Molecular and Morphological Data Support the Existence of a Sexual Cycle in Species of the Genus *Paracoccidioides*

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The genus *Paracoccidioides* includes the thermomorphic species *Paracoccidioides brasiliensis* and *P. lutzii*, both of which are etiologic agents of paracoccidioidomycosis, a systemic mycosis that affects humans in Latin America. Despite the common occurrence of a sexual stage among closely related fungi, this has not been observed with *Paracoccidioides* species, which have thus been considered asexual. Molecular evolutionary studies revealed recombination events within isolated populations of the genus *Paracoccidioides*, suggesting the possible existence of a sexual cycle. Comparative genomic analysis of all dimorphic fungi and *Saccharomyces cerevisiae* demonstrated the presence of conserved genes involved in sexual reproduction, including those encoding mating regulators such as MAT, pheromone receptors, pheromone-processing enzymes, and mating signaling regulators. The expression of sex-related genes in the yeast and mycelial phases of both *Paracoccidioides* species was also detected by real-time PCR, with nearly all of these genes being expressed preferentially in the filamentous form of the pathogens. In addition, the expression of sex-related genes was responsive to the putative presence of pheromone in the supernatants obtained from previous cocultures of strains of two different mating types. *In vitro* crossing of isolates of different mating types, discriminated by phylogenetic analysis of the α-box (*MAT1-1*) and the high-mobility-group (HMG) domain (*MAT1-2*), led to the identification of the formation of young ascocarps with constricted coiled hyphae related to the initial stage of mating. These genomic and morphological analyses strongly support the existence of a sexual cycle in species of the genus *Paracoccidioides*.

Fungi can reproduce asexually or sexually. This characteristic, called pleomorphism (1), is commonly observed in members of the host-associated dimorphic fungal pathogens of the family Ajellomycetaceae (2). These species can reproduce asexually by hyphal fragmentation or conidium production in specialized hyphae or by budding in the yeast form. Also, a sexual cycle may occur. Mating via pheromone exchange in a heterothallic bipolar system in the presence of mycelial cells of different mating types has been described for *Ajellomyces dermatitidis* (3), *Ajellomyces capsulatus* (4, 5), *Ajellomyces parva*, and *Ajellomyces crescens* (6). The initial step of mating in species of the Ajellomycetaceae family is triggered by hyphal constriction of cells of different mating types, coiling with one another several times to produce young ascocarps. Once hyphal aggregations become tighter, the cleistothecium is formed, with evanescence of the ascus, which harbors meiosporic spores (4). Sexual reproduction followed by meiosis and recombination plays an important role in the evolution of pathogenic fungi, by fitting populations and altering their virulence traits (7, 8).

Mating processes in filamentous ascomycetes are controlled by the presence of the *MAT1-1* idiomorph, which encodes an alpha-domain transcription factor, while the *MAT1-2* idiomorph encodes a high-mobility-group (HMG)-domain transcription factor that confers sexual identity to a haploid cell (9). These alleles or idiomorphs exhibit low comparative sequence similarity and are responsible for fungal sexuality and control of mating. Dimorphic pathogenic fungi, including *Histoplasma capsulatum*, *Coccidioides immitis*, *Coccidioides posadasii*, *Paracoccidioides brasiliensis*, and *Paracoccidioides lutzii*, exhibit a syntenic organization which has previously been characterized by comparative genomic analysis (10, 11). Besides the MAT locus, molecules such as pheromones, pheromone receptors, and pheromone-processing and response enzymes are involved in the sexual process, acting in opposite cell recognition through nuclear regulation of the mating process.

*Paracoccidioides* species are thermomorphid fungal pathogens of humans; they are also adapted to other mammalian hosts, such as armadillos (12). Once infective propagules are inhaled by immunocompetent or immunodepressed humans, paracoccidioidomycosis can be induced (13). This systemic mycosis affects mainly rural workers in Latin America, frequently found in Brazil, Venezuela, and Colombia (14). Based on molecular phylogenetic tools, extensive genetic divergence has been detected among *Paracoccidioides* isolates. Applying the method of genealogical concordance for phylogenetic species recognition, two major species have been proposed: *P. brasiliensis*, with three phylogenetically cryptic species, i.e., S1, PS2, and PS3 (15); and *P. lutzii*, harboring “Pb01-like” strains, which have so far been collected mainly in Ecuador and the northern-central region of Brazil (16). Also, recombination events have been shown within these two *Paracoccidioides* species that could explain their highly genotypic and phenotypic diversity and which have prompted the hypothesis of a sexual cycle in these pathogens.

To test this hypothesis, the sexual genomic traits of the teleomorphic species *A. capsulatus* (anamorph *Histoplasma capsula-
tunt) and A. dermatitidis (anamorph Blastomyces dermatitidis) were searched in silico in Paracoccidioides species. The presence of a Paracoccidioides mating type locus, MAT1-1, which is a homologue of that in Aspergillus nidulans and other components of the pheromone response pathway, was previously reported from transcriptomic analyses of both morphological phases of P. lutzii strain Pb01 (17, 18). Torres et al. (19) identified the presence and expression of MAT1-1 and MAT1-2 in the P. brasiliensis phylotypic species complex, suggesting a sexual cycle in this fungus.

In the present report, we provide additional information about (i) a comparative genomic analysis of mating regulator genes in all dimorphic fungi with available genome data that supports vertical transfer of the genomic trait for sexual reproduction through the Ajellomycescaceae family; (ii) the predominant expression of six sex-related genes in mycelial cells, including the MAT locus and genes for the pheromone receptors and components of the pheromone response cascade; (iii) the distribution of mating type idiomorphs in clinical and environmental strains; and (iv) strong evidence of direct activation of the mating pathway in cocultured strains of opposite mating types and the in vitro development of the initial steps of sexual reproduction, namely, the formation of young ascocarps in mated strains of P. brasiliensis and P. lutzii, with the possibility of homothallism in the Paracoccidioides genus.

MATERIALS AND METHODS

Maintenance of Paracoccidioides strains. Environmental and clinical strains used in this study (see Table S1 in the supplemental material) were kept in the mycelial phase in semisolid potato dextrose agar (PDA; Acumedia) at 25°C and were subcultured every 21 days. Yeast cells were maintained in yeast-peptone-dextrose agar (YPD: 0.5% casein peptone, 0.5% yeast extract, 1.0% glucose) at 37°C and were subcultured every 15 days.

Quantitative expression of sex-related genes in yeast and mycelial forms. To assess the levels of expression of sex-related genes (the α-box gene MAT1-1, the HMG gene MAT1-2, the pheromone receptor genes ste2 and ste3, the mitogen-activated protein kinase [MAPK] gene fus3, and the transcriptional factor gene ste2), we used real-time reverse transcription-PCR (RT-PCR). P. brasiliensis strains Pb18, Pb5, T15LN1, T1F1, and T9B1 and P. lutzii strains Pb01 and EE were cultured in yeast and mycelial forms in YPD for 14 days at 37°C and 25°C, respectively. Cells were harvested by centrifugation (5,000 × g for 10 min), and RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. To remove any genomic DNA contamination, RNA was treated with RNase-free DNase I (Promega), precipitated, and used for real-time RT-PCR as described above.

DNA extraction and population analysis of MAT locus distribution in Paracoccidioides species. DNA extractions were performed as described by Raeder and Broda (20), with modifications. Briefly, about 100 mg of either yeast cells or mycelium from each isolate was suspended in 500 µl of extraction buffer (25 mM EDTA, 250 mM NaCl, 0.5% [wt/vol] sodium dodecyl sulfate [SDS], 200 mM Tris-HCl, pH 8.5), to which 500 mg of 425- to 600-µm-diameter glass beads (Sigma-Aldrich) was added. The mixture was shaken vigorously for 15 min. A mixture of phenol-chloroform-isooamyl alcohol (25:24:1 [vol/vol/vol]; 500 µl) (BioAgency, Germany) was added to the solution, briefly mixed, and centrifuged for 60 min at 12,000 × g. The aqueous phase was treated with RNase A (final concentration, 100 µg/ml; Fisher Scientific) for 15 min at 37°C. An equal volume of chloroform was added and roughly mixed, and the solution was centrifuged for 10 min at 12,000 × g. The aqueous phase was precipitated with 0.5x (vol/vol) isopropanol by centrifugation for 20 min at 12,000 × g and 4°C. DNA was washed with 70% ethanol, dried at room temperature, and eluted with ultrapure water. The DNA integrity was confirmed by 0.8% agarose gel electrophoresis with ethidium bromide (0.5 µl/ml) staining.

For PCR amplification, 20 to 50 ng of genomic DNA from each isolate (see Table S1 in the supplemental material) was used for PCR amplification. Primers were designed (see Table S2) to target regions inside the open reading frames (ORFs) of MAT1-1 (α-box domain) and MAT1-2 (HMG domain) sequences taken from the P. brasiliensis genome (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html). PCR amplifications were done in a Veriti 96-well thermocycler (Applied Biosystems) and using PyroStart PCR master mix (Fermentas) as recommended by the manufacturers. The annealing temperature for both regions was 60°C. PCR samples were loaded into 0.8% agarose gels and analyzed by electrophoresis to identify the sexual idiomorph of each isolate.

Sequencing and phylogenetic analysis. Sixteen amplicons from MAT1-1 strains and 11 amplicons from MAT1-2 strains were selected for sequencing. Sequences of the amplicons were determined by automatic capillary Sanger sequencing in a MegaBACE 500 machine, using a DYEnamic ET dye terminator kit (GE Healthcare, United Kingdom). Fragments were sequenced on both strands, and bases with phred scores of >30 were considered for alignments, increasing the quality of the sequence data. The MAT1-1 (GenBank accession numbers HQ687769 to HQ687784) and MAT1-2 (GenBank accession numbers HQ687785 to HQ687795) sequences were aligned using the PRANK algorithm (21) implemented in PRANKSTER software and manually corrected in order to avoid mispaired bases. Maximum parsimony analyses were carried out using PAUP, version 4.0b10a (22). Unweighted parsimony was chosen, and character transformation states were considered disordered. Heuristic searches were performed by random stepwise sequence addition in order to obtain the initial tree; branch rearrangements were made by the tree bisection and reconnection (TBR) technique. One thousand bootstrap replicates were used to estimate confidence values for individual clades. Significant branches with bootstrap values of ≥50% were consid-
Mating tests. Mating assays were performed on pairwise MAT1-1 and MAT1-2 strains by slide culture and direct streaking of isolates on agar medium (see Table S3 in the supplemental material). The media selected for both mating methods were V8 juice agar (20% [vol/vol] Campbell’s V8 juice, 0.3% CaCO3, and 1.5% agar), soil extract agar (SEA; 0.2% glucose, 0.1% yeast extract, 1.5% agar, and 50% [vol/vol] soil extract collected from armadillo burrows), and oatmeal tomato paste agar (OTA; 2% Elefante’s tomato paste, 2% Dr. Oetker’s oatmeal flakes, 0.3% CaCO3, and 1.5% agar). Crossing combinations were performed with isolates from the same phylogenetic species: *P. brasiliensis* (15) and *P. lutzii* (16). For slide culture mating tests, plugs of mycelia from strains of each mating type were planted onto an agar block pairwise in a side-by-side pattern. For mating assays performed in plates, mycelial fragments were planted about 1 cm apart. Plates were sealed with Parafilm (Pechiney Plastic Packaging) and kept at 25°C in the dark. Mating crossings were visualized by stereomicroscopy using a Stemi 2000C apparatus (Zeiss) and by light microscopy with lactophenol cotton blue staining monthly for up to 8 months. The presence or absence of sex-related structures was documented in a model PM-30 photomicrographic system (Olympus, Japan) by inspection of at least five random microscopic fields.

Confocal microscopy. Slide cultures made as described above were also used for confocal microscopy experiments. The cocultured strains used were JAL × EE (*P. lutzii*) and T1F1 × T15LN1 (*P. brasiliensis*). After mating of the strains, the coverslips were removed, fixed, and permeabilized with 70% ethanol at 4°C overnight. The coverslips were then stained for 24 h with 1× SYBR green and for 1 h with 20 μg/ml calcofluor white. After washing, the coverslips were mounted with Prolong Gold antifade medium (Invitrogen), sealed, and imaged in a Leica SP5 laser scanning confocal microscope equipped with a 63×/1.4-numerical-aperture (NA) objective. Confocal images were processed using ImageJ and Adobe Photoshop software, without any nonlinear processing.

RESULTS

Identification of sex-related genes. The majority of components of the pheromone response pathway were inferred by comparative analysis of the genome sequences of the dimorphic fungi *P. brasiliensis*, *P. lutzii*, *H. capsulatum*, *B. dermatitidis*, *C. immitis*, and *C. posadasii* (see Table S4 in the supplemental material). The data revealed a conserved mating machinery that was vertically transmitted in all dimorphic fungal genomes. These data also helped in the selection of isolates from the same phylogenetic cluster for mating assays. Twenty-one isolates of *P. brasiliensis* and 24 isolates of *P. lutzii* were examined for the *MAT* locus, and they exhibited both homothallic and heterothallic profiles. Fifty-five isolates possessed the *MAT1-1* locus and 30 isolates possessed the *MAT1-2* locus, as demonstrated by single-locus amplification (Fig. 1A). Unexpectedly, 13 isolates presented both *MAT1-1* and *MAT1-2* amplified loci, suggesting that homothallicism could exist in the *Paracoccidioides* genus. The prevalences of heterothallic *MAT1-1* and *MAT1-2* and homothallic *MAT1-1*/ *MAT1-2* populations were 56%, 31%, and 13%, respectively, which approximated a ratio of 4:2:1 (Fig. 1B). Amplicons from 16 *MAT1-1* strains and 11 *MAT1-2* strains were sequenced and submitted to phylogenetic analysis. The *MAT* locus cladogram clearly revealed the genetic divergence of the isolates belonging to the *P. brasiliensis* species complex and *P. lutzii*, in agreement with the new species proposal (16). The *P. lutzii* and *P. brasiliensis* standard strains Pb01 and Pb18, respectively, were clustered apart from each other, which helped in species recognition (Fig. 1C and D). These data also helped in the selection of isolates from the same phylogenetic cluster for mating assays. Twenty-one isolates of *P. lutzii* and 24 isolates of *P. brasiliensis* (S1 and PS2 group) were analyzed separately to verify whether the distribution of mating type ratios was equivalent inside each species of *Paracoccidioides*. Considering the two species apart, the ratio of heterothallic...
MAT1-1 (12 isolates of *P. lutzii* and 16 isolates of *P. brasiliensis*) and MAT1-2 (6 isolates of *P. lutzii* and 4 isolates of *P. brasiliensis*) strains and homothallic strains (3 isolates of *P. lutzii* and 4 isolates of *P. brasiliensis*) was 4:2:1 for *P. lutzii* and 4:1:1 for *P. brasiliensis*. Also, we evaluated 12 isolates, belonging to *P. brasiliensis* species S1 and PS2, recovered from armadillos in Botucatu City and surrounding neighborhoods located in the central-south region of São Paulo State, Brazil (11). The idiomorphic distribution of the MAT locus for these isolates was 4:2:1. The MAT locus data revealed a slight predominance of heterothallic MAT1-1 isolates, followed by MAT1-2 and putative homothallic MAT1-1/MAT1-2 isolates, in *Paracoccidioides* species, even between species or in isolates recovered from armadillos.

Expression of sex-related genes in yeast and mycelial cells of *Paracoccidioides* species. To assess the expression of genes involved in the mating process, the mRNA levels of MAT1-1 (α-box), MAT1-2 (HMG box), the pheromone receptor genes ste2 and ste3, the MAPK gene fus3, and the transcriptional factor gene ste12 were quantified by real-time RT-PCR analysis of yeast and mycelia of seven isolates of *P. lutzii* and *P. brasiliensis*. MAT1-1 and MAT1-2 genes were expressed exclusively, in a cell type-specific manner, in the heterothallic strains. Surprisingly, we detected differential expression of the MAT genes in the putative homothallic isolate Pb18: MAT1-1 was expressed only in the yeast phase, while MAT1-2 was expressed only in the mycelium phase. In general, all genes were expressed more exuberantly in the mycelium phase, in a species-independent fashion and irrespective of whether the isolate was homothallic or heterothallic (Fig. 2). The MAPK gene (fus3) was differentially expressed in six of seven analyzed isolates. The higher level of fus3 mRNA in the mycelial phase could be related to the saprophytic lifestyle required for the mating process in *Paracoccidioides*.

**Evidence of pheromone induction in *P. brasiliensis* strains T9B1 and T15LN1.** The cocultivation of haploid compatible a- and α-cells in minimal medium induces pheromone production in some ascomycetes, as already reported for *S. cerevisiae* (36) and for the dimorphic fungus *H. capsulatum* (37). We tested the effect of mating stimulation by the CM of cocultured T9B1 (MAT1-1) and T15LN1 (MAT1-2) strains. After 24 h of stimulation of mycelial cells, total mRNA was extracted and the sex-related genes were quantified by real-time RT-PCR (Fig. 3). All genes of the mating cascade were highly upregulated after CM stimulation, except for the pheromone receptor gene (ste3) in the T9B1 isolate, indicating an activation of the mating machinery by a- and α-pheromone molecules, probably due to their presence in the CM. The pheromone-induction test was carried out only on T9B1 and T15LN1 isolates of the *P. brasiliensis* S1 cryptic species, because these two strains were able to produce initial ascocarps in mating assays. More experiments are in progress to extend the results to other strains of *P. brasiliensis* (the PS2 cryptic species) and *P. lutzii*. Even so, the available data are extremely important in guiding further studies of mating in dimorphic fungi.

**Mating assays.** The coincubation of MAT1-1 and MAT1-2 strains of *P. brasiliensis* (S1) and *P. lutzii* revealed coiled hyphae, indicating the formation of young ascocarps (Fig. 4; see Table S3 in the supplemental material). These results were also obtained previously by laboratory crossings with their relatives *H. capsulatum* and *B. dermatitis* (3, 4). Coiled hyphae were observed in the paired T1F1 × T15LN1 and T9B1 × T15LN1 *P. brasiliensis* iso-
lates (Fig. 4A to F). In *P. lutzii*, the coiled hyphae were also observed for JAL/H11003 EE, 66/H11003 EE, and PLRO1/H11003 ED01 strains (Fig. 4G to J). Undifferentiated hyphal tips began to wind around and multiply encircle hyphae of the opposite mating type beginning 2 months after cocultivation (Fig. 4A and G). The hypha constriction tightened into numerous coils 4 to 6 months after cocultivation (Fig. 4B and F). In Fig. 4J, many coils are observed in one field for the 66/H11003 EE *P. lutzii* crossing. The paired isolates, whether *P. brasiliensis* or *P. lutzii*, produced sexual structures, and no morphological alterations in young ascocarp formation were observed in the three different media (OTA, V8 juice agar, and SEA) used for mating experiments. Mating assays with *P. brasiliensis* isolates belonging to the PS2 phylogenetic species—Pb2 × Pb3 and Pb4 × Pb3—or with the putative homothallic isolate Pb18 did not produce any coiled hyphae, indicating an absence of sexual structure formation (see Table S3). No mature cleistothecium or ascus formation was observed, even 8 months after mating of cocultures. Mating of the standard laboratorial strains Pb18 and Pb01 also did not result in the production of sexual structures, likely because they were maintained in culture for a long time and lost their mating ability, as has clearly been demonstrated for *H. capsulatum* (5, 37, 38).

To further evaluate the coiled hypha structure, we stained JAL/H11003 EE (*P. lutzii*) (Fig. 5A) and T1F1 × T15LN1 (*P. brasiliensis*)

![FIG 2 Quantitative expression of the sex-related genes MAT1-1 (α-box), MAT1-2 (HMG), ste2 and ste3 (pheromone receptors), fus3 (MAPK), and ste12 (transcriptional factor) in yeast and mycelial phases of the homothallic strain Pb18 (A) and the heterothallic strains Pb3 (B), Pb01 (C), T1F1 (D), EE (E), T9B1 (F), and T15LN1 (G).](image-url)
Phylogenetic analysis revealed that the MAT1-1 and MAT1-2 loci of *P. brasiliensis* Pb3 and Pb18 and *P. lutzii* Pb01 were identified based on comparative genomic analyses with other dimorphic fungal pathogens and dermatophytes (11). The MAT locus of the Paracoccidioides genus represents a syntenic organization and locus expansion compared with the case in *H. capsulatum*, *C. immitis*, and *C. posadasii* (10, 11, 37). The presence of a single copy of each mating type gene (MAT1-1 and MAT1-2) in the Paracoccidioides genome (Pb18, Pb3, and Pb01) points to a potential bipolar mating system. The genomic identification of the mating components in Paracoccidioides and other dimorphic fungi led us to hypothesize that the genetic apparatus for sexual reproduction is conserved and vertically transferred among these fungi. The sexual cycle has been demonstrated for *H. capsulatum* and *B. dermatitidis*, and all orthologs were identified in their genomes compared to *P. brasiliensis* and *P. lutzii* (see Table S4 in the supplemental material).

The ratio of MAT1-1/MAT1-2 idiomorphs in bipolar fungal species is often 1:1 in sexual populations; this was observed for *C. immitis*, *C. posadasii* (40), *Aspergillus fumigatus* (41, 42), and, more recently, *P. brasiliensis* (19). In contrast, a high prevalence of MAT1-2 was observed in patients with disseminated histoplasmosis (43). Also, α-cells of the pathogenic basidiomycete *Cryptococcus neoformans* are 30 to 40 times more frequent than α-cells in both environmental and clinical samples (44). The high frequency of α-strains in clinical isolates has been correlated with their capacity to infect or colonize hosts (45). In the present study, there was a slight predominance of the MAT1-1 mating type, as well as the presence of both idiomorphs in a single genome, when *P. lutzii* and *P. brasiliensis* populations were considered together or individually (56% MAT1-1, 31% MAT1-2, and 13% MAT1-1/MAT1-2).

In this work, expression analysis of the mating components (MAT1-1, MAT1-2, ste2, ste3, fus3, and ste12) in yeast and mycelial cells of several *Paracoccidioides* isolates revealed the preferential expression of these genes in the filamentous phase (Fig. 2). This study also investigated the ability of the supernatant (CM) of cocultured mycelia from the T9B1 (MAT1-1) and T15LN1 (MAT1-2) strains in YEM medium after 21 days of growth to stimulate pheromone production. CM stimulation in individual strains provoked an upregulation of the components of the mating/pheromone pathway, providing evidence that α- and α-cells of *P. brasiliensis* respond to CM stimulation (Fig. 3). These results agree with those reported for *S. cerevisiae* (46) and *H. capsulatum* (37). The present results are also consistent with the mating behavior of the Ajellomycetaceae species *H. capsulatum* and *B. dermatitidis*, since functional analysis of sex-related genes revealed an upregulation in the mycelium stage and the capability to respond to CM containing pheromone.

Homothallism is an intrinsic characteristic of fungi in which a single individual harbors both mating types and can undergo sexual reproduction. In *Aspergillus* species, the homothallic characteristic appears to be an ancestral state and heterothallism is acquired after speciation (47), as has also been reported for the closely related *Cochliobolus* and *Stemphylium* genera (48). The present study provides the first evidence of the possibility of homothallism in the Ajellomycetaceae family. Both MAT1-1 and MAT1-2 idiomorphs were present in a single individual, as confirmed by PCR analysis of 15 *Paracoccidioides* isolates (Fig. 1). Phylogenetic analysis revealed that the MAT1-1 and MAT1-2 loci from isolates Pb18 and 7455 clustered with their respective species, *P. brasiliensis* and *P. lutzii* (Fig. 1). Phylogenetic analysis of MAT1-1 positioned isolate 133 (which contains both MAT loci) within *P. brasiliensis*, while the MAT1-2 idiomorph clustered it together with *P. lutzii*, so this isolate shows genomic signatures of both *Paracoccidioides* species. Thus, consistent with a recent suggestion (16), the evidence supports isolate 133 as a hybrid or an ancestral state of *Paracoccidioides*. Our results suggest that the *Paracoccidioides* genus, or at least its common ancestor, could be homothallic. In the homothallic species *A. nidulans* (a filamentous fungus), both MAT1-1 and MAT1-2 are coexpressed under *in vitro* mating conditions (41). In contrast, gene expression analysis of the MAT genes in the *P. brasiliensis* Pb18 strain (putatively homothallic) revealed a differential pattern, with the α-box gene (MAT1-1) detected only in the yeast phase and the HMG gene (MAT1-2) detected only in the mycelial cells. Thus, what we determined to be “homothallic” isolates (MAT1-1/MAT1-2) could

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**FIG 3** Pheromone stimulation after coculture of strains of opposite mating types (T9B1 [MAT1-1] and T15LN1 [MAT1-2]) in Erlenmeyer flasks containing YEM medium (CM). mRNA levels of the sex-related genes MAT1-1 (α-box), MAT1-2 (HMGI), ste2 and ste3 (pheromone receptors), fus3 (MAPK), and ste12 (transcriptional factor) were evaluated in isolates T9B1 (A) and T15LN1 (B), grown independently and stimulated for 24 h with the previously collected CM or with YEM medium (negative control).
be hybrid strains. We listed isolate 133 as an example of strains where, based on sequence information, MAT1-1 and MAT1-2 are derived from *P. brasiliensis* and *P. lutzii* (47). Thus, these strains are not likely “true” homothallic strains but are fused products of interspecies or intraspecies matings between MAT1-1 and MAT1-2 strains. This is consistent with these species being heterothallic. These particular isolates are stuck in the hybrid state, probably due to an impaired ability to undergo meiosis, consistent

**FIG 4** Initial stages of sexual development in *Paracoccidioides* species. The sexual structures were obtained by mating experiments using V8, SEA, or OTA medium, and the samples were monitored for 6 months. The description of each panel gives the following information: isolates crossed, time of incubation (months), medium used, and magnification. (A) T9B1 × T15LN1, 2, SEA, ×400; (B) T9B1 × T15LN1, 4, SEA, ×400; (C) T9B1 × T15LN1, 4, SEA, ×400; (D) T9B1 × T15LN1, 6, SEA, ×400; (E) T1F1 × T15LN1, 4, SEA, ×400; (F) T1F1 × T15LN1, 6, SEA, ×400; (G) ED01 × PLRO1, 2, OTA, ×400; (H) ED01 × PLRO1, 4, OTA, ×400; (I) 66 × EE, 6, OTA, ×400; (J) 66 × EE, 4, V8, ×100.
The mating interactions led us to suggest an exchange of genetic material between cells of opposite mating types (Fig. 5). Such observations are well described for *A. nidulans*, *Schizopyllum commune*, and *Ustilago maydis* during the initial steps of mating, suggesting that it is a consequence of nuclear migration (56–58). These results were obtained in mating experiments using V8, SEA, and OTA media, with samples being monitored for 6 months. The resemblance of the coiled hypha aggregates to immature ascocarps echoes previous observations in *H. capsulatum*, *B. dermatitidis*, *Emmonsia* spp., *A. fumigatus*, *A. nidulans*, and *Trichophyton mentagrophytes*, whose sexual cycles are known (3, 4, 59–62).

FIG 5 Confocal microscopy examination of *Paracoccidioides* species sexual structures. Samples from mating experiments using SEA medium and JAL × EE cocultures (*P. lutzii*) (A) or T1F1 × T15LN1 cocultures (*P. brasiliensis*) (B) were stained with calcofluor white, which labels cell wall chitin and is pseudocolored green, and SYBR green, which labels DNA in nuclei and is pseudocolored red. The arrows show coiled hyphae (CH) and three knob-like structures (K). In the coiled hyphae shown in detail in panel B, blue arrowheads point to single nuclei in adjacent cells and the yellow arrowhead points to a single cell in the sexual structure with four individual nuclei.

With the increase in fungal genome sequencing projects, many questions regarding the biology of mating in fungi are being answered. Components of the pheromone-dependent pathway involved in the mating system in ascomycetes are well characterized, as demonstrated for *Saccharomyces cerevisiae* (63), *Neurospora crassa* (6), and *Aspergillus fumigatus* (64). Combining structural and functional genomics and mating assays, we have shown that the sexual cycle of *Paracoccidioides* must occur during its sapro-

with speciation. Strains of the basidiomycete *C. neoformans* serotype hybrid AD, which are intervarietal or interspecies hybrids, cannot undergo meiosis due to genetic divergence (49).

The ploidy of *Paracoccidioides* was assessed by different methods, leading to controversial results. PFGE combined with microfluorometry (50–53) revealed that genome sizes vary from 23 to 31 Mb, and microfluorometric data indicated twice the genome content (46 to 61 Mb), indicating that *P. brasiliensis* yeast cells could be diploid, haploid, or at least aneuploid (50, 51). Also, by means of flow cytometry combined with the haploid genome size previously measured by electrophoretic karyotyping, the ploidy was assessed in several *P. brasiliensis* isolates, revealing haploid or aneuploid nuclei (54). Another possibility for the amplification of both mating type genes is that these isolates represent mixed populations of two heterothallic strains. This consideration should not be discarded, but we demonstrated a differential expression of *MAT1-1* and *MAT1-2* for Pb18, recovered from single colonies, indicating a single genotype. Since the diploid and/or aneuploid state was observed for Pb18 isolates by Feitosa et al. (51) and Almeida et al. (54), “homothallism” in *Paracoccidioides* species must be investigated further.

Fungal sexual development is initiated by cell chemoattraction that is governed by pheromone induction. In filamentous and dimorphic fungi, the initial ascocarp is developed when hyphae of opposite mating types match and start to coil with each other (4). The present study offers the first evidence for initial *in vitro* formation of ascocarps under mating conditions for *P. brasiliensis* (S1 group) and also for *P. lutzii* (Fig. 4). Knob-like structures were found in regions of coiled hyphae in the mating assay (Fig. 5). Similar results were observed during ascogenesis of *H. capsulatum* and also *Mucor circinelloides*, suggesting that these structures are important for the maintenance of ascocarp development (4, 55). Moreover, the occurrence of a multinucleated single hypha cell in the mating interactions led us to suggest an exchange of genetic material between cells of opposite mating types (Fig. 5). Such observations are well described for *A. nidulans*, *Schizopyllum commune*, and *Ustilago maydis* during the initial steps of mating, suggesting that it is a consequence of nuclear migration (56–58). These results were obtained in mating experiments using V8, SEA, and OTA media, with samples being monitored for 6 months. The resemblance of the coiled hypha aggregates to immature ascocarps echoes previous observations in *H. capsulatum*, *B. dermatitidis*, *Emmonsia* spp., *A. fumigatus*, *A. nidulans*, and *Trichophyton mentagrophytes*, whose sexual cycles are known (3, 4, 59–62).

Coiled hyphae were detected after 2 months of mating assays of isolates of opposite mating types for both species of *Paracoccidioides*; in fact, this is the first step of ascogenesis, as also reported for *H. capsulatum* and *Aspergillus* spp. (4, 60). However, no cleistothecium or ascus production was detected, even after 1 year of cocultivation. Further studies are being performed to find key environmental or physical conditions that could allow for cleistothecium and recombinant ascospore formation in *Paracoccidioides*, resulting in the description of a complete sexual stage. The balanced regulation of *MAT* locus expression could be an important factor for the progression of a sexual cycle, and specific environmental conditions might have to be present to allow sexual differentiation. Despite the detection of increased levels of transcripts of mating genes in hyphae, no correlation was observed between the expression levels of the sex-related genes and the ability to produce young ascocarps.

With the increase in fungal genome sequencing projects, many questions regarding the biology of mating in fungi are being answered. Components of the pheromone-dependent pathway involved in the mating system in ascomycetes are well characterized, as demonstrated for *Saccharomyces cerevisiae* (63), *Neurospora crassa* (6), and *Aspergillus fumigatus* (64). Combining structural and functional genomics and mating assays, we have shown that the sexual cycle of *Paracoccidioides* must occur during its sapro-
phytic life cycle. In addition, we have identified all orthologs of the mating machinery in both C. immitis and C. posadasii (see Table S4 in the supplemental material), although no evidence of sexual structures has yet been uncovered. It is well known that the Coccioidioides genus represents a cryptic speciation (65), and there is also evidence of a recombination process in this genus (66), supporting the existence of a sexual cycle. Furthermore, the ratio of MAT1-1/MAT1-2 idiomorphs is 1:1, providing molecular support for the presence of a sexual stage in the life cycle (40). The human pathogen A. fumigatus was long considered asexual. Genomic comparisons with S. cerevisiae and other ascomycetes and mRNA expression analyses of sex-related genes were revealed prior to the discovery of the sexual state of A. fumigatus (teleomorph = Neosartorya fumigata), thus showing the relevance of genomic data in defining the mating behavior of fungi (61, 64).

The other example is Trichophyton rubrum, which was also once considered asexual, since its mating machinery was discovered by comparative genomic analysis (11) and was recently confirmed by mating assays (67). Collectively, these data provide important information about the sexual behavior of fungi, especially for Paracoccidioides, and could be useful for further studies regarding the biology and speciation of this genus.

ACKNOWLEDGMENTS

We thank Rosane Christine Hahn, Eduardo Honda, and Patricia Cisalpino for supplying F. lutzii isolates and Erika Seki Kishishima for helping in improvement of the final artwork. This work was supported by Fundação de Amparo à Pesquisa do Distrito Federal (FAP-DF) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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