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Presence and expression of the mating type locus in *Paracoccidioides brasiliensis* isolates

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ABSTRACT

Paracoccidioides brasiliensis has been classified in the phylum Ascomycota, order Onygenales, family Ajellomycetaceae, even in the absence of a known sexual cycle or mating system. The objective of this work was to determine the presence of the mating type locus in 71 *P. brasiliensis* isolates from various sources. A PCR assay using specific primers for the *MAT 1* gene was developed and applied for the detection of such genes. Two heterothallic groups (*MAT1-1* or *MAT1-2*) were recognized and, in some isolates, gene expression was confirmed indicating the existence of a basal gene expression. The distribution of two mating type loci in the studied population suggested that sexual reproduction might occur in *P. brasiliensis*. This finding points towards the possibility of applying a more precise definition of the concept of biological species to *P. brasiliensis*. Further studies should be conducted to confirm the sexual capacity of this fungus and its implications among phylogenetic species and geographical distribution.

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1. Introduction

Paracoccidioides brasiliensis is the causative agent of paracoccidioidomicosis (PCM), a systemic disorder geographically restricted to Central and South America, and one of the most important endemic mycoses in these regions, especially among the male rural populations. The disease is most likely caused by the inhalation of asexual spores (conidia) produced by the mycelia form of the fungus, propagules that once in the lungs undergo differentiation towards the parasitic yeast form (Restrepo et al., 2001). It has been estimated that approximately 10 million individuals have been infected with the fungus, with a minor proportion (2%) developing the disease (Restrepo et al., 2001). Furthermore, skin tests studies with paracoccidioidin in healthy residents of the endemic areas have shown reactivity close to 20%, indicating a significant rate of infection by *P. brasiliensis* (Brummer et al., 1993; Restrepo et al., 2008).

Precise details concerning *P. brasiliensis* growth in the environment are lacking, and this uncertainty also applies to its sexual reproduction, which has not yet been observed. On the other hand, asexual reproduction has been described for the mycelia form, where it takes place via arthro- and micro-conidia, and for the yeast form through blastoconidia either in the host or in cultures at 36–37 °C (Brummer et al., 1993; Restrepo et al., 2001, 2008).

Matute et al. (2006), based on the sequence of eight nuclear loci, undertook a phylogenetic approach to study cryptic speciation and recombination in *P. brasiliensis*. Their study indicated that *P. brasiliensis* consists of at least three distinct, previously unrecognized phylogenetic species and provided the first evidence for sexual reproduction in at least one of these species, suggesting that the reproduction of *P. brasiliensis* is not only asexual, as recombination was shown to occur (Matute et al., 2006).

Due to the fact that *P. brasiliensis* teleomorph has not yet been described, classic systematic has classified this fungus in the broad artificial group of mitosporic fungi, class Hyphomycetes (San-Blas et al., 2002), although morphological, molecular, and phylogenetic studies placed the fungus in the phylum Ascomycota (Bowman et al., 1996; Kwon-Chung, 1972; Leclerc et al., 1994; Morais et al., 2000; Nino-Vega et al., 2004). Recently, a newer classification of this phylum has emerged with a clade distinct to the *Onygenaceae sensu lato* proposed as a new family (*Ajellomycetaceae*) to encompass the monophyletic group *Ajellomyces*, which includes the anamorphic genera *Blastomyces*, *Emmonsia*, *Histoplasma*, and *Paracoccidioides* (Bagagli et al., 2008; Hibbett et al., 2007; Untereiner et al., 2004). In the latter

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genus, the presence of a sexual cycle and mating type system remain unknown.

One criterion required for fungal sexual reproduction, albeit insufficient, is the presence of mating type genes that have been previously identified in a number of the Ascomycetes. Sexual reproduction in fungi is regulated by a specialized genomic region known as the mating type (*MAT*) locus. This system is composed of two idiomorphs, *MAT1-1* and *MAT1-2*, which are distinguished from each other by the presence in *MAT1-1* of an ORF that encodes for a protein with an alpha box motif protein while in *MAT1-2* a single ORF encoding an HMG motif protein is present (Turgeon and Yoder, 2000). Opposite *MAT* show a rather low similarity in sequence with each other even when placed in the same chromosomal locus, and for this reason they are called idiomorphs (Paoletti et al., 2005). In heterothallic species, isolates with compatible *MAT1-1* and *MAT1-2* genotypes must be present for sexual reproduction to occur (Fraser et al., 2007; Heitman et al., 2007). In addition, within the filamentous Ascomycetes there are a large number of homothallic species, many of which appear closely related to heterothallic isolates. The former contains genes from both *MAT* idiomorphs both of which are required for self-mating (Heitman et al., 2007).

Among the human pathogens, the mating process could well play a potential role in virulence, as recombination between two compatible isolates may result in a new strain with increased virulence. This phenomenon has been documented for the parasite *Toxoplasma gondii* (Grigg et al., 2001), and may also have occurred in the fungus *Cryptococcus gattii* during the Vancouver Island outbreak (Fraser et al., 2005).

The occurrence and distribution frequency of the *MAT 1* idiomorphs in a population may also constitute an indication of the reproductive behavior of a pathogen. Thus, in populations in which sexual reproduction prevails, the two *MAT 1* idiomorphs occur in approximately equal frequencies; in contrast, skewed ratios are indicative of asexual populations (Stergiopoulos et al., 2007). However, the presence of the mating type idiomorphs alone in a given species is insufficient to prove the existence of a sexual stage, as has been demonstrated for the filamentous ascomycetes *Alternaria alternata* and *Fusarium oxysporum* (Arie et al., 2000).

In this study, we describe the population distribution of the mating type idiomorphs from *P. brasiliensis* isolates. It is presently accepted that this pathogen reproduces only asexually but here we have demonstrated that isolates of the fungus contain the *MAT1-1* and *MAT1-2* genes that show high similarity with the homologous gene from other filamentous Ascomycetes fungi. In addition, by using real time PCR (qRT-PCR), we explored the basal expression of *MAT* gene in some of the *P. brasiliensis* isolates studied. Additionally, certain outcrosses between potentially compatible partner strains were attempted by plating on appropriate culture media in order to search for sexually reproductive structures.

2. Materials and methods

2.1. Isolates studied and culture conditions

Seventy-one *P. brasiliensis* isolates from a variety of sources (geographic, clinical, environmental, and phylogenetic species) (Table 1) were analyzed for the presence of mating type loci. For maintenance, the mycelia form was grown in the modified synthetic MacVeigh and Morton medium (MMSV) (Restrepo and Jimenez, 1980) at room temperature (18–23 °C). For DNA extraction, the yeast form of the organism was grown at 36 °C in Brain Heart Infusion (BHI) agar supplemented with 1% glucose (Diez et al., 1999). For the qRT-PCR assays, six *P. brasiliensis* isolates (Table 1) were chosen and grown in liquid BHI media at 36 °C for 5 days for the yeast form or at room temperature (22–25 °C) to obtain the mycelial form.

2.2. Analysis of the fungal mating loci

2.2.1. MAT 1 locus PCR

DNA was isolated from yeast form cultures according to the glass beads protocol described by Van Burik et al. (1998) or by maceration of previously frozen cells according to Morais et al. (2000), followed by purification steps with phenol:chloroform:isoamyl alcohol (Sambrook and Russell, 2001).

Initially, a PCR assay was conducted using specific pairs of 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 of the *Histoplasma capsulatum* *MAT* genes kindly provided by Dr. George Smulian from the University of Cincinnati, Cincinnati, Ohio. For *MAT1-1* these pairs were called MAT1-1 EST2-1 F and R and for *MAT1-2* gen MAT1-2 EST G-F and R (Table 2 and Fig. 1). Subsequently, using the *P. brasiliensis* genomic data published by the Broad Institute (www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html), two pairs of primers called GMAT1-1 and GMAT1-2 (genomic primers, Table 2, Fig. 1) capable of amplifying a large region of both genes were designed. Primers sequences and their related features are listed in Table 2, and primers location is shown in Fig. 1.

PCR amplifications were performed in a MyCycler IQ from BioRad®, using 0.5 μg of each DNA preparation in a 12.5 μl reaction mixture containing 1× PCR buffer (Tucan Taq, Corpogen® Bogotá, Colombia), 3.0–3.5 mM $MgCl_2$ (depending on the primer used), 0.5 mM of each dNTP, 0.25 μM of each primer, and 1 U of *Taq* polymerase (Tucan Taq, Corpogen®). The following amplification conditions were used: an initial denaturing of 2 min at 95 °C, followed by 30 cycles consisting of 30 s at 94 °C, 1 min at 50 °C, and 1 min at 72 °C, with a final elongation step at 72 °C for 10 min. Sterile water was used as a negative control in each assay. The amplification products were separated by agarose gel electrophoresis, and then stained with ethidium bromide and visualized with UV light, as described by Sambrook and Russell (2001). The mating genotype was assigned according to the primers used. For the PCR assays with genomic primers, the same reaction ratios and amplification conditions were used, changing only the annealing temperature to 56 °C. Several amplification products from each mating type were sequenced and analyzed by the BLAST tools available in the GenBank database.

2.3. Analysis of P. brasiliensis MAT sequences

Sequence similarity search of the *P. brasiliensis* *MAT1-1* and *MAT1-2* fragments obtained from the PCR assay utilizing genomic primers were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The intron and exon regions were assigned using the genome sequences available for the *P. brasiliensis* Pb03 (*MAT1-2*) and Pb01 (*MAT1-1*) strains deposited at the Broad Institute and described on their website. Protein sequence analysis was conducted at the PROSITE from ExPASy (http://us.expasy.org/prosite/).

2.4. Statistical analyses

The null hypothesis was set at a 1:1 ratio for the two mating types as characteristic for a random mating in a population of haploid organisms (Milgroom, 1996). The hypothesis was tested with Pearson's chi-square test for fit (χ^2), with $p < 0.05$ considered significant.

Table 1
Main features and classification of *P. brasiliensis* isolates studied according to their mating type.

Isolates	Species code[b]	Phylogenetic species[b]	Country of origin	Source	Mating type
T1F1	B1	S1	Brazil	Environment	*MAT1-1*
T4B14[a]	B3	S1	Brazil	Environment	*MAT1-1*
T7F6[a]	B4	S1	Brazil	Environment	*MAT1-1*
T8B1	B5	S1	Brazil	Environment	*MAT1-1*
T13LN1	B9	S1	Brazil	Environment	*MAT1-1*
BT60	B14	S1	Brazil	Clinical	*MAT1-1*
BT84	B15	PS2	Brazil	Clinical	*MAT1-1*
Pb339[a]	B18	S1	Brazil	Clinical	*MAT1-1*
Pb4	B23	PS2	Brazil	Clinical	*MAT1-1*
Pb6	B24	S1	Brazil	Clinical	*MAT1-1*
15632		ND	Brazil	Clinical	*MAT1-1*
Pb89		ND	Brazil	Clinical	*MAT1-1*
DO2		ND	Brazil	Clinical	*MAT1-1*
Pb01		ND	Brazil	Clinical	*MAT1-1*
Pb7		ND	Brazil	Clinical	*MAT1-1*
Pb192		ND	Brazil	Clinical	*MAT1-1*
PbS1		ND	Brazil	Environmental	*MAT1-1*
14-121	B16	S1	Brazil	Clinical	*MAT1-1*
4154		ND	Brazil	Clinical	*MAT1-1*
P46	C11	PS3	Colombia	Clinical	*MAT1-1*
P161	C12	PS3	Colombia	Clinical	*MAT1-1*
H45	C15	PS3	Colombia	Clinical	*MAT1-1*
P206	C17	PS3	Colombia	Clinical	*MAT1-1*
CIB44197[c]	C19	PS3	Colombia	Environment	*MAT1-1*
P176		ND	Colombia	Clinical	*MAT1-1*
P178		ND	Colombia	Clinical	*MAT1-1*
P175		ND	Colombia	Clinical	*MAT1-1*
P166		ND	Colombia	Clinical	*MAT1-1*
Pb73	C20	PS3	Colombia	Clinical	*MAT1-1*
Pb305	V3	S1	Venezuela	Clinical	*MAT1-1*
Pb15	V6	S1	Venezuela	Clinical	*MAT1-1*
Pb2	V2	PS2	Venezuela	Clinical	*MAT1-1*
Pb304	V4	S1	Venezuela	Clinical	*MAT1-1*
Pb Bolivia		ND	Bolivia	Clinical	*MAT1-1*
A2	A2	S1	Argentina	Clinical	*MAT1-1*
A3	A3	S1	Argentina	Clinical	*MAT1-1*
A1	A1	S1	Argentina	Clinical	*MAT1-1*
T10B1	B7	PS2	Brazil	Environment	*MAT1-2*
T5LN1	B8	S1	Brazil	Environment	*MAT1-2*
Ibia	B12	S1	Brazil	Environment	*MAT1-2*
Uberlandia[a]	B13	PS2	Brazil	Environment	MAT1-2
Pb18	B17	S1	Brazil	Clinical	MAT1-2
Pb3	B26	PS2	Brazil	Clinical	MAT1-2
Pb9	B20	S1	Brazil	Clinical	MAT1-2
Pb11	B21	S1	Brazil	Clinical	MAT1-2
DO1		ND	Brazil	Environment	MAT1-2
15601		ND	Brazil	Clinical	MAT1-2
Pb14	B22	S1	Brazil	Clinical	MAT1-2
Pb10	PE1	S1	Perú	Clinical	MAT1-2
60855[a]	C4	PS3	Colombia	Clinical	MAT1-2
P141	C5	PS3	Colombia	Clinical	MAT1-2
P196	C6	PS3	Colombia	Clinical	MAT1-2
P204	C7	PS3	Colombia	Clinical	MAT1-2
P68	C9	PS3	Colombia	Clinical	MAT1-2
76533	C13	PS3	Colombia	Clinical	MAT1-2
H47	C16	PS3	Colombia	Clinical	MAT1-2
P167		ND	Colombia	Clinical	MAT1-2
P168		ND	Colombia	Clinical	MAT1-2
P171		ND	Colombia	Clinical	MAT1-2
P172		ND	Colombia	Clinical	MAT1-2
P173		ND	Colombia	Clinical	MAT1-2
P174		ND	Colombia	Clinical	MAT1-2
CIB40392[c]	C21	PS3	Colombia	Environment	MAT1-2
P72	C10	PS3	Colombia	Clinical	MAT1-2
P151[a]	C18	PS3	Colombia	Clinical	MAT1-2
P169		ND	Colombia	Clinical	MAT1-2
Pb300	V1	S1	Venezuela	Environment	MAT1-2
Pb381	V5	S1	Venezuela	Clinical	MAT1-2
U1	U1	S1	Antarctic	Environment	MAT1-2
P2	P2	S1	Paraguay	Clinical	MAT1-2
P1	P1	S1	Paraguay	Clinical	MAT1-2

[a] Isolates tested by qRT-PCR.
[b] Code and phylogenetic species as defined by Matute et al. (2006).
[c] These isolates were obtained from armadillos: CIB40392: *Dasypus novemcinctus* CIB44197: *Cabassous centralis*.

Table 2
P. brasiliensis primer pairs used for mating or for qRT-PCR determination of mating type genes.

Primer name	Primer sequence (5′–3′)	Primer name	Primer sequence (5′–3′)	Genes
GMAT1-1 F	GCAATTGTCTATTTCCATCAGT	GMAT1-1 R	CTAGATGTCAAGGTACTCGGTA	*MAT1-1* alpha domain assignment
MAT1-1 EST2-1 F	GGCATTTAACAAATCTTTACG	MAT1-1 EST2-1 R	CCCAGTTTGTAGCAATGAGT	
GMAT1-2 F	TTCGACCGTCCACGCCTATCTC	GMAT1-2 R	TCATTGCGAAAAGGTGTCAAG	*MAT1-2* HMG domain assignment
MAT1-2 EST G-F	CATGTCTCTGTCATTGTTCCA	MAT1-2 EST G-R	GGAACAAGGAGGTTGAAGTT	
MAT1-1 E2 F	ATCAGGTTGGTTATGTCGCAGG	MAT1-1 E2 R	GGAAATGAGCGTGGTTTGGTT	qRT-PCR of *MAT1-1* gene
MAT1-2 E2 F	TGGAAGTCAGGGTTAGATGCTT	MAT1-2 E2 R	CCGTCAAATGCGTTCATTC	qRT-PCR of *MAT1-2* gene
BTubE1RTF	GTGGACCAGGTGATCGATGT	BTubE1RTR	ACCCTGGAGGCAGTCACA	qRT-PCR normalizing gene (β-tubulin)

Fig. 1. Location of primers used in the classification of *P. brasiliensis* isolates according to presence of *MAT 1-1* and *MAT 1-2* genes. Black arrows represent position of the genomic primer GMAT1-1 F and R and GMAT1-2 F and R that produce amplicons of 1455 bp and 1208 bp, respectively. Regions in box correspond to the fragments amplified with pairs of primers designed from *P. brasiliensis* EST libraries (Goldman et al., 2003; Felipe et al., 2005) and denominated MAT1-1 EST2-1 F and R for MAT1-1 and MAT1-2 EST G-F and R producing amplicons of 400 bp and 1000 bp, respectively. The *MAT* genes representation was taken from the Broad Institute page (www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/).

2.5. qRT-PCR of mating gene expression at the basal stage in P. brasiliensis isolates

RNA was extracted from six isolates grown separately in their mycelia and yeast forms using TRIZOL (Qiagen®, Inc., Chatswoth, CA) and following the manufacturers' protocols. DNA-free total RNA (0.5–1 µg) was reversely transcribed using the Super script III platinum two-step qRT-PCR kit with SYBR green (Invitrogen, Carlsbad, CA). cDNA samples were diluted to a similar concentration with sterile water in order to be used as templates and measurement of the expression levels of the *MAT1-1* and *MAT1-2* genes were performed using the SYBR green PCR master mix (Invitrogen, Carlsbad, CA). The corresponding reactions were carried out in the iCycler IQ (BIORAD); each isolate was evaluated in triplicate with Beta-tubulin being used as the reference gene to normalize expression (Goldman et al., 2003). Relative quantification was performed using the $2^{-\Delta\Delta CT}$ method reported by Livak and Schmittgen (2001). The primers used for qRT-PCR are listed in Table 2.

2.6. Mating assays and observation of sexual structures by light microscopy

Some isolates (Table 3) were selected according to various criteria, e.g. mating type, phylogenetic species as defined by Matute et al. (2006), and country of origin (Table 1). The isolates to be tested were sub-cultured on MMSV agar and grown at room temperature until mycelia growth became apparent. A square plug of each partner culture was transferred to four different media (malt agar, alphacel agar, Gorodkowa's agar, and water agar) (McGinnis, 1980), and placed a distance of approximately 8 mm from one another (Kwon-Chung, 1972) in order to induce sexual reproduction. A total of 68 crosses were prepared in this form. Crosses were grown at room temperature (23–25 °C) for 20–25 days with 12 h/12 h light–dark cycles and then examined by light microscopy. Structures resembling fruiting bodies were examined with lactophenol and observed using a light microscope.

2.7. Nucleotide sequences accession numbers

MAT1-1 and *MAT1-2* sequences from several strains were submitted to GenBank with following accession numbers in Pb6 (B24) *MAT1-1* (GQ411377), T7F6 (B4) *MAT1-1* (GQ411378), P165 *MAT1-1* (GQ411379), Pb300 (V1)-*MAT1-2* (GQ411380), P173-*MAT1-2* (GQ411381), and 60855 (C4) *MAT1-2* (GQ411382).

3. Results

3.1. Presence of either of MAT1-1 or MAT1-2 genes in the studied isolates

The PCR assays done with the primers designed on the basis of the *P. brasiliensis* EST library and primers obtained from the genome sequences (genomic primers) allowed us to identify the presence of either one of the two mating type genes in the 71 *P. brasiliensis* isolates tested. The first primers produced amplicons of 400 bp and 1000 bp corresponding to the *MAT1-1* and the *MAT1-*

Table 3
P. brasiliensis isolates selected for outcrossing between potentially compatible partners according to mating type, phylogenetic species, and country of origin.

MAT1-2	*MAT1-1*	Phylogenetic species and country of origin
Outcrossing[a]		
60855	P161	PS3–PS3 (Colombia–Colombia)
60855	P206	PS3–PS3 (Colombia–Colombia)
60855	P46	PS3–PS3 (Colombia–Colombia)
60855	T13LN1	PS3–S1 (Colombia–Brazil)
60855	Pb01	Colombia–Brazil
60855	Pb89	Colombia–Brazil
H-47	P206	PS3–PS3 (Colombia–Colombia)
Pb18	Pb339	S1–S1 (Brazil–Brazil)
Pb18	T13LN1	S1–S1 (Brazil–Brazil)
Pb18	Pb01	Brazil–Brazil
Pb18	Pb89	Brazil–Brazil
CIB40392[b]	CIB 44197[b]	PS3–PS3 (Colombia–Colombia)
P68	P46	PS3–PS3 (Colombia–Colombia)
T5LN1	T4B14	S1–S1 (Brazil–Brazil)
IBIA	Pb01	Brazil–Brazil
IBIA	Pb89	Brazil–Brazil
IBIA	P46	Brazil–Colombia
P178	T13LN1	Colombia–Brazil
P161	Pb89	Colombia–Brazil

[a] Outcrossings were carried out using four different culture media, for a total of 68 outcrosses.
[b] These isolates were obtained from armadillos: CIB40392: *Dasypus novemcinctus* CIB44197: *Cabassous centralis*.

2 genes, respectively. The genomic primers produced larger amplicons for the two mating type genes with those corresponding to the *MAT1-1* gene having a size of 1208 bp while for *MAT1-2* size was 1455 bp (Fig. 2).

The BLAST tools available in the GenBank database allowed analysis of *P. brasiliensis* sequence similarity with the *MAT1-1* and *MAT1-2* genes; results indicated a high grade identity with *H. capsulatum* sequences, 79% identity for *MAT1-1* and 70% for *MAT1-2* with highly significant *e*-values (2e−46, 2e−84, respectively). Furthermore, comparison of our *MAT* sequences with those in the *P. brasiliensis* genomes as annotated by the Broad Institute revealed that they corresponded to the *MAT* gene annotated in the corresponding genome. Additionally, using the BLAST tools available in the ExPASy server showed that the *P. brasiliensis MAT1-1* and *MAT1-2* sequences reported in this work had the characteristics of α and HMG boxes, respectively.

3.2. Equivalent distribution of two mating types in the P. brasiliensis population

For the entire isolates collection, the distribution of the mating type ratio corresponding to the heterothallic genotypes *MAT1-1*:*MAT1-2* was 37:34, a 1:1 distribution From the Brazilian population carrying the *MAT1-1* and *MAT1-2* genotypes, the relationships between country and mating type were found to be 62% and 38% of the strains, respectively (Table 4). In the Colombian isolates, the predominant genotype was *MAT1-2* (63%), while the remaining isolates (37%) carried the *MAT1-1* genotype. In the population constituted by isolates from other PCM endemic areas (Venezuela, Argentina, Bolivia, Paraguay, and the Antarctic), the genotype distribution was 57% for *MAT1-1* and 43% for *MAT1-2*.

Analyses of the *MAT* gene distribution according to the phylogenetic species described by Matute et al. (2006) (Table 4) were performed for 48 out of the 71 isolates included in the study, with their inclusion in the analysis based on the previous assignment of a phylogenetic code. In S1, 54% of the isolates possessed the *MAT1-1* genotype, and the remaining 46% carried the *MAT1-2*; in PS2, both genotypes were present at the same ratio (50%:50%). On the other hand, in PS3 consisting only of Colombian isolates, a predominance of the *MAT1-2* genotype (62.5%) was observed (Table 4). Concerning the *MAT* gene distribution according to the country of origin and phylogenetic species (Matute et al., 2006) distribution of the isolates as determined by the χ^2 test revealed a *MAT1-1*:*MAT1-2* 1:1 ratio (Table 4).

3.3. Transcriptional level of the MAT locus genes

The qRT-PCR results revealed that the expression of *P. brasiliensis MAT* genes under basal conditions was low (no more than 1.5-fold that of the normalization gene) both in the mycelia and yeast forms, with the exception of isolate Pb339, for which the *MAT 1-1* genotypes displayed a significantly higher expression in the yeast than in the mycelia form. The opposite was observed in isolate T4B14, for which the expression of *MAT1-1* genes between the yeast and mycelia forms exhibited a slight difference (1.05 in mycelia and 0.64 in yeast) (Fig. 3). In regards to the *MAT1-2*, the expression levels were low in all isolates tested (Fig. 3).

Table 4
Distribution of *MAT1-1* and *MAT1-2* mating types among *P. brasiliensis* isolates according to PCM endemic areas and phylogenetic species[a].

Isolates source	Mating type frequency[b]		χ^2 [c]	*P*-value
	MAT1-1	*MAT1-2*		
Brazil	62 (18)	38 (11)	1.19	0.19
Colombia	37 (10)	63 (17)	1.81	0.17
Other endemic areas	57 (8)	43 (6)	0.28	0.59
S1	54 (14)	46 (12)	0.15	0.69
PS2	50 (3)	50 (3)	NA	NA
PS3	37.5 (6)	62.5 (10)	1	0.31
Total	52 (37)	48 (34)	0.12	0.72

[a] As defined by Matute et al. (2006).
[b] Percentage of frequency. Numbers in parentheses refer to the number of isolates.
[c] Based on a 1:1 ratio with 1 degree of freedom. NA: not apply.

3.4. Mating compatibility test

After 20–25 days of incubation of the mating compatibility assays between different *P. brasiliensis* isolates, observation revealed some structures resembling fruiting bodies on malt agar corresponding to crosses of the strains 60855 + Pb01, 60855 + Pb89, and 60855 + T13LN1. However neither asci nor ascospores were to be found upon further inspection; there were only round compact structures surrounded by a plectenchyma (Fig. 4).

4. Discussion

Similarities between the *MAT* loci from several Ascomycetes strains have made it possible to identify the corresponding loci in microorganisms for which the genomes have not been previously sequenced. Thus, the use of *H. capsulatum MAT1-1* and *MAT1-2* sequences allowed us to find out that certain *P. brasiliensis* EST sequences in the libraries examined shared high similarity with both *H. capsulatum MAT* genes (http://www.ebi.ac.uk/embl/) (Arie et al., 1997; Bubnick and Smulian, 2007; Felipe et al., 2005; Goldman et al., 2003).

Additionally, synteny analysis performed by Li et al. (2009) revealed features of the *MAT* loci that were shared among several

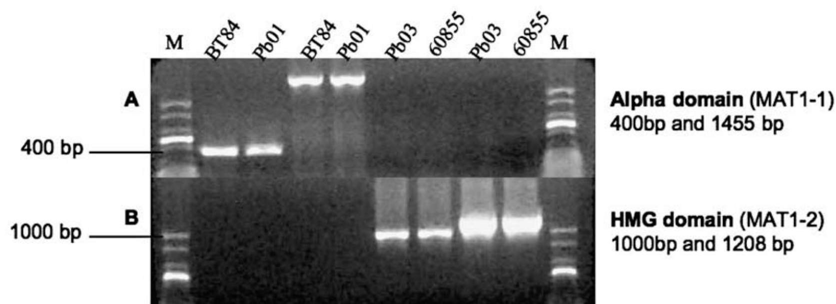
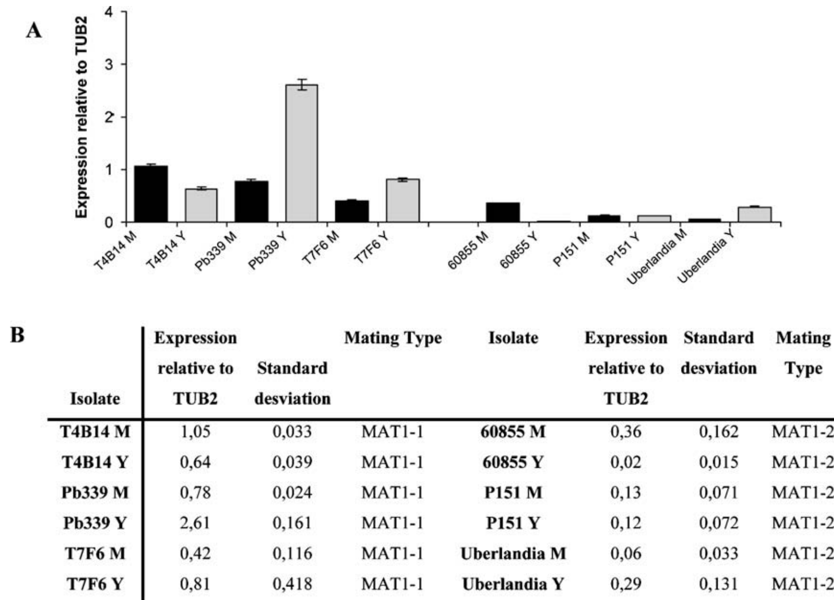


Fig. 2. PCR-based identification of MAT locus idiomorphs in 71 *P. brasiliensis* isolates. The 400 bp and 1000 bp bands were obtained using the primers designed according the *P. brasiliensis* EST libraries; the 1455 bp and the 1208 bp bands were amplified using the genomic primers. M: molecular weight markers-CIB. (A) A 400 bp and 1455 bp portion of the *MAT1-1* gene alpha domain were obtained from isolates BT84 and Pb01. (B) The *MAT1-2* gene HMG domain amplified 1000 bp and 1208 bp fragments from the Pb03 and 60855 isolates.



Isolate	Expression relative to TUB2	Standard desviation	Mating Type	Isolate	Expression relative to TUB2	Standard desviation	Mating Type
T4B14 M	1,05	0,033	MAT1-1	**60855 M**	0,36	0,162	MAT1-2
T4B14 Y	0,64	0,039	MAT1-1	**60855 Y**	0,02	0,015	MAT1-2
Pb339 M	0,78	0,024	MAT1-1	**P151 M**	0,13	0,071	MAT1-2
Pb339 Y	2,61	0,161	MAT1-1	**P151 Y**	0,12	0,072	MAT1-2
T7F6 M	0,42	0,116	MAT1-1	**Uberlandia M**	0,06	0,033	MAT1-2
T7F6 Y	0,81	0,418	MAT1-1	**Uberlandia Y**	0,29	0,131	MAT1-2

Fig. 3. *MAT1-1* and *MAT1-2* gene expression levels (qRT-PCR) of six *P. brasiliensis* isolates in both yeast and mycelial forms. (A) Comparative expression of *MAT1-1* and *MAT1-2* gene of micelia and yeast form in six *P. brasiliensis* were determine by qRT-PCR analysis. Relative quantification represents the normalized difference between the target gene expression and that of β tubulin (Goldman et al., 2003). Error bars indicate standard deviations. (B) Summary of the data.

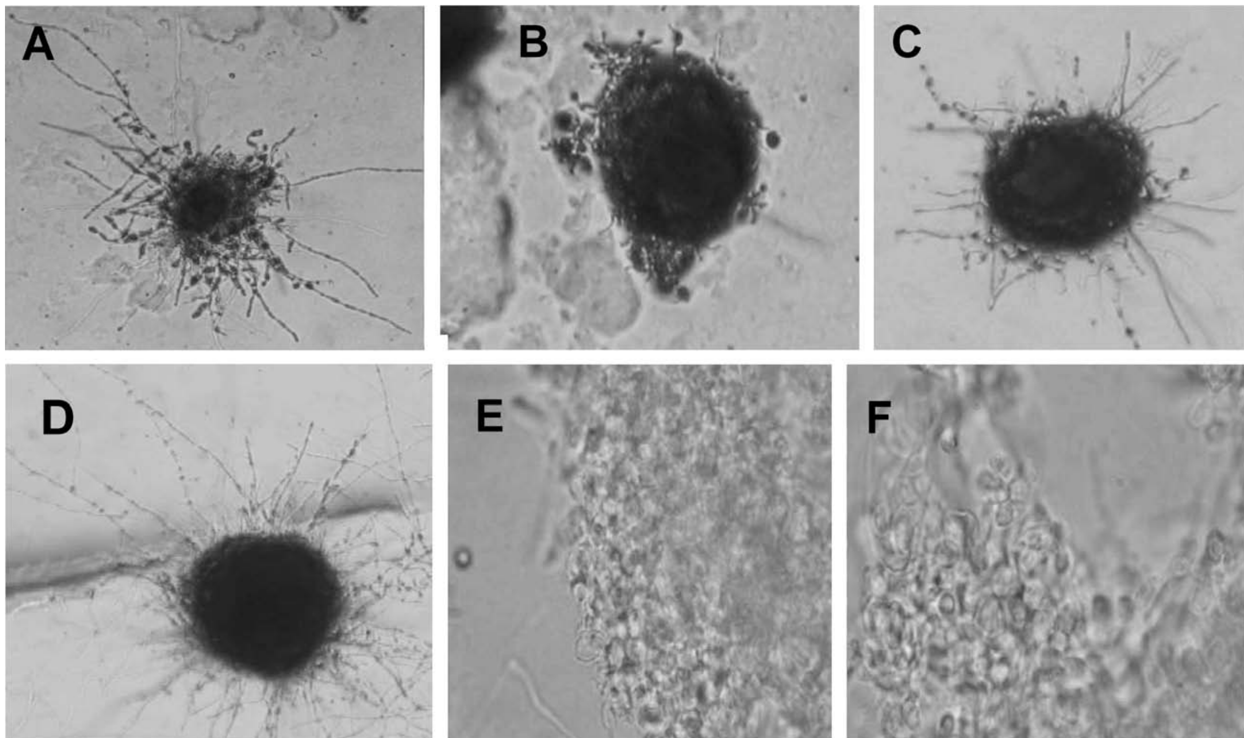


Fig. 4. Structures observed in crosses between *P. brasiliensis* isolates. A and B: Partners 60855 + T13LN1, C: partners 60855 + Pb89, D: partners 60855 + Pb01. E and F: Contents of the structures found in C. Observe rounded compacts structures conformed by a plectenchyma devoid of asci or ascospores.

of the dermatophytes studied (*Microsporum gypseum*, *Microsporum canis*, *Trichophyton equinum*). The *SLA*, *COX13*, and *APN2* genes flanking the *MAT* locus in other Ascomycota were linked instead on one side of the dermatophyte *MAT* locus. Furthermore, the transcriptional orientations of the *APN2* and *COX13* genes were in reverse positions when compared to *H. capsulatum*, *Coccidioides inmmitis*, and *C. posadassi*. The *MAT1-1* or the *MAT1-2* idiomorphs were also identified in the available genomic sequences of *H. capsulatum*, *C. immitis*, *C. posadassi*, and *P. brasiliensis*. The *P. brasiliensis MAT* locus structure displayed greater similarity to that of *H. capsulatum* than to those of *Coccidioides* species. The presence of these genes and of their variable organization could have implica-

tions concerning the effectiveness of the mating process (Li et al., 2009).

The above finding confirm that *P. brasiliensis* shares both the *MAT* locus structures and mating models described for other dimorphic fungi.

In this study and based on a suitable PCR method, it was possible to identify mating types in a large number of *P. brasiliensis* isolates and in representative cultures of the newly described phylogenetic species of this fungus (Matute et al., 2006) for which no sexual stages have been described. By means of PCR primers designed on the basis of *P. brasiliensis* EST libraries (Felipe et al., 2005; Goldman et al., 2003), as well as on the *H. capsulatum MAT* genes (Bubnick and Smulian, 2007) sequences comprehensively described in this and other fungi (Heitman, 2006), and that have been annotated in the *P. brasiliensis* genome, we were able to identify the *MAT* gene in the 71 *P. brasiliensis* isolates tested. These results indicate that a substantial similarity with the conserved motifs from *H. capsulatum* does exist suggesting that these partial sequences do, in fact, represent the mating type idiomorphs of *P. brasiliensis*, a fungus with no known sexual stage despite the evidence for recombination previously reported by Matute et al. (2006).

The PCR methods developed in the present study for mating type assessment in *P. brasiliensis* facilitated recognition of potentially compatible strains and as such could also be used in outcrossing experiments aimed at detecting teleomorphic structures (Kerenyi et al., 2004). The *MAT* gene represents valuable tools for the study of the biology and the genetics of fungal populations. The presence of both genes in *P. brasiliensis* suggests that sexual reproduction probably does occur in this fungus.

Analysis of the distribution of the *MAT* genes according to the isolates' country of origin revealed a bias towards the *MAT1-1* genotype in the Brazilian population (62%) and a similar bias towards the *MAT1-2* in the Colombian isolates (63%). The *MAT1-1* and *MAT1-2* disequilibria, as observed in both Brazilian and Colombian isolates, is not as marked as in the case of clinical isolates of *C. neoformans*, an organism with a predominance of the alpha mating type (Fraser et al., 2005; Heitman et al., 2007). Nonetheless, this observation may suggest important behavioral differences between the two idiomorphs. Given the former finding, sexual reproduction may alter fungal virulence (Bubnick and Smulian, 2007; Heitman et al., 2007; Kerenyi et al., 2004) and mating of environmental or commensal fungi in human pathogens could be associated with antifungal resistance or increased virulence (Heitman, 2006; Heitman et al., 2007). Nevertheless, whether or not the latter observations could be attributed to virulence differences between strains of opposite mating type remains to be defined (Bubnick and Smulian, 2007; Heitman et al., 2007).

Mating type genes are frequently used in population studies, as their presence, relative frequency and distribution within a population may indicate the reproductive modes of a fungus. In a sexual population, negative frequency-dependent selection is expected to retain equilibrium in the two mating type idiomorphs while in a asexual population, this ratio would be skewed (Stergiopoulos et al., 2007; Paoletti et al., 2005; Yun et al., 2000). A conserved 1:1 ratio in the *MAT* genes distribution for the *P. brasiliensis* isolates studied is consistent with natural sexual reproduction. This is similar to the distribution reported for *Aspergillus fumigatus*, where a 1:1 ratio of *MAT1-1*:*MAT1-2* isolates was found among a worldwide screening of 290 isolates (O'Gorman et al., 2009).

Pyrzak et al. (2008) reported the expression of putative mating type genes, as well as other sex-related genes, in *A. fumigatus* during mycelia growth. Similarly, in our studies the qRT-PCR test revealed low levels of *MAT* gene expression (Fig. 3), consistent with the lack of sexual activity of the fungus during its asexual cycle. New assays are in progress to additionally evaluate other *P. brasiliensis* isolates in both growth forms, studies that may provide new insights into the sexual behavior of this fungus.

In the present study, efforts were made to obtain mating compatibility in culture, with only three from the 68 crosses producing structures resembling fruiting bodies that contained neither asci nor ascospores. Nonetheless, under the assay conditions used these structures resembling fruiting bodies might indicate that *P. brasiliensis* has the capacity to produce ascocarps but that other factors would be needed to permit the production of fertile offsprings (Fig. 4). These factors may include, among others, the absence of compatibility between strains due to the divergence of the species outcrosses, inadequate functionality of the genes (Pyrzak et al., 2008), and inappropriate environmental conditions for successful mating, aspects that should be addressed in further studies (Bubnick and Smulian, 2007).

In human pathogenic fungi, limited sexual reproduction tends to generate clonal populations, with recombination occurring only rarely. Heitman (2006) proposed that limiting sex enables pathogenic microbes to generate clonal populations that are adapted to certain hosts and environmental niches while retaining their ability to reproduce sexually in response to environmental challenges in nature, in the host, or as a consequence of antimicrobial therapy. This means that fungi retain the machinery to engage in sexual or parasexual reproduction but that a variety of mechanisms limit the expression in nature of this sexual reproduction. This phenomenon generates highly clonal populations in which, concomitantly, recombination remains active with both features being likely advantageous for the fungus in response to different selective pressures (Heitman, 2006; Heitman et al., 2007).

The mechanisms responsible for limiting sexual reproduction are diverse and often unique for each organism (Heitman, 2006). Leslie and Klein explained the absence of sexual reproduction in local populations of the *Giberella fujikuroi* species complex by the presence of mutations that concomitantly resulted in female sterility with increased vegetative propagation capability (Kerenyi et al., 2004). Selection for an increased number of asexual propagules may result in the selective accumulation of sterile female strains, which could become prevalent even in large geographic areas. Under such conditions, mating is limited by the absence of normal fertile female partners. A similar phenomenon might occur in the case of *P. brasiliensis* phylogenetic specie PS3, for which a higher proportion of the *MAT1-2* genotype is present suggesting that this type of clonal selection would tend to increase this specific idiomorph.

Finally, the demonstration of the presence of the *MAT1-1* and *MAT1-2* genes in *P. brasiliensis* has provided new tools for the study of sexual reproduction in this fungus, leading to a better understanding of its biology both as a saprobe and as a pathogen, as well as to its precise taxonomic classification.

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References

Arie, T., Christiansen, S.K., Yoder, O.C., Turgeon, B.G., 1997. Efficient cloning of Ascomycete mating type genes by PCR amplification of the conserved MAT HMG Box. Fungal Genet. Biol. 21, 118–130.

Arie, T., Kaneko, I., Yoshida, T., Noguchi, M., Nomura, Y., Yamaguchi, I., 2000. Mating-type genes from asexual phytopathogenic ascomycetes *Fusarium oxysporum* and *Alternaria alternata*. Mol. Plant Microbe Interact. 13, 1330–1339.

Bagagli, E., Theodoro, R.C., Bosco, S.M., McEwen, J.G., 2008. *Paracoccidioides brasiliensis*: phylogenetic and ecological aspects. Mycopathologia 165, 197–207.

Bowman, B.H., White, T.J., Taylor, J.W., 1996. Human pathogeneic fungi and their close nonpathogenic relatives. Mol. Phylogenet. Evol. 6, 89–96.

Brummer, E., Castaneda, E., Restrepo, A., 1993. Paracoccidioidomycosis: an update. Clin. Microbiol. Rev. 6, 89–117.

Bubnick, M., Smulian, A.G., 2007. The MAT1 locus of *Histoplasma capsulatum* is responsive in a mating type-specific manner. Eukaryot. Cell 6, 616–621.

Diez, S., Garcia, E.A., Pino, P.A., Botero, S., Corredor, G.G., Peralta, L.A., Castano, J.H., Restrepo, A., McEwen, J.G., 1999. PCR with *Paracoccidioides brasiliensis* specific primers: potential use in ecological studies. Rev. Inst. Med. Trop. Sao Paulo 41, 351–358.

Felipe, M.S., Andrade, R.V., Arraes, F.B., Nicola, A.M., Maranhao, A.Q., Torres, F.A., Silva-Pereira, I., Pocas-Fonseca, M.J., Campos, E.G., Moraes, L.M., Andrade, P.A., Tavares, A.H., Silva, S.S., Kyaw, C.M., Souza, D.P., Pereira, M., Jesuino, R.S., Andrade, E.V., Parente, J.A., Oliveira, G.S., Barbosa, M.S., Martins, N.F., Fachin, A.L., Cardoso, R.S., Passos, G.A., Almeida, N.F., Walter, M.E., Soares, C.M., Carvalho, M.J., Brigido, M.M., 2005. Transcriptional profiles of the human pathogenic fungus *Paracoccidioides brasiliensis* in mycelium and yeast cells. J. Biol. Chem. 280, 24706–24714.

Fraser, J.A., Giles, S.S., Wenink, E.C., Geunes-Boyer, S.G., Wright, J.R., Diezmann, S., Allen, A., Stajich, J.E., Dietrich, F.S., Perfect, J.R., Heitman, J., 2005. Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. Nature 437, 1360–1364.

Fraser, J.A., Stajich, J.E., Tarcha, E.J., Cole, G.T., Inglis, D.O., Sil, A., Heitman, J., 2007. Evolution of the mating type locus: insights gained from the dimorphic primary fungal pathogens *Histoplasma capsulatum*, *Coccidioides immitis*, and *Coccidioides posadasii*. Eukaryot. Cell 6, 622–629.

Goldman, G.H., dos Reis Marques, E., Duarte Ribeiro, D.C., de Souza Bernardes, L.A., Quiapin, A.C., Vitorelli, P.M., Savoldi, M., Semighini, C.P., de Oliveira, R.C., Nunes, L.R., Travassos, L.R., Puccia, R., Batista, W.L., Ferreira, L.E., Moreira, J.C., Bogossian, A.P., Tekaia, F., Nobrega, M.P., Nobrega, F.G., Goldman, M.H., 2003. Expressed sequence tag analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: identification of putative homologues of *Candida albicans* virulence and pathogenicity genes. Eukaryot. Cell 2, 34–48.

Grigg, M.E., Bonnefoy, S., Hehl, A.B., Suzuki, Y., Boothroyd, J.C., 2001. Success and virulence in *Toxoplasma* as the result of sexual recombination between two distinct ancestries. Science 294, 161–165.

Heitman, J., 2006. Sexual reproduction and the evolution of microbial pathogens. Curr. Biol. 16, R711–R725.

Heitman, J., Kronstad, J.W., Taylor, J.W., Casselton, L.A., 2007. Sex in fungi: molecular determination and evolutionary implications. ASM Press, Washington, DC.

Hibbett, D.S., Binder, M., Bischoff, J.F., Blackwell, M., Cannon, P.F., Eriksson, O.E., Huhndorf, S., James, T., Kirk, P.M., Lucking, R., Thorsten Lumbsch, H., Lutzoni, F., Matheny, P.B., McLaughlin, D.J., Powell, M.J., Redhead, S., Schoch, C.L., Spatafora, J.W., Stalpers, J.A., Vilgalys, R., Aime, M.C., Aptroot, A., Bauer, R., Begerow, D., Benny, G.L., Castlebury, L.A., Crous, P.W., Dai, Y.C., Gams, W., Geiser, D.M., Griffith, G.W., Gueidan, C., Hawksworth, D.L., Hestmark, G., Hosaka, K., Humber, R.A., Hyde, K.D., Ironside, J.E., Koljalg, U., Kurtzman, C.P., Larsson, K.H., Lichtwardt, R., Longcore, J., Miadlikowska, J., Miller, A., Moncalvo, J.M., Mozley-Standridge, S., Oberwinkler, F., Parmasto, E., Reeb, V., Rogers, J.D., Roux, C., Ryvarden, L., Sampaio, J.P., Schussler, A., Sugiyama, J., Thorn, R.G., Tibell, L., Untereiner, W.A., Walker, C., Wang, Z., Weir, A., Weiss, M., White, M.M., Winka, K., Yao, Y.J., Zhang, N., 2007. A higher-level phylogenetic classification of the Fungi. Mycol. Res. 111, 509–547.

Kerenyi, Z., Moretti, A., Waalwijk, C., Olah, B., Hornok, L., 2004. Mating type sequences in asexually reproducing Fusarium species. Appl. Environ. Microbiol. 70, 4419–4423.

Kwon-Chung, K.J., 1972. Sexual stage of *Histoplasma capsulatum*. Science 175, 326.

Leclerc, M.C., Philippe, H., Gueho, E., 1994. Phylogeny of dermatophytes and dimorphic fungi based on large subunit ribosomal RNA sequence comparisons. J. Med. Vet. Mycol. 32, 331–341.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Li, W., White, T.C., Heitman, J., 2009. Organization and evolutionary trajectory of the mating type (MAT) locus in the dermatophyte and dimorphic fungal pathogens. Eukaryot. Cell. [Epub ahead of print].

Matute, D.R., McEwen, J.G., Puccia, R., Montes, B.A., San-Blas, G., Bagagli, E., Rauscher, J.T., Restrepo, A., Morais, F., Nino-Vega, G., Taylor, J.W., 2006. Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies. Mol. Biol. Evol. 23, 65–73.

McGinnis, M.R., 1980. Media and Reagents. Laboratory Handbook of Medical Mycology. Academic Press, Inc., New York. pp. 523–587.

Milgroom, M.G., 1996. Recombination and the multilocus structure of fungal populations. Annu. Rev. Phytopathol. 34, 457–477.

Morais, F.V., Barros, T.F., Fukada, M.K., Cisalpino, P.S., Puccia, R., 2000. Polymorphism in the gene coding for the immunodominant antigen gp43 from the pathogenic fungus *Paracoccidioides brasiliensis*. J. Clin. Microbiol. 38, 3960–3966.

Nino-Vega, G.A., Sorais, F., Calcagno, A.M., Ruiz-Herrera, J., Martinez-Espinoza, A.D., San-Blas, G., 2004. Cloning and expression analysis of the ornithine decarboxylase gene (PbrODC) of the pathogenic fungus *Paracoccidioides brasiliensis*. Yeast 21, 211–218.

O'Gorman, C.M., Fuller, H.T., Dyer, P.S., 2009. Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. Nature 457, 471–474.

Paoletti, M., Rydholm, C., Schwier, E.U., Anderson, M.J., Szakacs, G., Lutzoni, F., Debeaupuis, J.P., Latge, J.P., Denning, D.W., Dyer, P.S., 2005. Evidence for sexuality in the opportunistic fungal pathogen *Aspergillus fumigatus*. Curr. Biol. 15, 1242–1248.

Pyrzak, W., Miller, K.Y., Miller, B.L., 2008. Mating type protein Mat1-2 from asexual *Aspergillus fumigatus* drives sexual reproduction in fertile *Aspergillus nidulans*. Eukaryot. Cell 7, 1029–1040.

Restrepo, A., Jimenez, B.E., 1980. Growth of *Paracoccidioides brasiliensis* yeast phase in a chemically defined culture medium. J. Clin. Microbiol. 12, 279–281.

Restrepo, A., McEwen, J.G., Castaneda, E., 2001. The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle? Med. Mycol. 39, 233–241.

Restrepo, A., Tobón, A.M., Agudelo, C.A., 2008. Paracoccidioidomycosis. In: Hospenthal, A.M., Rinaldi, M.G. (Eds.), Infectious Diseases: Diagnosis and Treatment of Human Mycoses. Humana Press, Totowa, New Jersey, pp. 331–342.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: a Laboratory Manual. Harbor Laboratory Press, New York, Cold Spring.

San-Blas, G., Nino-Vega, G., Iturriaga, T., 2002. *Paracoccidioides brasiliensis* and paracoccidioidomycosis: molecular approaches to morphogenesis, diagnosis, epidemiology, taxonomy and genetics. Med. Mycol. 40, 225–242.

Stergiopoulos, I., Groenewald, M., Staats, M., Lindhout, P., Crous, P.W., De Wit, P.J., 2007. Mating-type genes and the genetic structure of a world-wide collection of the tomato pathogen Cladosporium fulvum. Fungal Genet. Biol. 44, 415–429.

Turgeon, B.G., Yoder, O.C., 2000. Proposed nomenclature for mating type genes of filamentous ascomycetes. Fungal Genet. Biol. 31, 1–5.

Untereiner, W.A., Scott, J.A., Naveau, F.A., Sigler, L., Bachewich, J., 2004. The Ajellomycetaceae a new family of vertebrate-associated Onygenales. Mycologia 96, 812–821.

Van Burik, J.A., Schreckhise, R.W., White, T.C., Bowden, R.A., Myerson, D., 1998. Comparison of six extraction techniques for isolation of DNA from filamentous fungi. Med. Mycol. 36, 299–303.

Yun, S.H., Arie, T., Kaneko, I., Yoder, O.C., Turgeon, B.G., 2000. Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella/Fusarium* species. Fungal Genet. Biol. 31, 7–20.