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Functional and genetic analysis of sexual reproduction in the thermodimorphic fungal pathogen *Paracoccidioides* spp

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Functional and genetic analysis of sexual reproduction in the thermodimorphic fungal pathogen *Paracoccidioides* spp

Tese de Doutoramento em Ciências da Vida e Saúde

Trabalho efetuado sob a orientação do
Professor Fernando Rodrigues
e do
PhD. Marcus Sturme

janeiro de 2017

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I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 15/01/2017

Full name: Jéssica Angélica Rezende Gomes

Signature: 

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Resumo

Paracoccidioides brasiliensis e *Paracoccidioides lutzii* são fungos multinucleares e termo dimórficos, sendo também os agentes etiológicos da Paracoccidioidomicose (PCM), uma micose que é endémica da América do Sul. Estima-se que a PCM afeta 10 milhões de pessoas em países da América Latina, sendo que o Brasil apresenta cerca de 80% dos casos relatados. Este fungo sofre uma transição morfológica da sua forma não-patogénica, micelar e conídeos, existentes em temperaturas ambientes, para a forma de levedura que é patogénica na temperatura corporal dos mamíferos. *Paracoccidioides* apresenta um ciclo de vida assexuado. Entretanto, estudos recentes apoiam a existência de ciclo sexual em *Paracoccidioides*, pela: i) a existência no seu genoma de praticamente todos os genes homólogos necessários para a reprodução sexual em Ascomicetos, incluindo recetores de feromonas, membros da via de sinalização para reprodução sexuada e isoformas dos loci *MAT1-1/MAT1-2*, e ii) identificação de eventos de recombinação genética entre isolados do meio ambiente. Apesar destas evidências, a reprodução sexuada no género *Paracoccidioides* nunca foi observada na natureza ou em condições laboratoriais; contrariamente ao observado em espécies de géneros filogeneticamente próximos como *Histoplasma* e *Aspergillus*.

Nesta tese, estudámos o processo de reprodução sexuada em *Paracoccidioides* sob diversos aspetos do processo de reprodução sexuada em Ascomicetos. Os nossos resultados mostram a baixa expressão dos genes que expressam os recetores das feromonas **a** e α (*PREA* e *PREB,* receptivamente), o gene da feromona α (*PBα*) e os genes *MAT*, nas formas de levedura e micélio em vários isolados de *Paracoccidioides*. O ensaio de indução realizado com a feromona sintética Pbα nas estirpes MAT1-2 de *Paracoccidioides* tinha como objetivo avaliar a resposta transcricional dos genes relacionados com a reprodução sexuada, entretanto a presença de Pbα falhou e não serviu como estímulo para a resposta da ativação da transcrição dos genes *MAT1-2*, *PREB* e *STE12*, sugerindo que as estirpes testadas são insensíveis à Pbα. As funcionalidades dos genes relacionados com a reprodução sexuada em *Paracoccidioides* foram analisadas através da expressão heteróloga desses genes em estipes mutantes de *Saccharomyces cerevisiae* que não possuíam os genes homólogos correspondentes. Nós demonstramos que as estirpes de *S. cerevisiae* que expressavam de forma heteróloga *PREB* respondiam à Pbα sintética, tanto como à Pbα extraída do sobrenadante de culturas de *Paracoccidioides* através da formação de shmoos e pela paragem de crescimento e ciclo celular. Estes resultados nos levaram a concluir que as estirpes de *Paracoccidioides* secretam a feromona alfa ativa no meio de cultura, que é capaz de ativar seu recetor cognitivo.

Em *A. nidulans* e *A. fumigatus*, um grupo específico de proteínas reguladoras que respondem à presença da luz, denominadas proteínas Velvet, apresentam um papel crucial no ciclo sexual na ausência de luz.

Através de análises *in silico*, nós demonstramos que *Paracoccidioides* tem proteínas que apresentam os domínios Velvet, demonstrando elevada homologia com os domínios das proteínas de *Aspergillus*. Além disso, o regulador negativo GprD e os reguladores positivos NsdD e LaeA da reprodução sexuada apresentam funções importantes durante o desenvolvimento sexual. Homólogos destes componentes também estão presentes no género *Paracoccidioides*. Neste estudo, nós analisamos os padrões de expressão génica destes genes na presença e ausência de luz em diferentes estirpes de *Paracoccidioides*. Nós concluímos que a expressão do *MAT* locus, dos genes Velvet, e dos reguladores *LAEA*, *GPRD* e *NSD*, ocorrem independente da presença de luz.

Neste trabalho, nós também realizamos ensaios de reprodução sexuada com isolados de *Paracoccidioides*. Nós delineamos nossos ensaios, através das informações previamente publicadas relacionadas com os ensaios de reprodução sexuada em *Aspergillus* e *Paracoccidioides*, também usamos estirpes MAT1-1 e MAT1-2 de *Paracoccidioides* que expressam genes responsáveis pela resistência à dois antibióticos diferentes. Após oito meses de incubação em diferentes meios e no escuro, analisamos as células recolhidas da intersecção das duas estirpes de MAT locus opostos, co-cultivadas que eram resistentes à ambos antibióticos. Através de análise microscópica, encontramos estruturas semelhantes à esporulação, ondulação de hifas, típicas em estágios iniciais da reprodução sexuada, e ascomas revestidos por células Hülle. As morfologias observadas nessas estruturas são comuns entre Ascomicetos durante o desenvolvimento sexual. O cultivo de ascósporos das estruturas semelhantes à ascomata para posterior análise não foi possível ser realizado. Também efetuamos análise por PCR das células que se desenvolveram em meio seletivo com ambos antibióticos de forma a confirmar a recombinação genética resultante da reprodução sexuada, que seria demonstrada pela presença de genes parentais e dos genes responsáveis pela resistência aos antibióticos, derivados das estirpes parentais. Contudo, os resultados dessa análise não foram conclusivos.

De forma geral, nossos resultados fornecem novos conhecimentos relativos à existência de um sistema de reprodução sexuada em *Paracoccidioides*, através da demonstração da ativação da via de sinalização da reprodução sexuada pelo reconhecimento de feromona-recetor, no sistema heterólogo. Também identificamos as proteínas Velvet e a homologia de seus domínios, assim como demonstramos que a expressão de tais genes é independente da luz. Finalmente, em nossos ensaios de reprodução sexuada fomos capazes de obter em *Paracoccidioides* estruturas comuns ao início da reprodução sexuada. Estes resultados apresentam novas indicações para: i) a identificação de experimentos que possuam as condições ideais para a reprodução sexuada de *Paracoccidioides* em condições laboratoriais; ii) descobrir os mecanismos moleculares e os componentes envolvidos neste processo.

Abstract

Paracoccidioides brasiliensis and *Paracoccidioides lutzii* are multinuclear thermodimorphic fungi and the etiological agents of paracoccidioidomycosis (PCM), a prevalent mycosis in South America. PCM is estimated to affect 10 million individuals in countries from Latin America, and Brazil present 80% of the diagnosed disease. The fungus undergoes a morphological transition from a non-pathogenic mycelial or conidial form at environmental temperatures to a multiple-budding pathogenic yeast form at the mammalian host temperature. *Paracoccidioides* is known to present an asexual cycle, but sexual reproduction in the *Paracoccidioides* genus was never observed, neither in nature nor under laboratory conditions. However, recent studies have revealed indications that support the existence of a sexual cycle in *Paracoccidioides* isolates. The first indication is that this fungus harbors homologues of most of the genes necessary for sexual reproduction in Ascomycetes, including pheromone receptors, mating signaling pathway components and *MAT1-1/MAT1-2* idiomorphs. A second indication came from the discovery of recombination events in *Paracoccidioides* strains isolated from nature. This information supports the idea of the occurrence of sexual reproduction in the *Paracoccidioides* genus. Despite the presence of mating loci and all essential mating-pheromone signaling pathway genes and the discovery of such a mode of reproduction in the closely-related phylogenetic genera *Histoplasma* and *Aspergillus*, sexual reproduction in *Paracoccidioides* was never uncovered.

In this thesis, we studied several aspects that are relevant for sexual reproduction in Ascomycetes. We present data showing low expression levels of the **a**- and α-pheromone receptor genes (*PREA* and *PREB*), the α-pheromone gene (*PBα*) and the *MAT* genes, in yeast and mycelia forms of *Paracoccidioides* isolates. Induction assays with synthetic α-pheromone were performed in *P. brasiliensis* MAT1-2 strains to assess the transcriptional response of mating-related genes, however Pbα failed to elicit transcriptional activation of *MAT1-2*, *PREB* and *STE12*, suggesting that the strains tested are insensitive to Pbα. Functionality of *Paracoccidioides* mating genes was further assessed by their heterologous expression in the corresponding *Saccharomyces cerevisiae* null mutants. We show that *S. cerevisiae* strains heterologously expressing *PREB* respond to Pbα either isolated from *Paracoccidioides* culture supernatants or in its synthetic form, by shmoo formation and by growth and cell cycle arrests. This led us to conclude that *Paracoccidioides* species secrete an active α-pheromone into the culture medium that is capable to activate its cognate receptor.

In *A. nidulans* and *A. fumigatus*, a particular group of light-responsive regulatory proteins, named Velvet proteins, play a crucial role in the sexual cycle in the absence of light. In addition, the negative regulator GprD and positive regulator NsdD of sexual reproduction play an important role during the sexual

development. Homologues of these components are also present in the *Paracoccidioides* genus. In this study, we therefore analyzed the gene expression patterns of these genes in the presence and absence of light in different *Paracoccidioides* strains. Through *in silico* analysis, we show that *Paracoccidioides* Velvet proteins harbor the Velvet domains present in the homologous proteins from *Aspergillus.* We conclude that expression of the mating locus (*MAT*), the Velvet genes, and regulators *LAEA*, *GPRD* and *NSDD*, occurs in a light-independent manner. Based on the presence and expression of these genes in *Paracoccidioides* isolates and their protein interactions as described in *A. nidulans*, a protein interaction model was also proposed for *Paracoccidioides*, that may be uncovered by further functional genomics and protein analysis.

In this work, we also performed mating assays with *Paracoccidioides* isolates. We designed our mating assays taking advantage of previous information regarding mating assays in *Aspergillus* and *Paracoccidioides*, and used MAT1-1 and MAT1-2 *Paracoccidioides* strains that were marked with two different antibiotic resistance genes. After eight months of incubation on different media in the dark, we analyzed cells from the intersection of the two co-cultivated opposite mating types that were resistant to both antibiotics. By microscopic analysis we found structures that are similar to sporulation, the hyphae loops classical in early stages of sexual reproduction, and the ascoma structures surrounded by Hülle cells. The morphology observed in these structures is common among ascomycetes during sexual reproduction development. Cultivation of ascospores from the ascomata-like structures for further analysis however could not be achieved. We also performed PCR analysis on cells that had developed on both selective antibiotics in order to confirm genetic recombination resulting from sexual reproduction, as would be indicated by the presence of parental and antibiotic genes derived from both mating strains. However, the result of this analysis was not conclusive.

Overall, our results provide new insights into the existence of a functional mating system in *Paracoccidioides*, by demonstrating signal activation by the mating α-pheromone-receptor system in a yeast model. Moreover, we identified Velvet proteins motifs in *Paracoccidioides* and showed that their gene expression is independent of light. Finally, we also achieved the development of early-stage sexual structures in *Paracoccidioides* mating assays. These data therefore provide novel directions for the experimental identification of the ideal conditions for *Paracoccidioides* sexual reproduction in laboratory and consequently uncover the molecular mechanisms and the components involved in this process.

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Objectives and outline of the thesis

The work presented in this thesis was developed in the context of the project "Identification of sexual reproduction mechanisms in the fungal pathogen *Paracoccidioides brasiliensis*", funded by *Fundação para a Ciência e a Tecnologia* (Grant Number: SFRH/BD/86759/2012). This work was supervised by Professor Fernando Rodrigues and co-supervised by Dr. Marcus Sturme (Postdoctoral researcher). All the work was performed in the Microbiology and Infection Research Domain of the Life and Health Sciences Research Institute (ICVS/3B's), School of Health Sciences, University of Minho, Braga, Portugal.

The main objectives of the research developed in this thesis were directed to the study of sexual reproduction in *Paracoccidioides* and aimed to: i) describe the *Paracoccidioides* mating alpha-pheromone and establish its recognition by the *Paracoccidioides* alpha-pheromone receptor PreB in *Saccharomyces cerevisiae* as a heterologous model, as well as the response of the MAPK signaling pathway to alpha-pheromone induction, ii) identify Velvet proteins in *Paracoccidioides* and observe the expression patterns of the Velvet genes and other transcriptional regulators of fungal sexual development in the dark and in the presence of light, and iii) perform mating assays with *Paracoccidioides* strains of opposite mating types under different growth conditions and evaluate the development of sexual structures and occurrence of genetic recombination.

Chapter 1 consist of a general introduction, presenting a review of the current knowledge and recent literature on *Paracoccidioides* biology and paracoccidioidomycosis. This chapter also emphasizes the sexual reproduction process among fungi, the molecular machinery and pathways involved for the achievement of sexual reproduction in several Ascomycetes, and the information related to the sexual components that are described in *Paracoccidioides.*

Chapter 2 demonstrates the uncovering of the *Paracoccidioides* α-pheromone and its interaction with the receptor PreB. This study was conducted using *S. cerevisiae* as a heterologous model, in which we validated the sexual response of the alpha-pheromone interaction with PreB through several functional assays. We also established that the *Paracoccidioides* MAPK signaling pathway genes are expressed at low levels, and that their expression does not increase upon the addition of synthetic *Paracoccidioides* alpha pheromone.

In **Chapter 3**, we analyzed the Velvet-domain containing proteins present in *Paracoccidioides* by *in silico* analysis and comparison with the light-responsive Velvet proteins from *Aspergillus nidulans*. We show that these proteins are highly similar and demonstrate for the first time that expression of the Velvet genes and interacting sexual development genes is not influenced by the presence of light, as it is in *A. nidulans*.

In **Chapter 4** we describe the mating assays performed with *Paracoccidioides* strains of opposite mating types in different media. The extended co-cultivation time used for this experiment led to the formation of structures that resemble Ascomycetes sexual structures, and give insight in the conditions necessary for accomplishing mating in *Paracoccidioides*. Conclusive genetic confirmation of recombination resulting from sexual reproduction however could not be made.

In **Chapter 5**, concluding remarks are presented, linking Chapters 2, 3 and 4 in the context of the originally proposed aims. Additionally, future scenarios for the analysis of sexual reproduction in *Paracoccidioides* are given, to obtain relevant information about the occurrence of mating and the mechanisms involved in the process.

List of abbreviations

ATMT	*Agrobacterium tumefaciens* mediated-transformation
A-YEM	Alphacel yeast extract medium
BHI	Brain heart infusion
BLASTN	Basic Local Alignment Search Tool
BSL	Biosafety Level 3 laboratory
CAAX	motif C = cysteine, A = aliphatic, X= any
Cdna	Complementary DNA
CFUs	Colony Forming Units
CLR	C-type lectin receptor
CNS	Central nervous system
CT	Cycle threshold
d	Days
DAPI	4',6-Diamidino-2-Phenylindole
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorting
FITC-ConA	Fluorescein isothiocyanate/concanavalin A
h	hours
HMG	High-mobility group
HMM	*Histoplasma* macrophage medium
HPH	Hygromycin B cassette
HR	Homologous recombination
KanMx	Geneticin cassette
LB	Lysogeny broth

m	Minutes
MAT	Mating-type
MAT	Methionine Adenosyl Transferases
MMcM	McVeigh Morton
mRNA	Messenger RNA
MTL	Mating-type like
ORF	Open Reading Frame
PAK	Protein activated kinase
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
qRT-PCR	Quantitative RT-PCR
RNA	Ribonucleic acid
s	Seconds
SOC	Super Optimal Broth
TI	Tumor-inducing
TLR	Toll-like receptors
TPP	Vacuum filtration system
YEPD	Yeast extract peptone dextrose
YNB	Yeast nitrogen base

Chapter 1

General introduction

1. *Paracoccidioides brasiliensis*: cell biology and pathogenesis

Adolf Lutz in 1908 first described *Paracoccidioides brasiliensis* as the etiological agent of a novel "American hyphoblastomycosis", a disease that originally was referred to as "mycosis pseudococcidica" (Lutz 1908). Although Alfonse Splendore proposed in 1911 the denomination *Zymonema brasiliense* to the fungus, Floriano de Almeida in 1930 changed it to *Paracoccidioides brasiliensis* (reviewed in Palmeiro, Cherubini, and Yurgel 2005). Recently, phylogenetic analysis of *Paracoccidioides* isolates established a new specie in the *Paracoccidioides* clade, giving rise to two species belonging to the *Paracoccidioides* genus: *P. brasiliensis* and *P. lutzii* (Teixeira et al., 2009).

Paracoccidioides spp. are thermodimorphic ascomycete fungi, classified into the order Onygenales, family Ajellomycetaceae (Bagagli et al., 2008). The thermodimorphic characteristic in *Paracoccidioides* spp., is displayed as the non-pathogenic mycelium phenotype at environmental temperatures (below 28 °C), and a complex morphological transition to the pathogenic multiple budding yeast form, with a raise in temperature to that of host (28-37°C) (Carbonell 1967). These fungi are responsible for the manifestation of paracoccidioidomycosis (PCM), which occurs through the inhalation of conidia or mycelia fragments (airborne propagule) by the host. Upon inhalation, the thermoregulation of the dimorphism of *Paracoccidioides* spp. triggers its transition to the pathogenic yeast form, and after establishment of infection the fungus can further disseminate to different organs originating a systemic mycosis (McEwen et al., 1987). For this reason, according to European Community regulations, the manipulation of *Paracoccidioides* spp. needs to be performed in a Biosafety Level (BSL) 3 laboratory (DIRECTIVE 93/88/EEC, Oct. 1993).

Paracoccidioides brasiliensis presents intriguing features in its cellular biology, namely its multiple-budding and multi-nucleate nature, as well as the conditions where it is able to reproduce sexually (Brummer, Castaneda, and Restrepo 1993). Similar to other fungal species, there is strong evidence from population genetic studies for a sexual cycle in *Paracoccidioides* spp., although this was never observed under natural or laboratory conditions (Felipe, Torres, et al., 2005). It is known that sexual reproduction is associated with the generation of novel strains, thereby increasing the diversity that is naturally associated with different phenotypes. In

pathogenic fungi, these events can account for the generation of novel strains with increased virulence and fitness (San-Blas, Niño-Vega, and Iturriaga 2002). An active sexual cycle has been known for years for pathogenic ascomycete fungi such *C. albicans* (Bennet and Johnson 2003) and *Histoplasma capsulatum* (Kwon-Chung 1972). Other pathogenic fungi once considered as "asexual" are nowadays recognized as fertile and thus able to develop a complete sexual cycle as well, such as *Aspergillus fumigatus* (O'Gorman, Fuller, and Dyer 2009) and *Candida glabrata* (Muller et al., 2008).

1.1 Morphological features of *Paracoccidioides* spp.

Paracoccidioides spp. cells present two major morphologies, the yeast and the mycelium form, that are interconvertible upon temperature alterations (Carbonell 1967). As such, this fungus presents a mycelia/conidia form when the surrounding temperature is below 28°C, whereas at temperatures around 37°C, the host temperature, its cellular form resembles the yeast form with a multi-budded appearance. Thus, when temperature alterations occur, transition between the two forms takes place and several intermediate stages can be observed (Figure 1), this characteristic is referred to as thermodimorphism.

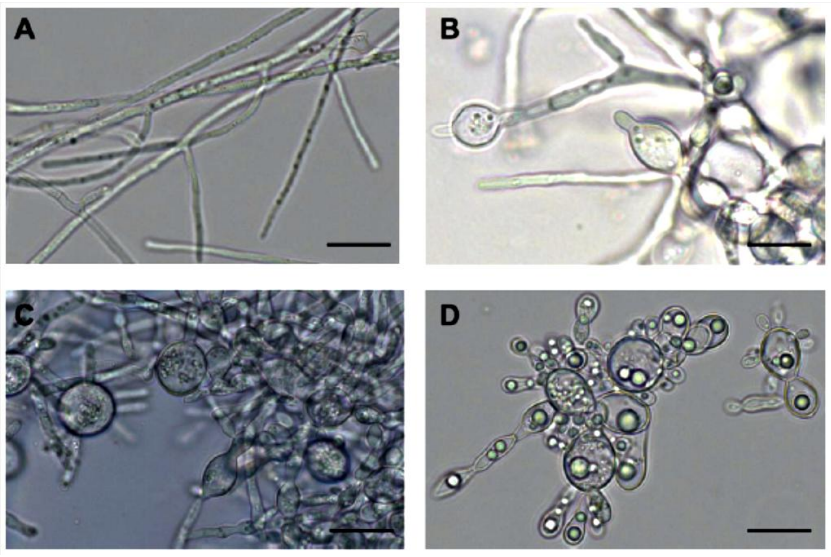


Figure 1 - Morphology of *Paracoccidioides spp.* Cells grown in liquid culture were induced to undergo mycelium-to-yeast transition by an increase in temperature from 26 °C to 37 °C. The cellular forms observed were (A) hyphae (0 days) (B) differentiating mycelium (3 days) (C) transforming yeast (5 days), and (D) yeast (10 days). Black bars correspond to 10 mm (Sturme et al., 2011).

In terms of macroscopic features, mycelial colonies are normally white, presenting a soft and velvet surface, and usually have a regular circular shape (Figure 2A). The colony growth is slow, and can take around 20 days to be formed. Observed by light microscopy, hyphal cells are elongated (1 to 3 μm), septated and multinucleated, and forming long and clear segments of cells (Figure 1A and 2B).

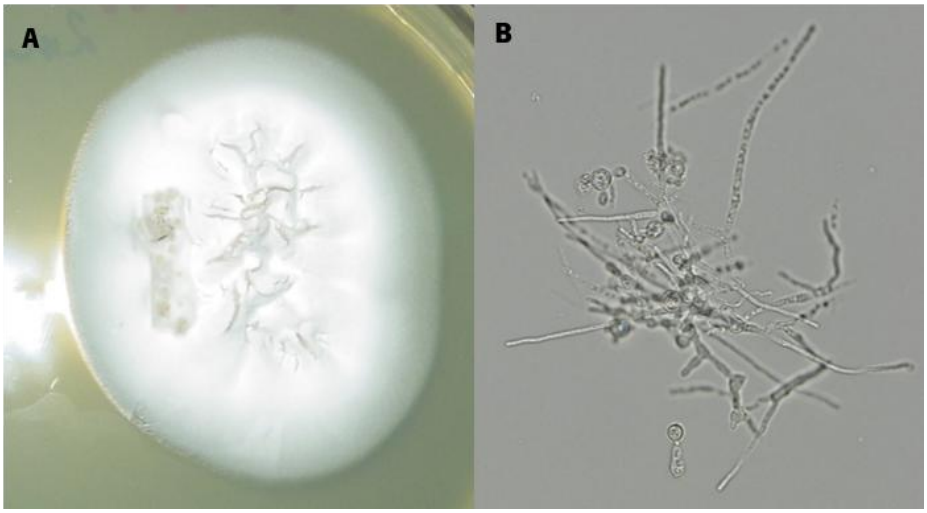


Figure 2 - *Paracoccidioides* mycelial form. Morphological aspect of a colony (A) and microscopic observation of mycelial cells (B).

Paracoccidioides spp. yeast colonies normally present a wrinkled brain aspect, a pink to yellow-brownish color with an oval or circular shape (Figure 3A). Yeast cells have a doubling time of 17-21 hrs in rich medium and on solid rich medium BHI it takes around 5 days to grow from a small patch of cells to full colony development. *Paracoccidioides* spp. yeast cells are distinguished by their "pilot wheel" aspect, multiple budding and multi-nucleated cells, variable cell sizes of 4 to 40 μm and a thick cell wall (Figure 3B-C). The multiple budding feature is uncommon in nature and the underlying genetic mechanisms are not well understood, although one study showed that activity of the cell division control gene 42 (*CDC42*) plays a role in the budding pattern and bud morphology (Almeida, 2009). Microscopic observations show that during cell division, the mother cell is always localized in the center and surrounded by multiple daughter cells (blastoconidia), measuring around 2 to 4 μm (Figure 3B-C) (Brummer, Castaneda, and Restrepo 1993).

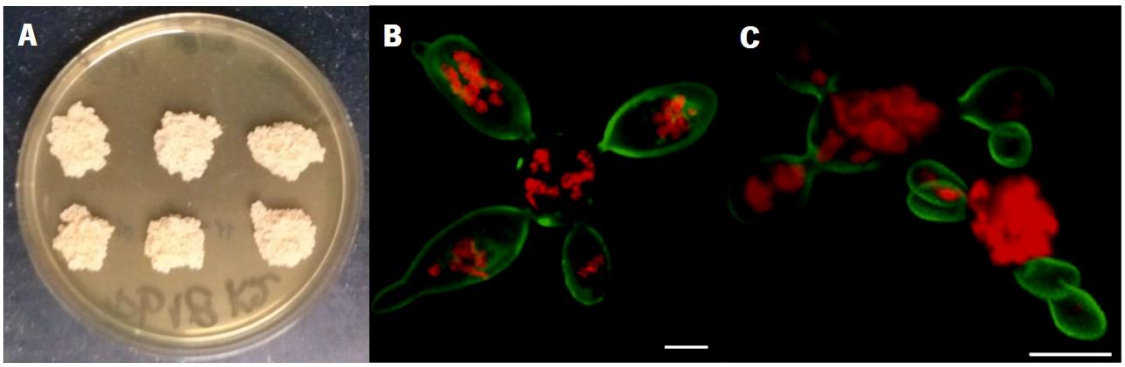


Figure 3 - *Paracoccidioides* yeast morphology. Morphological aspect of yeast colonies (A). Multiple-budding and multinucleate nature of the *Paracoccidioides* yeast form. (B and C). Confocal microscopy observation of yeast cells double-stained for nuclei with PI (propidium iodide; red) and the cell wall with FITC-ConA (B and C) (fluorescein isothiocyanate/concanavalin; green). White bars correspond to 5 mm (Adapted from Almeida et al., 2009) .

Under nutrient-limited growth conditions, such as lack of oxygen, water, carbon- or nitrogen-sources, mycelia can give rise to different forms of propagula, namely conidia and chlamydospores (Figure 4A). Conidia are uninucleated small cells (3.5 to 5.0 µm) formed under temperatures below 28°C and stress or nutrient deprivation (Figure 4B). Conidiation is very difficult to achieve under laboratory conditions and in appropriate growth conditions, conidia can form mycelia and yeast cells. Conidia can be classified in intercalary septate, pedunculated conidia, terminal hyphal conidia and arthroconidia, according to their morphology (Figure 4A-C). On the other hand, chlamydospores are multinucleated and possess several mitochondria, that may suggest the ability for further self-governing development (Brummer, Castaneda, and Restrepo 1993).

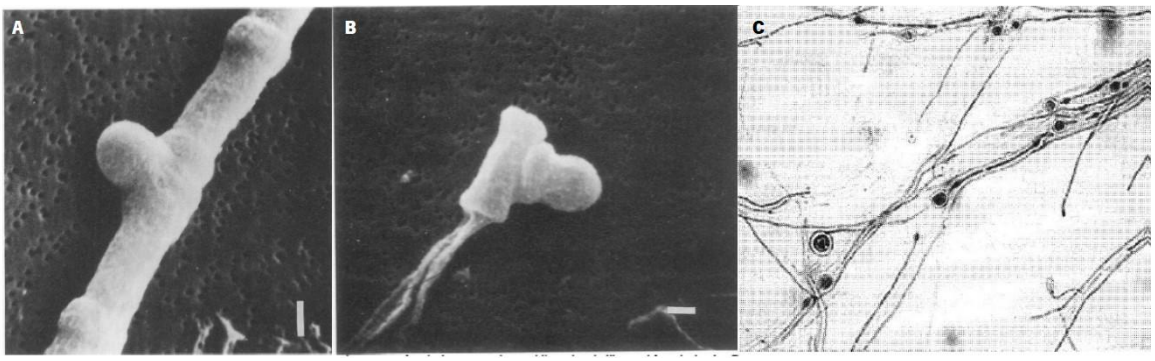


Figure 4 - Scanning electron microscopy of *Paracoccidioides* conidia: (A) conidia in early stage for the formation of an intercalary arthroconidium. (B) Typical mature arthroconidium already liberated from its hypha. Bars, 1 µm.

(Adapted from Brummer, Castaneda, and Restrepo 1993). (C) Chlamydospores formation (Adapted from Miyaji et al., 2003).

In the dimorphic *Paracoccidioides* spp., in both yeast and mycelia form, the cell wall composition is similar to that of other fungi, with an arrangement of proteins, lipids, and the polysaccharides glucan and chitin. The lipid content corresponds to 5-10% and glucan to 36-47% of the cell wall content. While α-glucan is common in the yeast form, it is mainly β-glucan that is found in the mycelia form (Kanetsuna and Carbonell 1970). The mycelia form also harbors a alkali and water-soluble galactomannan, however the galactomannan molecules are not present during mycelia to yeast transition (San-Blas et al., 2005). The yeast form holds a higher amount of chitin, 37 to 48%, while the mycelial form harbors 7 to 18% (Kanetsuna and Carbonell 1970). Through the blended activity of β-glucanase and the disulfide reductase the cell is shaped around discrete islets of β-glucan, assembling the classic bud form. The disulfide reductase belongs to the oxidoreductases family, and has been proposed as the enzyme that is essential in the formation of yeast buds in *Paracoccidioides* spp. cells (San-Blas et al., 2005). Although the importance of those molecules in the cell wall, a few years ago their presence was also confirmed in the mitochondria (Batista et al., 2006; Kanetsuna, Carbonell, and Azuma 1972)

The complex transition from yeast to mycelia and vice versa (Figure 1), comprises changes in the cell wall conformation, namely restructuring and relocation of lipids, particularly glycosphingolipids (Carbonell 1969; San-Blas et al., 2005). Extracellular vesicles are also involved in this transition and associated with the secretion of at least 120 proteins from the cell to the supernatant in the culture media. Around 10 % of these proteins are related to the typical secretory signal peptide, the rest of the secreted proteins are implicated in basic metabolic pathways, protein expression, fungal pathogenesis and cell wall maintenance (Karkowska-Kuleta and Kozik 2015).

The *Paracoccidioides* spp. yeast cell is characterized by its multiple buds and multiple nuclei nature that harbor abundant ribosomes and glycogen. The morphological switch from yeast to mycelia (Y-M), occurs when the temperature is below 28 °C, and the process happens by bud elongation, stretching of the cell wall and the occurrence of more interseptal structures formations. Between bud elongation and the development of several interseptal spaces, the formation of hyphae is accomplished (Carbonell 1969). The transition from mycelia to yeast (M-Y)

initiates when the diameter of the hyphae increases at the interseptal space (Figure 1B). As the transition evolves, the cell wall thickness increases. After the diameter increase in the interseptal space, there is a separation of the cell, and it becomes rounded (Figure 1D) (Carbonell 1969). In order to identify functional genetic alterations occurring during morphological transition, Nunes and co-workers monitored the morphological shift from M-Y form, using a microarray carrying the sequences of 4692 genes from *P. brasiliensis* related to growth, morphology and cell division (Nunes et al., 2005). They showed that, 2583 genes related to genome structure, development and growth control, amino acid catabolism enzymes and signal transduction proteins exhibited significant modulations during the yeast to mycelia transition.

Among the genes that encode ribosomal proteins, during M-Y transition, 60 genes were overexpressed during the initial first 24 hours of the temperature elevation (Nunes et al., 2005; Felipe, Andrade, et al., 2005). This and the overexpression of the genes encoding RNA polymerase I transcription factor, demonstrated that the M-Y transition involves major protein synthesis. At the cell surface level, the M-Y transition is characterized by increased expression of the genes that encodes chitin synthases, and at the same time the reduced expression of endochitinases and chitinases, which could reflect an increase in chitin content in the cell wall (Nunes et al., 2005). In addition, 22 heat shock related genes were affected by the temperature elevation. These data show that the main transcriptional adjustments during the M-Y transition are related to cellular differentiation and virulence processes (Felipe, Andrade, et al., 2005).

1.2 Paracoccidioidomycosis

As referred to previously, infection by *Paracoccidioides* spp. initiates by inhalation of airborne conidia derived from the mycelial form, resulting in the systemic mycosis paracoccidioidomycosis (PCM). Whenever the conidia reach the epithelial cells of the lungs of the host, the morphological switch to the yeast form happens. After the establishment of infection the yeast cells begin to spread into the blood system, developing a disseminated infection (Figure 5) (McEwen et al., 1987; Brummer et al., 1990). Epidemiological and clinical data have shown that the disease is presented as two main forms: the acute or sub-acute form, also known as juvenile type, and the chronic form, the adult type. The juvenile type of PCM progresses in weeks or months after the first contact with the fungus and is more severe, presenting higher rates of mortality. However,

the adult type of PCM can be in latency for several years and presents more than 90% of the cases (Borges-Walmsley et al., 2002). PCM establishment, its symptoms and severity are dependent on the fungus' intrinsic features, such its antigenic composition and virulence, as well the effectiveness of the host immune response (Calich et al., 2008). It was estimated that PCM affects 10 million young adults in South America (Brummer, Castaneda, and Restrepo 1993) Furthermore, immunological skin test studies in healthy inhabitants within endemic areas have shown reactivity close to 20%, indicating a substantial rate of infection or previous contact with *Paracoccidioides* spp. (Brummer, Castaneda, and Restrepo 1993; González et al., 2008).

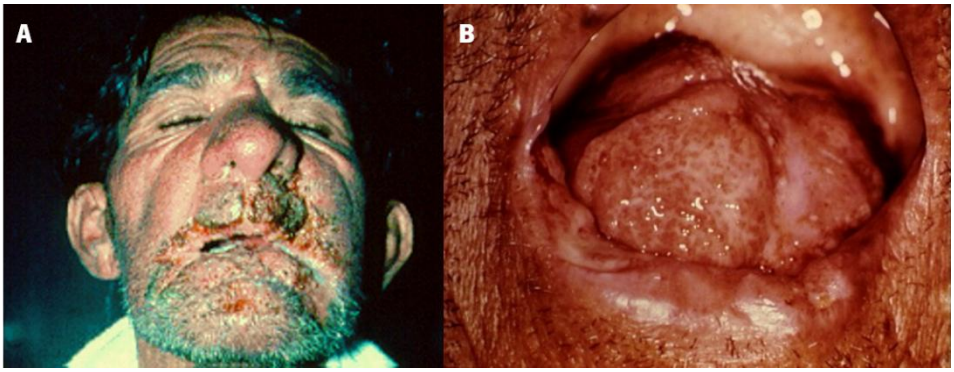


Figure 5 – PCM forms. The acute form (A) and the chronic form (B) (Adapted from Marques 2012).

Paracoccidioidomycosis arises mostly in rural populations and is more common in males. The mechanisms underlying this gender vulnerability is assumed to be related to the hormonal regulation of the *Paracoccidioides* spp. morphological switch. *In vitro* studies have revealed that female hormones such as estrogens, block the conidia- or M-Y transition, possibly through a cytosolic steroid-binding G-protein (Fortes et al., 2011). *In vivo* studies with mice validate these results, and showed that 96h after conidia inoculation, the transition to the yeast form is complete in the lungs of the male mice, but not in female mice (Salazar, Restrepo, and Stevens 1988). An immunological study showed that the interference by the sexual hormones in the immune response against *Paracoccidioides* spp. influence the variances in the clinical incidence and progression of PCM between males and females (Pinzan et al., 2010; McEwen et al., 1987). This fact is supported by the results obtained from PCM-speficic gp43 antigen tests in rural areas that show an equal number of positive results in both sexes, indicating no difference in exposure or infection rate (Brummer, Castaneda, and Restrepo 1993; Araújo, Espindola, and Pedroso 2012). It was suggested that the sex discrepancy in infection outcome could be explained by the

inhibitory action of estrogens during conidia or M-Y morphological transition (Brummer, Castaneda, and Restrepo 1993; Martinez 2015).

Whenever the host immune system is suppressed, a later reactivation of the infection of the organs can affect the nervous systems. Two decades ago, infection of the Central Nervous System (CNS) was detected in a patient through immunological and computerized skull tomography. This diagnosis of neural paracoccidioidomycosis (NPCM) in the past was confused with meningoencephalitis in individual from rural areas (Magalhaes et al., 1993). The frequency of NPCM is variable, from 9.6% to 25.4% of the confirmed PCM cases. However, recently an increase of NPCM cases was observed through neurosurgical and anatomopathological findings samples (Magalhaes et al., 1993; Pedroso et al., 2012; Martinez 2015).

The fungus presents a complex antigenic structure that exposes epitopes related to the pathogen-host interaction. The main antigenic component is glycoprotein Gp43, present in its cell wall, and is associated with the virulence and evasion mechanisms towards the host defense system (Camargo et al., 1994). At the initial stages of infection, the immunological response involves the activity of neutrophils, macrophages and monocytes (Benard 2008). The participation of those cells in the inflammatory response and anti-fungal activity is induced by the fungus, and cytokines are produced during the interaction with the phagocytes. Some receptor molecules have shown to be essential in the recognition of *Paracoccidioides* spp. by the host, such as Toll-like receptors (TLRs), C-type lectin receptor (CLR), and Dectin-1 (Hardison and Brown 2012; Acorci-Valério et al., 2010). Dectin, TLR2, TLR4 and TLR9 are involved in the recognition and internalization of *Paracoccidioides* spp. with the consequent activation of neutrophils (Acorci-Valério et al., 2010; Menino et al., 2013). Low-virulent isolates are especially recognized by Dectin-1 and TLR2 and result in the production of TNF-α and IL-10. Meanwhile, more virulent strains induce a major production of TNF-α, IL-10, Th1 Th2, Th9, Th17 and Th22 , inducing a less damaging and a more controlled response by the host (Bonfim, Mamoni, and Blotta 2009). During the infection, cytokines TNF-α, IFN-γ and IL-12 protect the host by reducing fungal spreading. Patients with the disease present a decrease in the immunological cell response, characterized by the low production of Th1 cytokines like IFN-γ, IL-12 e IL-2 and an increase of Th2 cytokines IL-4, IL-5 e IL-10, that does not represent an effective response to control the mycosis (Zielinski et al., 2012; Oliveira et al., 2002).

1.3 Ecology of *Paracoccidioides* spp.

The natural habitat of *Paracoccidioides* spp. has not yet been exactly determined and its isolation from the environment is still a challenge (Puccia, McEwen, and Cisalpino 2008). Yet, it is commonly accepted that the natural environment of *Paracoccidioides* spp. is humid, within the tropical and agricultural areas of Central and South America (Brummer, Castaneda, and Restrepo 1993; Desjardins et al., 2011). *Paracoccidioides* isolates have been frequently isolated from the nine-banded armadillo, *Dasypus novemcinctus*, representing 70-100% of mammals captured in endemic PCM areas in Brazil (Figure 6) (Puccia, McEwen, and Cisalpino 2008; Bagagli et al., 2008). Regardless of the high incidence of *Paracoccidioides* spp. in the armadillo *D. novemcinctus*, their infection is not gender or age dependent, and the animals do not exhibit perceptible signs of PCM disease (Bagagli et al., 2008). The fungus has been detected by PCR in soil samples, and found in burrows and feces of naturally infected armadillos, and it was possible to isolate *Paracoccidioides* spp. from its saprobic form (Franco et al., 2000). The low body temperature of armadillos, around 32-35°C, makes these animals the ideal host for *Paracoccidioides* spp. In the infected animals, unusual granulomas containing fungal cells were found (Figure 6) (Bagagli et al., 1998). The conservation of a parasitic and saprobic form would contribute to sexual reproduction, since *Paracoccidioides* isolates were found in delimited areas, increasing the possibility to meet individuals of the same species or strain (Figure 6) (Bagagli et al., 2006). Torres and coworkers found an equivalent distribution of *MAT* loci among different clinical and environmental *Paracoccidioides* spp. isolates (Torres et al., 2010). As well, a phylogenetic analysis of several *Paracoccidioides* isolates demonstrated a high phylogenetic relationship between the S1, PS2, PS3 and ‘‘Pb01-like” groups. Those evidences indicate that the environmental conditions may contribute to the occurrence of sexual development (Torres et al., 2010; Teixeira et al., 2009).

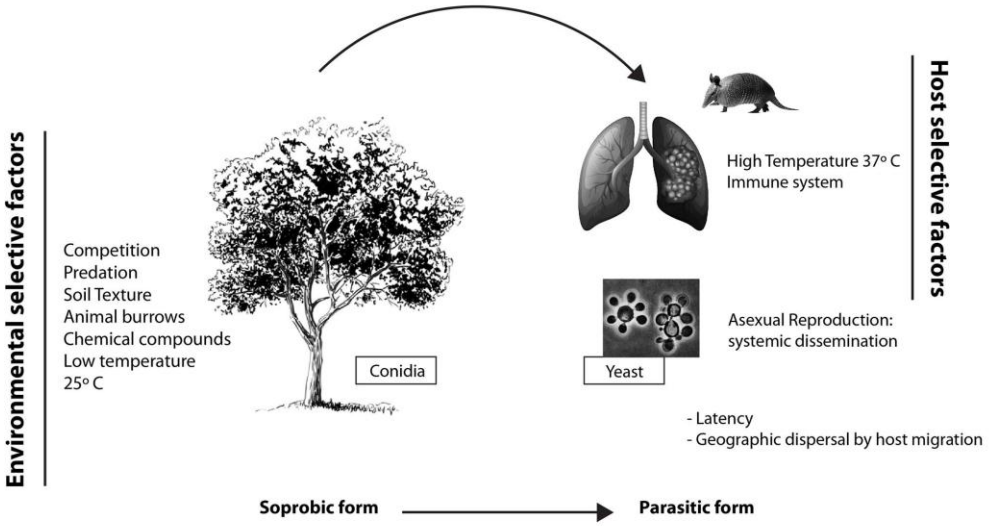


Figure 6 - Hypothetical biological cycle of *Paracoccidioides*. Representation of the niches, supposed ecological niches in hosts and in saprobic environment.

The molecular, genetic and virulence profiles of *Paracoccidioides* isolates collected from wild mammals showed to be extremely variable (Bagagli et al., 1998; Richini-Pereira et al., 2009). The high incidence of PCM in rural areas and farmworkers is due to their constant contact with the soil and armadillo environments (Restrepo, McEwen, and Castañeda 2001) and most of the clinical *Paracoccidioides* isolates are collected from PCM patients that live in rural areas. These are indications that both humans and animals present the optimal growth condition for *Paracoccidioides* spp. development inside the host (Bagagli et al., 1998; Terçarioli et al., 2007).

1.4 Phylogeny, Cryptic Speciation and Genetics

In classic phylogeny, the *Paracoccidioides* genus was placed in the anamorphic phylum Deuteromycota in the Hyphomycetes class, due to the lack of an observable sexual phase. However, morphological and phylogenetic studies placed *Paracoccidioides* spp. in the phylum Ascomycota, order Onygenales, family *Onygenaceae sensu lato* (Gioconda San-Blas and Niño-Vega 2008; Bagagli et al., 2006). Recently, a new family denominated Ajellomycetaceae was proposed, distinct from *Onygenaceae sensu lato*. This family comprises the related fungi of the genera *Blastomyces*, *Histoplasma*, *Emmonsia*, and *Paracoccidioides* (Figure 7) (Bagagli et al., 2006; Bagagli et al., 2008; Untereiner et al., 2004). All those members are saprobic and pathogenic vertebrate-associated taxa (Untereiner et al., 2004).

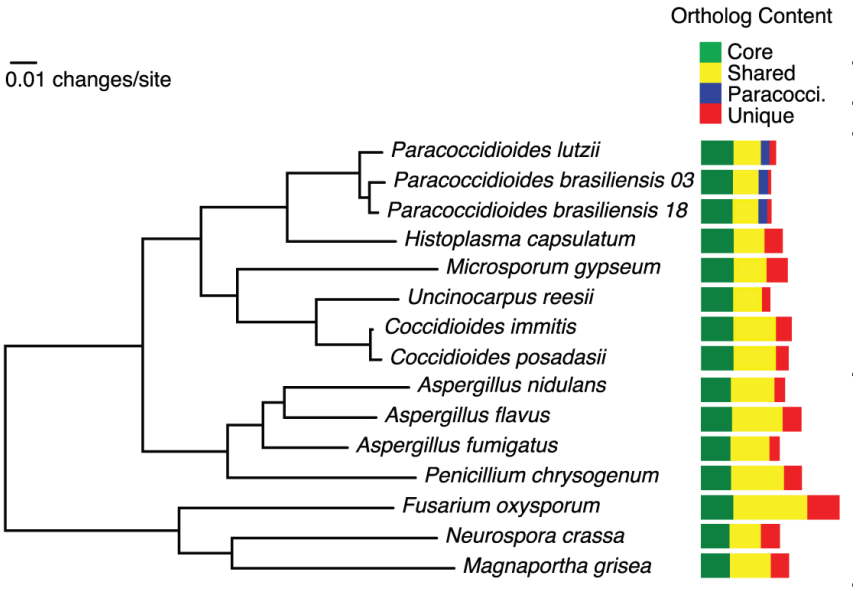


Figure 7 - Phylogeny of the dimorphic fungi, shared gene content, and clade-specific carbohydrate and protein metabolism. The phylogeny was inferred by maximum likelihood. All nodes on the phylogenetic tree were supported with bootstrap values of 100%. Colored bars indicate orthologues shared across all taxa (green), orthologues shared across some taxa (yellow), *Paracoccidioides*-specific (blue) and species-specific genes (red) (Adapted from Desjardins et al., 2011).

In a study performed through the analysis of eight regions from five nuclear coding genes, it was concluded that *P. brasiliensis* is stratified in at least three distinct species (Figure 8), namely S1 (phylogenetic species from Brazil, Argentina, Paraguay, Peru and Venezuela), PS2 (phylogenetic species from Brazil and Venezuela) and PS3 (phylogenetic species from Colombia) (Matute et al., 2006). A different study proposed the existence of a new *Paracoccidioides* specie by showing that 17 genotypically similar isolates, including the strain Pb01, were distinct from the three cryptic species formerly described. For this reason, the new "Pb01-like" cluster was proposed as a new species, *P. lutzii* (Teixeira et al., 2009). A teleomorphic form of *Paracoccidioides* spp. has not yet been isolated, although recombination and phylogenetic analysis, *MAT* locus distribution, and the presence of mating related genes indicated that the fungus recombines in nature (Torres et al., 2010; Teixeira et al., 2009; Desjardins et al., 2011; Gomes-Rezende et al., 2012). However, its sexual development was never observed neither in natural nor in laboratory conditions (Matute et al., 2006).

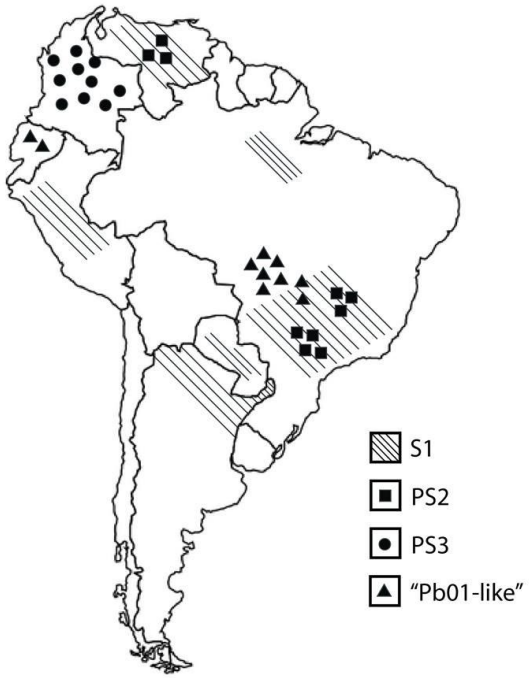


Figure 8 – Phylogenetic distribution of *Paracoccidioides* in Latin America. Distribution of species S1, PS2, PS3 and "Pb01-like" in Latin America (Teixeira et al., 2009).

The chromosomal mapping and genome characterization of *P. brasiliensis* and *P. lutzii* were assessed by Pulse Field Gel electrophoresis (PFGE), flow cytometry (FCM) and microfluorometry (Isabel et al., 1998; Almeida, Matute, et al., 2007; Feitosa et al., 2003). Based on PFGE, the existence of 4-5 chromosomes was discovered, and the complete genome was estimated to be around 23-31 Mb (Isabel et al., 1998; Feitosa et al., 2003). Taking advantage of microfluorometry it was demonstrated that the *Paracoccidoides* spp. genome size is around 45.7 to 60.9 Mb. This demonstrated a possibility of the existence of haploid, diploid and possible anaploid isolates (Almeida, Matute, et al., 2007; Feitosa et al., 2003). The genome and ploidy of the fungus was asssessed by FCM and the results obtained showed that the ploidy ratio is between 1.0 and 1.1, meaning that the isolates are haploid. The genome size was demonstrated to be composed by about 26.3 to 35.5 Mb per uninucleated yeast cell (Almeida, Matute, et al., 2007). Complete genome sequences of *P. lutzii* (Pb01) and *P. brasiliensis* strains (Pb03 and Pb18) were later published (https://data.broadinstitute.org/annotation/genome/paracoccidioidesbrasiliensis /MultiHome.html), indicating that the Pb03 and Pb18 genomes are

similar in size, around 30.0 Mb and 29.1 Mb respectively, and the *P. lutzii* Pb01 genome is 3 Mb larger at aproximately 32.9 Mb (Desjardins et al., 2011). Recently, the genomes of another 31 *Paracoccidioides* strains were sequenced (Munoz, mSphere 2016). The genomic characteristics of sequenced *Paracoccidoides* spp. and its carefull analysis were compared with several relatives from the phylum Ascomycota. It represented a major achievement into the molecular and proteomics aspects of *Paracoccidoides*, providing crucial insights into genomic attributes that may have contributed to the separation of the *Paracoccidioides* lineage from other dimorphic fungal species. These attributes comprise a catalogue of unique genes and metabolic proteins that are conserved among close dimorphic relatives.

To uncover the biology of *Paracoccidioides* spp. several genetic tools have been developed and applied over the last years. An important molecular technique for functional genomics in *Paracoccidoides* was the development of random insertion mutagenesis via *Agrobacterium tumefaciens* mediated-transformation (ATMT) (Bundock et al., 1995). The plant pathogen *A. tumefaciens* is a bacteria that harbours a tumor-inducing (Ti) vector comprising a transfer DNA sequence (T-DNA) that is separately and arbitrarily introduced into the plant genome throughout infection (Hoekema et al., 1984). Taking advantage of these *A. tumefaciens* features, a protocol was developed to allow the integration of the T-DNA sequence from *A. tumefaciens* into eukaryotic organisms, such as fungi. It was shown that the path of addition of extraneous DNA into the receiver organism is host factor dependent, since its integration in *S. cerevisiae* was achieved by homologous recombination, as replacement for of random integration in the genome (Bundock et al., 1995). Fundamented in this technique, an efficient protocol was established for the insertion of exogenous DNA into the genome of *P. brasiliensis* (Almeida et al., 2007) by co-culturing of *P. brasiliensis* and *A. tumefaciens*. Important conditions to achieve ATMT transformation of *P. brasileinsis* were shown to be time of co-cultivation, exposure to light and ratio of cells between the two organisms (Almeida et al., 2007). The ATMT protocol allows that an artificial plasmid harbouring an antibiotic-resistance marker, such as the hygromycin B phosphotransferase gene and the specific aRNA sequence can be introduced into *P. brasiliensis* genome. Later on, this technique was used to accomplish genetic manipulation in *P. brasiliensis* for down-regulation of gene expression (gene silencing) using antisense-RNA sequences directed against specific genes of the fungus (Menino et al., 2012) (Menino, Almeida et al., 2012). This technique is usefull to obtain knowledge of *P. brasiliensis* biology using e.g. fluorescent-tagged

proteins, down and over expression of target genes and introducing antibiotic resistance markers. The recent development of specific genetic tools for *P. brasiliensis* genetics allows to obtain further knowledge regarding M-Y and Y-M transitions, metabolic pathways, and genes involved in meiosis and in sexual development, and thereby represents a whole new genetic perspective of *Paracoccidioides* (Desjardins et al., 2011).

1.5 Sexual development in fungi

Sexual development costs energy, representing a significant event in an organism's life cycle (Gonalves-Sá and Murray 2011). For decades, it was established that sexual reproduction obliges the purge of genetic defects and deleterious mutations in the genome and the generation of genetic variation. Therefore recombination is beneficial to the sexual offspring, since the mixed gene pool from both parents increases fitness in novel or changing ecological niches by producing a progeny that is more adequately adapted to the environment (Fisher 2007).

Ascomycetes can produce sexual and asexual spores. Asexual spores are produced through asexual reproduction, are unsealed and exposed to the environment. This phenomenon is well described in *A. nidulans* (Fisher 2007). Approximately 18h after spore germination, the aerial hyphal divisions are assembled in the middle of the colony and some branches afterwards differentiate into conidiophores (asexual spores). The process for the initial aerial growth takes ≈8 hours until the development of the first asexual spore or conidium. In this manner, the new spore is formed and the asexual cycle is reinitiated within ≈24h after the spore germinates (Park and Yu 2012).

During the sexual cycle of filamentous ascomycetes, morphogenetic changes take place, such as formation of specialized sexual structures and sporulation. Sexual spores (ascospores) are produced through mating followed by meiosis, and are formed enclosed in a specialized structure called ascus. Asci are composed of four or eight ascospores, depending on the specie. In Pezizomycotina (Euascomycetes) the asci consist of a ascocarp, that is classified in structures denominated (i) cleistothecium (found in dermatophytes and *A. nidulans*), (ii) ascostroma, a hole-like structure that is named a locule (found in filthy mold), an (iii) apothecium (totally exposed structure found in cup fungi such as morels) and (iv) the perithecium (an opened-bottle-like structure found in *Neurospora* spp.) (Figure 9) (Debuchy, Berteaux-lecellier, and Silar 2010).

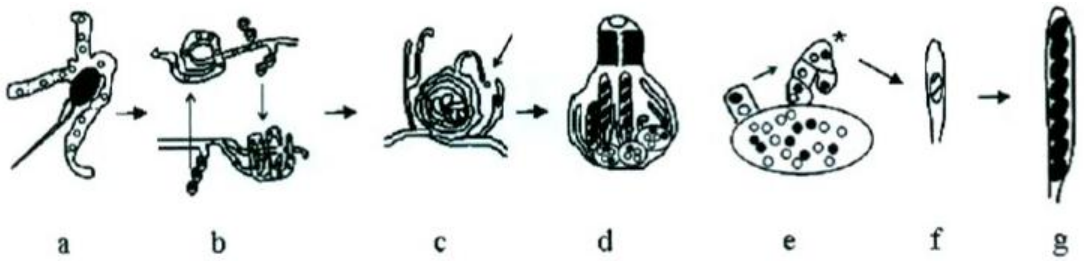


Figure 9 – Lifecycle and fruiting body development in Pezizomycotina. The germinating ascospores (a) gives rise to the mycelia on which cells of both mating type gametes differentiates (b). Fertilization occurs only between opposite mating types gametes (c). The trichocyte reaches a mycelium (c) and the ascogonium develops into a perithecium (d). Once inside the perithecium, dikaryotic ascogonium hyphae emerges from the plurinucleated dikaryotic cells. After two cycles of mitosis, the ascogonium hyphae give rises to a crozier (e). Karyogamy takes place in the upper cell (*) and is immediately followed by meiosis, mitosis and ascospores formation (Debuchy, Berteaux-lecellier, and Silar 2010).

Unfortunately, events associated to mating and meiosis have not been fully established in many pathogenic fungi. Nevertheless, it is known that sexual reproduction and several distinct patterns of sexual recombination have been described in Ascomycota, Microsporidia, Chytridiomycota, Basidiomycota and Mucoromycotina (Goodenough and Heitman 2014; Lee et al., 2010). It is well established that fungal sexual development is orchestrated by transcription factors encoded by a genetic locus called the mating-type or *MAT* locus, that determines the sex of the fungus (Lee et al., 2010).

Due to the development of new strategies, genetic tools and protocols, many pathogenic fungi previously considered asexual, nowadays have a sexual cycle described. These insights came from complete genome sequencing allowing the identification of *MAT* loci, establishment of active sexual machinery, presence of mating pheromones and its recognition by the cells, and by uncovering the singular conditions for the occurrence of sexual reproduction, which in many cases is the absence of light (Nielsen and Heitman 2007; Lengeler et al., 2002; Laskowski 2010; Findley et al., 2012; Dyer, Paoletti, and Archer 2003; Dyer and O' Gorman 2012; Wong et al., 2003).

Mating in fungi is linked to either a homothallic (self-fertile) or a heterothallic (self-sterile) process. In homothallic processes, each strain can mate with itself, discharging the necessity for a partner. In heterothallic processes mating occurs between two sexually compatible groups, morphologically identical and distinguished by their mating type. However, some fungal strains

are self-fertile, but the nuclei undergoing fusion are from opposite mating types, this feature in fungi is denominated pseudo-homothallic.

1.5.1 Mating and *MAT* locus

The sexual process in fungi is entirely orchestrated by a genetic region named mating type or *MAT* locus. *MAT* locus location and size are variable in fungi and the locus is an essential requirement for sexual reproduction to happen, due to the presence of genes encoding transcription factors that are master regulators of sexual reproduction (Fraser et al., 2007; Lee et al., 2010). Mating and mating type were first characterized at a molecular level in *S. cerevisiae* (Astell et al., 1981). For this reason, all the aspects regarding sexual reproduction in this fungus was discovered along the years (Fisher 2007).

1.5.2 Regulation of mating in the model ascomycete *Saccharomyces cerevisiae*

S. cerevisiae develops generally as yeast and can exist as a haploid or diploid cell and under specific conditions also as a polyploid, (Lee et al., 2010). At a molecular level, mating was first characterized in *S. cerevisiae*, and nowadays this is still the most and best studied organism among fungi (Li et al., 2010). In *S. cerevisiae*, the *MAT* locus was first described though evidence that this fungus possesses two isoenzymatic Methionine Adenosyl Transferases (Cherest and Surdin-Kerjan 1978). Taking advantage of this information, it was possible to perform mating under laboratory conditions with two opposite mating types strains of *S. cerevisiae* (Klar, Fogel, and Radin 1979).

Two haploid (**a** and α) and one diploid (**a**/α) mating type exists among *S. cerevisiae* populations (Lindegren and Lindegren 1943). The **a** cells possess the *MAT**a*** allele that harbors the **a**1 gene, which produces an HD2 class homeodomain (HD) transcriptional factor. The α cells harbor the *MAT*α locus that carries the *α1* and *α2* genes. The *α1* gene encodes the α box transcriptional factor Matα1, while the *α2* gene encodes the HD1 class domain transcriptional factor Matα2. In α mating type cells both *α1* and *α2* genes are responsible for the regulation of ***a*** and *α* cell specific gene expression (Figure 11) (Herman and Roman 1966). As a transcriptional factor, the Matα1 protein interacts with the Mcm1 protein in order to recognize the promoter of several *α*

specific genes. The α1 domain is conserved among several Mat proteins in filamentous ascomycetes. This proposes that α1 may also act as transcriptional activator and might collaborate with different transcriptional factors associated to Mcm1, which remain unidentified (Figure 10) (Grayhack 1992). All the *MAT**a*** specific genes (*MAT**a*** loci), are expressed constitutively. Their expression is yet restricted to **a** cells since in α cells, the **a**-specific gene expression is suppressed by the α2 repressor, while the *α*-specific genes are induced by the α1 activator. Consequently, *S. cerevisiae* cells lacking the *MATα* loci, display an **a** mating type specificity (Herskowitz 1989). Some *S. cerevisiae* strains are homothallic, meaning that a haploid cell can switch its mating type and undergo a self-fertile sexual cycle. The mating type switching (Figure 11) happens through a gene switching event, where information at the *MAT* locus nearby the centromere of chromosome III is replaced with information from one of the silent *HMR* and *HML* cassettes, present at the ends of the chromosome. *HMLα* and *HMR**a*** are silent loci that contain non-transcribed copies of the ***a*** or *α* genes, respectively. *HMLα* and *HMR**a*** harbor sequences that are very alike to their MAT counterparts. However, they are located in the heterochromatin and are not expressed (Cosma, Tanaka, and Nasmyth 2016).

Switching between mating types begins through the activity of the HO endonuclease, that introduces a staggered double stranded disruption at a specific recognition site within *MAT* (Nasmyth 1993). The switch happens only in mother cells, cells that have suffered mitotic division at least on one occasion. This is largely controlled by the expression of the *HO* gene, that is repressed in daughter cells by asymmetric localization of Ash1 (Sil and Herskowitz 1996). *ASH1* encodes a zinc finger protein and its presence in daughter cells is responsible for limiting *HO* expression to mother cells (Bobola et al., 1996). *HO* expression is also repressed in diploid cells by the a1/α2 repressors, and in haploid mother cells it is expressed only at G1 phase, via the synchronized action of transcription factors and chromatin remodeling machines (Nasmyth 1993; Mathias et al., 2004; Cosma, Tanaka, and Nasmyth 2016).

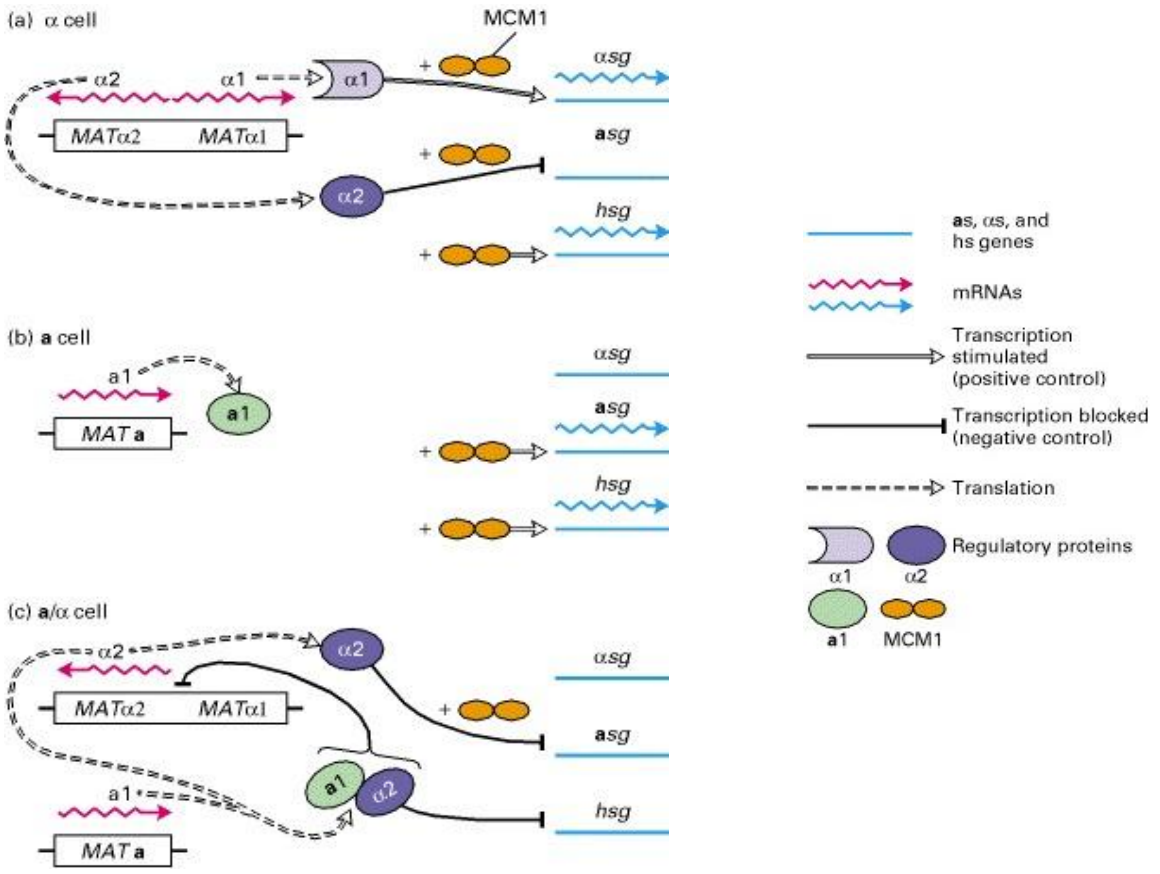


Figure 10 - Regulation of *S. cerevisiae* cell-type specific genes. Transcription factors encoded by each MAT locus act with *MCM1* transcription factor to regulate the expression of asg (**a** specific genes/mRNAs), αsg (α specific genes/mRNAa), and hsg (haploid specific genes/mRNAss). *MAT*α cell mating type expression (a), *MAT***a** cell mating type expression (b) and the diploid *MAT***a**/α mating type (c).(Lodish et al., 2000)

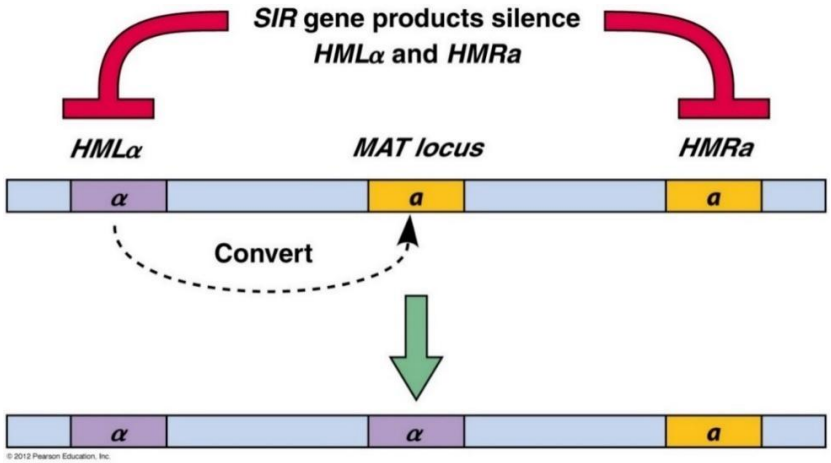


Figure 11 – *S. cerevisiae* mating type switch. The *HMLα* and *HMRa* loci contain complete copies of the α and **a** forms of the *MAT* locus (Copied from Hardin and Bertoni 2012).

The **a**-factor produced by *MAT**a*** cells is produced by the *MFa1* and *MFa2* genes. The initial **a**-factor passes several phases of posttranslational modification and proteolytic cleavage before its secretion. The mature **a**-pheromone is composed of 13 amino acid residues with the sequence WHWLGLKPGQPMY. The **a**-pheromone is primarily translated as a precursor protein of 165 amino acids composed of three regions; i) a 19 amino acid signal sequence, ii) a 64 amino acid region containing several glycosylation sites: iii) a region containing four (*MFa1*) or two (*MFa2*) repeat domains of the mature **a** pheromone disconnected by short linker sequences The sequence is translocated to the endoplasmic reticulum and the signal peptide is cleaved off and an extensive glycosylation of the peptide happens. After transportation to the Golgi system, the freshly added carbohydrate groups are modified and three cleavage reactions takes place. First, the Kex2 protease separates the **a**-factor repetitions by cleavage after the conserved residues lysine-arginine (KR). Second, Kex1 eliminates the two KR amino acids by carboxypeptidase activity. The diaminopeptidase Ste13 concludes the maturing development by removing the additional residues at the N-terminal part of the peptide (Chen et al., 1997).

S. cerevisiae α-factor is secreted as a mature α-pheromone and is resultant from one of two similar precursors encoded by *MFα1* and *MFα2* genes. The MFα1 precursor is composed of 165 amino acids, containing an N-terminal signal sequence called "pro" region; and four tandem copies, separated by sequences that comprise cleavage sites for several proteases. The MFα1 precursor also undergoes a posttranslational translocation across the membrane of the ER. The precursor is traced by a signal sequence that cleaves on three asparagine residues at the pro region in the endoplasmic region lumen (Figure 12). After the vesicular transportation from the endoplasmic reticulum to the Golgi complex, the glycan chains of the MFα1 precursor are modified in the Golgi lumen and three proteolytic cleavage stages occur within the MFα1 spacers. The cleavages happen through the action of the Kex1, Kex2, and Ste13 enzymes, in order to produce four copies of the mature unmodified α-factor. Secretory transportation vesicles that comprise the completely processed α-factor bud from the Golgi complex (Figure 12) and fuse with the plasma membrane in order to release α-factor to the outside of the cell (Fuller, Sterne, and Thorner 1988; Fuller, Brake, and Thorner 1989)

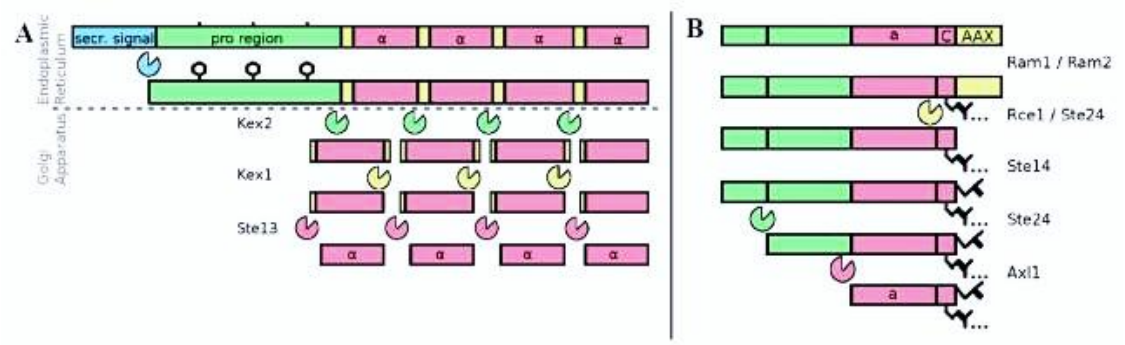


Figure 12 – Processing of a and α pheromones. The secretion sequence (blue) is cleaved to produce α pheromone, that is glycosylated in the ER. After the transport to the Golgi complex, three proteolytic phases eliminate the pro-region (green) and linker regions (yellow) that separate copies of the mature α-factor (pink) (A). Processing of **a**-factor: In the cytosol, **a** pro-pheromone is farnesylated at a conserved cysteine in the C-terminal CAAX motif. The amino acids (yellow) behind the cysteine are detached and substituted with a carboxymethyl group. Two proteolytic events remove N-terminus amino acids (green) for the production of the mature **a**-factor (pink) (B) (Adapted from Jones and Bennett 2011).

Mating occurs when **a** cells encounter α cells. Haploid **a** cells produce **a** factor (pheromone) to signal to α cells. In the same way, haploid α cells produce α pheromone to signal to **a** cells. The **a** cells produce the α pheromone receptor Ste2, and α cells produce the **a** factor receptor Ste3 (Bender and Sprague 1986). Upon responses by a compatible mating partner, both cells develop projection tips called shmoos, and cell fusion occurs between the **a** and α cell. This event is followed by nuclear fusion to form a **a**/α diploid (Figure 13) (Butler 2010).

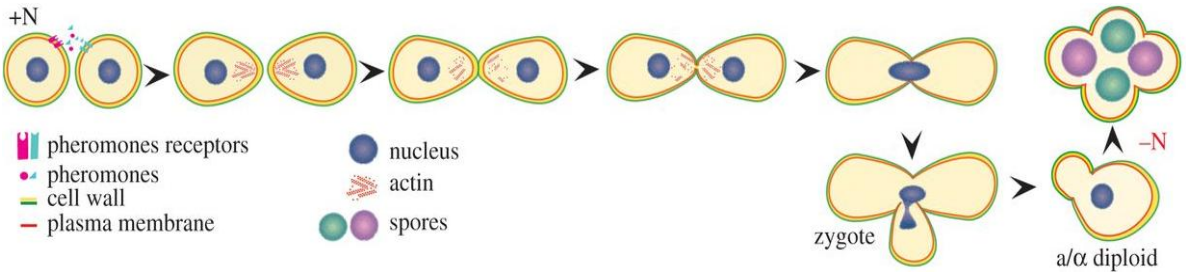


Figure 13 - *S. cerevisiae* mating phases. Budding yeast cells of opposite mating type can mate spontaneously to form stable diploids that undergo sporulation upon starvation (-N). After pheromone exchange, cells grow in a polarized manner in the direction of their partner and undergo fusion, karyogamy and sporulation (Adapted from Merlini, Dudin, and Martin 2013).

The **a** and α pheromones bind to one of two G-protein-coupled transmembrane receptors, Ste3 and Ste2, respectively. A recent study showed that pheromone concentrations are not essential for effective mating of ascomycete fungi in general, confirming that in budding yeasts, the major determinant of mating are the specificity between the receptors and their corresponding pheromones (Gonçalves-Sá and Murray 2011). After the recognition of the pheromone by its respective pheromone receptor, there are three major responses in the cell: transcriptional induction of genes involved in mating, arrest of cells in G1 phase, and morphological changes (shmoo formation, Figure 13) (Fujimura 1998). The expression of the pheromone receptors is highly controlled at the transcriptional level by the MAT**a,** MATα and Mcm1 key transcriptional regulators (Grayhack 1992).

Interestingly all intracellular components of the pheromone response pathway are common to both **a** and α cells (Figure 14) (Bender and Sprague 1986). Pheromone recognition results in the dissociation of the Gα subunit (Gpa1), that consequently results in the dissociation of the Gβ (Ste4) and Gγ (Ste18) subunits, leading to the activation of the mitogen-activated protein (MAP) kinase cascade (Sprague and Thorner 1992) and conferring the activation of the p21-activated protein kinase Ste20. Ste20 phosphorylates Ste11, a MAP kinase kinase (MEKK); that phosphorylates and thereby activates Ste7, the MEK family member; Ste7 in turn activates MAPK proteins Fus3 and Kss1 by phosphorylation (Errede and Levin 1993). Ste5 is a scaffold protein and supports positioning of Ste11, Ste7, Fus3, and Kss1 (Figure 14). Ste5 acts to chain the components of the MAPK cascade to one another; a mechanism that avoids signaling crosstalk in the mating pathway and with other pathways that use the equal stock of MAPK components. MAP kinases are activated by phosphorylation of both tyrosine and threonine (Yablonski, Marbach, and Levitzki 1996). The signal is then shifted to the cyclin-dependent kinase inhibitor Far1 and to the transcription factor Ste12, that by its phosphorylation is responsible for the regulation of the expression of genes required for mating (Figure 15). Ste12 is frequently in association with the general transcription factor Mcm1, and then activates the transcription of several genes encoding components of the pheromone response pathway and the genes required for cell fusion (Oehlen, McKinney, and Cross 1996). The two MAPKs in this pathway, Fus3 and Kss1, deliver an excellent example of functional redundancy: either kinase is sufficient for Ste12 activation individually (Madhani, Styles, and Fink 2001).

Cell cycle arrest is one of the steps in the pheromone-receptor recognition process; and it is triggered via MAPK pathway. It results ultimately from action of the Far1 protein, that directly binds to the cyclin-dependent kinases CDC28-CLN1 and CDC28-CLN2, in order to inhibit their activity (Galgoczy et al., 2004). Far1 activity is governed in two ways by the pheromone response pathway and, in particular, by Fus3. First, the transcription of the *FAR1* gene is induced 3- to 5-fold by mating pheromones. Second, Far1 is an excellent substrate for both Fus3 and Kss1 *in vitro* and *in vivo* (Strickfaden et al., 2007; Dorer, Pryciak, and Hartwell 1995).

Cassette-based mating type switching is not restricted to *S. cerevisiae,* but is also found in closely related pathogenic species such as *Candida albicans* (Butler 2010). *S. cerevisiae* has been used as a model for the study of mating in several fungi, however, recent studies in human fungal pathogens showed novel mating paradigms that contrast significantly from the *S. cerevisiae* model. The mating type switching event happens by a gene conversion incident. In this process, the information of the *MAT* locus neighboring the centromere of chromosome number III is substituted with information from one of the two silent cassettes *HMR* or *HML*. Such cassettes are present in the final part of the chromosome.

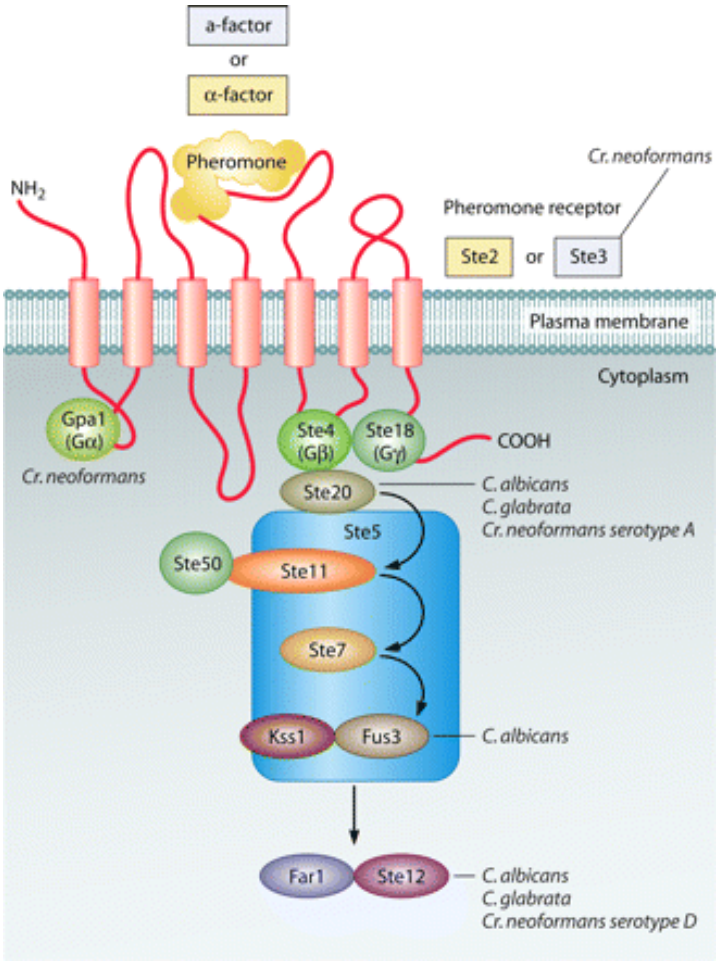


Figure 14 – *S. cerevisiae* MAPK signaling pathway. The pathway in *S. cerevisiae* is demonstrated, as well as the differences in other fungi in which the fundamentals of the MAP kinase pathway are conserved. Deletion of genes that affect virulence of human pathogens are indicated (Butler 2010).

1.5.4 Mating in the ascomycetes *Aspergillus fumigatus* and *A. nidulans*

A. fumigatus is an opportunistic human pathogen, that belongs to the phylum Ascomycota, family Eurotiales (Geiser et al., 2007). The genus *Aspergillus* is one of the most widespread groups of fungi on Earth, including about 300–350 species assigned to various subgenera and sections (Debets et al., 1993). This genus represents some of the most common fungi found in nature, with only a minor number of species responsible for human disease. It can cause potentially lethal invasive infections in immunocompromised individuals. Like all the others *Aspergillus* species, it produces conidia that correspond to the infectious propagules, that when released into the atmosphere can be inhaled by the host (Chapman et al., 2008).

Aspergilli that naturally comprise both known asexual and sexual species, provide an excellent system to study the genetic basis of reproductive modes in fungi. All *Aspergillus* species can reproduce asexually by forming a conidiophore, which has a vesicle head bearing long chains of asexual spores (conidia) (Figure 18). Around 70 named species may also proliferate through sexual development, displaying a homothallic or heterothallic patterns of mating (Geiser et al., 2007; Geiser, Timberlake, and Arnold 1996; Lee et al., 2010). Phylogenetic analysis of the nuclear and mitochondrial genomes proposes that most of the studied species have arisen from a common ancestor, and that the asexual species arise frequently from sexual ones (Geiser et al., 2007).

Aspergillus spp. can reproduce asexually, sexually, and parasexually. However, most *Aspergillus* species are notorious to produce asexual propagula (conidia) from branching conidiophores borne with the characteristics conidial heads. The majority of *Aspergillus* spp. (≈60 %) are only known to reproduce via an asexual cycle (Dyer and O'Gorman 2011). The asexual reproductive cycle (Figure 15), is divided into two main phases: vegetative growth and development. The vegetative growth phase initiates with the germination of a conidium and is followed by the formation of mycelia composed of hyphal cells. After the period of vegetative growth, under appropriate conditions, some hyphal cells conclude their normal growth and initiate the asexual development, that includes conidiophore formation and conidial maturation (Mah and Yu 2006). *Aspergillus* spp. can suffer a parasexual cycle that allows recombination during mitosis (Figure 15). The parasexual cycle begins with the development of a dikaryon by hyphal fusion, followed by a haploid (N) nuclear fusion, that results in the establishment of diploid (2N) hyphae. As a substitute for meiosis, the vegetative cells remain dividing mitotically, and the haploid chromosome number (N) is restored by random chromosome loss (Levadoux, Gregory, and Taylor 1981).

Although a parasexual cycle has been identified in several *Aspergillus* species, relatively little is known about its evolutionary importance (Levadoux, Gregory, and Taylor 1981; Schoustra et al., 2007). The growth fitness of both haploid and diploid hyphae was studied in *A. nidulans* isolates. The results showed that diploid strains have higher fitness than isogenic haploid strains (Schoustra et al., 2007). After ≈3.000 mitotic divisions, these faster-growing isolates that evolved from a diploid progenitor, had undergone parasexual reduction and returned to the haploid state. Consequently,

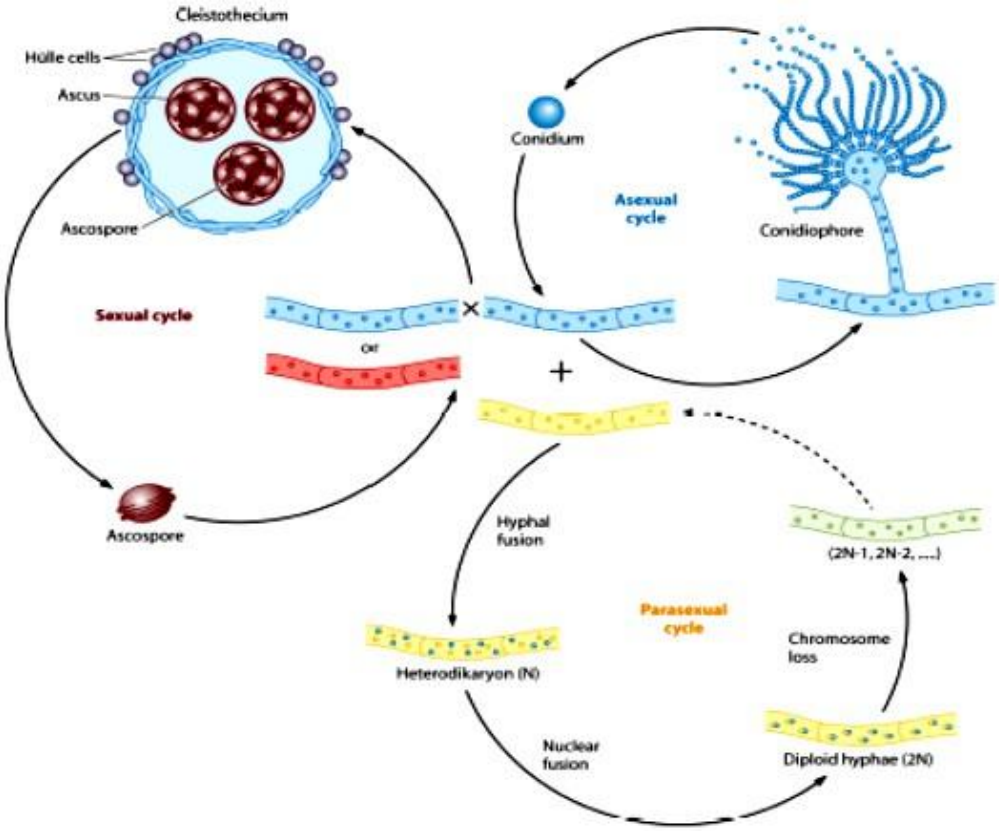


Figure 15 – Reproduction cycles for *A. nidulans*. Conidia are produced during the asexual cycle. The conidium may germinate to form hyphae, from which conidiophores develop to produce more conidia. The homothallic sexual cycle involving self or out-crossing to generate fruiting bodies (cleistothecia) containing thousands of ascospores, which germinate to form hyphae. In the parasexual cycle heterohyphae fuse to form a heterodikaryon, followed by nuclear fusion to generate diploid hyphae (Copied from Lee et al., 2010)

mitotic recombination occurring during the parasexual cycle can accelerate the adaptation under laboratory conditions. The advanced fitness is due to effects of the mutations occurring in diploid nuclei. It might be neutral or harmful on their own in a haploid cell, but are advantageous when combined (Debets et al., 1993; Firon et al., 2002). In order to confirm the benefits of mitotic recombination through the parasexual cycle, the advantageous mutations were selected based on faster growth and revealed that the parasexual cycle can act as a capacitor for evolution and might generate genotypic variations (Montiel-Gonzalez et al., 2002).

Later, several other well-known "asexual" *Aspergillus* spp., including *A. fumigatus* and *A. niger* were discovered to have extant heterothallic sexual cycles (O'Gorman, Fuller, and Dyer 2009; Braumann, Van den Berg, and Kempken 2008). The description of the conditions required for the sexual cycle in *A. fumigatus* is curious, since the mating under laboratory condition occurred

only in the dark with an incubation period of six months (O'Gorman, Fuller, and Dyer 2009). Later, the light-responsive components involved in the sexual cycle (Velvet proteins) were elucidated (Bayram et al., 2010; Park et al., 2012) The comparison of the asexual development and sexual development, especially outcrossing, demonstrated that the sexual cycle has many potential benefits. These benefits include the generation of new genotypes that may be beneficial in novel environments, purging the genome from the accumulation of deleterious mutations, and allowing the formation of thick-walled fruiting bodies that are resistant to severe conditions. The sexual development initiates with the formation of a cleistothecium that are ascogonial coils formed by hyphae. Two ascogonial coils from two hyphal partners fuse to produce a dikaryon that is enveloped in a structure similar to nets, embraced by thick-walled cells named Hulle cells, which serve as nurse cells. Inside the bigger protective structure, the Hulle cells (Figure 18), each dikaryon suffers a nuclear fusion followed by meiosis, post-meiotic and mitosis processes, resulting in the formation of an ascus containing eight haploid ascospores. To conclude, a fruiting body called a cleistothecium is formed and serves to house thousands of ascospores (Figure 15) (Sohn and Yoon 2002). Most sexual *Aspergillus* species are self-fertile (homothallic) but are also capable of outcrossing (heterothallism) (Scazzocchio 2006).

Aspergillus fumigatus and *A. nidulans* harbor both mating type loci, *MAT1-1* and *MAT1-2*. These loci encode transcription factor proteins that regulate sexual identity and seem to regulate later stages of the sexual development. The *MAT1-1* genes family encode an α-domain protein, and the *MAT1-2* genes family encode a HMG box protein (Pyrzak, Miller, and Miller 2008). The *MAT1-1* and *MAT1-2* loci resemble features in other heterothallic and homothallic ascomycetes and highlight the role of *MAT* gene products as master regulators of sexual development (Rydholm, Dyer, and Lutzoni 2007). It was described that *MAT1-1* is mandatory for correct transcription of the alpha-pheromone gene *PPGA*, that resembles structural features of those from other Ascomycetes; and *MAT1-2* appears to suppress its expression. The pheromone receptor genes *PREA* and *PREB* were also expressed, with no differences between the MAT-1 and MAT-2 isolates. However, the expression level of the α-pheromone precursor gene was higher in the MAT-1 than the MAT-2 isolate. Both *MAT1-1* and *MAT1-2* are expressed under the growth conditions tested in a mating-type-specific manner (Paoletti et al., 2005).

Indeed, similar pheromone sensing pathway components regulating sexual or asexual spore germination have been identified in other *Aspergillus* fungi (Desjardins et al., 2011). Regarding

the molecular mechanisms controlling spore germination in *A. nidulans* and *A. fumigatus*, it is known that the protein FlbA is essential for the activation of the sporulation process (Hicks et al., 1997). The FadA homologue GpaA intermediates the signaling for vegetative growth, that negatively controls conidiation processes. GpaA signaling is attenuated by AfuFlbA. The homologues of SfaD and GpgA in *A. fumigatus* presented a series of evidence that these proteins play a crucial role in governing vegetative growth, spore germination, conidiation and production of certain secondary metabolites (Kong et al., 2013)

The pheromone responsive MAP kinase pathway and sexual mating activation is well studied in *A. nidulans* (Saito 2010). AnFus3 dislocates along the membrane and crosses from the cytoplasm into the nuclear envelope, assembling complexes with AnSte7, AnSte11 and the adaptor protein AnSte50. In the nucleus, AnFus3 interacts with the transcription factor AnSte12, responsible for triggering sexual development (Bayram et al., 2012; Bayram et al., 2010). As the importance of the MAPK signaling pathway, another group of proteins, denominated Velvet proteins play an essential role during the sexual development of *A. fumigatus*. The MAPK pathway protein AnFus3 interacts with the Velvet protein VeA, and yet unidentified proteins (Beyhan et al., 2013; Webster and Sil 2008) that allow the formation of the VeA-VelB complex with the Velvet protein VelB, and is essential for coordination and development of secondary metabolism. *A. nidulans* Fus3 is specifically assembled at diverse intracellular positions and its membrane localization of the module is presumably important to notice external signals in yeast cultures. The septal localization is important for the hyphal fusions and cell-cell contacts. MAP kinases AnSte7 and AnSte11 interact in the septa, suggesting additional phosphorylation functions at septa, an interaction independent of AnFus3. The AnSte50 might also contribute to Ste11 activation (Bayram et al., 2012). In yeast, Fus3 dissociates from the Ste5 to secure that the module enters into the nucleus. The mobilization of the AnFus3 in the AnSte50-Ste11-Ste7 complex into the nuclear envelope, functions as an additional signal transduction step in *A. nidulans* and might secure that AnFus3 can be kept active over larger distances until it reaches the nucleus (Bayram et al., 2012). Once inside the nucleus, AnFus3 interacts with AnSte12, and probably phosphorylates it. AnFus3 phosphorylates the velvet protein VeA, which efficiently associates with the Velvet protein VelB and the global regulator LaeA (Bayram et al., 2010). VelB-VeA contributes with AnSte12 to sexual fruiting body development and the trimeric VelB-VeA-LaeA

complex induces the expression of distinct genes for secondary metabolites (Bayram and Braus 2012).

O'Gorman and co-workers accomplished sexual reproduction in *A. fumigatus* through the incubation of the opposite mating type strains AfRB23 (MAT1-1) and AfIR956 (MAT1-2). The methodology applied was to incubate the strains for 6 months at 30°C on Oatmeal agar medium, in the dark (O'Gorman, Fuller, and Dyer 2009). The structures observed under microscope were the ones expects in sexual reproduction, such as spores, ascospores and cleistothecia (Figure 16) (O'Gorman, Fuller, and Dyer 2009).

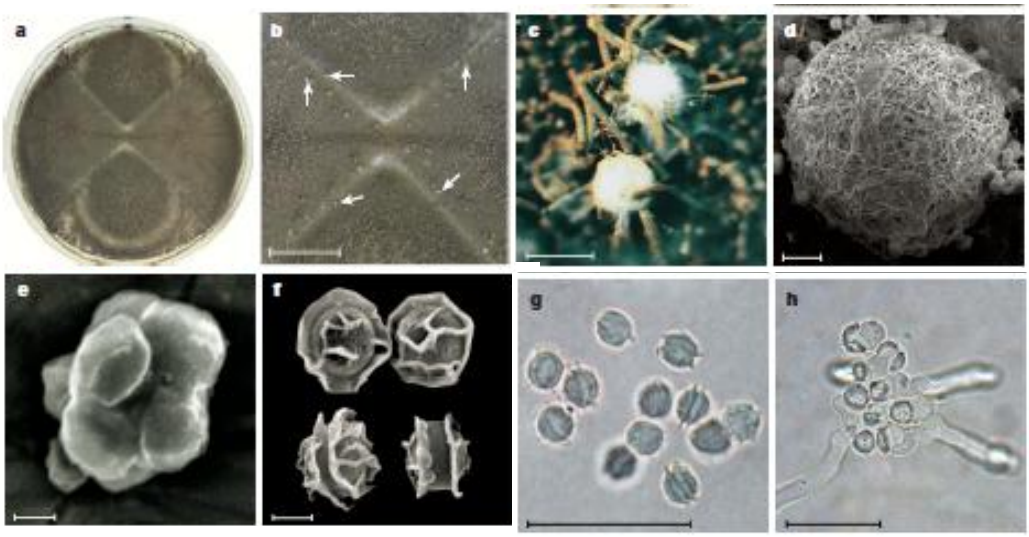


Figure 16 –Development of sexual structures in *A. fumigatus*. AfRB23 x AfIR956 (a); Cleistothecia (arrows) along the junctions of intersecting colonies of opposite mating type (b) (scale bar, 1 cm.); Cleistothecia among chains of conidia (c) (scale bar, 400 mm). Scanning electron micrograph of a cleistothecium, showing the period of interwoven hyphae (d) (scale bar, 100 mm); Scanning electron micrograph of an eight-spored ascus (e) and ascospores (f). (scale bars, 2 mm); Ascospores ungerminated (g) and germinating after exposure to high temperature (h) (scale bars, 20 mm) (O'Gorman et al., 2009).

Supermater strains were recently uncovered by Sugui and co-workers using the AFIR928 (MAT1-2) and two different MAT1-1 strains, where mating was accomplished in only 4 weeks. A possible explanation for this shorter time frame is the media composition (Sugui et al., 2011). The authors discovered that oatmeal agar medium was the most variable factor. Different batches of oatmeal obtained from the same producer did not always yield the same degree of fertility in certain crosses. Since the oatmeal used by O'Gorman et al., 2009, and the oatmeal used in the

supermaters study were from different sources, the discrepancy in fertility results obtained with the tested strains is most probable due to changes in the composition of the oatmeal (Sugui et al., 2011). Due to the results obtained in that study, the *A. fumigatus* isolates can be divided in high fertility (HF), normal fertility (NF) and low fertility (LF) types. The HF type was not associated with a specific mating type, the NF type was more prominent in MAT1-2 strains and the LF type was slightly more prominent in the MAT-11 strains. The strains were submitted to *MAT* locus sequencing, and the insertion of three nucleotides was found (GCC). Whether this insertions affects the transcription of the *MAT* locus genes is being currently investigated (Sugui et al., 2011).

1.5.5 Mating in the thermodimorphic ascomycete *Histoplasma capsulatum*

Histoplasma capsulatum is a thermodimorphic fungus that exists as mycelia and airborne conidia forms at environmental temperatures, and shifts to the budding yeast form at the mammalian host temperature (Conant 1941). It can be isolated from soil (Zeidberg et al., 1952), and the fungus is found in high concentrations in the areas where there are bird or bat droppings, such as bridges and caves (Meals and McKinney 1998). If inhaled, conidia or hyphae fragments once inside the lungs, lead to a progression of an asymptomatic or self-limiting disease (Kauffman 2007). The symptomatic severe acute pulmonary histoplasmosis, is responsible for pneumonia development in those with impaired cell mediated immunity (Meals and McKinney 1998).

H. capsulatum is a heterothallic organism with two described mating types: + and - (Fraser et al., 2007). Mating between + and – strains happens at the mycelial stage, and was demonstrated under laboratory conditions (Kwon-Chung 1973).

The *MAT1-1* (+) mating type harbors a predicted transcription factor characterized by the occurrence of an α1 domain. The *MAT1-2* idiomorph contains a transcription factor characterized by an HMG domain that is common among ascomycete MAT1-2 strains. The ascomycete α1 and HMG domains contain introns at conserved spots inside the domains, which were also recognized in the sequences predicted for the MAT transcription factors in *H. capsulatum* (Coppin et al., 1997). The fungus' heterothallic mating system, and its mating morphology, has been described in detail using isolates of opposite mating types (Kwon-Chung 1973). The mating-

specific gene regulation suggests that the pheromones produced by both mating types are constantly present in the cultures (Kwon-Chung 1973; Bubnick and Smulian 2007).

In 1972, mating was confirmed morphologically in two *H. capsulatum* isolates of opposite mating type. As a result of sexual reproduction in *H. capsulatum* strains, asci and ascospores were not observed, however, cleistothecia and closed mating structures were found, as well as the formation of diploid cells (Kwon-Chung 1972). The *H. capsulatum* mature cleistothecia produce characteristic coiled hyphae that assemble a hyphae net. Inside this structure several asci are found, with each ascus containing eight ascospores (Kwon-Chung 1972).

Bubnick and Smulian experimented with a mating assay in which the putative pheromone-containing suspensions were used. The pheromones were isolated using a protocol previously described (Strazdis and Mackay 1982). The opposite mating type isolates UH1 (MAT1-1) and VA1 (MAT1-2) were tested and cultivated on HMM plates and grown at 25°C until mycelial growth was observed (Figure 20). A mass of each organism was transferred onto A-YEM agarose. The mycelia pieces were placed about 8 mm apart. The cultures were grown at 25°C for 3 weeks and then examined under the microscope. Both UH1 and VA1 strains were stimulated with each of the putative pheromone extracts (Bubnick and Smulian 2007). The resultant structures from this assay were the formation of the ascocarp, that has been described previously by Kwon-Chung. The structures observed included hyphal coiling and cleistothecia covered by short branching hyphae (Figure 17) (Kwon-Chung 1973). Several regulators are involved in the development of sexual structures in *H. capsulatum*, such as the Velvet protein VeA that is essential for cleistothecia assembly (Laskowski-Peak et al., 2012).

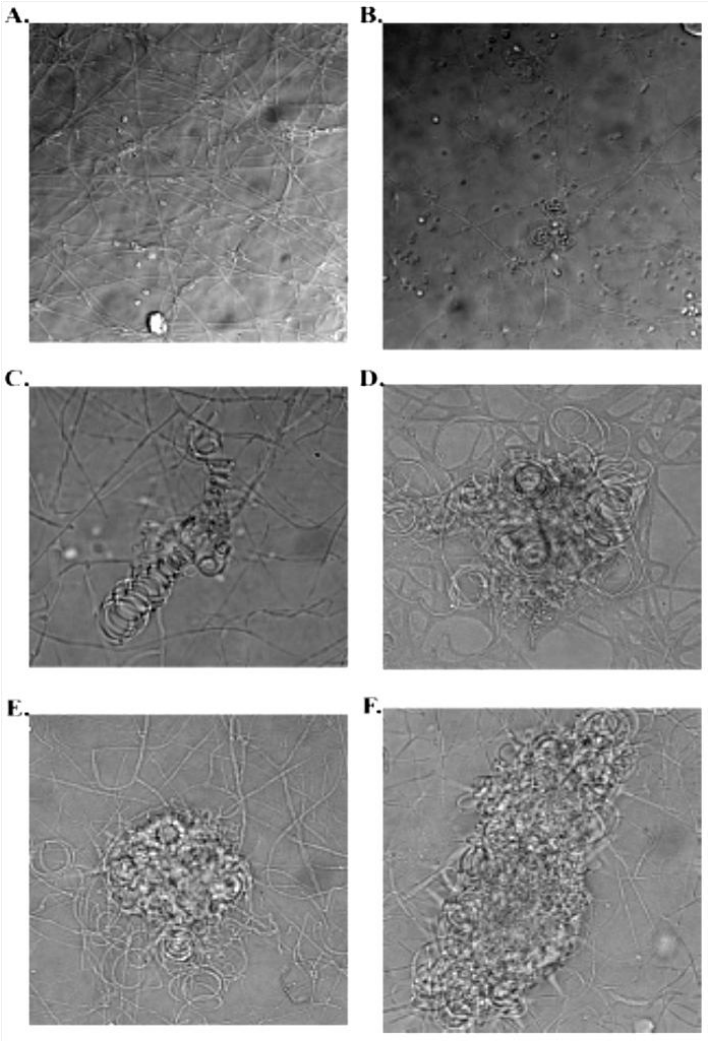


Figure 17 - Results of mating assay in *H. capsulatum.* Strains were plated on A-YEM (A); UH1 strain self-cross. (B to F); UH1and VA1 strains crossed. (B and C) Coiled hyphae, consistent with younger mating structures. (D to F) Coiled hyphae covered consistent with mature mating structures (Copied from Bubnick et al., 2007).

1.6.6 Mating in the thermodimorphic ascomycete *Paracoccidioides* spp.

Although sexual development was never observed under nature or laboratory conditions, several evidences for the existence of sexual reproduction in *Paracoccidioides* spp. have been described in the past years. Torres and co-workers demonstrated the presence, distribution and structure of *MAT1-1* and *MAT1-2* idiomorphs in *Paracoccidioides* spp. (Torres et al., 2010). It was established that *MAT1-1* and *MAT1-2* genes showed an elevated similarity to the homologous genes from other filamentous Ascomycetes. The *MAT* locus identification was carried out taking advantage of specific primers designed on the basis of the sequences available in the previously published *P. brasiliensis* EST libraries (http://www.ebi.ac.uk/embl/), as well as on the sequences from *H.*

capsulatum MAT genes (Torres et al., 2010). The genomic products from the PCR, produced amplicons for both mating types that corresponded to the *MAT1-1* and *MAT1-2* loci (Figure 18). The *Paracoccidioides* spp. *MAT1-1* and *MAT1-2* genes sequences indicated an elevated identity with *H. capsulatum* sequences, 79% identity for *MAT1-1* and 70% for *MAT1-2.* Moreover, it was shown that the *Paracoccidioides* spp. Mat1-1 and Mat1-2 protein sequences harbor features of α-box and HMG domains, respectively (Torres et al., 2010).

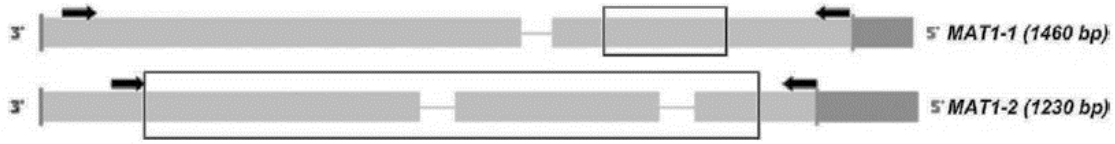


Figure 18 - *Paracoccidioides spp. MAT* locus. Position of the primers used in the *Paracoccidioides* isolates classification according to presence of *MAT1-1* and *MAT1-2* genes. Black arrows represent position of the genomic primer Regions in box correspond to the fragments amplified (Copied from Torres et al., 2010).

The complete genomes sequences of 3 *Paracoccidioides* strains confirmed the conservation of genes required for meiosis and mating in the sequenced *Paracoccidioides* strains (Pb01, Pb03 and Pb18). Most of the genes involved in chromosome cohesion and recombination are found in *Paracoccidioides* strains. The detection of the expression of 78% of the mating and 72% of the meiosis genes was detected in the EST library (Table 1) (Desjardins et al., 2011) .

The genome composition provided a major support for the existence of a sexual cycle in *Paracoccidioides* spp, as the majority of the genes involved in the mating process or mating signaling are highly conserved. Orthologues of known regulators of the MAPK signaling pathway (Table 1), cell and nuclear fusion related genes (meiosis), genes related to positive and negative regulators of sexual development, light-sensitive related genes and genes involved in other process were all identified by Desjardins and coworkers (Desjardins et al., 2011).

Table 1: Conservation of mating and mating-related genes in *Paracoccidioides* spp. (Adapted from Desjardins et al., 2011)

Gene	Organism	Annotation	*P. lutzii*	Pb03	Pb18
ANID_02755	*A. nidulans*	mating type alpha box protein	**PAAG_05873**	none	none
ANID_04734	*A. nidulans*	mating type HMG-box protein	PAAG_02636	**PABG_05778**	**PADG_06118**
SPBC25B2.02c	*S. pombe*	M-factor transporter Mam1	PAAG_07335	PABG_05520	PADG_05835
YGL203C	*S. cerevisiae*	*KEX1*; Protease involved in the processing of α factor precursor	**PAAG_00330**	**PABG_06619**	**PADG_02348**
YNL238W	*S. cerevisiae*	*KEX2*; lysin-like protease (protein convertase), involved in the activation of proteins of the secretory pathway	**PAAG_07022**	**PABG_03025**	**PADG_01553**
YOR219C	*S. cerevisiae*	*STE13*; Dipeptidyl aminopeptidase, Golgi integral membrane protein, required for maturation of α factor	PAAG_01724	PABG_03771	PADG_00053
YMR274C	*S. cerevisiae*	*RCE1*; Type II CAAX phenyl protease involved in the maturation **a**-factor	**PAAG_03593**	**PABG_06930**	**PADG_06229**
YJR117W	*S. cerevisiae*	*STE24*; Highly conserved zinc metalloprotease that functions in two steps of **a**-factor maturation	**PAAG_00744**	**PABG_01483**	**PADG_04087**
YDR410C	*S. cerevisiae*	*STE14*; Farnesyl cysteine-carboxyl methyltransferase, mediates the carboxyl methylation **a**-factor	**PAAG_07523**	**PABG_07253**	**PADG_05218**
YKL209C	*S. cerevisiae*	*STE6*; Plasma membrane ATP-binding cassette; transporter required for the export of **a**-factor; expressed only in MAT**a** cells	PAAG_03754	**PABG_03725**	**PADG_05835**
YFL026W	*S. cerevisiae*	*STE2*; Receptor for α-factor pheromone; heterotrimeric G protein to initiate the signaling response that leads to mating	**PAAG_05858**	**PABG_05794**	**PADG_06133**
ANID_02520	*A. nidulans*	pheromone receptor *PREB*	**PAAG_05858**	**PABG_05794**	**PADG_06133**
YKL178C	*S. cerevisiae*	*STE3*; Receptor for **a** factor pheromone, transcribed in α cells and required for mating by α cells	**PAAG_07295**	**PABG_05483**	**PADG_05797**

ANID_07743	*A. nidulans*	pheromone receptor *PREA*	**PAAG_07295**	**PABG_05483**	**PADG_05797**
ANID_00651	*A. nidulans*	G-protein α subunit	**PAAG_08675**	**PABG_03841**	**PADG_07281**
YHR005C	*S. cerevisiae*	*GPA1*; GTP-binding α subunit of the heterotrimeric G protein that couples to pheromone receptors	PAAG_08675	PABG_03841	PADG_07281
ANID_00081	*A. nidulans*	G-protein β subunit	**PAAG_02472**	**PABG_04961**	**PADG_05621**
YOR212W	*S. cerevisiae*	*STE4*; G protein β subunit; forms a dimer with Ste18 and a heterotrimer with Gpa1 and Ste18 to dampen signaling	**PAAG_02472**	**PABG_04961**	**PADG_05621**
YDR103W	*S. cerevisiae*	*STE5*; Pheromone-response scaffold protein; binds Ste11, Ste7, and Fus3 kinases, forming a MAPK cascade complex; allosteric activator of Fus3 that facilitates Ste7-mediated activation	None	none	none
YJR086W	*S. cerevisiae*	*STE18*; G protein γ subunit, forms a dimer with Ste4 and a heterodimer with Gpa1p and Ste4 to dampen signaling	**PAAG_00817**	none**	**PADG_04014**
YHL007C	*S. cerevisiae*	*STE20*; Cdc42-activated signal transducing kinase of the p21-activated kinase family; involved in pheromone response	**PAAG_09053**	**PABG_07870**	**PADG_08706**
ANID_02269	*A. nidulans*	MAPKK kinase	**PAAG_00450**	**PABG_06721**	**PADG_02230**
YLR362W	*S. cerevisiae*	*STE11*; Signal transducing MEK kinase involved in pheromone response; phosphorylates Ste7; regulated by Ste20 and Ste50	**PAAG_00450**	**PABG_06721**	**PADG_02230**
YDL159W	*S. cerevisiae*	*STE7*; Signal transducing MAP kinase kinase involved in pheromone response, where it phosphorylates Fus3	**PAAG_06020**	**PABG_05652**	**PADG_05980**
ANID_03719	*A. nidulans*	mitogen-activated protein kinase	**PAAG_05033**	**PABG_04490**	**PADG_04880**
YBL016W	*S. cerevisiae*	*FUS3*; Mitogen-activated serine/threonine protein kinase involved in mating; phosphoactivated by Ste7	**PAAG_05033**	**PABG_04490**	**PADG_04880**
YHR084W	*S. cerevisiae*	*STE12*; Transcription factor that is activated by a MAP kinase	**PAAG_00406**	**PABG_06687**	**PADG_02268**

		signaling cascade, activates genes involved in mating			
YJL157C	*S. cerevisiae*	*FAR1*; Cyclin-dependent kinase inhibitor that mediates cell cycle arrest in response to pheromone; forms a complex with Cdc24, Ste4p, and Ste18p that specify the direction of polarized growth during mating	None	none	none
YCL032W	*S. cerevisiae*	*STE50*; Protein involved in mating response; protein associated with Cdc42-Ste20 complex to the effector Ste11 to modulate signal transduction	**PAAG_05534**	**PABG_05032**	**PADG_05695**
SPBC24C6.06	*S. pombe*	G-protein α subunit	**PAAG_04436**	**PABG_03841**	**PADG_04598**
SPAC1565.04c	*S. pombe*	adaptor protein Ste4	PAAG_05534	**PABG_05032**	PADG_05695

*may fall in contig gap; **weak hit found (with higher e-value)

Bold: Best bidirectional hit.

EST matches are counts of best blast hits to *P. brasiliensis* (Pb18) transcripts, requiring expect values of 1e-10 and 90% identity. EST matches for MATα are based on blast to *P. lutzii* transcripts.

The absence of a sexual cycle in *Paracoccidioides*, despite the existence of most of the homologous genes necessary for sexual development and evidence for genetic recombination, indicates that there might be (other) genetic, biological or environmental constraints that prevent mating in *Paracoccidioides*. In order to identify genetic barriers that could prevent a functional sexual cycle it is important to evaluate e.g. the expression of MAPK signaling pathway genes and genes that encode light-sensitive proteins (essential for mating in other fungi) under different conditions. Moreover, the MAPK signaling components or their biochemical interaction might be non-functional or hampered, and mating pheromones have not been identified despite the presence of pheromone receptors. Therefore, it is important to identify any missing factors for mating (such as mating pheromones) and to demonstrate the functionality of all mating components, e.g. by their ability to restore mating mutations in a heterologous yeast model. Biological and environmental factors that induce mating also require further study, and includes testing the ability of a large set of opposite mating types to mate under different growth conditions, such as medium composition or the effects of light. The validation of a sexual cycle in

the *Paracoccidioides* genus and the molecular characterization of the mating system are of great importance, because it will provide the essential knowledge on basic biological and evolutionary aspects of these fungi. Moreover, the identification and characterization of the sexual mechanisms will support genetic studies in this fungus. On a wider scale the information obtained will contribute valuable new insights into the long standing problem of sexuality/asexuality, which has not been completely clarified in several fungi.

Chapter 2

Functionality of the *Paracoccidioides* mating alpha-pheromone-receptor system

The results presented over this chapter were published:

(i) in an international peer reviewed journal:
Gomes-Rezende J., Gomes-Alves A., Menino J., Coelho M., Ludovico P., Gonçalves P., Sturme M., Rodrigues F. (2012). "Functionality of the Paracoccidioides Mating α- Pheromone-Receptor System". PLoS One. 7(10):00e 47033.

(ii) in conference proceedings:
Gomes-Rezende. J. "Functional analysis of mating genes of the dimorphic fungal pathogen Paracoccidioides brasiliensis". XXXVII Portuguese Genetics Conference, from the 28th to the 30th of May 2012, Lisbon. *Oral communication.*

Gomes-Rezende J., Gomes-Alves A., Menino J., Coelho M., Ludovico M., Gonçalves P., Sturme M. and Rodrigues F. "Identification of Paracoccidioides mating alpha-pheromone receptor system functionality". Fifth FEBS Advanced Lecture Course: "Human Fungal Pathogens: Molecular Mechanisms of Host-Pathogen Interactions and Virulence". 25-31 May, 2013, La Colle sur Loup, France. *Poster presentation.*

Abstract

Recent evidence suggests that *Paracoccidioides* species have the potential to undergo sexual reproduction, although no sexual cycle has been identified either in nature or under laboratory conditions. In the present work, we detected low expression levels of the heterothallic MAT loci genes *MAT1-1* and *MAT1-2*, the α-pheromone (*PBα*) gene, and the a- and α pheromone receptor (*PREB* and *PREA*) genes in yeast and mycelia forms of several *Paracoccidioides* isolates. None of the genes were expressed in a mating type dependent manner. Stimulation of *P. brasiliensis* MAT1-2 strains with the synthetic α pheromone peptide failed to elicit transcriptional activation of *MAT1-2*, *PREB* or *STE12*, suggesting that the strains tested are insensitive to α -pheromone. In order to further evaluate the biological functionality of the pair α pheromone and its receptor, we took advantage of the heterologous expression of these *Paracoccidioides* genes in the corresponding *S. cerevisiae* null mutants. We show that *S. cerevisiae* strains heterologously expressing *PREB* respond to Pbα pheromone either isolated from *Paracoccidioides* culture supernatants or in its synthetic form, both by shmoo formation and by growth and cell cycle arrests. This allowed us to conclude that *Paracoccidioides* species secrete an active a-pheromone into the culture medium that is able to activate its cognate receptor. Moreover, expression of *PREB* or *PBα* in the corresponding null mutants of *S. cerevisiae* restored mating in these non-fertile strains. Taken together, our data demonstrate pheromone signaling activation by the *Paracoccidioides* α-pheromone through its receptor in this yeast model, which provides novel evidence for the existence of a functional mating signaling system in *Paracoccidioides*.

Introduction

Paracoccidioides species are thermodimorphic ascomycete fungi that occur in a non-pathogenic mycelial form at environmental temperatures (below 25°C), and switch to a pathogenic multiple budding yeast-form at the mammalian host temperature (37°C). This fungus is the etiological agent of paracoccidioidomycosis (PCM), a systemic mycosis that is prevalent in Latin America and has high incidence in Colombia, Venezuela and Brazil (Brummer, Castaneda, and Restrepo 1993; Restrepo, McEwen, and Castañeda 2001). Host infection occurs through inhalation of infective airborne conidia (asexual spores) or mycelial propagula from the environment that differentiates into the pathogenic yeast form in the lungs and may disseminate to other organs, thereby producing a disseminated mycosis (McEwen et al., 1987).

While many studies have addressed key virulence factors [reviewed in (Puccia et al., 2011)] and the cell cycle of *P. brasiliensis* (Almeida et al., 2006), several other basic aspects of its biology still remain to be elucidated, in particular its ecology and whether or not it is capable of sexual reproduction. Sexual structures have not been observed for this fungus and it is therefore thought to only reproduce asexually. This is in contrast to the phylogenetically closely related dimorphic fungus *H. capsulatum*, which exhibits a clear heterothallic sexual reproduction mode (Kwon-Chung 1972, Kwon-Chung 1973). The absence of a sexual cycle has been noted for over 20% of fungal species (Dyer, O' Gorman, and Céline M. 2011), which is surprising considering the proposed advantages of a sexual cycle over solely asexual reproduction (Lee et al., 2010; Nielsen and Heitman 2007). Nonetheless, in the last decade several studies have uncovered extant sexual cycles in several pathogenic fungi that were previously considered to be ''asexual'' (viz. *Aspergillus fumigatus*, *Candida albicans* and *Candida tropicalis*) (Hull, Raisner, and Johnson 2000; Magee and Magee 2000; O'Gorman, Fuller, and Dyer 2009; Porman et al., 2011). *C. albicans* was shown to undergo mating in vitro as well as in vivo in a mammalian host (Hull, Raisner, and Johnson 2000; Magee and Magee 2000). The identification of sexual reproduction in *A. fumigatus* required lengthy incubations of mating pairs under particular conditions (O'Gorman, Fuller, and Dyer 2009), though a recent study identified some highly fertile *A. fumigatus strains*, that were capable of completing the sexual cycle more rapidly (Sugui et al., 2011). Moreover, a study by Pyrzak et al., (Pyrzak, Miller, and Miller 2008) showed that low transcriptional activity of mating-type regulators might be the cause of the low fertility observed in *A. fumigatus*. These studies suggest that the discovery of sexual reproduction in asexual fungi may require specific culturing conditions, and show as well that fungi may have regulatory constraints that hinder expression of the mating system.

Even though sexual reproduction has not yet been observed in *Paracoccidioides*, there are indications that it may occur. Firstly, phylogenetic and population genetics studies of *Paracoccidioides* species brought to light the existence of four different phylogenetic lineages, namely: S1 (species 1 from Brazil, Venezuela, Peru, Paraguay and Argentina), PS2 (phylogenetic species from Brazil and Venezuela), PS3 (phylogenetic species from Colombia), and the ''Pb01-like'' group, the latter of which has been proposed to constitute a new species named *Paracoccidioides lutzii* (Matute et al., 2006; Teixeira et al., 2009). Genealogical analysis of isolates from these lineages presented evidence for genetic recombination within and between

lineages of the *Paracoccidioides* genus (Matute et al., 2006), indicative of sexual reproduction. Additional support for the existence of a sexual cycle came from the identification of two idiomorphic mating type genes (*MAT1-1* and *MAT1-2*) in *Paracoccidioides* isolates, exhibiting high similarities to mating type genes of other filamentous ascomycetes (Li et al., 2010; Torres et al., 2010). A 1:1 distribution of the *MAT* loci was observed among the isolates consistent with a heterothallic sexual reproduction mode, and gene expression of *MAT* genes was confirmed. Torres et al., also tested *in vitro* mating of opposite mating types of *Paracoccidioides* under different conditions, but were unable to confirm the production of true sexual structures (Torres et al., 2010). This could mean that the production of functional sexual structures requires additional stimuli and/or different environmental conditions or that *Paracoccidioides* mating mechanisms are not functioning efficiently at a genetic or biochemical level. Besides the identification of two *MAT* idiomorphs (Li et al., 2010; Torres et al., 2010), *MAT1-1* comprising an α-domain gene and *MAT1-2* containing an *HMG* gene, whole genome sequencing of three *Paracoccidioides* isolates further revealed the presence of most components of the mating-pheromone mitogen-activated protein kinase (MAPK) signaling pathway and meiosis specific genes described in other fungi (Butler 2010; Desjardins et al., 2011). The MAPK pathway involved in sexual reproduction is well described in *S. cerevisiae* and conserved among fungi (Figure 1A and B).

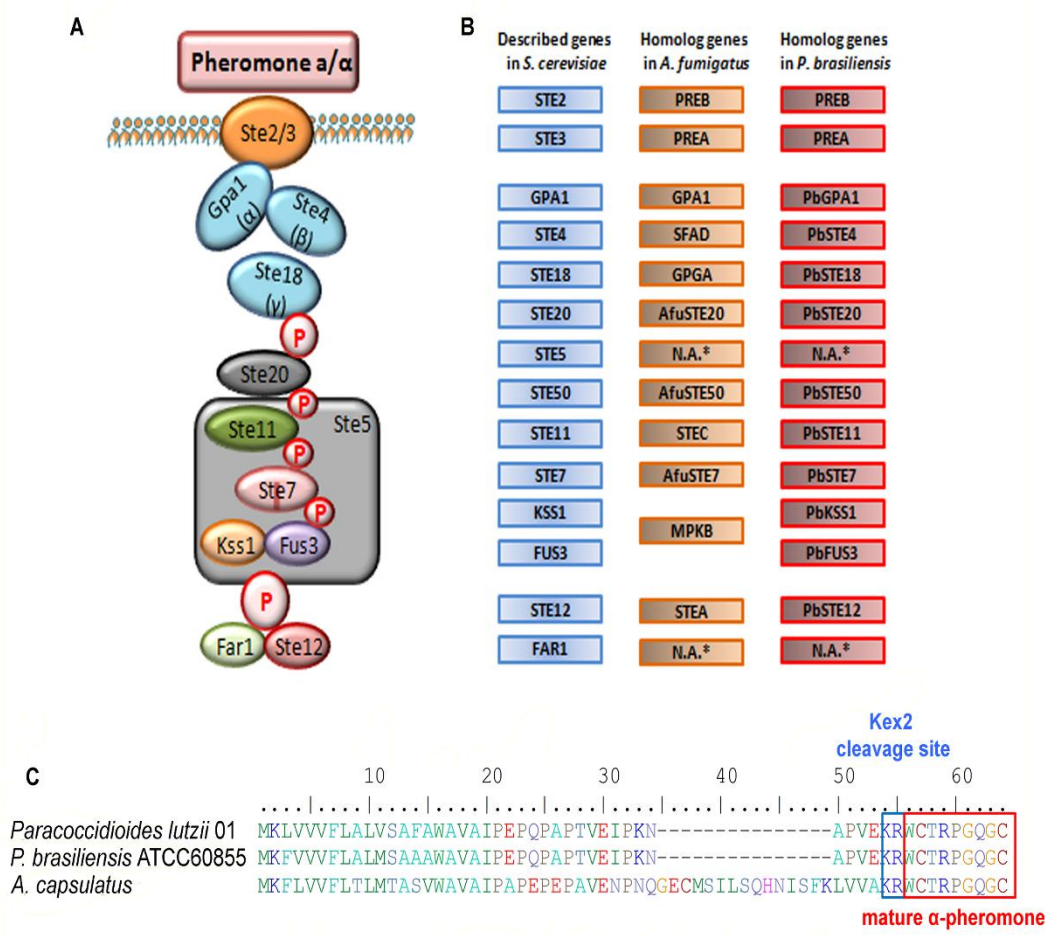


Figure 1 - The genome of *Paracoccidioides* encodes an α-pheromone. (A) Mating pheromone signaling pathway described in *S. cerevisiae* (Butler 2010). (B) *S. cerevisiae* mating pheromone signaling pathway components and the homologous genes annotated in *Paracoccidioides* and *A. fumigatus* databases. Genes not annotated in databases are denoted N.A. (C) Alignment of a-pheromone precursor peptide sequences from *Paracoccidioides* strains and *H. capsulatum.* The Kex2 peptidase recognition site (KR) and the mature pheromone are indicated by blue and red boxes, respectively.

This pathway is activated when the mating pheromone binds to the respective membrane receptor, leading to the dissociation of the Gα subunit of the G-protein coupled to the membrane receptor. The cascade is composed of Ste20 (protein kinase), Ste11 (MAP kinase kinase kinase), Ste7 (MAP kinase kinase) and Fus3 and Kss1 (MAP kinases). These kinases are supported on a scaffold provided by the Ste5 protein. The signal from this cascade is transferred to Far1 (cyclin-dependent kinase inhibitor) and Ste12 (transcription factor) leading to transcription of mating-related genes (Butler 2010). *Paracoccidioides* harbors all the components of the cascade found in *S. cerevisiae*, except for the Ste5 and Far1 homologs (Desjardins et al., 2011) (Figure 1B). In this work, we identified a non-annotated gene encoding a mating α pheromone (*PBα*) and

evaluated its expression in *Paracoccidioides*, as well as the expression of its cognate α-pheromone receptor (PreB). As the observation of mating in *Paracoccidioides* remains elusive, despite the apparent availability of the entire genetic makeup required for pheromone signaling, associated with the fact that genetic tools for functional studies in this fungus are limited (Almeida, Carmona, et al., 2007; Almeida et al., 2009), we set out to determine whether or not components of the mating-pheromone signaling would be able to function in the heterologous host *S. cerevisiae*.

Although mating gene expression could not be induced in the tested *P. brasiliensis* MAT1-2 strains stimulated with synthetic Pbα pheromone, we were able to provide evidence for the functionality of the Pbα pheromone and its receptor PreB in the heterologous expression host *S. cerevisiae*. Our study provides a framework for further studies into the functionality of mating components present in *Paracoccidioides* aiming to identify potential restrictions at the molecular level that reduce mating fertility in this pathogenic fungus.

Materials and Methods

Strains and culture conditions

Paracoccidioides and *S. cerevisiae* strains used in this study are listed in Table 1. *S. cerevisiae* deletion strains were obtained from the EUROSCARF deletion strain collection. For maintenance, *S. cerevisiae* strains were grown at 30°C on YEPD solid medium (0.5% yeast extract, 1% peptone, 2% glucose and 2% agar) or YNB minimal medium dropout plates (6.7% Yeast Nitrogen Base without amino acids, 2% glucose and 2% agar), supplemented to meet auxotrophic requirements. For experimental procedures, *S. cerevisiae* strains were grown in YEPD or YNB broth at 26 °C and 150 rpm. *Paracoccidioides* yeast strains were maintained at 37 °C by periodic subculturing on brain heart infusion (BHI) solid medium supplemented with 1% glucose (1.6% agar). For assays the yeast form was grown in BHI broth supplemented with 1% glucose at 37 °C and 200 rpm. The mycelium form was grown in synthetic McVeigh Morton (MMcM) medium (Restrepo and Jimenez 1980) at 24°C, 150 rpm. *Escherichia coli* strains were grown in Lysogeny broth (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37°C, 220 rpm, and supplemented with selective antibiotics where appropriate.

Table 1 – Strains used in this study.

Strain	Genotype[a]	Source
S. cerevisiae		
BY4741	*MATa (his3Δ1 leu2Δ0 met15Δ0 ura3Δ0)*	EUROSCARF
BY4742	*MATα (his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0)*	EUROSCARF
BY4741 STE2Δ	*MATa STE2::kanMX4*	EUROSCARF
BY4742 MF(α)1Δ	*MATa MF(α)1::kanMX4*	EUROSCARF
BY4742 MF(α)2Δ	*MATα MF(α)2::kanMX4*	EUROSCARF
AGDScα	*MATα MF(α)1::kanMX4 MF(a)2::hph*	This study
AGLPbα	*MATα MF(α)1::kanMX4 MF(α)2::hph pLPbα*	This study
AGMPbα	*MATα MF(α)1::kanMX4 MF(α)2::hph pMPα*	This study
AGLPreB	*MATα STE2::kanMX4 pLPreB*	This study
Paracoccidioides isolates		
P. lutzii Pb01	MAT1-1 (Pb01)b	Chronic PCM/Goia´s - Brazil
P. brasiliensis T8B1	MAT1-1 (S1)b	Armadillo/Botucatu
P. brasiliensis Pb03	MAT1-2 (PS2)b	Chronic PCM/São Paulo - Brazil
P. brasiliensis ATCC60855	MAT1-2 (PS3)b	Sputum/Colombia

a–Plasmids listed in Table 2.

b–Phylogenetic species as defined by Matute et al., 2006 and Teixeira et al., 2009.

Construction of a *MF(α)1/2* double mutant in *S. cerevisiae*

For heterologous expression of the *Paracoccidioides* α-pheromone (*PBα*) in *S. cerevisiae*, a double mutant for both *S. cerevisiae* α pheromones *MFα1* and *MFα2* genes was constructed. To obtain a double mutant with two different antibiotic resistance cassettes replacing the *MF(α)1* and *MF(α)2* genes, we first replaced the geneticin cassette (*KanMx*) in *S. cerevisiae* strain BY4742 *ΔMF(α)2* by a hygromycin B cassette (*hph*). To this end plasmid pAG34, carrying the *hph* gene under the control of a TEF promoter and terminator, was used. This plasmid allows for homologous recombination of the *hph* cassette with the *KanMX* cassette in EUROSCARF deletion strains, as this cassette carries the same TEF promoter and terminator. Plasmid pAG34 was digested with restriction enzyme XhoI (FastDigest, Fermentas), and subsequently transformed in

strain BY4742 *ΔMF(α)2* using the lithium acetate method as described (Gietz and Woods 2006). Transformants were isolated on medium with hygromycin as a selective marker. Replacement of the *KanMX* cassette in gene *MF(α)2* by a *hph* cassette was confirmed by PCR. Subsequently, the strain BY4742 *ΔMF(α)1* was transformed with the *hph* cassette with *MF(α)2 flanking* regions (amplified using primers MFa2-HR-HPH-Fw and MFa2-HR-HPH-Rev, and Phusion DNA Polymerase (Finnzymes). Double mutants for the *MF(α)1/2* genes of *S. cerevisiae* were isolated on YEPD selective plates (supplemented with hygromycin and geneticin) and correct genomic insertion was confirmed by PCR. PCR reactions were performed with primers described in Table S1, using the DyNAzyme II DNA polymerase kit (Finnzymes) and genomic DNA from transformants as a template.

Construction of heterologous expression plasmids

All vectors reported in this study are listed in Table 2. For heterologous expression of *Paracoccidioides* mating-related genes the tetracycline-repressed expression vectors pCM189 (low-copy) and pCM190 (high-copy) were used, both containing the uracil marker (*URA3*). *P. brasiliensis* α-pheromone (GenBank accession number JX429928) and *PREB* genes were amplified from Pb01 mycelium specific cDNA, using proof-reading Phusion DNA Polymerase (Finnzymes). The amplified PCR products included short terminal sequences homologous to the vector cloning site, to allow for cloning by homologous recombination (primers - Table S1). PCR products and linearized vectors were transformed in the respective *S. cerevisiae* deletion strain BY4741 *ΔSTE2* (for *PREB*) or *AGΔScα* (for *Paracoccidioides* α-pheromone). *PREB* cloned in the low-copy vector pCM189 was designated pLPreB, while *Paracoccidioides* α-pheromone cloned in both low and multi-copy vectors, were designated pLPba and pMPba, respectively. Transformants were isolated on YNB uracil dropout medium and confirmed by PCR. PCR reactions were performed with primers described in Table S1, using the DyNAzyme II DNA polymerase kit (Finnzymes) and genomic DNA from transformants as a template.

Table 2 – Plasmids used in this study.

Plasmid	Construction	Resistance/auxotrophic marker	Source
pAG34	-	Ampicillin, Hygromycin B	EUROSCARF
pLPbα	pCM189::PBα	Ampicillin/Uracil	This study
pMPbα	pCM190::PBα	Ampicillin/Uracil	This study
pLPreB	pCM189::PREB	Ampicillin/Uracil	This study

Identification of *Paracoccidioides* mating related genes

Identification of *Paracoccidioides* mating-related genes was performed using BLAST searches against a genome database of isolates Pb01, Pb03 and Pb18, available at the Broad Institute (http://www.broadinstitute.org/). In order to identify the α-pheromone gene we performed TBLASTN searches against *Paracoccidioides* transcripts and genomic sequences (E-value cut-off 1e-1) using the precursor protein sequence of the *Histoplasma capsulatum* α-pheromone *PPG1* as a query (GenBank: ACU27365.1). Subsequently, transcript hits were translated in all six reading frames using ORF Finder, to identify the position and reading frame of the pheromone sequence. The **a** and α pheromone receptor genes (*PREA* and *PREB*, respectively) are annotated by the Broad Institute and the genes involved in the intracellular signaling pathway were obtained after performing a BLASTN using the known sequences of other ascomycete fungi. Pheromone receptor topology was predicted using TMHMM 2.0 and visualized with TMRPres2D.

To confirm the *in silico* identified sequences for the *Paracoccidioides* α-pheromone and PreB, their respective transcript sequences were amplified from cDNA (primers - Table S1) using a combination of DyNAzyme II and Phusion DNA polymerases (Finnzymes). The PCR products were cloned with the TOPO TA Cloning Kit for sequencing (Invitrogen) following the manufacturer's protocol. Plasmid was extracted from *E. coli* strain DH5α using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced at STAB VIDA (Portugal). Alpha-pheromone transcript sequences have been deposited at GenBank under accession numbers JX429928 and JX429929.

Growth arrest assays

For growth arrest assays, the mature *Paracoccidioides* α-pheromone (Pbα–WCTRPGQGC) was synthesized at Metabion (Germany) and synthetic *S. cerevisiae* α-pheromone (MFα-WHWLQLKPGQPMY) was obtained from GenScript (USA). Halo assays to monitor pheromone-induced growth arrest were performed to establish the biological activity of Pbα in a *S. cerevisiae STE2* deletion strain expressing the *Paracoccidioides* α-pheromone receptor PreB (AGLPreB). The strains *S. cerevisiae* BY4741 and AGLPreB were grown for 16 h in YNB minimal drop-out medium (supplemented with the appropriate amino acids) to an optical density at 640 nm (OD_{640}) of 1.0 to 1.4. Yeast cells at 10^6 and $5x10^6$ cells/mL were embedded in soft agar (0.8%), spread on YEPD plates and dried. Subsequently, 10 mL of synthetic Pbα or MFα (at 2 mg/mL), and 10 ml of Pbα pheromone extracted from *P. brasiliensis* cultures and from AGLPba was spotted on plates and incubated for 16–24 h at 30 °C. Halo development was monitored and halo areas measured. Each experiment was repeated four times.

Cell growth arrest upon pheromone stimulation was also assessed by measuring Colony Forming Units (CFUs) at different time points along incubation with synthetic pheromones. Cultures at an initial concentration of $3.3x10^7$ cells/mL were incubated with Pbα (4 and 40 mg/mL) for strain AGLPreB or MFα (2 and 20 mg/mL) for strain BY4741. At indicated time-points samples were taken and serial dilutions plated on the respective selective media. CFU values were determined after 48 h of incubation at 30°C. Growth rate constants (m) were determined from the respective CFU growth curves.

Shmoo assay

Different strains of *S. cerevisiae* were grown in YNB minimal drop-out medium (supplemented with the appropriate amino acids) to an optical density at 640 nm (OD_{640}) of 1.0 to 1.4. Cells were resuspended in YEPD to $4x10^6$ cells/mL and incubated at 26°C with either Pbα (at 4 and 40 mg/mL) or MFα (at 2 and 20 mg/mL), respectively. The percentage of cell shmooing was determined at different times and cell images were taken on a Zeiss Axioskop equipped with a Carl Zeiss AxioCam (Carl Zeiss, Jena). Shmoo counts were done for 200–300 cells, and each experiment was repeated five times.

α-pheromone isolation

α-pheromone from Pb01/Pb03 and Pb01/ATCC60855 mycelium co-cultures, Pb01, ATCC60855, Pb03 and T8B1 monoculture or AGLPbα culture was extracted using the resin AmberliteH XADH2, according to the protocol described elsewhere (Strazdis and Mackay 1982). In brief, resin was washed in distilled water and conditioned for a-factor isolation in methylene chloride-methanol (1:3 v/v). Pheromone isolation from *Paracoccidioides* mycelium co-cultures was performed by direct addition of resin to the cocultures and incubation of co-cultures for 14 d at 24 °C, 150 rpm. For Pbα pheromone extraction from AGLPbα the resin was directly added to a stationary phase culture and incubated for 2 d at 30 °C, 200 rpm. After incubations, resin was washed several times with distilled water to remove mycelium or yeast cells and α-pheromone subsequently extracted by incubation in 40% methanol for 2 h at 40 °C. Methanol extracts were dried using SpeedVac lyophilisation and resuspended in sterile distilled water.

Cell cycle analysis by flow cytometry

Strains BY4741 and AGLPreB were grown in YNB minimal drop-out medium YNB (supplemented with the appropriate amino acids) to an optical density at 640nm (OD_{640}) of 1.0–1.4. Cells were resuspended in YEPD to $4x10^6$ cells/mL and incubated at 26°C with either Pbα or MFα at 40 or 20 mg/mL, respectively. Cell cycle analysis was performed for different time points. Cell treatment was adapted from Fortuna et al., (Fortuna et al., 2001). Briefly, cells were fixed by resuspending in 500 ml (70% ethanol), and subsequently centrifuged and washed with 1 mL of sodium citrate buffer

(50 mM pH 7.5). Cells were resuspended in 850 ml of sodium citrate buffer and 125 mL of RNase A (2 mg/mL in Tris-EDTA pH 8.0) and incubated at 50 °C for 1 h. After the addition of 50 mL of proteinase K (20 mg/mL) samples were incubated at 50 °C for 1 h. Cells were transferred to a cytometry tube and stained overnight at 4 °C with SYBR Green I (final concentration ranging 0.01X to 100X in Tris-EDTA pH 8.0). Triton X-100 (0.25% v/v in 50 mM sodium citrate buffer pH 7.5) was added and the sample was sonicated with three consecutive ultrasound pulses at 40W for 2 s with an interval of 2 s between each pulse. Cell cycle analysis was performed by flow cytometry (FCM) on a BDTM LSR II flow cytometer. A minimum of 50,000 cells per sample were acquired at low/medium flow rate. Offline data were analyzed with the flow cytometry analysis software package FlowJo 7.6.1. Each experiment was repeated four times.

Quantitative mating assays

Quantitative mating assays were performed based on the protocol by Guthrie and Fink (Guthrie and Fink 2004). Cells were grown in YNB minimal drop-out medium (supplemented with the appropriate amino acids) to exponential phase. Cells were mixed on a 0.45 mm pore membrane (Amersham Hybond-N nylon membranes, GE Healthcare), using a vacuum filtration system (TPP), at different concentrations (16 corresponds to $1.5x10^7$ cells). Membranes were placed on the surface of YEPD plates and incubated at 30 °C for 5 h. For mating assays with the addition of the synthetic pheromone, cells were not filtrated, but incubated in YEPD media for 5 h. Cells were resuspended in sterile water and plated on YNB drop-out plates without methionine and lysine to select for diploids, and on plates without methionine or lysine in order to select for haploid cells. Mating efficiency was calculated as (Gonçalves-Sá 2010):

$$\%mating = \frac{\#Diploid\ cells}{\#\ min\ Haploids} x100$$

Real-time-PCR gene expression analysis and Pbα pheromone induction

For basal expression analysis yeast cultures were inoculated from a single colony and grown to exponential phase, by incubation for 7 d at 37 °C, 200 rpm, during which medium was refreshed once after 4 d. Mycelium cultures were obtained by incubating these yeast cultures for 7 d at 24°C, 150 rpm, to allow for complete yeast-to-mycelium conversion. Total RNA was extracted from *Paracoccidioides* yeast and mycelial cells using Trizol (Invitrogen) standard method for cellular disruption, complemented with heat shock treatment (45 min at 65 °C followed by 60 min at -80°C) and bead-beating using 0.1 mm silica/zirconium beads. Total RNA was treated with DNase I (Ambion) and DNA-free total RNA (1 mg) reverse transcribed using the DyNAmoTM cDNA Synthesis Kit (Finnzymes). cDNA samples were used as templates in order to measure the basal level expression of the *MAT1-1*, *MAT1-2*, *PREA*, *PREB*, *GPA1*, *STE4*, *STE18*, *STE20*, *STE50*, *STE11*, *STE7, KSS1*, *STE12* and α pheromone genes in yeast and mycelium. Quantitative RT-PCR (qRT-PCR) was performed on the CFX96 Real-Time PCR Detection System (Bio-Rad), and qRT-PCR amplification performed using the Sso Fast EvaGreen Supermix kit (Bio-Rad), according to the manufacturer's protocol. For all samples DNA contamination was

discarded, as the utilization of the isolated RNA as a template gave no amplification in qRT-PCR. The thermal cycling conditions comprised: initial denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s and primer annealing and elongation for 5 s at appropriate temperature. After PCR cycling, melt-curves were obtained from 65–95 °C at 0.5 °C increments. For pheromone induction experiments, *Paracoccidioides* strains were grown as mycelium to exponential phase in MMcM medium (Restrepo and Jimenez 1980) and synthetic Pbα pheromone was added to 50 mg/mL. Samples were taken at intervals, and RNA extraction and real-time PCR were performed as described above. All measurements were performed in triplicate and relative expression levels determined using the ΔCT method versus b-tubulin (*TUB2*) as a reference gene (Marques et al., 2004). The primers used are listed in Table S1.

Statistical analysis

Data are reported as the mean 6 standard error of the mean of at least four independent repetitions of each assay. The statistical analyses were performed using the SPSS program version 19 and GraphPad software.

Results

The *Paracoccidioides* spp. genome encodes a mating α pheromone

The genome of *Paracoccidioides* harbors most of the mating- and meiosis-specific genes, with the exception of *S. cerevisiae STE5* and *FAR1* homologues (Desjardins et al., 2011) (Figure 1). Interestingly, while the genus appears to be heterothallic, based on the presence of either the MAT1-1 or MAT1-2 idiomorphs in all strains examined, all three fully sequenced isolates (Pb01, Pb03 and Pb18) encode both the receptors for the mating **a**-pheromone (PreA) and the α-pheromone (PreB). The corresponding mating pheromone genes were however not annotated. To this end we used the α pheromone precursor sequence of the phylogenetically closely related species *H. capsulatum* (PPG1: GenBank ACU27365.1) as a query to perform a TBLASTN database search in all six reading frames against transcripts and genomic sequences of *P. brasiliensis/P. lutzii*. Results showed an ORF encoding an a-pheromone homologue in *P. lutzii* Pb01 and *P. brasiliensis* Pb03 and Pb18 (data not shown). To evaluate the biological significance of this in silico predicted pheromone gene we confirmed the sequence of cDNA generated from

P. lutzii Pb01 and ATCC60855 mycelium cultures (Figure S1). Sequence analysis of the genomic DNA and the cDNA revealed that the pheromone gene appears to be interrupted by an intron of 79 bp (Figure S1), where exon-intron boundaries obey the canonical GT-AG rule (Breathnach and Chambon 1981).

The alignment between the *Histoplasma* and *Paracoccidioides* precursor pheromone protein sequences (Figure 1C) showed highly conserved regions along the entire sequence (53% identity), and both contain a Kex2 protease (prototypical eukaryotic prohormone-processing enzyme) recognition sequence (KR). Cleavage at this site would release the mature pheromone sequence (WCTRPGQGC), which shows an identity of 100% with *H. capsulatum* and 77% with *A. fumigatus*, respectively, and contains residues that are conserved among other fungal α pheromones in general (Martin et al., 2011). The predicted small ORF encoding the α-pheromone (designated *PBα*) overlaps with the annotated hypothetical genes PAAG_00855, PABG_01384 and PADG_03979 of Pb01, Pb03 and Pb18 respectively, and for Pb01 the *PBα* gene is located on the opposite DNA strand of PAAG_00855.

Mating-related gene expression in *Paracoccidioides* sp. yeast and mycelium

As described above, *Paracoccidioides* harbors most genes implicated in mating, but it has not been demonstrated that this fungus undergoes a sexual cycle. Therefore, we set out to assess if *Paracoccidioides* isolates produce mating-specific transcripts. To this end, expression levels of the in silico identified mating-related genes were monitored by qRT-PCR in yeast and mycelial cultures of strains representing both mating types (MAT1-1 and MAT1-2), and including isolates for each of the four phylogenetic groups previously described (Figure 2).

Expression of *Paracoccidioides MAT1-1* or *MAT1-2* genes under basal conditions was shown to occur at low levels in both yeast and mycelium (Figure 2). For the strains encoding the MAT1-1 idiomorph, expression of the α pheromone and the α-pheromone receptor *PREA* was expected, whereas for MAT1-2 strains α-pheromone and the a-pheromone receptor *PREB* expression was envisaged. However, instead we observed low expression levels of both receptors in strains of both mating types. The MAT1-1 strains expressed both pheromone receptors at low levels but there is a clear difference between yeast and mycelial forms (Figure 2E, G). Although overall expression is low, the MAT1-2 strains show significantly higher expression levels of both receptors (*PREA* and *PREB*) in the mycelium when compared with the yeast form (Figure 2F, H). Moreover,

the MAT1-2 strains in mycelial form have higher expression levels of both receptors when compared to the MAT1-1 strains. Previous studies have shown that some heterothallic ascomycetes express pheromone receptors in a mating-type independent manner, as is the case for *A. fumigatus*, *N. crassa* and C. *glabrata* (Muller et al., 2008; Paoletti et al., 2005). Similarly, the α-pheromone is expressed by strains of both mating-types, although with higher values for the MAT1-1 strains (Figure 2C, D). In addition, the expression levels the α-pheromone are significantly higher in the mycelium form, with the exception of Pb03 (Figure 2C, D). However, expression levels are very low for all strains, except for Pb01 that shows a high level of expression in mycelium form.

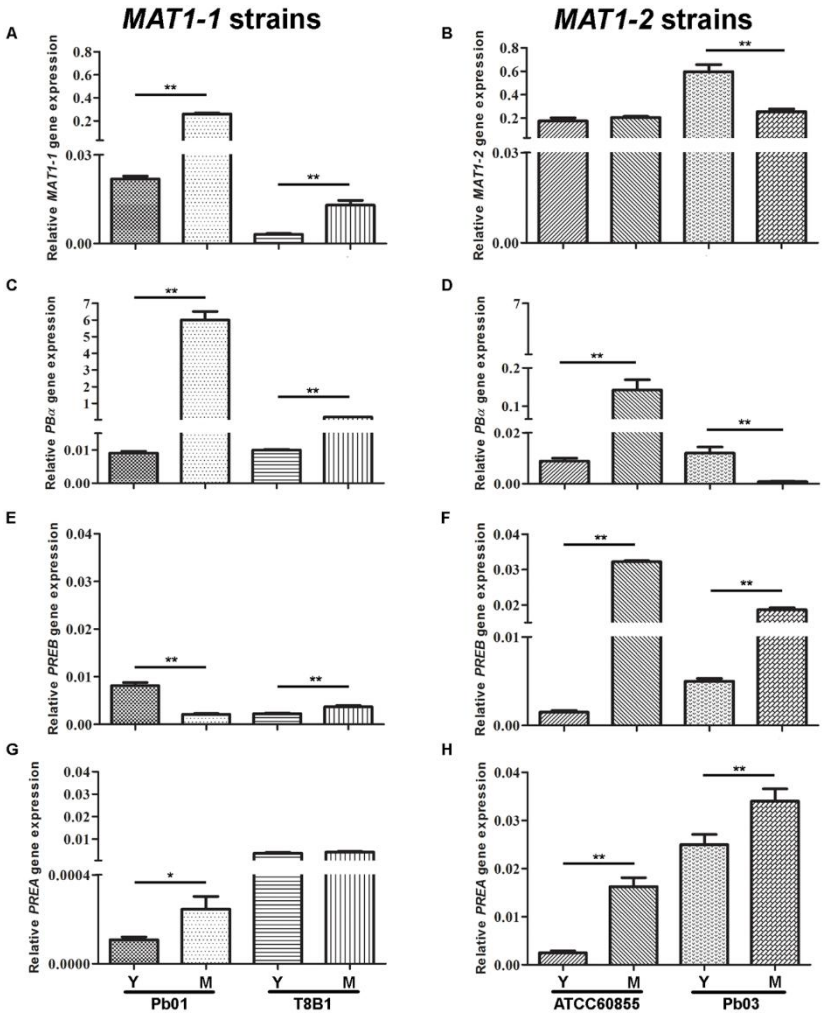


Figure 2 - *Paracoccidioides* expresses the α-pheromone and the a- and α-receptor genes independently of the mating type. Mating gene expression of Paracoccidioides MAT1-1 strains (Pb01 and T8B1) and MAT1-2 strains (ATCC60855 and Pb03) in yeast (Y) and mycelial (M) form. Expression levels of *MAT1-1* (A) and *MAT1-2* (B), *PBα*

(B, C), *PREB* (E, F) and *PREA* (G, H) genes were measured. Error bars are indicated, and asterisks show significant differences (*p,0.05 or **p,0.01).

The α-pheromone does not elicit mating response in *Paracoccidioides*

To assess the biological activity of the identified α-pheromone of *Paracoccidioides* sp. we tested the ability of a synthetic peptide identical to the predicted mature pheromone to trigger a transcriptional response of mating-related genes in *P. brasiliensis* MAT1-2 strains ATCC60855 and Pb03 in mycelium form. Our data show that expression of *MAT1-2*, *PREB* and *STE12* genes did not increase upon pheromone stimulation in *P. brasiliensis* ATCC60855 (Figure 3A) and Pb03 (data not shown). Taking into consideration that MAT1-1 strains also express *PREB*, we further evaluate the transcriptional response of *P. lutzii* Pb01 to the α-pheromone. Our results show that, as for the MAT1-2 strains, the pheromone did not trigger a transcriptional response of the MAT-related genes analyzed (Figure 3B). The nonresponse of *Paracoccidioides* species to α-pheromone led us to question whether or not the molecular players involved in the signaling cascade were being expressed under basal condition in mycelium form. Our data shows that all the components

(Figure 1 A) are expressed at low levels (Figure 3 C–K).

Moreover, comparative analysis of the expression levels (Figure 2 and 3) highlight that in particular the upper components of the signaling cascade (*PREB*, *GPA1*, *STE4* and *STE18*) show the lowest expression levels, which could explain the inability of *Paracoccidioides* to respond to the α-pheromone.

To further evaluate the functionality of the pair α-pheromone and its cognate receptor PreB and considering the observed low mating gene expression and the inability to induce pheromone signaling in *Paracoccidioides*, we continued our studies using the well-established *S. cerevisiae* system as a heterologous model.

Paracoccidioides α-pheromone promotes shmoo formation in *S. cerevisiae* expressing α-pheromone Receptor

To assess the functionality of the predicted *Paracoccidioides* α-pheromone in the heterologous model *S. cerevisiae*, the *PREB* gene, encoding the a-pheromone receptor, was cloned into a constitutive expression vector and was used to transform a *S. cerevisiae STE2* null mutant. The resulting strain is hereafter referred to as AGLPreB.

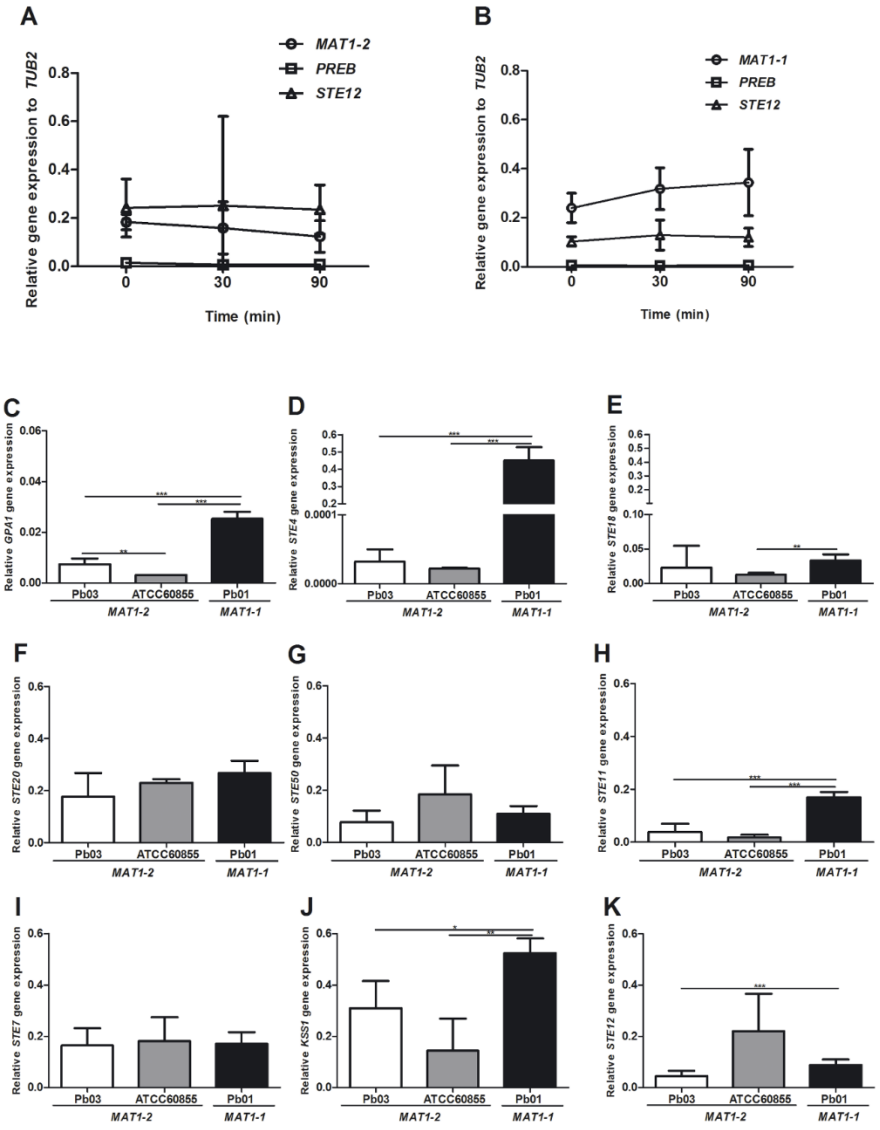


Figure 3 - α-pheromone does not induce mating gene expression in *Paracoccidioides*. Mating gene expression at different time points in *P. brasiliensis* ATCC60855 (A) and *P. lutzii* Pb01 (B) in mycelium form, upon induction with 50 mg/mL of synthetic Pbα pheromone. Basal expression levels of MAPK signaling pathway components in the mycelium form of *P. brasiliensis* Pb03 and ATCC60855 (MAT1-2) and *P. lutzii* Pb01 (MAT1-1) (C-K). Error bars are indicated, and asterisks show significant differences (*p,0.05, **p,0.01, ***p,0.001).

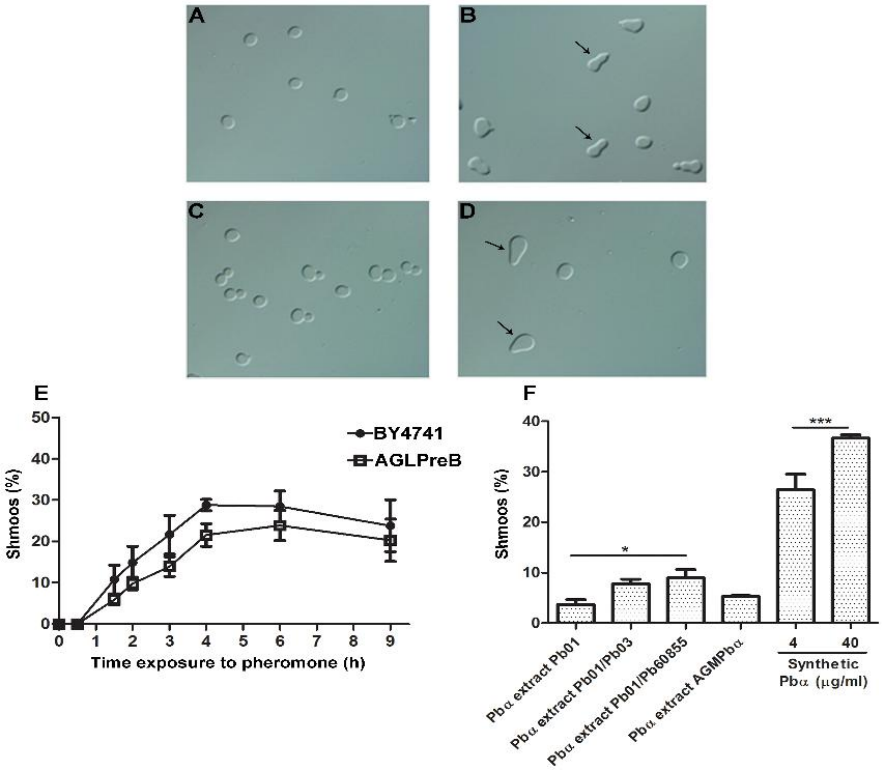


Figure 4 - α-pheromone activates PreB receptor and induced shmoo formation in the *S. cerevisiae* model. Shmoo formation in *S. cerevisiae* BY4741 induced with 20 mg/ml synthetic MFα pheromone after 0 and 5 h of induction (A and B), and *S. cerevisiae* AGLPreB induced with 40 mg/ml synthetic Pbα pheromone after 0 and 5 h induction (C and D). Quantification of shmoo formation using *S. cerevisiae* BY4741 stimulated with MFα (2 mg/mL) or *S. cerevisiae* AGLPreB stimulated with Pbα (4 mg/mL) (E). Induction of S. cerevisiae AGLPreB using Pbα pheromone extracted from *P. lutzii* Pb01 monoculture or *Paracoccidioides* co-cultures (Pb01/Pb03 and Pb01/Pb60855), Pbα pheromone extracted from AGMPbα culture, as well stimulation with the synthetic Pbα pheromone (40 mg/mL) (F). Error bars are indicated, and asterisks show significant differences (*p,0.05,***p,0.001).

In *S. cerevisiae*, it is known that after pheromone recognition, the opposite mating type cell develops projections called shmoos, characterized by a polarized growth along a pheromone gradient towards a mating partner (Gustin et al., 1998). Therefore, the response of AGLPreB cells to synthetic Pbα (WCTRPGQGC) was first monitored by observation of shmoo formation. Maximum shmoo formation was observed after 5 h for AGLPreB exposed to synthetic Pbα (Figure 4C, D, E, F), with a decrease after 9 h (Figure 4E). A similar pattern was observed for the wild-type *S. cerevisiae* MAT**a** strain BY4741 upon exposure to the cognate synthetic α-pheromone (MFα) (Figure 4A, B, E). We also examined the potential for inter-species signaling between α pheromones and receptors from *S. cerevisiae* and *Paracoccidioides*. Our data shows that the responses were species-specific since the synthetic Pbα was unable to induce shmoo formation

through activation of Ste2, while synthetic MFα failed to activate PreB (data not shown). Overall these data demonstrate the effectiveness of the Pbα pheromone in the activation of PreB, and establish the proficiency of *S. cerevisiae* as a heterologous system to study *Paracoccidioides* mating machinery, paving the way for further studies.

As shown previously, expression of *PBα* in *Paracoccidioides* is independent of mating type (Figure 2). However, it could not be discounted that mating type-specific post-transcriptional mechanisms contribute to the production of a fully functional pheromone. Hence, we also investigated if strains of both mating types could express a functional α-pheromone. For this, Pbα was isolated from the supernatants of *Paracoccidioides* cell cultures of Pb01, Pb03, ATCC60855 and T8B1 as well as various co-cultures. Pbα pheromone extracted from *P. brasiliensis* strains Pb03, T8B1 and ATCC60855 did not trigger shmoo formation in strain AGLPreB (data not shown). However, Pbα extracted from *Paracoccidioides* co-cultures Pb01/Pb03 and Pb01/ Pb60855 as well as from the Pb01 mono-culture, induced shmoo formation in AGLPreB (Figure 4F) in a manner dependent on the expression of *PREB* (not shown). Moreover, Pbα isolated from the supernatant of *S. cerevisiae* cultures overexpressing *PBα* (AGMPbα strain) also induced shmoo formation (Figure 4F). The quantification of shmoo formation revealed an induction ranging from 1% to 10%, and was significantly higher for pheromone isolated from co-cultures (Figure 4F). Taken together, the data presented suggests that at least *P. lutzii* Pb01 produces an active α-pheromone.

The *Paracoccidioides* α-pheromone induces cell cycle and growth arrest in *S. cerevisiae* expressing the α-pheromone receptor

Efficient cell fusion during mating requires cell synchronization, which is achieved by cell cycle arrest in the G1 phase, ultimately resulting in growth arrest (Cherkasova, Lyons, and Elion 1999). Pheromone-induced growth and cell cycle arrest were assessed in strain AGLPreB exposed to Pbα (Figure 5).

Our results show that AGLPreB undergoes growth arrest, with increased halo areas upon exposure to higher amounts of synthetic pheromone (Figure 5B–D, H). Pbα extracted from strain Pb01 cultures (Figure 5A) also induced growth arrest, while this was not the case for MAT1-1 strain T8B1 or MAT1-2 strains Pb60855 and Pb03 (data not shown). These results are in line with those obtained for the same set of strains for the induction of shmoo formation. In addition,

Pbα isolated from *S. cerevisiae*, AGMPbα cultures also resulted in halo formation (Figure 5 E), confirming that *S. cerevisiae* is capable of producing a functional Pbα pheromone.

Flow cytometric analysis of DNA content demonstrated pheromone-induced cell cycle arrest, as indicated by the increase of the G0/G1 DNA peak (n) after the addition of Pbα synthetic pheromone to the AGLPreB strain (Figure 5J, M and N). The maximum cell cycle arrest was achieved 60 min after pheromone stimulation, with an increase of <30% of cells arrested in the G1- phase. Growth arrest was also confirmed by the decrease of cell growth rate upon pheromone stimulation (Figure 5P).

Identical results were obtained for wild-type *S. cerevisiae* exposed to its cognate synthetic MFα pheromone (Figure 5F, H, I, K, L and O).

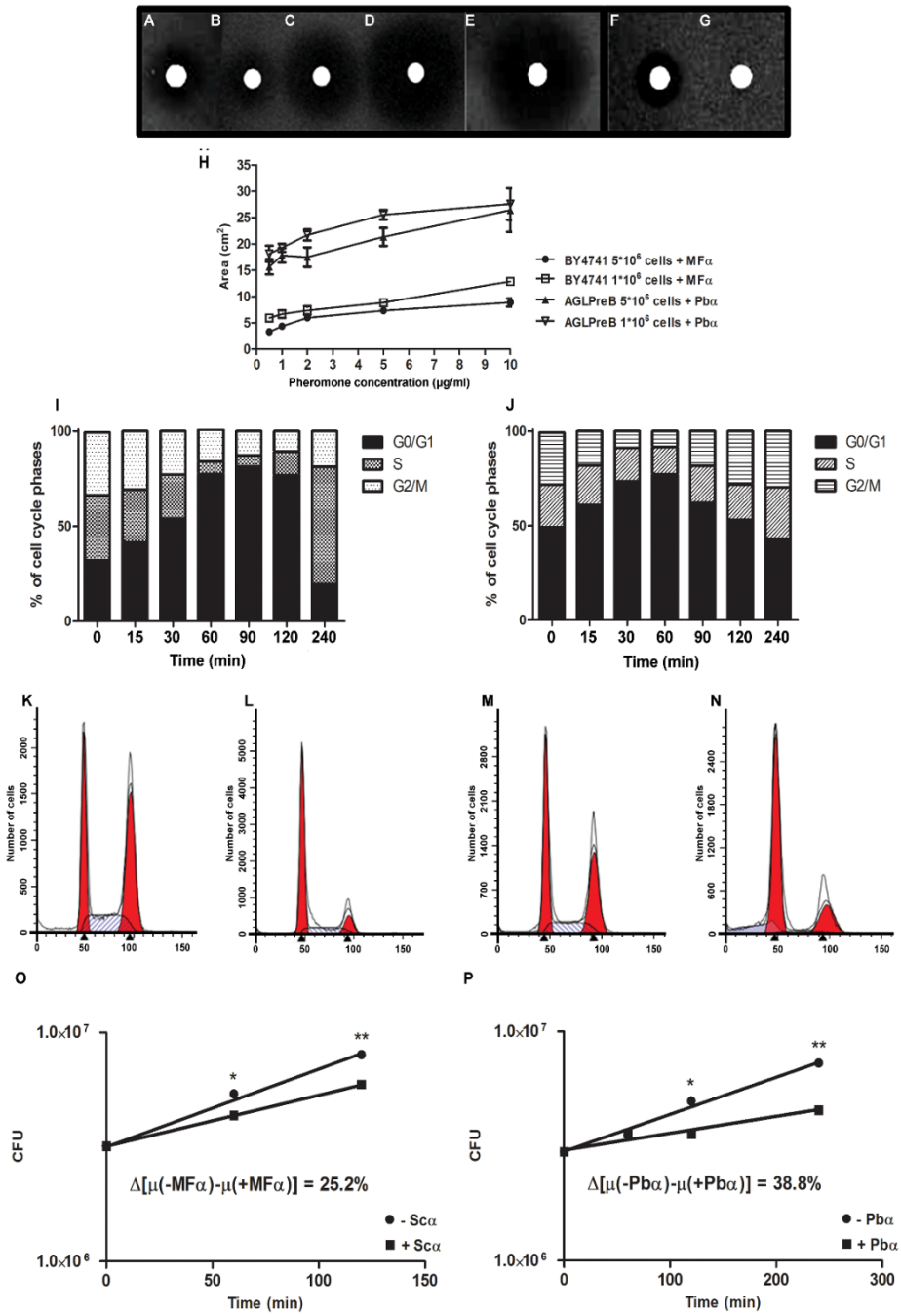


Figure 5 - α-pheromone activates PreB receptor, inducing growth and cell cycle arrest in the *S. cerevisiae* model. Halo formation in *S. cerevisiae* AGLPreB stimulated with Pbα pheromone extracted from *P. lutzii* Pb01 monoculture (A); stimulated with 0.8, 4.0 and 40 mg synthetic Pbα pheromone (B–D respectively) or Pbα pheromone extracted from *S. cerevisiae* AGMPba (E). *S. cerevisiae* BY4741 stimulated with 10 mg synthetic Mfα (F) and with 20 mg synthetic Pbα (G). Quantification of growth inhibition zones (cm^2) upon exposure to α-pheromones. Halos were recorded after 24 h of incubation and standard deviations indicated by error bars (H). Cell cycle arrest analysis for *S. cerevisiae* BY4741 upon addition of synthetic MFα (20 mg/mL) (I) and *S. cerevisiae* AGLPreB upon

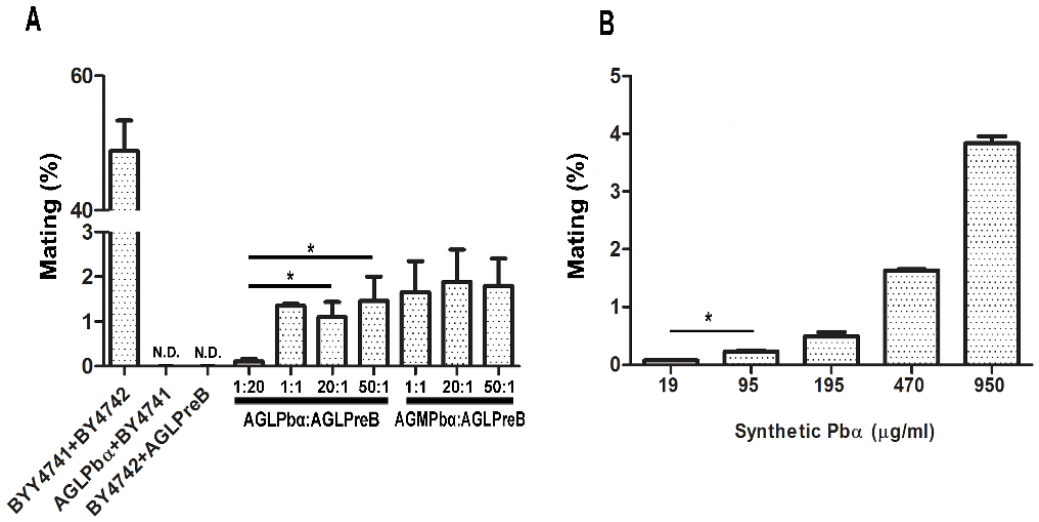


Figure 6 - α-pheromone and its cognate receptor functionally complement mating in the corresponding *S. cerevisiae* mutants. (A) Mating efficiency of *S. cerevisiae* null mutants, expressing Paracoccidioides α-pheromone (AGLPba and AGMPbα) and its cognate receptor (AGLPreB), crossed at different ratios after incubation for 5 h. (B) Mating efficiency after 5 h incubation of *S. cerevisiae* strains AGDScα and AGLPreB, upon the addition of different concentrations of *Paracoccidioides* synthetic α-pheromone. Error bars are indicated, and asterisks show significant differences(*p,0.05).

Mating efficiencies were, in general, relatively low for the heterologous mating pairs (below 10%), similar to those described for other heterologous systems expressed in *S. cerevisiae* (Gonçalves-Sá and Murray 2011), compared with 50% diploids observed for the control system (*S. cerevisiae* wild-type strains). These results, together with those obtained with mixtures of AGLPbα and AGLPreB at different cell number ratios, suggested that α pheromone was a limiting factor for mating (Figure 6A). To assess this, we evaluated mating following the addition of exogenous synthetic pheromone at different concentrations to the BY4742 and AGLPreB mating cross. The results obtained show a dose response effect, although the mating efficiencies are still low (Figure 6B). A similar assay for the heterologously expressed Ste2 homolog from *C. albicans* previously showed diploid formation of up to 80% (Janiak et al., 2005), although it has to be noted that the *C. albicans* homolog CaSte2 is more similar to Ste2 than PreB (36 vs 28% identity).

Discussion

In this work, we investigated the functionality of the sexual reproduction machinery of the genus *Paracoccidioides*. We demonstrate that the genomes of these fungi encode a functional α pheromone that is able to activate its cognate receptor PreB, but we also show that *P. brasiliensis* ATCC60855 and Pb03, both MAT1-2 strains, as well as the MAT1-1 strain *P. lutzii Pb01* are apparently insensitive to α-pheromone. This suggests that pheromone signaling is somehow blocked in these *Paracoccidioides* strains, probably due to low transcription levels of the upper components of the signaling pathway.

Notably, we could demonstrate that *P. lutzii* Pb01 expresses a functional α-pheromone. The identified mature pheromone sequence was shown to be identical to the one described in *H. capsulatum*, although the precursor was slightly different. In both *Paracoccidioides* and *Histoplasma,* the precursor peptide encodes only one copy of the mature pheromone, which is in contrast to the majority of ascomycetes that encode two or more copies of the mature pheromone in the precursor (Martin et al., 2011).

The predicted α-pheromone and its cognate receptor were individually overexpressed in *S. cerevisiae* null mutants of the homologous genes (constructed in strains of opposite mating types), and the functionality of this signaling pair was confirmed by their ability both to restore the hallmarks of pheromone signaling (shmoo induction and cell cycle and growth arrest) and to complement (at least partially) the mating deficiency of the host. Moreover, we investigated the specificity of the α-pheromone– receptor interaction that regulates the pheromone-sensing pathway. We demonstrate that there is considerable specificity in the interaction of Ste2 and PreB receptors with their cognate pheromone ligands. Thus, incubation of BY4741 and AGLPreB with Pbα and MFα synthetic pheromones respectively did not show evident shmoo formation, cell cycle and growth arrest, contrarily to what was observed when stimulation was performed with the corresponding pheromone. The observed pheromone specificity can be attributed to the differences in protein sequences and topology of Ste2 and PreB. In particular the N-terminal region and trans-membrane domain 1, which have been implicated in pheromone binding (Clark, Palzkill, and Botstein 1994), show low similarity between these receptors (Figure S2).

Functioning of the *Paracoccidioides* α-pheromone/α-pheromone receptor pair in the heterologous host was evident, although the observed mating efficiencies were low. Similar low mating efficiencies of heterologously expressed mating genes in *S. cerevisiae* models have been shown

in other studies (Janiak et al., 2005; Gonçalves-Sá and Murray 2011). Interestingly, also expression of endogenous pheromones and pheromone receptors leads to reduced mating efficiencies that are most probably related to the expression system used (Gonçalves-Sá and Murray 2011). In addition, the low identity between PreB and Ste2 (28%) might cause a less efficient interaction of the PreB receptor with the pheromone response pathway G-protein of the host. In fact, topology predictions for these α-pheromone receptors suggested that the third intracellular loop, important for the interaction of Ste2 with the G-protein, is different. Nevertheless, the known essential markers in the third intracellular loop needed for the activation of the intracellular mating pathway in *S. cerevisiae* are shared between Ste2 and PreB. In particular, the two essential amino acids R233 and F241 in Ste2 (Clark, Palzkill, and Botstein 1994) are also present and separated by the same number of amino acids in PreB (R223 and F231), and the triplet LGL is located at the same position in between these residues for both receptors (Figure S2). The topology prediction however places phenylalanine residue F231 into the transmembrane domain of PreB. Furthermore, the second loop in PreB is much longer than that of Ste2, which can also contribute to a general structural alteration of the intracellular receptor domain that culminates in a reduced signaling efficiency. Accordingly, *PbGPA1* was unable to rescue the mating ability of the corresponding *S. cerevisiae GPA1* null mutant. However, mating was rescued by simultaneous expression of both *PREB* and *PbGPA1* in a *S. cerevisiae* double mutant (Figure S3).

In the heterologous mating experiments, we could show that αpheromone was a limiting factor because higher concentration of pheromone resulted in increased mating efficiency. Even so, with the exogenous addition of the synthetic Pbα pheromone at a very high concentration, a mating efficiency of only 3.7% was achieved. It is likely that either the binding affinity of the ligand for the receptor or the interaction of the receptor with the signal transduction pathway, or both, contribute to the reduced pheromone bioactivity. Further studies are therefore needed, eventually using pheromone analogs, to evaluate their ability to activate the signaling machinery.

One of the most revealing aspects of the current work is the demonstration of the biochemical proficiency of the pheromone signaling machinery of *Paracoccidioides* genus, at the level of α pheromone and its receptor, while at the same time signaling seems to be impaired. In fact, our results also show that mating gene expression in *Paracoccidioides* species is very low, especially the upper components of the signaling cascade (*PREB*, *GPA1*, *STE4* and *STE18*). Moreover,

expression of mating related genes was not inducible through stimulation with synthetic *Paracoccidioides* α pheromone. This could suggest that due to the low basal gene expression the number of PreB receptor molecules present on the cell surface is too low to elicit a mating response, as well as the other components of the signaling pathway. Therefore, further research is underway to identify the environmental conditions that stimulate mating gene expression, as well as to detect low frequency mating events in this fungal pathogen via genetic approaches with dominant-marked strains. The confirmation of a sexual cycle in *Paracoccidioides* and the molecular characterization of its mating system are of major importance, since it will provide essential knowledge on basic biological and evolutionary aspects of these fungi. In addition, identification of sexual mechanisms will support genetic studies in this fungus and this information will contribute valuable new insights into the long-standing problem of sexuality/asexuality, which has not been fully elucidated in many fungi.

Supporting Information

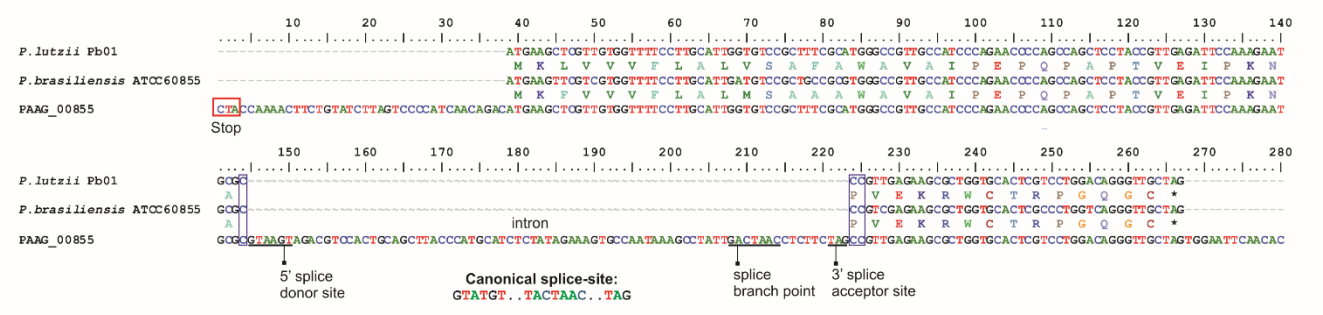


Figure S1 Alignment of a-pheromone cDNA sequences of *P. lutzii* Pb01 and *P. brasiliensis* ATCC60855 and partial genomic DNA sequence (minus strand) of the predicted ORF PAAG_00855 from *P. lutzii* Pb01. The location of the stop codon of PAAG_00855 is indicated by a red box. Nucleotides in the minus strand DNA sequence of PAAG_00855 obeying the canonical mRNA splicing motifs are underlined. Nucleotide translation to pheromone precursor is shown below the DNA sequence. Splicing of the 79 bp intron results in the proline (P) codon CCC detected in the cDNA (boxed positions).

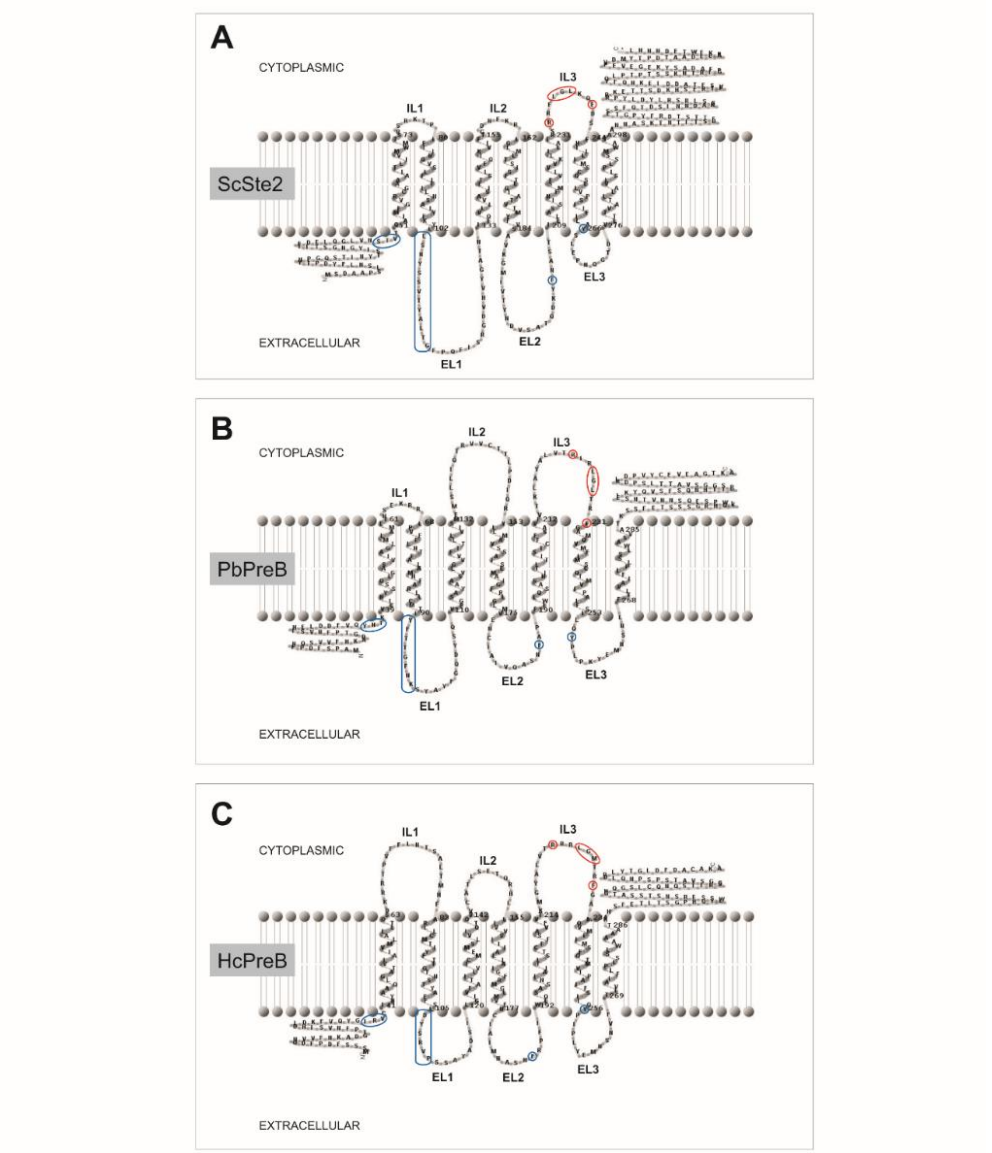


Figure S2 Topology of a-pheromone receptor Ste2 from *S. cerevisiae* (A), PreB from *P. lutzii* Pb01 (B), and PreB from *H. capsulatum* (C). Topology predictions were made using TMHMM Software and visualization done using the TMRPres2D tool. Essential residues for Gpa1 interaction of Ste2 in the third intracellular loop (IL3) are highlighted in red. Regions and residues involved in pheromone recognition and specificity of Ste2 are highlighted in blue. Corresponding regions and putative residues in PreB are indicated in similar colors.

A)

Strains/Plasmids	Genotype	Source
Strains		
BY4741 *ΔGPA1*	*MAT***a** *GPA1*::KanMX4	EUROSCARF
AG*ΔGPA1ΔSTE2*	*MAT***a** *GPA1*::hph; *STE2*::kanMX4	This study
AGLPbGpa1	*MAT***a** *GPA1*::hph pLPbGpa1	This study
AGLPbGpa1PreB	*MAT***a** *GPA1*::hph pLPbGpa1; *STE2*::kanMX4 pLPreB	This Study
Plasmids		
pLPbGpa1	pCM189::PbGPA1 Ampicilin^{R}/Histidine^{+}	This study

(B)

Mate pair[a]	% Mating[b]
BY4741 X BY4742	48.8 ± 4.53
BY4741 X AGLPbα	Not detected
AGLPreB X BY4742	Not detected
AGLPreB X AGLPbα	2.53 ± 0.87
AGLPbGpa1 X BY4742	Not detected
AGLPreBPbGpa1 X AGLPbα	2.92 ± 0.56

[a] Strains as described in (A)
[b] Percentage mating ± standard deviation

Figure S3 Mating efficiencies of *S. cerevisiae* null mutants heterologously expressing the *Paracoccidioides* a-pheromone receptor PreB and G-protein PbGpa1. Strains and plasmids used in mating assays (A). Mating efficiencies of mate pairs (B). Mating assays were performed as described in the Materials and Methods. PbGpa1 on its own is not able to restore mating in a *BY4741ΔGPA1* strain, but the *Paracoccidioides* α-pheromone receptor PreB and G-protein PbGpa1 collectively restore the mating ability in the corresponding *S. cerevisiae* double mutant.

Table S1 Primers used in this study. Sequences for homologous recombination (HR) in *S. cerevisiae* are indicated by shading. Start and stop codons are underlined.

Table S1. Primers used in this study. Sequences for homologous recombination (HR) in *S. cerevisiae* are indicated by shading. Start and stop codons are underlined.

Primer	Sequence 5´-3´	Target	Reference
Homologous recombination			
PREB-HR-Fw	CACTAAATTACCGGATCAATTCGGGATG GCACCCTCATTCGACCCCTTC	pCM189 / *PREB*	This study
PREB-HR-Rev	TCGATGTTAACAGGCCTGTTTAAACTCA GGCCTTTGTGCCAGCTTC	pCM189 / *PREB*	This study
Pbα-HR-Fw	CACTAAATTACCGGATCAATTCGGGATG AAGTTCGTCGTGGTTTTCCTTGC	pCM189-pCM190 / *PBα*	This study
Pbα-HR-Rev	TCGATGTTAACAGGCCTGTTTAAACCTA GCAACCCTGACCAGGGCGAGTG	pCM189-pCM190 / *PBα*	This study
MFα2-HR-HPH-Fw	GCTAGTGTTCACTTGCTCATTGATGTCC CGAGAAAGGAAGGGAAGAAAGCGAAAG GAG	*MFα2* flank / HPH	This study
MFα2-HR-HPH-Rev	GGCCAATTATTACTGCTAAAGATAAACTC CACAGGAAACAGCTATGACCATGATTAC	*MFα2* flank / HPH	This study
Gene replacement confirmation			
pCM-Conf-Fw	GCATGCATGTGCTCTGTATG	pCM189 / pCM190	This study
pCM-Conf-Rev	TTTCGGTTAGAGCGGATGTG	pCM189 / pCM190	This study
HPH-Conf-Fw	CGCAAGGAATCGGTCAATAC	HPH	This study
HPH-Conf-ver	AAAGCATCAGCTCATCGAGA	HPH	This study
MFα-Conf-ver	GTCCGAAAAATTGAAAGTC	*MFα2* flank	This study
Real-time PCR			
MAT11-RT-Fw	CATTCAGAAGCTTCTACTCTAC	*MAT1-1*	This study
MAT11-RT-Rev	CCTTCGCAAGGATTGCCCAC	*MAT1-1*	This study
MAT12-RT-Fw	AACGACATATCGATACTCCTTG	*MAT1-2*	This study
MAT12-RT-Rev	GATAGTAAGGGTGATCTTTG	*MAT1-2*	This study
Pbα-RT-Fw	GTCGTGGTTTTCCTTGCATTG	*Pbα*	This study
Pbα-RT-Rev	TCTCGACGGGCGCATTC	*Pbα*	This study
PREB-RT-Fw	GTGATCCCAGCCATCTTCTC	*PREB*	This study
PREB-RT-Rev	GTAGCCACTGAAGCCCATAG	*PREB*	This study
PREA-RT-Fw	TCCCCAAGAAACATCAGTCC	*PREA*	This study
PREA-RT-Rev	CATGACCATGCTAGAGGGATG	*PREA*	This study

Real-time PCR

Primer	Sequence 5´-3´	Target	Reference
GPA1-RT-Fw	ACTGAAGAGGGATAAGATG	*GPA1*	This study
GPA1-RT-Rev	CATAGCCTCGAGAATAAC	*GPA1*	This study
STE4-RT-Fw	CTGGGAAATCAGTACAAAC	*STE4*	This study
STE4-RT-Rev	ACCTTCTCTCCTCTTAGAAC	*STE4*	This study
STE18-RT-F1	CTATCCCACACCATTCTAG	*STE18*	This study
STE18-RT-R1	GTACGTTGAGCTCATTTAG	*STE18*	This study
STE20-RT-F1	TGGATATGATAACGAGAC	*STE20*	This study
STE20-RT-R1	TAATATCCACCATAGTCTGC	*STE20*	This study
STE11-RT-F1	CAGCTCACAAATCATCTAC	*STE11*	This study
STE11-RT-R1	CGTATACTATGTAGCCAATC	*STE11*	This study
STE50-RT-F1	GCCAAGAACTATCATCAC	*STE50*	This study
STE50-RT-R1	ACTCTTTAGAATCGTCAGTC	*STE50*	This study
STE7-RT-F1	GCCTATTCTTGATGACTTTG	*STE7*	This study
STE7-RT-R1	GTAGGATTTGCGGTTGTG	*STE7*	This study
KSS1-RT-F1	GACAACTCTGGATTTATGAC	*KSS1*	This study
KSS1-RT-R1	GATAAGGGTAAGTTGGTG	*KSS1*	This study
STE12-RT-Fw	AGAAACCTTCATGCTCTC	*STE12*	This study
STE12-RT-Rev	CAACTGGTGCAGATATAAG	*STE12*	This study
Tub2F	AGCCTTGCGTCGGAACATAG	*β-tubulin (TUB2)*	Marques *et al.*,, 2004
Tub2R	ACCTCCATCCAGGAACTCTTCA	*β-tubulin (TUB2)*	Marques *et al.*,, 2004

Author Contributions

Conceived and designed the experiments: FR MHJS PG MAC PL. Performed the experiments: JAGR AGGA JFM MHJS. Analyzed the data: FR MHJS PG MAC. Contributed reagents/materials/analysis tools: FR. Wrote the paper: FR MHJS PG MAC JAGR AGGA.

Chapter 3

The effect of light on gene expression of sexual machinery components from *Paracoccidioides* spp.

The results presented over this chapter were presented in conference proceedings:

Gomes-Rezende, J., Sturme, M., Rodrigues, F. "Effect of light on sexual gene expression sexual machinery in Paracoccidioides spp". Microbiotec'15 10-12 December. University of Évora. Oral communication.

Abstract

Paracoccidioidomycosis (PCM) is a systemic mycosis that is prevalent in Latin America. The etiological agent of PCM is the thermodimorphic fungus *Paracoccidioides*, comprised of the species *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii.* Sexual reproduction in this fungus was never uncovered in nature or under laboratory conditions, despite the presence of mating loci and all essential mating pheromone signaling pathway genes and the discovery of such a mode of reproduction in the closely-related phylogenetic genera *Histoplasma*, *Blastomyces* and *Aspergillus*. In *A. nidulans* and *A. fumigatus,* a particular group of light-responsive regulatory proteins, named Velvet proteins, play a crucial role in the sexual cycle in the absence of light. In addition, the negative regulator GprD and positive regulator NsdD play an important role during sexual reproduction. Homologues of these components are also present in the *Paracoccidioides* genus. In this study, we therefore analyzed the gene expression patterns of these genes in the presence and absence of light in different *Paracoccidioides* strains. We conclude that expression of the mating locus (*MAT*), the Velvet genes, and regulators *LAEA, GPRD* and *NSD,* occurs in a light-independent manner. We conclude that expression of the *MAT* locus, the Velvet genes, and regulators GPRD and NSDD, occurs in a light-independent manner. Based on the gene expression levels, we also propose a model of interaction of these sexual reproduction proteins in *Paracoccidioides* strains that may be uncovered by further functional protein analysis.

Introduction

Paracoccidioidomycosis (PCM), is a systemic mycosis that predominantly occurs in Latin America, with a high incidence in Colombia, Venezuela and Brazil (Brummer, Castaneda, and Restrepo 1993). The etiological agents of PCM are the dimorphic ascomycetes *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*. These fungi exist as a mycelial form at environmental temperatures (around 25°C) and shift to the yeast form at the mammalian host temperature (37°C) (Restrepo, McEwen, and Castañeda 2001). The infection in mammals occurs through inhalation of infective airborne conidia (asexual spores) or mycelial propagula from the environment, which switches into the pathogenic yeast form in the lungs and may disseminate to different organs, thereby generating a disseminated mycosis (McEwen et al., 1987). Many aspects of the biology of *Paracoccidioides* spp. are not yet elucidated, in particular whether this

fungus is capable of sexual reproduction. Sexual mature structures have not been observed in this fungus, neither in nature nor under laboratory conditions (Teixeira et al., 2013; Matute et al., 2006). However, genealogical analysis of *Paracoccidioides* isolates showed evidence for genetic recombination within and between lineages of the *Paracoccidioides* genus, indicative of sexual reproduction (Matute et al., 2006). Further evidence for the occurrence of a sexual cycle was derived from the identification of two idiomorphic mating-type genes (*MAT1-1* and *MAT1-2*) in *Paracoccidioides* isolates, that revealed high similarities to mating-type genes of other filamentous ascomycetes (Li et al., 2010; Torres et al., 2010; Desjardins et al., 2011). In addition, homologues of most of the components of the mitogen activated-protein kinase (MAPK) pathway - the pathway indispensable for pheromone responsive signaling during sexual reproduction, were described to be present in *Paracoccidioides* spp., with the exception of genes that express Ste5 and Far1 proteins (Desjardins et al., 2011; Gomes-Rezende et al., 2012). Moreover, a gene encoding the mating α-pheromone (*Pbα*) was discovered in *P. lutzii,* and its functionality in downstream activation of the MAPK signaling pathway was established using *Saccharomyces cerevisiae* as a heterologous expression host for *Paracoccidioides* spp. proteins (Gomes-Rezende et al., 2012).

During the last years, many phylogenetically related dimorphic fungi such as *Histoplasma capsulatum* (Webster and Sil 2008)*, Aspergillus fumigatus* (Bayram et al., 2010)*, Aspergillus nidulans* (Szewczyk and Krappmann 2010) and *Candida albicans* (Dyer and O'Gorman 2012; Park et al., 2012), that were once considered to reproduce asexually, were shown to possess a sexual cycle. The sexual cycle in *A. fumigatus* and *A. nidulans* occurs only under specific conditions, of which one of the most intriguing is the absence of light. Sexual development in *A. nidulans* creates spherical closed fruiting bodies named cleistothecia, where meiotic spores are produced. The mature fruiting bodies are surrounded by a layer of globose Hülle cells that provide protection and nutrients for the fruiting bodies (Osiewacz 2002). After the discovery of a sexual cycle in *A. fumigatus* and *A. nidulans*, many regulators involved in this event have been studied in order to clarify the mechanisms for the development of sexual structures. Recently, the effect of light on the sexual cycle of *A. fumigatus* and *A. nidulans* has been explored in detail. In this sense, a complex network of specific regulators for activation of sexual reproduction and secondary metabolism have been studied and their activity was shown to be associated with the absence of light in *A. fumigatus* and *A. nidulans* (Szewczyk and Krappmann 2010; Bayram,

Biesemann, et al., 2008; Bayram, Krappmann, et al., 2008; Blumenstein et al., 2005; Bok and Keller 2004; Purschwitz et al., 2008).

In fungi light can be detected by several light receptors: blue light is detected by receptors LreA-LreB and CryA (Bayram, Biesemann, et al., 2008; Purschwitz et al., 2008) while red light is detected by FphA (Blumenstein et al., 2005). The light receptor CryA is a negative regulator of transcription of sexual development genes through its interaction with other nuclear proteins and transcriptional factors, or by binding directly to the DNA, thereby repressing expression of genes essential to sexual development (Bayram, Krappmann, et al., 2008).

Another complex of proteins involved in light-sensing is the Velvet complex that plays an essential role downstream in the light-sensing regulation. The activity of the Velvet proteins (VeA, VelB, VelC and VosA) is stimulated by the absence of light and fundamental for the activation of sexual development (Dyer and O'Gorman 2012; Kim et al., 2002). Overall, the formation of Velvet complexes between Velvet proteins is due to three specific motifs that are characteristic of this type of proteins (Figure 1), and the *Velvet* domain is well conserved among filamentous and dimorphic fungi (Webster and Sil 2008; Szewczyk and Krappmann 2010).

The formation of the VeA-VelB complex occurs in the cytoplasm and is the major path that leads to VelB entry into the nucleus in the absence of light (Bayram et al., 2010; Park et al., 2012). VelB is a primary component of the velvet complex and plays an essential role in secondary metabolism and sexual development. VelB interacts with VosA, which is necessary for the formation of trehalose in sexual spores and enhances their viability (Park et al., 2012). The expression of *VELB* and *VOSA* are under control of AbaA, a transcriptional activator that binds directly to *VELB* and *VOSA* promoters (Park et al., 2012). The transport of the Velvet protein complexes into the nucleus is mediated by the α-importin KapA, which is expressed and functional in a light-independent manner (Park et al., 2012). The global regulator of the secondary metabolism, LaeA interacts with VeA, VelB and VelC (Bayram, Krappmann, et al., 2008; Park et al., 2014), controlling the VelB complex allocation between VosA-VelB and VeA-VelB, as well as the levels of VelB and VosA in a light dependent manner (Bayram et al., 2010). These interactions are essential during the shift from filamentous cells to Hülle cells surrounding cleistothecia, being key factors in light-controlled fungal development.

In *H. capsulatum*, orthologues of VeA, VosA and VelB genes have been identified as Ryp1, Ryp2 and Ryp3 respectively (Webster and Sil 2008). The regulators Ryp1, Ryp2, and Ryp3 are required

for growth in the yeast-phase and Ryp1 is also involved in the regulation of asexual and sexual development and secondary metabolism (Webster and Sil 2008; Nguyen and Sil 2008; Laskowski-Peak et al., 2012).

NsdD is a GATA-type transcriptional factor that has been described as a positive regulator of sexual development in *Aspergillus* spp. (Laskowski-Peak et al., 2012). There is evidence that NsdD is involved in the early stages of the sexual development where it might present a regulatory role in cell wall integrity and in hyphal anastomosis in early mating phases in the dark (Szewczyk and Krappmann 2010).

GprD is a heterotrimeric G-protein that negatively controls/impairs downstream signaling via the MAPK signaling pathway after recognition of **a** and α pheromones by their specific receptors in *Aspergillus* spp. (Han, Seo and Yu 2004; Seo, Han and Yu 2004). Upstream control by GprD over the MAPK signaling does not always lead to impairment of the signaling cascade activity and sexual development, as there are other positive sexual development regulatory proteins involved as well. The balance between both negative and positive regulators present determines the final outcome for asexual or sexual development in *Aspergillus* spp. (Seo, Han and Yu 2004). There is no evidence that GprD activity is determined by the presence or absence of light (Dyer and O'Gorman 2012).

Paracoccidioides strains harbor homologous genes of all the above-mentioned Velvet, LaeA, GprD, and NsdD proteins, and other mating-related genes, except for the *CRYA* light receptor. Most of the activities, interaction and pathways are described in *Aspergillus* spp., however, due to the lack of specifics antibodies to analyze the protein expression, in this work we evaluated the expression levels of mating loci *MAT1-1* and *MAT1-2*, *KAPA*, the Velvet genes *VEA, VELB*, *VELC,* and *VOSA* and regulators *LAEA, NSD* and *GPRD* in the presence and absence of light in *Paracoccidioides* spp.. Through this analysis a correlation between their gene expression and their role under the conditions tested was evaluated. This analysis is expected to give new insights for genetic factors that constrain the presence of an active sexual reproduction cycle in *Paracoccidioides* spp..

Materials and methods

Strains and culture conditions

Paracoccidioides wild-type strains used in this study are listed in Table 1. The strains were maintained in the yeast-form at 37°C by periodic subculturing on brain heart infusion (BHI) solid medium supplemented with 1% (w/v) glucose. For the yeast-to-mycelium conversion, the yeast cells were inoculated in McVeigh Morton (MMcM) synthetic medium (Restrepo and Jimenez 1980), and grown at 24°C, 150 rpm. Once converted, the cultures were centrifuged and 5 mL of each strain were transferred to 250 mL shake flasks containing 50 mL of fresh MMVM medium. Cultures were grown for 25 days in the presence or absence of light, and samples for RNA extraction collected during this period. The samples from the cultures growing in the dark were collected in the absence of light. At each collection point, the cultures were refreshed with the same volume that was collected for RNA extraction. In order to test whether velvet proteins are involved in the transition between mycelia and yeast form, the cultures were cultivated in the presence of light and samples for RNA extraction were collected during this period.

Table 1: *Paracoccidioides* strains used in this study.

Strain	Mating type (phylogenetic group)	Source
P. lutzii Pb01	MAT1-1 (Pb01)[a]	Chronic PCM / Goiás - Brazil
P. brasiliensis Pb18	MAT1-2 (S1)[a]	Chronic PCM / Goiás - Brazil
P. brasiliensis ATCC60855	MAT1-2 (PS3) [a]	Sputum/Colombia

a– Phylogenetic species as defined by Matute et al., 2006 and Teixeira et al., 2009.

Identification of *Paracoccidioides* light-responsive mating genes

Identification of *Paracoccidioides* spp. light-responsive mating possess homologues of the *A. nidulans* velvet genes (Table 2), was performed using BLAST searches against a genome database of isolates Pb01 and Pb18, available at the Broad Institute (http://www.broadinstitute.org/). In order to identify the *Paracoccidioides* homologous genes we performed TBLASTN searches against *Paracoccidioides* transcripts and genomic sequences (E-value cut-off 1e-3) using the precursor protein sequences of *A. nidulans* available in the Aspergillus Genome Database (http://www.aspergillusgenome.org/). The homologues analyzed in this study and their annotation numbers are listed in Table 2. The three specific regions that

are important for the interaction with the other velvet proteins in complex formation are also present in the *Paracoccidioides* homologues.

Table 2. Genes from *A. nidulans* and their homologues in *Paracoccidioides* spp. analyzed in this study.

	Gene Annotation numbers		
Gene	*A. Nidulans*	Pb01	Pb18
KAPA	AN2142	PAAG_06657	PADG_01114
LAEA	AN0807	PAAG_05395	PADG_06744.2
VELB	AN0363	PAAG_06081.2	PADG_08037.2
VOSA	AN1959	PAAG_02671	PADG_01695
VEA	AN1052	PAAG_03070.2	PADG_02949.2
VELC	AN2059	PAAG_08283.2	PADG_11359.2

Quantitative real-time polymerase chain reaction (qRT-PCR)

For basal expression analysis, mycelial cultures exposed to or protected from light, were collected at 7, 14 and 25 d of incubation. Total RNA was extracted from mycelial cells using a Trizol (Invitrogen) standard method for cellular disruption, complemented with heat shock treatment (10 min at 65°C followed by 60 min at -80°C) and bead-beating using 0.1 mm silica/zirconium beads. Total RNA was treated with DNase I (Ambion) and DNA-free total RNA (1 µg) reverse transcribed using the NZY First-Strand cDNA Synthesis Kit (NZYTech). The cDNA samples were used as templates in order to measure the basal level expression of the *MAT1-1*, *MAT1-2*, *KAPA*, *LAEA*, *VOSA*, *VEA*, *VELB*, and *VELC* genes in the mycelium form and the Velvet genes during transition. Quantitative RT-PCR (qRT-PCR) was performed on the CFX96 Real-Time PCR Detection System (Bio-Rad), and qRT-PCR amplification performed using the Sso Fast EvaGreen Supermix kit (Bio-Rad), according to the manufacturer's protocol. For all samples, DNA contamination was absent, as the use of isolated RNA only as a template gave no amplification in qRT-PCR. The thermal cycling conditions were: initial denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s and primer annealing and elongation for 5 s at the appropriate temperature. After PCR cycling, melt-curves were obtained from 65–95°C at 0.5 °C increments. All measurements were performed in triplicate and relative expression levels determined using the

ΔCT method (Livak and Schmittgen 2001), with the expression of the ribosomal protein encoded by *L34* gene as a reference (Livak and Schmittgen 2001).

Table 3. Primers used in this study for qt-PCR.

Primer	Sequence 5' 3'	Target Gene
FW VELB	GGATCTCAGTGTCCGCACAGAAGG	*VELB*
RV VELB	GGCCAGAACGGGTGCGGATCC	*VELB*
FW VELC	GCGACTTGGTTCTAAAGGACGG	*VELC*
RV VELC	GGCACGAGCTGCTATAGGTTGC	*VELC*
FW LAEA	CCCATAGAAAAGGCGCCTTCCC	*LAEA*
RV LAEA	GGCGAAACTCGTGATACTGCC	*LAEA*
FW VEA	CGAGACCGAAAATAGTGTGTCTCG	*VEA*
RV VEA	CCACAGGAGGTGGGTCAACTGGTCG	*VEA*
FW VOSA	GCGCCACGACATATCCTGAGG	*VOSA*
RV VOSA	GGATAATAGGTGGAGGATCTACC	*VOSA*
FW KAPA	CGTGATGGAGGCATCGGTGTAGG	*KAPA*
RV KAPA	CGATAGGAGGGTTCCGTTCTTTGG	*KAPA*
FW GPRD	GGCATGTGTGTGTGGTGTTTGGGCG	*GPRD*
RV GPRD	GTGTGTCCACAACCTGTCCG	*GPRD*
FW NSDD	GGTGGTGGAGGCGGTTTTG	*NSDD*
RV NSDD	CTGCTGCAGGCACAGATCG	*NSDD*

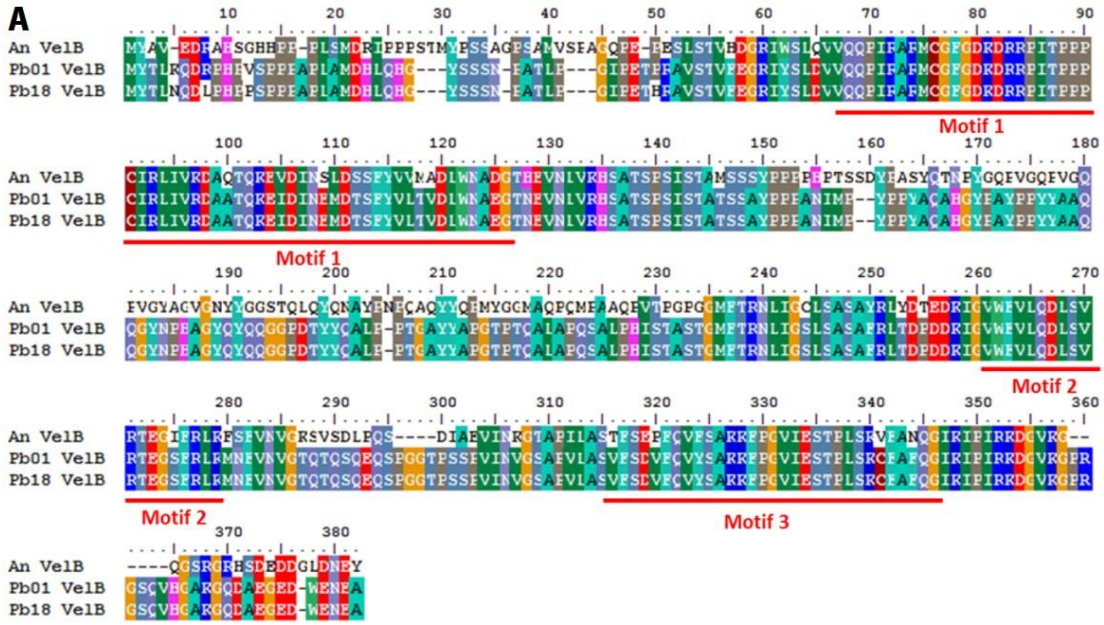
Statistical Analysis

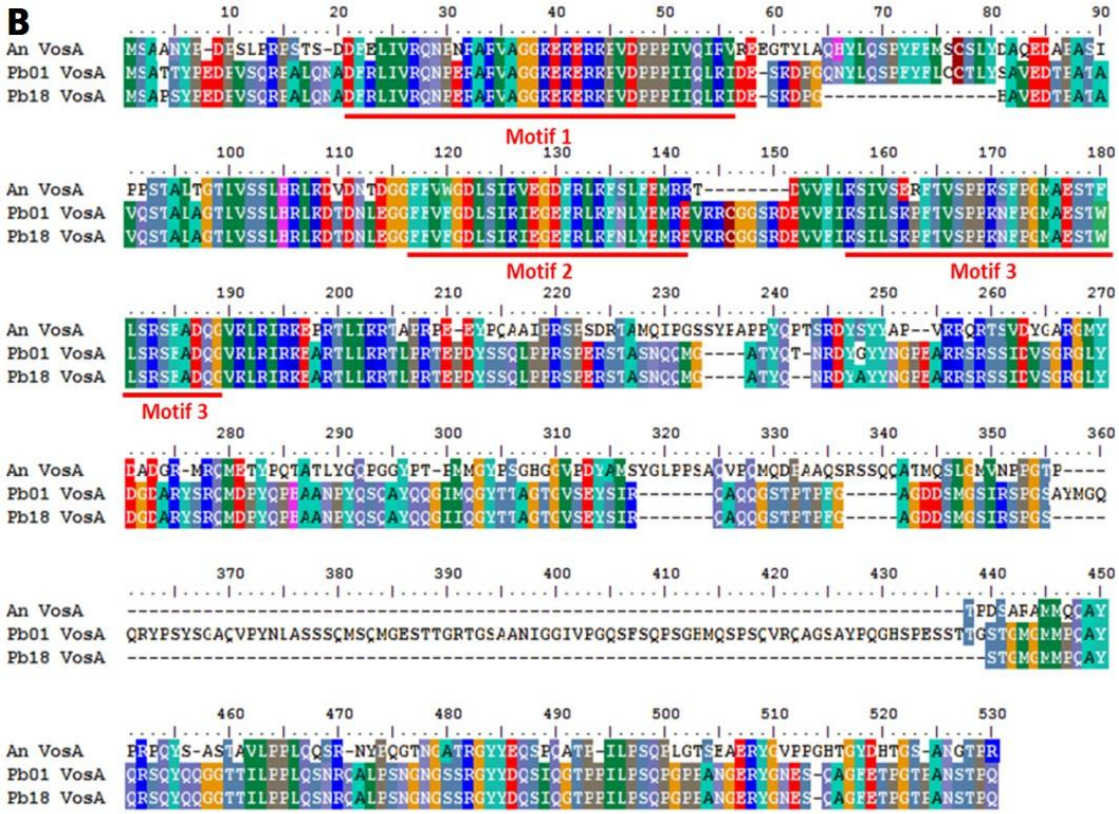
Data are reported as the mean standard error of the mean of at least four independent repetitions of each assay. The statistical analyses were performed using the SPSS program version 19 and Graph Pad Prism 5 software. The statistical analysis used was One way Anova.

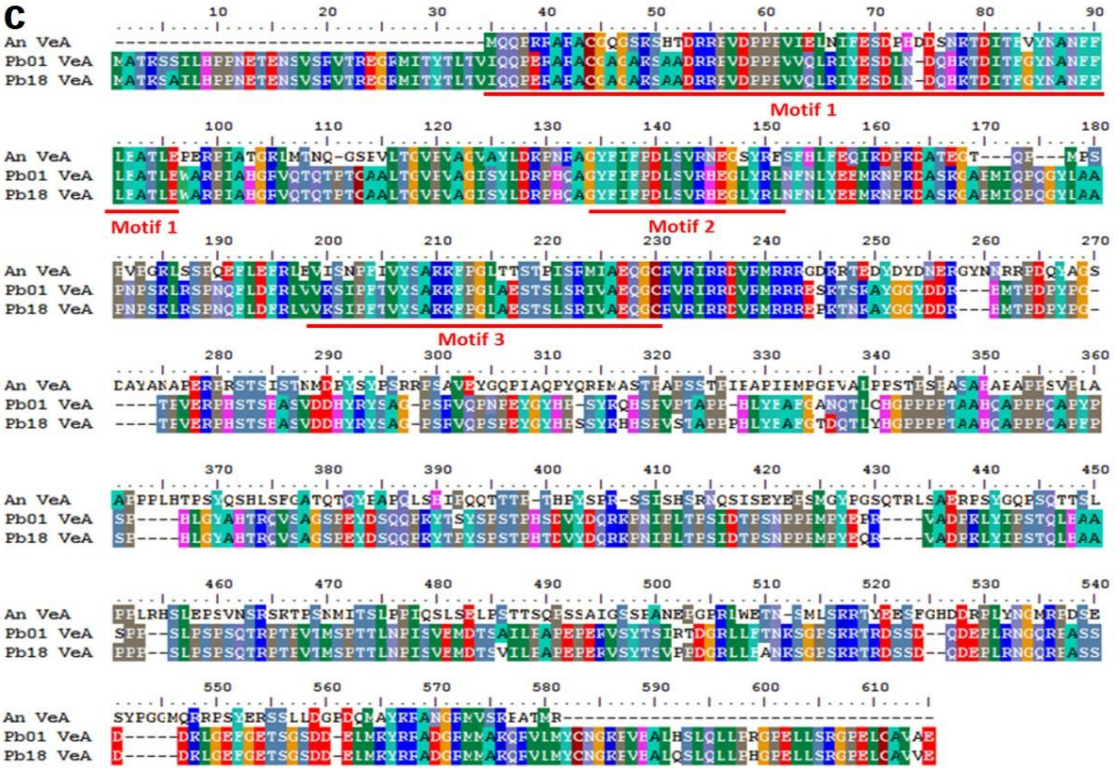
Results

Paracoccidioides Velvet proteins

The *in silico* analysis of the velvet genes and proteins from *A. nidulans* compered to *P. brasiliensis* strains Pb01 and Pb18 resulted in homology of the proteins, and demonstrated the presence of the velvet domains in *Paracoccidioides* velvet proteins, that are responsible for the interaction and formation of velvet proteins complexes (Figure 1).




B
An VosA
Pb01 VosA
Pb18 VosA
Motif 1
Motif 2
Motif 3
Motif 3


C
An VeA
Pb01 VeA
Pb18 VeA
Motif 1
Motif 1
Motif 2
Motif 3

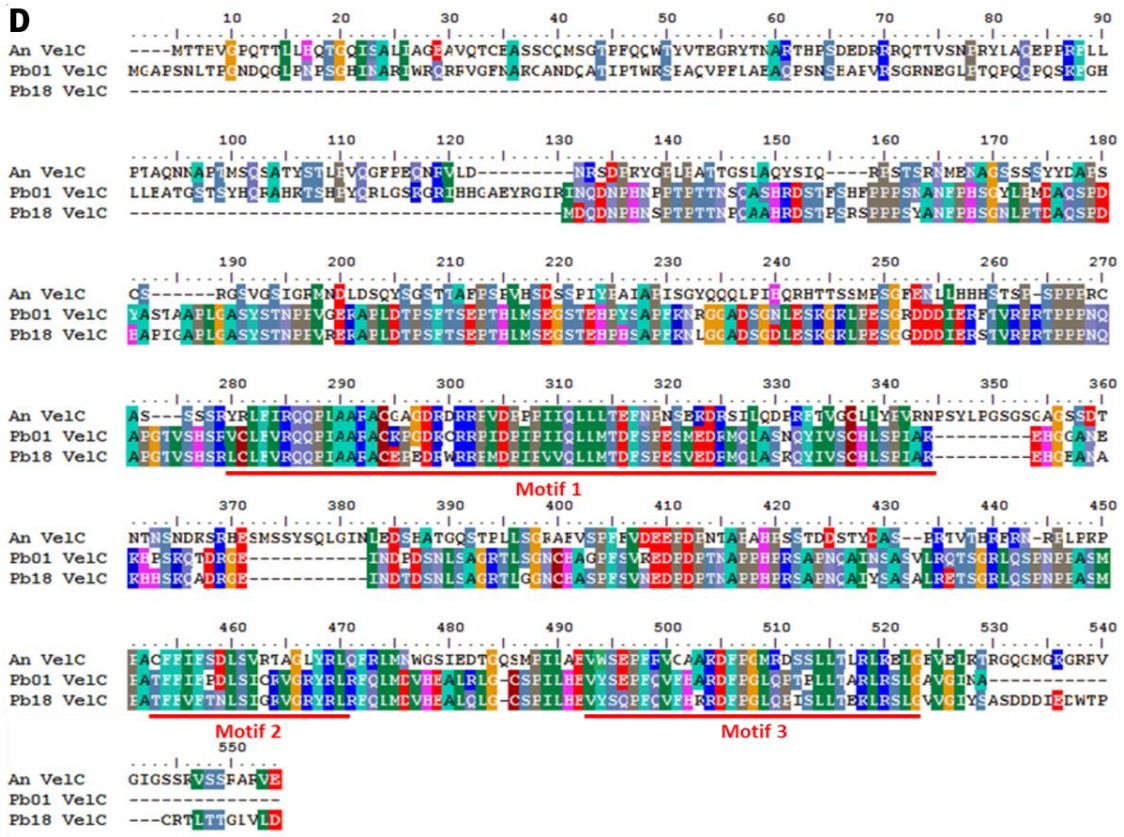


Figure 1 - Velvet proteins alignment. VelB (A), VosA (B), VeA(C) and VelC (D) proteins alignment between velvet proteins from *A. nidulans* and their homologues from the sequenced *Paracoccidioides* strains Pb01 and Pb18.

MAT locus expression is independent of light

Studies have shown that sexual events in filamentous fungi phylogenetically related to *Paracoccidioides* spp. occur in the absence of light. Since sexual reproduction in fungi is dependent on the activity of the *MAT* locus, gene expression of this gene in *Paracoccidioides* spp. under the absence or presence of light was evaluated. *MAT* locus expression (*MAT1-1* and *MAT1-2*) was quantified in the cultures of the strains Pb01, Pb18 and ATCC60855 (Figure 2A, B and C, respectively). The expression level of *MAT1-2* in strains Pb18 (Figure 2B) and ATCC60855 (Figure 1C) showed no differences between light and dark conditions. In *P. lutzii* Pb01, differences in *MAT1-1* expression were observed between light and dark conditions at 7 and 14 d.

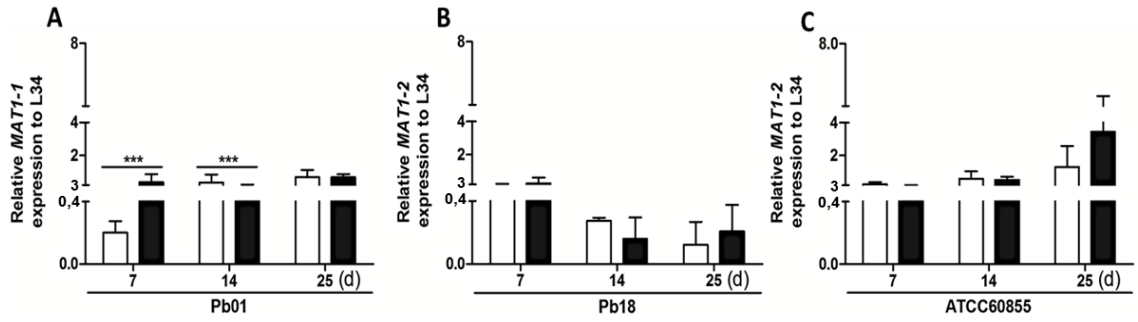


Figure 2: *MAT* locus expression in *Paracoccidioides* mycelium cultures. Relative expression of *MAT1-1* in *P. lutzii* Pb01 culture (A), *MAT1-2* in ATCC60855 (B) and *MAT1-2* in Pb18 (C). White bars represent cultures exposed to light. Black bars represent cultures grown in the dark. (***p<0.001).

GPRD and *NSDD* expression in the presence of light is variant in *Paracoccidioides* strains

GPRD expression and functionality are independent of light (Dyer and O'Gorman 2012; Han, Seo, and Yu 2004). GprD downregulates activity of key positive regulators of sexual reproduction at many levels, while NsdD is a positive regulator and inductor of the fungi sexual reproduction. The expression levels of this gene in the strains tested shows a correlation between gene expression level and presence or absence of light along the course of the experiment. *GPRD* expression level in *P. lutzii* Pb01 is higher in the presence of light at 7 and 14 d of the experiment (Figure 3A), and the same pattern is also observed in *P. brasiliensis* Pb18 strain at 7 and 14 days (Figure 3C). There are no differences between *GPRD* expression in the light and dark in strain ATCC60855 in all time points tested (Figure 3E). *NSDD* expression in Pb01 is higher in the presence of light at 7 and 14 days, however, at 25 days *NSDD* expression is higher in the dark (Figure 3B). In Pb18, *NSDD* expression presents similar levels between light and dark at 7 and 14 days, but at 25 days the expression increases significantly in the dark (Figure 3D). ATCC60855 strain at 7 and 14 days, presents no differences in expression between light and dark, however at 25 days *NSDD* expression is lower in the dark (Figure 3F). When comparing expression levels of *GPRD* and *NSDD* (Figure 3) it is possible to affirm that the pattern of *GPRD* and *NSDD* expression is similar in strains Pb01 and ATCC60855, however this does not occur with Pb18 strains.

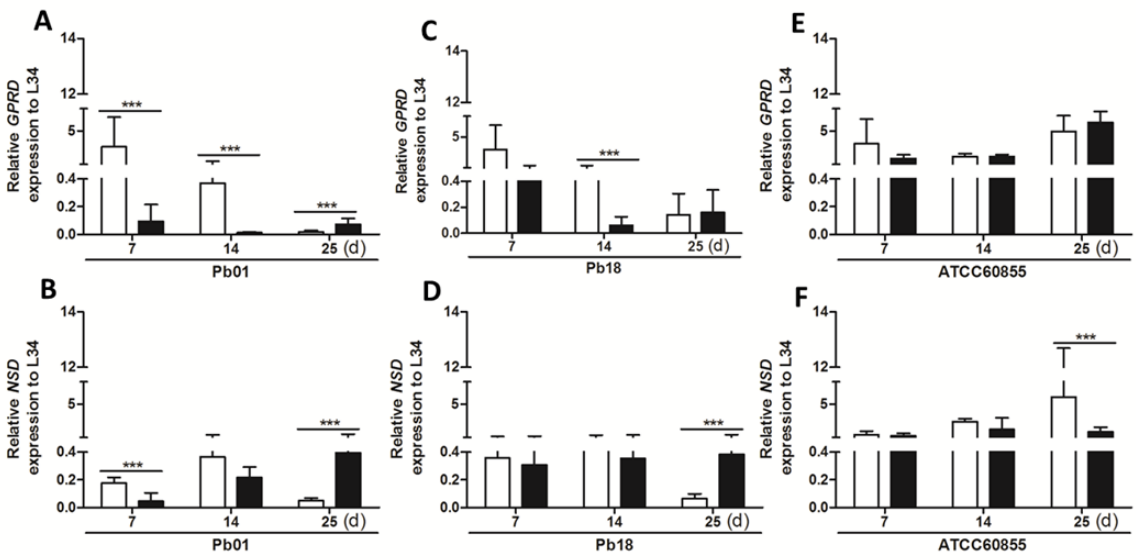


Figure 3: *NSDD* and *GPRD* expression in *Paracoccidioides* mycelium cultures. Relative *GPRD* and *NSDD* expression in *P. lutzii* Pb01 (A-B), Pb18 (C-D) and ATCC60855 (E-F), respectively. White bars represent cultures exposed to light. Black bars represent cultures grown in the dark (***p<0.001).

LAEA and *KAPA* gene expression is independent of light

In *A. nidulans*, LaeA is a global regulator of secondary metabolism and sexual development, and responsible for the amount of VelB and VeA available in the cytoplasm (Bayram et al., 2010). Our gene expression analysis shows that contrarily to what is described in the literature for other systems (Bok and Keller 2004), the expression of *LAEA* is not affected by the presence or absence of light (Figure 4 A-C). The pattern of *LAEA* expression observed in Pb01, Pb18 and ATCC60855 (Figure 4A, B and C, respectively), shows similar expression levels for all time points in the presence or absence of light, except for Pb01 at 7 days (Figure 4A). No statistically significant differences were found in the expression levels (Figure 4).

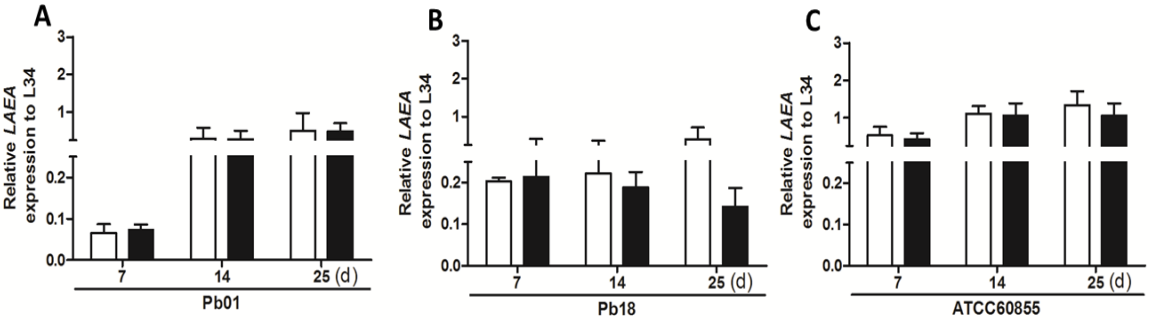


Figure 4: *LAEA* expression in *Paracoccidioides* mycelium cultures. Relative *LAEA* expression in *P. lutzii* Pb01 (A), *P. brasiliensis* Pb18 (B) and ATCC60855 (C). White bars represent cultures exposed to light. Black bars represent cultures grown in the dark.

Since the α-importin KapA plays an essential role in transporting the velvet complex into the nucleus in order to interact with LaeA, and its gene expression is independent of the presence of light (Bok and Keller 2004), we tested whether this also is the case in *Paracoccidioides* spp.. Our results show that *KAPA* is expressed at high levels in *Paracoccidioides* strain, but the expression pattern is different between strains (Figure 5). Differences in *KAPA* expression between light and dark are observed for the strains Pb01 and ATCC60855 (Figure 5A and 4C), *KAPA* expression is mainly higher in the dark for Pb01, while in ATCC60855 for the same time points this gene expression is higher in the light. At 7 and 14 days, the strain Pb18 presents no significant differences between light and dark, however, at 25 days *KAPA* expression is higher in the presence of light. The expression levels in most of the strains and time points tested are statistically significant between light and dark conditions, however it is not possible to affirm if the presence of light in general induces or inhibits its expression.

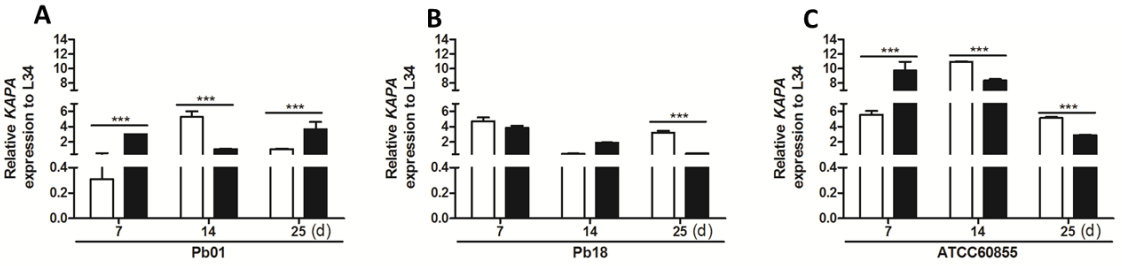


Figure 5: *KAPA* expression in *Paracoccidioides* mycelium cultures. Relative *KAPA* expression in *P. lutzii* Pb01 (A), Pb18 (B) and ATCC60855 (C), respectively. White bars represent cultures exposed to light. Black bars represent cultures grown in the dark (***p<0.001).

Expression of Velvet genes is independent of light

The light-sensitive Velvet proteins are essential for the formation of sexual structures in *Aspergillus* species. These proteins can form complexes in the cytoplasm which are then transported into the nucleus, where they can interact with LaeA, a process that is well characterized in *A. nidulans* (Bayram et al., 2010; Park et al., 2012; Bayram, Krappmann, et al., 2008; Bok and Keller 2004; Braus et al., 2002). In *Paracoccidioides* spp., the function of VeA, VelB, VelC and VosA is to activate the sexual development stimulated by the absence of light (Dyer and O'Gorman 2012). We observed that expression of the Velvet genes in *Paracoccidioides*

spp. is not affected by light, and might be similar to what happens in *H. capsulatum* where they are active during phase transition (data not shown) (Nguyen and Sil 2008). As presented in Figure 6, it is clear that the expression levels of velvet genes are similar whether light is present or not. Another possible explanation is that all genes expression changes occur on a shorter time scale, below 48 hours as described in Bayram, 2016. There is an evident pattern in *VELB* expression between light and dark, time points and in the different strains tested (Figure 6A, B, and C), with similar expression levels in all cases, except for Pb01 in which *VELB* is highly expressed in the dark. *VOSA* expression in *P. lutzii* (Pb01) is almost null in light and dark at 7 and 14 days (Figure 6D). However, after 25 days of experiment, the *VOSA* expression increases and it is significantly higher in the dark (Figure 6D). In Pb18 strain, *VOSA* expression along the time of the experiment is similar between light and dark, however its expression is higher at 7 days, and at the 14 and 25 days, expression decreases to nearly zero (Figure 6E). In strain ATCC60855, the expression levels in light or dark are similar over time, however after 7 days, expression levels increase substantially (Figure 6F). *VEA* expression in strain Pb01 (Figure 6G) is constant at 7 and 14 days and no differences were observed in expression

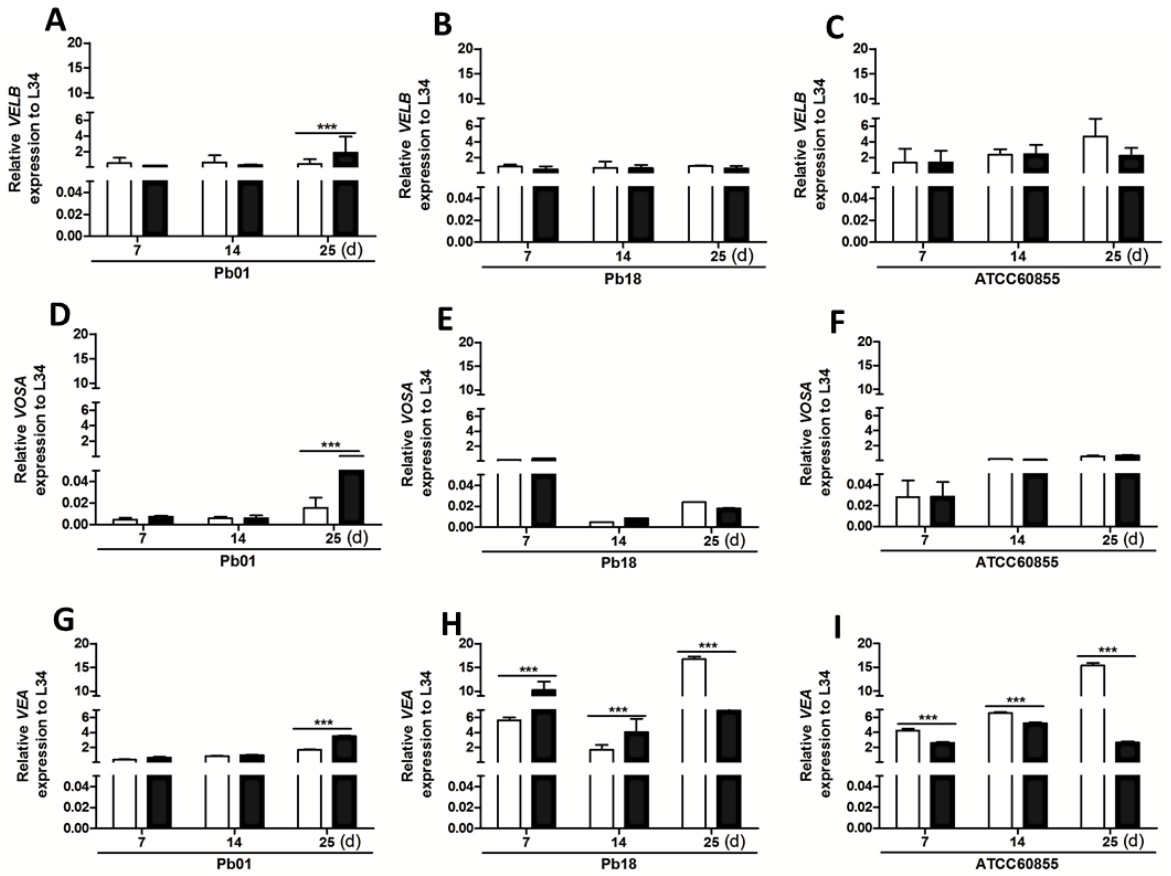


Figure 6: *VELB, VOSA and VEA* expression in *Paracoccidioides* mycelium/strains. Relative *VELB* expression in *P. lutzii* Pb01 (A), Pb18 (B) and ATCC60855 (C). Relative *VOSA* expression in Pb01 (D), Pb18 (E) and ATCC60855 (F). Relative *VEA* expression in Pb01 (G), Pb18 (H) and ATCC60855 (I). White bars represent cultures exposed to light. Black bars represent cultures grown in the dark. (***p<0.001).

between light and dark, but at 25 days *VEA* expression increases in the absence of light. At 7 and 14 days of the experiment, Pb18 presents a higher expression of *VEA* in the dark, however, at 25 days this pattern is reversed (Figure 6H). In strain ATCC60855, *VEA* expression is higher in the presence of light in all time points tested (Figure 6I). It is essential to mention that *Paracoccidioides* spp. also harbors a homologue for *VELC*, however expression of this gene was not observed in any of the conditions tested (data not shown).

Discussion

Since the discovery of sexual reproduction in *A. fumigatus* (Szewczyk and Krappmann 2010) and *A. nidulans* (Osiewacz 2002), several studies have addressed the molecular mechanisms involved, thereby highlighting aspects of the mating signaling pathways (Kim et al., 2009), mode of action of negative and positive regulators of sexual development (Bayram et al., 2010; Park et al., 2012; Osiewacz 2002), and the effect of light (Matute et al., 2006; Teixeira et al., 2009). A key finding was the essential role of the Velvet proteins in this event (Webster and Sil 2008).

While *Paracoccidioides* strains harbor all the genetic components for sexual reproduction (Purschwitz et al., 2008; Gomes-Rezende et al., 2012), for which the functionality of some has been confirmed (Park et al., 2014), a sexual cycle in *Paracoccidioides* spp. could not yet be confirmed. As *Paracoccidioides* spp. also encode homologues of the light-responsive Velvet proteins (Table 2; Figure 1), as well as other regulatory proteins implicated in sexual reproduction, we set out to study expression of these genes under the presence/absence of light. The role played by Velvet in *Paracoccidioides* spp., based on their expression may resemble the role of the Velvet genes (and proteins) *VEA* (*RYP1*) and *VOSA* (*RYP2*) in the related fungus *H. capsulatum*, based on their expression. However, is not possible to affirm that the Velvet proteins expression in *Paracoccidioides* spp. is not triggered by temperature variations. Our results showed that expression levels of the *MAT1-1* and *MAT1-2* are not influenced by the presence or absence of light, while in *A. nidulans*, *MATB* expression is also dependent of the absence of light (Bayram et al., 2016). In Gomes-Rezende et al.,, we proposed that *Paracoccidioides* strains apparently are insensitive to the presence of sexual pheromones and that the pathway for activation of *MAT* locus expression is somehow blocked. Therefore, it might be that also light-effects are indirectly blocked the sexual development in *Paracoccidioides* spp.

Several models of protein interaction in the regulation of the development of sexual reproduction have been proposed in the literature for *A. nidulans*. The models represented the impairment of this process by the negative regulator GprD, the activation of this process in the dark supported by the positive regulator NsdD (Han et al., 2001), and the involvement of the velvet proteins in the absence of light (Bayram et al., 2010; Park et al., 2012; Bayram, Krappmann, et al., 2008; Bok and Keller 2004; Kim et al., 2009; Han, Seo, and Yu 2004). Herein we analyzed several key genes involved in this process at expression level (*MAT1-1*, *MAT1-2*, *LAEA*, *KAPA*, *VELB*, *VOSA*,

VEA and *VELC*) in *Paracoccidioides* and we propose a hypothetical model of interaction based on the results that we obtained (Figure 7).

We were able to demonstrate the expression of all genes tested, except for *VELC*, and the expression results obtained may indicate that these components play different roles when compared to *A. nidulans*. Keeping in mind that in *Paracoccidioides* expression of these genes was not affected by the presence of light and no evident pattern of expression between light and dark was detected, we propose the following model to interpret the findings based on the expression response obtained in this work (Figure 7). According to the literature, the coordination of sexual reproduction is accomplished by the formation of velvet protein complexes, *MAT* loci expression and the activation of NsdD, the positive regulator of mating. The lack of alpha pheromone response in the MAPK signaling, upon Pbα stimulus (Gomes-Rezende et al., 2012), at this point can be blocked by the interference of GprD. As well, GprD may also be blocking the positive effect of NsdD effect on the start of sexual development.

As the model proposed in Figure 7, besides the recognition of the pheromone by its receptor, the triggering of the MAPK signaling pathway may be blocked by the blocking function of the coupled G-Protein GprD on the MAPK signaling cascade. The involvement of NsdD, a positive sexual development regulator (Park et al., 2012; Osiewacz 2002) in early stages of sexual reproduction is impaired by the presence of light. However, our results show no relation between *NSDD* gene expression and the presence or absence of light in the *Paracoccidioides* strains, differing from the profile observed in *A. nidulans* (Osiewacz 2002). Only in *P. lutzii* it is possible to conclude that light does affect *NSDD* expression. Future analysis will aim to uncover if there is an impairment of NsdD activity by GprD, at the protein level. As well, the mechanisms behind the regulation of expression of both genes, and the interaction of the codified proteins must be further analyzed in order to understand the expression of *GPRD* and *NSD*.

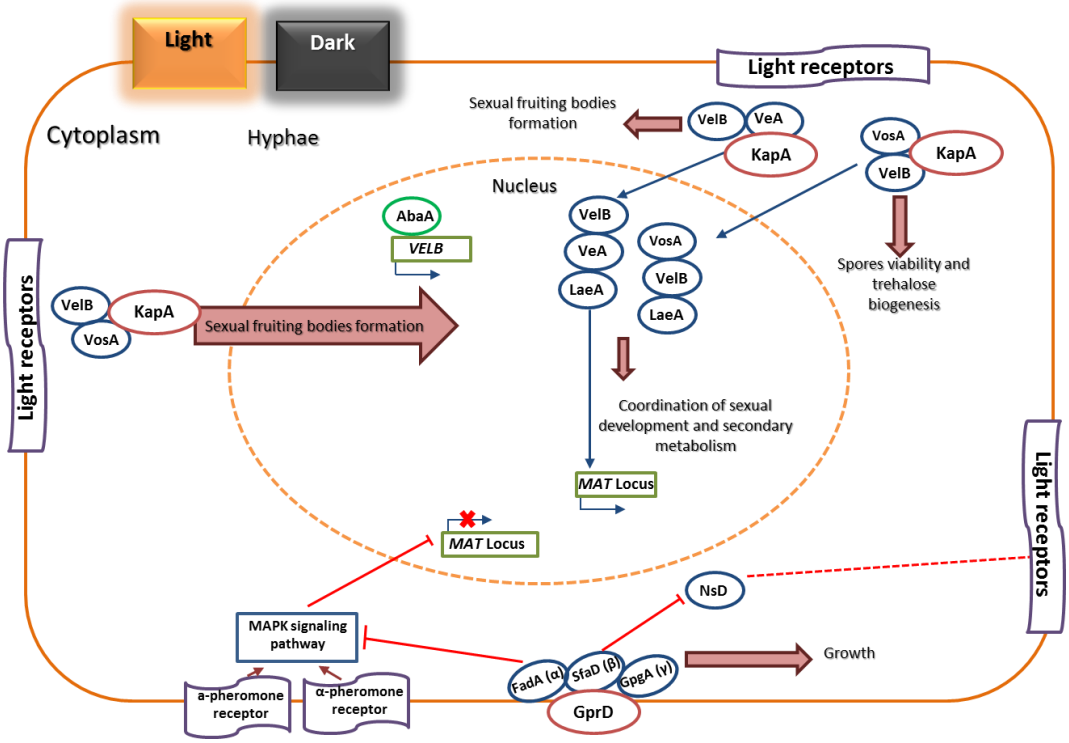


Figure 7: *Paracoccidioides* spp. model for interaction of mating components based on gene transcriptional levels. The protein model of interaction for *Paracoccidioides* spp. based on predicted interaction of proteins from *A. nidulans.* Red arrows represent impairment of functionality. Blue arrows represent interaction and progress of the process.

The nuclear-localized protein LaeA is described as a global regulator of both sexual reproduction and secondary metabolism (Szewczyk and Krappmann 2010), whose function is impaired in the presence of light in *A. nidulans* (Webster and Sil 2008; Szewczyk and Krappmann 2010). However, our results indicated that in all *Paracoccidioides* strains tested, *LAEA* expression is independent of the presence of light. In fact, the patterns of its expression are very similar in each time point whether or not light is present (Figure 4). The occurrence of expression balance in such an important sexual reproduction regulator is intriguing, because it is very different from de expression patterns observed in *A. nidulans*, except for LaeA (Desjardins et al., 2011; Gomes-Rezende et al., 2012). Our results are different from the model described in *A. nidulans*, but due to the lack of information regarding a sexual cycle in *Paracoccidioides* spp. LaeA may still play a key role in *Paracoccidioides* strains in early sexual development, but under conditions other than light. LaeA also controls the amount of VelB, VeA and VosA available in the cytoplasm (Szewczyk and Krappmann 2010). Once these proteins are in the cytoplasm, in the absence of light, they

form complexes such as VelB-VosA and VeA-VelB leading to the entry of the complexes into the nucleus by the activity of the α-transportin KapA, and activate the expression of genes and activity of proteins essential for mating. Since in the strains tested here, *KAPA* is highly expressed in a light-independent manner, most probably the transport of the velvet complexes into the nucleus is accomplished. The expression of all Velvet genes was analyzed, but our results showed no VelC expression. Therefore, we propose that NsdD functionality is blocked by GprD interaction and presence of light and MAPK signaling pathway is blocked by GprD.

Our results also show that in contrast to *A. nidulans*, in *Paracoccidioides* genus there are no differences between the velvet genes expression in light or dark (Figure 6). It is interesting that *VELB* and *VEA* expression are much higher when compared to *VOSA*. The complete lack of *VELC* expression is very intriguing, because in *A. nidulans*, VelC and the other Velvet proteins play an essential role in early stages of sexual reproduction (Szewczyk and Krappmann 2010). It is possible that the lack of sexual reproduction in *Paracoccidioides* spp. is due to the absence of VelC expression. For this reason, it would be important to overexpress *VELC* in *Paracoccidioides* spp., as well *NSDD*, or downregulate *GPRD*, in order to test their roles in sexual reproduction and spore formation.

Paracoccidioides spp. harbor most of the homologues required for sexual reproduction. However, this event was never observed under nature or laboratory conditions. In this work, we demonstrate for the first time the existence of the Velvet genes, encoding putatively light-responsive regulators of sexual reproduction, and their gene expression profile. We conclude that expression of none of the genes tested is under the control of light, which is surprising given the fact that for mating to occur in other phylogenetically-related fungi, the absence of light is crucial. Further genetic analysis using mutants and the overexpression of these genes, should clarify the role they play in sexual reproduction and/or thermal dimorphism in *Paracoccidioides* spp., and uncover the molecular mechanisms for sexual reproduction to occur in *Paracoccidioides* spp..

Chapter 4

Mating assays in *Paracoccidioides* spp.

Abstract

The genus *Paracoccidioides* includes the two thermodimorphic species *P. brasiliensis* and *P. lutzii*. These species are the agents of paracoccidioidomycosis (PCM), a serious systemic mycosis that affects especially male individuals in Central and South America. While the occurrence of sexual reproduction is common amongst phylogenetically closely related fungi, a sexual cycle has not been detected within the *Paracoccidioides* species, neither in nature nor under laboratory conditions. Nevertheless, evolutionary studies have revealed that recombination events may have occurred between populations in the genus *Paracoccidioides*, suggesting the existence of a sexual cycle. One essential criterion for fungal sexual reproduction is the presence of an active specialized genomic region denoted mating type locus *(MAT* locus*)*. This system in ascomycete fungi is composed of two idiomorphic genes, *MAT1-1* and *MAT1-2*, that are differentiated by the existence of a gene that encodes a protein with an α box motif protein (*MAT1-1)* and a gene encoding a protein with a High-Mobility Group (HMG) motif (*MAT1-2)*. The presence of such *MAT* loci was described in the *Paracoccidioides* genus. Comparative genome analysis with phylogenetically related fungi and *Saccharomyces cerevisiae* also demonstrated the presence of the mitogen-activated protein kinase (MAPK) signal transduction pathway, a conserved set of genes involved in pheromone-induced signal transduction required for sexual reproduction. The presence of the components for the orchestration of MAPK signaling supports the idea of a functional sexual cycle in *P. brasiliensis,* and the functionality of the alpha-pheromone-receptor pair Pbα -PreB was demonstrated before. In this work we assessed the ability to mate between different *Paracoccidioides* isolates with opposite mating type, when cultivated on different potentially mating-inducing growth media. Mating assays with strains *P. lutzii* Pb01 (*MAT1-1*) and *P. brasiliensis* Pb18 (*MAT1-2*) led to the identification of putative early stage structures of sexual reproduction in *Paracoccidioides* isolates, but actual viable ascospores or genetic recombination could not be confirmed. Further genetic studies will be necessary to confirm recombination through mating, while the morphological analyses support that the conditions tested may be appropriate for initiating sexual reproduction.

Introduction

Paracoccidioides species are the etiological agents of paracoccidioidomycosis (PCM), a systemic disease restricted to Central and South America, and one of the most significant endemic mycoses in these regions (Bagagli et al., 2008). PCM affects especially male individual in rural areas. The disease is caused by the inhalation of mycelia fragments and asexual spores (conidia) (Bagagli et al., 2008; Matute et al., 2006), that once inside the system propagate in the lungs and undergo differentiation to the parasitic yeast form (McEwen et al., 1987). It has been predicted that approximately 10 million individuals have been infected, however only a minor fraction (≈2%) develop PCM (Restrepo, McEwen, and Castañeda 2001). The pathogenicity and virulence of *Paracoccidioides* isolates is variable among the different strains (Carvalho et al., 2005), and the existence of a sexual cycle is associated with increased virulence in pathogenic fungi (Hsueh and Heitman 2008). In fact, due to genetic recombination, fungi have the ability to increase their genetic variation that leads to the emergence of novel strains with increased virulence and improved adaptive responses during infection (McEwen et al., 1987; Torres et al., 2010; Brummer, Castaneda, and Restrepo 1993). Pathogenic fungi such as *Candida albicans* and *Aspergillus fumigatus* are responsible for several important mycoses in humans, and in the past these fungi were considered to reproduce only by an asexual mechanism (Wong et al., 2003; O'Gorman, Fuller, and Dyer 2009). However, genetic and morphological studies demonstrated the existence of a sexual cycle in these species which can be associated with their virulence (Fraser et al., 2007; O'Gorman, Fuller, and Dyer 2009; Janiak et al., 2005; Hull, Raisner, and Johnson 2000; Magee and Magee 2000). For these fungi it was found that often very specific conditions are required to achieve mating. For example, mating of *A. fumigatus* strains with opposite mating types was initially accomplished after six months of incubations in the dark on an Oatmeal agar medium (O'Gorman, Fuller, and Dyer 2009). Subsequently, also *A. fumigatus* supermater pairs were identified that proved to generate ascospores in only four weeks under the same conditions (Sugui et al., 2011). These studies showed the importance of identifying the right environmental conditions for uncovering sexual cycles in pathogenic fungi.

The main indicator for sexual reproduction in fungi is the existence of a mating type or *MAT* locus. This region coordinates the majority of the processes involved in sexual development and is responsible for the determination of the sex of the fungus (Lee et al., 2010). The system is composed of two idiomorphic genes, *MAT1-1* and *MAT1-2*, that are differentiated by the

existence of a gene that encodes a protein with an α box motif protein (*MAT1-1)* and a gene encoding a protein with a HMG motif (*MAT1-2)*. The presence of two mating types was described in the *Paracoccidioides* genus (Torres et al., 2010). Further genealogical analysis was performed and demonstrated possible sexual recombination between the distinct phylogenetic species S1 (from Brazil, Venezuela, Peru, Paraguay and Argentina), PS2 (from Brazil and Venezuela), PS3 (from Colombia) and the newly described "Pb01-like" group, currently denominated as the new specie *P. lutzii* (Brazil, Colombia and Venezuela) (Theodoro et al., 2008; Matute et al., 2006; Brummer, Castaneda, and Restrepo 1993).

Initiation of mating requires the perception of mating pheromones via the MAPK signaling cascade. Haploid cells harbor one of two different mating-types: MAT**a** or MATα (Duntze, MacKay, and Manney 1970). The pheromones are recognized by specific membrane receptors of the opposite mating type cell: **a**-cells encode the α-receptor gene and α-cells encode the a-receptor gene (I. R. A. Herskowitz and Herskowitz 1988). In the mating pheromone pathway, pheromones are recognized by specific G-coupled-protein receptors that activate a mitogen activated the MAPK cascade (Figure 1). The recognition of the pheromone promotes the dissociation of Gpa1, Ste4 and Ste18 complex, which binds Ste20, leading to the phosphorylation of sub sequential kinases involved in the MAPK pheromone response (Figure 1) (Casselton 2002; Butler 2010). Once the MAPK is activated, the cell develops projections called shmoos, characterized by a polarized growth in the direction of the opposite mating type partner leading to growth and cell cycle arrest, cellular fusion, nuclear fusion and consequently formation of diploid cells (**a**/α cells) (Debuchy, Berteaux-lecellier, and Silar 2010).

It has already been shown that *Paracoccidioides* encodes most of the components of the pheromone signaling MAPK pathway described for other fungi, as well as an alpha-pheromone (Torres et al., 2010; Gomes-Rezende et al., 2012; Desjardins et al., 2011). Gomes-Rezende et al., found a high genetic conservation and homology between the sequences of the α-pheromone from *H. capsulatum* and *Paracoccidioides* and characterized the Pbα pheromone, establishing the recognition by its cognate receptor PreB, as well as the interaction of PreB with the G-protein PbGpa1 (Gα subunit). These protein functionalities were confirmed by restoring the mating ability in *S. cerevisiae* null mutants of the respective genes using as an heterologous expression system (Gomes-Rezende et al., 2012).

In Ascomycetes adverse environmental conditions, such as lack of nutrients and oxygen, may trigger sexual development and influence secondary metabolism (Bahn et al., 2007; Lee et al., 2010; Ö. Bayram and Braus 2012). In *A. fumigatus* and *A. nidulans* stress conditions initiate sexual development via a sequence of morphological transitions in asexual spores or hyphae that allow for mating between opposite mating types and lead to the development of sexual fruiting bodies called cleistothecium in which the ascospores are formed (Ö. Bayram and Braus 2012). The fruiting bodies need specific cells in order to protect and feed the ascospores. The maturing cleistothecium is surrounded by Hülle cells which function is to shelter and sustain the maturing cleistothecium (Figure 1A and B) (Bayram and Braus 2012). The external coating of the cleistothecium protects the ascospores, supporting their survival under severe conditions. Several proteins have been found to be essential for sexual development, cleistothecium maturation and secondary metabolism. These are LaeA and the two velvet proteins VeA and VelB, that were found to form a trimeric complex that is indispensable to synchronize secondary metabolism and sexual? development in the dark (Bok and Keller 2004). It was shown that VelB and VeA are indispensable for fruiting body establishment, while LaeA is indispensable to form sexual Hülle cells and to assist the formation of asexual spores. LaeA is as well a major regulator of secondary metabolism, that is probably modulated by activity of VelB and VeA proteins during the development in the dark (Bok & Keller 2004; Bayram et al., 2008).

Species from the genus *Aspergillus* produce their ascospores in cleistothecia, the only ascomata type that completely enfolds the asci and ascospores (Sohn and Yoon 2002). Cleistothecia can comprise up to 100 000 ascospores. Among the teleomorphic genera, eight can be associated with the aspergilli. The four most shared and widely distributed teleomorphic genera are *Petromyces*, *Emericella*, *Eurotium* and *Aporothielavia* (Figure 1) (Stchigel and Guarro 2007). They can be distinguished through their cleistothecia morphology, with the major differences found in the cell wall of the cleistothecia (peridium) (Figure 1) (Stchigel and Guarro 2007). The complete sexual cycle among ascomycete fungi is demonstrated in Figure 1E.

The recent data regarding sexual components in the *Paracoccidioides* genus, such as: the existence of opposite mating-types (Torres et al., 2010); the genes encoding the MAPK signaling pathway homologues (Desjardins et al., 2011); the expression and functionality of Pbα pheromone and its receptor PreB in a heterologous system, leading to mating restoration

(Gomes-Rezende et al., 2012) and initial formation of sexual structures in *Paracoccidioides* mating assays (Teixeira et al., 2013), forms a complex of valuable information regarding a possible active sexual cycle in *Paracoccidioides*.

Taking all this information into consideration, in this study we aimed to identify the environmental conditions that induce sexual reproduction in *Paracoccidioides* by performing *in vitro* mating assays between different mate pairs, followed by screening for the formation of mating structures and confirmation of genetic recombination.

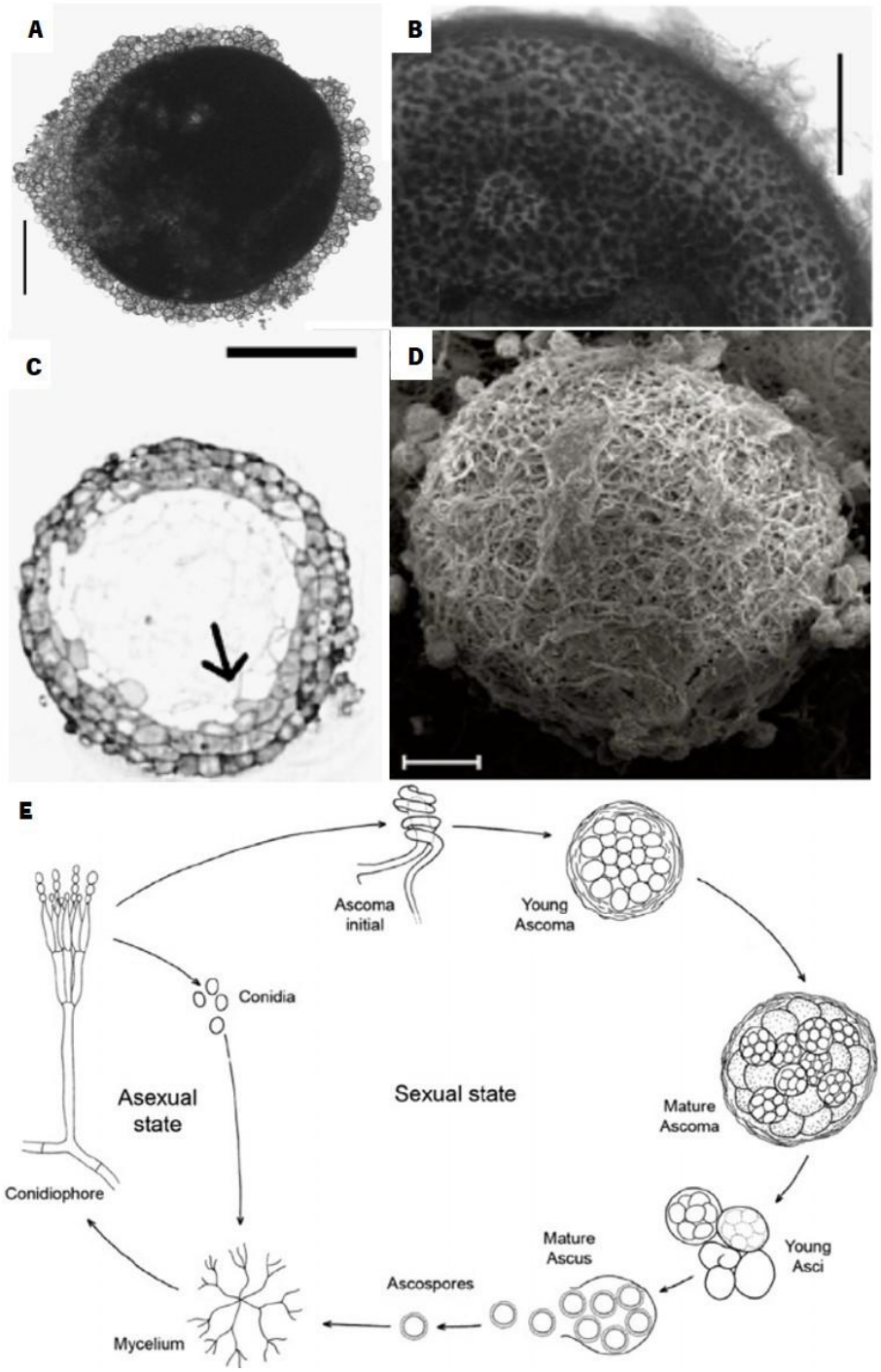


Figure 1. Microscopic details of different cleistothecia and sexual cycle in ascomycetes. (A) *E. indica* FMR 6232 ascomata surrounded by Hülle cells (scale bar 50 µm) (Stchigel and Guarro 2007). (B) *E. nidulans* FMR 8815 peridial morphology (scale bar 25 µm) (Stchigel and Guarro 2007) (Greif and Currah 2007). (C) Cross-section of the cleistothecium in *Aporothielavia leptoderma* showing a branching hypha in the developing peridium (arrow; scale bar 40 µm). (D) SEM of a cleistothecium in *A. fumigatus*, showing the peridium of interwoven hyphae (Scale bar, 100 mm) (O'Gorman, Fuller, and Dyer 2009). (E) Sexual cycle in filamentous ascomycete fungi (Wyatt, Wösten, and Dijksterhuis 2013).

Materials and methods

Microorganisms and culture media

Paracoccidioides strains were maintained at 37°C by periodic subculturing in brain heart infusion (BHI) solid media (supplemented with 1% glucose, 1.6% agar). For mating assays *Paracoccidioides* was grown as mycelium at 24°C in Malt medium. For subsequent genetic analysis, colonies were grown as yeast cells in BHI broth at 37°C and 200rpm (Restrepo and Jimenez 1980).

Table 1 – *Paracoccidioides* spp. strains used in this study.

Strain	Genotype (phylogenetic group)	Source
P. lutzii Pb01	MAT1-1::*KanMX* (Pb01-like)	Chronic PCM/Brazil
P. brasiliensis Pb18	MAT1-2:: *HPH* (S1)	Sputum/Colombia
P. brasiliensis T8B1	MAT1-2:: *HPH* (S1)	Clinical isolate/Brazil
P. brasiliensis Garcia	MAT1-2:: *HPH* (S3)	Clinical isolate/Brazil
P. brasiliensis ATCC60855	MAT1-2:: *HPH* (S1)	Clinical isolate/Colombia

DNA extraction from *Paracoccidioides* cells

Genomic DNA was isolated from *Paracoccidioides* mycelia grown in BHI broth for 3d at 37°C, 200 rpm or from mating assay plates. Mycelium was harvested by centrifugation (3000xg for 10 min at 4 °C), resuspended in 200μL of lysis buffer (1mM EDTA, 10mM Tris-HCl pH 8.0, 1% SDS, 100mM NaCl) and 200μL phenol/chloroform (1:1), followed by a heat shock treatment for 45 min at 65 °C – with intermittent vortexing every 10 min - and freezing for 60 min at -80 °C. After centrifugation at 12000 x *g* for 15 min at 4 °C, the upper aqueous phase was transferred, and an additional chloroform extraction was performed. DNA in the upper aqueous phase was precipitated with isopropanol, washed with 70% ethanol, dried and resuspended in water. The genomic DNA was used as a template in a PCR assay to identify *MAT1-1* or *MAT1-2* loci and the *KanMX* and *HPH* markers in the *Paracoccidioides* strains used in the mating assays. PCR was performed in a 20μl reaction volume containing 1x reaction buffer, 2 mM $MgCl_2$, 200μM dNTPs, 200μM of each primer (Table 2) and 0.5U DyNAzyme II DNA polymerase (Finnzymes). Thermal

cycling conditions were: an initial denaturing step at 94°C for 10 min, followed by 35 cycles at 94°C for 30 s, 40 s at the best annealing temperature previous tested and at 72°C for 1min/Kb, and a final elongation cycle at 72 °C for 10 min. Amplification products were analyzed by agarose gel electrophoresis and stained with Green Safe (NZYTech).

Table 2. Primers used in this study.

Primer	Sequence 5' 3'	Target	Reference
Mating type			
TO MAT1-1-Fw	GCAATTGTCTATTTCCATCAGT	MAT1-1	Torres et al., 2009
TO MAT1-1-Rev	CTAGATGTCAAGGTACTCGGTA	MAT1-1	Torres et al., 2009
TO MAT1-2-Fw	TTCGACCGTCCACGCCTATCTC	MAT1-2	Torres et al., 2009
TO MAT1-2-Rev	TCATTGCGAAAAGGTGTCAA	MAT1-2	Torres et al., 2009
TX MAT1-1-Fw	GCCAGGATGATAGGAAACGAAGTC	MAT1-1	Teixeira et al., 2012
TX MAT1-1-Rev	GGGAAAGATCCGGGAATACAGTAG	MAT1-1	Teixeira et al., 2012
TX MAT1-2-Fw	GAAGGCTTTCGAGGAGCATATTGA	MAT1-2	Teixeira et al., 2012
TX MAT1-2-Rev	GAGTGCTATGAACCATCTGAAGTC	MAT1-2	Teixeira et al., 2012
Internal transcribed spacer (ITS)			
ITS1	TCCGTAGGTGAACCTGCGG	ITS1	Luo and Mitchell 2002
ITS4	TCCTCCGCTTATTGATATGC	ITS4	Luo and Mitchell 2002

Mating assays between *Paracoccidioides* isolates

Mating tests were performed between pairs of MAT1-1 and MAT1-2 strains in the mycelia form. Mycelium from a liquid *Paracoccidioides* culture was spotted onto agar media plates, with opposite mating type strains about 5mm apart. The media selected for mating tests are found in Table 3. Plates were sealed with Parafilm (Pechiney Plastic packaging, USA) and kept at 24°C in the dark for up to 8 months. Every month the samples from the mating assay were collected and analyzed in the light and fluorescence microscope in order to observe the formation of fungal sexual structures. The pairs of opposite mating types assembled were: Pb01+ATCC60855, Pb01+Pb18, Pb01+T8B1 and Pb01+Garcia. A total of 4 different crosses on 4 different media were prepared in this arrangement. Crosses were grown at 24°C for 8 months in the dark. Samples from the plates were collected with a needle and spread on a microscope slide. Fungal structures were examined with lactophenol cotton blue and observed using a light microscope. In order to observe possible nuclear fusion, the samples were collected and fixed in 8% formaldehyde for 1 h, and treated with 20µL RNase A (2 µg/mL) (Thermo Fisher Scientific) and

10µL proteinase K (20µg/mL) (Thermo Fisher Scientific). Before observation, Sybr Green was added to the samples for DNA staining.

Table 3: Media composition used for *Paracoccidioides* mating assays.

Media	Composition
Malt Agar	Malt extract 10%, Agar 2%
Gorodkowa Agar	Glucose 0.1%, Sodium chloride 0.5%, Peptone 1%, Agar 2%
OTA Agar	Tomato paste 2%, Oatmeal flakes 2%, Agar 1.5%
SEA	Soil extract 50%, Glucose 0.2%, Yeast extract 0.1%, Agar 1.5%

In order to select for possible sexual reproduction progeny, we marked *Paracoccidioides* strains of opposite mating types with different antibiotic markers: MAT1-1 strains carried the *KanMx* marker (geneticin resistant) and MAT1-2 strains the *HPH* marker (hygromycin B resistant) (Table 1). Whereas parental strains will be resistant to one antibiotic only, successful mating will result in double-resistant offspring. Using *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Menino, Almeida, and Rodrigues 2012) *Paracoccidioides* strains carrying either the *KanMx* or *HPH* gene cassettes were constructed. In the case of successful mating, double-resistant *Paracoccidioides* colonies can be selected on double-selective media (hygromycin and geneticin) and analyzed for sexual structures using light microscopy. Controls included plating on medium without antibiotics or medium with each antibiotic separately.

Confirmation of genetic markers and *MAT* loci in mating progeny

To confirm the presence of both mating types and the presence of both *HPH* and *KanMx* cassettes in double-resistant colonies, the intersection area was cultivated in BHI with only hygromycin (75 µg/mL), only geneticin (100 µg/mL), or both hygromycin and geneticin (75 µg/mL and 100 µg/mL, respectively). From the colonies obtained from the double selective media DNA was extracted and PCR performed for detection of the *HPH* and *KanMX* cassettes and the *MAT1-1* and *MAT1-2* loci and visualized by agarose gel electrophoresis. To discard possible contamination of another fungus in a specific colony, ITS gene sequences were amplified from cDNA of the selected samples (after eight months mating), using ITS1 and ITS4 primers pair (Table 2), and a combination of DyNAzyme II and proofreading Phusion DNA

polymerases (Finnzymes). The PCR product was cloned with the TOPO TA Cloning Kit for sequencing (Invitrogen) following the manufacturer's protocol. Plasmid was extracted from *E. coli* strain DH5α using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced at STAB VIDA (Portugal).

Results

Paracoccidioides mating assays

Mating assays between pairs of opposite mating type cells were performed, whereby mycelial cells were placed close to each other on solid media of different composition. Mycelium from both strains will grow towards each other, meet, and recognize cells expressing opposite sexual pheromones to initiate development of their sexual cycle. At the end of the 8-month mating assay, we collected *Paracoccidioides* samples from the plates (Figure 4), and in order to confirm that the samples collected were not contaminated with other fungi, we performed PCR with universal ITS-specific primers (Table 2). The results obtained from the amplicon sequencing showed that the sequence indeed belonged to the *Paracoccidioides* genus, and there was no microbiological contamination (data not shown).

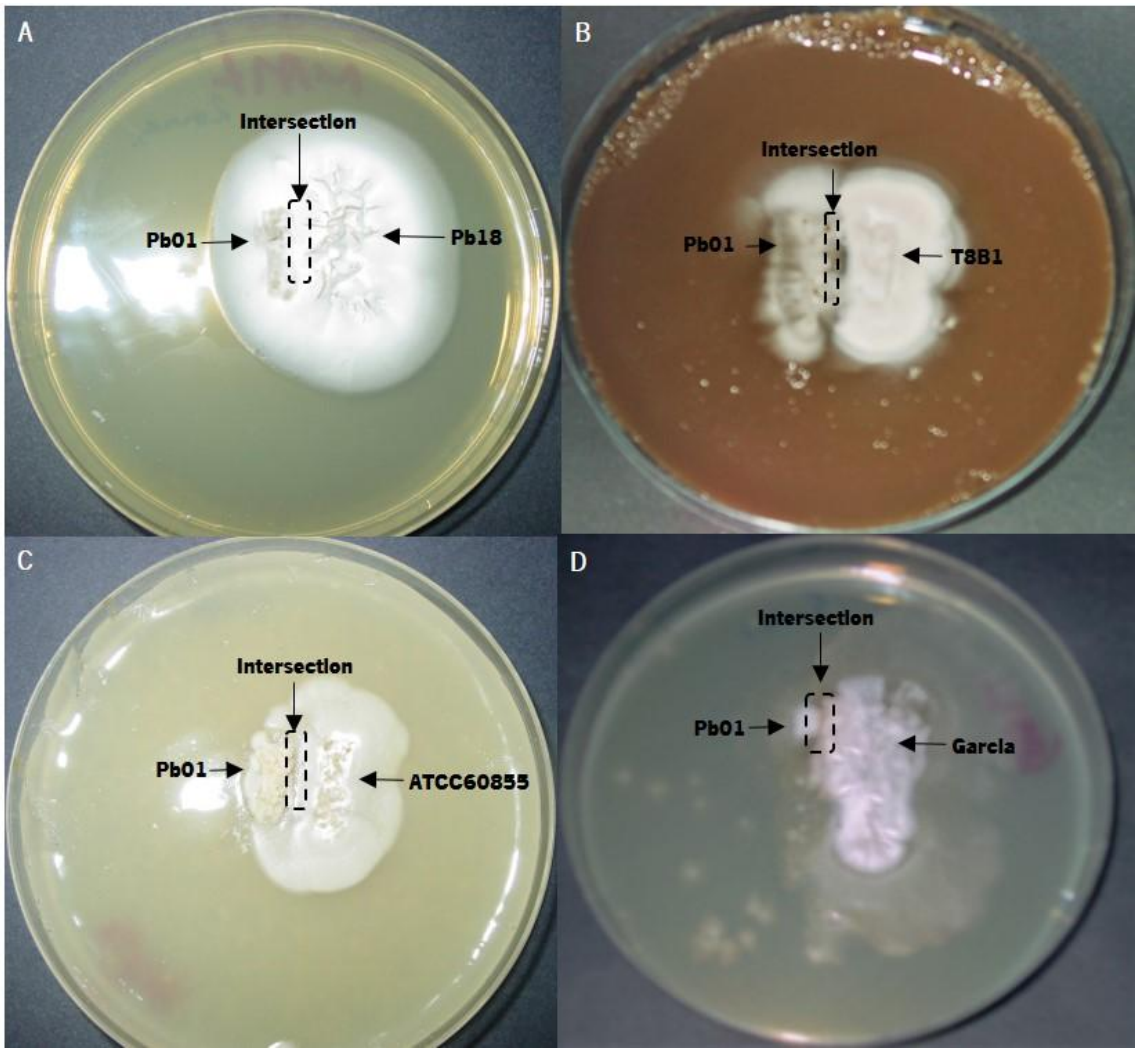


Figure 2 – *Paracoccidioides* mating assay plates at 8 months. Malt media with Pb01+Pb18 (A), Soil media with Pb01+T8B1 (B), Gorodkowa media with Pb01+ATCC60855 (C) and OTA media with Pb01+Garcia (D). The samples cultivation and analysis were collected from the intersection area demonstrated.

After 8 months of incubation in the dark, the cultures in the mating assay with the intersection of the pairs were collected for sub-cultivation and analysis. It was observed that the growth development of the Pb01 colony was less evolved in the OTA media (Figure 2D), which might be due to the medium, while in the Malt, soil and Gorodkowa media its development was better (Figure 2A-C). The biomass from the intersection was sub-cultivated on BHI media with hygromycin only, geneticin only and both hygromycin and geneticin in order to select double resistant cells. We then analyzed the surviving biomass samples under the microscope. In the samples from the pairs Pb01+Pb18 (Figure 3A-C), Pb01+T8B1 (Figure 3D-F) and Pb01+Garcia (Figure 3H-J) we observed structures that resemble conidiophore-like sporulation structures that are common in ascomycete sexual development (Figure 3). In order to confirm that the samples

collected were not contaminated by other fungi, we performed a PCR using the universal ITS primers for fungi (Table 2). The product was sequenced and the results demonstrated that in fact the sequences were from the *Paracoccidioides* genus (data not shown).

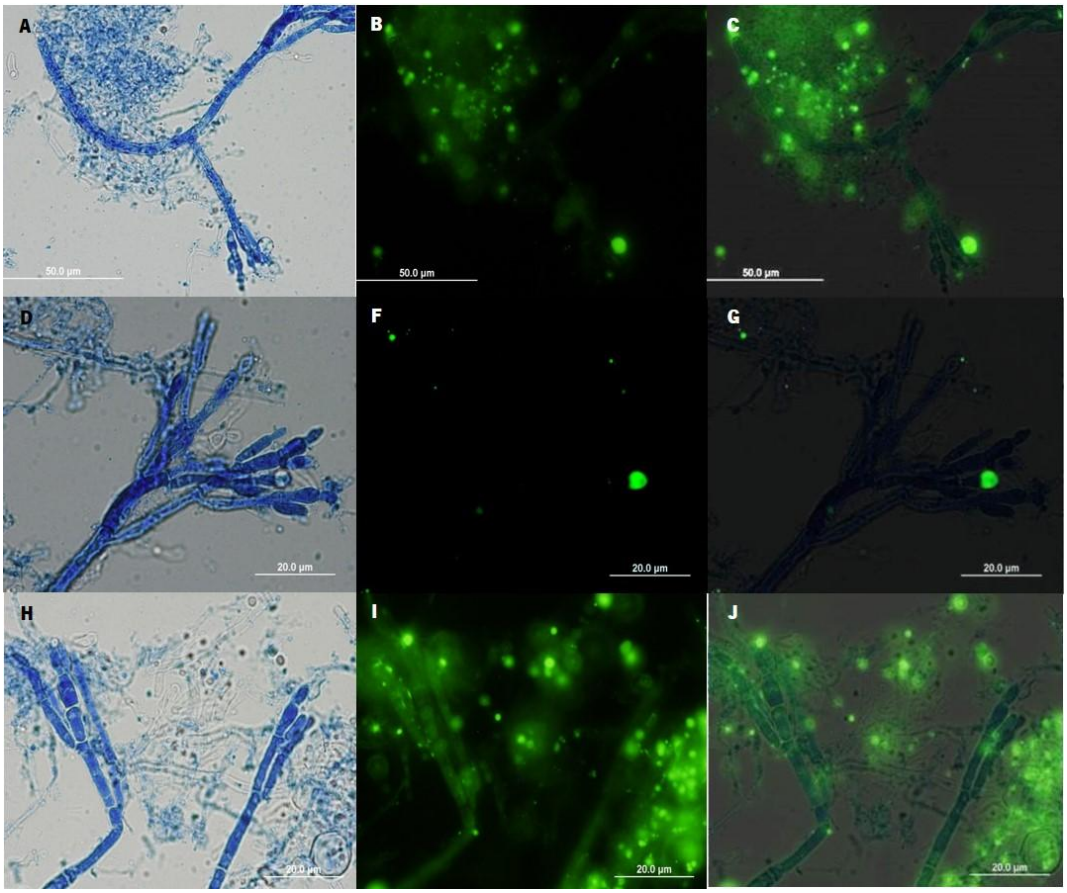


Figure 3 – Sporulation-like structures. Sporulation-like structures in the sample from Pb01+Pb18 on Malt medium (A, B and C), Pb01+T8B1 on Soil medium (E, F and G) and Pb01+Garcia on OTA medium (H, I and J), after 8 months of incubation in the dark. Bright field microscopy of fungal cell walls stained with lactophenol in (A, D and H: blue), green fluorescence of nuclear DNA with SybrGreen (B, E and I) and overlapping images (C, G and J).

We tried to isolated and cultivate the spore-like structures (Figure 3), however cultivation showed no growth. A protocol for staining spores was then used (Champe and El-Zayat 1989) in the mating assay samples and we could observe structures that resemble the Hülle cells that surround ascospores-like structures (Figure 5). The result obtained from the staining was according to what is indicated by the protocol, spores on the inside colored green while Hülle cells on the outside colored red/purple (Champe and El-Zayat 1989).

An interesting observation in the morphology of the cells from the Pb01+Pb18 mating assay, were loops formed by the mycelium (Figure 4). Such structures and disposition were observed

when sexual reproduction was achieved under laboratory conditions for *Histoplasma capsulatum* (Bubnick and Smulian 2007). Although we cannot confirm that the loop structures are initial stages of sexual structures, the fact that the loops formed close to a ascoma structure is suggestive of a sexual structure (Stchigel and Guarro 2007).

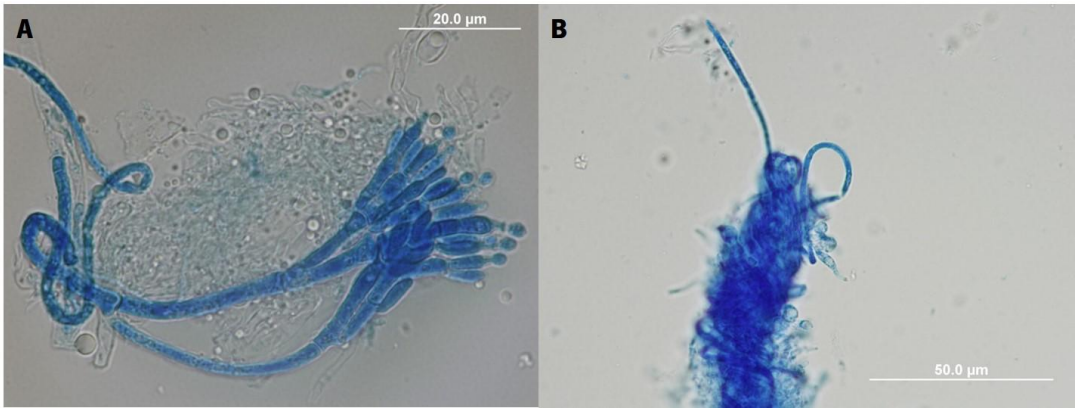


Figure 4– Hyphae loops. After 8 months mating assay between Pb01 and Pb18 on Malt medium in the dark, these loop-like structures were observed in several samples.

After collecting the samples from the intersection area from the mating assay plates, we sub-cultured the samples from Pb01+Pb18, Pb01+T8B1 and Pb01+Garcia on BHI plates complemented with hygromycin only, geneticin only and both hygromycin and geneticin. The samples from Pb01+T8B1 and Pb01+Garcia stopped growing after 1 week in double-selective media. However, the sample from Pb01+Pb18 developed for four weeks, before growth was stopped. In order to obtain conclusive results about a possible genetic recombination resulting from sexual reproduction, a double-resistant strain was grown in media containing hygromycin and geneticin. We performed several PCRs in order to confirm the presence of the individual genes that belong to each strain. Pb01 strain harbors the *MAT1-1* locus, the *KanMx* marker and a *HSP70* gene sequence specific for *P. lutzii* Pb01 (Teixeira et al., 2009), while Pb18 strain harbors the *MAT1-2* locus and the *HPH* marker. The results of the amplifications obtained from the Pb01+Pb18 sample can be observed in Table 4. The results obtained were inconclusive, since from the Pb01+Pb18 crossing we only obtained amplification of the *MAT1-2* locus but not *MAT1-1*. However, there was amplification with primers specific for the *P. lutzii*-like *HSP70* sequence (Pb01-like) (Table 4). We obtained amplification of the marker *KanMX,* but no amplification from the hygromycin marker *HPH*.

Table 4- Genes amplified from the Pb01+Pb18 mating assay sample- Gene amplifications of the parental strains and from the sample after the mating assay.

	MAT1-1	*MAT1-2*	*KanMx*	*HPH*	*HSP70*
Pb01	x	-	x	-	x
Pb18	-	x	-	x	-
Pb01+Pb18	-	x	x	-	x

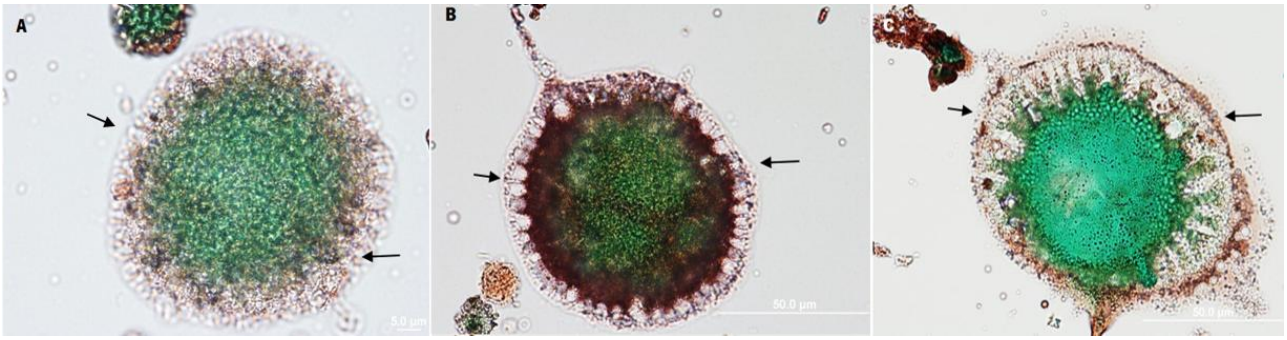


Figure 5– Ascospores staining. After 8 months mating assay samples were stained in order to observe the presence of ascospores. Samples from Pb01+ Pb18 (A), Pb01+T88B1 (B) and Pb01+ Garcia in Malt media (C). Arrows indicate Hülle cells (purple) and ascospores (green).

Discussion

Although sexual reproduction has not been reported in *Paracoccidioides*, several data suggest the existence of a sexual cycle in this fungus. The presence in *Paracoccidioides* spp. of most components of the mating pheromone MAPK pathway described in *S. cerevisiae* and the indication of genetic recombination between different strains strongly suggest that *Paracoccidioides* can reproduce sexually (Felipe et al., 2005; Teixeira et al., 2013; Desjardins et al., 2011; Gomes-Rezende et al., 2012). Studies performed by Gomes-Rezende et al., already demonstrated an interaction between the Pbα pheromone and its cognitive receptor PreB. This indicated a possible functionality of the mating pheromone MAPK pathway in *Paracoccidioides*.

Our mating assays were based on successful mating conditions for *A. fumigatus* and *H. capsulatum*, and a previous study for *Paracoccidioides* (Torres et al., 2010; Teixeira et al., 2012). Since the ascomycetes *A. fumigatus* and *H. capsulatum* mate in the dark, we also performed our

mating assays in the dark. As for the media it is important to mention that Teixeira and co-workers used soil from an PCM epidemic area in their attempts to induce mating (Teixeira et al., 2013), while in our work we used common garden soil from Portugal. Nevertheless, in our soil medium as well as in malt and OTA medium we observed putative early stage sexual structures.

During the mating assay experiments we encountered several unexpected problems, such as plate contaminations along the tests, lack of growth of the strains used, condensation inside the plates and a diminishing thickness of the plates along the tests. For this reason, we had to adapt several conditions in order to keep the plates with a thick agar layer for the entire period of eight months. To avoid contaminations, we used spotted mycelium cells previous cultured in Malt liquid medium and for reproducibility reasons we assembled the plates in triplicate, for each mate pair and each month of the assay. We had to perform all the assays inside a BSL3 laboratory, a condition that was limiting when we got to the point to collect the samples for observation. Since biosafety regulations state that *Paracoccidioides* samples must be fixed to be taken out of a BSL3 laboratory, it meant that due to the fragile nature of the sexual structures collected from the samples, it is possible that the structures could be damaged during the fixation.

The major achievement of this work was the observation of the putative ascospores-forming structures, never demonstrated in *Paracoccidioides* before. To confirm that the structures observed were not a contamination from different fungi, the PCR amplification with the universal ITS primers and amplicon sequencing proved that no contamination was present. However, we were not able to cultivate such structures. We also observed structures that resembles ascomas/early stage cleistothecia, Hülle cells, as well as those forming accentuated loops in the mycelia of *Paracoccidioides* mating assays (Figure 4), resembling the sexual structures from *H. capsulatum* mating. Such results were only obtained in the Malt agar media for the Pb01+Pb18 pair. PCR analysis demonstrated that no *MAT1-1/MAT1-2* diploid strains were formed. However, it is possible that a chromosomal recombination occurred, in order to explain the results from the gene amplifications (Andrade et al., 2005; Tavares et al., 2015) (Table 4).

The previously observed absence of mating can be due to several factors such as i) inter- and intra-incompatibilities between *P. lutzii* and *P. brasiliensis* strains due to genetic divergence of the species, ii) too low gene expression or lack of functionality of the *MAT* genes and MAPK components and their products (Pyrzak, Miller, and Miller 2008)(Gomes-Rezende et al., 2012)

and iii) inappropriate environmental conditions for inducing successful mating (Bubnick and Smulian 2007). The apparent formation of ascomas suggest early stages of sexual reproduction, however, the lack of further developed structures and genetic confirmation may indicate that the environmental conditions were not optimal yet or that there are genetic constrains that prevent completion of sexual reproduction.

Under the conditions used in the mating-pair assay, the structures resembling fruiting bodies might indicate that *Paracoccidioides* has the ability to produce ascocarps, however other factors may be necessary for the development of fertile offspring. Those factors may be the absence of ideal conditions for spore germination and inappropriate functionality of the genes and proteins necessary for completing the sexual cycle. Additional studies must be performed in order to find the crucial environmental conditions that could allow for development of cleistothecia and ascospores in *Paracoccidioides* spp., to confirm the presence of a fully active sexual cycle. Genetic approaches also must be developed in order to confirm sexual recombination, namely the presence of both mating types in heterothallic strains, as well the sexual machinery and pathways involved in sexual reproduction. In order to improve the techniques to identify rare events of *Paracoccidioides* sexual reproduction under laboratory conditions, the opposite mating type cells could e.g. also be marked with fluorescent proteins in order to observe double staining of recombinant diploid cells. In order to manipulate the sex-related genes, the down-regulation of the negative sexual reproduction regulator GprD or overexpression of the positive regulator NsdD could lead to an improvement of the chances for the occurrence of a sexual cycle (Han, Seo, and Yu 2004; Han et al., 2001).

Overall, mating assays performed by Torres et al., 2010 and Teixeira et al., 2012 and this study provide clues about the environmental conditions necessary for a complete sexual cycle in *Paracoccidioides*. Moreover, in this study in particular we observed ascomycete-like sexual structures in *Paracoccidioides* that were not observed before. We hope that these results can be useful for the achievement of a full sexual reproduction cycle in *Paracoccidioides* under laboratory conditions.

Concluding remarks and future perspectives

Paracoccidioides is known to reproduce asexually, but in contrast to related fungi from the Ajellomycetaceae family, no sexual reproduction has been observed in nature or under laboratory conditions (Brummer, Castaneda, and Restrepo 1993). The life cycle of *Paracoccidioides* has long been considered to be asexual (Brummer, Castaneda, and Restrepo 1993), until in 2010 two studies demonstrated genetic indications that are considered landmarks for the presence of a sexual cycle in fungi in general. First, the discovery, description and distribution of two opposite mating types (MAT1-1 and MAT1-2) was described in an extensive number of *Paracoccidioides* isolates (Torres et al., 2010). Secondly, the *in silico* analysis that was performed in the sequenced *Paracoccidioides* strains demonstrated the existence of sex-related genes with high homology to those of other Ascomycetes (Desjardins et al., 2011). Despite these genetic markers sexual reproduction could not be demonstrated in *Paracoccidioides* mating assays (Torres et al., 2010; Teixeira et al., 2012).

In fungi, sexual reproduction leads to the generation of strains with increased fitness, i.e. that are adapted or resistant to certain hosts and environmental conditions, and show increased pathogenicity (Butler 2010). The essential requirements for ascomycete mating are the presence of cells of opposite mating type, the production and release to the environment of opposite mating pheromones and the presence of the respective pheromone receptors in the cell membrane. After the recognition of the pheromone, the signal is transmitted through the cell membrane into the nucleus via the MAPK signaling cascade, and in the nucleus, several sex-related transcriptional factors and the sexual machinery are activated in order to start the sexual cycle (Coppin et al., 1997; Gustin et al., 1998; Bardwell et al., 1996).

The basis of sexual reproduction is the existence of opposite mating types, the expression of mating pheromones and their recognition by the cognate membrane receptor in the opposite mating type cell. In chapter 2, through *in silico* analysis we were able to identify the previously non-annotated *Paracoccidioides* α-pheromone, one of the two pheromones essential for mating, by using the *Histoplasma* α-pheromone precursor protein sequence as a query (Chapter 2). The mature Pbα-pheromone protein sequence (WCTRPGQGC) displayed 100% identity to the *Histoplasma* α-pheromone, while the precursor peptide harbored most important residues that are conserved between different fungal α-pheromones in general. The presence of the Kex2

protease (prototypical eukaryotic prohormone-processing enzyme) recognition sequence (KR), whose cleavage would result in a mature pheromone was also confirmed.

Taking advantage of heterologous expression of *Paracoccidioides* mating related genes in *S. cerevisiae* mating gene mutants we then demonstrated the interaction between the Pbα pheromone and the *Paracoccidioides* α-pheromone receptor PreB (Chapter 2). In addition, interaction between pheromone receptor PreB and G-protein PbGpa1(G-protein α subunit) was demonstrated. All interactions were confirmed in the *S. cerevisiae* null mutant strains heterologously expressing Pbα, PreB and PbGpa1, through the observation of: i) mating restoration, ii) pheromone-induced shmoo formation, iii) pheromone-induced growth arrest (halo assays) and iv) pheromone-induced cell cycle arrest (flow cytometry) (Chapter 2). We also demonstrated that PbGpa1 does not interact with the α-pheromone receptor Ste2 from *S. cerevisiae,* and requires the presence of the cognate PreB receptor. Although the interaction between Pbα-PreB was successful in the heterologous system, we were not able to increase mating-related gene expression in *Paracoccidioides* strains using synthetic Pbα pheromone. The reason for the lack of response and the general low mating gene expression may be due to the impairment by other sexual regulators. In *A. fumigatus* and *A. nidulans* for example, the negative regulator of sexual reproduction GprD, and light-responsive Velvet proteins play regulatory roles in sexual reproduction (Han, Seo and Yu 2004). The negative regulator GprD in *Aspergillus* is responsible for the impairment of MAPK signaling and thereby blocking the sexual development. However, there are positive regulatory proteins for sexual development involved as well, such as NsdD. GprD levels are constant in *Aspergillus* since its gene expression is independent of the presence of light (Dyer and O'Gorman 2012), while the positive regulator of sexual reproduction NsdD in *Aspergillus* is expressed in the dark and is involved in primary phases of sexual development (Szewczyk & Krappmann 2010; Laskowski-Peak et al., 2012; Han et al., 2001). Therefore, in Chapter 3, we analyzed the gene expression of these transcriptional regulators and demonstrated that in *P. lutzii* Pb01 and *P. brasiliensis* Pb18 *GPRD* expression is elevated in the presence of light and its expression is practically zero in the dark. Literature describes that *GPRD* expression is independent of the presence of light (Gehrke et al., 2010; Han, Seo and Yu 2004), but our results suggest that in *Paracoccidioides* it is down-regulated in the dark. NsdD plays a positive regulatory role in early stages of sexual reproduction in *Aspergillus* (Szewczyk and

Krappmann 2010; K. H. Han et al., 2001), and its function is impaired in the presence of light. Our results show no clear relation between *NSDD* gene expression and the presence or absence of light in the *Paracoccidioides* strains. In *P. lutzii* Pb01 light does affect *NSDD* expression all over the 25-day experiment, while in *P. brasiliensis* Pb18 and ATCC60855 there are differences in expression in dark and light conditions only at 25 days of cultivation. Overall, we did not observe clear patterns in *GPRD* and *NSDD* expression in the *Paracoccidioides* strains tested. Future analysis will need to uncover if there is an impairment of NsdD activity by GprD at the protein level, since in this study protein levels and phosphorylation states were not measured due to the lack of *Paracoccidioides* spp. specific antibodies for these proteins. Moreover, the mechanisms behind the regulation of expression of these genes, and the interaction of the proteins must be further analyzed in order to understand the function of GprD and NsdD in *Paracoccidioides*.

In Chapter 3 of other regulators of sexual reproduction in *Paracoccidioides*, we also analyzed the expression levels. In particular, the light-responsive and sex-modulating Velvet proteins might play a role in this, and we therefore analyzed the expression of these genes in the presence and absence of light (Chapter 3). Our results showed no correlation between *MAT1-1* and *MAT1-2* gene expression and presence or absence of light during cultivation, leading to the conclusion that their expression is light-independent. *MAT* expression might be activated by real light-activated genes, that at this moment are unknown. Regulation of sexual development in *A. nidulans* involves multiple protein interactions with the Velvet proteins (Park et al., 2014; Yu 2010). Next to the Velvet genes *VELB, VOSA, VEA* and *VELC* we therefore also examined the gene expression of the interacting regulators LaeA and KapA in *Paracoccidioides* spp. Our data showed that in the *Paracoccidioides* strains tested, *LAEA* gene expression is independent of light. The constitutive gene expression of the sexual reproduction regulator LaeA in *Paracoccidioides* is intriguing because it is very different from the differential expression patterns observed in *A. nidulans* (Bayram et al., 2010).

In *Aspergillus*, Velvet proteins form the complexes VelB-VosA and VeA-VelB in the cytoplasm in the dark, (Bayram, Krappmann, et al., 2008), that then enter into the nucleus through the activity of the α-transportin KapA, and trigger the expression of genes indispensable for the mating process (Bayram et al., 2010; Park et al., 2012; Bayram and Braus 2012; Bayram, Krappmann, et al., 2008). In the strains tested in this work, *KAPA* is highly expressed in a light-independent

manner. While we assessed that *Paracoccidioides* expresses the genes that encode the Velvet proteins, we in fact have no information about their protein levels and activation states. This requires further investigation in future studies. The *Paracoccidioides* genome also encodes the *VELC* gene, but this gene was not expressed in any of the conditions tested. The lack of *VELC* expression is surprising, because in *A. nidulans* VelC and the other Velvet proteins play a crucial part in the early stages of sexual development (Szewczyk and Krappmann 2010). It is therefore possible that the lack of sexual reproduction in *Paracoccidioides* spp. is due to the absence of VelC expression. Nevertheless, in this work we demonstrate the presence and expression profiles of the other Velvet genes and genes of Velvet interacting proteins in *Paracoccidioides*, concluding that gene expression of none of the Velvet proteins essential for sexual reproduction is under the control of light in *Paracoccidioides*.

The results obtained in Chapters 2 and 3 and previously published data demonstrate several evidences that *Paracoccidioides* may present an active sexual cycle: i) the presence of opposite mating types (Torres et al., 2010); ii) the presence of homologues of the MAPK signaling cascade (Desjardins et al., 2011); iii) the existence of the alpha pheromone and its recognition by the PreB alpha pheromone receptor, demonstrated in Chapter 2; iv) the mating assays performed by Teixeira and co-workers that show structures that are indicative of early stages of sexual reproduction in *Paracoccidioides* (Teixeira et al., 2013); and v) the Velvet gene expression (Chapter 3). However, actual sexual reproduction has not been shown, and the mating results from Teixeira et al., 2013 are not conclusive. Therefore, in Chapter 4 we performed several mating assays in different conditions, based on the requirements for the occurrence of sexual reproduction in *A. fumigatus* (O'Gorman, Fuller, and Dyer 2009) and the mating assays previously performed in *Paracoccidioides* (Teixeira et al., 2013). By plating several mate-pairs of two opposite mating-type strains on four different media and cultivation in the dark for eight months, in Chapter 4 we could observe the formation of putative early-stage sexual structures that are common among ascomycetes. We obtained sporulation-like structures, the coiled loops in pre-mating hyphae and the presence of ascospore-containing structures, suggesting the beginning of sexual development. The ascospore-like structures were confirmed by using a staining protocol that differentially marks the ascospores and the cell wall. In our mating assays the MAT1-1 strain Pb01 was geneticin resistant (harboring the cassette *KanMx*), and the MAT1-2

strains used were hygromycin resistant (harboring the *HPH* cassette). The antibiotic resistant strains were constructed in order to confirm possible mating, by the occurrence of hygromycin/geneticin double-resistant cells, and also provide an additional PCR target to confirm that the colonies formed, presented double antibiotic resistance. Despite the structures obtained that resembled ascomycete-like sexual structures, we were not able to confirm the presence of one mating type and both antibiotic resistance cassettes by PCR. However, the PCR results obtained from the mating assays from Pb01+Pb18 were intriguing as the PCRs from the colonies that grew on both hygromycin and geneticin were positive for the presence of *MAT1-2, KanMx* and a Pb01-like *HSP70* sequence, indicating some form of genetic recombination between the MAT1-1 and MAT1-2 strain. In this sense, we were not able to confirm the occurrence of mating.

The media in sexual reproduction plays a major role in the development and progression of mating, as it is established that mating in ascomycetes occurs under stress, nutrient or oxygen deprivation (Ni et al., 2011). Finally, since the cells were cultured for a long time, it is possible that the media themselves were depleted in several nutrients. The medium composition also plays a major role in this experiment. It may induce the development of sexual reproduction, but it remains unknown what nutrients and factors are present and essential for sexual development. Agar and yeast extracts can present differences in composition for different suppliers, and these can show a high effect on the phenotype of the culture. Additionally, agar media based on natural components can differ between manufactures and labs, and even within one lab batch to batch variances can occur. Ascomycetes may require exogenous minerals, vitamins and other natural materials for ascomata production, and these ingredients are not present in synthetic media. This might be one of the explanations why sexual reproduction is more often found on media made from natural substrates. With this information, when performing mating assays with ascomycetes, it is better to use a range of media, which should include basal media supplemented with the natural growth substrate of the species. Teixeira et al., 2013, in their experiments used soil from the area where *Paracoccidioides* isolates are found for the media composition. OTA medium inhibited the growth and development of strain Pb01, while the Malt, SEA and Gorodkowa media did not inhibit the development of any of the strains tested. In contrast to studies in other fungi OTA might therefore not be an inducing medium for *Paracoccidioides*.

In this thesis, we set out to demonstrate the functionality of mating-related genes and identify missing components (Chapter 2) and for the first time we analyzed the expression of the Velvet genes in *Paracoccidioides* (Chapter 3). We also performed several mating assays with *Paracoccidioides* isolates, and we were able to find structures that resemble structures that are developed and essential for sexual reproduction in other ascomycetes (Chapter 4). The major task to identify an active sexual cycle in *Paracoccidioides* will be the execution of mating assays with a large number of strains from all phylogenetic groups and geographical locations in different media. After achieving the appropriate conditions for mating, it is essential to cultivate the ascospores and to test the fate of the *MAT* locus and other strain-specific genes in the ascospores to understand possible genetic recombination, variation and mutation due to meiotic events. It is also important to cultivate the newly formed strains to perform further genetic analysis and to test if their virulence is different from the parental strains. It is also crucial to understand the presence and functionality of all pheromone-responsive MAPK signaling pathway components. While the *Paracoccidioides* α-pheromone was identified in this thesis, the **a** pheromone still remains to be discovered. Fungal **a**-pheromones in general are difficult to identify in genomes due to their high sequence diversity, and requires advanced bioinformatics or degenerate PCR approaches. It is pivotal to identify the **a**-factor and assess its functionality in a similar way as was done for Pbα, to confirm the presence of a fully functional mating pheromone signaling system. Identification and isolation of the Pb **a**-pheromone and interaction with its receptor PreA was attempted during this thesis work, but proved not successful (data not shown). Once the **a**-factor is described, is important to assess the sexual pheromones recognition by their cognate receptors, the triggering of sexual reproduction and its molecular players, as well its effect during mating assays, and the typical response to pheromone recognition. Finally, the discovery of an active sexual cycle in *Paracoccidioides* will aid in the functional analysis of the genetic processes involved through e.g. mutational approaches and their effect on mating. Furthermore, once sexual reproduction is active it will be able to analyze the possible genetic recombination that may have occurred in the fungus, as well as perform a profound study of the molecular mechanisms involved, in particular the role of the Velvet proteins, the MAPK signaling components, and other genes that are related to sexual development. Several aspects that need to be further investigated to this end include the confirmation of the activity of all the components of the MAPK signaling pathway that trigger sexual reproduction, and testing the genetic

mechanisms and components of mating-related genes through mutagenesis, silencing or overexpression, and their effect on the development of sexual structures. For example the down-regulation of *GPRD* expression, as well as overexpression of *NSDD* are strategies that have been successful to prove their function in sexual development in *Aspergillus* (Han et al., 2001).

In all, this study supplies further indications for the existence of an active sexual cycle in *Paracoccidioides*, although sexual reproduction could not be demonstrated. The work developed in this thesis uncovered several novel aspects of *Paracoccidioides* biology and further insights into the key factors responsible for the occurrence of sexual reproduction. The data herein described will therefore be helpful for the achievement of *Paracoccidioides* sexual reproduction in the future.

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