strains. Different isolate pairings varied in observed compatibility outcome, which includes pre- and post- fusion incompatibility – i.e., the production of septa before or after hyphal fusion, ranging from 0.5 % to 13.6 % and 0.7 % to 38.2 % of the samples respectively. Overall, all isolate pairings had many non-interacting hyphae (ranging from 39.6 % to 98.5 %), and notably only the pairing of MAT-3 isolate A1 and MAT-6 isolate C2 produced perfect fusions (5.1 %).

Identification of AMF dikaryotic spore progeny in co-cultures of homokaryotic relatives

The *in vivo* hyphal interaction assays provided evidence that, among all heterologous pairings, the isolates A1 and C2 are most likely to undergo plasmogamy. We thus hypothesized that these isolates could exchange nuclei to create dikaryotic offspring. To investigate this, the isolates A1 and C2 were grown within a tri-compartment carrot ROCs that allows each isolate's hyphae to meet and potentially undergo heterologous anastomosis (**Figure S3.1**).

After six months of growth, a total of 375 individual spores were collected within the middle compartment of these ROCs and their genetic content was analyzed using both restriction fragment length polymorphism (RFLP) and droplet digital PCR (ddPCR) to determine whether some contained two parental genotypes (**Figure 3.2, Figure S3.2**).

Of the 213 spores analyzed by RFLP, which were independently obtained from 3 replicated experiments, 8 spores (3.8 %) carried the A1 and C2 genotypes, providing evidence that compatible homokaryons can produce a dikaryotic spore progeny. The overwhelming majority of

spores analyzed using RFLP were, however, homokaryotic, with 67.1 % (143 of 213 spores analyzed) carrying the MAT-3 (A1) genotype and 29.1 % (62 of 213 spores analyzed) carrying the MAT-6 (C2) genotype, indicating that the production of dikaryons is not a common occurrence in ROCs when A1 and C2 hyphae interact.

To validate this observation, independent ddPCR based on the MAT-3 and MAT-6 probes was performed on an additional 162 spores. This ddPCR analysis, which was performed using an established protocol that allows reliable MAT-3 and MAT-6 genotyping of single nuclei (Kokkoris et al. 2021a, b), resulted in the identification of 3 spores (1.9 %) containing two parental genotypes (at a 1:1 ratio), 31 spores (19.1 %) carrying the MAT-3 (A1) genotype and 128 spores (79.0 %) carrying the MAT-6 (C2) genotype.

Overall, this analysis revealed that 2.9 % of the 375 spores isolated in the middle compartment and analyzed using distinct methods contained two parental genotypes, while 46.4 % carried the MAT-3 genotype and 50.7 % carried the MAT-6 genotype. An additional 2000 spores from co-cultures were also harvested with the aim to establish new dikaryotic single spore ROCs. Of these 331 spores germinated, and only 5 (1.5 %) produced viable ROCs, none of which showed evidence of carrying two parental genotypes.

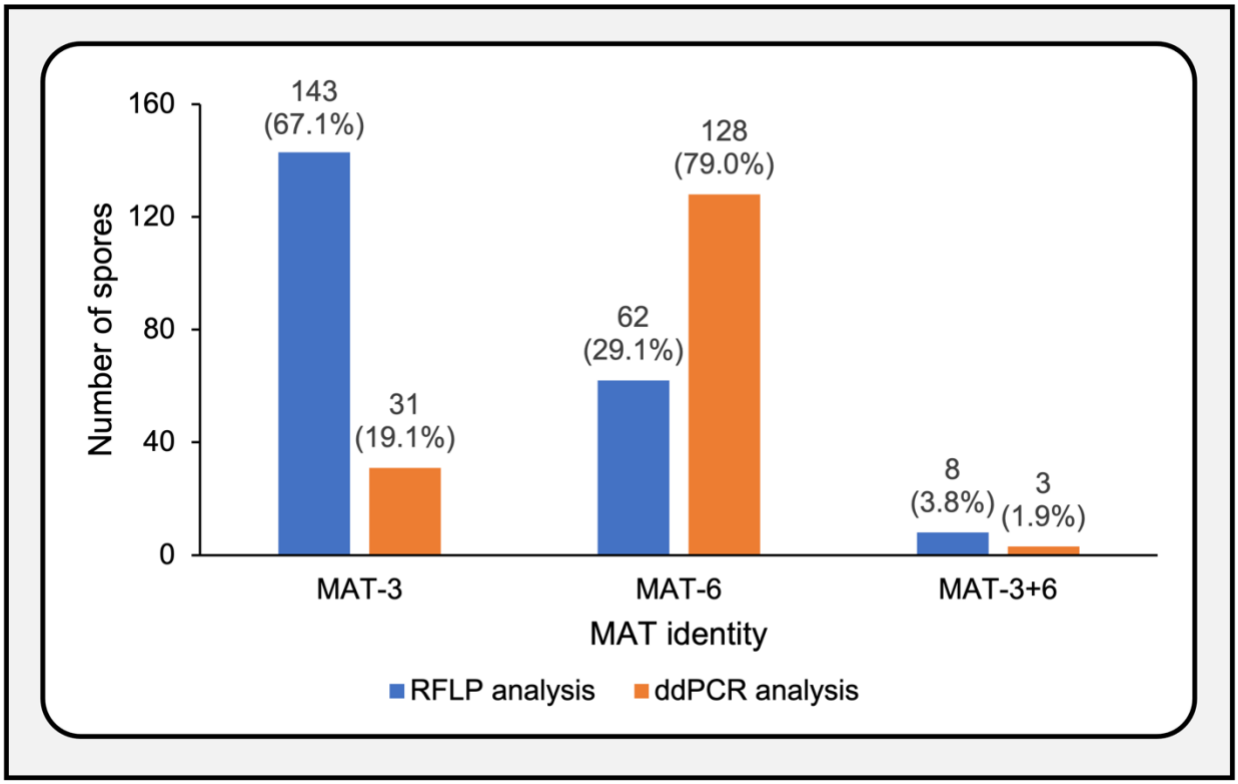

Figure 3.2. Identity of spore progenies collected from *in vitro* crossings of the isolates A1 and C2. Spores were isolated from the middle compartment and analyzed using RFLP (blue, n = 213) or ddPCR (orange, n = 162) for the identity of their MAT-locus.

Recombination analysis of homokaryotic spore progeny from co-cultured isolates

The current model that describes AMF genetics indicates that crossed AMF homokaryons should eventually also produce a recombined homokaryotic spore progeny. To test this, 84 spores previously confirmed to be homokaryotic using RFLP and ddPCR were genotyped using PCR at eight single copy genomic regions. This analysis showed evidence of putative recombination between the A1 and C2 genomes in all eight distinct genomic locations (**Figure 3.3a**). Specifically, a total of 18 homokaryotic spores (21%) showed putative recombination, where 14 MAT-3 spores carried evidence of some MAT-6 genotypes within their genome and, conversely,

a total of 4 MAT-6 spores carried some MAT-3 genotypes, indicating potential inter-isolate recombination within the co-culture (**Figure 3.3b**). In all cases, negative controls showed no evidence of amplification, and identical analyses performed on parental isolates showed no evidence for recombination (**Figure S3.3**). However, none of the 5 ROCs newly established using homokaryotic single spores showed evidence of recombination.

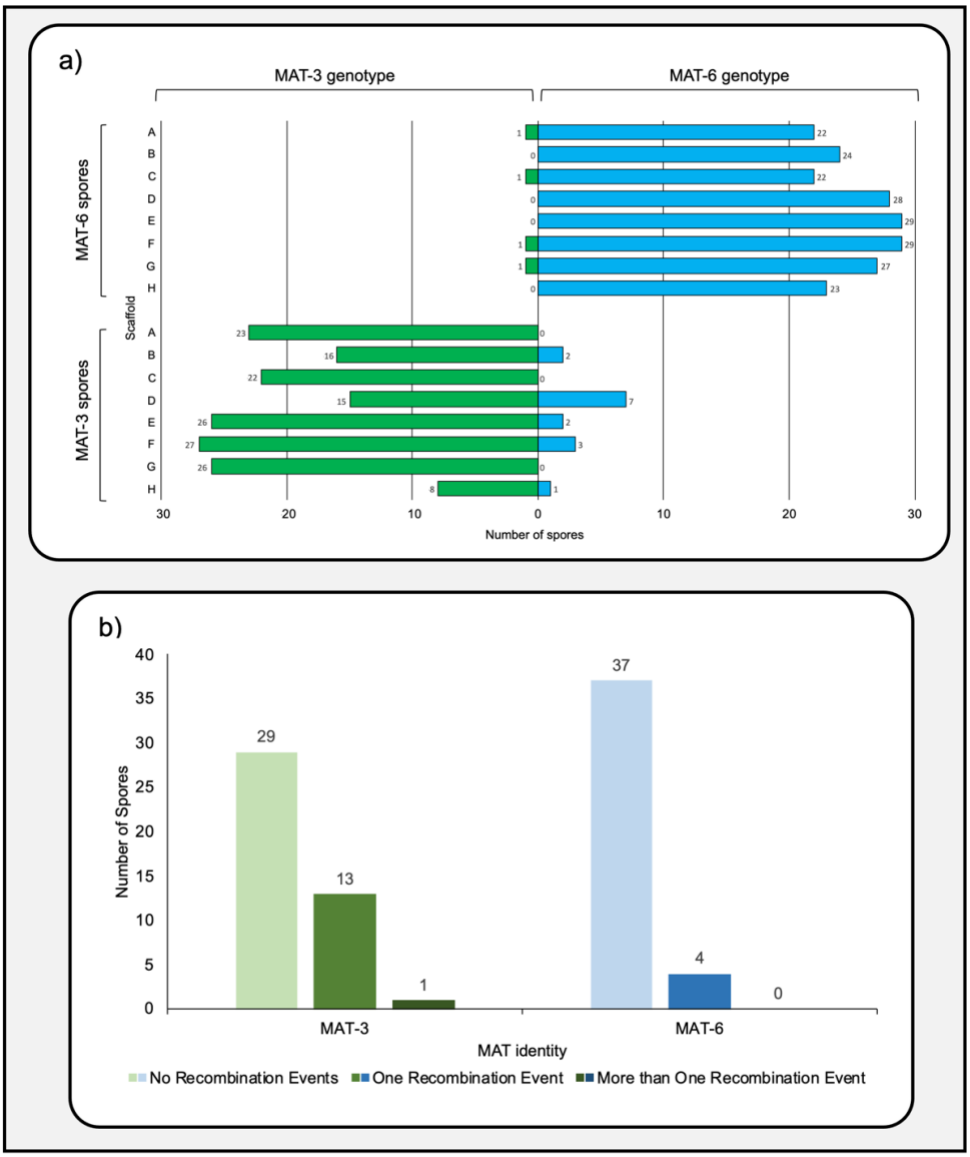

Figure 3.3. Genotype of single copy genomic regions in MAT-3 and MAT-6 homokaryotic spores indicate putative-recombination. a) The different scaffolds are indicated by the letters A-H (full details can be found in **Table S3.1**). Green bars indicate the presence of the MAT-3 genotype while blue bars indicate the presence of the MAT-6 genotype. b) The number of spores of each MAT (3 or 6) with no recombination events (light green/blue), one recombination event (medium green/blue) or more than one recombination event (dark green/blue). The analysis was conducted on a total of 84 spores.

Discussion

Overall, this study provides evidence that plasmogamy between homokaryotic AMF isolates can produce spores with two parental genotypes. These findings support the model first proposed by Ropars *et al.* (2016) that AMF undergo two tightly linked life stages – a clonal homokaryotic stage that can sometimes lead to a sexual or parasexual stage where two parental genotypes co-exist as a result of hyphal compatibility, as seen in sexual basidiomycetes. Furthermore, the rare cases we observe of spores with new allele combinations are consistent with the notion that plasmogamy and the production of dikaryons can generat recombinant nuclear types during strain interactions, thus supporting recent evidence of past genomic rearrangements between the A1 and C2 isolates (Chen et al. 2018b).

A surprising finding from the anastomosis assay is that perfect fusions between the MAT-3 and the MAT-6 isolates are not highly consistent. Specifically, although perfect fusions were found between A1 and C2 in our anastomosis assays, perfect fusions between isolates C2 and B3 were not observed, despite these carrying the same putative MAT-locus variants – i.e., the B3 isolate carries the MAT-3 variant, as does isolate A1 (**Figure 3.1**). An earlier study based on ROCs (Croll et al. 2009) did demonstrate perfect fusions between these isolates, so these contrasting results suggests that perfect fusions may be a plastic response that depends on the conditions in which isolates are grown, or that genomic regions beyond the MAT-locus play a role in AMF hyphal compatibility. This compatibility determinant may take the form of a somatic/vegetative incompatibility locus or a pheromone encoding locus, amongst others, but remains to be identified. The increase in post-fusion incompatibility – i.e., formation of septa after anastomosis – between pairings of different isolates may suggest a weakened selection on pheromone receptors, which direct the initial stages of mate recognition and cell fusion

(Raudaskoski and Kothe 2010). Such effects have been observed in the basidiomycetes *Schizophyllum commune* and *Coprinopsis cinerea* (Fraser and Heitman 2003; Ni et al. 2011), and thus might also be at play in AMF.

The overall low number of spores carrying two parental genotypes mirrors recent reports of rare AMF dikaryons in ROCs collections (Kokkoris et al. 2021a). Within this context, it is possible that the stable ROCs and the MSR medium used in our study do not reflect the environmental variability that triggers a need to increase gene shuffling within natural terrestrial ecosystems. Future studies should now aim to determine if the levels of inter-strain nuclear exchange increase in more stressful growth conditions, or in-planta, or if co-culturing other putatively compatible strains with different hosts produce different genetic outcomes for AMF crosses. Future analyses should also test if other genotype combinations – e.g., MAT-1/2 or MAT-1/5 – trigger higher rates of plasmogamy, nuclear exchange and ultimately recombination between the co-existing genotypes.

Whether the inter-strain recombination events we observed in some homokaryotic spores are the result of meiotic or parasexual events (or both; Yildirir et al. 2020) is still unclear at this time. However, since most spores exhibited only a single recombination event, and the overall frequency and genomic location of recombination that we identified varied among the spores analysed, it is very likely that these events occurred somatically and independently across the mycelium, as opposed to having risen through a single meiotic event. Somatic mutations are common in fungal dikaryons (Parag 1962; Frankel and Ellingboe 1977; Deng et al. 1985; Xu et al. 1996; Clark and Anderson 2004; Anderson and Kohn 2007), and these results support the idea that they also occur between parental nuclei in AMF.

In conclusion, this study provides strong support for the model proposed by Ropars *et al.* (2016). What now needs to be better understood is whether the production of dikaryotic and recombined homokaryotic progeny is mainly driven by their putative mating types, or whether other important factors also regulate these events. For example, the strains A1 and C2 possess significant genomic differences, including different genome sizes (Ropars et al. 2016; Chen et al. 2018b), and it is thus plausible that more genetically similar strains may generate higher recombination rates due to their overall higher sequence homology – i.e. the latter may also produce more viable ROCs generated using single spores.

The recombination identified in this study also supports the Ropars *et al.* (2016) model, and is consistent with past reports of inter-strain recombination in AMF (Chen et al. 2018b, a). However, although cross contamination cannot explain the findings of our recombination analyses, we argue that conclusive evidence of inter-strain recombination would still require the production of ROCs from recombined single spores generated from AMF crosses. The inability to produce viable ROCs from individual spores carrying two parental genotypes (and recombined homokaryotic spores) is disappointing, although in our view this result is likely due to stochastic events. Specifically, with only 2.9 % of all spores carrying two genotypes, and only 1.5 % of all spores producing viable ROCs, the likelihood of obtaining a ROCs of a novel AMF dikaryon is infinitesimal and may require the isolation of several thousands of individual spores from AMF crossing experiments. Though tedious, such work will be needed to reveal the genome-level changes that occur following nuclear exchange between compatible isolates, and uncover the molecular mechanisms involved in these changes. Ultimately, the generation of genetically novel AMF strains could pave the road for the production of inoculum optimized for different plant species or environments.

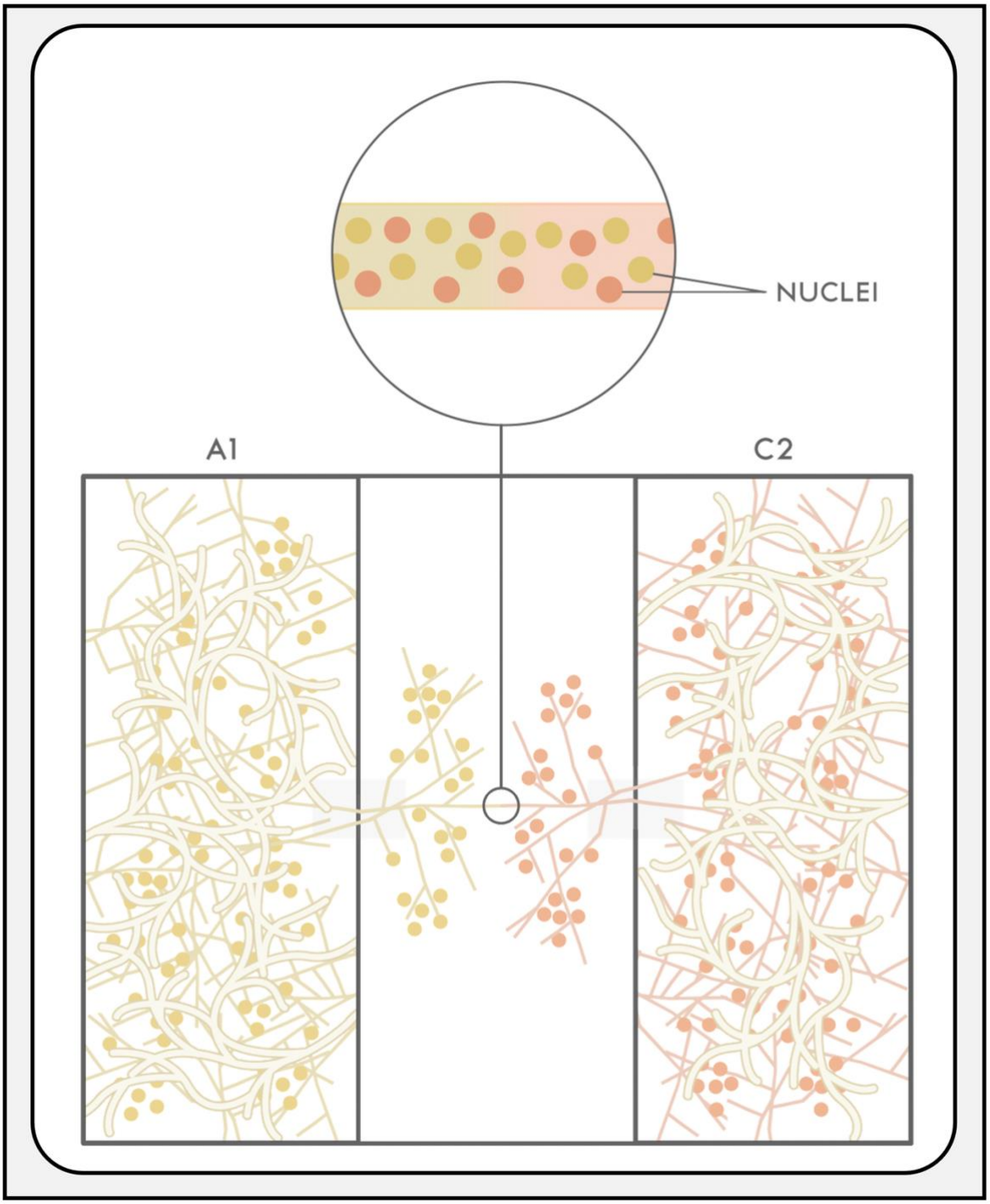

Figure S3.1. Depiction of the *in vitro* set-up in a three compartment Petri dish. Isolates A1 and C2 in the outer chambers and their hyphae allowed to grow together in the middle chamber. At the site of plasmogamy (shown in the circle) nuclei of both isolates will flow between the fused hyphae. Image generated by and used with the permission of Matthew Villeneuve-Laroche.

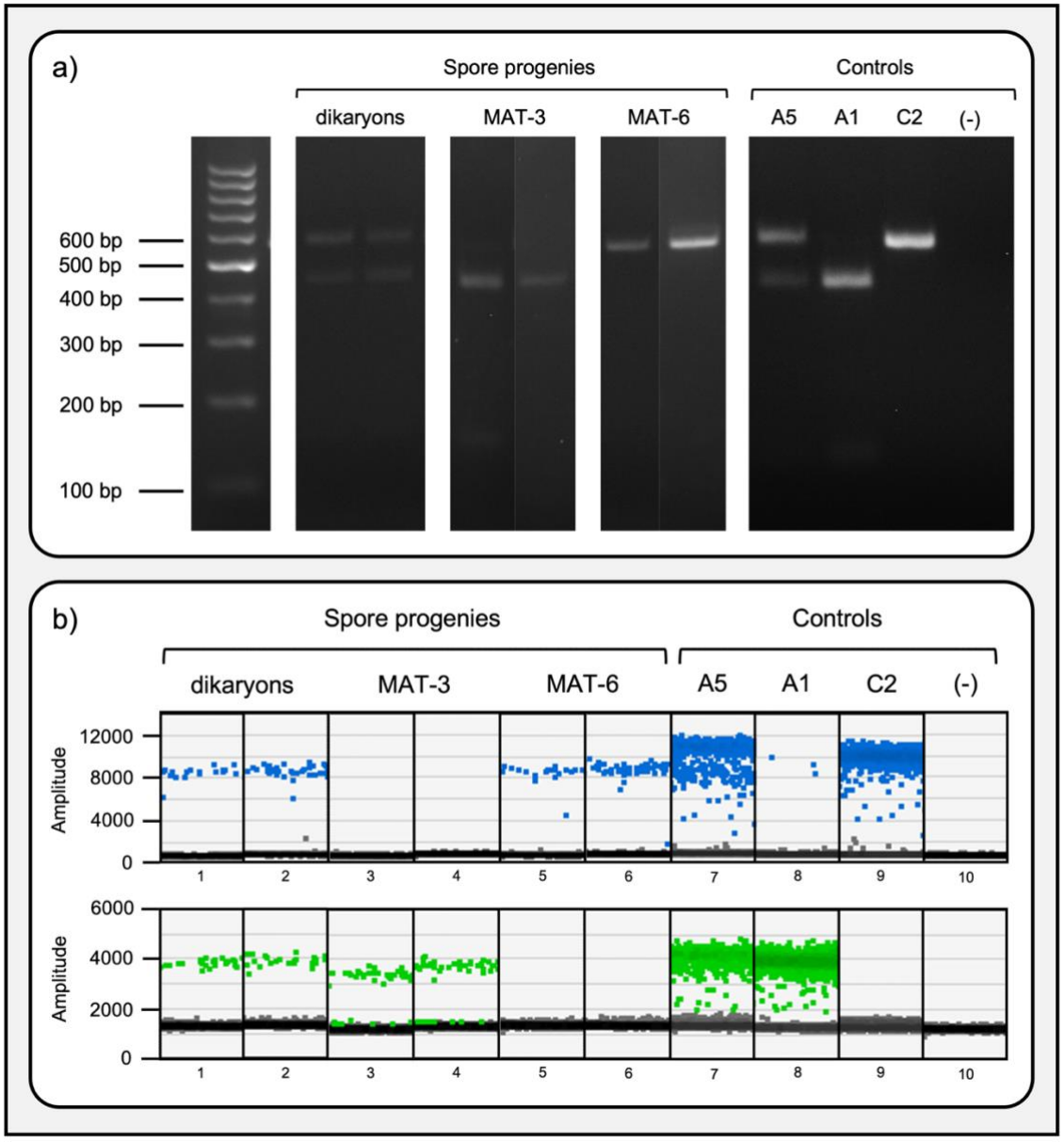

Figure S3.2. Example karyosis analysis of spore progenies from crossings. a) Example of RFLP analysis with dikaryotic and homokaryotic MAT-3 and MAT-6 spore progenies. b) Example of ddPCR analysis with dikaryotic and homokaryotic MAT-3 and MAT-6 spore progenies. Numbers along the bottom of the graph represent different sample numbers. Blue dots represent positive droplets of the MAT-6 variant, green dots represent positive droplets of the MAT-3 variant, grey dots represent droplets containing no template, which are below the threshold, and no droplets represents a negative reading.

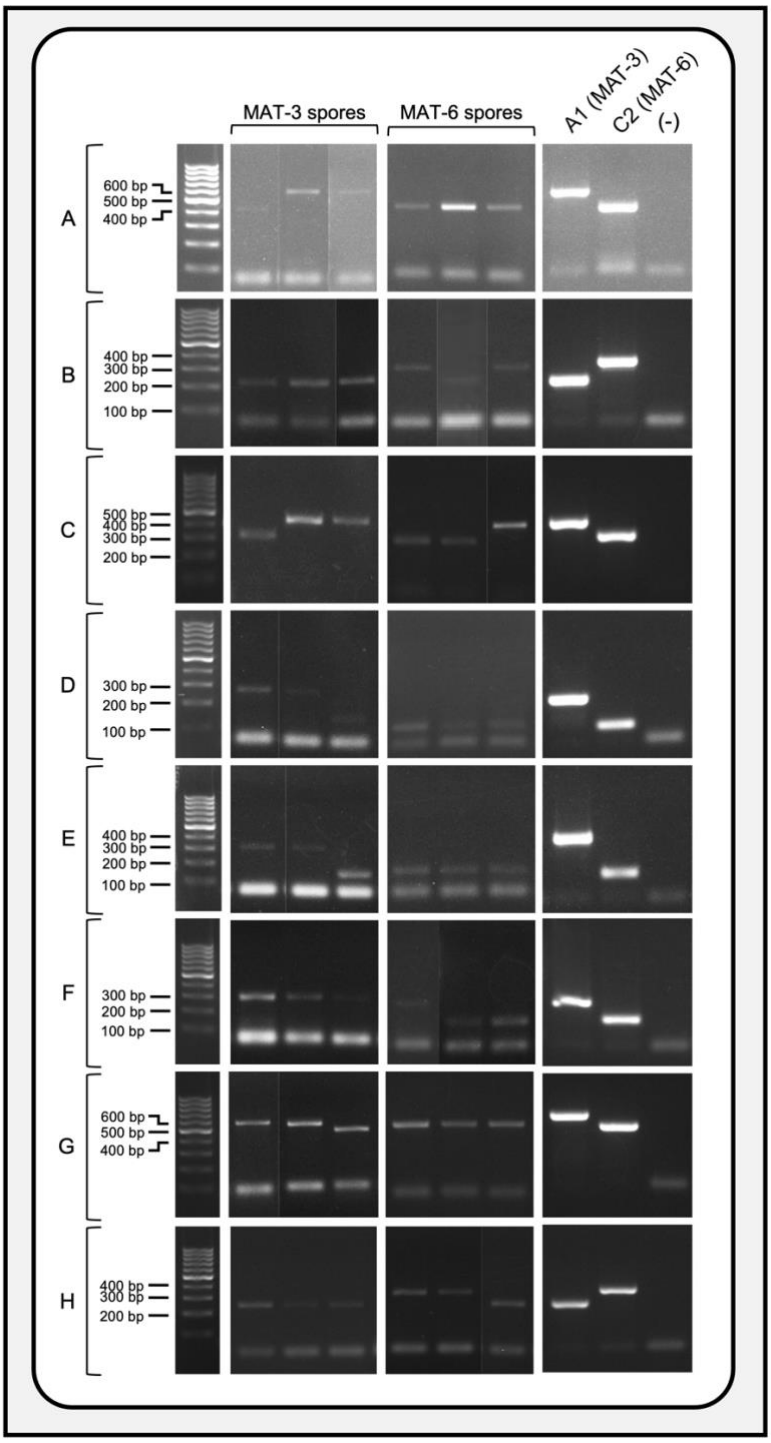

Figure S3.3. Example of homokaryotic progeny spores analyzed for recombination at the eight single copy regions. MAT-3 and MAT-6 refer to the karyotype of the progeny spore, while A1 and C2 represent positive controls of the parental isolates. Detailed information about regions A-H, including expected band sizes can be found in **Table S3.1**.

Table S3.1. PCR primers designed based on single copy homologous regions between A1 and C2 genomes. Genome information (scaffold and accession number) is provided for each genome in addition to the amplicon start and end position within the scaffold and the length of the amplicon that is expected in gel electrophoresis.

	Primer Pairs	
	Forward Primer	**Reverse Primer**
A	5'–CAGAAAAAATGCTTTCGTGATTGG–3'	5'–CACTATCGTGACAATCTACTGGG–3'
B	5'–GTGATTTTAGAATTGGTTGCACG–3'	5'–GGTGTAGCTTCAAACTTGGC–3'
C	5'–GGTCTGACATATATCGATAACATCG–3'	5'–GCTTTTGCTACAAAACTTAAATTCC–3'
D	5'–GAAAAATAATTTGGCTTTTTTCGTACGC–3'	5'–CACGACCACGTGATTGGC–3'
E	5'–CAAGGAGGTGTGGAACTAATTCC–3'	5'–CGTCTGGGAAGTTCGAAGC–3'
F	5'–GCTTCGTCAGTTTTAGAAATATAACTAAGG–3'	5'–GATCAATAAATTTTGCTACGGCG–3'
G	5'–GATTCTATATTCAAAATTTCACAGTTCTAGCC–3'	5'–GGTAATTGTTACATTATTTAGACAAAAATAGTGG–3'
H	5'–CTAAGGAAATTTTAGTGCTTTTAATGTCC–3'	5'–GGTTCGCTATTCTTAACAGAAGAAATG–3'

	A1 genome				
	Scaffold number	**Accession number**	**Amplicon start position (bp)**	**Amplicon end position (bp)**	**Amplicon length (bp)**
A	41	LLXH01000041	117831	118415	585
B	97	LLXH01000096	48555	48760	206
C	251	LLXH01000249	66140	66548	409
D	48	LLXH01000048	120150	120373	224
E	121	LLXH01000120	84843	85160	318
F	102	LLXH01000101	31390	31656	267
G	342	LLXH01000339	50398	50982	585
H	101	LLXH01000100	13397	13652	256

	C2 genome				
	Scaffold number	**Accession number**	**Amplicon start position (bp)**	**Amplicon end position (bp)**	**Amplicon length (bp)**
A	5	LLXL01000005	101399	101818	420
B	6	LLXL01000006	197352	197667	316
C	21	LLXL01000021	69820	70122	303
D	25	LLXL01000025	139567	139667	101
E	43	LLXL01000043	85622	85759	138
F	55	LLXL01000055	14319	14491	173
G	102	LLXL01000101	163104	163563	460
H	104	LLXL01000103	50945	51294	350

Table S3.2. *In vivo* interactions between different isolate pairings of *R. irregularis* in symbiosis with chicory. Isolates selected were all of MAT-3 (A1, B3) or MAT-6 (C2, 4401) based on the previously identified dikaryon (A5) of this combination. MAT type represents the identity of the MAT-locus variant, pairing represents the identity of the isolates that were matched together in the assay, n represents the number of interactions counted, the different interaction types are presented as percentages out of 100. ± symbol represents standard error.

MAT type	Pairing	n	Average contacts per replicate	Perfect fusions	Pre-fusion incompatibility	Post-fusion incompatibility	No interaction
3|3	A1|A1	203	25.4 ± 3.5	45.8 ± 1.5	0.5 ± 0.5	1.4 ± 0.9	52.3 ± 2.4
3|3	B3|B3	293	36.6 ± 2.7	48.1 ± 2.0	0.0 ± 0.0	0.0 ± 0.0	51.9 ± 2.0
6|6	4401*|4401	505	63.1 ± 4.8	60.4 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	39.6 ± 1.3
6|6	C2|C2	171	21.4 ± 2.5	54.6 ± 2.0	1.1 ± 0.7	0.7 ± 0.7	43.6 ± 2.1
3|3	A1|B3	418	52.3 ± 6.0	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.6	98.5 ± 0.6
6|6	4401|C2	475	59.4 ± 6.1	0.0 ± 0.0	2.8 ± 1.0	38.2 ± 1.4	59.0 ± 1.0
3|6	A1|C2	265	33.1 ± 2.4	5.1 ± 1.9	1.0 ± 0.5	15.3 ± 2.3	78.6 ± 3.1
3|6	B3|C2	224	28.0 ± 2.6	0.0 ± 0.0	13.6 ± 1.8	4.2 ± 2.0	82.2 ± 3.0
3|6	A1|4401	561	70.1 ± 3.3	0.0 ± 0.0	0.0 ± 0.0	26.9 ± 0.8	73.1 ± 0.8
3|6	B3|4401	326	40.8 ± 2.1	0.0 ± 0.0	3.1 ± 0.6	0.0 ± 0.0	96.9 ± 0.6

* also known as DAOM240446

Conclusion

Identifying reproductive strategies provides insight into how diverse traits emerge within populations of organisms, influencing intra- and inter- specific interactions within ecological niches. In particular, when mechanisms of reproduction are unclear, testing hypotheses becomes challenging as it is impossible to fully understand how organisms maintain genetic diversity to adapt to changing environments. Overall, this lack of knowledge results in less accurate inferences about the basic biology of a given species, and this is one of the main dilemmas faced by scientists studying arbuscular mycorrhizal fungi (AMF).

For several decades, the existence of a continuous multinucleate cytoplasm in AMF, coupled with the absence of observable sexual structures, resulted in hypotheses of a biologically unique reproductive lifecycle for AMF. It was hypothesized that the presence of heterokaryosis, that is, the existence of highly diverging co-existing nuclei, has allowed these fungi to evolve without sex for over a hundred million years. (Sanders 1999; Kuhn et al. 2001; Hijri and Sanders 2005). We now know that the "AMF heterokaryosis hypothesis" did not accurately represent the reproductive life cycle of AMF, thus it is not surprising that studies measuring the effects of AMF inoculum on plants based on this hypothesis have produced conflicting results and left gaps in our understanding of AMF biology and ecology.

Research suggests that AMF could be a vital component of more sustainable agricultural practices as well as conservation efforts. However key aspects of their biology need to be addressed first, particularly their reproductive life cycle (der Heijden et al. 1998; Berruti et al. 2016; Basu et al. 2018; Chen et al. 2018c). My thesis has tackled this, enriching our understanding of the fundamental biological principles in AMF, including genetic incompatibility, inter-strain nuclear exchange, and recombination.

Summary of main findings

I first performed a literature review that brings together several recent studies reporting the presence of high intraspecific diversity and phenotypic plasticity in AMF. In that chapter, I review several findings showing that the phenotype of conspecific isolates can vary significantly depending on the plant host, resulting in downstream functional effects for plant growth. Furthermore, I discuss how the genome of model AMF *R. irregularis* is highly variable and it has been suggested to constitute a pangenome, where the total genes available to a species can exceed those encoded by one individual. This work highlights the idea that mechanisms underlying plastic effects and pangenomes in AMF may be partially facilitated by sexual reproduction.

To evaluate the link between this diversity and the recently discovered putative MAT-locus in the genome of *R. irregularis*, in chapter 2 I developed an RFLP based protocol to identify the genotype and genetic organization of *R. irregularis* isolates. This protocol used a variable region within the HD2 domain of the MAT-locus to effectively distinguish different MAT-locus variants and determine sample karyosis. The technique was applied to both whole mycelium DNA and single spores and verified independently, making it suitable to analyze genetic crosses.

In chapter 3 the RFLP method was used to identify compatibility among *R. irregularis* isolates known to carry potentially compatible genomes, based on MAT-locus variants found in AMF dikaryons. The results of this study revealed for the first time that a dikaryotic spore progeny can indeed emerge by co-culturing genetically distinct homokaryotic lineages. Furthermore, preliminary evidence of recombination in homokaryotic progeny spores suggests that AMF may follow a more typical meiotic-driven or parasexual, reproductive life cycle than previously hypothesized.

Future Directions

Overall, this thesis contributes markedly to our understanding of reproduction in AMF but also generates many exciting new questions. One such question arises from the anastomosis results in my third chapter in respect to current knowledge of fungal MAT-loci, particularly those of Basidiomycota (**Figure 3.1**). As previously discussed, basidiomycetes have a tetrapolar mating system, in which one locus encodes homeodomains that regulate the sexual cycle, while the second locus encodes pheromone proteins and pheromone receptors (Casselton and Olesnicky 1998). The anastomosis results shown in chapter 3 evaluated the interactions between pairs of isolates with different MAT variants. The isolates A1 (MAT-3) and C2 (MAT-6) were found to anastomose, while other similar MAT-locus combinations between MAT-3 and MAT-6 isolates did not produce evidence of anastomosis. Although this result could be due to chance – i.e. some hyphal fusions may have been detected with more replicates – it also suggests that compatibility between isolates may be dictated by genomic regions that go beyond the MAT-locus. A detailed survey of genomic regions carrying divergent, distinct nucleotype-specific alleles in AMF dikaryons may reveal the existence of other genomic locations that play a role in mating and could include pheromone receptor genes, similar to the second MAT-locus of Basidiomycetes or even *het* loci if some of these interactions were the result of vegetative hyphal fusions (Saupe 2000). Future studies that consider additional diverging regions may thus elucidate the role of sexual versus parasexual interactions in AMF.

Another possibility is that the genetic background of the isolates may play an important role in determining partner compatibility. Specifically, isolates that are more genetically similar may be more likely to anastomose, while it can be expected that even if isolates contain a compatible MAT-locus, that at a certain genetic distance from each other they will still be unable

to interact (Bruns et al. 2017). Currently, markers used to phylogenetically distinguish *R. irregularis* isolates with different MAT-locus variants are mainly limited to the H+ATPase gene. Nevertheless, this single copy gene has been shown to reliably distinguish isolates, producing phylogenetic trees that mirror phylogenies based on large genome data sets (Ropars et al. 2016; Chen et al. 2018b; Kokkoris et al. 2021a). Using this gene and the MAT-locus as markers, we could not find evidence that higher genetic similarity plays a key role in anastomosis and nuclear exchange. Specifically, the isolates A1 and B3 are both equally distant phylogenetically from C2 yet show very distinct rates of anastomosis with C2. Furthermore, other isolates used for the anastomosis assay also did not show significant evidence that anastomosis rates are driven by genetic similarities. However, as discussed in chapter 3, it is likely that these interactions have a plastic response directly related to their growth environment, which could alter the interaction rates between isolates. To test this hypothesis, future experiments should be designed to tease apart the contributions of growth environment and genetic background versus MAT-locus identity, as the main drivers of mate recognition, anastomosis, and nuclear exchange in AMF.

The ability of compatible homokaryotic isolates to share nuclei, as demonstrated in this thesis, also raises the interesting question of whether pairings between compatible homokaryotic and dikaryotic isolates may also be able to share nuclei. This dikaryon-monokaryon (di-mon) mating is known as the Buller phenomenon, in which the dikaryon isolate can act as a nucleus donor, effectively giving one type of nucleus to the homokaryon isolate and retaining the other, leading to new combinations of parental nuclei (Buller 1930; Quintanilha 1937). In these types of interactions, it is often observed that one nuclear type performs better as a donor while other nuclear types are better able to thrive as monokaryons (Nieuwenhuis et al. 2013). Though this process has been observed in Basidiomycete fungi such as *S. commune* (Ellingboe and Raper

1962), it would be interesting to see if a similar process exists when AMF mycelia with thousands of co-existing nuclei encounter and anastomose.

More importantly, however, the next major goal in studying dikaryon formation in AMF must be successfully generating *in vitro* cultures. To demonstrate the viability of these newly created dikaryons and putative recombinants as well as study both their genetics and functional effects, we must first be able to culture them. In my studies, despite collecting 2000 spores, only five of the collected spores produced viable cultures, none of which contained two parental genotypes. In future efforts, culture conditions of isolated spores should be adjusted to optimize germination and improve symbiosis initiation. Previous studies have shown that placing *in vitro* cultures containing isolated spores in CO_2 incubators and in the presence of certain root exudates or flavonoids has increased the *in vitro* germination rate of other AMF species, such as *Gigaspora margarita* and *Glomus macrocarpum* (Siqueira et al. 1982; Bécard and Piché 1989; Gianinazzi-Pearson et al. 1989; Tsai and Phillips 1991). Adopting some of these conditions may incur some beneficial effects on the *in vitro* culturing of *R. irregularis* dikaryon isolates.

Lastly, as much as *in vitro* studies allow us to address questions in sterile and controlled conditions, one of the primary objectives of AMF research is to apply our findings to natural environments. Research using whole plant systems in soil would better represent these conditions and could allow for more direct inferences from the results obtained. As a result, it would be important to repeat the compatibility experiments in these mesocosms to verify the conclusions obtained in this thesis.

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