Alexandra Sofia Rodrigues Cabrita

Licenciada em Biologia Celular e Molecular

Molecular Mechanisms of Sexual Reproduction in the Order Cystofilobasidiales

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientadora: Professora Doutora Paula Gonçalves,
Universidade Nova de Lisboa
Co-orientador: Doutor Marco Coelho, Universidade Nova
de Lisboa

Júri:

Presidente: Professora Doutora Margarida Casal Ribeiro Castro Caldas Braga

Arguente: Doutora Maria Teresa Proença Mendes Maia

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Novembro, 2018

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Acknowledgments

First, I would like to thank my supervisor, Professor Paula Gonçalves, for having hosted me in the Yeast Genomics lab and for all the help and support throughout this year.

I would also like to thank all the members from the Yeast Genomics lab, for making me feel welcome from the very beginning, for all the discussions and suggestions throughout this year, and for the always uplifting spirit.

A special thanks is in order to the former members of the Yeast Genomics lab, Márcia David-Palma and Marco Coelho, for all the help and guidance during my initial months in the lab, and for all the materials they left me and that were used in this work. I've learned so much with you all, thank you.

I would also like to express my gratitude to Nicole Soares, our lab technician.

Also acknowledged is PYCC (Portuguese Yeast Culture Collection) for providing some of the yeast strains used throughout this work.

Some of the *Cystofilobasidium capitatum* and *Cystofilobasidium ferigula* sequences used in this work, namely the *HD* locus and rDNA locus of *C. capitatum* strain CBS 7420 and the *HD* locus of *Cystofilobasidium ferigula* strains PYCC 5628 and PYCC 4410, were obtained in collaboration with Professor Joseph Heitman's research group.

Of course, I could never forget those who have supported me in the backstage.

My friends and family were, doubtlessly, essential during this year, and I will be forever grateful to those who have spent countless hours listening through those waves of insecurity and those of excitement.

A very special thanks to my parents and my dearest grandmother, for supporting me and believing in me always.

To Pedro Pais as well, words can't express how grateful I am, for everything.

I couldn't have accomplished this without you all.

Thank you.

Resumo

Em fungos, *mating-type* é definido pelos *loci MAT.* Espécies heterotálicas só se reproduzem sexuadamente após cruzamento com uma estirpe de *mating-type* distinto, possuindo diferentes alelos nos *loci MAT*. Pelo contrário, espécies homotálicas podem reproduzir-se sexuadamente sem necessidade de cruzamento com um indivíduo compatível. No filo Basidiomycota, os *loci MAT* codificam feromonas, recetores de feromonas e fatores de transcrição *homeodomain* HD1 e HD2, que normalmente atuam como heterodímeros HD1/HD2, sendo todos estes geralmente essenciais à reprodução sexuada.

Este estudo teve como objetivo elucidar quanto aos sistemas de reprodução sexuada dentro da ordem Cystofilobasidiales, uma linhagem do filo Basidiomycota com uma proporção de espécies homotálicas superior ao comum neste filo. Assim, duas vertentes foram abordadas neste trabalho.

Primeiramente, os genes pertencentes aos *loci MAT* de espécies do género *Cystofilobasidium* foram analisados filogeneticamente, revelando novos dados, em particular, relacionados com as espécies *Cystofilobasidium ferigula* e *Cystofilobasidium macerans*. Resultados relativos a *C. macerans* sugerem que esta espécie se encontra aparentemente num estado de transição entre heterotalismo e homotalismo.

Numa segunda vertente, a interação entre as proteínas HD1 e HD2, normalmente indispensável na reprodução sexuada de espécies heterotálicas, foi estudada em duas espécies homotálicas: *Phaffia rhodozyma*, como continuação do estudo iniciado por David-Palma *et al*, 2016; e *Cystofilobasidium capitatum*, por comparação com uma espécie heterotálica do mesmo género, *C. ferigula.* Para *C. capitatum*, a expressão heteróloga do seu *locus HD* num mutante de deleção do *locus* equivalente de *P. rhodozyma* conseguiu reestabelecer o fenótipo de esporulação, indicando que estas proteínas são funcionais e detêm um papel no comportamento homotálico. No entanto, resultados de um ensaio *Yeast Two-Hybrid* não revelaram uma forte heterodimerização destas proteínas, antes uma clara homodimerização de HD2, deixando em aberto futuras investigações em relação às funções moleculares destas proteínas em *C. capitatum*.

Termos-chave: Cystofilobasidiales, *loci MAT*, Homotalismo, *Homeodomain*, Reprodução Sexuada.

Abstract

In fungi, mating-type is defined by the *MAT* loci. Heterothallic fungi can only reproduce sexually through mating with a second strain of distinct mating-type, which carry different alleles at the *MAT* loci. On the contrary, homothallic fungi complete their sexual life cycle without the need for a compatible partner. In Basidiomycota, *MAT* loci encode pheromones, pheromone receptors and the homeodomain transcription factors HD1 and HD2, which normally function as HD1/HD2 heterodimers and are all usually required for sexual reproduction.

This study aimed to shed some light on the sexual reproduction systems within the order Cystofilobasidiales, a lineage in the Basidiomycota with an unusually high proportion of homothallic species. To this end, two lines of study were conducted.

Firstly, the genes contained in the *MAT* loci from species of the *Cystofilobasidium* genus were examined from a phylogenetic standpoint, revealing novel findings pertaining in particular to the species *Cystofilobasidium ferigula* and *Cystofilobasidium macerans*. The findings concerning the latter species suggest that it may be in transition between heterothallism and homothallism.

Secondly, the interaction between HD1 and HD2 proteins, normally indispensable for sexual reproduction in heterothallic species, was studied in two homothallic species: *Phaffia rhodozyma*, continuing the study initiated by David-Palma *et al*, 2016; and *Cystofilobasidium capitatum*, in comparison to a heterothallic species of the same genus, *C. ferigula*. For *C. capitatum*, the heterologous expression of its *HD* locus in a *P. rhodozyma* cognate deletion mutant was able to restore sporulation, indicating that these proteins are functional and play a role in homothallism. However, the results from the Yeast Two-Hybrid assay with *C. capitatum* proteins did not reveal a strong interaction between HD1 and HD2 in this species, but rather a clear homodimerization of HD2, leaving plenty of room for further investigation of the molecular functions of HD proteins in *C. capitatum*.

Keywords: Cystofilobasidiales, *MAT* loci, Homothallism, Homeodomain, Sexual Reproduction.

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Abbreviations

bp	base pair
CBS	Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands
cDNA	Complementary deoxyribonucleic acid
CDS	Coding DNA sequence
cfu	Colony forming unit
CRUB	Centro Regional Universitario Bariloche, Argentina
DNA	Deoxyribonucleic acid
HD	Homeodomain
ITS	Internal transcribed spacer
MAT	Mating-type
mRNA	Messenger ribonucleic acid
ms	millisecond
NCBI	National Centre for Biotechnology Information
OD	Optical density
PCR	Polymerase chain reaction
PYCC	Portuguese Yeast Culture Collection, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal.
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid

1. Introduction

1.1. General Concepts of Sexual Reproduction in Fungi

Mechanisms of sexual development are spread across all eukaryotic lineages, crucially affecting the evolution of species through the contribution to the genetic diversity within a population, eventually acting on the adaptation to given environments and contributing to the long-term maintenance of living organisms (Lee *et al*, 2010; Stanton and Hull, 2007).

Within the Fungal kingdom, most species can undergo asexual and sexual reproduction (Coelho *et al*, 2017), where for sexual reproduction three crucial steps are common for all species: cell fusion of two compatible partners, karyogamy (or nuclear fusion) and finally meiosis, culminating in the development of haploid progeny (Lee *et al*, 2010; Lin and Heitman, 2007). Despite these key common steps, sexual development in fungi is extremely diverse, both morphologically and molecularly (Lee *et al*, 2010; Heitman *et al*, 2007).

Concerning sexual reproduction within fungi, information regarding cell type identity is encoded within a specific region of the genome, designated *MAT* locus, encoding for sex-specific proteins that regulate sexual development through a self/non-self recognition system and allow the definition of distinct mating-types (Lee *et al*, 2010; Stanton and Hull, 2007).

Typically, for sexual reproduction to occur in fungal species, mating must take place between haploid cells belonging to strains of different mating-types (i.e. encompassing different alleles at the *MAT* locus). Such sexual behaviour is defined as heterothallism, where a single strain is self-sterile and requires a second genetically distinct strain to complete its sexual cycle (Lin and Heitman, 2007).

However, some fungi have the ability to undergo sexual reproduction without mating with a second strain that is genetically different at the *MAT* locus, and this behaviour is designated homothallism, where strains are considered self-fertile (Lin and Heitman, 2007; Wilson *et al*, 2015).

As a complex sexual behaviour, and of great relevance to the present work, homothallism can occur through very distinct strategies and molecular mechanisms that allow a single strain to undergo its sexual cycle independently (Wilson *et al*, 2015; Lin and Heitman, 2007).

Some of these strategies responsible for the homothallic sexual behaviour of some species have been described, and will be assessed here in general terms.

One of these strategies is defined as primary homothallism (Figure 1.1), where a single strain possesses, within its genome, all the necessary *MAT* genes for sexual reproduction to take place. All these *MAT* genes would, in a heterothallic species, compose two distinct *MAT* loci in two strains of different mating-types. Therefore, with the presence of a compatible set of *MAT* genes within its genome, primary homothallic species can undergo sexual reproduction without a second strain (Wilson *et al*, 2015; Lin and Heitman, 2007).

Other mechanisms responsible for homothallism include, for instance, pseudohomothallism (Figure 1.1), where each spore possesses two different nuclei, each containing distinct *MAT* loci of different compatible mating-types, and therefore a single individual can start the process of sexual reproduction independently (Wilson *et al*, 2015).

Mating-type switching presents another mechanism responsible for a homothallic sexual behaviour (in this case defined as secondary homothallism), that has been extensively studied. In this strategy, the mating-type of a certain individual can be switched (reversibly or irreversibly) to a different mating-type (Wilson *et al*, 2015; Lin and Heitman, 2007). First described in *Saccharomyces cerevisiae*, this type of mating behaviour allows the emergence of two different mating-types in a single isolated colony, where sexual reproduction can occur (Figure 1.1) (Wilson *et al*, 2015; Lin and Heitman, 2007).

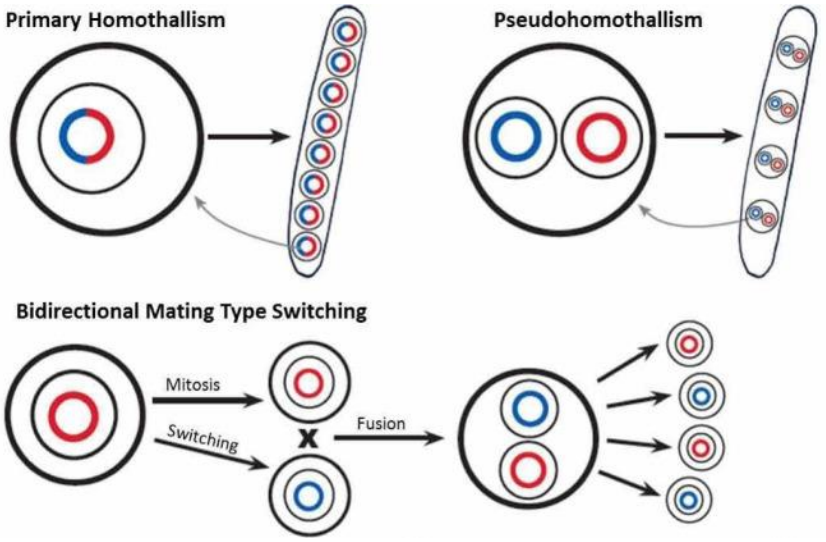


Figure 1.1: Schematic representation of some of the described molecular mechanisms of homothallism in fungi. Coloured circles represent haploid genomes. Figure adapted from Wilson *et al*, 2015.

Certainly, other molecular mechanisms such as bypasses or constitutively active pathways can be underlying the homothallic behaviour present in some fungal species that have yet to be studied in more detail.

In the following subsections, the basis underlying sexual reproduction in the phylum Basidiomycota will be analysed in more detail, where key concepts such as heterothallism and homothallism, as well as the definition of mating-types, will be essential for a clearer exposure of such matters.

1.2. Sexual Reproduction in Basidiomycetes

1.2.1. General Concepts

The phylum Basidiomycota contains a great diversity of fungi of ecological and economical relevance, like mushrooms, plant parasites and human pathogens, as is the case for the species of the *Cryptococcus* genus (Fraser *et al*, 2007). Together with the Ascomycota, these two phyla constitute the Dikarya sub-kingdom, with the Basidiomycota being composed of three sub-phyla, namely

Ustilaginomycotina, Pucciniomycotina and Agaricomycotina, the latter comprising the mushroom-forming species and a large diversity of yeast species (Kües *et al*, 2011; Grigoriev *et al*, 2014).

Concerning sexual reproduction, a distinctly common characteristic in basidiomycetous fungi is the development of very particular structures during mating, with the development of a basidium where the spores - in this case named basidiospores - mature externally (Fraser *et al*, 2007; Jones and Richards, 2011).

Many fungi belonging to the Basidiomycota have a haploid yeast state, resulting from the germination of the basidiospores (Boekhout *et al*, 2011). Hence, from a general point of view, the life cycle of basidiomycetes starts after the fusion of two haploid cells, leading to the dikaryotic state with the development of a structure named dikaryon where the two haploid nuclei coexist before fusion (Kües *et al*, 2011; Boekhout *et al*, 2011; Fraser *et al*, 2007). The dikaryon then grows in hyphal form, culminating in the development of a structure named basidium. It is in the basidium that karyogamy occurs, with the fusion of the two haploid nuclei. Later, and still within the basidium, meiosis takes place, leading to the formation of haploid basidiospores and completing the life cycle of these fungi (Kües *et al*, 2011; Fraser *et al*, 2007; Coelho *et al*, 2017), as depicted in Figure 1.2.

These structures will suffer slight variations, depending on the species in question and on the type of sexual behaviour that it presents. For instance, fungi that alternate from a haploid yeast state to a dikaryotic hyphal state are known as dimorphic (Boekhout *et al*, 2011). There are however species where their haploid state occurs in the form of homokaryotic hyphae (Coelho *et al*, 2017; Meinhardt *et al*, 1990). In order to simplify the discussed matters ahead, and given the relevance for this work, all references to the haploid state of the basidiomycetous sexual cycle will be henceforth referred to as the haploid yeast (or haploid cell) state.

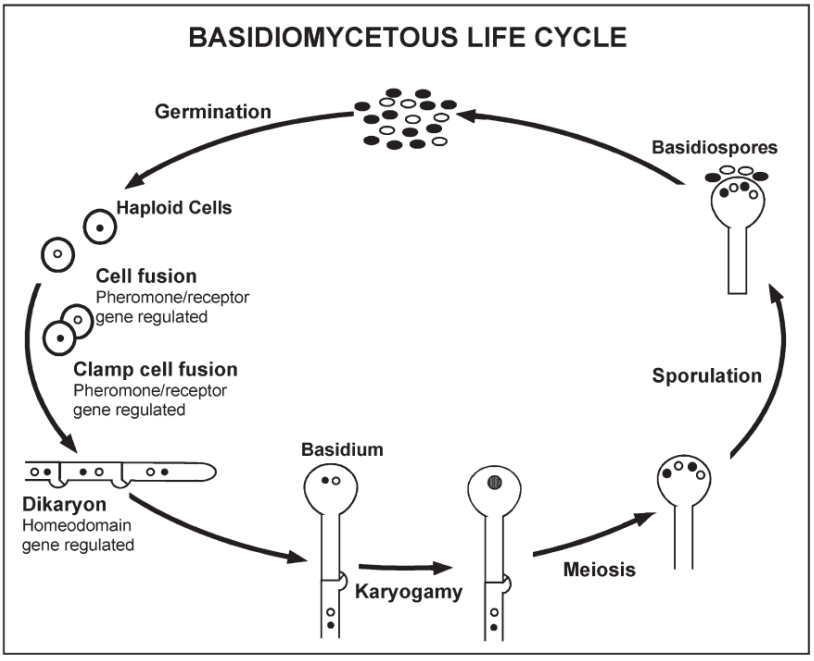


Figure 1.2: General representation of the life cycle of fungi belonging to the phylum Basidiomycota. The main stages of the life cycle represented in this figure correspond to those generally occurring in heterothallic basidiomycete species. Figure adapted from Fraser *et al*, 2007.

Independently of the structural variations, when it comes to heterothallic behaviour (where strains are self-sterile), sexual reproduction can only occur after fusion between two haploid cells belonging to strains of different mating-types. In species comprising this sexual behaviour (around 90% of all basidiomycete species (Kües et al, 2011)), the fusion of the two haploid cells is usually regulated through the recognition between pheromones and pheromone receptors, leading to the formation of dikaryotic hyphae, usually with clamp connections, that allow the two different haploid nuclei to be correctly distributed during duplication of the nuclei and elongation of the hyphae (Coelho *et al*, 2017; Meinhardt *et al*, 1990). Regulating morphological differentiation following fusion is the heterodimerization between the HD1 and HD2 homeodomain transcription factors from both haploid progenitors, through the formation of a transcription regulation complex to regulate the transcription of essential genes in this stage of the sexual cycle (Raudaskoski and Kothe, 2010; Kües *et al*, 2011; Coelho *et al*, 2017). The roles of pheromones and pheromone receptors and of the homeodomain transcription factors in the self/non-self recognition system of heterothallic basidiomycetes, as well as the *MAT* loci where the genes encoding these mating-type specific proteins are present, will be addressed in more detail in the following sub-sections.

1.2.2. First Level of Recognition – Pheromones and Pheromone Receptors

In heterothallic basidiomycetes, the molecular pathways concerning sexual reproduction that are initiated through the interaction between pheromone and pheromone receptor and between the homeodomain transcription factors make up a self/non-self recognition system, comprising several steps (Fraser *et al*, 2007).

Concerning the interaction between the pheromone and the pheromone receptor, this constitutes the first recognition point between the two strains during sexual reproduction. These proteins must be compatible for two strains to successfully mate, and for that to happen each strain must possess distinct alleles encoding pheromones (*MFA*) and pheromone receptors (*STE3*) (Fraser *et al*, 2007).

The *MFA* gene encodes a pheromone precursor, a small peptide (40 to 100 amino acids) that suffers post-transcriptional modifications at both termini of the peptide, like farnesylation at the C-terminal region. This process will lead to the formation of an active pheromone, a 9 to 14 amino acid diffusible lipopeptide with hydrophobic properties (Fraser *et al*, 2007; Kües *et al*, 2011; Coelho *et al*, 2017; Raudaskoski and Kothe, 2010). This maturation process will have slight variations, depending on the pheromone (Fraser *et al*, 2007).

Concerning the pheromone receptor, this protein possesses seven transmembrane domains and is coupled to a G-protein (Kües *et al*, 2011; Raudaskoski and Kothe, 2010). After the pheromone receptor recognizes and interacts with its respective compatible pheromone (through the N-terminal region of the receptor), this interaction will induce conformational alterations to the C-terminal region of the receptor. The C-terminal region will then interact with the heterotrimeric G-protein in the plasma membrane, leading to its activation (Kües *et al*, 2011). The activated subunits of the G-protein will then be able to trigger a signalling cascade (mediated by a protein kinase A and a mitogen-activated protein kinase) that will lead to the regulation of the fusion between the two haploid cells and dikaryotization (Kües *et*

al, 2011; Coelho *et al*, 2017; Raudaskoski and Kothe, 2010). The activation of this signalling cascade will also lead, together with the homeodomain transcription factors, to the transcriptional regulation of specific genes that are crucial in the subsequent steps of sexual reproduction (Fraser *et al*, 2007; Kües *et al*, 2011).

1.2.3. Second Level of Recognition – Homeodomain Transcription Factors

A second check point in sexual reproduction concerns the compatibility between the homeodomain (HD) transcription factors. The *HD1* and *HD2* genes encode two proteins of different homeodomain classes, named HD1 and HD2 (Coelho *et al*, 2017). Usually, in basidiomycetes, the *HD1* and *HD2* genes possess a less conserved region at the 5' end, which corresponds to the N-terminal region of the HD protein which is usually involved in the heterodimerization between the HD1 and HD2 proteins (Fraser *et al*, 2007; Kües *et al*, 2011; Kämper *et al*, 1995; Hull *et al*, 2005). Opposingly, the homeodomain region (usually pertaining to the DNA binding domain) and the C-terminal region of these proteins are significantly more conserved between HD proteins of the same class (Kües *et al*, 2011).

Therefore, the N-terminal region of these proteins is a determinant domain in terms of mating-type specificity, establishing the interaction, or lack thereof, between the HD1 and HD2 proteins (Kües *et al*, 2011; Fraser *et al*, 2007). When heterodimerization occurs, the resulting HD1/HD2 heterodimer will act as a transcription factor that will activate a homeodomain regulated pathway, regulating the transcription of genes that are essential to the morphological developments in the sexual cycle, such as the development in the dikaryon stage and the formation of the basidium (Kües *et al*, 2011; Fraser *et al*, 2007; Coelho *et al*, 2017).

As with the pheromones and pheromone receptors, in a heterothallic behaviour, strains must differ at both *HD1* and *HD2* alleles so that heterodimerization is possible, as the HD1 and HD2 proteins encoded in the same strain are not compatible. These conclusions have been taken through experiments involving the assessment of heterodimerization between the HD proteins of *Ustilago maydis,* a heterothallic plant pathogen belonging to the phylum Basidiomycota, resorting to a technique denominated Yeast Two-Hybrid assay (Kämper *et al*, 1995). Briefly, this technique is based on the molecular interaction existent between the transcription activator Gal4 with specific promoter sequences (UAS, or "upstream activating sequence"), naturally occurring in the genome of yeasts, to activate the expression of genes involved in the metabolism of galactose in *S. cerevisiae* (Brückner *et al*, 2009). Considering that the two domains of Gal4 (the DNA binding domain and the activation domain) maintain their respective functions when physically separated (Brückner *et al*, 2009), fusion constructions can be performed involving the Gal4 domains and the proteins under study (in this case HD1 and HD2). In case an interaction between the proteins under study is present, it allows the reconstitution of the Gal4 transcription activator, which in turn activates the transcription of reporter genes that are being regulated by UAS promoter sequences (Figure 1.3). Depending on the reporter gene in question, its expression can be detected in appropriate medium through a colorimetric reaction (Figure 1.3), the quantification

of the activity of a certain enzyme, the acquisition of resistance to a certain antibiotic or the complementation of an auxotrophic marker (Brückner *et al*, 2009).

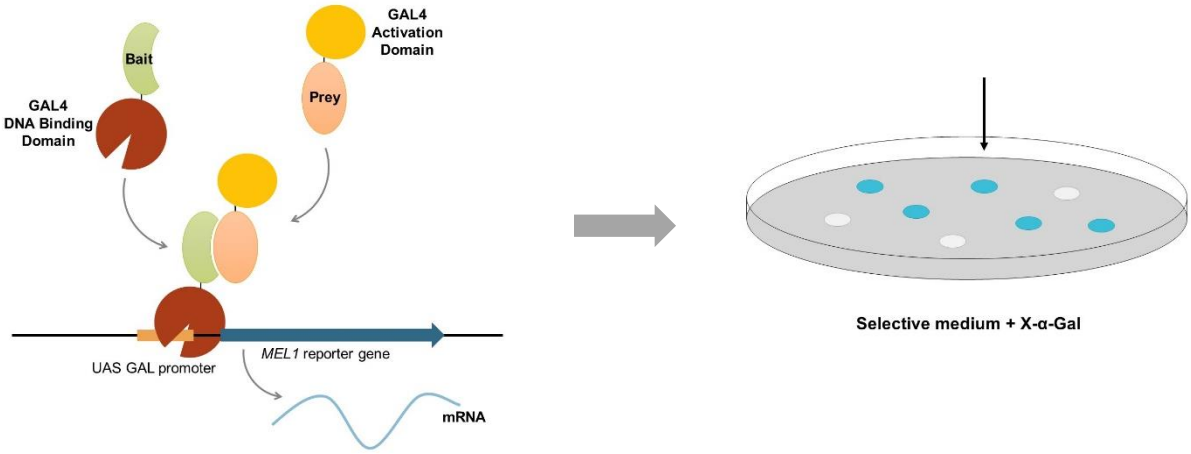


Figure 1.3: Molecular basis for the detection of interaction between two proteins (Bait and Prey) using the Yeast Two-Hybrid assay. The figure shows the example of a positive interaction between the proteins under study, with the activation of the *MEL1* reporter gene, encoding a α-galactosidase. In medium supplemented with X-α-Gal, the expression of *MEL1* can be detected through the growth of blue coloured colonies.

Through the Yeast Two-Hybrid assay, Kämper *et al*, 1995, were able to demonstrate that the bE and bW proteins of *U. maydis* (corresponding to HD1 and HD2, respectively) do heterodimerize, but only when these derive from two strains of different mating-types (possessing different alleles at the *HD* locus).

Therefore, the heterodimerization between HD1 and HD2 from two different strains establishes a second stage of self/non-self recognition in sexual reproduction for heterothallic basidiomycetes (Fraser *et al*, 2007; Raudaskoski and Kothe, 2010).

1.2.4. The *MAT* loci in Basidiomycetes: Bipolar and Tetrapolar Breeding Systems

The different mating-types found in basidiomycetes with a heterothallic behaviour are defined based on the allelic content of the *MAT* loci, which contain three distinct types of genes. One of these loci (*HD* locus) contains the two divergently transcribed genes that encode for the homeodomain transcription factors, *HD1* and *HD2*. The second *MAT* locus contains genes that encode for at least one pheromone precursor (*MFA*) and pheromone receptor (*STE3*), comprising the *P/R* locus (Fraser *et al*, 2007).

The content and organization of the *MAT* loci (*P/R* and *HD*) will be instrumental to establish the bipolar and tetrapolar breeding systems present in the phylum Basidiomycota. Related to the basidiospore formation upon meiosis, species with a bipolar system will give rise to basidiospores of two distinct mating-types, whilst species with a tetrapolar system will originate basidiospores of four distinct mating-types (Fraser *et al*, 2007; Coelho *et al*, 2017). Figure 1.4 shows a simplified schematic representation of the bipolar and tetrapolar systems governing the heterothallic sexual behaviour in basidiomycetes, concerning *MAT* loci organization and the generation of distinct mating-types upon meiosis. Indeed, in a tetrapolar system, two genetically unlinked *MAT* loci (*P/R* and *HD*) are at the basis of the generation

of four distinct mating-types upon meiosis. In a bipolar system, however, a single *MAT* locus is at the basis of the generation of only two distinct mating-types, and that could be due to a linkage between the *P/R* and *HD* loci, impeding the independent segregation of *P/R* and *HD* (as depicted in Figure 1.4), or due to a loss of function of one of the *MAT* loci (*P/R* or *HD*) in discriminating between different mating-types (Coelho *et al*, 2017). This loss of discriminating potential can be due to (i) mutations that allow self-recognition, i.e. interaction between the pheromone and pheromone receptor or between the HD1 and HD2 proteins encoded in the same haploid strain, or (ii) mutations that lead to constitutively active pathways involved in the sexual cycle and that are normally activated after a successful interaction between the mating-type specific proteins originated from strains of different mating-types (Coelho *et al*, 2017; Fraser *et al*, 2007).

While in a bipolar breeding system a single *MAT* locus is considered (either through linkage or loss of function of one of the original *MAT* loci), in a tetrapolar system two genetically unlinked *MAT* loci are present and, for sexual reproduction to occur and the sexual cycle to be successfully completed, two strains must differ in terms of gene content at both *P/R* and *HD* loci (Fraser *et al*, 2007; Coelho *et al*, 2017). Therefore, there is 25% chance of a haploid strain resulting from a tetrapolar progeny to successfully mate with a second strain from the same progeny (for example, following the nomenclature in Figure 1.4, the strain of mating-type Aa can only mate with the strain Bb, and the strain Ab can only

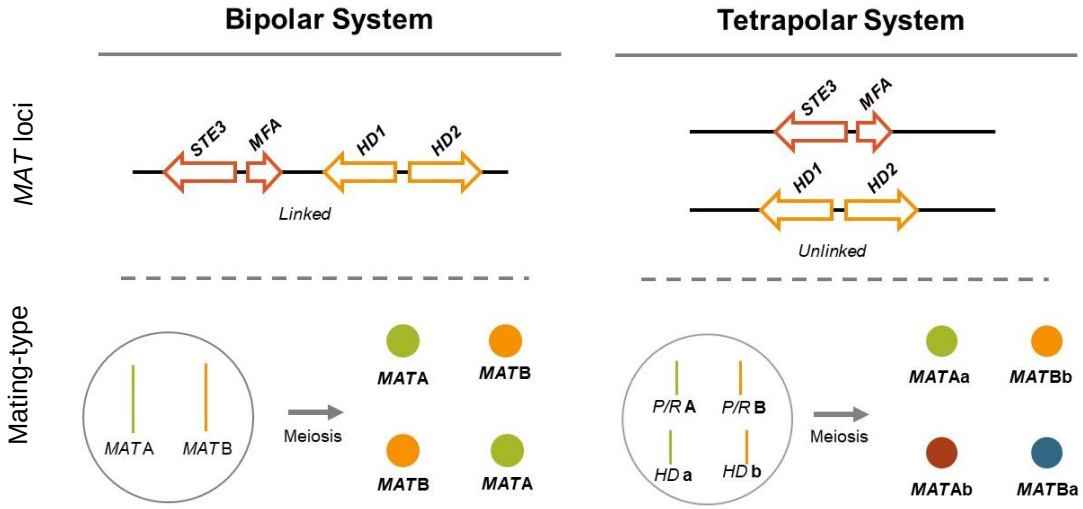


Figure 1.4: General representation of the bipolar and tetrapolar breeding systems governing the heterothallic sexual behaviour in the phylum Basidiomycota. For each breeding system, a simplified organization of the *MAT* loci in a single haploid genome is represented, with the consequent number of generated mating-types after meiosis.

mate with the strain Ba). Opposingly, strains resulting from a bipolar progeny have 50% chance to mate with strains from the same progeny (Fraser *et al*, 2007).

Although some species are biallelic at the *MAT* loci (leading to only two or four potential mating-types in a bipolar or tetrapolar systems, respectively), many species yield the potential to generate several distinct mating-types in a population. Multiallelism at the *MAT* loci occurs most notably in the mushroom species in the sub-phylum Agaricomycotina, like *Coprinopsis cinerea*, with at least 160 variants of the *A* locus (*HD* locus) (Coelho *et al*, 2017; Asante-Owusu *et al*, 1996).

While within the Ascomycota, heterothallic species are predominantly bipolar (Butler, 2007; Fraser *et al*, 2007), in the phylum Basidiomycota most species seem to be tetrapolar, with an approximate preponderance of 65% of species exhibiting this system in the Agaricomycotina sub-phylum (Coelho *et al*, 2017; Fraser *et al*, 2007; Kües *et al*, 2011). Indeed, and although the bipolar system still seems to be predominant in the other two sub-phyla of the Basidiomycota (results from classical mating studies), tetrapolarity is thought to be the ancestral form of breeding systems in the phylum Basidiomycota (Coelho *et al*, 2017). Supporting such hypothesis is the fact that the bipolar breeding system exists throughout the whole Basidiomycota phylum, scattered together with tetrapolar species (Coelho *et al*, 2017; James *et al*, 2013). Given the *MAT* genes present in bipolar species throughout all three sub-phyla of the Basidiomycota, their general genomic organization and the general complexity of the tetrapolar system, it is likely that the bipolar breeding system in basidiomycetes has arisen from a tetrapolar system (Coelho *et al*, 2017; Heitman *et al*, 2013; Kües *et al*, 2011), through mutations or recombination events that would lead to a linkage between the *P/R* and *HD* loci or loss of function of one of these loci (Coelho *et al*, 2017), as previously mentioned.

1.2.5. Homothallism in Basidiomycota

As was referred previously, in homothallic behaviour, sexual reproduction can occur and the life cycle of a fungal species can be completed by a single haploid strain, with no need for mating with a second strain, given their self-fertilization capability (Kües *et al*, 2011; Lin and Heitman, 2007).

Specifically concerning the phylum Basidiomycota, homothallism is quite uncommon, with very few homothallic species studied so far. Indeed, and although homothallism can be favourable at times by providing universal compatibility of strains (Coelho *et al*, 2017; Lin and Heitman, 2007), the complex reproductive system in this phylum, both molecularly and at the genome level (concerning the organization of the *MAT* loci), could be at the basis of such low preponderance of this sexual behaviour (Coelho *et al*, 2017). Such matters, however, remain to be clarified.

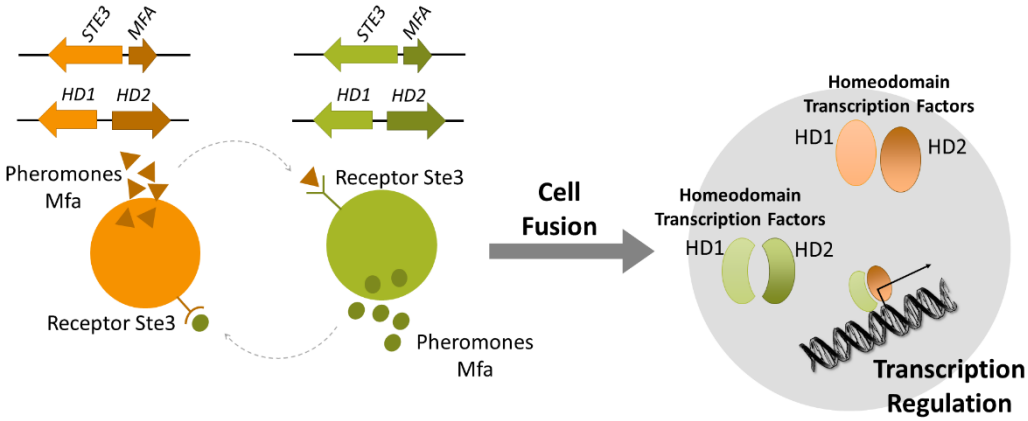
Trying to apply some of the mechanisms of homothallism described in Section 1.1 to the general reproductive mechanisms of the Basidiomycota, primary homothallism, for instance, would require the presence of a compatible pair of pheromone and pheromone receptor encoded at the *P/R* locus and a compatible pair of HD1 and HD2 homeodomain transcription factors encoded at the *HD* locus, to allow self-recognition and trigger sexual reproduction (Wilson *et al*, 2015). In Figure 1.5 is schematized a simplified comparison between the molecular interactions governing the initiation of sexual reproduction in heterothallic (Figure 1.5.A) and in primary homothallic basidiomycetes (Figure 1.5.B). Recently proposed to possess a mechanism of primary homothallism, and most relevant to the present work, is the basidiomycete species *Phaffia rhodozyma* (David-Palma *et al*, 2016), to be described in more detail in Section 1.3.1.

Still, a homothallic behaviour could potentially result from several different mechanisms in Basidiomycota. Besides some of the mechanisms described in Section 1.1, another form of homothallism defined as unisexual reproduction has been described for a particular mating-type of *Cryptococcus neoformans* (typically a heterothallic species with a bipolar breeding system). This strain

can complete its life cycle without mating with the strain of opposite mating-type in response to lack of nutrients (α-α mating), with no form of mating-type discrimination occurring at the *MAT* loci level (Lin *et al*, 2005; Wilson *et al*, 2015; Coelho *et al*, 2017). It is also possible that, for some basidiomycete species, homothallism can be achieved through constitutively active pathways, that would only be activated through the molecular interactions known to trigger sexual reproduction in heterothallic species of this phylum (i.e., the mitogen-activated protein kinase cascade triggered by the interaction between compatible pheromone and pheromone receptors, or the pathways initiated by the HD1/HD2 heterodimer to regulate gene expression). However, and as stated above, these mechanisms have yet to be studied for many homothallic basidiomycete species.

It is important to note that, when it comes to homothallic basidiomycetes, there is no definition of mating-types, since these strains can initiate sexual reproduction without need of a compatible partner. However, the *MAT* loci can be present all the same, (as described for primary homothallism) (Wilson *et al*, 2015), with the possibility of outcrossing.

A. Heterothallism in Basidiomycota



B. Primary Homothallism in Basidiomycota

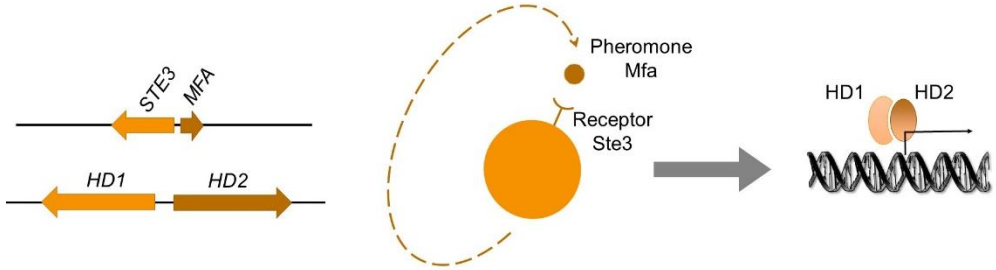


Figure 1.5: Main molecular interactions responsible for triggering sexual reproduction in basidiomycetes. A) Molecular interactions in control of the self/non-self recognition system in heterothallic sexual behaviour, with indication of the functions in sexual reproduction associated with each interaction. B) Potential molecular interactions responsible for primary homothallic sexual behaviour in basidiomycetes.

1.2.6. Transitions between Heterothallism and Homothallism

Throughout the fungal kingdom, transitions between heterothallic and homothallic sexual behaviours are not uncommon, with cases where both behaviours are present within the same genus not being entirely unusual (Lin and Heitman, 2007). However, the evolutionary history of sexual behaviour in fungi is still unclear, and there are studies supporting either the hypothesis of homothallism having evolved from a heterothallic ancestor or the hypothesis pertaining to the opposite course of evolution (Lin and Heitman, 2007).

Some evidences seem, however, to suggest that homothallism is an evolutionary dead end (Gioti *et al*, 2012), i.e. there would be an unidirectional transition from heterothallism to homothallism.

Concerning the phylum Basidiomycota, even considering the genetic make-up of the mating-type governing loci and the potential transitions from tetrapolar to bipolar breeding systems previously mentioned (Section 1.2.4), one could argue that after reaching bipolarity (through attainment of self-compatibility of one of the *MAT* loci) a single event that would lead to self-compatibility at the second *MAT* locus would be enough for a species to transition from a heterothallic to a homothallic sexual behaviour (depicted in Figure 1.6) (Fraser *et al*, 2007; Lin and Heitman, 2007). It is, however, true, that the reverse process would also be viable (Fraser *et al*, 2007).

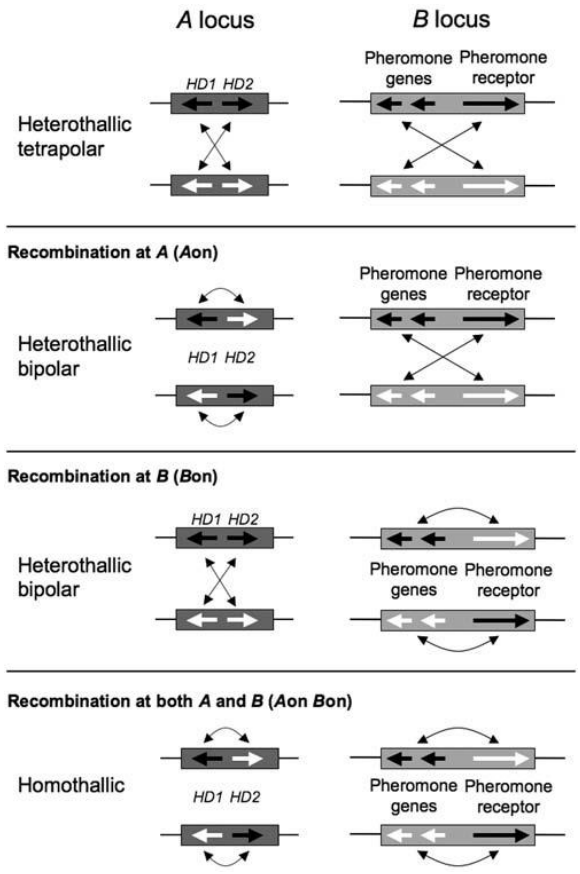


Figure 1.6: Mechanisms of transition between breeding systems and sexual behaviours in basidiomycetes. Representation of how recombination at the *MAT* loci can be at the basis for the transitions between tetrapolar and bipolar systems, and between heterothallism and homothallism. Figure adapted from Fraser *et al*, 2007.

Although a consensus regarding the evolutionary direction from which heterothallism and homothallism have arisen is yet to be reached, the hypothesis concerning a transition from heterothallism to homothallism seems to be supported by the fact that several heterothallic fungi can attain a homothallic behaviour through pseudohomothallism or unisexuality (Wilson *et al*, 2015; Lin *et al*, 2005; Lin and Heitman, 2007), and by studies involving the artificial conversion from a heterothallic to a homothallic sexual behaviour through expression of compatible genes of the original mating-types (Lin and Heitman, 2007; Hull *et al*, 2005). Also, the fact that some of the mechanisms underlying homothallism seem to point to the presence of all the necessary *MAT* genes to allow sexual reproduction without mating with a different strain (primary homothallism) seem to be in accordance with a

homothallic behaviour having evolved from a heterothallic ancestor, instead of several independent insurgences of heterothallism from a homothallic behaviour (Lin and Heitman, 2007).
Several more studies concerning the evolution of sexual behaviours and of the *MAT* loci certainly ought to be performed, especially involving lineages with a great variety of sexual behaviours, to better understand the evolutionary tendencies of such behaviours, not only in the phylum Basidiomycota but throughout the whole fungal kingdom.

1.3. The Order Cystofilobasidiales

Belonging to the Basidiomycota, the order Cystofilobasidiales comprises a lineage in the Tremellomycetes class (within Agaricomycotina), composed of seven genera of yeasts (Kurtzman *et al*, 2011; Liu *et al*, 2015). This order presents a greater incidence of species with a homothallic sexual behaviour, by comparison with other lineages in the Tremellomycetes class and even within the phylum Basidiomycota (Kurtzman *et al*, 2011).
Indeed, homothallism is spread across at least three of the seven genera composing the order Cystofilobasidiales (namely *Phaffia*, *Cystofilobasidium* and *Mrakia*), while, for some species and strains throughout the genera composing this order, a sexual state is yet to be identified (Kurtzman *et al*, 2011).
Given the preponderance and distribution of homothallism within this order, the Cystofilobasidiales constitutes quite an interesting lineage for the study of homothallism in basidiomycetes, not only in terms of the molecular mechanisms governing such behaviour in the considered species, but also from an evolutionary point of view.
Concerning this order, and of interest to the present work, recent findings regarding the molecular interactions governing the homothallic behaviour of *P. rhodozyma*, as well as the current understanding of the distribution of sexual behaviours and breeding systems in the *Cystofilobasidium* genus, will be approached in more detail in the following sub-sections.

1.3.1. *Phaffia rhodozyma*

P. rhodozyma is a homothallic species belonging to the Cystofilobasidiales order (Kurtzman *et al*, 2011), with great biotechnological and economic value due to its ability to produce astaxanthin, a carotenoid that has been widely used in the cosmetic industry, for its antioxidant properties, and in aquaculture feed supplements, for its pigmentation (Fell *et al*, 2011; Fell and Johnson, 2011; Schmidt *et al*, 2011; Johnson *et al*, 1980; Barredo *et al*, 2017). Given its biotechnological potential, *P. rhodozyma* has been extensively studied, leading to the development of methodologies that allow this species to be genetically transformed (Visser *et al*, 2005).
Concerning sexual reproduction, *P. rhodozyma* possesses a homothallic sexual behaviour, with the development of elongated slender basidia, with basidiospores formed terminally to the basidium (Figure 1.7), when incubated in a minimal polyol medium (Fell *et al*, 2011).

In a recent study from our research group (David-Palma *et al*, 2016), the molecular interactions governing the homothallic life cycle of *P. rhodozyma* have been uncovered, and the main findings of the study will be explained in this section.

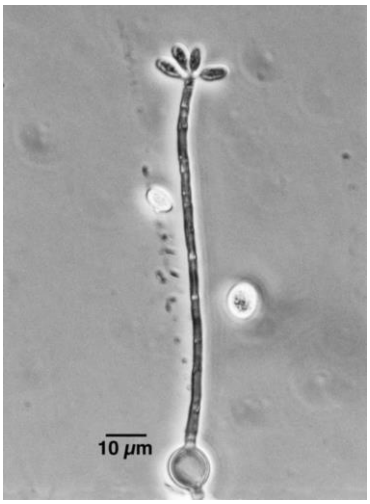


Figure 1.7: Depiction of an elongated basidium and terminal basidiospores of *P. rhodozyma* (*Xanthophyllomyces dendrorhous* CBS 9090). Sporulation in polyol medium, at 18ºC. Figure adapted from Fell *et al*, 2011 (Photograph by N. Pinel).

P. rhodozyma possesses two pairs of different pheromone and pheromone receptor encoding genes (*STE3-1/MFA1* and *STE3-2/MFA2*) in its *P/R* locus, and a single pair of divergently transcribed *HD1* and *HD2* genes in its *HD* locus (David-Palma *et al*, 2016). Through experiments involving the construction of deletion mutants of the *MAT* loci, David-Palma *et al*, 2016, demonstrated a reciprocal compatibility between the pheromones and pheromone receptors encoded in the two pheromone/receptor gene pairs (where Ste3-1 is compatible with Mfa2 and vice versa), demonstrating as well that a single pheromone/receptor compatible pair is necessary and sufficient for sexual reproduction to occur in this species.

Also, through the construction of deletion mutants, the authors demonstrated that both HD1 and HD2 are necessary for sexual reproduction (where a deletion mutant of *HD2* was still able to vestigially sporulate) (David-Palma *et al*, 2016). However, studies on the heterodimerization between HD1 and HD2 were not entirely consistent, with only a weak interaction being detected in cases when only the N-terminal region of one of the proteins was considered (typically the dimerization domain of these proteins in heterothallic basidiomycetes (Kämper *et al*, 1995; Hull *et al*, 2005)), leading to the conclusion that the HD1 and HD2 proteins of *P. rhodozyma* might interact weakly (David-Palma *et al*, 2016).

These findings taken together, suggested that primary homothallism is the behaviour governing sexual reproduction in *P. rhodozyma* (as depicted in Figure 1.8), potentially having derived from a heterothallic tetrapolar ancestor, given the genomic organization of the *MAT* loci in this species (David-Palma *et al*, 2016).

Although a weak interaction between the HD1 and HD2 proteins of *P. rhodozyma* has been proposed, no studies pertaining to the interaction between both complete proteins was performed by David-Palma *et al*, 2016. As information regarding the role and mode of action of the HD proteins in homothallic sexual behaviours within Basidiomycota is lacking, the possibility of the results obtained by our research group (David-Palma *et al*, 2016) to suffer variations when considering the complete HD1 and HD2 proteins should not be disregarded.

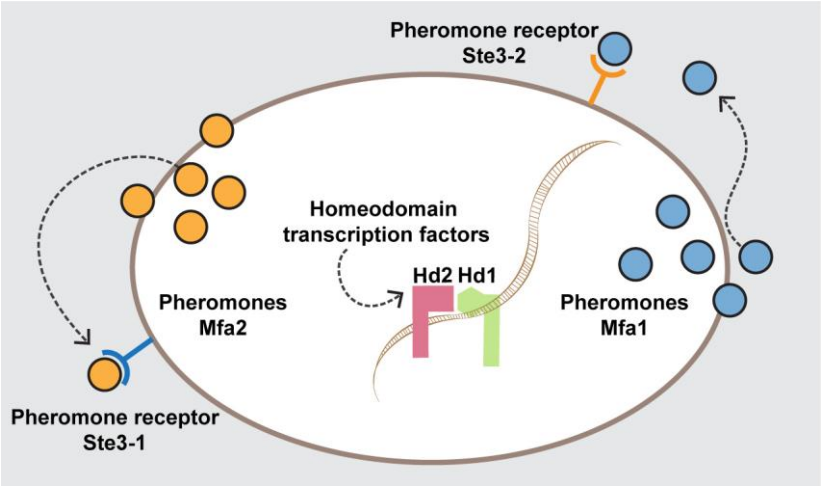


Figure 1.8: Proposed model for molecular interactions responsible for the homothallic sexual behaviour of *P. rhodozyma.* Depicted in the figure are the interactions between the two reciprocally compatible pairs of pheromone and pheromone receptors, and the potential weak interaction between the HD1 and HD2 homeodomain transcription factors. Figure adapted from David-Palma *et al*, 2016.

1.3.2. *Cystofilobasidium* genus

Within the *Cystofilobasidium* genus, a great diversity of sexual behaviours is readily observable, from heterothallic species, with tetrapolar and bipolar breeding systems, to exclusively homothallic species (Sampaio, 2011).

One exclusively homothallic species within this genus, and of utmost relevance to the present work, is *Cystofilobasidium capitatum*. Differently from what occurs in *P. rhodozyma*, during the sexual cycle of *C. capitatum* uninucleate hyphae are developed, with the formation of teliospores laterally or terminally on the hyphae (Sampaio, 2011). Although no information is available concerning the molecular mechanisms responsible for the homothallic sexual behaviour of this species, analysis of the genome of *C. capitatum* strain CBS 7420 revealed the presence of a single pheromone (*MFA*) and single pheromone receptor (*STE3*) genes, as well as one pair of divergently transcribed *HD1* and *HD2* genes (David-Palma, 2017). Therefore, and given how close this species is to *P. rhodozyma*, a hypothesis could be proposed for a mechanism of primary homothallism, where the pheromone receptor would be activated by the pheromone encoded in the genome of *C. capitatum*, and the HD1 and HD2 proteins encoded in the *HD* locus would heterodimerize and form an active transcription factor. Several studies concerning the role and mode of action of these proteins in the sexual cycle of *C. capitatum* should, certainly, be performed to help validate or reject such hypothesis (to be further addressed in the Results and Discussion Section of the present work).

Concerning the exclusively heterothallic species, an example is *Cystofilobasidium ferigula* where only two mating-types have been reported, leading to the consideration that this species has a bipolar breeding system (Sampaio, 2011). Hyphae containing clamp connections are developed, with the formation of teliospores (Figure 1.9.B). Also, as opposed to what occurs in *C. capitatum*, where basidia are elongated (Figure 1.9.A), in *C. ferigula* basidia are short and non-elongated (Sampaio, 2011). Other

exclusively heterothallic species in the genus *Cystofilobasidium* comprise *Cystofilobasidium bisporidii*, *Cystofilobasidium infirmominiatum* and *Cystofilobasidium lacus-mascardii* (Sampaio, 2011).

An interesting case within the *Cystofilobasidium* genus, concerning sexual reproduction, seems to be the species *Cystofilobasidium macerans*. This species comprises both heterothallic and homothallic strains, as well as strains for which the sexual state is yet to be defined (Sampaio, 2011). Regarding homothallic strains, hyphae are developed without clamp connections, with the formation of teliospores (Figure 1.9.C). Similarly to *C. capitatum*, basidia are elongated and slender (Sampaio, 2011). The analysis of the genomes of two heterothallic *C. macerans* strains of distinct mating-types (CBS 6532 and CBS 2425) revealed (besides the *P/R* locus) a single pair of *HD1* and *HD2* genes on each strain and, upon analysis of the polymorphic pattern of the HD1 and HD2 proteins, a distinct pattern of conservation was revealed compared to what was expected for heterothallic strains, where the homeodomain and C-termini of these proteins presented a degree of conservation much lower than expected (David-Palma, 2017).

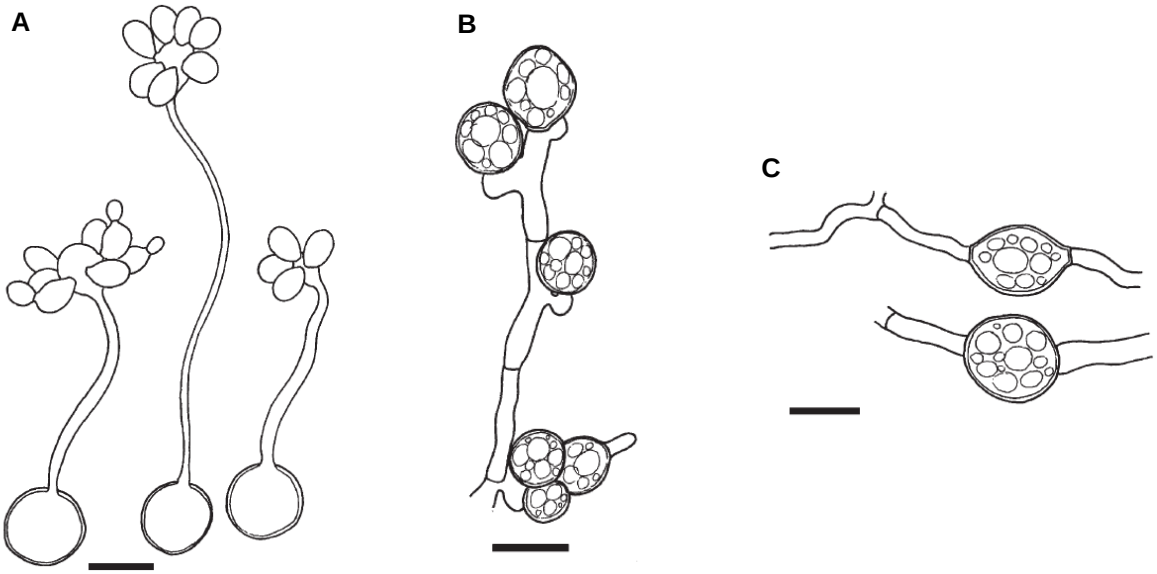


Figure 1.9: Development of sexual structures during the life cycle of species of the *Cystofilobasidium* genus. A) *C. capitatum* strains PYCC 5626 and A 301 with germinated teliospores containing basidia and basidiospores. B) *C. ferigula* showing clamped hypha with teliospores. C) *C. macerans* strain CBS 6984 showing hyphae (without clamp connections) with teliospores. Scale bars = 10 µm. Figure adapted from (Sampaio, 2011).

Indeed, the *Cystofilobasidium* genus comprises a quite diverse group of species regarding sexual reproduction, being of the utmost relevance for the study of transitions between sexual behaviours, most evidently when considering the complex sexual state of the *C. macerans* species.

1.4. Main Objectives of this Work

In the current study, the order Cystofilobasidiales was selected to help elucidate the molecular mechanisms and evolutionary tendencies of sexual reproduction, given the variability in sexual behaviours and the incidence of homothallism throughout this lineage. Considering these factors, two main lines of work were defined for this study.

Firstly, given the diversity of sexual behaviours in the *Cystofilobasidium* genus, the *MAT* loci of *Cystofilobasidium* species were analysed and studied, aiming at, on the one hand, trying to elucidate the current state of these loci in terms of allele content. On the other hand, a study of the variability of sexual behaviours and breeding systems would be imperative to shed light on the evolutionary transitions between heterothallism and homothallism that might have taken place within this genus, and eventually try to extrapolate and compare such results to the current hypothesis for these evolutionary transitions within the Basidiomycota.

Secondly, this work focused on understanding the molecular mechanisms governing the homothallic sexual behaviour of two species within the Cystofilobasidiales order, *P. rhodozyma* and *C. capitatum*, with a focus on the role of the HD1 and HD2 proteins in sexual reproduction.

For *P. rhodozyma*, this constitutes a direct continuation of the study initiated by our research group (David-Palma *et al*, 2016) this time considering both complete HD1 and HD2 proteins, to understand if the proposed weak interaction is still valid when considering the complete proteins or if any dimerization domains could be present in other locations of the proteins.

For *C. capitatum*, this study constitutes the first attempt at understanding the molecular mechanisms underlying the homothallic behaviour of this species. Still focussing on the HD1 and HD2 proteins, this study aimed at not only uncovering their functionality in sexual reproduction but also at the ability of these proteins to heterodimerize.

To detect the interactions between the HD proteins for both these species, the Yeast Two-Hybrid assay was performed, in line with the methodology used by David-Palma *et al*, 2016.

Hopefully the current study will help elucidate on the above-mentioned matters and incite thought and discussion on such a complex matter as is sexual reproduction in fungi, as well as help bring to attention the order Cystofilobasidiales and its variability in sexual behaviours as an incredibly important lineage for the study of the molecular mechanisms behind homothallism and of the behavioural transitions regarding sexual reproduction within the Basidiomycota.

2. Materials and Methods

2.1. Phylogenetic study of the *MAT* loci in the *Cystofilobasidium* genus

2.1.1. Species, strains and growth conditions

The considered *Cystofilobasidium* strains were maintained in YMA solid medium (Table 6.1, in Appendix) at 17ºC. A complete list of the *Cystofilobasidium* strains used can be found in Table 6.2, in Appendix.

2.1.2. Amplification of *MAT* genes, purification and sequencing

Genomic DNA was extracted through a Phenol-Chloroform precipitation method, with a phenol solution saturated with 10mM of Tris-HCl (pH 8.0) and 1mM of ethylenediaminetetraacetic acid (EDTA). A lysis buffer solution (100mM NaCl; 10mM Tris-HCl pH 8.0; 1mM EDTA; 2% (v/v) Triton X-100; 1% (v/v) sodium dodecyl sulphate (SDS)) was used for cell lysis, which was performed by mechanic agitation with glass beads at 1000 rpm (VXR basic Vibrax by IKA). The genomic DNA extracted was precipitated with ethanol (70%) and resuspended in a solution of 50 ng/µL of RNase in 1X TE, following incubation at 55ºC for 15 minutes.

PCR reactions were performed to amplify the *STE3* gene, a region comprising the 5' ends of the *HD1* and *HD2* genes as well as the intergenic region between these, and the ITS rDNA region. The primers used were designed based on the *MAT* gene sequences from the available genomes of the following strains: *C. capitatum* CBS 7420; *C. ferigula* PYCC 4410 and PYCC 5628; *C. macerans* CBS 2425 and CBS 6532 (David-Palma, 2017) (Table 6.2, in Appendix). Concerning the *STE3* gene, the primers were designed for the 5' and 3' ends of the gene, whilst for the *HD* region the primers were designed for the homeodomain regions of *HD1* and *HD2,* so that the amplified region included the 5' ends of both *HD1* and *HD2* and the intergenic region between these genes (henceforth, this region will be referred to as 5'-*HD1/HD2*). Concerning the rDNA region, the universal primers ITS5 and LR6 (Sampaio *et al*, 2001a; Libkind *et al*, 2010) were used. The complete list of primers used in this phylogenetic study can be found in Table 6.4, in Appendix**.** All PCR reactions were performed using DreamTaq thermosensitive DNA Polymerase (Thermo Scientific).

All PCR products were analysed by electrophoresis in 1% agarose gel (in 1X TAE and 0.5X GelRed (by Biotium)), using GeneRuler (Thermo Scientific) as a molecular marker. The PCR products were purified using *Illustra GFX PCR DNA and Gel Band Purification Kit* (GE Healthcare Life Sciences)*,* which were then sequenced by Sanger Sequencing, at STABVida (Portugal), using the same primers used to amplify the regions of interest, with the exception of the ITS region that was sequenced using universal primer ITS4 (Sampaio *et al*, 2001a).

2.1.3. Construction of phylogenies

All phylogenies were constructed using the software MEGA v7.0.26 (Kumar *et al*, 2016). The sequences obtained for each region were aligned using the software MUSCLE (implemented in MEGA7). For the ITS, 5'-*HD1/HD2* and *STE3* DNA sequences, as well as for the *HD1* partial gene sequences of *C. ferigula*, the evolutionary history was inferred using the Neighbour-Joining method and the evolutionary distances were computed using the Kimura 2-parameter method, in units of the number of base substitutions per site. For the of Ste3 and HD2 protein sequences, the evolutionary history was inferred using the Neighbour-Joining method and the evolutionary distances were computed using the Jones-Taylor-Thornton (JTT) matrix-based method, in units of the number of amino acid substitutions per site. In all analyses, the bootstrap test was applied with 1000 replicates.

2.2. Assessing the interaction between the complete HD1 and HD2 proteins of *P. rhodozyma*

2.2.1. Species, strains and growth conditions

Two *S. cerevisiae* strains were used for the Yeast Two-Hybrid assay, namely Y187 and Y2HGold (host strains from Matchmaker Gold Yeast Two-Hybrid System, by Clontech). The growth conditions used for the *S. cerevisiae* strains were as described in the Yeastmaker Yeast Transformation System 2 protocol (Clontech). The strains were grown in YPD liquid medium (Table 6.1, in Appendix) at 30ºC, 180 rpm (Gallenkamp Incubator, by B. Braun-Biotech), for the preparation of competent yeast cells and, after transformation, the cells went through a recovery step in YPD medium supplemented with 0.1 g/L adenine sulphate at 30ºC, 250 rpm, for 30 minutes (Thermoblock by Frilabo). YNB medium with amino acid supplements (Table 6.1, in Appendix) was used as selective medium for both the haploid and the diploid strains that were obtained, instead of the SD drop out medium.

2.2.2. Yeast Two-Hybrid assay to assess the interaction between HD1 and HD2 of *P. rhodozyma*

In order to assess the interaction between the HD1 and HD2 proteins of *P. rhodozyma*, Matchmaker Gold Yeast Two-Hybrid System kit (by Clontech) was used, where the complete synthetic *HD1* and *HD2* genes used by David-Palma *et al*, 2016 - corresponding to the CDS of the *HD* genes of *P. rhodozyma* – were separately cloned into a pGADT7 plasmid (Table 6.3, in Appendix).

For that, the *HD1* and *HD2* complete synthetic genes were first amplified using primers that contained 40 bp tails at their 5' ends that correspond to the flanking regions of the Multiple Cloning Site present in pGADT7 (Table 6.4, in Appendix). Plasmid pGADT7 was then linearized at the Multiple Cloning Site by digestion with Cla I (Thermo Scientific), using 20 units of enzyme. The preparation of competent *S. cerevisiae* strain Y187 and its transformation was performed as described in the Yeastmaker Yeast Transformation System 2 protocol (by Clontech) during which the constructions were assembled by homologous recombination (plasmid/insert proportion of (1:2)). For transformation, a 10mg/mL solution of single stranded DNA (by Sigma-Aldrich) was used instead of Yeastmaker Carrier DNA. After the transformation protocol, the cells were plated in appropriate selective media (Table 6.1, in Appendix) and incubated at 30ºC until the colonies were visible to the naked eye (4 to 5 days). Transformants were tested for the presence of the *HD* fragments of interest through *S. cerevisiae* colony PCR, using as

template for the reaction the suspension of a transformant colony in 0.02 M of NaOH, after heating the suspension for 10 minutes at 90ºC.

Mating was performed by incubating a single colony from each of the two haploid transformants to be mated in 200 µl of YPD medium at 30ºC, for 24 hours, at 250 rpm (Thermoblock by Frilabo). After incubation, the cells were recovered and thoroughly washed with distilled sterile water and plated in appropriate selective media (Table 6.1, in Appendix). The cells were incubated at 30ºC until the colonies were visible to the naked eye (4 to 5 days). DNA was extracted from the obtained diploid strains for PCR confirmation of the presence of the two relevant plasmids. DNA extraction was performed as described in Section 2.1.2.

To test the activation of *ADE2* and *HIS3* reporter genes, haploid transformants and diploids were plated in the appropriate selective medium without adenine nor histidine, respectively, whilst to test the activation of *MEL1* the haploid transformants and diploids were plated in selective medium supplemented with X-α-Gal (Takara Bio) at a final concentration of 40 µg/ml (Table 6.1, in Appendix).

2.2.3. Liquid culture β-galactosidase assay using ONPG as substrate

In order to quantify the activation of the *LacZ* reporter gene, a β-galactosidase activity assay was performed, using o-nitrophenyl β-D-galactopyranoside (ONPG) as the substrate. Briefly, strains were grown overnight in 5 ml of the appropriate selective media (Table 6.1, in Appendix), from which 2 ml were subsequently inoculated in 8 ml of YPD medium and incubated at 30ºC with shaking until mid-log phase. At this point 1.5 ml of each culture were collected, the cells were pelleted, and thoroughly washed in Z-buffer (16 g/L $Na_2HPO_4 \cdot 7H_2O$; 5.50 g/L $NaH_2PO_4 \cdot H_2O$; 0.75 g/L KCl; 0.246 g/L $MgSO_4 \cdot 7H_2O$; pH 7). The washed pellet was then resuspended in Z-buffer, so as to achieve a concentration factor of 5-fold. Of the resulted cell suspension, 0.1 ml were collected for cell lysis, through 3 cycles of frosting and thawing of the suspension in liquid nitrogen and in a 37ºC water bath, respectively. After lysis, 0.7 ml of a solution of Z-buffer with 0.27% (v/v) of β-mercaptoethanol was added to each suspension, followed by 160 µL of a solution of 4 mg/ml of ONPG in Z-buffer (pH 7). Each suspension was then incubated at 30ºC. All reactions were performed in triplicates, so that the reaction could be stopped at 3 different points in time (after 2 hours, 6 hours, and 23 hours and 30 minutes), by adding 0.4 mL of 1M Na_2CO_3 to each suspension. After centrifugation (10 min, at 10000 xg) the supernatants were collected, and the absorbance was measured at 420nm to calculate the β-galactosidase units, using the following formula:

$$\beta - \text{galactosidase units} = \frac{1000 \times A420nm}{t \times V \times OD600nm}$$

where: *t* is the time of incubation at 30ºC, in minutes, up to the point when the reaction was stopped; *V* is the product of the volume of initial cell suspension and the concentration factor; and OD_{600nm} is the OD at which the initial culture was collected.

2.3. Assessing the role of the HD1 and HD2 proteins of *C. capitatum*

2.3.1. Species, strains and growth conditions

P. rhodozyma CBS 6938 and *C. capitatum* CBS 7420 were grown in YPD medium (Table 6.1, in Appendix) at 20ºC for both the preparation of electrocompetent cells and the Minimum Inhibitory Concentration assays, the latter concerning only *C. capitatum* strain CBS 7420. Transformants were incubated at 17ºC in selective medium, consisting of YPD supplemented with the appropriate antifungal drugs (100 µg/ml of geneticin and/or 100 µg/ml of zeocin for *P. rhodozyma*).

For RNA extraction, *C. capitatum* strains CBS 7420, PYCC 5626 and A 301 were incubated in CMA (Difco), GSA and MYP solid media (Table 6.1, in Appendix), at 17ºC for up to 27 days, to assess their ability to sporulate in the mentioned media. All three strains were then inoculated in GSA liquid medium and incubated at 20ºC, 90 rpm (Sartorius Certomat IS incubator), for 8 days in 10% the volume of the flask.

Escherichia coli strain DH5α (Gibco-BRL, Carlsbad, CA, USA) was used as a cloning host and was grown at 37ºC in LB medium (Table 6.1, in Appendix) supplemented with ampicillin at 100 µg/ml when appropriate.

2.3.2. Construction of recombinant plasmids and gene deletion cassettes

For the transformations of *P. rhodozyma* by electroporation, the construction of recombinant plasmids and gene deletion cassettes was performed, where the latter was specifically used to generate deletion mutant *hd1hd2*Δ::Zeo (deletion of the *HD* locus). To design the primers relevant to *P. rhodozyma*, the genome of strain CBS 6938 was used (from NCBI project PRJEB6925 (Sharma *et al*, 2015)). The original plasmids used for the constructions of the recombinant plasmids and deletion cassettes were pJET1.2/blunt (Thermo Scientific) and pPR2TN (Verdoes *et al*, 1999), the latter containing a geneticin resistance cassette. The primers designed to amplify the fragments used in all cloning steps, namely the rDNA fragment of *C. capitatum* (Figure 6.1, in Appendix) (used for the assessment of the ability to genetically transform *C. capitatum*), the *HD1/HD2* fragment of *P. rhodozyma* and the zeocin resistance cassette (Figure 6.3, in Appendix) (used for the construction of the deletion mutant *hd1hd2*Δ::Zeo), and the *HD* locus of *C. capitatum* (Figure 6.2, in Appendix) (for the construction of the complementation plasmid, in the generation of mutant *hd1hd2*Δ::Zeo+*HD1HD2*::G418), contained the restriction sites required to allow for cloning in the relevant plasmids. The complete list of primers used in this work can be found in Table 6.4, in Appendix. All fragments used for cloning and deletion cassettes were amplified by PCR using Phusion High-Fidelity DNA polymerase (Thermo Scientific) and the amplified products were purified using either *Illustra GFX PCR DNA and Gel Band Purification Kit* (GE Healthcare) or *GeneJET Gel Extraction Kit* (Thermo Scientific).

The constructions were performed using standard molecular cloning methods (Sambrook *et al*, 2001) involving the digestion of plasmids and amplified fragments with restriction enzymes (all by Thermo Scientific), all according to manufacturer's instructions. Ligation of the digested fragments and plasmids was performed using T4 DNA ligase (Thermo Scientific) for the ligation reaction, using 2 units of enzyme and 50 ng of linearized plasmid in a reaction volume of 20 µl, to a proportion of plasmid/insert of (1:3),

or (1:5) whenever required. All ligation reactions were performed over-night, at 17ºC. Subsequent transformations of the resultant recombinant plasmids were performed in *E. coli* strain DH5α, through a heat shock transformation method (Ausubel *et al*, 1987). All recombinant plasmids were recovered from *E. coli* DH5α using the *NZYMiniprep kit* (NZYTech).
A complete list of all plasmids used and constructed in this work can be found in Table 6.3, in Appendix.

2.3.3. Transformation of *P. rhodozyma* by electroporation

P. rhodozyma strains were transformed with the linearized recombinant plasmids and deletion cassettes using the protocol described by Visser *et al*, 2005. Briefly, for the preparation of electrocompetent cells, the strains were grown in YPD liquid medium in 10% of the volume of the flask, at 20ºC and 180 rpm (Sartorius Certomat IS incubator), until the OD_{600nm} of the culture reached 1.2. The pelleted cells were incubated in a solution of 50mM potassium phosphate buffer (pH 7) and 25mM dithiothreitol (DTT) for 15 minutes at room temperature. The cells were then thoroughly washed in ice-cold STM buffer (270mM of sucrose, 10mM of Tris-HCl (pH 7.5), 1mM of $MgCl_2$) and then resuspended in 500 µL of STM buffer. Transformation of electrocompetent cells was performed using 60 µL of the suspension of electrocompetent cells and 5 to 10 µg of the DNA of interest concentrated in a volume of 5 to 10 µL. For transformations involving recombination at the rDNA locus, the amount of DNA used was reduced to 600 ng, and up to 2 µg when necessary. The electroporation conditions consisted of an internal resistance of 1000 Ω, an electric pulse of 0.8 kV, a capacitance of 25 µF, resulting in a pulse ranging from 18 to 20 ms (Rodriguez-Saíz *et al*, 2008; Wery *et al*, 1998). The electroporation apparatus used was Gene Pulser II Electroporation System, from Bio-Rad. The cells went subsequently through a recovery step in YPD liquid medium, for a minimum of 2.5 hours at 17ºC before being plated on YPD medium supplemented with the appropriate antifungal drug and incubated upside-down at 17ºC.
The transformants obtained were grown on selective medium for genomic DNA extraction (as described in Section 2.1.2) and PCR was used to ascertain their genotypes, through determination of the presence or absence of the genes of interest in each case, as well as the place of integration.

2.3.4. Sporulation assays

To determine the ability of the *P. rhodozyma* mutants to sporulate, sporulation assays were performed, in which the *P. rhodozyma* mutants were grown in DWR solid medium with 0.5% of ribitol (Table 6.1, in Appendix). The sporulation assay was performed as described by David-Palma *et al*, 2016. Briefly, the cells were grown over-night in YPD liquid medium in 10% of the volume of the flask, at 18ºC and 180 rpm (Sartorius Certomat IS incubator), until the OD_{600nm} of the culture reached 1.0. The cells were then pelleted and thoroughly washed with distilled sterile water and then resuspended in 1 mL of distilled sterile water. The cell suspension was plated in triplicate in sporulation medium (DWR+0.5% ribitol) with 10 µL drops. All plates were incubated at 17ºC and observed regularly, for up to 31 days, using an optical microscope to search for basidia on the border and on top of the colonies.

2.3.5. Minimum Inhibitory Concentration assay for *C. capitatum*

A Minimum Inhibitory Concentration assay was performed to determine the resistance of *C. capitatum* strain CBS 7420 to the antifungal drugs geneticin (G418), hygromycin B and zeocin. *C. capitatum* was grown in the same conditions as those used for the preparation of electrocompetent cells for the transformation of *P. rhodozyma* (Section 2.3.3), until the culture reached an OD_{600nm} of 1.2. At this point, an aliquot of 60 µL of the culture was washed twice with sterile water and the pellet was resuspended in the same volume of sterile water, after which 60 µL of the cell suspension were plated on YPD medium supplemented with different concentrations (50 µg/ml, 100 µg/ml, 150 µg/ml and 250 µg/ml) of each of the antifungal drugs that were being studied. The plates were incubated upside down, at 17ºC, and were observed for up to 7 days of incubation.

2.3.6. Test of the ability to genetically transform *C. capitatum* by electroporation

Transformation of *C. capitatum* CBS 7420 with linearized plasmid pPR2TN'+AC025/AC026, was performed using the same protocol as for *P. rhodozyma* (Visser *et al*, 2005) (Section 2.3.3) with minor adjustments. The amount of DNA used for transformation was 1.5 µg and 3.3 µg, and the selective medium used consisted of YPD supplemented with geneticin at a concentration of 50 µg/ml and 40 µg/ml on each of the two different attempts at transformation, respectively. The recovery step in YPD liquid medium was performed over-night at 17ºC. The electroporation parameters were not altered.

2.3.7. RNA extraction and cDNA synthesis for the *HD1* and *HD2* genes of *C. capitatum*

C. capitatum strains CBS 7420, PYCC 5626 and A 301 were incubated in GSA liquid medium at 20ºC, 90 rpm (Sartorius Certomat IS incubator), for 8 days for RNA extraction. Total RNA extraction was performed using the *ZR Fungal/Bacterial RNA MiniPrep kit* (by ZYMO Research), performing a single step of in-column DNaseI Digestion to free the RNA samples of genomic DNA. From the extracted RNA, cDNA was synthesized from total mRNA using Maxima H Minus Reverse Transcriptase (by Thermo Scientific) and oligo $(dT)_{20}$ as primer. For the synthesis of the second DNA strand, specific primers for the complete *HD1* and *HD2* genes were designed (Table 6.4, in Appendix). These same primers were used in a PCR reaction using the RNA sample as template, to confirm the absence of genomic DNA in the sample. The fragments corresponding to the CDS of *HD1* and *HD2* of strain PYCC 5626 were then amplified by PCR directly from the cDNA samples using Phusion High-Fidelity DNA Polymerase (by Thermo Scientific) and then cloned into the pJET1.2/blunt plasmid using the *CloneJET PCR Cloning Kit* (by Thermo Scientific). The plasmids carrying the fragments of interest were then used to transform *E. coli* DH5α through a heat shock transformation method (Ausubel *et al*, 1987) and recovered using the *NZYMiniprep kit* (by NZYTech). The cloned fragments were sequenced by Sanger Sequencing, at STABVida (Portugal), and aligned with the CDS of *HD1* and *HD2* available for strain CBS 7420 (Figure 6.2, in Appendix, for the DNA sequence of *HD* locus of *C. capitatum* strain CBS 7420). The alignments were performed using the software ClustalW (implemented in BioEdit Sequence Alignment Editor v7.0.5.3 (Hall, 1999)). The protein sequences of HD1 and HD2 of strain PYCC 5626 were also aligned

with those of strain CBS 7420 using the software MUSCLE (implemented in the software Unipro UGENE v1.30.0 (Okonechnikov *et al*, 2012)).

2.3.8. Design of synthetic *HD1* and *HD2* genes of *C. ferigula*

In order to perform the Yeast Two-Hybrid assay for HD proteins originating from *C. ferigula*, *HD1* and *HD2* synthetic genes from two strains of different mating-types were designed, corresponding to the CDS of the *HD1* gene from strain PYCC 5628 and of the *HD2* gene from strain PYCC 4410. Available CDS of the mentioned genes were optimized for the codon usage of *S. cerevisiae* using the software GENEius (by Eurofins Genomics). The synthetic genes (Figure 6.5, in Appendix) were synthesized at Eurofins (Germany), each of them cloned into a pEX-A258 plasmid.

2.3.9. Yeast Two-Hybrid assay to assess the interaction between HD1 and HD2 of *C. capitatum* and comparison with the Yeast Two-Hybrid results for the interaction between HD1 and HD2 of *C. ferigula*

To assess the existence of interaction between the HD1 and HD2 proteins of *C. capitatum*, the Yeast Two-Hybrid assay was performed, using the same methods as described for the Yeast Two-Hybrid assay performed for the HD1 and HD2 proteins of *P. rhodozyma* (Section 2.2.2). Concerning the HD1 and HD2 proteins of *C. capitatum*, the sequences corresponding to the *HD1* and *HD2* CDS of strain PYCC 5626 were used to perform the fusion constructions with both the activation and DNA binding domains of Gal4. The designed primers containing the 40 bp flanking regions of the multiple cloning sites of the plasmids pGADT7 and pGBKT7 (Table 6.4, in Appendix) were used to amplify the complete *HD1* and *HD2* CDS, using either Phusion High-Fidelity DNA Polymerase (by Thermo Scientific), Q5 High Fidelity DNA Polymerase (by NE BioLabs) or LongAmp Taq DNA Polymerase (by NE BioLabs). The linearized pGADT7 and pGBKT7 (linearized with Cla I and Pst I restriction enzymes at the multiple cloning site, respectively, using 20 units of enzyme in both cases) were then used to transform *S. cerevisiae* strains Y187 and Y2HGold respectively, together with the relevant amplified *HD1* and *HD2* fragments.

Similar methods were implemented to perform the Yeast Two-Hybrid assay between the complete HD1 and HD2 proteins of *C. ferigula* (strains PYCC 5628 and PYCC 4410 respectively), using the designed synthetic genes to perform the fusion constructions, where only *HD1* fused with the DNA binding domain of Gal4 and *HD2* fused with the activation domain of Gal4 constructions were performed.

The transformation of the *S. cerevisiae* strains, the mating experiments and the testing for the activations of the reporter genes were performed as described in Section 2.2.2.

3. Results and Discussion

3.1. Phylogenetic study of the *MAT* loci in the *Cystofilobasidium* genus

The *Cystofilobasidium* genus encompasses a lineage with a great variety of sexual behaviours, where not only exclusively heterothallic and exclusively homothallic species are present, but there is also the particular case of *C. macerans*, a species encompassing both homothallic and heterothallic strains (Sampaio, 2011).

Therefore, this genus within the order Cystofilobasidiales is an interesting lineage, where a more profound study of the *MAT* loci would be relevant to help elucidate the evolutionary transitions between heterothallic and homothallic sexual behaviours not only within the *Cystofilobasidium* genus, but possibly also within the order Cystofilobasidiales.

In this work, a phylogenetic study of specific regions of the *MAT* loci was performed, namely the *STE3* gene and the 5'-*HD1/HD2* region of the *HD* locus, involving several strains of three species of the *Cystofilobasidium* genus: *C. capitatum*, *C. ferigula* and *C. macerans* (Table 6.2, in Appendix). The results from this study will be presented and discussed in the following sections, each species in turn.

Initially, the species *C. bisporidii* was also included in this study. However, given the limited amount of strains available (four strains) and given the fact that two of them (CBS 6348 and CBS 6349) resulted from the mating between the other two strains (PYCC 5604 and PYCC 4415^{T}), whose genomes had been previously sequenced and analysed (David-Palma, 2017), no information would be added concerning the *MAT* loci in this species. Therefore, this species was not included in the present study.

3.1.1. *MAT* loci in *C. capitatum*

The *C. capitatum* species is solely composed of strains with a homothallic sexual behaviour (Sampaio, 2011), where the sexual cycle of a single strain can be completed without mating with a second strain. Through analysis of the genome of *C. capitatum* strain CBS 7420 (David-Palma, 2017), a study of the *MAT* loci revealed the existence of a single *STE3* gene in the *P/R* locus and a single pair of *HD1* and *HD2* genes in the *HD* locus. These findings formed the basis for the phylogenetic study of the *MAT* loci presented in this work.

Initially, to try and discriminate the organization of the *C. capitatum* strains considered in this study in different phylogenetic groups, a phylogeny of the ITS region of rDNA obtained for all the strains was performed, with the results presented in Figure 3.1.

In Figure 3.1.A, a significant divergence of strain PYCC 5627 from the remaining *C. capitatum* strains analysed in this study can be observed, which raised the question of whether strain PYCC 5627 belonged to the *C. capitatum* species. Indeed, when aligning the ITS sequence of strain PYCC 5627 with the obtained sequences of this region of strains belonging to the other *Cystofilobasidium* species considered in the present study, PYCC 5627 seemed to group unequivocally with the *C. ferigula* strains

(also discussed in Section 3.1.2 of this work). The ITS region of this strain was reamplified and re-sequenced twice, with similar results obtained for this sequence.

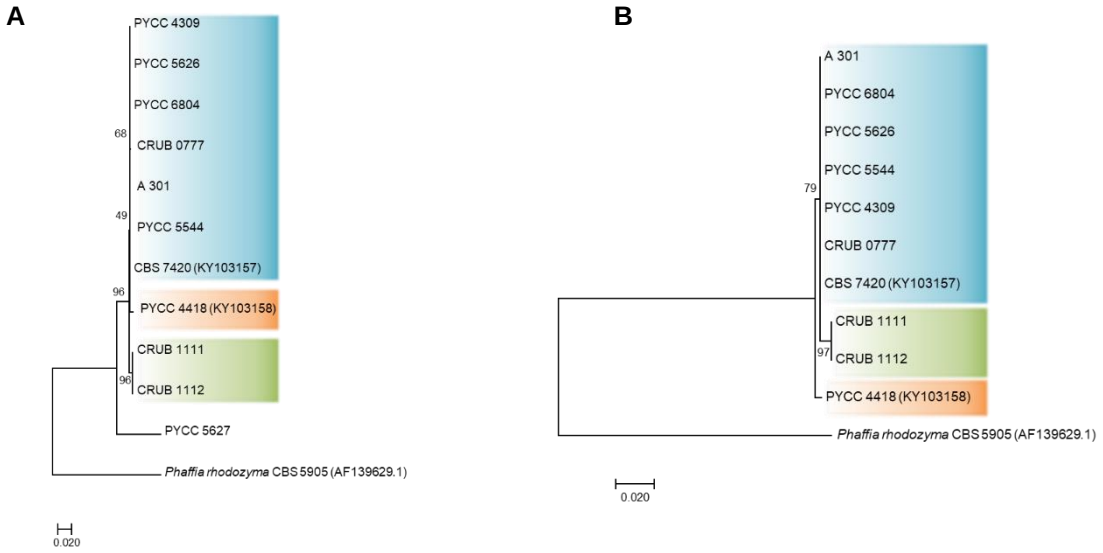


Figure 3.1: Phylogenies of the ITS region of the rDNA for the *C. capitatum* strains under study. In both phylogenies, *P. rhodozyma* was used as an outgroup. Between parentheses are the GenBank accession numbers for the sequences used in these analyses, for all the strains for which this applies. Bootstrap values are shown next to the branches. In both analyses, all positions containing gaps and missing data were eliminated. A) Phylogeny includes strain PYCC 5627, where a total of 517 positions were present in the final dataset. B) Phylogeny excludes strain PYCC 5627, where a total of 516 positions were present in the final dataset.

A second phylogeny of the ITS region was performed for the *C. capitatum* strains excluding PYCC 5627 (Figure 3.1.B) where, similarly to what was obtained in the phylogeny of Figure 3.1.A, the *C. capitatum* strains seem to be organized in distinct groups, which are in accordance with the geographical origins for most of the strains. Clear exceptions to this, are strains CBS 7420 and CRUB 0777 (originating from France and Patagonia, respectively) that group with the blue clade in the phylogenies of Figure 3.1, which is comprised of strains originating from Portugal (Table 6.2, in Appendix). Throughout the phylogenies involving the regions of the *MAT* loci, all strains will be coloured in accordance to the groups formed in the phylogeny of the ITS region.

Starting with the analysis of the phylogenies that were obtained for both the *STE3* gene and the protein sequence of the Ste3 pheromone receptor for the *C. capitatum* strains (Figure 3.2), a moderate degree of divergence is observed between the different strains under study. Still, when in comparison with the divergence observed in the sequences for both *STE3-1* and *STE3-2* alleles of *P. rhodozyma*, it is possible to assume that, with a high probability, the different *STE3* sequences obtained for the *C. capitatum* strains represent the same allele, as the evolutionary distances are not as prominent as those observed for the two different alleles of *P. rhodozyma*. Analysing the phylogeny of the protein sequence of the pheromone receptor Ste3 (Figure 3.2.B), the evolutionary relationships seem to be equivalent to those obtained in the phylogeny of the *STE3* gene sequence (Figure 3.2.A), except for PYCC 5626 which seems to be more closely related to the group formed by CRUB 1112 and CRUB 1111 in the phylogeny of the Ste3 protein sequence as opposed to what is observed in the phylogeny of the *STE3* gene sequence. Still, and given the evolutionary distances observed in both phylogenies (Figure 3.2),

the assumption that all *C. capitatum* strains possess the same *STE3* allele remains valid. However, since the variations in the Ste3 pheromone receptors of the different *C. capitatum* strains were not studied in this work, it is possible that these would result in distinct compatibilities in case they lead to alterations in the pheromone recognition centre.

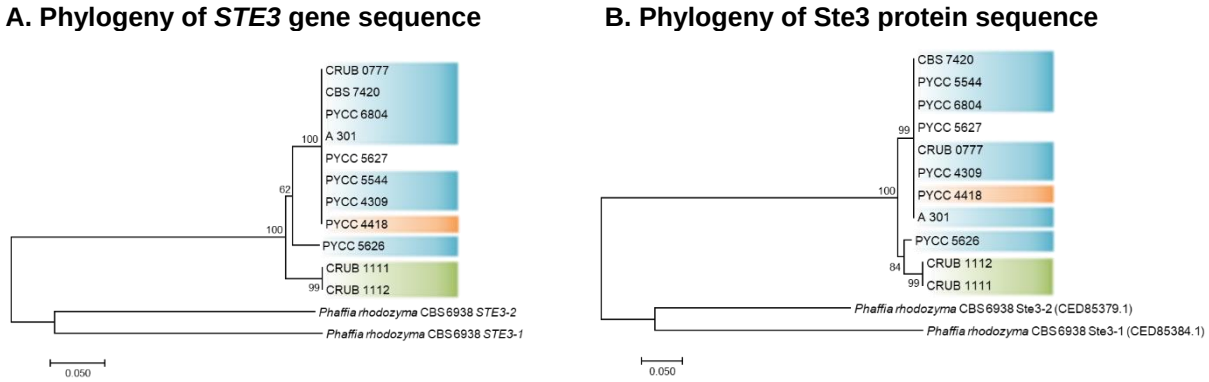


Figure 3.2: Phylogenies of the *STE3* gene (A) and of the pheromone receptor Ste3 protein (B) for the *C. capitatum* strains under study. In both phylogenies, *P. rhodozyma* was used as an outgroup. Between parentheses are the GenBank accession numbers for the sequences used in these analyses, for all the strains for which this applies. Bootstrap values are shown next to the branches. In both analyses, all positions containing gaps and missing data were eliminated. A) Phylogeny of the *STE3* gene, where the codon positions included were 1st+2nd+3rd+Noncoding, and a total of 770 positions were present in the final dataset. B) Phylogeny of the Ste3 protein where a total of 225 positions were present in the final dataset.

Interestingly, and as can be observed in the phylogenies depicted in Figure 3.2, strain PYCC 5627 (that did not group with the remaining *C. capitatum* strains in the phylogeny of the ITS region of rDNA, in Figure 3.1.A) groups unequivocally with the *C. capitatum* strains concerning the pheromone receptor gene (*STE3*) and protein (Ste3) sequences, leading to the conclusion that this strain possesses, indeed, a *STE3* allele belonging to *C. capitatum*.

Concerning the phylogeny of the region 5'-*HD1/HD2* (Figure 3.3.A), a stronger divergence between strains was already expected, by comparison with what is observed in the *STE3* phylogeny. Firstly, this region includes the intergenic region between the *HD1* and *HD2* genes, which is already more variable than what is expected of a coding sequence. Secondly, the 5' ends of both *HD1* and *HD2* genes are also being considered for this phylogeny, which have been described as the most variable regions of these genes for heterothallic basidiomycete species, as the N-terminal domain of the HD proteins constitutes the dimerization domain in species with this sexual behaviour (Fraser *et al*, 2007; Kües *et al*, 2011).

The divergence pattern observed in the phylogeny of the 5'-*HD1/HD2* region (Figure 3.3.A) is also observed in the phylogeny of the N-terminal region of the HD2 protein (Figure 3.3.B), which suggests that the complete HD2 protein will present a similar phylogenetic pattern, since, as stated above, the N-terminal region is normally the least conserved region in the HD proteins.

Given the great divergence existent between the *HD* locus of *P. rhodozyma* and that of *C. capitatum*, *P. rhodozyma* could not be used as outgroup in these phylogenies (Figure 3.3), since an alignment of these sequences could not be adequately performed. Therefore, it is not clear whether the analysed *C. capitatum* strains comprise different *HD* loci. It is, however, interesting to note that the strain PYCC 5626

and strains CRUB 1112 and CRUB 1111 group separately in these phylogenies (Figure 3.3), in line with what was observed in the phylogenies of the pheromone receptor (Figure 3.2). Still, and although it seems likely that strain PYCC 5626 and strains CRUB 1112 and CRUB 1111 may harbour two different alleles of the *HD* loci, the present results are not sufficient to support this conclusion.

Concerning strain PYCC 5627, the sequence of the 5'-*HD1/HD2* region could not be obtained. Although a fragment of the expected size for the 5'-*HD1/HD2* region could be obtained using primers AC016 and AC017 (designed to amplify the 5'-*HD1/HD2* region of *C. capitatum*) (Table 6.4, in Appendix), several

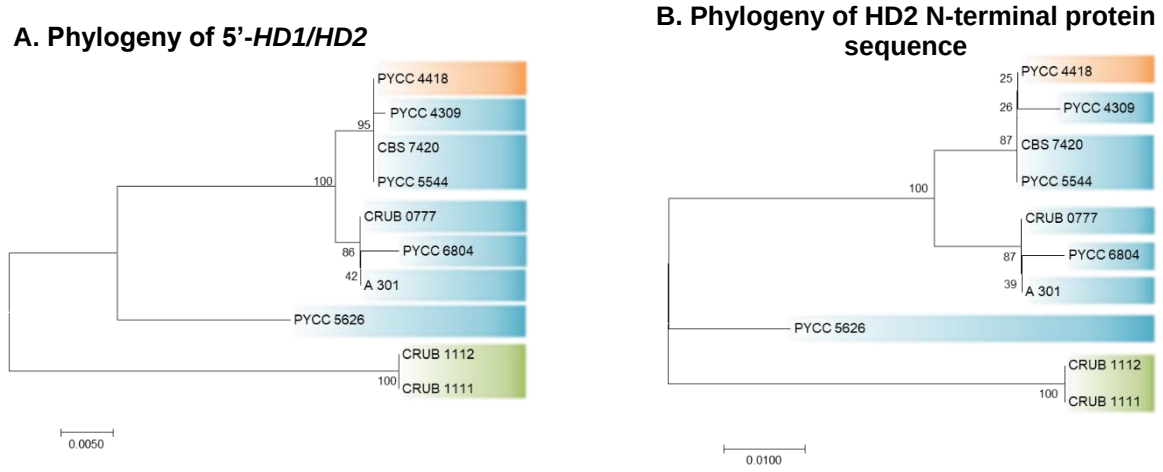


Figure 3.3: Phylogenies of the 5'-*HD1/HD2* region (A) and of the N-terminal region of the HD2 protein (B) for the *C. capitatum* strains under study. Bootstrap values are shown next to the branches. In both analyses, all positions containing gaps and missing data were eliminated. A) Phylogeny of the 5'-*HD1/HD2* region, where a total of 829 positions were present in the final dataset. B) Phylogeny of the HD2 N-terminal protein where a total of 192 positions were present in the final dataset.

rounds of optimization of the PCR conditions were necessary for such a fragment to be amplified. This region was not properly sequenced in either of the two attempts that were performed.

The obtained results concerning strain PYCC 5627 are indeed thought provoking, as no events of hybridization have been reported for species of the order Cystofilobasidiales. More profound studies should be made, involving repetition of the sequencing steps for both the ITS region and the *STE3* gene of this strain and, later, with a larger sample of both *C. capitatum* and *C. ferigula* strains to try to understand what is occurring in strain PYCC 5627, as no illations can be taken from the present study concerning these observations.

3.1.2. *MAT* loci in *C. ferigula*

C. ferigula encompasses only heterothallic strains, described as possessing a bipolar system of sexual reproduction, as only two mating-types have been described (Sampaio, 2011). Through analysis of the genome sequences of the strains PYCC 4410 and PYCC 5628, comprising two distinct mating-types, the sequences of two different *STE3* alleles were obtained (*STE3.A1* and *STE3.A2*, respectively), each strain containing a single *STE3* gene in their genome (David-Palma, 2017). Analysis of these genomes also revealed a single pair of *HD1* and *HD2* genes in the *HD* locus of each of these strains (David-Palma, 2017).

Similarly to what was performed for the analysed *C. capitatum* strains (Section 3.1.1 of this work), an initial phylogeny of the ITS region of rDNA was performed for all the *C. ferigula* strains considered (Figure 3.4). Indeed, as can be observed in Figure 3.4.A, all five *C. ferigula* strains group together, independently of their geographical origins (Table 6.2, in Appendix).

As mentioned in Section 3.1.1 of this work, a second phylogeny of the ITS region was performed for the *C. ferigula* strains, this time including the ITS sequence obtained for strain PYCC 5627 (described as

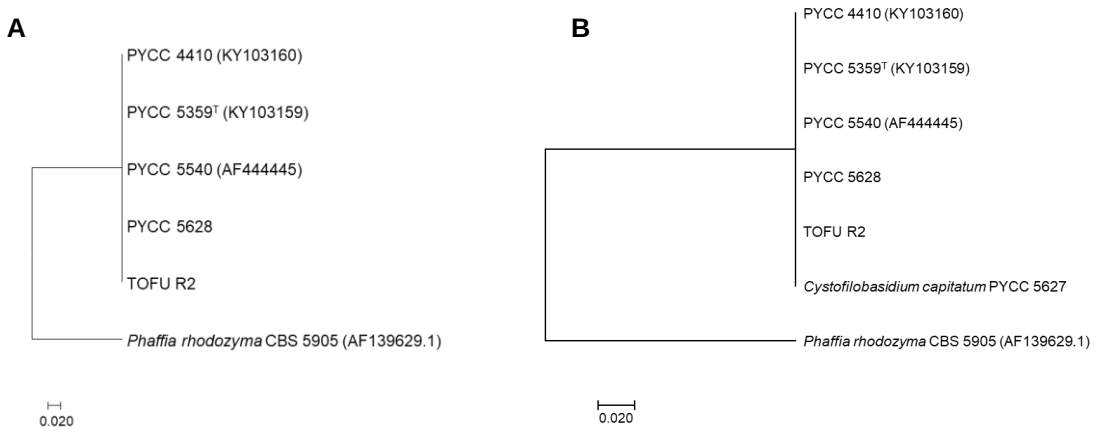


Figure 3.4: Phylogenies of the ITS region of the rDNA for the *C. ferigula* strains under study. In both phylogenies, *P. rhodozyma* was used as an outgroup. Between parentheses are the GenBank accession numbers for the sequences used in these analyses, for all the strains for which this applies. Bootstrap values are shown next to the branches. In both analyses, all positions containing gaps and missing data were eliminated. A) In this phylogeny a total of 511 positions were present in the final dataset. B) Phylogeny includes strain PYCC 5627, where a total of 526 positions were present in the final dataset. ^{T} is indicative of the “type strain”.

C. capitatum) (Figure 3.4.B). Indeed, and as stated in Section 3.1.1, PYCC 5627 unequivocally groups with the remaining *C. ferigula* strains. However, and as has been discussed in the mentioned section of this work, no conclusions can be taken regarding the status of strain PYCC 5627 given the present results and, therefore, this strain will not be taken into consideration throughout the rest of the study of the *MAT* regions for *C. ferigula.*

Through analysis of the phylogenies for both the *STE3* gene and the Ste3 protein sequences (Figure 3.5.A and 3.5.B), two very distinct groups are formed for the *C. ferigula* strains, comprising the two different *STE3* alleles. Indeed, strains PYCC 5359 and PYCC 5540 group together with PYCC 4410, sharing the same pheromone receptor (Ste3.A1), while strain TOFU R2 groups with PYCC 5628, sharing the second pheromone receptor (Ste3.A2).

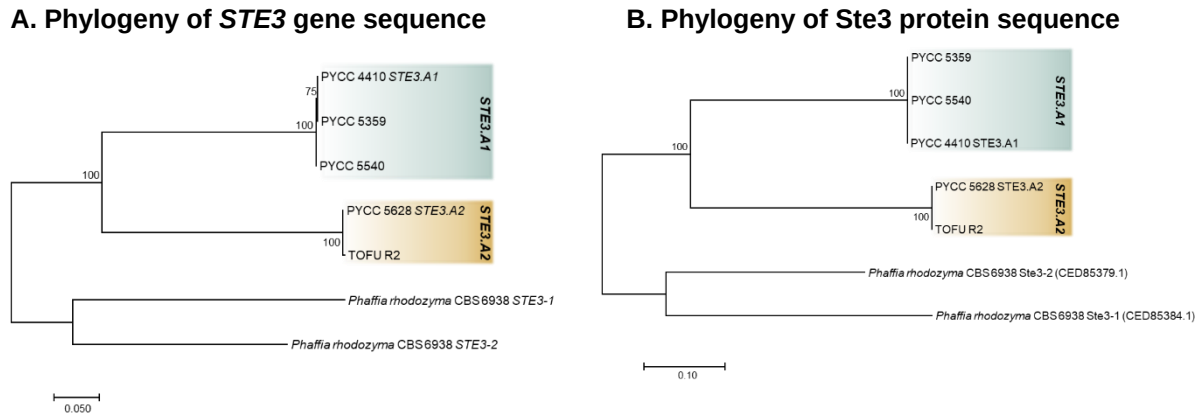


Figure 3.5: Phylogenies of the *STE3* gene (A) and of the pheromone receptor Ste3 protein (B) for the *C. ferigula* strains under study. In both phylogenies, *P. rhodozyma* was used as an outgroup. Between parentheses are the GenBank accession numbers for the sequences used in these analyses, for all the strains for which this applies. Bootstrap values are shown next to the branches. In both analyses, all positions containing gaps and missing data were eliminated. A) Phylogeny of the *STE3* gene, where the codon positions included were 1st+2nd+3rd+Noncoding, and a total of 772 positions were present in the final dataset. B) Phylogeny of the Ste3 protein where a total of 226 positions were present in the final dataset.

However, the most striking result obtained for this species derives from the analysis of the phylogenies obtained for the *HD* region of these strains (Figure 3.6.A and 3.6.B), most notably concerning the 5' end of the *HD1* gene (Figure 3.6.B). In these phylogenies, strain TOFU R2 groups with strains PYCC 5359 and PYCC 4410 and not with strain PYCC 5628, as what occurred in the phylogenies of the *STE3* gene and Ste3 protein sequences. This result implies that the *P/R* and *HD* loci in *C. ferigula* are unlinked, segregating separately during meiosis and enabling the generation of mating-types as those of strains PYCC 4410 (A1.B1), PYCC 5628 (A2.B2) and TOFU R2 (A2.B1).

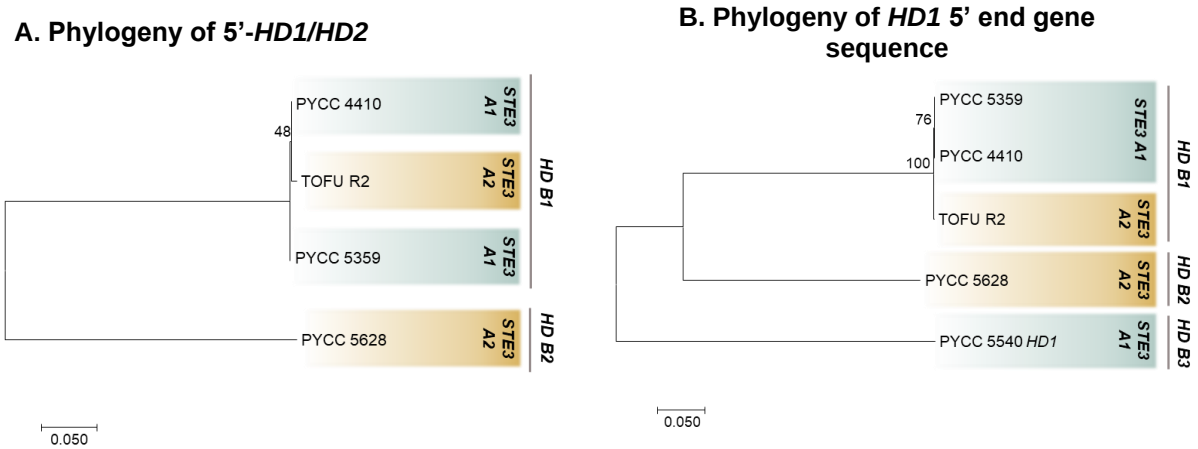


Figure 3.6: Phylogenies of the 5'-*HD1/HD2* region (A) and of the 5' end of the *HD1* gene (B) for the *C. ferigula* strains under study. Bootstrap values are shown next to the branches. In both analyses, all positions containing gaps and missing data were eliminated. A) Phylogeny of the 5'-*HD1/HD2* region, where a total of 1332 positions were present in the final dataset. B) Phylogeny of the 5' end of the *HD1* gene, where codon positions included were 1st+2nd+3rd+Noncoding, and a total of 445 positions were present in the final dataset.

Concerning strain PYCC 5540, the full sequence for the 5'-*HD1/HD2* region could not be obtained, and only the region comprising the 5' end of the *HD1* gene was retrieved from the data. Therefore, PYCC 5540 is only being considered in the phylogeny of the *HD1* 5' end (Figure 3.6.B), where this strain forms a third group, separated from all other strains This result suggests the existence of a third *HD1* allele, and strain PYCC 5540 seemingly possesses a third mating-type (A1.B3), distinct from all other *C. ferigula* strains that were analysed in this study.

Taken together, the results of the phylogenies of *STE3* and of the *HD* region of the *C. ferigula* strains seem to invalidate the possibility of a bipolar breeding system (Fraser *et al*, 2007). Given the mating-type of the strain TOFU R2 (A2.B1) - and potentially of strain PYCC 5540 (A1.B3) - and the fact that three distinct *HD* alleles seem to be present in the *C. ferigula* strain sampling used in this study, these results support instead a tetrapolar system, potentially triallelic for the *HD* locus.

It is, however, important to note that no additional mating experiments were performed for the considered *C. ferigula* strains, nor were the HD protein sequences functionally studied. Given the fact that bipolarity can be achieved through the loss of function of one of the *MAT* loci (Coelho *et al*, 2017), a more profound study of the *C. ferigula* strains should be performed, taking these factors into consideration, to confirm the hypothesised breeding system of this species.

3.1.3. *MAT* loci in *C. macerans*

The *C. macerans* species presents a great variety of strains when it comes to sexual behaviour, being composed of homothallic and heterothallic strains and even some strains that do not apparently sporulate (Sampaio, 2011) (Table 6.2, in Appendix).

In line with the study of the previous *Cystofilobasidium* species considered in this work, an initial analysis of the genomes of strains CBS 2425 and CBS 6532 revealed the existence of a single *STE3* gene and a single pair of *HD1* and *HD2* genes in the *MAT* loci of these *C. macerans* strains (David-Palma, 2017).

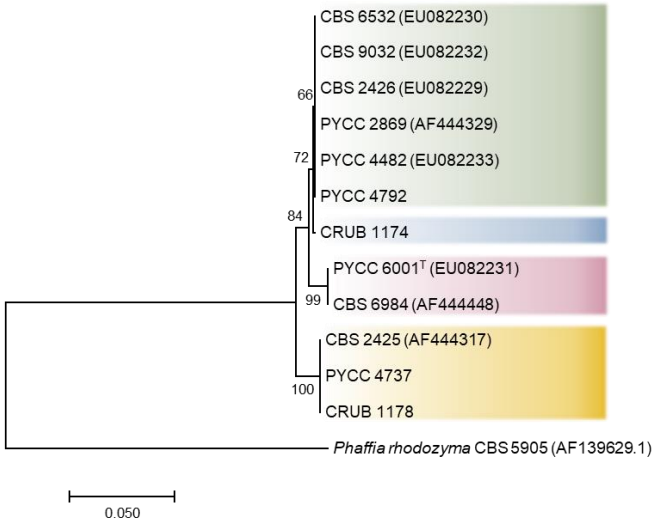


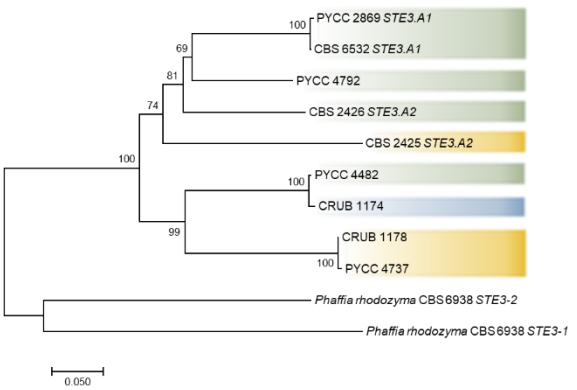
Figure 3.7: Phylogeny of the ITS region of the rDNA for the *C. macerans* strains under study. In this phylogeny, *P. rhodozyma* was used as an outgroup. Between parentheses are the GenBank accession numbers for the sequences used in this analysis, for all the strains for which this applies. Bootstrap values are shown next to the branches. All positions containing gaps and missing data were eliminated. In this phylogeny, a total of 521 positions were present in the final dataset. ^{T} is indicative of the "type strain".

Through analysis of the phylogeny of the ITS region of rDNA for the considered *C. macerans* strains (Figure 3.7), some significant divergence is observable between some of them. Since the geographical origin of some of the considered strains is not known (Table 6.2, in Appendix) not much can be inferred from these results, as all the considered strains have been described as belonging to *C. macerans* (Sampaio, 2011). However, the two strains originating from Patagonia (CRUB 1174 and CRUB 1178) are grouping quite distantly from each other.

Concerning the study of the *MAT* regions, in general there were some difficulties in amplifying the *STE3* and 5'-*HD1/HD2* regions through PCR. This was most notable for the 5'-*HD1/HD2* region, which is due to the fact that the homeodomain region of the HD1 and HD2 proteins in this species showed a lower conservation compared to what happens in other *Cystofilobasidium* species, through the analysis of the genomes of strains CBS 2425 and CBS 6532 (David-Palma, 2017). Taking this into consideration, there is a considerable amount of *C. macerans* strains for which the 5'-*HD1/HD2* region was not obtained (five strains in a total of twelve), and so no phylogeny was constructed based on this region.

The phylogeny of the *STE3* gene and of the Ste3 protein sequence (Figure 3.8.A and 3.8.B), shows considerable divergence between strains of *C. macerans*, and the evolutionary distances between sequences (when compared to those between *STE3-1* and *STE3-2* of *P. rhodozyma*) seem to be enough to assume the existence of several different *STE3* alleles. It is, however, important to note that some of the strains being analysed in the phylogenies of Figure 3.8 do not apparently sporulate, or their sexual behaviour has not been described yet (Sampaio, 2011). Adding to that fact, the *STE3* sequences of the only two homothallic strains of *C. macerans* involved in this study (CBS 6984 and PYCC 6001) could not be obtained through amplification.

A. Phylogeny of *STE3* gene sequence



B. Phylogeny of Ste3 protein sequence

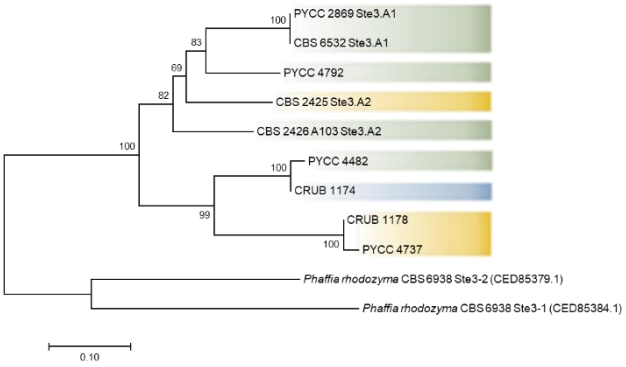


Figure 3.8: Phylogenies of the *STE3* gene (A) and of the pheromone receptor Ste3 protein (B) for the *C. macerans* strains under study. In both phylogenies, *P. rhodozyma* was used as an outgroup. Between parentheses are the GenBank accession numbers for the sequences used in these analyses, for all the strains for which this applies. Bootstrap values are shown next to the branches. In both analyses, all positions containing gaps and missing data were eliminated. A) Phylogeny of the *STE3* gene, where the codon positions included were 1st+2nd+3rd+Noncoding, and a total of 770 positions were present in the final dataset. B) Phylogeny of the Ste3 protein where a total of 220 positions were present in the final dataset.

Considering how incomplete the study of the *MAT* loci for *C. macerans* turned out, in terms of sequences of the *MAT* regions that could not be obtained and given the lack of support for some of the branches in

the phylogenies in Figure 3.8 (bootstrap values below 70), it is extremely difficult to draw any conclusion regarding the organization and number of alleles in the *MAT loci* of this species. However, the fact that for some strains the *MAT* regions considered in this study could not be amplified through PCR, given the methodology implemented for the obtention of such sequences (Section 2.1.2 of the Materials and Methods Section), really is revealing of the state of evolution of the *MAT* loci, especially concerning the 5'-*HD1/HD2* region, and eventually of the evolution of the sexual behaviours of the strains that comprise this species.

C. macerans seems to be indeed a peculiar case in the *Cystofilobasidium* genus in terms of sexual reproduction, being quite the reflection of the great variability of sexual behaviours present in this genus. More exhaustive studies concerning the evolution of the *MAT* loci, genomic and otherwise, would be very important for a better understanding of the transitions between sexual behaviours that are potentially ongoing within this species, eventually helping to enlighten evolution throughout the order Cystofilobasidiales.

3.2. Molecular interactions of homothallism in the order Cystofilobasidiales: Interaction between HD1 and HD2 in *P. rhodozyma*

In a previous study from our research group, a model of the molecular interactions governing the homothallic sexual behaviour of *P. rhodozyma* was proposed (David-Palma *et al*, 2016). According to this model, both pairs of pheromones and pheromone receptors encoded in its genome present a reciprocal compatibility, with the role of these compatible pairs in sexual reproduction being redundant. Concerning the HD1 and HD2 homeodomain transcription factors, this study revealed that the presence of both these proteins is required for sexual reproduction, and results from a Yeast Two-Hybrid assay showed a weak interaction between the HD1 and HD2 N-terminal domains, corresponding to some activation of only one of the three reporter genes analysed (*MEL1*) (David-Palma *et al*, 2016).

As the N-terminal domains of the HD1 and HD2 homeodomain transcription factors correspond to the dimerization domains of these proteins in heterothallic basidiomycetes (Fraser *et al*, 2007; Kämper *et al*, 1995; Hull *et al*, 2005), these findings for *P. rhodozyma* seem to suggest that a weak interaction exists between these domains, eventually being sufficient to form the HD1/HD2 heterodimer necessary to regulate the expression of genes that play essential roles in later stages of the sexual cycle (David-Palma *et al*, 2016).

In the present work, in order to better characterize the interaction between the HD1 and HD2 proteins of *P. rhodozyma*, a Yeast Two-Hybrid assay was performed, in line with the methodology used by David-Palma *et al*, 2016. In this assay, the complete HD1 and HD2 proteins were studied for their ability to interact, using qualitative results for the activation of the *MEL1, ADE2* and *HIS3* reporter genes, as well as quantitative results for the expression of the *LacZ* reporter gene, through a quantitative β-galactosidase assay. These results were then confronted with the results previously obtained for the interaction between the N-terminal regions of these proteins.

3.2.1. Yeast Two-Hybrid assay: Interaction between the complete HD1 and HD2 proteins of *P. rhodozyma*

In line with the study initiated by our research group (David-Palma *et al*, 2016), two new fusion proteins were constructed in this study, corresponding to the fusion between the activation domain of Gal4 and the complete HD1 and HD2 proteins of *P. rhodozyma*, giving rise to the Y187 strain transformants 12 and 13 respectively (Table 3.1).

An initial comparison between the sizes of the colonies corresponding to haploid transformants 12 and 13 with the sizes of the colonies corresponding to haploid transformant 2 (containing the pGADT7 empty plasmid) led to the conclusion that these fragments were not toxic to the yeast cells, as their presence did not seem to affect the growth of the transformant colonies. Indeed, as is observable in Figure 3.9, all colonies from all transformant plates seem to be of roughly identical size, being indicative that the potential expression of the *HD1* and *HD2* genes of *P. rhodozyma* is not affecting the viability of the Y187 cells.

Table 3.1: List of *S. cerevisiae* Y2HGold and Y187 haploid transformants used in the Yeast Two-Hybrid assay for the interaction between the complete HD1 and HD2 proteins of *P. rhodozyma*. Containing information regarding the plasmids present in each transformant. Haploid transformants 12 and 13 correspond to those generated in this study. Adapted from David-Palma *et al*, 2016.

Haploid ID	Plasmid in the haploid *S. cerevisiae* strain	*HD* fragment
1	pGBKT7 in Y2HGold cells	-
2	pGADT7 in Y187 cells	-
3	pGBKT7-53 in Y2HGold cells	-
4	pGADT7-T in Y187 cells	-
5	pGBKT7-Lam in Y2HGold cells	-
7	pGBKT7+MP181/MP183 in Y2HGold cells	*HD1* complete
8	pGBKT7+MP184/MP185 in Y2HGold cells	*HD2* partial
9	pGBKT7+MP184/MP186 in Y2HGold cells	*HD2* complete
10	pGADT7+MP187/MP188 in Y187 cells	*HD1* partial
12	pGADT7+MP187/MP201 in Y187 cells	*HD1* complete
13	pGADT7+MP189/MP202 in Y187 cells	*HD2* complete

After confirmation of the presence of the fragments of interest in each of the Y187 transformants through *S. cerevisiae* colony PCR, haploid transformants 12 and 13 were used to perform mating experiments with three already existent haploid transformants from the previous study by David-Palma *et al*, 2016, corresponding to the haploid transformants 7 and 9, possessing the fusion constructions corresponding to the HD1 and HD2 complete proteins each fused with the DNA binding domain of Gal4, respectively, as well as haploid transformant 8, containing the fusion construction of the N-terminal region of HD2 fused with the DNA binding domain of Gal4 (Table 3.1).

Transformant 2 **Transformant 12** **Transformant 13**

Figure 3.9: Toxicity assay for the presence of *HD1* and *HD2* fragments of *P. rhodozyma* in *S. cerevisiae* haploid transformants of strain Y187. Growth in the appropriate selective medium for haploid transformants of *S. cerevisiae* strain Y187.

In Table 3.2, the mating experiments 7x13, 8x12, 9x12, 7x12, and 9x13 correspond to the diploids constructed and analysed for the activation of the Yeast Two-Hybrid reporter genes for the first time in this study, allowing the study of both heterodimerization and homodimerization of the HD1 and HD2 complete proteins of *P. rhodozyma*

Table 3.2: List of *S. cerevisiae* diploids, generated through mating between the Y2HGold and Y187 haploid transformants, used in the Yeast Two-Hybrid assay for the interaction between the complete HD1 and HD2 proteins of *P. rhodozyma*. Containing relevant information regarding the interactions being tested. Diploids 7x13, 8x12, 9x12, 7x12, and 9x13 correspond to those generated in this study. Adapted from David-Palma *et al*, 2016.

Mating experiments using the haploid strains	Objective
1x2	Negative control with empty plasmids
3x4	Positive control for interaction
4x5	Negative control for interaction
8x10	Testing for heterodimerization hd2xhd1
9x10	Testing for heterodimerization HD2xhd1
7x13	Testing for heterodimerization HD1xHD2
8x12	Testing for heterodimerization hd2xHD1
9x12	Testing for heterodimerization HD2xHD1
7x12	Testing for homodimerization HD1xHD1
9x13	Testing for homodimerization HD2xHD2

Before proceeding with the assays for the activation of the reporter genes, all the diploids generated were tested for the presence of both recombinant plasmids, each corresponding to the fusion between the Gal4 activation and DNA binding domains with the *HD* fragments of interest. This confirmation was performed by PCR amplification of the *HD1* and *HD2* fragments used for each construction, using the primers designed to produce the *HD* fragments for the construction of the recombinant plasmids. As can be observed in Figure 3.10, all generated diploids contained the fragments of interest, leading to the conclusion that all diploids contained the expected fusion genes.

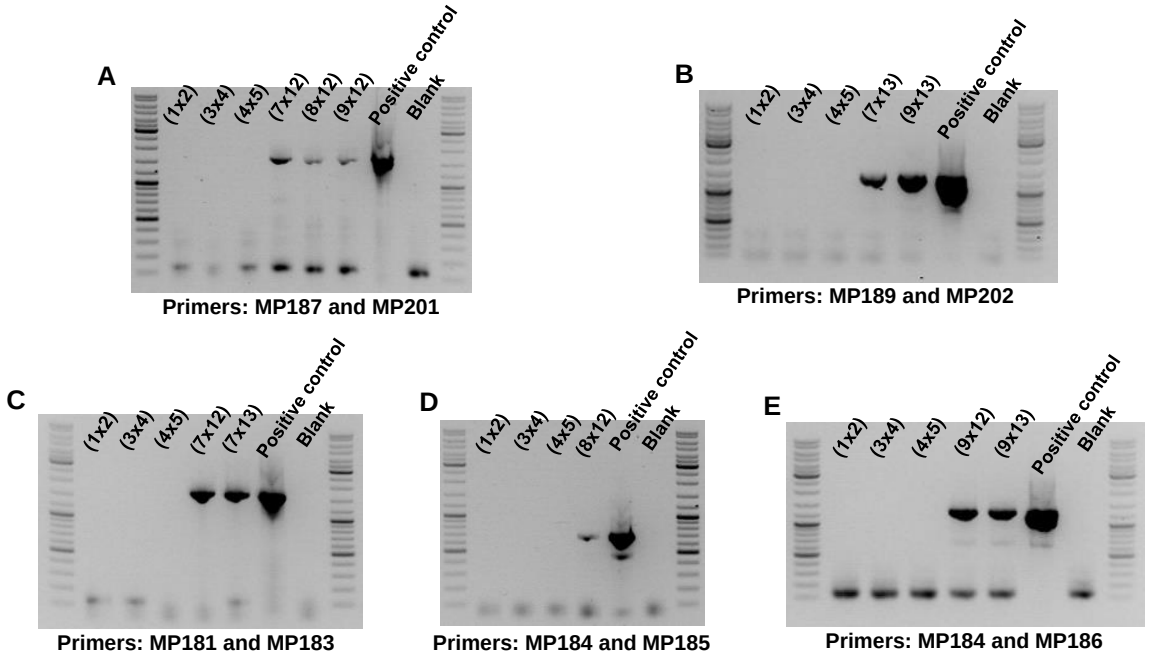


Figure 3.10: Confirmation of the presence of the *HD1* and *HD2 P. rhodozyma* fragments of interest in each of the generated diploids. In all agarose gels, the positive control constitutes the original plasmid containing the *HD* synthetic gene of interest. A) Confirmation of the presence of the *HD1* complete synthetic gene as used for the fusion construction with the activation domain of Gal4. B) Confirmation of the presence of the *HD2* complete synthetic gene as used for the fusion construction with the activation domain of Gal4. C) Confirmation of the presence of the *HD1* complete synthetic gene as used for the fusion construction with the DNA binding domain of Gal4. D) Confirmation of the presence of the *HD2* partial synthetic gene as used for the fusion construction with the DNA binding domain of Gal4. E) Confirmation of the presence of the *HD2* complete synthetic gene as used for the fusion construction with the DNA binding domain of Gal4.

Afterwards, all generated diploids were tested for the activation of the reporter genes of the Yeast Two-Hybrid assay, namely *MEL1, ADE2* and *HIS3.* In Figure 3.11, the results of the activation of all three reporter genes are shown for all the generated diploids, as well as the results of their activation for all the haploid generated transformants (auto-activation).

Concerning the heterodimerization of the HD proteins of *P. rhodozyma*, there seems to be some activation of the reporter gene *MEL1* in the diploids 8x12 and 9x12, shown through the blue tinge of the growth patch (Figure 3.11.A). However, through the analysis of the intensity of the blue colour, a weak interaction can be inferred, as it is much less intense when compared with the positive control for interaction in the Yeast Two-Hybrid assay (3x4). A weak activation of *MEL1* also seems to be present for diploid 7x13, although not to the extent that is observed for the diploid 9x12. These results seem to

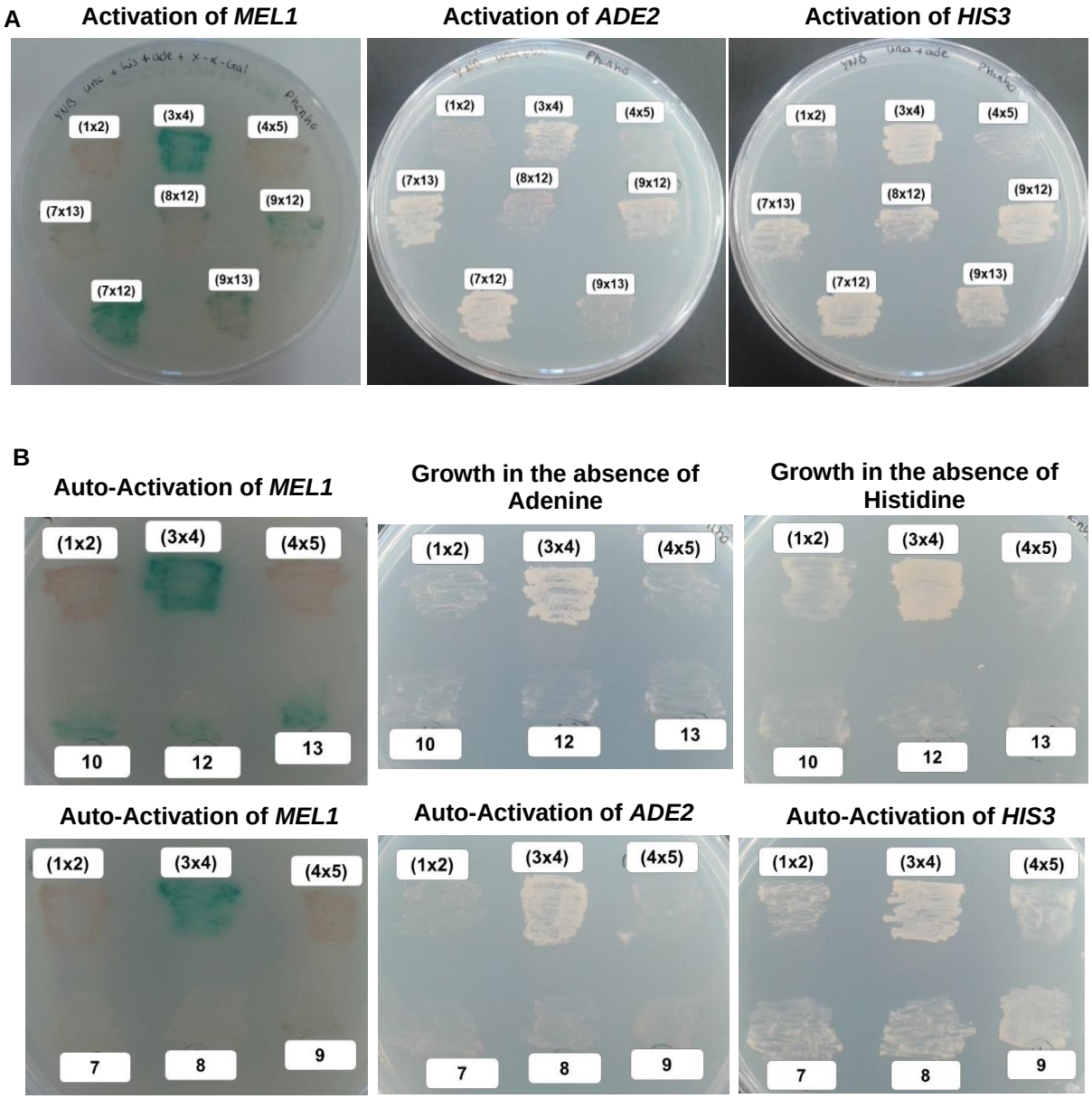


Figure 3.11: Yeast Two-Hybrid assay to test interactions between the HD proteins of *P. rhodozyma* – Results of the activation of the reporter genes *MEL1*, *ADE2* and *HIS3*. A) Activation of the reporter genes in the generated diploids, corresponding to the detection of heterodimerization and homodimerization of the HD proteins of *P. rhodozyma*. B) Activation of the reporter genes in the haploid transformants used in this study. All results represented in this figure correspond to three days of incubation, at 30ºC, in the appropriate selective medium in each case.

be somewhat consistent with the activation of the reporter *HIS3* (allowing the growth of diploids 8x12 and 9x12 in the absence of histidine), but not entirely with what is observed for the activation of *ADE2*, where the diploid 7x13 seems to be presenting a more intense growth in the absence of adenine than the diploids 8x12 and 9x12 (Figure 3.11.A).

Concerning the studies of homodimerization between the HD complete proteins, diploid 9x13 seems to be weakly activating *MEL1* reporter gene, and activating *HIS3* more evidently (homodimerization of HD2). The diploid 7x12, however, seems to be able to activate all three reporter genes, equivalently to

what is observed for the positive control of interaction in the Yeast Two-Hybrid assay (3x4), revealing a stronger homodimerization of HD1 than what is inferred for HD2 (Figure 3.11.A).

When analysing Figure 3.11.B, regarding the auto-activation of the reporter genes in the haploid transformants tested, there seems to be some activation of the *MEL1* reporter gene in haploids 10, 12 and 13, which coincides with the fusion constructions involving the Gal4 activation domain. In this study, although *S. cerevisiae* strain Y187 does not possess the *ADE2* and *HIS3* reporter genes, haploids 10, 12 and 13 were still tested for their ability to grow in the absence of adenine and histidine (Figure 3.11.B), serving as a second control for the absence of growth in this experiment. Concerning the fusion constructions involving the Gal4 DNA binding domain, only haploid 9 seems to be able to activate the reporter gene *HIS3*, where the growth observed in haploids 7 and 8 regarding the activation of this gene is equivalent to that obtained for the negative controls of the Yeast Two-Hybrid assay (1x2 and 4x5). Haploids 7, 8 and 9 do not seem to be able to activate any of the other reporter genes tested in this system (Figure 3.11.B). Interestingly, in the course of the previous study, no auto-activation of the haploid transformants generated was reported (David-Palma *et al*, 2016).

The above stated results taken together, it is not easy to establish a direct correlation between the observed activations of the reporter genes and the strength of interaction between the HD1 and HD2 proteins of *P. rhodozyma*. On the one hand, the existence of activation is not entirely consistent through the three reporter genes, where activation in the same diploid analysed seemed to have clearly different intensities for the different reporter genes (diploids 7x12, 8x12 and 9x13). Also, to be taken into account is the existence of activation of some of the reporter genes in the haploid transformants (auto-activation of *HIS3* in haploid 9 and auto-activation of *MEL1* in haploids 10, 12 and 13).

Upon repetition of the experiments for both the activation and auto-activation of the reporter genes, there seemed to be some slight variations of the observed results for all three reporter genes. Without altering the concentrations of the supplemented amino acids, the ability of the diploids and haploid transformants to grow in the absence of adenine or histidine seemed to vary, given the same amount of incubation time at 30ºC, where even the negative controls of the Yeast Two-Hybrid system (1x2 and 4x5) seemed to present some growth in these conditions, in some cases. This can be observed in Figure 3.11.B, concerning the study of the auto-activation of *HIS3* in haploids 7, 8 and 9. Similar results were also obtained concerning the activation and auto-activation of *MEL1*. This seeming inconsistency of the activation of the reporter genes, given the selective media used in both this study and in the study by David-Palma *et al*, 2016, could be the reason for why the latter did not report any auto-activations in the haploid transformants used, when these same transformants presented activation of some of the reporter genes when they were tested in the present study.

In general, the results from the present study seem to indicate the existence of interaction between the complete HD1 and HD2 proteins of *P. rhodozyma* (7x13 and 9x12), however weak, potentially through the N-terminal region of these proteins (8x12), in line with what has been described for heterothallic basidiomycetes (Hull *et al*, 2005).

Homodimerization of the HD proteins also seems to be present, more evidently for the HD1 protein (7x12), a result that had also been reported by David-Palma *et al*, 2016. However, and as previously discussed by David-Palma *et al*, 2016, concerning the evidences for homodimerization of HD1 and HD2

in *P. rhodozyma*, no functions have been described so far for potential homodimers of the HD proteins in basidiomycetes.

3.2.2. Quantitative β-galactosidase assay: Assessing the relative strength of interaction between the complete HD1 and HD2 proteins of *P. rhodozyma*

After several replicates of the Yeast Two-Hybrid assay were performed, concerning the interaction between the HD1 and HD2 complete and truncated proteins of *P. rhodozyma*, the results for the activation of the reporter genes *MEL1, ADE2* and *HIS3* seemed to be reasonably consistent for four diploids: 8x10, 8x12, 9x10 and 9x12. In all these cases, the HD2 protein (complete and truncated) was fused with the DNA binding domain of Gal4, while the HD1 protein was fused with the activation domain of Gal4 (Tables 3.1 and 3.2). As can be observed in Figure 3.12, some activation of the *MEL1* reporter

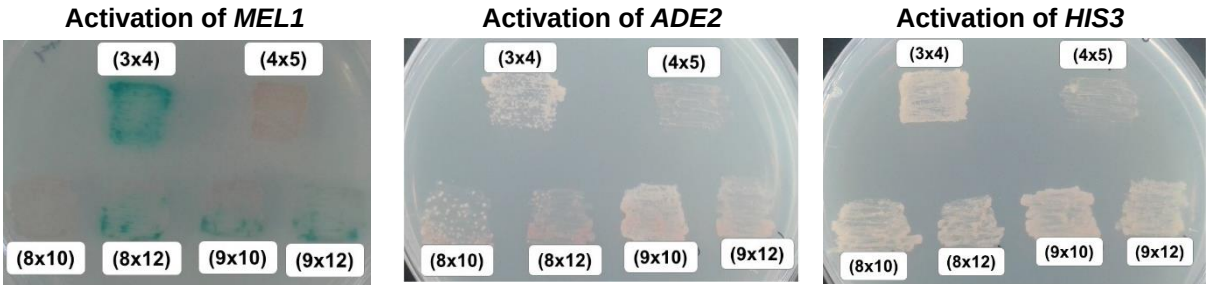


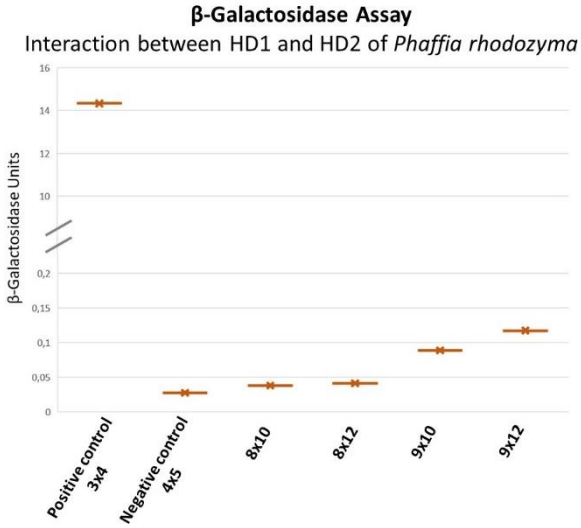
Figure 3.12: Results for the activation of the reporter genes *MEL1, ADE2* and *HIS3* for the relevant diploids used in the quantitative β-galactosidase assay. All results represented in this figure correspond to three days of incubation, at 30ºC, in the appropriate selective medium in each case.

gene is present in diploids 8x12, 9x10 and 9x12. Concerning the reporter gene *ADE2,* the diploid 9x10 seems to be growing with more intensity, but some activation is also observed for 8x10, 8x12 and 9x12. The reporter gene *HIS3* is activated by all four studied diploids.

To set more insight on the significance of these results, the activation of the reporter gene *LacZ* was tested, through a quantitative assay of the activity of β-galactosidase (encoded in the *LacZ* gene). This assay was performed in liquid culture and using ONPG as substrate of β-galactosidase. In quantifying the activity of this enzyme, the level of expression of the *LacZ* reporter gene can be determined for each diploid being tested. In doing so, it is possible to assess the relative strength of the potential interactions observed between the HD1 and HD2 proteins of *P. rhodozyma*, establishing a comparison between the interactions involving the N-terminal regions of these proteins and the interactions involving the complete proteins.

As described in the Materials and Methods section of this work (Section 2.2.3), the β-galactosidase assay was performed in triplicate for all the tested diploids, where in each of the replicates the reaction was stopped at three different times by addition of sodium carbonate (Na_2CO_3). For each of the replicates, the β-galactosidase units were calculated as described in the Materials and Methods section of this work, and the mean of the three values of β-galactosidase units was calculated for each corresponding diploid being tested.

The results obtained from this assay are schematically represented in Figure 3.13. When comparing the β-galactosidase units obtained for the diploids tested with those obtained for the positive and negative controls, it is possible to infer the existence of a weak interaction between HD1 and HD2. Indeed, the β-galactosidase units obtained for the diploids 9x10 and 9x12 are only 4 to 5 times larger than the value obtained for the negative control of interaction (4x5), and still much lower than what was obtained for the positive control of interaction (3x4). On another hand, the β-galactosidase units obtained for the diploids 8x10 and 8x12 are roughly within the same range as the value obtained for the negative control.



Diploid ID	β-galactosidase Units (mean)
3x4	14,334
4x5	0,0273
8x10	0,0378
8x12	0,0412
9x10	0,0888
9x12	0,117

Figure 3.13: Results of the quantitative β-galactosidase assay to study the relative strength of the interaction between HD1 and HD2 proteins of *P. rhodozyma*. The mean of the β-galactosidase units obtained in each of the three replicates is represented in the table to the right for each of the considered diploids, with a graphical representation of these values in the graphic to the left.

These results are in accordance with what was observed in the Yeast Two-Hybrid assay (Section 3.2.1), as well as with the findings reported by David-Palma *et al*, 2016. Indeed, a weak interaction seems to be present between the HD1 and HD2 proteins of *P. rhodozyma* and, through analysis of the results from the quantitative β-galactosidase assay, the relative strength of the interaction between the complete HD1 and HD2 proteins is not significantly greater than what is observed for the N-terminal regions of these proteins.

One aspect that was not considered when performing the β-galactosidase assay was the possibility of activation of the *LacZ* reporter gene in the haploid transformants. Through the results of the auto-activation of the reporter genes *MEL1*, *ADE2* and *HIS3* in Section 3.2.1 of this work it could be observed that haploid transformants 10 and 12 had the ability to activate *MEL1*. As the strain Y187 possesses the reporter gene *LacZ*, it is possible that the expression of this gene is also being activated in haploid transformants 10 and 12.

So, based on these results, the existence of interaction between the HD1 and HD2 proteins of *P. rhodozyma* could be argued, and it would be important to perform a β-galactosidase assay involving haploid transformants 10 and 12 to properly validate the existence of interaction between these proteins and its relative strength.

Previous studies can, however, provide support to the hypothesis of a weak interaction between the HD1 and HD2 proteins of *P. rhodozyma* being existent and sufficient to assure the later stages of this specie's sexual cycle.

Firstly, and specifically concerning *P. rhodozyma,* David-Palma *et al*, 2016, showed, through experiments with deletion mutants of the *HD* locus, that both HD1 and HD2 proteins are necessary for sexual development and sporulation in this species. Although the absence of HD2 did not completely abolish the ability to sporulate, it was considered vestigial when comparing with the rate of sporulation of the wild-type strain, suggesting that indeed both proteins are necessary for sporulation (David-Palma *et al*, 2016).

Given the results obtained in the present study pertaining to a weak interaction, and especially given the existence of auto-activation of the reporter genes in some of the haploid transformants used, the possibility of the HD1 and HD2 proteins of *P. rhodozyma* having a different mode of action in their role in sexual reproduction, other than the heterodimerization and regulation of gene expression described in heterothallic basidiomycetes (Hull *et al*, 2005; Kämper *et al*, 1995), cannot be discarded. However, the weak nature of the potential interaction should not be enough reason to disregard the formation of a heterodimer as the mode of action of the HD1 and HD2 proteins in sexual reproduction. Indeed, previous studies in *U. maydis* involving the mutagenesis of the dimerization region of *bE* (corresponding to *HD1* in this species) showed that a weak interaction between the homeodomain proteins was sufficient to allow the formation of an active heterodimer and potentiate the sexual development in *U. maydis* (Kämper *et al*, 1995).

Therefore, and as stated previously, the present results seem to indicate that a weak interaction exists between HD1 and HD2 of *P. rhodozyma*, potentially involving the N-terminal region of these proteins (as has been reported for heterothallic basidiomycete species, such as *C. neoformans* (Hull *et al*, 2005) and *U. maydis* (Kämper *et al*, 1995)), being the regulation of transcription through heterodimer formation the most probable mode of action of these proteins in sexual reproduction.

3.3. Molecular interactions of homothallism in the order Cystofilobasidiales: The role of HD1 and HD2 in *C. capitatum*

To find out to which extent the molecular interactions governing the homothallic behaviour of species in the order Cystofilobasidiales tended to resemble the findings for *P. rhodozyma*, a second homothallic species was selected to study the roles of the HD1 and HD2 proteins in sexual reproduction: *C. capitatum.*

In line with the studies performed concerning the molecular mechanisms of homothallism of *P. rhodozyma* (David-Palma *et al*, 2016), initial studies of the HD1 and HD2 proteins of *C. capitatum* pertained to their role in sexual reproduction, specifically their requirement for sporulation. With such objectives in mind, initial experiments involved testing the possibility to genetically transform *C. capitatum* through electroporation (with the main goal of generating *C. capitatum* deletion mutants of the *HD* locus) and the heterologous expression of the *HD* locus of *C. capitatum* in a *P. rhodozyma* cognate deletion mutant (to assess the capacity of the HD1 and HD2 proteins of *C. capitatum* to restore the sporulation ability of the *P. rhodozyma* mutant).

A second stage aimed at studying the interactions between the HD1 and HD2 proteins of *C. capitatum* and of *C. ferigula*, the latter species being the first heterothallic species to be studied in this respect in the Cystofilobasidiales. Taken together with the results already obtained for *P. rhodozyma*, these experiments will provide important insight into functioning of the HD proteins in homothallic vs heterothallic species in this order.

3.3.1. Testing the ability to genetically transform *C. capitatum*

As this study constituted the first attempt at genetically transforming *C. capitatum*, the same methodology as that used to genetically transform *P. rhodozyma* was attempted, since this is the closest related species to *C. capitatum* to have been reported to be transformable.

Therefore, and as described in the Materials and Methods section of this work, the methodology described by Visser *et al*, 2005, to transform *P. rhodozyma* was applied to test the transformation of *C. capitatum*.

Initially, a Minimum Inhibitory Concentration assay was performed to assess the resistance of *C. capitatum* strain CBS 7420 to the antifungal drugs commonly used as selective markers for *P. rhodozyma* transformants: geneticin, hygromycin B and zeocin. The plates were observed for up to 7 days to determine the ability of *C. capitatum* to grow in the presence of the above-mentioned antifungal drugs at four different concentrations. Figure 3.14 shows the results from the Minimum Inhibitory Concentration assay, after 7 days of incubation at 17ºC, revealing no growth in the presence of the

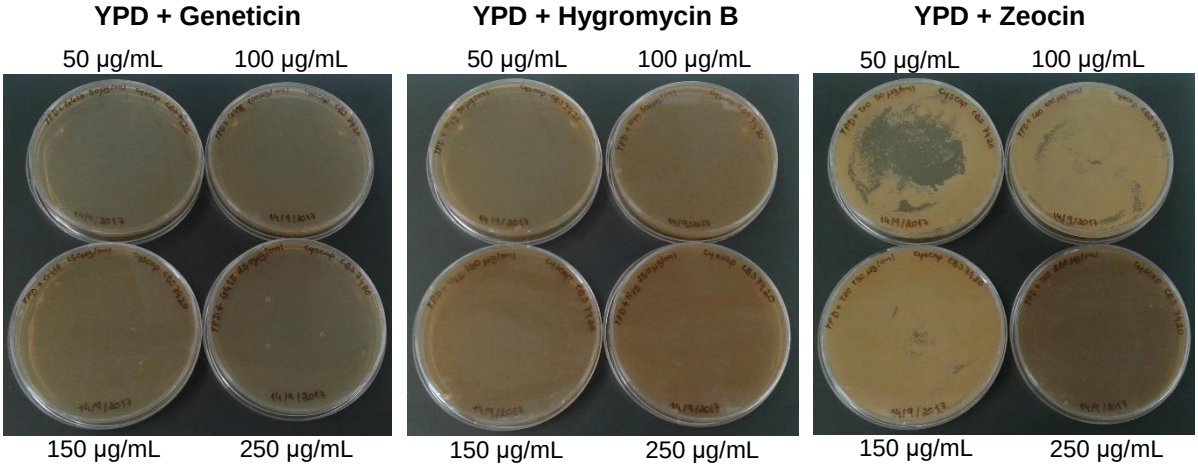


Figure 3.14: Minimum Inhibitory Concentration assay of the antifungal drugs geneticin, hygromycin B and zeocin for *C. capitatum* strain CBS 7420. After 7 days of incubation at 17ºC.

antifungal drugs geneticin and hygromycin B at concentrations as low as 50 µg/mL. In the presence of zeocin, however, *C. capitatum* seemed to be able to grow at concentrations of up to 150 µg/mL, with vestigial growth at 250 µg/mL. The apparent weaker growth at 50 µg/mL of zeocin could be due to an uneven spread of the cell suspension at the time of inoculation.

Given these results, a strategy to test the ability to transform *C. capitatum* was devised in order to obtain a *C. capitatum* mutant resistant to geneticin.

In this strategy, the pPR2TN plasmid (Verdoes *et al*, 1999) was used as a basis to generate a construction to be used in the transformation of *C. capitatum,* that allowed homologous recombination at the rDNA locus, allowing the insertion of a geneticin resistance cassette in multiple copies within the genome of this species. The original pPR2TN plasmid (Figure 3.15) contains an ampicillin resistance gene, a geneticin resistance cassette ($G418^R$) regulated by a *P. rhodozyma* promoter (Pgpd) and terminator (Tgpd), and a *P. rhodozyma* rDNA fragment of approximately 3000 bp.

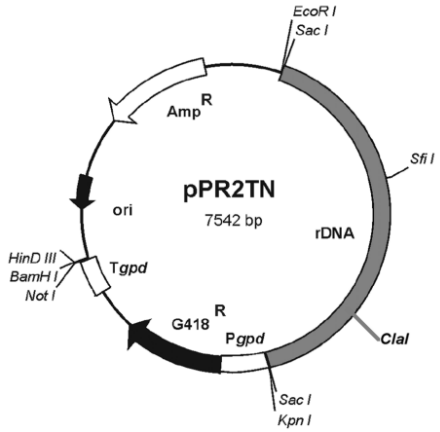


Figure 3.15: Schematic representation of the plasmid pPR2TN used for transformations of *P. rhodozyma*. Used as a basis for the recombinant plasmid used to assess the ability to transform *C. capitatum*. Figure adapted from Visser *et al*, 2005.

To perform the construction used to attempt the generation of a *C. capitatum* geneticin resistant mutant, the rDNA fragment of *P. rhodozyma* was initially excised from pPR2TN through an enzymatic digestion using Sac I. The resultant plasmid, henceforth referred to as pPR2TN', was then used to clone a rDNA fragment belonging to *C. capitatum* CBS 7420 using the same Sac I restriction site. The resulting recombinant plasmid was named pPR2TN'+AC025/AC026. The *C. capitatum* rDNA fragment comprised a 3036 bp region including the 18S and the 5' end of the 26S genes, with a single recognition site for the restriction enzyme Cla I, allowing for the linearization of pPR2TN'+AC025/AC026 with Cla I within rDNA fragment, prior to transformation of *C. capitatum*.

Two separate attempts to transform *C. capitatum* were performed using the strategy described above, the two varying in the amount of linearized pPR2TN'+AC025/AC026 used for transformation and the concentration of geneticin used in the transformant selective medium.

In a first attempt, 1.5 µg of linearized pPR2TN'+AC025/AC026 was used for transformation, being in line with the amount of DNA usually used for transformations in *P. rhodozyma* involving recombination at the rDNA locus (Wery *et al*, 1998).

Concerning the electroporation conditions, the only parameter where the values changed, compared to what is used with *P. rhodozyma*, was the time of the electric pulse. Whilst for *P. rhodozyma* it usually ranges from 18 to 20 ms (Wery *et al*, 1998; Rodriguez-Saíz *et al*, 2008), for *C. capitatum* it consisted of 10.7 and 9.22 ms for the actual transformation and for the negative control (strain CBS 7420 in the presence of sterile nuclease free water instead of DNA), respectively (Figure 3.16). This could be due to the morphology of the cells, as they differ both in size and shape when comparing *C. capitatum* with *P. rhodozyma* (Sampaio, 2011; Fell *et al*, 2011; Fell and Johnson, 2011).

The selective medium used in this first attempt contained geneticin at a concentration of 50 µg/mL, as this was the lowest concentration of the antifungal drug tested in the Minimum Inhibitory Concentration assay where *C. capitatum* CBS 7420 did not appear to grow. Where generally for *P. rhodozyma* (Visser

et al, 2005), after four to five days of incubation, transformant colonies are already visible to the naked eye, for *C. capitatum* no colonies were present in neither of the plates corresponding to the transformation (CBS 7420 + pPR2TN'+AC025/AC026) after six days of incubation at 17ºC.
Incubation was prolonged at the same temperature, and after nineteen days colonies were present in all the plates corresponding to the transformation, as well as in the negative control. The colonies in the transformation plates and in the negative control were very similar in both size and number. Replica plating was performed for all the transformation and negative control plates in the same selective medium, and all colonies presented growth, including the ones in the negative control, as early as after two days of incubation at 17ºC (data not shown).

Transformation	Internal Resistance	Electric Pulse	Capacitance	Time of Pulse
CBS 7420 **+** pPR2TN'+AC025/AC026	1000 Ω	0.8 kV	25 µF	10.7 ms
Negative Control (CBS 7420 + H_2O)	1000 Ω	0.8 kV	25 µF	9.22 ms

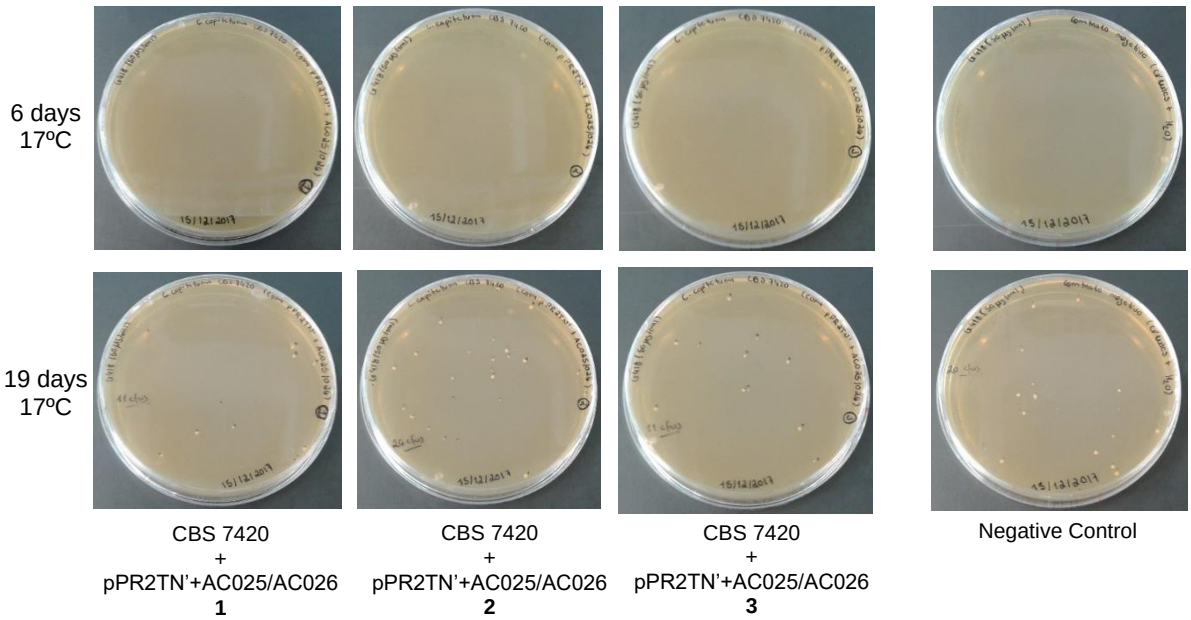


Figure 3.16: Results from the first attempt at generating a *C. capitatum* mutant resistant to geneticin. Electroporation parameters used are indicated in the table above, with the transformation results in YPD medium supplemented with geneticin depicted below, after 6 and 19 days of incubation at 17ºC.

These results seem to indicate the existence of individuals within the population that are naturally more resistant to geneticin, having a delayed growth in medium supplemented with this antifungal drug, at the tested concentration, since the colonies in the negative control were still able to grow after replica plating. However, this growth could also be due to the degradation of geneticin over time, as the time of incubation was much longer than what is necessary when transforming *P. rhodozyma.*
Failure to obtain *C. capitatum* transformants resistant to geneticin in this first attempt could, certainly, be due to many variables throughout the transformation protocol, as it is optimized for *P. rhodozyma.*

These variables could be, among others, the amount of DNA used for transformation, the concentration of geneticin being used in the selective medium or the electroporation parameters.

Concerning the concentration of geneticin in the selective medium, since 50 µg/mL was the lowest concentration tested in the Minimum Inhibitory Concentration assay and no growth was observed for *C. capitatum* CBS 7420, this was selected as the concentration to use in the first transformation attempt. However, this concentration could still be much higher than the minimum inhibitory concentration of geneticin for *C. capitatum*, and that could be at the basis for the reason why potential transformants did not start growing earlier, and eventually ended up growing at the same rate as potential spontaneous geneticin resistant colonies. Given this possibility, a similar process as that used for the Minimum Inhibitory Concentration assay was performed, using lower concentrations of geneticin in the selective medium, to try to understand if lower concentrations were still sufficient to inhibit the growth of *C. capitatum.*

In line with what was above mentioned, to try to understand if some of these variables could be affecting the transformation, a second attempt at transforming *C. capitatum* was performed, where the amount of linearized pPR2TN'+AC025/AC026 used for transformation was increased (3.3 µg) and the concentration of geneticin in the selective medium was decreased to 40 µg/mL. Results of the second transformation attempt can be analysed in Figure 3.17.

Transformation	Internal Resistance	Electric Pulse	Capacitance	Time of Pulse
CBS 7420 **+** pPR2TN'+AC025/AC026	1000 Ω	0.8 kV	25 µF	10.0 ms
Negative Control (CBS 7420 + H_2O)	1000 Ω	0.8 kV	25 µF	9.8 ms

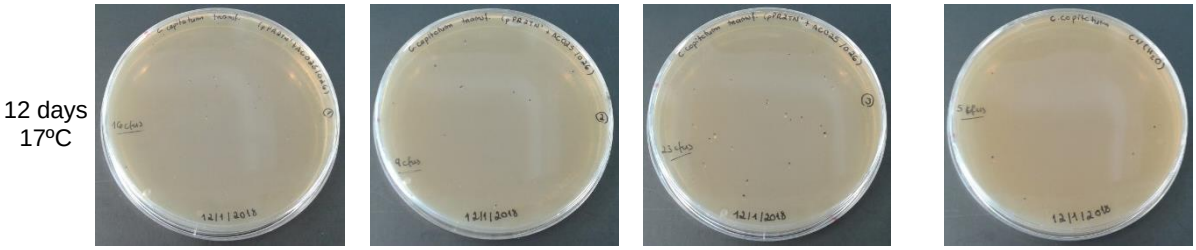


Figure 3.17: Results from the second attempt at generating a *C. capitatum* mutant resistant to geneticin. Electroporation parameters used are indicated in the table above, with the transformation results in YPD medium supplemented with geneticin depicted below, after 12 days of incubation at 17ºC.

As with in the first attempt, the time of the pulse obtained was still much lower than what would be expected for *P. rhodozyma* (10.0 and 9.8 ms for the transformation and for the negative control, respectively). Even with the reduction of the concentration of geneticin in the selective medium, colonies still exhibited delayed growth as compared to what is expected for *P. rhodozyma* transformants, and only after twelve days of incubation at 17ºC were the colonies visible to the naked eye. Colonies were, however, present in both transformation and negative control plates in similar numbers, again

suggesting an unsuccessful transformation. Some of the colonies in the transformation plates were, however, larger sized than those obtained in the negative control suggesting that these had started growing before those in the negative control. So, in order to confirm if these colonies corresponded to successful recombinant transformants, six of these were selected for DNA extraction and PCR confirmation of the presence of the geneticin resistance cassette.

As observed in Figure 3.18, none of the tested colonies correspond to recombinant transformants, as the pattern of unspecific bands amplified is equivalent to that of *C. capitatum* strain CBS 7420.

As stated above, the results obtained for both attempts seem to suggest that, within the population of *C. capitatum*, some individuals may be more resistant to geneticin in the medium, being able to grow at the tested concentration in a period of twelve days of incubation, which was not tested in the Minimum Inhibitory Concentration assay. However, it is also possible that some individuals within the population spontaneously gained resistance to geneticin, eventually through spontaneous mutation. The possible degradation of geneticin in this period of time could also be a factor, allowing non-transformants to start growing.

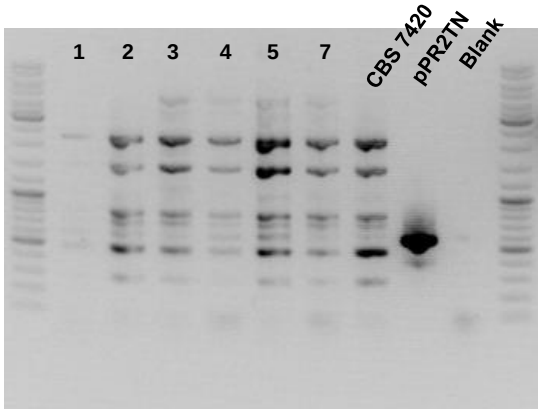


Figure 3.18: Confirmation of the potential *C. capitatum* transformants. Results from the PCR amplification of the geneticin resistance gene, present in the pPR2TN'+AC025/AC026 recombinant plasmid. Primers used: MP091 and MP092. Expected fragment size: 587 bp.

No other attempts were performed at transforming *C. capitatum.* However, it would be relevant to perform more attempts to try to understand if the transformation of *C. capitatum* is possible.

Concerning potential variables to consider when testing the ability to transform *C. capitatum* once again, a more profound study on the effects of the protocol used to prepare electrocompetent cells in *C. capitatum* could be relevant. Although *P. rhodozyma* is phylogenetically close to *C. capitatum*, their morphologies vary, and the slight variations present in the cell wall composition of both these species (Sampaio, 2011; Fell *et al*, 2011; Fell and Johnson, 2011) could eventually be sufficient to allow *P. rhodozyma* to become electrocompetent through the methodology described by Visser *et al*, 2005, but not so for *C. capitatum.*

Also considering the variations in morphology, the electroporation parameters may not be correctly adjusted for the transformation of *C. capitatum.* As observed in the obtained results from both attempts at transforming *C. capitatum* (Figures 3.16 and 3.17), the time of the pulse (the only electroporation parameter that cannot be set) was already quite distinct from what is expected when transforming *P. rhodozyma*. As these parameters seem to vary greatly considering the transformation through electroporation of other yeast species, testing with different values of the internal resistance, the voltage of the electric pulse or the capacitance could be key to improve the possibilities of successfully transforming *C. capitatum* through electroporation.

It would indeed be quite valuable to investigate the ability to genetically transform *C. capitatum* in a more in-depth way. In case a procedure to successfully transform *C. capitatum* can be developed, a more profound and thorough study of the molecular pathways and interactions governing this species' homothallic sexual behaviour could be achieved. For instance, as previously performed in the study of the homothallic sexual behaviour of *P. rhodozyma* (David-Palma *et al*, 2016), the generation of knock out mutants of the *MAT* loci in *C. capitatum* would be of great value and a much more direct way to better understand the roles of not only the HD1 and HD2 proteins but also those of the pheromone and pheromone receptor.

3.3.2. Heterologous expression of the *HD1* and *HD2* genes of *C. capitatum* in a *P. rhodozyma* cognate deletion mutant

As the transformation of *C. capitatum* CBS 7420 through electroporation was not successful, a new approach was taken in order to try to understand if the HD1 and HD2 proteins of *C. capitatum* have a functional role in sexual reproduction.

This new approach involved the heterologous expression of the *HD1* and *HD2* genes of *C. capitatum* in a *P. rhodozyma* deletion mutant of the *HD* locus. As previously shown (David-Palma *et al*, 2016), both HD1 and HD2 proteins are required for sexual reproduction in *P. rhodozyma*, where a deletion mutant of the *HD* locus (knock out of both *HD1* and *HD2* genes) loses the ability to sporulate. So, considering these findings, after complementing a *P. rhodozyma* deletion mutant of the *HD* locus with the *HD1* and *HD2* genes of *C. capitatum*, results from a sporulation assay should reveal the ability of such a complementation mutant to restore sporulation.

3.3.2.1. Generation of a *P. rhodozyma* deletion mutant of the *HD* locus

Initially, the strategy to generate a *P. rhodozyma* deletion mutant of the *HD* locus was devised, involving the interruption of the *HD* locus with a zeocin resistance cassette. This interruption would then lead to the deletion of the promoters and 5' ends of both *HD1* and *HD2* genes, up to part of the homeodomain region. A schematic representation of the resulting *HD* locus of the generated deletion mutant (*hd1hd2*Δ::Zeo) is present in Figure 3.19.A, where in the absence of both the promoters and the 5' ends of these genes, no expression of *HD1* and *HD2* is expected.

The construction of the deletion cassette was performed using pJET1. 2/blunt plasmid (Figure 3.19.B). Throughout the construction of the deletion cassette used to transform *P. rhodozyma*, some difficulties arose in regard to the confirmation of the correct cloning of the zeocin resistance cassette interrupting the *HD1/HD2* fragment, as (after performing a local alignment with the DNA sequence of the zeocin resistance cassette) the primers used to amplify the *HD1/HD2* fragment (primers AC027 and AC028) seemed to be hybridizing in an unspecific way within the zeocin resistance cassette (data not shown), possibly leading to the amplification of a fragment of approximately 700 bp instead of the expected *HD1/HD2* interrupted fragment of approximately 3000 bp (Figure 3.20.A). However, the zeocin resistance cassette seemed to be present within the recombinant plasmid (Figure 3.20.B). Therefore,

and to confirm that the fragments of interest were correctly cloned, two different confirmations were performed.

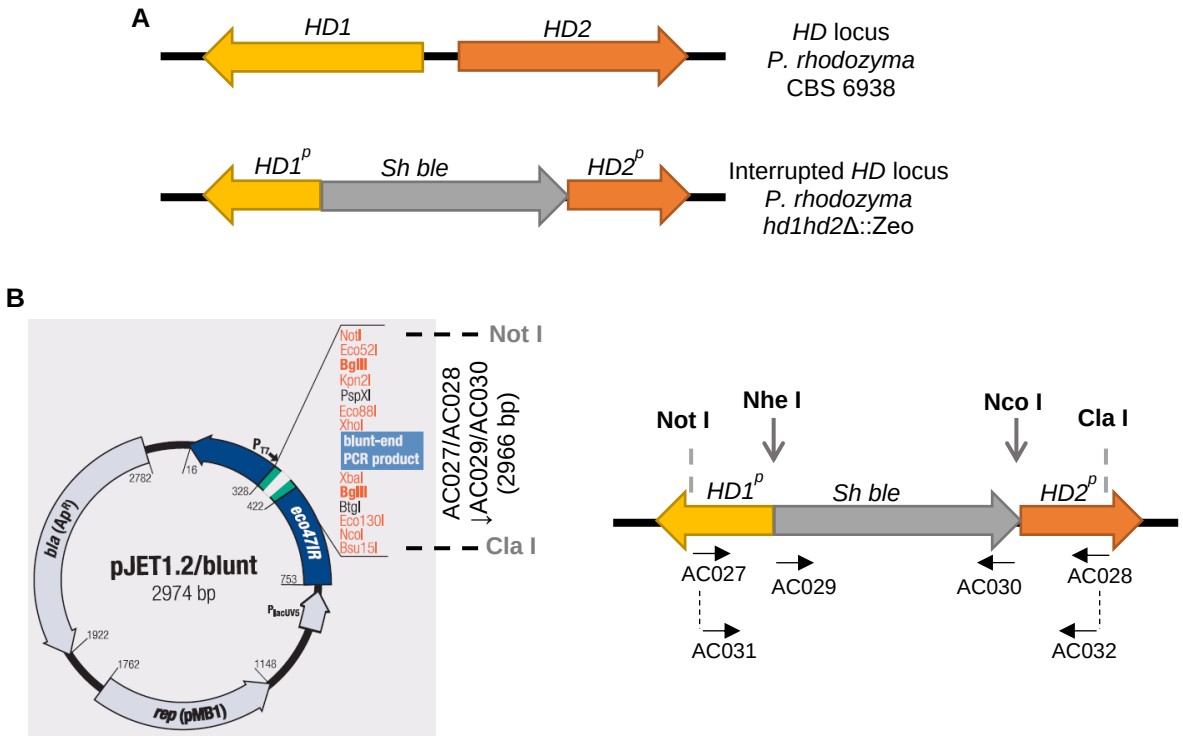


Figure 3.19: Schematic representation of the strategy used to generate the *P. rhodozyma* deletion mutant of the *HD* locus: *hd1hd2*Δ::Zeo. A) Representation of the interrupted *HD* locus in the genome of *hd1hd2*Δ::Zeo, where $HD1^{p}$ and $HD2^{p}$ correspond to the partial *HD1* and *HD2* genes and *Sh ble* corresponds to the zeocin resistance cassette. The original *HD* locus of *P. rhodozyma* strain CBS 6938 is also represented. B) Representation of the construction of the recombinant plasmid pJET1.2+AC027/AC028↓AC029/AC030, constructed for the generation of the deletion cassette AC031/AC032. Relevant restriction sites and primers are indicated in the figure. pJET1.2/blunt figure was adapted from *Thermo Scientific CloneJET PCR Cloning Kit* user manual.

First, a digestion of the potential recombinant plasmid was performed using restriction enzymes Cla I and Not I, initially used to clone the *HD1/HD2* fragment in pJET1.2. From this digestion, the presence of a single band of approximately 3000 bp after electrophoresis in an agarose gel was expected, given the correct cloning of the fragments (corresponding to the *HD1/HD2* interrupted fragment with 2966 bp, and the remaining pJET1.2 plasmid with 2974 bp). Given the already demonstrated presence of the zeocin resistance cassette in the recombinant plasmid, were the *HD1/HD2* and the zeocin resistance cassette fragments not correctly cloned, fragments of different sizes from those expected would arise from the double digestion.

Second, nested primers designed for the amplification of the deletion cassette used in the transformation of *P. rhodozyma* (primers AC031 and AC032) were used with the recombinant plasmid as template to confirm the correct assembly of the construction, as the resulting fragment would be distinguishable through electrophoresis in agarose gel when compared to the amplification with these same primers using the genomic DNA of *P. rhodozyma* strain CBS 6938 as template (deletion cassette would be 2859 bp, while using the genomic DNA as template would amplify a fragment with 3373 bp).

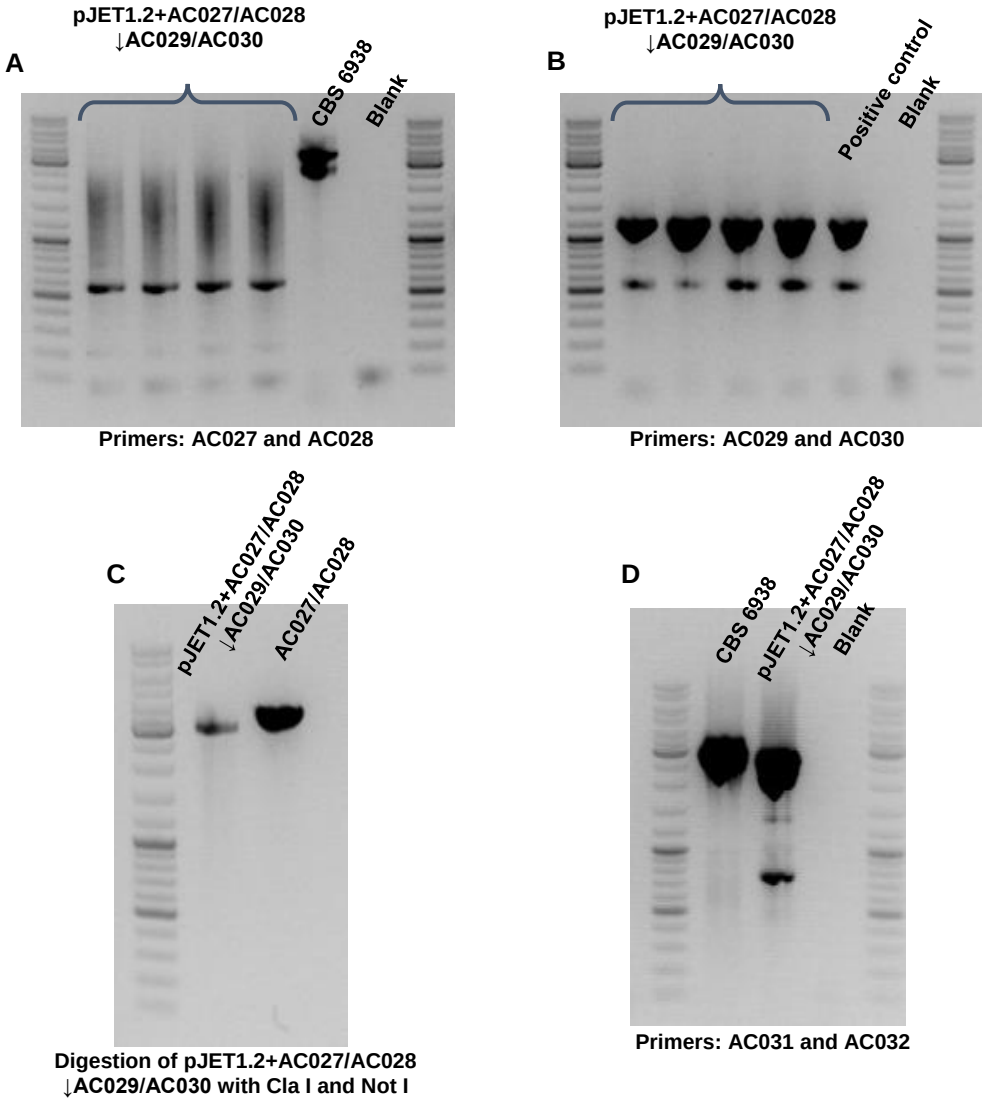


Figure 3.20: Confirmation of the construction of deletion cassette AC031/AC032, cloned in the recombinant plasmid pJET1.2+AC027/AC028↓AC029/AC030. All amplified and digested fragments were analysed by electrophoresis in agarose gel. A) Amplification of the *HD1/HD2* fragment used in the first cloning step. Expected fragment size: 3480 bp for CBS 6938 and 2966 bp for pJET1.2+AC027/AC028↓AC029/AC030. B) Amplification of the zeocin resistance cassette, where the positive control corresponds to the original plasmid used to amplify the zeocin resistance cassette (pJET1.2+Zeo). Expected fragment size: 1181 bp. C) Digestion of pJET1.2+AC027/AC028↓AC029/AC030 with the restriction enzymes Cla I and Not I, where AC027/AC028 corresponds to the *HD1/HD2* fragment as amplified using primers AC027 and AC028 with the genome of strain CBS 6938 as template. D) Amplification of the deletion cassette AC031/AC032, using nested primers AC031 and AC032. Expected fragment size: 3373 bp for CBS 6938 and 2859 bp for pJET1.2+AC027/AC028↓AC029/AC030.

As can be observed through the results of the agarose gels in Figures 3.20.C and 3.20.D, the fragments seem to be correctly cloned in pJET1.2, providing the confirmation that the deletion cassette, corresponding to the *HD* locus interrupted by a zeocin resistance cassette, was correctly assembled.

P. rhodozyma strain CBS 6938 was then used for transformation by electroporation, using the constructed deletion cassette. Obtained transformants were confirmed by DNA extraction and PCR amplification of the zeocin resistance cassette and of the complete *HD1* and *HD2* genes. Although the

amplification of the zeocin resistance cassette was quite faint or even absent for some of the tested transformants (Figure 3.21.A), the fact that these transformants were still able to grow in YPD medium supplemented with zeocin at 100 µg/mL after several passages allowed to confirm the acquisition of resistance to zeocin of these transformants. In addition, the results of the amplification using specific primers for the complete *HD1* and *HD2* genes of *P. rhodozyma* revealed the absence of amplification of the fragments corresponding to these genes in all tested transformants (Figure 3.21.B and 3.21.C)

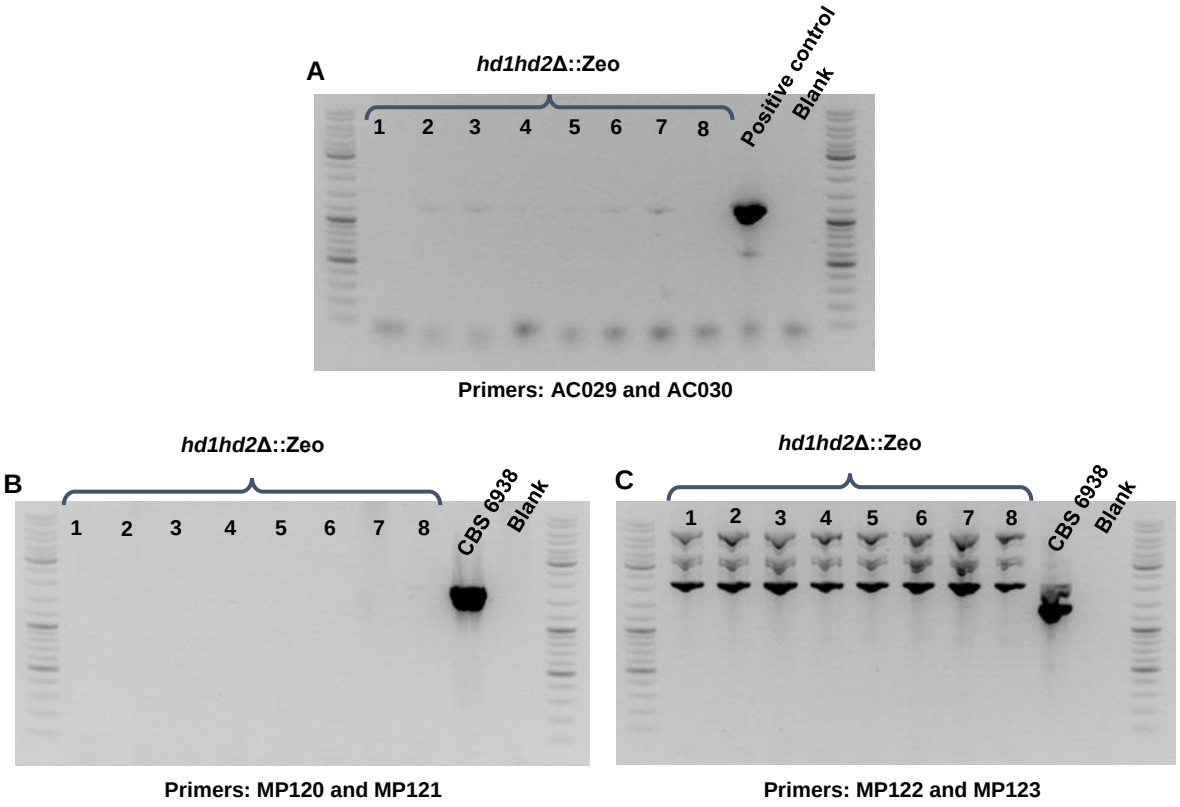


Figure 3.21: Confirmation of the generated *hd1hd2*Δ::Zeo mutants. All amplified fragments were analysed by electrophoresis in agarose gel. A) Amplification of the zeocin resistance cassette, where the positive control corresponds to the original plasmid used to amplify the zeocin resistance cassette (pJET1.2+Zeo). Expected fragment size: 1181 bp. B) Amplification of the *HD1* complete gene of *P. rhodozyma.* Expected fragment size: 1861 bp for strain CBS 6938. C) Amplification of the *HD2* complete gene of *P. rhodozyma*. Expected fragment size: 1459 bp for strain CBS 6938.

as was expected for a successful transformation, given the deletion of the 5' ends of both *HD1* and *HD2* genes. These results supported the conclusion that *P. rhodozyma* mutant *hd1hd2*Δ::Zeo was successfully generated.

A sporulation assay was then performed, using one of the transformants obtained (transformant 7, in Figure 3.21), to confirm the inability of the *P. rhodozyma* mutant *hd1hd2*Δ::Zeo to sporulate. Sporulation plates were observed for up to thirty days of incubation at 17ºC to search for basidia and basidiospores, and after this period of time none of these structures were observed for the *hd1hd2*Δ::Zeo in DWR+0.5% ribitol. The results of the sporulation assay for the mutant *hd1hd2*Δ::Zeo are presented in Figure 3.22, as observed with an optical microscope after twenty and thirty days of incubation at 17ºC, with *P. rhodozyma* strain CBS 6938 as a positive control of sporulation.

As no sporulation was observed for *P. rhodozyma* mutant *hd1hd2*Δ::Zeo, this generated mutant was then used as host to test the ability of the HD1 and HD2 proteins of *C. capitatum* to complement the absence of their *P. rhodozyma* counterparts.

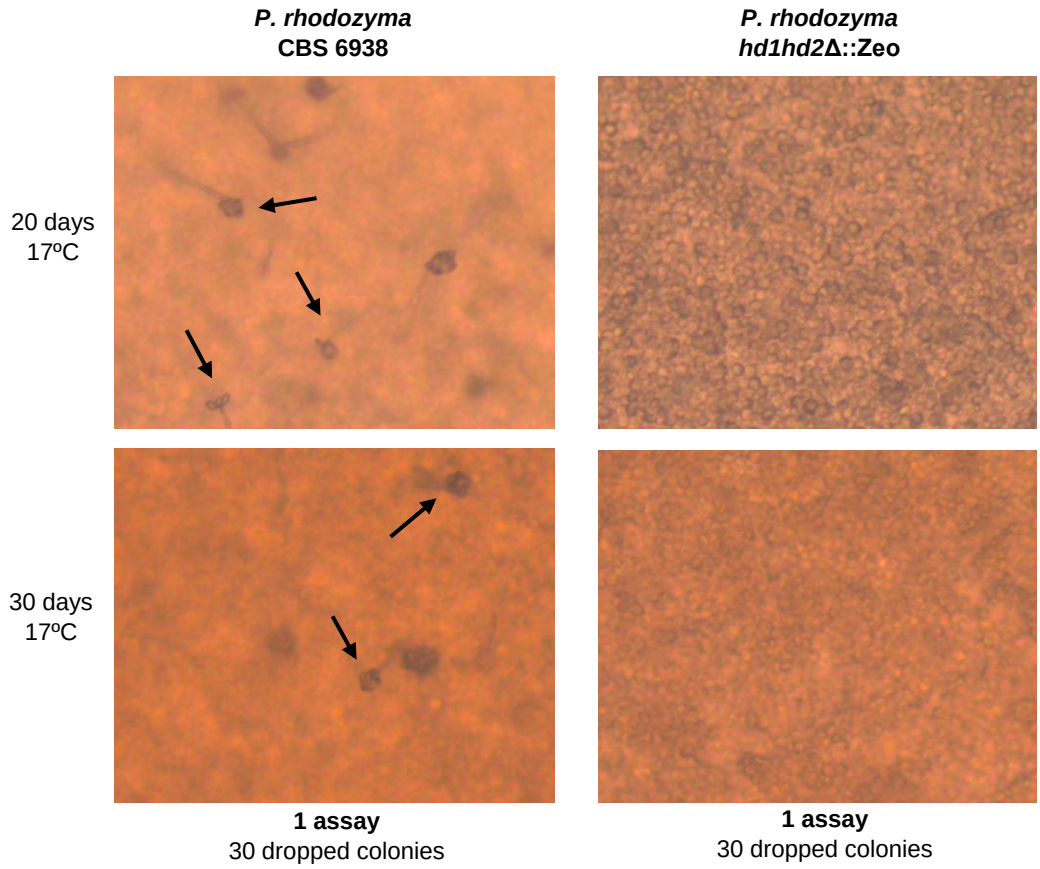


Figure 3.22: Sporulation assay of *P. rhodozyma* deletion mutant *hd1hd2*Δ::Zeo. Results correspond to the sporulation plates as observed from the top of the colonies with the optical microscope, with an amplification of 150x, for both the *hd1hd2*Δ::Zeo mutant and *P. rhodozyma* strain CBS 6938, after 20 and 30 days of incubation at 17ºC. Black arrows point to some of the basidia and basidiospores present in the *P. rhodozyma* strain CBS 6938 sporulation plate.

3.3.2.2. Complementation of a *P. rhodozyma hd1hd2*Δ::Zeo mutant by cognate *C. capitatum* genes

Heterologous expression of the *HD* locus of *C. capitatum* in the *P. rhodozyma hd1hd2*Δ::Zeo mutant provides a means to study the roles of the HD1 and HD2 proteins of *C. capitatum* in sexual reproduction, by assessing their ability to restore sporulation in the *P. rhodozyma* mutant *hd1hd2*Δ::Zeo.

A strategy was devised to transform *P. rhodozyma* mutant *hd1hd2*Δ::Zeo with the *HD1* and *HD2* genes of *C. capitatum* strain CBS 7420, using plasmid pPR2TN (Verdoes *et al*, 1999) to clone the whole *HD* locus of strain CBS 7420. The resulting recombinant plasmid, named pPR2TN+AC033/AC034, can be linearized to allow transformation of *hd1hd2*Δ::Zeo by integration of the plasmid at the rDNA locus.

The construction of the recombinant plasmid pPR2TN+AC033/AC034 used for the complementation of *hd1hd2*Δ::Zeo is schematically shown in Figure 3.23.

As the genomic sequence of the *HD* locus of *C. capitatum* strain CBS 7420 (Figure 6.2, in Appendix) contains a recognition site for the Cla I restriction enzyme, the recombinant plasmid pPR2TN+AC033/AC034 could not be linearized using this enzyme. Therefore, the plasmid was initially digested with restriction enzyme Sfi I for linearization in the rDNA fragment. However, and after several

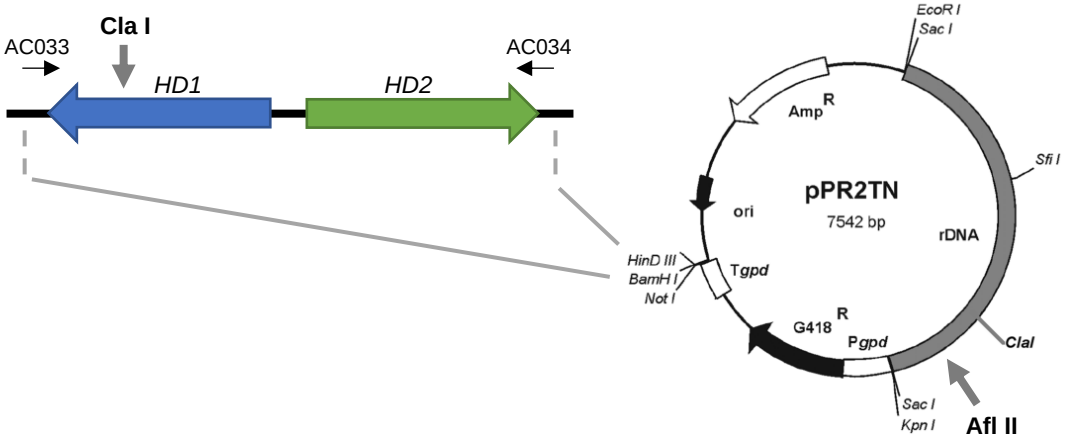


Figure 3.23: Schematic representation of the strategy used to generate the *P. rhodozyma* complementation mutant *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA. The figure depicts the cloning of the *HD* locus of *C. capitatum* strain CBS 7420 in the pPR2TN plasmid at the HinD III and BamH I sites, generating the recombinant plasmid pPR2TN+AC033/AC034 used for transformation of the *P. rhodozyma* mutant *hd1hd2*Δ::Zeo through recombination at the rDNA locus. pPR2TN figure adapted from Visser *et al*, 2005.

attempts, pPR2TN+AC033/AC034 could never be linearized through digestion with Sfi I. As observed in Figure 3.24.A, after digestion of both pPR2TN and pPR2TN+AC033/AC034, no linearization seems to have occurred, which could be due to a poor performance of Sfi I in digesting the plasmids or potentially the loss of its restriction site in pPR2TN. However, as neither of the plasmids were sequenced, the reason for the lack of linearization with Sfi I could not be confirmed.

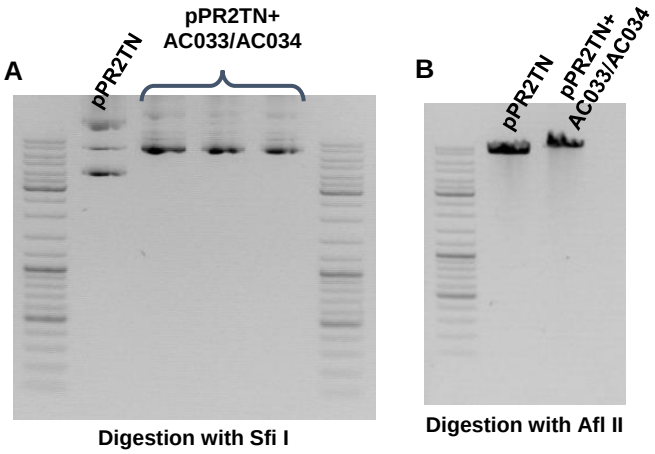


Figure 3.24: Confirmation of the linearization of plasmid pPR2TN+AC033/AC034. Results from the digestions were analysed by electrophoresis in agarose gel, where pPR2TN was used as control of the linearization. A) Enzymatic digestion of pPR2TN and of pPR2TN+AC033/AC034 with Sfi I. B) Enzymatic digestion of pPR2TN and of pPR2TN+AC033/AC034 with Afl II.

As a unique restriction site for the enzyme Afl II is present at the rDNA sequence of plasmid pPR2TN (Figure 3.23), this enzyme was selected to linearize pPR2TN+AC033/AC034. Confirmation of the linearization through digestion with Afl II is presented in Figure 3.24.B.

The linearized pPR2TN+AC033/AC034 plasmid was successfully used to transform the *P. rhodozyma* mutant *hd1hd2*Δ::Zeo through electroporation. The generated *P. rhodozyma* transformant, containing the *HD* locus of *C. capitatum* strain CBS 7420, was named *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA. In Figure 3.25, all the PCR confirmations for the presence of the genes of interest are shown, having as the negative control a reaction using *hd1hd2*Δ::Zeo genomic DNA as template. All three tested

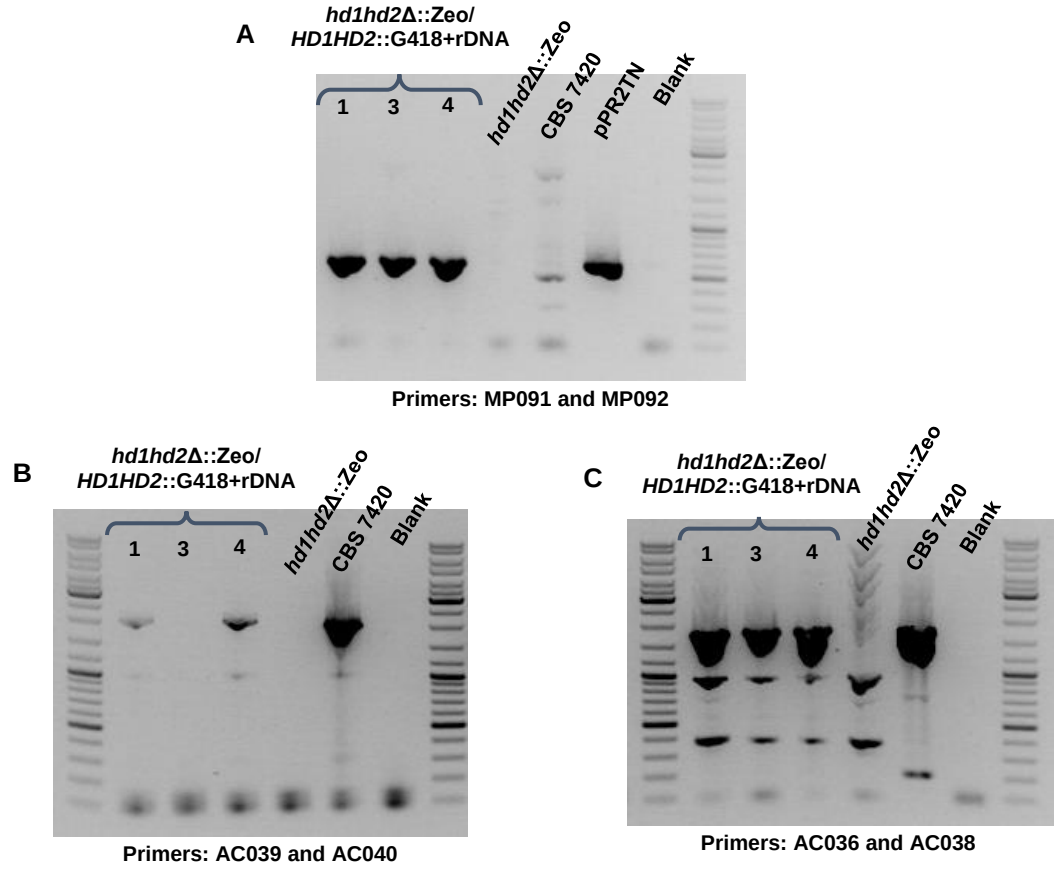


Figure 3.25: Confirmation of the generated *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA complementation mutants. All amplified fragments were analysed by electrophoresis in agarose gel. A) Amplification of the geneticin resistance gene, using the genomic DNA of *hd1hd2*Δ::Zeo and of CBS 7420 as negative controls of amplification. Expected fragment size: 587 bp. B) Amplification of the *HD1* complete gene of *C. capitatum.* Expected fragment size: 1957 bp. C) Amplification of the *HD2* complete gene of *C. capitatum*. Expected fragment size: 1729 bp.

transformants presented amplification of the geneticin resistance gene (Figure 3.25.A) and of the complete *HD2* gene of *C. capitatum* (Figure 3.25.C). However, one of the tested *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA transformants did not present amplification of the *HD1* complete gene of *C. capitatum* (Figure 3.25.B). Given the presence of both the geneticin resistance cassette and the *HD2* complete gene of *C. capitatum* in the tested *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA transformant number 3, it seems unlikely that the transformation was unsuccessful, and this result could be due to possible manipulation errors when performing PCR for the amplification of the *HD1* gene of *C. capitatum.* Transformants 1 and 4 were, however, considered successful, and *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA transformant number 1 was used for the sporulation assay.

The use of the whole *HD* locus of *C. capitatum*, exactly as is present in the genome of strain CBS 7420, to complement the *P. rhodozyma* mutant *hd1hd2*Δ::Zeo, could be perceived as a rather ambitious strategy, as this constitutes the first attempt at expressing *C. capitatum* genes in *P. rhodozyma.* Although the two species are closely related, no previous studies exist to shed any light regarding the ability of *P. rhodozyma* to recognize promoters nor terminators of *C. capitatum* or the ability to correctly process the introns present in the genes of this species. Therefore, it was possible that *P. rhodozyma* would not be able to correctly express the *HD1* and *HD2* genes of *C. capitatum*, in which case a possible inability of the complementation mutant to restore sporulation could potentially be the result of the above-mentioned factors and not a direct indication of the role of the HD1 and HD2 proteins of *C. capitatum* in sexual reproduction. Moreover, a considerable divergence exists between the *HD* genes of *P. rhodozyma* and those of *C. capitatum*, as has been mentioned in Section 3.1. Since their sequences are considerably different, the DNA binding sites of the HD1 and HD2 proteins of *C. capitatum* may not be able to correctly recognize the promoter elements of the *P. rhodozyma* genes that are regulated by the complex formed by the homeodomain transcription factors during this species' sexual cycle. Considering these factors, the results from the sporulation assay for mutant *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA, presented in Figure 3.26, are quite promising.

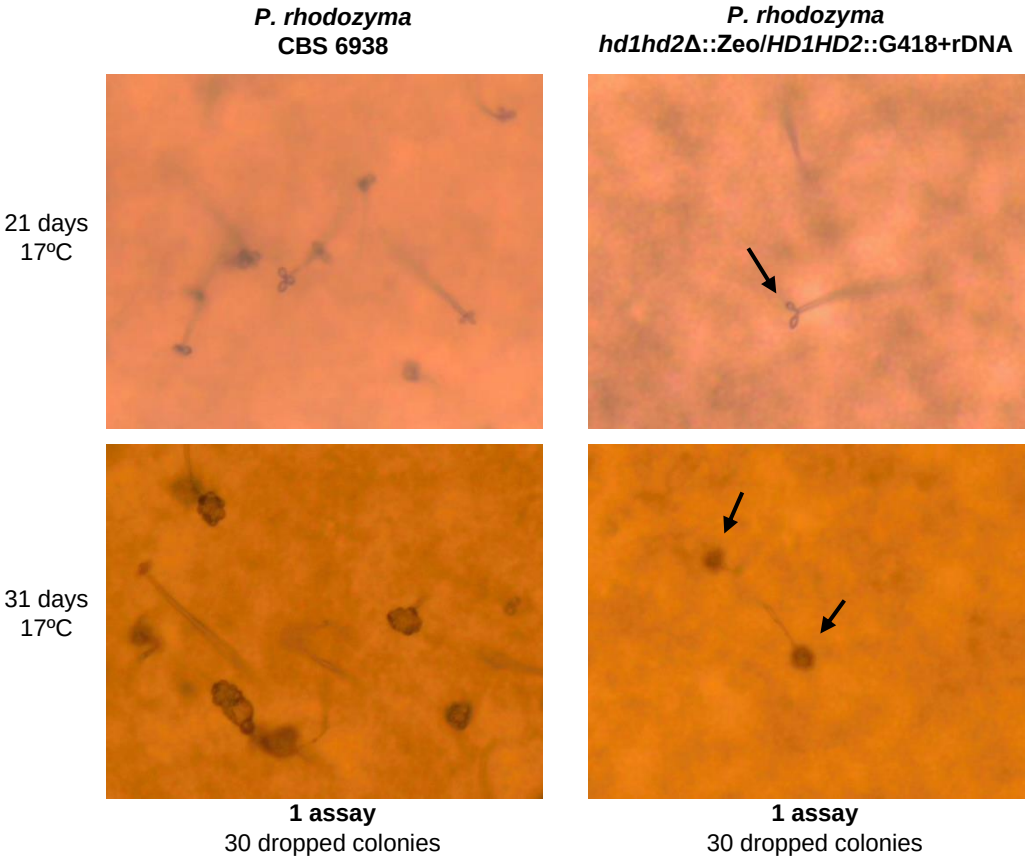


Figure 3.26: Sporulation assay of *P. rhodozyma* complementation mutant *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA. Results correspond to the sporulation plates as observed from the top of the colonies with the optical microscope, with an amplification of 150x for both the *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA mutant and *P. rhodozyma* strain CBS 6938, after 21 and 31 days of incubation at 17ºC. Black arrows point to the basidia and basidiospores present in the *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA mutant sporulation plate.

Indeed, after twenty-one days of incubation at 17ºC in DWR+0.5% ribitol, basidia and basidiospores are visible for the *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA mutant, although not in the same proportion as observed for wild-type strain CBS 6938 of *P. rhodozyma*. This proportion is maintained after thirty-one days of incubation (Figure 3.26).

Some conclusions can be taken from these results. On the one hand, *P. rhodozyma* seems to be able to correctly recognize the promoters and terminators of *C. capitatum*, allowing the expression of this species' *HD1* and *HD2* genes. As sporulation, although not as efficient as in the wild-type *P. rhodozyma* strain CBS 6938, was restored in the complemented mutant, it can also be deduced that not only are the *HD1* and *HD2* genes of *C. capitatum* being expressed but also that the introns in their corresponding mRNA are being correctly processed, allowing functional HD1 and HD2 proteins to be produced.

Therefore, the HD1 and HD2 proteins of *C. capitatum* seem to be functional, allowing the sexual cycle of *P. rhodozyma* to be completed in a mutant lacking its original *HD* locus, which in turn leads to the assumption that these proteins possess a similar mode of action in sexual reproduction as those of *P. rhodozyma*, with the ability to correctly recognise the promoter elements of the genes involved in the later stages of the sexual cycle of *P. rhodozyma* and regulate their expression.

It is important to note that, although the HD1 and HD2 proteins of *C. capitatum* seem to be able to restore sporulation in a *P. rhodozyma* deletion mutant, this does not necessarily mean that these proteins would have an essential role in the sexual cycle of *C. capitatum*. As a homothallic species, it is possible that *C. capitatum* may have constitutively active pathways, or even bypasses to the HD1/HD2 regulated pathway. Also, in the results from the heterologous expression of the *HD* locus of *C. capitatum* in a *P. rhodozyma* mutant presented in this study, both HD1 and HD2 proteins are being considered. The possibility of only one of the HD proteins of *C. capitatum* being sufficient to allow sporulation must also be considered (eventually similarly to what has been reported for *P. rhodozyma* concerning the HD1 protein (David-Palma *et al*, 2016)), not having been tested in the present study.

Regardless of the above-mentioned factors, the present results allow the following conclusions: one or both of the HD proteins of *C. capitatum* is involved in the reposition of sporulation in a *P. rhodozyma* deletion mutant of the *HD* locus, which in turn leads to the assumption that these proteins have the potential to be involved in the homothallic sexual cycle of *C. capitatum,* eventually through a similar mode of action as that described for the HD proteins of *P. rhodozyma* (David-Palma *et al*, 2016).

3.3.3. Yeast Two-Hybrid assay: Assessing the interaction between the complete HD1 and HD2 proteins of *C. capitatum* and establishing a comparison with a heterothallic species from the order Cystofilobasidiales

Following the results of the heterologous expression of *HD1* and *HD2* genes of *C. capitatum* in a *P. rhodozyma* cognate deletion mutant (Section 3.3.2), a Yeast Two-Hybrid assay involving the complete HD1 and HD2 proteins of *C. capitatum* was performed to determine the existence of interaction between these proteins, so as to understand if heterodimerization would be at the basis of the role of these proteins in sexual reproduction. As the Yeast Two-Hybrid assay is expressed in *S. cerevisiae*, the fragments corresponding to the CDS of the *HD1* and *HD2* genes of *C. capitatum* had first to be obtained,

through RNA extraction and cDNA synthesis, to be used for the transformation of the *S. cerevisiae* strains of the Yeast Two-Hybrid system.

In parallel, and to establish a comparison with the expected interaction between the HD1 and HD2 proteins of a heterothallic species from the order Cystofilobasidiales, the Yeast Two-Hybrid system was also used to test the interaction between the HD1 and HD2 proteins from two *C. ferigula* strains of different mating-types (HD1 from PYCC 5628 and HD2 from PYCC 4410), using synthetic *HD1* and *HD2* genes to perform the transformations of the *S. cerevisiae* strains of the Yeast Two-Hybrid assay.

3.3.3.1. Obtention of the CDS of *HD1* and *HD2* of *C. capitatum*

Taking into consideration the stage of a basidiomycete's life cycle when the genes encoding the homeodomain transcription factors are usually more actively being expressed (Raudaskoski and Kothe, 2010), a strategy was devised in order to perform a total RNA extraction during the hyphal stage of the sexual cycle of *C. capitatum.*

C. capitatum has been described as having the ability to sporulate in CMA (Corn Meal Agar) medium (Sampaio, 2011). However, as it would be experimentally more convenient to perform RNA extraction from a liquid culture, *C. capitatum* was tested for its ability to sporulate in two other minimal media with the potential to induce sporulation in basidiomycetes - GSA and MYP – as these could be prepared in a liquid form.

C. capitatum strain CBS 7420 was initially tested for sporulation in GSA and MYP solid media, being also inoculated in CMA medium as a positive control for sporulation. However, after twenty-seven days of incubation at 17ºC, no teliospores were observable in either of the three media tested, as can be observed in Figure 3.27 for CMA and GSA solid medium.

C. capitatum strain CBS 7420 was originally classified as *Leucosporidium lari-marini* (Saëz and Nguyen, 1989). It was later classified as *Cystofilobasidium lari-marini,* through analysis of rRNA sequences (Fell and Statzell-Tallman, 1992), and then finally re-classified as *C. capitatum*, after analysis of microsatellite-primed PCR fingerprints and DNA-DNA reassociation experiments (Sampaio *et al*, 2001b). Strain CBS 7420 has, however, been described as being able to produce teliospores in CMA (Fell and Statzell-Tallman, 1992) and, therefore, the fact these were not observable after twenty-seven days of incubation was not an expected result. It could be possible that this strain takes a longer period of time to sporulate in CMA than what is usually expected for *C. capitatum* (Sampaio, 2011).

Two other *C. capitatum* strains (PYCC 5626 and A 301) were tested for sporulation in all three of the above-mentioned media. After twelve days of incubation at 17ºC, teliospores were observed for strain PYCC 5626 in all tested media (Figure 3.27, for results in CMA and GSA).

For strain A 301, however, after twelve days of incubation, no teliospores were observed in either of the tested media (Figure 3.27, for results in CMA and GSA). Still, after twenty-one days of incubation, some teliospores were present in all media, although not to the extent that was observed for PYCC 5626 after twelve days (data not shown).

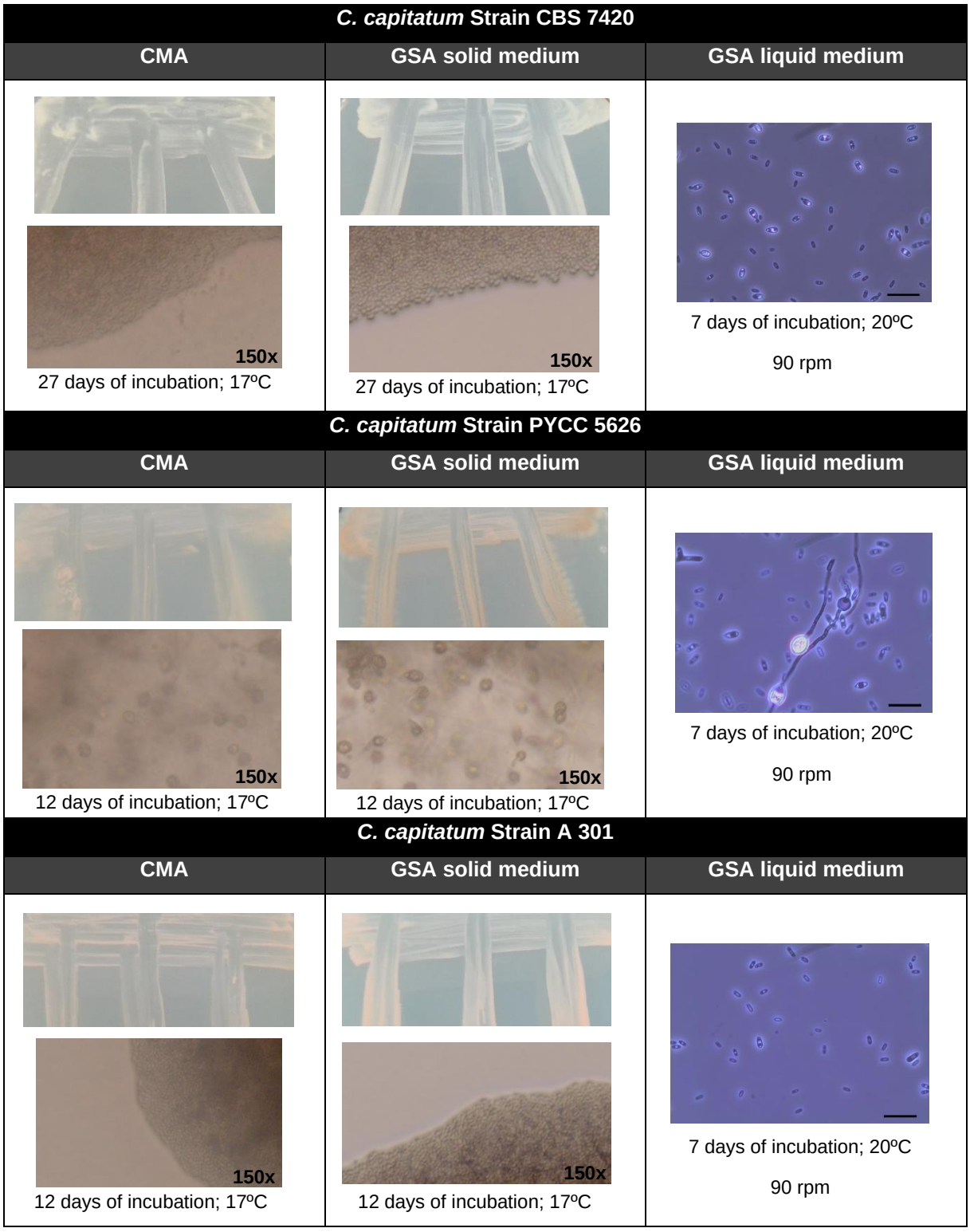


Figure 3.27: Sporulation results for *C. capitatum* strains CBS 7420, PYCC 5626 and A 301 in solid medium (CMA and GSA) and GSA liquid medium. For results concerning CMA and GSA solid media, photographs were taken of both the sporulation plates, and of the periphery of the growth patch with the optical microscope (amplification defined in the figure). For results concerning GSA liquid medium, the scale bars in the microscopy images correspond to 20 µm.

All three of the *C. capitatum* strains were then tested for sporulation in liquid GSA medium, where, as expected from the results of sporulation in solid medium, only PYCC 5626 developed teliospores, after only seven days of incubation with low agitation (Figure 3.27). Still, total RNA was extracted from all three *C. capitatum* strains from the GSA liquid cultures and tested for the presence of *HD1* and *HD2* mRNA.

After cDNA synthesis using anchored polydT primers (oligo $(dT)_{20}$), thereby producing cDNA from the entire mRNA pool, an initial second strand-synthesis of partial *HD1* and *HD2* CDS was performed for

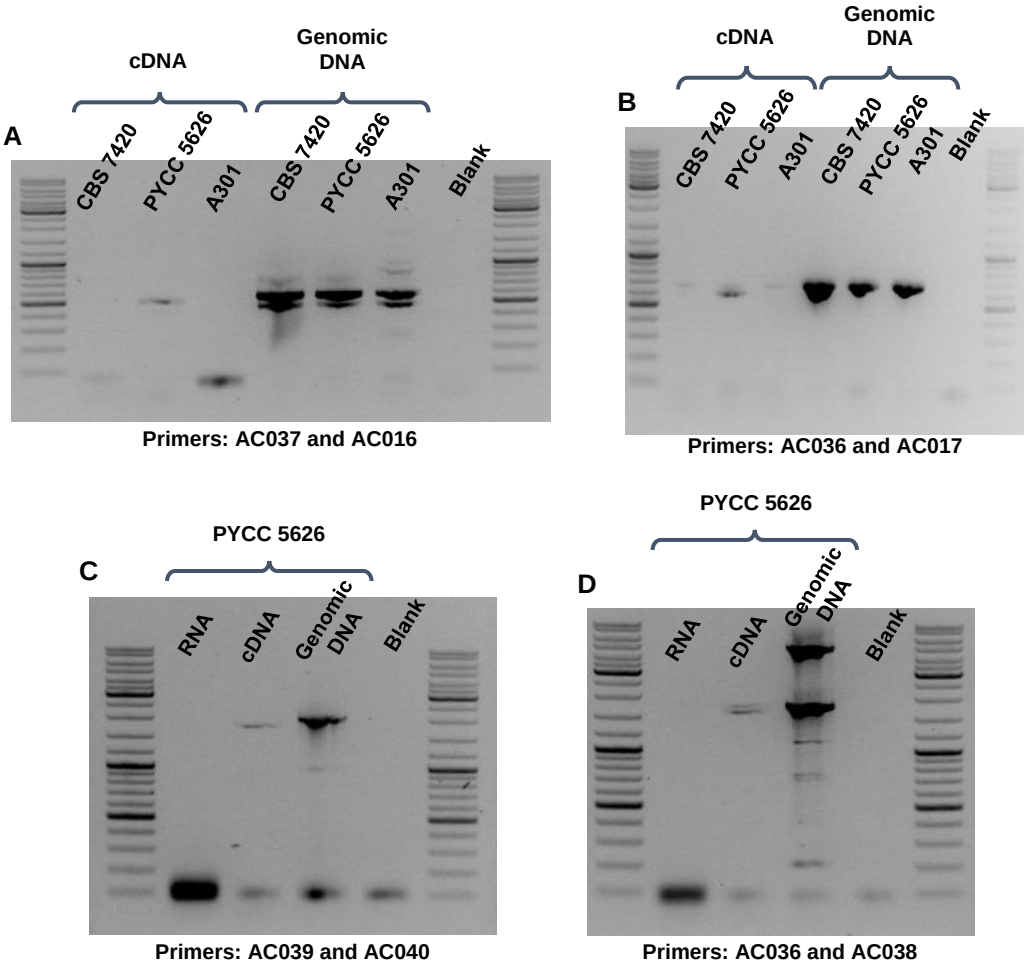


Figure 3.28: Results from second-strand synthesis of *HD1* and *HD2* cDNA of *C. capitatum* strains. All amplified fragments were analysed by electrophoresis in agarose gel. All expected fragment sizes are based on the data available for strain CBS 7420. A) Amplification of partial *HD1* fragment of *C. capitatum*. Expected fragment size: 562 bp for the gene fragment and 501 bp for the CDS fragment. B) Amplification of partial *HD2* fragment of *C. capitatum*. Expected fragment size: 645 bp for the gene fragment and 577 bp for the CDS fragment. C) Amplification of the complete *HD1* fragment of *C. capitatum*. Expected fragment size: 1957 bp for the gene fragment and 1842 bp for the CDS fragment. D) Amplification of the complete *HD2* fragment of *C. capitatum*. Expected fragment size: 1729 bp for the gene fragment and 1587 bp for the CDS fragment.

all three *C. capitatum* strains, where only the region from the 5' end up to the homeodomain of each gene was being considered. As can be observed in Figures 3.28.A and 3.28.B, only for strain PYCC 5626 was it possible to obtain the partial cDNA for both *HD1* and *HD2*. Although Figure 3.28.B reveals

faint bands corresponding to fragments of approximately the same size as the partial cDNA of *HD2* for both CBS 7420 and A 301, these results could never be reproduced after repetition and optimization of the second-strand synthesis reaction. Therefore, strain PYCC 5626 was selected to proceed with the synthesis of the complete *HD1* and *HD2* CDS.

Figures 3.28.C and 3.28.D show the confirmation of amplification of the complete CDS for *HD1* and *HD2* of *C. capitatum* strain PYCC 5626, having a control where a sample of the extracted RNA was used as template for the reaction, to ensure that there was no contamination with genomic DNA. Had that been the case, there would be no guarantee that the amplified fragments would have corresponded to the complete CDS of *HD1* and *HD2*. As no amplification was obtained for the samples where RNA was used as template, the fragments were most probably amplified from cDNA.

The amplified complete CDS fragments of *HD1* and *HD2* were cloned into a pJET1.2 plasmid, originating the recombinant plasmids pJET1.2+HD1cdsCyscap and pJET1.2+HD2cdsCyscap, respectively (Table 6.3, in Appendix). The cloned CDS fragments were then sequenced to confirm that these indeed corresponded to the coding sequences of the *HD1* and *HD2* genes. As no complete gene sequence was available for the *HD1* and *HD2* genes of *C. capitatum* strain PYCC 5626, the sequences of the CDS fragments were confirmed through an alignment of their corresponding protein sequences with the protein sequences derived from the putative CDS available for the *HD1* and *HD2* genes of *C. capitatum* strain CBS 7420 (Figure 6.2 in Appendix, for the DNA sequence of the *HD* locus of strain CBS 7420). A schematic representation of the obtained alignments is shown in Figure 3.29.

As discussed in Section 3.1.1 of this work, it is not clear whether the *C. capitatum* species possesses a single allele of the *HD* locus. In the alignments presented in Figure 3.29, some differences can be

Figure 3.29: Alignments of the protein sequences of HD1 and HD2 for the *C. capitatum* strains CBS 7420 and PYCC 5626. The protein sequences of the HD1 and HD2 proteins of PYCC 5626 used in these alignments were derived from the translation of the CDS experimentally obtained in this study. Sequence identity throughout the alignments is represented in shades of blue, according to the key in the bottom right corner of the figure.

observed between the HD1 and the HD2 protein sequences of strains CBS 7420 and PYCC 5626, even in the N-terminal region, which could potentially be sufficient to define distinct alleles of the *HD* locus in

these strains, as this region is usually responsible for heterodimerization. However, these evidences were not further explored in the present work.

Although we cannot be certain at this time that the HD proteins from *C. capitatum* PYCC 5626 are required for sexual reproduction in their normal setting, the obtained *HD1* and *HD2* CDS fragments of strain PYCC 5626 were still used to perform the Yeast Two-Hybrid assay. It would certainly be relevant to repeat this assay with the HD1 and HD2 proteins of the strain CBS 7420 and eventually perform heterologous expression of the *HD* locus of the strain PYCC 5626 in a *P. rhodozyma* mutant, for all the results to be in full accordance.

3.3.3.2. Yeast Two-Hybrid assay: Interaction between the HD proteins of *C. capitatum* and between the HD proteins of *C. ferigula*

To perform the Yeast Two-Hybrid assay involving the HD proteins of *C. capitatum*, the fusion proteins necessary for this study were first constructed (giving rise to the haploid *S. cerevisiae* transformants described in Table 3.3) aiming to assess the existence heterodimerization and homodimerization of the complete HD1 and HD2 proteins. In this study, and as previously mentioned, the CDS fragments of the *HD1* and *HD2* genes belonging to *C. capitatum* strain PYCC 5626 (Section 3.3.3.1) were used in the construction of the fusions.

Table 3.3: List of *S. cerevisiae* Y2HGold and Y187 haploid transformants used in the Yeast Two-Hybrid assay for the interaction between the complete HD1 and HD2 proteins of *C. capitatum* and of *C. ferigula*. Containing information regarding the plasmids present in each transformant and the *HD* fragment present in each plasmid.

Haploid ID	Plasmid in the haploid *S. cerevisiae* strain	*HD* fragment
1	pGBKT7 in Y2HGold cells	-
2	pGADT7 in Y187 cells	-
3	pGBKT7-53 in Y2HGold cells	-
4	pGADT7-T in Y187 cells	-
5	pGBKT7-Lam in Y2HGold cells	-
A	pGBKT7+AC047/AC048 in Y2HGold cells	*HD1* complete (*C. capitatum*)
B	pGBKT7+AC043/AC044 in Y2HGold cells	*HD2* complete (*C. capitatum*)
C	pGADT7+AC045/AC046 in Y187 cells	*HD1* complete (*C. capitatum*)
D	pGADT7+AC041/AC042 in Y187 cells	*HD2* complete (*C. capitatum*)
E	pGBKT7+AC049/AC050 in Y2HGold cells	*HD1* complete (*C. ferigula* PYCC 5628)
F	pGADT7+AC051/AC052 in Y187 cells	*HD2* complete (*C. ferigula* PYCC 4410)

Concerning the Yeast Two-Hybrid assay involving the HD proteins of *C. ferigula*, the fusions were constructed using the synthetic genes corresponding to the CDS of the *HD1* gene of strain PYCC 5628 and the *HD2* gene of strain PYCC 4410, optimized for expression in *S. cerevisiae* (Figure 6.5, in Appendix). As a strong interaction is expected between the HD1 and HD2 proteins from these *C. ferigula* strains of different mating-types, given the heterothallic sexual behaviour of this species (Hull *et al*, 2005), a single fusion construction was performed for each of the HD proteins, namely HD1 fused with

the DNA binding domain of Gal4 (transformant E) and HD2 fused with the activation domain of Gal4 (transformant F) (Table 3.3).

Similarly to the procedure in the Yeast Two-Hybrid assay involving the HD proteins of *P. rhodozyma*, after transformation of the *S. cerevisiae* strains with the plasmids expressing the fusion proteins, the sizes of the haploid transformant colonies were compared to those of the corresponding *S. cerevisiae* strain used for transformation containing the original empty plasmids pGBKT7 and pGADT7 (transformants 1 and 2, respectively – Table 3.3), to assess the toxicity of the *C. capitatum* and *C. ferigula HD* fragments. In Figure 3.30, the results for the toxicity assay for all the considered haploid transformants can be observed.

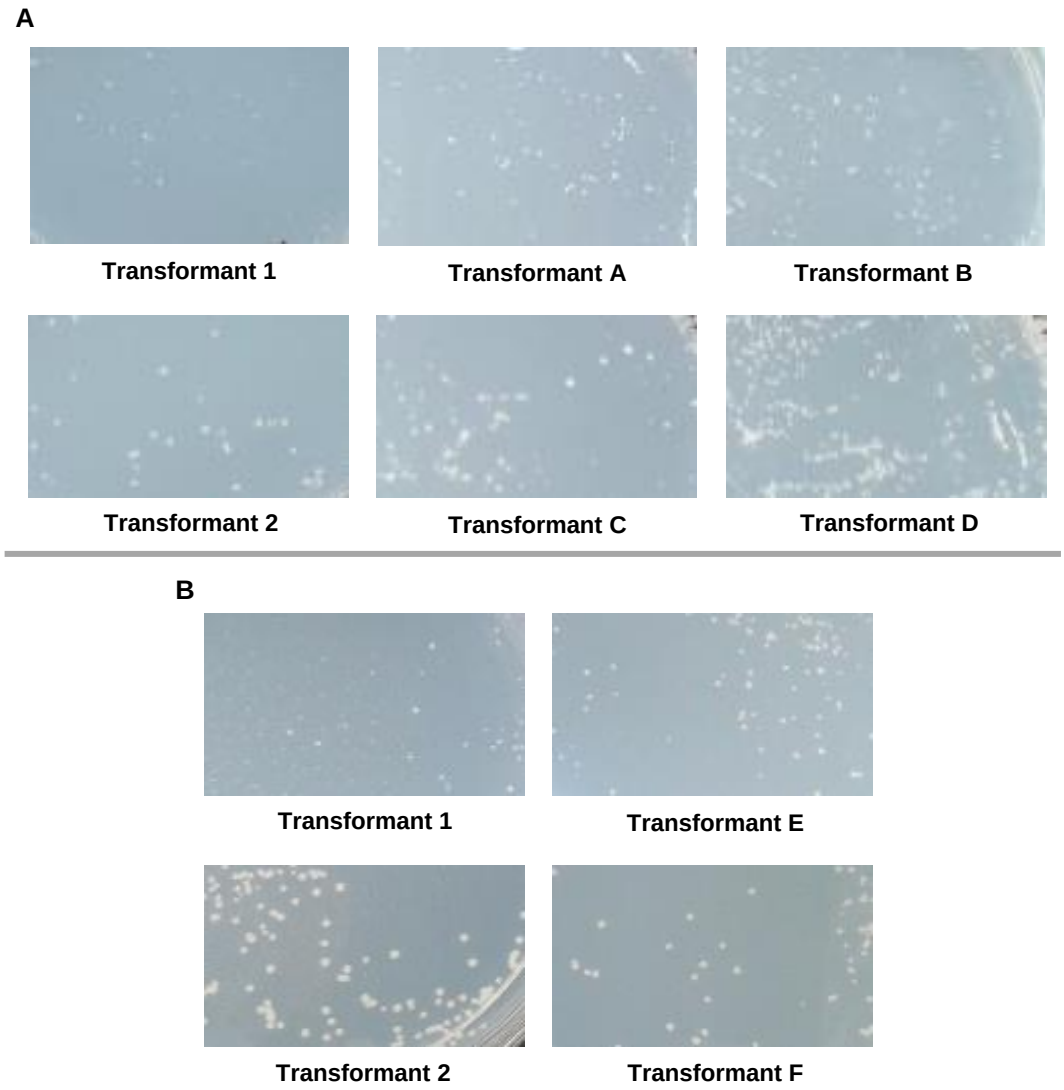


Figure 3.30: Toxicity assay of the presence of *HD1* and *HD2* fragments of *C. capitatum* and *C. ferigula* in the *S. cerevisiae* strains Y187 and Y2HGold. A) Results for the transformants containing the *HD1* and *HD2* fragments of *C. capitatum*. B) Results for the transformants containing the *HD1* and *HD2* fragments of *C. ferigula*. All transformants were grown in the appropriate selective medium in each case.

Indeed, the *HD* fragments belonging to *C. capitatum* do not seem to be toxic to the *S. cerevisiae* strains, as all colonies seem to be of roughly the same size when compared to the colonies of the transformants containing the empty plasmids (transformants 1 and 2) (Figure 3.30.A). However, the *HD* fragments

belonging to *C. ferigula* seem to be affecting the normal growth of the *S. cerevisiae* strains, as the colonies corresponding to transformants E and F seem to be slightly smaller than those of transformants 1 and 2, respectively (Figure 3.30.B). This is especially evident concerning transformant F, although it may not be readily perceivable in Figure 3.30.B.

Confirmation of the haploid transformants was performed through colony PCR, where positive colonies for the presence of the *HD* fragments of interest were obtained for all transformants except for transformant A (*HD1* of *C. capitatum* fused with the DNA binding domain of Gal4). Colony PCR for this particular transformation was performed using the specific primers for the *HD1* fragment of *C. capitatum* used in this transformation (AC047 and AC048) and also using the sequencing primers described for plasmid pGBKT7 (T7 sequencing primer and 3' DNA-BD sequencing primer). Results from the colony PCR using primers AC047 and AC048 did not reveal amplification of the expected *HD1* fragment (Figure 3.31.A), suggesting that all tested colonies corresponded to transformants with an auto-ligated pGBKT7.

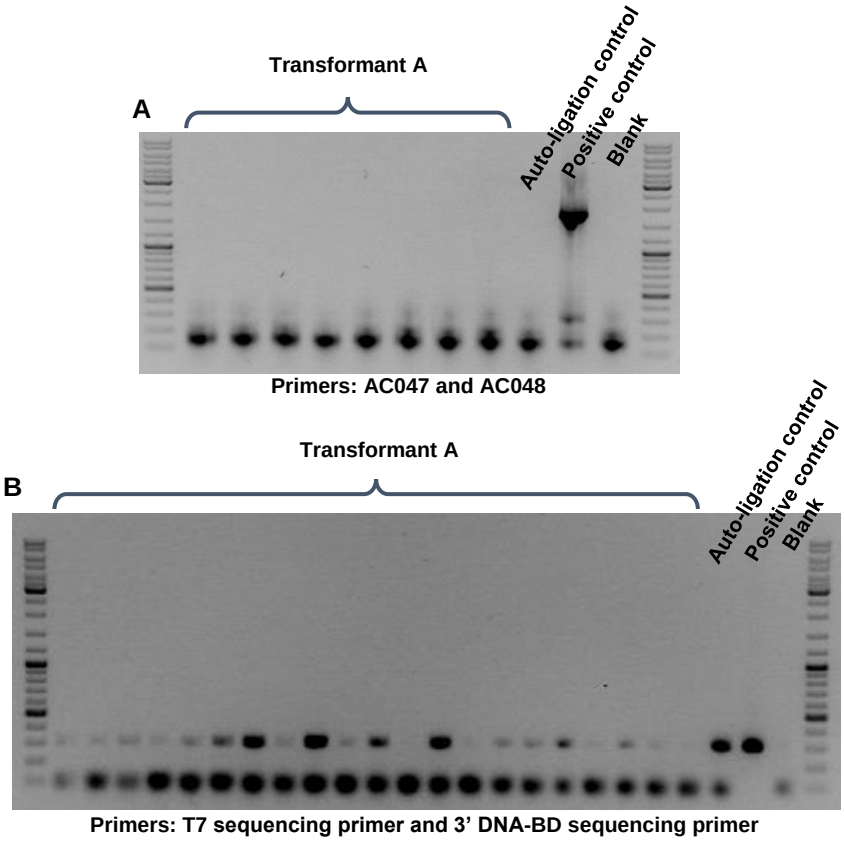


Figure 3.31: Colony PCR to confirm the presence of the *HD1* fragment of *C. capitatum* in the generated haploid transformant A. All amplified fragments were analysed by electrophoresis in agarose gel. A) Amplification of the *HD1* fragment of *C. capitatum* used for transformation. The auto-ligation control corresponds to the negative control of amplification (having used a colony from the control of transformation with linearized pGBKT7), and in the positive control pJET1.2+HD1cdsCyscap was used as template. Expected fragment size: 1922 bp. B) Results from colony PCR using the sequencing primers described for pGBKT7. In the auto-ligation control, a colony from the control of transformation was used as template, and in the positive control pGBKT7 plasmid was used as template. Expected fragment size: 2082 bp for the correct construction and 298 bp for the auto-ligation and positive controls.

This assumption was confirmed through colony PCR with the pGBKT7 sequencing primers (Figure 3.31.B), as the amplified fragments corresponded to that of the original pGBKT7 plasmid.

Oddly enough, the number of colonies obtained in this transformation (365 cfus) is much greater than that obtained for the control of auto-ligation of pGBKT7 (23 cfus), a proportion that would suggest that the *HD1* fragment would have been correctly cloned in pGBKT7 (data not shown).

The transformation of *S. cerevisiae* strain Y2HGold to generate transformant A was attempted three more times, with similar results in both the proportion of colonies in the transformation plate and the control of auto-ligation of the plasmid and in the confirmations through colony PCR using specific primers for the *HD1* fragment and the sequencing primers of pGBKT7.

Although speculations could be made as to why transformant A was unable to be generated, it should be taken into consideration the fact that the transformations involving this construct were performed simultaneously with the remaining haploid transformants for the Yeast Two-Hybrid assay for the interaction between HD1 and HD2 of *C. capitatum* and of *C. ferigula*. Therefore, and given that the remaining haploid transformants could be generated, it would then be improbable that the failure in generating transformant A would be due to any of the main steps of the *S. cerevisiae* transformation procedure or due to errors when preparing the media for the selection of haploid transformants. Also, the fact that the proportion of obtained colonies between the transformation plate and the control of auto-ligation of the plasmid is as expected for a correctly cloned fragment, seems to suggest that the *HD1* fragment was cloned in the plasmid, at least at some point. Since cloning of the *HD1* fragment in pGBKT7 within the *S. cerevisiae* cell would occur through homologous recombination at the 40 bp tails present in the 5' and 3' ends of the fragment (and flanking the multiple cloning site in pGBKT7), it would be highly improbable that the *HD1* fragment, once cloned, would have been excised from the plasmid. Such a phenomenon would be conceivably likely only if sequences prone to recombination would be present within the fragment, allowing it to be excised. However, given the results of the colony performed PCR (Figure 3.31.B), these sites would have to be such that would allow the reconstitution of the original pGBKT7 plasmid, which would be, once again, improbable. Further experiments should be performed so as to understand the reason why transformant A could not be generated. For the present study, the heterodimerization of the HD complete proteins of *C. capitatum* was assessed resorting solely to haploid transformants B and C (Table 3.3).

Table 3.4: List of *S. cerevisiae* diploids, generated through mating between the Y2HGold and Y187 haploid transformants, used in the Yeast Two-Hybrid assay for the interaction between the complete HD1 and HD2 proteins of *C. capitatum* and of *C. ferigula*. Containing relevant information regarding the interactions being tested.

Mating Experiments using the haploid strains	Objective
1x2	Negative control with empty plasmids
3x4	Positive control for interaction
4x5	Negative control for interaction
BxC	Testing for heterodimerization HD2xHD1 *C. capitatum*
BxD	Testing for homodimerization HD2xHD2 *C. capitatum*
ExF	Testing for heterodimerization HD1xHD2 *C. ferigula*

The mating experiments using the generated haploid transformants were performed as described in Table 3.4.

In line with the confirmations performed in the Yeast Two-Hybrid assay involving the complete HD proteins of *P. rhodozyma* (Section 3.2.1), all generated diploids were tested for the presence of both *HD* fragments of interest through PCR, using the specific primers designed to amplify each of the considered *HD* fragments used in the fusion constructions (Table 6.4, in Appendix). Results from the PCR confirmations for the generated diploids can be analysed in Figure 3.32 (3.32.A, 3.32.B, 3.32.C and 3.32.D). Indeed, diploids BxC and BxD, corresponding to the study of the heterodimerization and homodimerization of HD2 of *C. capitatum*, respectively, seem to contain both *HD* fragments of interest

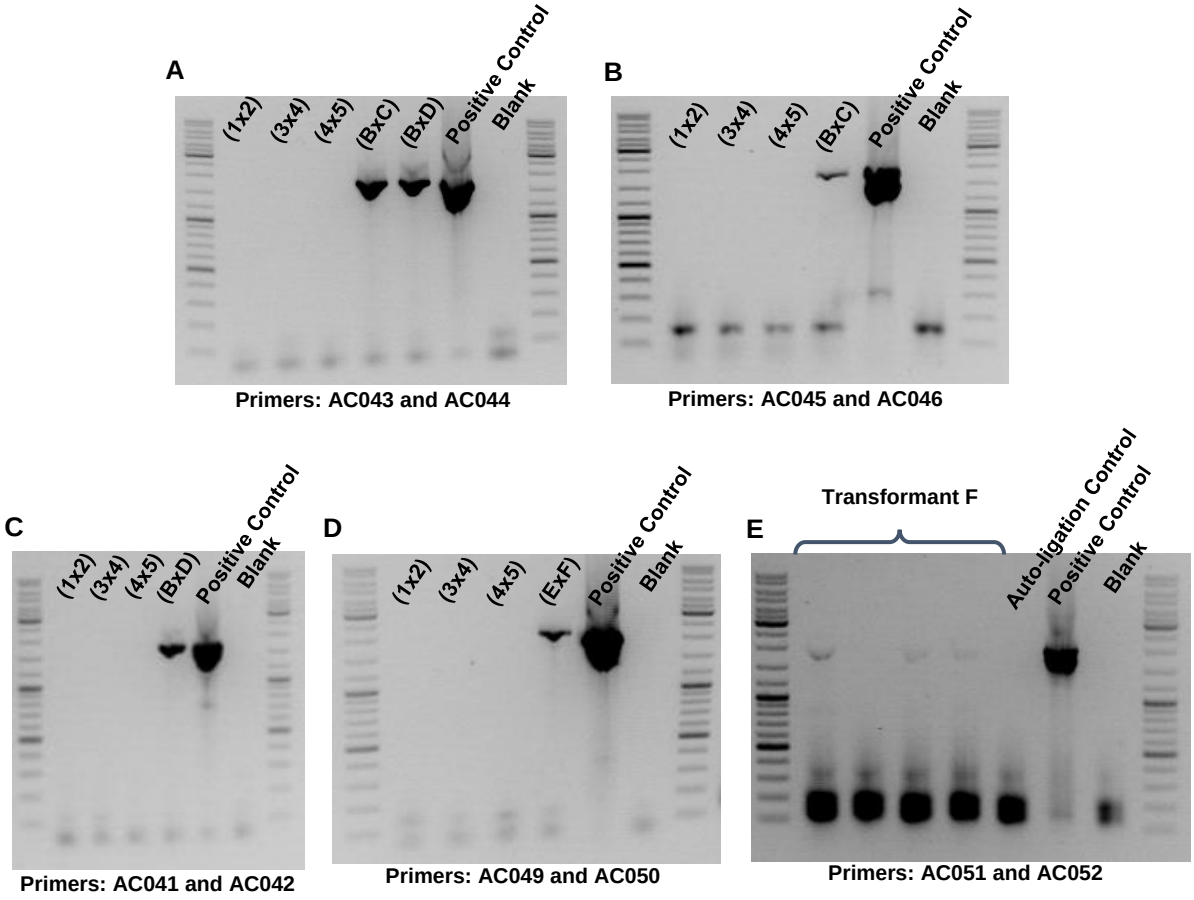


Figure 3.32: Confirmation of the presence of the *HD1* and *HD2* fragments of interest belonging to *C. capitatum* and *C. ferigula* in each of the generated diploids. In all agarose gels, the positive control constitutes the original plasmid containing the *HD* genes of interest. A) Confirmation of the presence of the *HD2* fragment of *C. capitatum* as used for the fusion construction with the DNA binding domain of Gal4. B) Confirmation of the presence of the *HD1* fragment of *C. capitatum* as used for the fusion construction with the activation domain of Gal4. C) Confirmation of the presence of the *HD2* fragment of *C. capitatum* as used for the fusion construction with the activation domain of Gal4. D) Confirmation of the presence of the *HD1* synthetic gene of *C. ferigula* as used for the fusion construction with the DNA binding domain of Gal4. E) Colony PCR for the confirmation of the presence of the *HD2* synthetic gene of *C. ferigula* in transformant F (fusion with the activation domain of Gal4).

in each case (Figures 3.32.A, 3.32.B and 3.32.C). Concerning diploid ExF, as can be observed in Figure 3.32.D, the fragment corresponding to *HD1* of *C. ferigula* PYCC 5628 is indeed present. However, the fragment corresponding to *HD2* of *C. ferigula* PYCC 4410 could not be amplified in observable amounts.

As mentioned previously, the *HD* fragments of *C. ferigula* seem to be toxic to the *S. cerevisiae* strains used for transformation, such toxicity being more evident for haploid transformant F (Figure 3.30.B). Therefore, and although the plasmids used in the Yeast Two-Hybrid system are multi-copy, it is possible that the amount of copies of the recombinant plasmid were reduced to the minimum possible in diploid ExF to allow the survival of the cells in selective medium for the *S. cerevisiae* diploids used in the Yeast Two-Hybrid system, which is likely the reason why the *HD2* fragment of *C. ferigula* PYCC 4410 could not be amplified using as template the extracted DNA of diploid ExF. However, and as mentioned before, haploid transformant F had been confirmed through colony PCR, and such results are present in Figure 3.32.E.

As was performed for the study of the interaction between the complete HD proteins of *P. rhodozyma*, all generated diploids were tested for their ability to activate the three reporter genes of the Yeast Two-Hybrid system. The results for the activation of the reporter genes in the generated diploids, as well as the auto-activation in the haploid transformants, are shown in Figure 3.33.

Regarding the results for the diploids concerning the interactions between the HD proteins of *C. capitatum*, the diploid BxC (which corresponds to the heterodimerization of HD1 and HD2) does not seem to be able to activate any of the three reporter genes, as no growth is observed in the absence of histidine or adenine and no blue tinge is observed in the medium supplemented with X-α-Gal (Figure 3.33.A). On another hand, the diploid BxD seems to be strongly activating *MEL1*, *ADE2* and *HIS3*, implying that homodimerization of the HD2 protein of *C. capitatum* is occurring (Figure 3.33.A).

Regarding the heterodimerization of the HD1 and HD2 complete proteins of *C. ferigula* strains PYCC 5628 and PYCC 4410, respectively, a strong interaction seems to be present, as all three reporter genes of the Yeast Two-Hybrid system were activated by diploid ExF (Figure 3.33.A). Concerning the activation of *MEL1* by the diploid ExF, a strong blue tinge was observable in the growth patch in less than 24 hours after inoculation. These results suggest that the HD proteins of *C. ferigula* belonging to strains of different mating-types do heterodimerize, in accordance to what has been reported for other heterothallic basidiomycete species (Hull *et al*, 2005; Kämper *et al*, 1995; Asante-Owusu *et al*, 1996). Therefore, the HD proteins of *C. ferigula* establish a great positive control for the interaction between the HD1 and HD2 proteins when performing studies involving these proteins in homothallic species of the order Cystofilobasidiales.

Regarding the results from the auto-activation of the reporter genes in the haploid transformants (Figure 3.33.B), some activation of *MEL1* seems to be occurring only in the haploid transformants C, D and B (used in the study of the interaction of the HD proteins of *C. capitatum*), where haploid transformant B also seems to be activating the reporter gene *ADE2*. Concerning the auto-activation of *HIS3*, a similar phenomenon seems to be occurring as described in Section 3.2.1 of this work, where the negative controls of the Yeast Two-Hybrid assay (1x2 and 4x5) seem to be able to grow in the absence of histidine, although not as prominently as the positive control for interaction (3x4) (Figure 3.33.B). Therefore, and trying to establish a comparison with the growth observed in the positive and negative controls, it is possible to deduce that haploids C, D and F are behaving similarly to the negative controls and that haploid E probably is not activating the expression of *HIS3*. The growth observed in haploid B,

however, seems to be more prominent than that of the negative controls, and some activation of *HIS3* may be occurring.

However, these stated auto-activations do not seem to be affecting the activation of the reporter genes by the generated diploids, since diploid BxC is not activating any of the three reporter genes (Figure 3.33.A). Hence, the results obtained for the activation of the reporter genes in the diploids generated in this study are considered valid.

Trying to establish a comparison between these results and those obtained for the interactions between

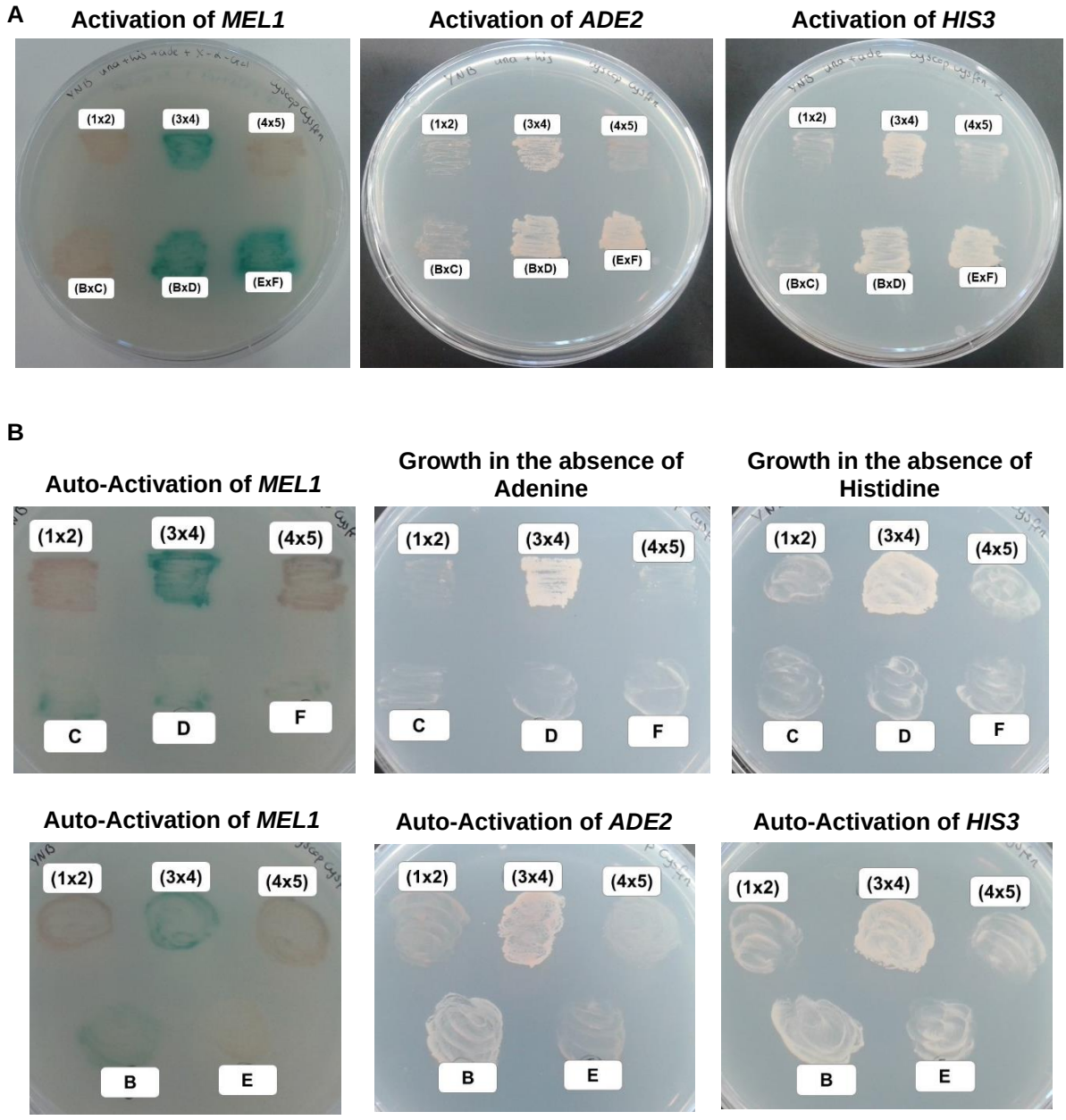


Figure 3.33: Yeast Two-Hybrid assay to test interactions between the HD proteins of *C. capitatum* and of *C. ferigula* – Results of the activation of the reporter genes *MEL1*, *ADE2* and *HIS3*. A) Activation of the reporter genes in the generated diploids, corresponding to the detection of heterodimerization of the HD proteins of *C. capitatum* and of *C. ferigula* and homodimerization of the HD2 protein of *C. capitatum*. B) Activation of the reporter genes in the haploid transformants used in this study. All results represented in this figure correspond to three days of incubation, at 30ºC, in the appropriate selective medium in each case.

the HD proteins of *P. rhodozyma* (Section 3.2), it is quite interesting to note that the results for the

interactions between the HD proteins of *C. capitatum* seem to be in accordance, to some extent. Weak interactions seem to be governing the heterodimerization of the HD proteins of *P. rhodozyma* (Section 3.2). Through the study of the interaction between the HD1 and HD2 proteins of *C. capitatum*, diploid BxC is not perceivably activating any of the reporter genes (Figure 3.33.A). However, it could be possible that a weak interaction is also present between the HD1 and HD2 proteins of this species, such that it would not be observable through a qualitative assay like the Yeast Two-Hybrid assay implemented in this study. Also, and since only one combination of the fusion constructions was tested for the detection of heterodimerization (given the inability to generate the haploid transformant A), eventual steric hindrance within the fusion proteins in diploid BxC could be obstructing a potentially weak interaction between HD1 and HD2 of *C. capitatum*, impeding the activation of the Yeast Two-Hybrid reporter genes. Supporting the hypothesis of a weak interaction between the HD1 and HD2 proteins of *C. capitatum* are the results from the heterologous expression of the *HD* locus of *C. capitatum* in a *P. rhodozyma* mutant (in Section 3.3.2.2 of this work), where, although concerning the *HD* locus of a different *C. capitatum* strain (CBS 7420), it was able to restore sporulation in a *P. rhodozyma* mutant lacking its native *HD* locus. Therefore, these results suggest that the HD proteins of *C. capitatum* have a similar role and mode of action in sexual reproduction as the HD proteins of *P. rhodozyma*. Still, it should always be taken into account that the results obtained in Sections 3.3.2.2 and 3.3.3.2 of this work pertain to two different *C. capitatum* strains, that, as discussed in Section 3.1.1, could potentially possess different alleles of the *HD* locus. Although one would expect the molecular mechanisms governing the homothallic sexual behaviour of these two strains to be equivalent (given they belong to the same species), no evidences supporting such hypothesis are existent at the moment.

Further studies must, then, be made to confirm the existence of interaction between the HD1 and HD2 proteins of *C. capitatum*, such as the completion of the Yeast Two-Hybrid assay initiated in this study through the generation of transformant A (Table 3.3), to try to understand if these results are maintained with the opposite pair of fusion proteins (testing the interaction between the HD1 and HD2 proteins, with HD1 fused with the DNA binding domain of Gal4 and HD2 fused with the activation domain of Gal4).

Regarding the homodimerization of the HD2 protein of *C. capitatum*, as mentioned above, the diploid BxD seems to be strongly activating all three reporter genes, with results being equivalent to the positive control of interaction of the Yeast Two-Hybrid system (3x4) (Figure 3.33.A). As homodimerization of both HD1 and HD2 proteins was also reported for *P. rhodozyma* (David-Palma *et al*, 2016, and Section 3.2 of this work), it would be relevant to try to understand the role of homodimers of the HD proteins in basidiomycetes and whether these could play a role in sexual reproduction in homothallic species such as *P. rhodozyma* and *C. capitatum*. Continuing this line of thought, for *P. rhodozyma* homodimerization of the HD1 protein seems to be more prominent than homodimerization of the HD2 protein (Section 3.2.1), while for *C. capitatum* only the homodimerization of the HD2 protein was tested (Figure 3.33.A). It would be quite interesting to try to understand if, for *P. rhodozyma*, the ability of the HD1 protein to homodimerize would be related to the fact the HD1 protein is sufficient to induce sporulation, albeit vestigial (David-Palma *et al*, 2016), and whether such a hypothesis could be extrapolated to *C. capitatum.*

4. Conclusions and Future Perspectives

The variability in sexual behaviours present in the order Cystofilobasidiales (Kurtzman *et al*, 2011) sets this lineage as a very good model for the study of the transitions between sexual behaviours and breeding systems in the phylum Basidiomycota, as well as the study of the molecular basis of homothallism that take place in the homothallic species of this order.

The main objectives of this work aimed at helping to elucidate these crucial aspects of sexual reproduction in basidiomycetes, on the one hand through the study of the *MAT* loci from a set of species belonging to the *Cystofilobasidium* genus from a phylogenetic point of view, and on another through the study of the role and mode of action of the HD1 and HD2 proteins of two homothallic species of this order (*P. rhodozyma* and *C. capitatum*).

Concerning the study of the *MAT* loci within the *Cystofilobasidium* genus, this could not be completed because it was not possible to obtain sequences of the *MAT* loci for some of the considered strains. This incompletion can, however, be already enlightening on the state of these loci given the methodology used in this study, especially regarding the *HD* locus of *C. macerans*. This species in particular seems to be in an intermediate stage between heterothallism and homothallism, where homothallic strains have been reported to be more sexually competent than heterothallic strains (Libkind *et al*, 2009). The fact that the homeodomain and C-terminal regions of the HD1 and HD2 proteins of the heterothallic strains CBS 6532 and CBS 2425 of *C. macerans* are significantly less conserved, given what is expected for these proteins (David-Palma, 2017), could potentially be in accordance with the hypothesis of this species being on the verge of a transition in sexual behaviour.

Further and more in-depth studies concerning the evolution of the *MAT* loci are of utmost relevance, not only concerning *C. macerans* but the whole of the *Cystofilobasidium* genus, to clarify the evolutionary transitions between sexual behaviours and breeding systems taking place in this genus. Future work on this matter could involve the study of the diversity of alleles of the *MAT* genes for each species, through the obtention of genomic data and phylogenetic analyses for a greater number of strains within this genus.

Of similar importance would be the study of the conservation of the protein sequences of HD1 and HD2, through determination of their polymorphic pattern, in line with what was performed by David-Palma, 2017, when comparing the sequences of several strains of the same species. Calculating the rate of synonymous and non-synonymous substitutions (dN/dS), for instance, could be of great relevance to try to understand if the selective pressures at work are tending for the maintenance or eventual loss of function (Cardoso-Moreira and Long, 2012; Kosiol and Anisimova, 2012) of the HD proteins (at least regarding what is common in a heterothallic behaviour). Comparing the results of such study for heterothallic and homothallic species, and especially within *C. macerans*, would be of great importance to understand the evolutionary tendencies of the transitions between sexual behaviours within this lineage.

4. Conclusions and Future Perspectives

Regarding the study of the role and mode of action of the HD1 and HD2 proteins of the two homothallic species analysed, the work performed concerning *P. rhodozyma* pertained to the continuation of the study initiated by our research group (David-Palma *et al*, 2016), helping to enlighten the mode of action of the HD1 and HD2 proteins in the homothallic sexual behaviour of this species. Indeed, when considering the complete HD proteins of *P. rhodozyma*, a weak interaction seems to be at the basis for the formation of the heterodimer, as reported by David-Palma *et al*, 2016, when considering the N-terminal region of these proteins. These results suggest that heterodimerization of these proteins indeed occurs, eventually through the N-terminal region, seemingly supporting the hypothesis of the sexual behaviour of *P. rhodozyma* having evolved from a heterothallic ancestor (David-Palma *et al*, 2016).

Results on the role of the HD1 and HD2 proteins of *C. capitatum* in sexual reproduction were, however, less clear. On the one hand, the *HD* locus of *C. capitatum* was able to restore the sporulation phenotype in a *P. rhodozyma* deletion mutant of the *HD* locus, through heterologous expression. On the other hand, no heterodimerization was detected between the HD1 and HD2 proteins of this species from the results of the Yeast Two-Hybrid assay, instead detecting homodimerization of the HD2 protein of *C. capitatum*. Although the results from the heterologous expression seem to suggest a similar role of the HD proteins of *C.* capitatum in sexual reproduction as those of *P.* rhodozyma, the fact that no heterodimerization was detected leaves the molecular mechanisms by which the HD1 and HD2 proteins of *C. capitatum* employ their function to be clearly ascertained.

The most direct and reliable way to clarify these questions would be through the generation of *C. capitatum* knock-out mutants of the *HD1* and *HD2* genes and following sporulation assays. For such study to be possible, it would be necessary to find a method to genetically transform *C. capitatum*. Although it was not possible to transform *C. capitatum* in this work, further studies to find such method could be relevant to understand not only the functions of HD1 and HD2 in the homothallic sexual behaviour in this species but also the role of the single pair of pheromone and pheromone receptor encoded in its genome.

Studies involving the heterologous expression of the *HD2* gene of *C. capitatum* in the *P. rhodozyma* deletion mutant of the *HD* locus could also be relevant to determine if the homodimer formed by the HD2 protein of *C. capitatum* plays a role in sexual reproduction that would allow the sporulation phenotype of *P. rhodozyma* to be restored.

The Yeast Two-Hybrid assay concerning the interactions governing the HD proteins of *C. capitatum* should, nevertheless, be completed. The generation of the fusion construction between the HD1 complete protein and the DNA binding domain of Gal4 would allow to confirm if the inability to detect heterodimerization was indeed reflective of what occurs within *C. capitatum* or if it could have resulted from eventual steric hindrance within the fusion constructions used in the Yeast Two-Hybrid assay of this work, as well as assess the homodimerization of the HD1 protein of *C. capitatum*. In any case, it would be relevant to assess the expression of each fusion construction in the generated diploids being used in the Yeast Two-Hybrid assay through western-blot, and to determine the interaction between the HD proteins through co-immunoprecipitation assays, allowing to support and eventually validate the results obtained in the Yeast Two-Hybrid assay.

Concerning the results of the Yeast Two-Hybrid assay obtained for the HD proteins of *C. ferigula*, a positive control for the interaction between HD1 and HD2 of a heterothallic species from the order Cystofilobasidiales could be established, showing that in the Cystofilobasidiales strong interactions are observed between HD1 and HD2 proteins of heterothallic species, as was previously observed in other lineages, and that therefore, the results obtained for *P. rhodozyma* and *C. capitatum* are likely to be related to their homothallism. Therefore, future work regarding the relative strength of the interaction between the HD1 and HD2 proteins from the mentioned homothallic species (and eventually for other homothallic species from the order Cystofilobasidiales to be studied in the future) could be performed, using the interaction between the HD proteins of *C. ferigula* as a positive control, for instance through a quantitative β-galactosidase assay from the Yeast Two-Hybrid system, as was performed in this work concerning the interaction between the HD proteins of *P. rhodozyma*.

Still concerning the HD proteins of *C. ferigula*, it could be interesting to perform a study of the homodimerization of HD1 and HD2 and try to understand if, given the ability of these to homodimerize, these homodimers would hold any function in sexual reproduction. Since homodimerization of the HD proteins seems to be preponderant in both *P. rhodozyma* and *C. capitatum*, a comparison with a heterothallic species of the same order could help clarify if these homodimers, in case they hold any function in sexual reproduction, are specific to homothallic species or if they could also take place in heterothallism within the Cystofilobasidiales.

The order Cystofilobasidiales is, indeed, a lineage with great potential for the study of sexual reproduction within the phylum Basidiomycota. Further studies within this order would be of great value to better understand its evolution and the mechanisms behind such complex matter as is sexual reproduction.

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6. Appendix

Table 6.1: Complete list of media used in this work. Containing information concerning the composition of each medium and their use throughout this work.

Medium	Composition	Use
YPD	2% of Peptone; 1% of Yeast Extract; 2% of Glucose (2% of Agar for solid medium)	Standard growth of yeasts
YMA	0.3% of Yeast Extract; 0.5% of Peptone; 0.3% of Malt Extract; 1% of Glucose (1% of Agar for solid medium)	Standard growth of yeasts
LB	1% of NaCl; 1% of Tryptone; 0.5% of Yeast Extract (2% of Agar for solid medium)	Standard growth of *E. coli* strain DH5α
YNB without amino acids + supplements	1x YNB without amino acids (Formedium); 2% of Glucose; 0.02mg/mL of Uracil; 0.02mg/mL of Adenine Sulphate; 0.02mg/mL of L-Histidine; 0.02mg/mL of L-Tryptophan; 0.02mg/mL of Methionine (2% of Agar for solid medium)	Yeast Two-Hybrid: Selective medium for haploid transformants of the *S. cerevisiae* strain Y187
	1x YNB without amino acids (Formedium); 2% of Glucose; 0.02mg/mL of Uracil; 0.02mg/mL of Adenine Sulphate; 0.02mg/mL of L-Histidine; 0.06mg/mL of Leucin (2% of Agar for solid medium)	Yeast Two-Hybrid: Selective medium for haploid transformants of the *S. cerevisiae* strain Y2H Gold
	1x YNB without amino acids (Formedium); 2% of Glucose; 0.02mg/mL of Uracil; 0.02mg/mL of Adenine Sulphate; 0.02mg/mL of L-Histidine (2% of Agar for solid medium)	Yeast Two-Hybrid: Selective medium for diploids from mating of the *S. cerevisiae* strains Y187 and Y2H Gold
	1x YNB without amino acids (Formedium); 2% of Glucose; 0.02mg/mL of Uracil; 0.02mg/mL of Adenine Sulphate (2% of Agar for solid medium)	Yeast Two-Hybrid: Selective medium for *S. cerevisiae* diploids to test the activation of *HIS3* reporter gene
	1x YNB without amino acids (Formedium); 2% of Glucose; 0.02mg/mL of Uracil; 0.02mg/mL of L-Histidine (2% of Agar for solid medium)	Yeast Two-Hybrid: Selective medium for *S. cerevisiae* diploids to test the activation of *ADE2* reporter gene
	1x YNB without amino acids (Formedium); 2% of Glucose; 0.02mg/mL of Uracil; 0.02mg/mL of Adenine Sulphate; 0.06mg/mL of Leucin (2% of Agar for solid medium)	Yeast Two-Hybrid: Selective medium to test the activation of *HIS3* reporter gene in haploid transformants of the *S. cerevisiae* strain Y2H Gold
	1x YNB without amino acids (Formedium); 2% of Glucose; 0.02mg/mL of Uracil; 0.02mg/mL of L-Histidine; 0.06mg/mL of Leucin (2% of Agar for solid medium)	Yeast Two-Hybrid: Selective medium to test the activation of *ADE2* reporter gene in haploid transformants of the *S. cerevisiae* strain Y2H Gold
	1x YNB without amino acids (Formedium); 2% of Glucose; 0.02mg/mL of Uracil; 0.02mg/mL of Adenine Sulphate; 0.02mg/mL of L-Tryptophan; 0.02mg/mL of Methionine (2% of Agar for solid medium)	Yeast Two-Hybrid: Selective medium to test the growth in the absence of Histidine in haploid transformants of the *S. cerevisiae* strain Y187
	1x YNB without amino acids (Formedium); 2% of Glucose; 0.02mg/mL of Uracil; 0.02mg/mL of L-Histidine; 0.02mg/mL of L-Tryptophan; 0.02mg/mL of Methionine (2% of Agar for solid medium)	Yeast Two-Hybrid: Selective medium to test the growth in the absence of Adenine in haploid transformants of the *S. cerevisiae* strain Y187
DWR + 0.5% Ribitol	0.5% of Ribitol; 2.5% of Agar	Sporulation assays using *P. rhodozyma* strains
CMA	1.7% of Corn meal agar (Difco); 0.5% Agar	Sporulation of *C. capitatum* strains
GSA	0.2% of Glucose; 0.2% of Soytone (1.5% of Agar for solid medium)	Sporulation of *C. capitatum* strains
MYP	0.7% of Malt extract; 0.05% of Yeast Extract; 0.25% of Soytone; 1.5% of Agar	Sporulation of *C. capitatum* strains

Table 6.2: Complete list of *Cystofilobasidium* strains used in the phylogenetic study of the *MAT* loci. Containing relevant information concerning the strains ID in PYCC and other culture collections, their sexual behaviour, origin of isolation and other relevant information.

Species	Strain		Sexual Behaviour	Origin	Relevant Information
	PYCC ID	Other collections			
Cystofilobasidium ferigula	PYCC 4410	CBS 7201	Heterothallic	*Rhea americana*, Paris Zoological Gardens, France	Sequenced Genome PRJNA371786 *
	PYCC 5359^{T}	CBS 7202^{T}		Dead male *Papio papio*, Paris Zoological Gardens, France	
	PYCC 5540	CBS 6954		Sawdust (location unknown)	
	PYCC 5628	ZP 322		Contamination of home-made sauerkraut, Caparica, Portugal	Sequenced Genome PRJNA371793 *
		TOFU R2			
Cystofilobasidium macerans	PYCC 2869	CBS 2206	Heterothallic Bipolar	Dew-retted flax straw, location unknown	
	PYCC 4482	CBS 12945	-	Flower of *Cineraria* sp., Portugal	
	PYCC 6001^{T}	CBS 10757^{T}	Homothallic	Estuarine water from Tagus River, Lisbon, Portugal	
		CBS 6984	Homothallic	Leaf of cereal, Locality unknown	
		CBS 2425	Heterothallic Bipolar	Dew-retted flax straw, Denmark	Sequenced Genome PRJNA371814 *
		CBS 2426	Heterothallic Bipolar	Dew-retted flax, locality unknown	
		CBS 6532	Heterothallic Bipolar	Mouth of newly born *Axis axis*, Paris Zoological Park, France	Sequenced Genome PRJNA371809 *
		CBS 9032	-	Flower of *Vicia villosa*, Germany	
		CRUB 1174	-	Subsurface water from Lake Mascardi, Nahuel Huapi National Park, Patagonia, Argentina	
		CRUB 1178	-	Meltwater from Manso glacial pond, Nahuel Huapi National Park, Patagonia, Argentina	
	PYCC 4737	CBS 12948	-	Grasshopper, Portugal	
	PYCC 4792	CBS 12952	-	Unknown	
Cystofilobasidium capitatum	PYCC 4530	CBS 7420	Homothallic	*Larus marinus*, France	Sequenced Genome PRJNA371774 *
	PYCC 4418^{T}	CBS 6358		Zooplankton, Antarctic Ocean	
	PYCC 5544			*Exidiopsis* sp. (fungi and yeasts), Caramulo, Portugal	
	PYCC 5626			*Trifolium* sp. Leaf infected with rust, Sesimbra, Portugal	
	PYCC 5627			Contamination of MYP agar plate, Caparica, Portugal	
	PYCC 6804			Soil, Arrábida Natural Park, Sesimbra, Portugal	
		CRUB 0777		Fruiting body of the ascomycetous plant parasite *Cyttaria hariotii*, Patagonia, Argentina	
		CRUB 1111		Patagonia, Argentina	
		CRUB 1112		Patagonia, Argentina	
		A 301		Water from Olo River, Portugal	
	PYCC 4309				

* Genomes used in this work (David-Palma, 2017). ^{T} = Type strain.

Table 6.3: Complete list of the relevant plasmids used in this work. Containing relevant information on each plasmid as well as their use throughout this work.

Plasmid ID	Relevant Information	Use
pGBKT7	Plasmid by Clontech (Cat. No. 630443)	Yeast Two-Hybrid: Transformation of *S. cerevisiae* strain Y2HGold
pGADT7	Plasmid by Clontech (Cat. No. 630442)	Yeast Two-Hybrid: Transformation of *S. cerevisiae* strain Y187
pGBKT7-53	Plasmid by Clontech	Yeast Two-Hybrid: Transformation of *S. cerevisiae* strain Y2HGold
pGADT7-T	Plasmid by Clontech	Yeast Two-Hybrid: Transformation of *S. cerevisiae* strain Y187
pGBKT7-Lam	Plasmid by Clontech	Yeast Two-Hybrid: Transformation of *S. cerevisiae* strain Y2HGold
pPR2TN	(Verdoes *et al*, 1999)	Generation of plasmids and recombinant plasmids relevant in this work
pJET1.2/blunt	Plasmid by Thermo Scientific (Cat. No. K1231; K1232)	Generation of recombinant plasmids relevant in this work
pPR2TN'	pPR2TN, with the rDNA fragment of *P. rhodozyma* having been excised at the Sac I restriction site	Generation of recombinant plasmid pPR2TN'+AC025/AC026
pPR2TN'+AC025/AC026	Recombinant plasmid of pPR2TN', containing a rDNA fragment of *C. capitatum* CBS 7420 (AC025/AC026) cloned at the Sac I restriction site	Testing the transformation of *C. capitatum* CBS 7420
pJET1.2+Zeo	Recombinant plasmid of pJET1.2, containing a zeocin resistance cassette cloned at the Cla I and Not I restriction sites	Template for the amplification of the zeocin resistance cassette used to generate the recombinant plasmid pJET1.2+AC027/AC028↓AC029/AC030
pJET1.2+AC027/AC028↓AC029/AC030	Recombinant plasmid of pJET1.2, containing the fragment AC027/AC028↓AC029/AC030 (*HD1*/*HD2* region of *P. rhodozyma* interrupted by a zeocin resistance cassette) cloned at the Cla I and Not I restriction sites	Amplification of the deletion cassette AC031/AC032 used to generate *P. rhodozyma* deletion mutant *hd1hd2*Δ::Zeo
pPR2TN+AC033/AC034	Recombinant plasmid of pPR2TN, containing a fragment corresponding to the *HD* locus of C. *capitatum* CBS 7420 (AC033/AC034) cloned at the Hind III and BamH I restriction sites	Generation of *P. rhodozyma* complementation mutant *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA
pJET1.2+HD1cdsCyscap	Recombinant plasmid of pJET1.2, containing the fragment corresponding to the *HD1* CDS of *C. capitatum* PYCC 5626	Template for the amplification of the *HD1* CDS of *C. capitatum* PYCC 5626 used in the Yeast Two-Hybrid assay
pJET1.2+HD2cdsCyscap	Recombinant plasmid of pJET1.2, containing the fragment corresponding to the *HD2* CDS of *C. capitatum* PYCC 5626	Template for the amplification of the *HD2* CDS of *C. capitatum* PYCC 5626 used in the Yeast Two-Hybrid assay
pEX-A258+HD1-PYCC5628	pEX-A258 containing the *HD1* synthetic gene of *C. ferigula* PYCC 5628	Template for the amplification of the *HD1* synthetic gene of *C. ferigula* PYCC 5628 used in the Yeast Two-Hybrid assay
pEX-A258+HD2-PYCC4410	pEX-A258 containing the *HD2* synthetic gene of *C. ferigula* PYCC 4410	Template for the amplification of the *HD2* synthetic gene of *C. ferigula* PYCC 4410 used in the Yeast Two-Hybrid assay

Table 6.4: Complete list of primers used in this work. Containing relevant information concerning the sequence of each primer, the region of amplification of each pair of primers, and their general use throughout this work.

Primer ID	Sequence (5' - 3')	Description	Use in this work
AC003	ATGGGCGACGTCCTCTTCATC	Amplification of *STE3* gene allele A1 of *C. ferigula*	Phylogenetic study of the *MAT* loci of the *Cystofilobasidium* genus
AC005	GACGAKGCCGAAGTYGTAATG		
AC004	ATGGGCGACATCATCTTCCTC	Amplification of *STE3* gene allele A2 of *C. ferigula*	
AC005	GACGAKGCCGAAGTYGTAATG		
AC006	GTMCTCTWCATCGTCTACTCG	Amplification of *STE3* gene of *C. macerans*	
AC007	GACGAGRCCGAAGTTGTAGTG		
AC008	CGTCCTCTTCATCGTCTATTCG	Amplification of *STE3* gene of *C. capitatum*	
AC009	GCTGAACCCATAGTGGACATC		
AC012	CRAGCRCGGCCTTCTCATC	Amplification of 5'-*HD1/HD2* region of *C. ferigula*	
AC013	ACCTGCTTGTAGTCCATCTGG		
AC014	TCGSACGCACCAGCCGTTG	Amplification of 5'-*HD1/HD2* region of *C. macerans*	
AC015	GTTMGGGTACGAGTTGATGAGG		
AC016	AACGCACCATCCGTTGACCTG	Amplification of 5'-*HD1/HD2* region of *C. capitatum*	
AC017	AGACCCGAACCTGCTTGTAGTC		
AC025	TTTTTGAGCTCTCATTCTCAAGCAGACTGG	Amplification of an rDNA region of *C. capitatum* CBS 7420 containing Sac I restriction sites	Transformation of *C. capitatum* CBS 7420
AC026	TTTTTGAGCTCTTCCCGCTTCACTCG		
AC027	ACAGGATCGATGGTTCTGCGATTGGG	Amplification of a region of the *HD* locus of *P. rhodozyma*, containing Not I and Cla I restriction sites	Generation of *P. rhodozyma* mutant *hd1hd2*Δ::Zeo
AC028	TTTTTGCGGCCGCTGAAATGATTCTCCG		
AC029	ATTAAGCTAGCATCGGCTCATCAGCCGACAG	Amplification of a Zeocin resistance cassette (*Sh ble*), containing Neh I and Nco I restriction sites	
AC030	ACCCACCATGGATCATGAGAGATGACGG		
AC031	TGGTTGATGAGTGAGCAAGC	Amplification of the deletion cassette used to generate the *P. rhodozyma* mutant *hd1hd2*Δ::Zeo	
AC032	AAGGGAAGGTTCGATTTGAGG		

AC033	TATGGAAGCTTGATGTTCGAGAACGC	Amplification of the *HD* locus of *C. capitatum* CBS 7420, containing Hind III and BamH I restriction sites	Generation of *P. rhodozyma* mutant *hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA
AC034	AAACCGGATCCACAATTCGAGAAACG		
AC036	ATGGACGACACCGACAAGTC	Amplification of partial *HD2* gene of *C. capitatum* (5' region up to the homeodomain)	cDNA Synthesis of the *HD1* and *HD2* genes of *C. capitatum*
AC017	AGACCCGAACCTGCTTGTAGTC		
AC037	ATGTCCTTCCACAACCCGAAAC	Amplification of partial *HD1* gene of *C. capitatum* (5' region up to the homeodomain)	
AC016	AACGCACCATCCGTTGACCTG		
AC036	ATGGACGACACCGACAAGTC	Amplification of the complete *HD2* gene of *C. capitatum*	
AC038	CTACCAAGCTGCAGCCGGT		
AC039	ATGTCCTTCCACAACCCGAAACCTCC	Amplification of the complete *HD1* gene of *C. capitatum*	
AC040	TCAGAGTCCAAACTCCCTCTCCTC		
AC041	CGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTATGGACGACACCGACAAGTC	Amplification of the *HD2* CDS of *C. capitatum* PYCC 5626 containing the 40 bp tails flanking the multiple cloning site of pGADT7	Yeast Two-Hybrid Assay: Interaction between the HD1 and HD2 complete proteins of *C. capitatum* and of *C. ferigula*
AC042	TTGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCACTACCAAGCTGCAGCCGGT		
AC043	CATCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGATGGACGACACCGACAAGTC	Amplification of the *HD2* CDS of *C. capitatum* PYCC 5626 containing the 40 bp tails flanking the multiple cloning site of pGBKT7	
AC044	TCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTACTACCAAGCTGCAGCCGGT		
AC045	CGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTATGTCCTTCCACAACCCGAAACCTCC	Amplification of the *HD1* CDS of *C. capitatum* PYCC 5626 containing the 40 bp tails flanking the multiple cloning site of pGADT7	
AC046	TTGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCATCAGAGTCCAAACTCCCTCTCCTC		
AC047	CATCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGATGTCCTTCCACAACCCGAAACCTCC	Amplification of the *HD1* CDS of *C. capitatum* PYCC 5626 containing the 40 bp tails flanking the multiple cloning site of pGBKT7	
AC048	TCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTATCAGAGTCCAAACTCCCTCTCCTC		
AC049	CATCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGATGGCAACCCAAACTATTCTC	Amplification of the *HD1* CDS of *C. ferigula* PYCC 5628 containing the 40 bp tails flanking the multiple cloning site of pGBKT7	
AC050	TCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTATTATTGACCACCACCAGAAC		
AC051	CGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTATGCCGGATGATACTGC	Amplification of the *HD2* CDS of *C. ferigula* PYCC 4410 containing the 40 bp tails flanking the multiple cloning site of pGADT7	
AC052	TTGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCATTACCAAACTGGTTCTGG		

MP091	AAGATGGATTGCACGCAGGTTCTCC	Amplification of the Geneticin resistance gene	Transformations of *C. capitatum* and of *P. rhodozyma*
MP092	TTCCACCATGATATTCGGCAAGCAGG		
MP120	CTAGCAGTCAAAAACCTCTCCAAGC	Amplification of *HD1* complete gene of *P. rhodozyma* CBS 6938	
MP121	ATGGAACCATCTCCAGCGTCAGATC		
MP122	ATGAGAATCATATGGATTTGTAGG	Amplification of *HD2* complete gene of *P. rhodozyma* CBS 6938	
MP123	TTATTTCCCGGTCGTCATG		
MP181	CATCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGATGGAACCGAGTCCGGCG	Amplification of the complete *HD1* synthetic gene of *P. rhodozyma* containing the 40 bp tails flanking the multiple cloning site of pGBKT7	<u>Yeast Two-Hybrid Assay:</u> Interaction between the Complete HD1 and HD2 complete proteins of *P. rhodozyma*
MP183	TCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTATTAGCAGTCGAATACTTCGCCC		
MP184	CATCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGATGTATAGCTTCACCAAGTTGCC	Amplification of the partial *HD2* synthetic gene of *P. rhodozyma* containing the 40 bp tails flanking the multiple cloning site of pGBKT7	
MP185	TCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTAAGAGCGCCGATTACGGCG		
MP184	CATCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGATGTATAGCTTCACCAAGTTGCC	Amplification of the complete *HD2* synthetic gene of *P. rhodozyma* containing the 40 bp tails flanking the multiple cloning site of pGBKT7	
MP186	TCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTATTATTTCCCCGTTGTCATGTTCAGG		
MP187	CGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTATGGAACCGAGTCCGGCG	Amplification of the partial *HD1* synthetic gene of *P. rhodozyma* containing the 40 bp tails flanking the multiple cloning site of pGADT7	
MP188	TTGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCACATCCAGCCGCTGCGACG		
MP187	CGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTATGGAACCGAGTCCGGCG	Amplification of the complete *HD1* synthetic gene of *P. rhodozyma* containing the 40 bp tails flanking the multiple cloning site of pGADT7	
MP201	TTGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCATTAGCAGTCGAATACTTCGCCC		
MP189	CGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTATGTATAGCTTCACCAAGTTGCC	Amplification of the complete *HD2* synthetic gene of *P. rhodozyma* containing the 40 bp tails flanking the multiple cloning site of pGADT7	
MP202	TTGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCATTATTTCCCCGTTGTCATGTTCAG		

All MP primers, with the exception of MP201 and MP202, originated from the work of David-Palma, 2017.

C. capitatum CBS 7420 rDNA region

TCATTCTCAAGCAGACTGGAACGATTAGAATCGTACACAGGGCCGAGTACTCTTTGGGGAAAGGTTGGTT
ACAACTGCTATTACAATTTTCAAGCTGTCGATTGGCTTCAAGCAAGTACTCTTTGGGGAACGCTTTTAGAG
AACAAGCCAGTTTGTGATTGTGAAGGCGACAGATGCGACTGCTGCATAACGCCGGTCTCCTGTCAAACA
AGTCCTATTGCTCGCAGTAGGCAAGGATTAGTAGTGTGGATTACTTCCCTCTGGGGTAACCTATCGCAATT
AATTAATCGGGGATCCGTCTGGATGTGACTGCTGCTTTTCGAAGCCGGTCCATTTATGATAGTCCTCACTT
TGGAACTTGAATCGAGTCGTATCTGAGTCCTTCGGGATGTGAAGTGCGACTGGATGACTAGTTTCAGAG
CTGGTAAGAACCGGGGGATAAGGCAGTCATTGAAGTGAAAGAGTCTACCAGATGTCCCGCAAGGGAATA
ATTGGTGCTCTGATCTTCCTTGATTTGTATAATTTTGGTAATCACCATTTTAACGAACACCTAAGTCGAGGG
GGTCGCTTGATTGCGACTCTCAAGGCGGTTGTAATCTTCGGATTACAGCTCAATTGGAATAGTTACCTGG
TTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAACAAATTCATA
CTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTACCTTACTACATGGATAACTGT
GGTAATTCTAGAGCTAATACATGCTGAAAAGCCCCGACTTCTGGAAGGGGTGTATTTATTAGATAAAAAAC
CAACGCAGGCAACTGCTCCCTTGGTGATTCATAATAACTTCTCGAATCGTATGACCTTGTGTCGACGATG
CTTCATTCAAATATCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTTTCAACGGGTAAC
GGGGAATAAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAG
GCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACAATATAGGGCTCTATGGGTC
TTATAATTGGAATGAGTACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAG
CCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACTTCAG
GTCTGGTGGGCCGGTCCGCCTCACGGTGTGTACTGGTCGACTGGACCTTACCTCTTGGTGAGCCGTCA
TGCCGTTTATTCGGTGTGGCGGGGAACCAGGACTTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCC
TATGCCCGAATACATTAGCATGGAATAATAGAATAGGACGTGCGGTTCTATTTTGTTGGTTTCTAGGATCGC
CGTAATGATTAATAGGGATAGTTGGGGGCATTAGTATTCAGTCGCTAGAGGTGAAATTCTTGGATTGACTG
AAGACTAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAGGTTAGGGGATCGA
AAACGATTAGATACCGTTGTAGTCTTAACAGTAAACTATGCCGACTAGGGATCGGGCGATGTTCTTCTTTT
GACTCGCTCGGCACCTTACGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAA
ACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGG
GAAACTCACCAGGTCCAGACAATATAAGGATTGACAGATTGATAGCTCTTTCTTGATTTATTGGGTGGTGG
TGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGATAACGAACGAGACCTTAACCTG
CTAAATAGCCCGGCTGGCTTTGGCTGGTCGCTGGCTTCTTAGAGGGACTATCTGTGTTTAGCAGATGGAA
GTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGAG
CCAGCGAGTTTTTTCCTTGACCGGAAGGTCTGGGTAATCTTGTGAAACTCTGTCGTGCTGGGGATAGAG
CATTGCAATTCTTGCTCTTCAACGAGGAATTCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCC
CTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGTTTCCGGATTGGCTTT
GAGGAGCTGGCAACGGCACCTTGTCGCTGAGAAGTTACCCAAACTTGGTCATTTAGAGGAAGTAAAAGT
CGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCACTAAAGAATTCGCCCTTCGGGGCTCTCTTTA
TTCACACACCCCTGTGCACTTTGGCCACCTCTTTGTTGAGGTGTGTCTTTTTAATTACCATACCCTATAAA
CACAAGTTATTGAATGTAAAATCGTTATAAACTAATATAACTTTCAACAACGGATCTCTTGGTTCTCGCATCG
ATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CATCTTGCGCTCTTTGGTATTCCGAAGAGCATGCCTGTTTGAGTGTCATGAAACTCTCACCTCCAATCTTT
TTTAACTAGAAGGTTGGGGCGTGGACGTGAGTGCTGCTGGTGCCCTGGTTGCATCGGCTCACTTGAAAT
TCATTAGCTGAATCCTTTAAAGTAGGTTCTACTCGACGTGATAAGATCTCCGTCGAGGACAGTGCTTCGG
CATTGGCCAACGATAAAGTTGATACGCTTCTAATTAGCGCAGACTTCGAGTGCTGGCAATTTTGACAACTT
GGCCTCAAATCAGGTAGGACTACCCACTGAACTTAAGCATATCAATAAGTGGAGGAAAAGAAACTAACAA
GGATTCCCCTAGTAACGGCGAGTGAAGCGGGAA

Figure 6.1: DNA sequence of the rDNA region of *C. capitatum* CBS 7420. rDNA region in the plasmid pPR2TN'+AC025/AC026, used to attempt the transformation of *C. capitatum.*

C. capitatum CBS 7420 *HD* locus

GATGTTCGAGAACGCAGCGAGAAGAGATCGAAGGGAGAACAAGATGGGTACCAGAGTTCGAGTCGAGGAAAGAAG
AAGAGCATAGCGGACGAGAGCAGGGGAGGGGGGCCTATTATTTAGTGTTCGTATCCAAGCAGAGGCAGAAGAAGG
GAAGTTCAGAGTCCAAACTCCCTCTCCTCCCGTTCAATCTCCTCCTCCCTCCTGATCCTCTCTCGTCGCCGTTC
CAACGCCTCCCTCCTCTCTATCTCATTCCCGCTGCCCTGGGGCACGATCGGTGCCAGAGGGATCGAGGCGTAGTC
GTACACTCCTTGATGGGGTTGGGGAGCGATTGGGGTGAGGGAAGCGAAGGAGGGTTGGGTTTGAGAGTGGGAGA
TCGCTCGTCTCTTCGAGGGACGTCGAGAAGGTCCCCCTACGGTGGTAGGGACTGAGGTAGAAGGGAGAGAGTACT
GTCTCGGGTGAGGACCTCGACTTCCTGCTTGGTGAGGGTTACCGTACGTCGAGTGGGAGCGCGAGTACCAAGGAG
GAGGGGGAGGGTGGTGTCCGTAATCTTGTCCTCCGTAGTAAGCCTGGGTGGGGTACTCTTGAGCAGGGGTTGGTG
CGTAGTAGTCCGTTTGTGGAGGATGAGGAGGGTGGGCATGAGGATAGAAAGCAGAGTGGGGGTTGAAAGCGTGGT
GTTCGTCGTACTCCTCCGAAGGAGTGAGTGGAATGGAGTCGTACGACGACCCAGAAGAGTTGCGGAGGGATGACG
TGCGATGGGCTGTCGAAGAATGTCCGTGAGACTGGATGGAGGGCATGATGGAGACCGATCGATGAGGGACAGAAG
GGGCAGTGATGGTTGACGAGGCAAGAGCGGTGGGAGGGGAAGAAGGGATCGAGACGGATCGGGAGACGTCGGA
GGAGGCTGAGACGCGCACTTTTGGGAGAGGCTTGAGGTGACGAGCGAACGAAGAGGCTGGTTCGGGCTTTTTGG
AGGAGGAGGAGGAGGTTGACCGTCGAGTTGGGCGAGCGGGGCGGCCGGACTCGGGAGCCTTGGGAACGACGTT
GCTGACTTTCCTCTTCCTCCCTCCACCAGCCGTCGAGCCATACTCCTCCTCGTCATCCTCGTCGTCCTCGTAGACTT
CGCAGTCCGGCTCAGAGTCCTCTCCGAGGACATCCAGTCCCTCCGCGACCTCTCTCGAAGTCTCCTCAATCACCC
CAACCATCCAATCCCTCACCCTCTCATGACCCCTCTCATCTACGTAGTCCTCCACTCGCTCGAGGGCGAGCTGAAC
GTCCCGTGCTAGAGTGAGAGGGTGAGCTTTGAAGGTCTTGATGAGGGTTCGCATGCGGGTGTGGTCCGAGTTGGC
AAAGTCGCGTTCTGCTTGAGTCCAGCCCTGTACACCCCGCACAACGAAGAAAGACGGTTCAGCAACTTGCGCTTG
GAAAAAGAAGAAGGAGGAGGACGTACAGAGCGTCGTCTCATGTTCGTCATCCTATAGAAAGAAATCTCATGTCAGTC
CGCTACTCGTTCAAAGGGGTGAGCGAACAATGGAAACAAAAGAGGACCGAACGCACCATCCGTTGACCTGGTTCA
CAGTAATCCCTCGAGTGAGAGCCATCTCCTTCTTCTGATTGGAGGTAGGAAAGGGGTGTTCGAGGTTCTTGAGAAA
CCAGTCGCGGAGGTGACGGAAAATTGAGGGAGGAGGGGGGAGGCCGGAGGAAGGGTCGTAGGGGGGTTCTTCG
ATCGGAGGGGGGGAGGGGGGTCGGCTTGAGGAAGAGGCCTGCAGGTCGCAATCCACATCTGGAGAGACTCAATA
CTGGTCAGTACATGCTTCGGGCCAGAAGGGTAGACTTAGACGCACCTCGTGTCAAGTCGCTCTTGATGTCCTCCAT
CTTCTGCTGCGCGTCCAGCAGGACGGAGCACATGTCCTTCAAGGCAGCAGCCACAGCAACCCCTACTTCCGATGT
CTGGGCATCCAAAAGACCTAGTCGCCGCTCTCGCTTGACCTCCTCCACCAGCGAACTCAGACGCTGAACAAACTCT
TGTGTTCCGTATCCGCGTCCAGCGAAGTCCAGGAGTGCTCCCTGGAGGCGTCCGACCAAAGGAGGTTTCGGGTTG
TGTAAGGACATGGCGTGGTGGAGTGTGTGTTTGATGGTCCTCTAGAATCGTTCGAGCTTCCCATCTACGTAAGTTGA
AGGGAGGATCAAGGGTAGCAGGGGGAGCAAGAGCGACGACTGGAAGCCCGTCGCAAGTGACCCCTGAAACTGCC
CCTTTTATTTTGTGAGTTTGAACCTCCGACAATCCTTTTGTCTCCCCTTCTGCCTTCAACCCATCACTCTCTCCGACA
AGAAGGTCCTCTCGCTCTGACCACCACCCGCTCCTCTGCCCCCTCTCTTTGCCTGTCAGCAACCACTCCTCCACG
GCTCTCGCTTTCGGTAAATCGACATGGACGACACCGACAAGTCTTTCCTTCGACAAGTCCTTCTCCTCGCCCAGAAT
CTCAAAGGCGAAGGGAGCTCAAGCACCCATCCTCCAACCTTTCATGGCAACATCCCCACCCTCGCACTTTCTCCTT
TGCCCGAAGTTGAAAGGACCATCCGAGGACTCGGTCTGTCAGGGGAAGTCGCACAGGCGTTGCAACGGACTTTCC
AAGGGCGTTTGACAAGCTACCAGGCGCTCCTCGTTGAGGCTTGCCAGCGGGAGGTTCCTCACGCGCAAGCGGCG
CTAGAGGGGATTCAAGGCGACAGAGGCAGCGACACCTACTCAGGGCGCGTGATTTGGGCATTCAAGTGTCGTGTA
GAGAAGCGCTTTGAGTCCCAAGTGCTGGAGATGCGGTCTCTTGTGTTGGACAAGGTCCAGTCCTGGCTCTCTCGC
AACGCCTCTCGTCCCTCTGCCCCCGTCTTCACCAAGGTGCGCGTCTCCTGTATTTTCTTCTGCCCTCCGAACAACA
CTGACGCGACGCGTCTCCTTTCTTGCAGGCCACCATCGACGTTCTCCGCAAGATTTACCTCCTCAACCCTCAGCCC
AACCCAGCCGAGGTCAAGCTGCTCGCTAGCAAAATCGGGATGGACTACAAGCAGGTTCGGGTCTGGGTCCGTGCT
CCTCCCCTCTCCTCTCACGCGCGTCTCGGCTCTGACTTTCGACTTTTGCTCGCCCTTCGCAGTTCCAGAATCGACG
CAACCGCAAGGCCGTCTCTGCCCGGACCATCGAGGAGGAGCGACGGATCGCCAAAGCCAGCGGCATCACCCTAG
CGAAAGCCCACAAGACACAGGTCAAGCAGGTGAAGAAGGTCGAGAAGAAGACGCAGGTCAAGAAAAAGGCTGCT
GCTCGGTCGACGTCGGTTAAGGTCCGACACTTCGCCCAAGAGGGAGATGAGAACGATCGGGAGATGGACGTCGCT
TGCCCAACGACGCACTACAAGCCTTCGCCACTCGACGCTCACTCGATCAACTACTACGCCTCGTCGAACCTCGGGT
ACGGTCGACCACCCGTCTCCACCTCGACCTCGCCTGACCCTTACTCGACGTCGGCCTCCTCGTCGCAGACCTCCT
TCAACGTCCCTTCCTCTTCGCACCCCTCCAACTCGATGTCGTATCAGGTGACTTCCCAGCAGCAGGCGGCCGCCTC
GCCCGAGCAGTCTTGGAACCTCGTCCCCATCCAGCCGGCGTACTACTCTCAGGCTCCGGACCCCTCCTTCCTCCC
TCCTCTGATGCAACGGTCAGCGTCGTACGCTTCATCCTCGACGTCGCCCTCGACCGTCTCGGACCTGGACTCCCT
CTGGTCCATCGCCTCCACCGACCCTGCATCGACGTACTCAAACTTCTGCGAGCCACCTTGCGTTCCCACCAGCCTC
GACTTTACACCCCCCCCTCCCTCAGACGAGTCAAGTTTGACGAACCCGATGTTCGAACCCCCAGAGTACCTCCTCT
CCTCCTCCATCCCGACATTCTCCTTTTCCCCTTCTCCACCGCCACTCCAACCACCCGCATTCTCAACCGACACCTTC
CGTGCCCTTCTCGACTCGTCCTCAGGCGACATGGAGACGCTTTGGGGAGGAGGGGACGTACTCGAGTTGACGGA
GGAGATCGACCAGGTCTTGAGGGAGTACACGCCCGAGGGAGAGACGTTCGACCTGTTTGACTTTAACATCCCTCAA
CCGGCTGCAGCTTGGTAGATTCATCTCATATTTCTTTCATTCGTCTTCGTCACTTGCATACCCACCCACACAACGACC
AGGTTCTCTCAGGAGACCACCTCTATATCTCGTCCGCTTTTCTCATCTCTGTCTCCGCTTCATCTCCTCGTTTCTCGA
ATTGT

Figure 6.2: DNA sequence of the *HD* locus of *C. capitatum* CBS 7420. Comprising the *HD1* and *HD2* genes of *C. capitatum* CBS 7420, this region is cloned in the plasmid pPR2TN+AC033/AC034, used to generate the complementation mutant of *P. rhodozyma hd1hd2*Δ::Zeo/*HD1HD2*::G418+rDNA.

Zeocin resistance cassette

ATCGGCTCATCAGCCGACAGTTCATCCGACACAAGCTCTTTGCCTAGATCGTCAAACGATCGACATCGACACGAA
AACAAATCCCGATCAGTCAATCAGTAGAACTGCCGAAGAATGAAACTGAAGACTGCTGTGACACGTGACTATAGA
AGCGGTGTCATCTGACTTGCGAATTTGCTTGTACAAAAGTCAGGTTGGCTGATTCGCTCGCGTTGTCGACAAGAA
TTAGGAAACCTCATTTCCAGCCTTCCTTTTTTTCCCCTCAGAACACCTCACATCTTCTCACAAAAAAGTAAGATATT
TTCACTTGGAGTTGGTGGATTTGGCGACTGAATGGAGAGACAGAAGCTTGATACTGATAAGACACTCTTTTGTAAT
CTATCTCGAACAGCTTCACCAGATCTATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTC
GCCGGAGCGGTCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGT
GTGGTCCGGGACGACGTGACCCTGTTCATCAGCGCGGTCCAGGACCAGGTGGTGCCGGACAACACCCTGGCC
TGGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGAC
GCCTCCGGGCCGGCCATGACCGAGATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGC
CGGCAACTGCGTGCACTTCGTGGCCGAGGAGCAGGACTGATCTAGAAGGCCTACGGTTCTCTCCAAACCCTCT
CCCCTTTTGCCCTGCCCATTGAATTGATTCCCTAAATAGAATATCCCACTTTCTTTTATGCTCTACCTATGATCAGTT
TATCTGTCTTTTTCTTTGTGCGTGTCGGTTGTGCGACTGTACCCACCTCTTGAGGGACAAGGCAAGAAGTGAGCA
AGATATGAACAAGAACAACAAAGAAAAAGAGACAAAGAAAAAAAAAGGAAAGAGAAAACAATCCCCCCCCCCCC
CCCCCAAAAAAATCTCTATCTTTATCTGATCAAGAGATTATATTGCATCTTTTGAATTTCCTATCGCCTTCATCCTCG
CCAGAGAAAAAAGTTTCAATCACATCTGTTGACCATCACCATCATCTCCGTCATCTCTCATGAT

Figure 6.3: DNA sequence of the Zeocin resistance cassette used in this work. The Zeocin resistance cassette consists of *Sh ble* gene (underlined in the sequence), a *Tef* promoter and a *Gpd* terminator. The Zeocin resistance cassette is cloned in the plasmid pJET1.2+AC027/AC028↓AC029/AC030, used to generate the *P. rhodozyma* deletion mutant *hd1hd2*Δ::Zeo.

***C. capitatum* PYCC 5626 HD1 protein sequence**

PPLVGRLQGALLDFAGRGYGTQEFVQHLSSLVEEVKRERRLGLLDAQTSEVGAAVAAALKDMCSVLLDAQQKMEDIK
SDLTRDVDCDLQASSSSRPPSPPPIEETPYDPSSGLPPPPSTFRHLRDWFLKNLEHPFPTSNQKKEMALTRGITVNQ
VNGWMTNMRRRSGWTQAERDFANSDHTRMRTLVKNFKAHPLTLARDVQLALERVEDYVDERGHERVRDWMVGVI
EETSREVAEGLDVLGEDSEPDCEVEEDDEDDEEEYGSMAGGERKRKVSNVVPKVAEFGRPARIRPTRRSTSSSSKK
PEAASSFARHLKPLPKLRVSASSDVSRSVSIPSSPPTAPSSSTNTAPSVPHRSVSIMPSIQSHGHSSTAHRTSSLRNS
SGSSYDSIPPTPSEEYDEHHAFNPHSAFYPHAHPPPPQTDYYAPTPAQEYPTQAYYGGQDYGHHPPPPPPWYSHS
HSTYGNPHQAGSRGPHPRQYSLPSTSVPTTVGGPSRRPSKRRAISHSQTKPSFASLPPIAPQPHQGVYDYASIPLAP
IVPQGSGSEIERREALERRRERIRREEE

***C. capitatum* PYCC 5626 HD2 protein sequence**

DTDKSFLRQVLLLAQNLKGEGSSSTQPPTFHGNIPTLALSPLPDIERTIRGLGLPGDVAQALQRTFQVCLTSYQALLVE
ACQREVPHAQAALEGIQGDRGSDTYSGRVIWAFKSRVEKRFESQVLEMRSLVLDKVQSWLSRNASRPSAPIFTKATI
DVLRKIYLLNPQPNPAEVKLLASKIGMDYKQVRVWFQNRRNRKAVSARTIEEERRIAKANGITLAKAHKTQAKQVKKV
EKKTQVKKKAAPRSTSVKAQHFAQEGDENDREMDIACPTTHYKPSPLDAHSFNYHDSSNLGYGRPPVSTSTSPDPY
STSASSSQTSFNVPSSSHPSNSMSYQSTPQQQAAASPEQSWNLVPIQPAYYSQAPDPSFLPPLMQRSASYASSSTS
PSTVSDLDSLWSIASTDPASTYSNFCEPPCVPTSLDFTPPPPSDESSLTNPMFEPPEYLLSSSIPQFSFSPSPPPLQPP
EFSTDTFRALLDSSSGDMETLWGGGDVLELTEEIDQVLREYTPEGETFDLFDFNIPQPAAA

Figure 6.4: HD1 and HD2 protein sequences of *C. capitatum* PYCC 5626. Sequences obtained through translation of the CDS of the *HD1* and *HD2* genes of PYCC 5626 obtained experimentally and used in the Yeast Two-Hybrid assay of the *C. capitatum* HD proteins. These protein sequences were used for the alignment with the HD1 and HD2 protein sequences of *C. capitatum* strain CBS 7420.

C. ferigula PYCC 5628 *HD1* synthetic gene sequence

ATGGCAACCCAAACTATTCTCAAAAACCGACTTCAGGACGGGCTGCAGAATTTCTTCAGTGTACTAAAAAGCGGTGAC
AACCAGTCATGTCGAAGTTTCCAGGCACAACTTCTTTCACTAGTCCAGGCTGTTGGACAAGCAGCACAGCAGGGGCG
GTTGGATGAGGAAACCAGAACGGTATGTGGTGCTGTAGCAGCCATACTTGAATCGAGCGCCACTGTCCTTCTTCAGG
TTAAGTCTGACGCCTGTGTTATGGAACAGCAGCTGCATTTTGATGTTAGCAAAGATCTAGGCTCGCATGCCGTGATAAG
GTCGTCCGTGGAAGAACACACGGCGCCATTGCCCTACCCTTGCCACTCCTCGCCTGACTCGTTTAGTTTAAGCCCAC
CGCCCCCGCCATTCTCCTCTAGCATCACACCAACGGCGGCACCCGCTCCGCCTCCACCATCTGCTTTTCGCCACATC
AGAAACTGGTTTTTGGAGCATCTGGAACACCCGTTCCCTACACCTGACGAAAAAGCCGCGTTAGCCAAAGCAAAAGG
TTTAACTGTTCCACAAGTTAACGGTTGGCTAACAAATATGAGGCGCAGGTCGGGTTGGACTCAAACAGAAAGGGATTT
CGCCAACGCAGATCATGAACAAATGAGAGCGCTCGTGCTAGCCTTTCGGAGAGGCCCTTCCGGTGGCGTAGCCAGA
GATGTCGTGTTGGCCCTAGAAAAAATAGAAGACTATGTGGATGAAAGGGGACAAGAAAGAGTCAGAGATTGGATGGTA
GGTGTGATCGAGCAAACCGCTAAAGACGTACAAGAAGGAAAGGACGTTCTGGGTGAGGATTCCGAGGATGAAGAAG
GAGCATTTGATGAGGATGACGGAGAAGCGCCCAGTACGAGTGCGGCCGTCAGCGAAGCTGTGGGGAAGAAACGTAA
AACAAGTTCTTTGGCTGCACCATCCGCCAGCTCAGTCAAACAACCTAAATCTAAACCAGCGCCAAAGACTGAAGATCG
TATTCTGAAGCCGTTGCCGAAGTTGAGGCTCGTAAGGCCTTTGTCATCAACTAGTTACTCTGCAACTGTTCCCGCCAG
TCCGCCACCATTGCCCCCAAATGCGAGGACCATTACCCCGCCCTACAGTCGTCACCCAAGTGAGCCTAGAAGTACTG
TTGGCTTGGGGTACCCATCTGTTTCAACTAATCCTCATCAACATAGAACCTCTTCTGTCAGAAATTCATCAGGAACATCA
TCACTAGAAACCGCCGTTGGACCGGGAACTCCGGAAGAATTCGAAGGCGACTTCGAGCCAAATGCTGTTTTTCAACA
ACAACATGAGTTAGGCGTTTTTTCAGCTCCCTTACCGCCTCCTTTGTCCCAATACCCCTCTCACGCCCCCCCCCCCA
TCAAGCATATTACCCTTCCCACTCACCATTGCCTCCCCCCCACTACTTTGCTCCTTTGGCTCCCGCATTACCACCTTCC
CATTCCGATGTTTTTCCTCACCCATACGCATATGATCCACCTTATTCCCACGCTAATTTTCCACCACCTCCTCCACCAAC
TAATGGCCGTTTAGCACACCCTCGTACATATTCTTGGTCAGCTGGTGAAGCTCATCCTCCACCACAACCACCAGCTAA
GAAGCATAAGAGAAGAGCTACATCTGATCAACATCCTTCTACATTATCATCTGCACATGCTTATTATCATCCAGCACCAC
AAGAACCTTATCCATATGCTCCACCTCATGCTTTTTCTCCTCCTCATCCATCTTATTTACATCCATTACCTCCACCTGCTC
ATGCTGCACCACCACCACCACAAACAGATGATGATAGACATGCAGCTTTATTAGCTAGAAAGGAAAGAATGAGAGAAG
AGCAAGAACAAATTAGAAGAGAGGAGGAAGCTTTAGCTTCTGGTGGTGGTGGTGGTTCTGGTGGTGGTCAATAA

C. ferigula PYCC 4410 *HD2* synthetic gene sequence

ATGCCGGATGATACTGCTCTTCTGCAGAATGTTTTGAAGCTTGCACAGGAGTTAGGTCAGTCTGCTGGAGTCCCGCTC
AGTACAGGAAGACCACTTACCTTACCGGAGTACCTCCCGCTGGATCTCCCACCTCCCCCCACCTTGTCGAGATATCTA
GAGTCGATTGGGATTTCACCGGCTTTGCAAGCAGCTCTGCAACTTGCATTCGAAAAAAGAATCCGAACATTTGCCATAT
CAACTCAACAATTGTATCAGCGATCGTCTGGGGGAGTGAGTGCACCATTGGGTGTCTTGGAGGAGGTCAATGGGTCT
AGGGGCTACGCGGCTAGATTGTTCCGTGACTTCCGTGCTCAAGTGATAGCCCACTTTGTAACCGAAGTTGCCAAATTG
CAAGCCATGGTTTTAAGCAAGGCAAGGGACCATGTACTGAAGGCTGGATCCACTAAACCGCCTCCCCAGTTCACTAAA
GCTCACCCATTGAGCAGTCATCCTCCCACTAGCGCTATAGAAACACTGCGGAAGCTGTACTTGTTAAATACTTACCCTA
ACCCTGCAGAAATACGTTTGATAGCTAAAAAAATCCAGATGGATTACAAACAGGTGAGGGTCTGGTTTCAAAACCGTAG
GAACAGAAAAGCTGTCTCCGCACGCGGACTAAAGGAAGAAGTAAGGATCGCACAGGCTTCCGGCATCAAATTACCGT
CCACGCACAAGCGTGAGTTGAGAAAACTTGAGACAGCCGCTGCAAAGCAAACTAGGAAGAAGGATGGCGAGAGAAG
ACGCAGAGGAGGCGTAGTCGTCACAGCTCAGGATGTACAAGATGAAGACAAAGAAGGCATGCATGTGGACGATGCC
GGCATCGCTCCTCCGACAGCCAAACATAGCGCTCTAGACACGGTATCCTTCAATTATGGGGATGTGTCAATTCTTGGTT
ATGGCAGACCACCTCCCGTTTATTTCGGAGCGGGAAGTTCAGAGACATCGCCAGCACCCCACAGTGCTAGTGCTGCT
AGCTCCTCTCTATCGTTGTCTGGTCAACCCCCTCCCCCATCATCCTTAGTGCCCAGTGGTTCTTGGAGCTATGATGCTT
CCCCCTCCCAATGTTCTCAATCGATGACTATTCCAGATGAACAATGGAATCTTGTTCCAGCTAGACCAGACTACTATCAA
CAAAGACATTCTGACGGTTCTGTTTTAGTTCCACCTCCAGCAACGGCACAACCTCATAGAACGACCTCATTCGGTAGC
AGCTCAAGTTCATCAGGTCTTTCGGATCTAGATTCCTTGTGGTCAATTGCAAGTACCGATCCAGCGGCGACTTACTCCT
CTTTCACATCAGCTCCTTGTGTTCCTACTGCTTTAGACTTTACCCCTCCTTTGCCTTCTGATGAAAGTACTTTGGCGGC
TGCACATGAAAACCCTCTATTCGAACCACCAGAATATTTATTACAATCTTCAATTCCTGAATTCAGTTTTTCTCCATCTCC
TCCACCAGCCCTAAATCCAGCATGTGCCTCTAATCTAGGTTTTTCTGCAGATGATTTTAGAGCCGTTTTAAACGCCGAT
CCTTTTTCAGACTCTTCTGCCGACGCGTTAGCGCAAGCTGACTCTAACGGTTTTTTTTGGAATTGCTCAGGTGGTGCG
GGTGCAGACGTTTTAGAATTATCAGCCGAAATTGATCAAGTTTTAAGAGCATTTACACCAGAATTTTTAGGTGCTCCAG
GTGCCGAAGGTGAAGCATTTGATCCATATGATTGGGGTATTCCAGCCCCAGCAGATACAACCTATCCAGAACCAGTTT
GGTAA

Figure 6.5: DNA sequences of the designed synthetic *HD1* and *HD2* genes of *C. ferigula* PYCC 5628 and PYCC 4410, respectively. Sequences correspond to the CDS of the *HD1* and *HD2* genes of PYCC 5628 and PYCC 4410, respectively, optimized for the codon usage of *S. cerevisiae*.