Characterization of a Paracoccidioides spp. strain from southeastern Brazil genotyped as Paracoccidioides restrepiensis (PS3) and review of this phylogenetic species

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Abstract

Phylogenetic species of Paracoccidioides brasiliensis complex (S1a and S1b, PS2, PS3, and PS4) and Paracoccidioides lutzii are agents of paracoccidioidomycosis, an endemic fungal disease in Latin America. P. restrepiensis (PS3 genotype) was classified as monophyletic and geographically restricted to Colombia and neighboring territories. BAT (or Pb-327B) was isolated from a patient living in the southeast region of Brazil but with genotype similar to Colombian Paracoccidioides spp. strains. This study aimed to define the phylogenetic species of BAT isolate by using additional genotyping methods, as well as reviewing the epidemiological and clinical studies related to P. restrepiensis isolates. Genomic DNA of BAT isolate and reference strains of P. brasiliensis sensu stricto (S1b), P. americana (PS2), P. restrepiensis (PS3), and P. lutzii were analyzed by conventional polymerase chain reaction (PCR) of partial gp43 exon 2 loci, by PCR-RFLP technique of tub1 gene, and by sequencing of the whole gp43 exon 2 loci. Here, we show that BAT isolate belongs to P. restrepiensis species, which is an unusual identification in southeastern Brazil, where P. brasiliensis sensu stricto is the prevalent genotype. This identification has relevance for geographical distribution and propagation of the genus Paracoccidioides in South America.

Keywords: Paracoccidioides restrepiensis, Paracoccidioides brasiliensis PS3, phylogenetic species, evolution, paracoccidioidomycosis epidemiology.

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Introduction

Paracoccidioidomycosis (PCM) is a systemic fungal infection endemic and restricted to Latin American countries such as Brazil, Argentina, Colombia, and Venezuela (Martínez, 2017). Pathogens that cause the acute and chronic forms of PCM are thermomorphic fungi belonging to the genus Paracoccidioides, family Ajellomyccetaeae, order Onygenales, class Eurotiomycetes, and species Paracoccidioides brasiliensis and Paracoccidioides lutzii (Gonzalez and Hernandez, 2016). P. brasiliensis clade is composed of five phylogenetic species, in which S1a and S1b belong to the paraphyletic group distributed in Brazil, Argentina, Paraguay, Peru, and Venezuela; PS2 belongs to the monophyletic group distributed in Brazil and Venezuela; PS3 belongs to the monophyletic group found mainly in Colombia; and the PS4 monophyletic group is found exclusively in Venezuela (Matute et al., 2006; Carrero et al., 2008; Teixeira et al., 2009; Teixeira et al., 2014; Muñoz et al., 2016). Turissini et al. (2017) analyzed microsatellites, mitochondrial and nuclear genes, proposing four new species belonging to the genus Paracoccidioides: P. brasiliensis sensu stricto (S1a and S1b), P. americana (PS2), P. restrepiensis (PS3), and P. venezuelensis (PS4). These species show among them genotypic and micromorphological divergences (Turissini et al., 2017). The P. lutzii clade contains exclusively P. lutzii (Teixeira et al., 2009).

Phylogenetic species 3 (PS3), now P. restrepiensis, was characterized by Matute et al. (2006) and classified as monophyletic, geographically restricted to Colombia, and considered an evolutionary lineage independent of other phylogenetic species of Paracoccidioides spp. complex. The same authors described the phylogenetic relationship of P. restrepiensis (PS3) with other species of P. brasiliensis.
complex, showing ancestral proximity to *P. brasiliensis sensu stricto* (S1a and S1b), but having a greater genetic distance from *P. americana* (PS2). Munõz et al. (2016), when analyzing genotypic divergences among the phylogenetic species, verified the ancestral proximity of Colombian *P. restrepiensis* (PS3) isolates with Venezuelan isolates of *P. venezuelensis* (PS4) and Argentinian and Brazilian isolates of *P. brasiliensis sensu stricto* (S1a and S1b). Besides the genetic proximity of *P. restrepiensis* (PS3) to other phylogenetic species of *P. brasiliensis* complex, Roberto et al. (2016) characterized two strains (human isolate chronic form PCM, and soil isolate) obtained in the Venezuelan territory as PS3 (now *P. restrepiensis*), suggesting its regional dissemination in South America.

This study aimed to characterize a clinical isolate from southeastern Brazil as *P. restrepiensis* (PS3), an unusual finding in such geographical area. Additionally, a review has been presented with studies on human and environmental isolates of the same genotype.

**Material and Methods**

*Paracoccidioides* spp. isolates and culture conditions

BAT (also known as Pb-327-B) clinical strain was isolated in 1985 from a suppurated lymph node of a patient resident in a city belonging to the metropolitan region of Ribeirão Preto, São Paulo State, Brazil (21°10’13.44” S and 47°48’37.17” W). The patient was a 33-year-old male rural worker who had the subacute form of PCM manifested by generalized lymphadenomegalgy, hepatosplenomegalgy, disseminated cutaneous lesion, fungal lesions in duodenal and colonic mucosa, and jaundice. The patient denied previous disease history or travel to other Brazilian states and South American countries. PCM diagnosis was supported by *Paracoccidioides* spp. isolation in culture, histopathological examination of intestinal lesions, and a 1:1024 serum titer in the counterimmunoelectrophoresis for anti-Paracoccidioides spp. antibodies. The patient obtained clinical cure after two years of treatment with sulfa drugs.

The following reference strains, whose genotypes were determined in other studies, were employed for BAT clinical isolate comparison: Pb 18 – representative of *P. brasiliensis sensu stricto* (S1b) species (Matute et al., 2006); Pb dog-EPN 194-representative of *P. americana* (PS2) species and T2-EPN 54-representative of *P. restrepiensis* (PS3) species (Roberto et al., 2016); and Pb 01 representative of *P. lutzii* (Teixeira et al., 2009). All the strains are maintained by successive subcultures on Sabouraud Agar Dextrose medium (Oxoid) plus 0.15 g l⁻¹ chloramphenicol sodium succinate (Blau Farmacêutica), and incubated at 25 °C. The study was approved by the Research Ethics Committee of the Hospital das Clínicas of Ribeirão Preto Medical School, University of São Paulo (Protocol HCRP nº 4456/2017).

Genomic DNA extraction of *Paracoccidioides* spp. strains

The genomic DNA of *Paracoccidioides* spp. strains were obtained from the fungal mycelia, which were grown in a synthetic modified McVeigh-Morton liquid medium for 35 days at 25 °C in an orbital shaker at 130 rpm (Infors HT-Ecotron) (Restrepo and Jimenez, 1980). The mycelia were subjected to extraction of genomic DNA according to the method I (treated glass beads and phenol-chloroform-isooamyl alcohol), with minimal modifications (van Burik et al., 1998). The genomic DNA was treated with 300 ng ml⁻¹ RNase A® (Thermo Fisher Scientific) at 37 °C for one hour. The concentration of genomic DNA was determined by using NanoDrop 2000® (Thermo Fisher Scientific) and its integrity checked in 1% agarose gel using SYBR® Safe DNA gel stain (Thermo Fisher Scientific) and visualized using the ChemiDoc XRS+ imager with Image Lab software (Bio-Rad).

Partial gp43 exon 2 loci PCR amplification

To identify and classify BAT clinical isolate into the genus *Paracoccidioides*, the genomic DNA of *Paracoccidioides* spp. reference strains and BAT isolate were submitted to partial amplification of the gp43 exon 2 loci by using the primers gp43-E2F: (5'-CCA GGA GGC GTG CAG GTG TTC C - 3') and gp43-E2R: (5'-GCC CCC TTC GTC TTC CAT GTG C - 3) (Cisalpino et al., 1996; Roberto et al., 2016) at 10 mM concentration, and annealing temperature at 58 °C. PCR reaction was performed with Taq polymerase enzyme-GoTaq® Green Master Mix (Promega) according to the manufacturer’s instructions. The final volume of PCR reaction was 25 µl containing 500 ng genomic DNA. Thermocycling was performed in the Vapo Protect® thermocycler (Eppendorf). PCR products, approximately 533 bp, had their integrity verified in 2% agarose gel by using SYBR® Safe DNA gel stain (Thermo Fisher Scientific). Its molecular weight was determined by 100-bp Ladder marker, Ready-To-Use (Sinapse), and visualized and photographed on the ChemiDoc XRS+ imager with Image Lab software (Bio-Rad).

Polymerase Chain Reaction – Restriction Fragment Length Polymorphism of tub1 gene – PCR-RFLP

Phylogenetic species identification of BAT clinical isolate was made according to Roberto et al. (2016). Briefly, PCR-RFLP of alpha-tubulin (*tub1*) gene was performed with Taq polymerase-GoTaq® Green Master Mix enzyme (Promega). The final volume of PCR reaction was 25 µl containing 500 ng genomic DNA and the primers *tub1*F: (5'-CTG GGA GGT ATG ATA GTC C - 3) and *tub1*R: (5'-CGT CGG GCT ATT CAG ATT TAA G - 3') (Kasuga et al., 2002; Roberto et al., 2016) at a concentration of 10 mM, and annealing temperature of 58 °C. PCR *tub1* products (263 bp) were cleaved with *Bcl* and *MspI* endonucleases (Thermo Fisher Scientific) at a concentration of 10 U µL⁻¹ each at 37 °C per 16 hours, according to manufacturer’s instructions.
Cleaved DNA fragments were visualized in 2.5% agarose gel at 70 V for 140 minutes in presence of SYBR® Safe DNA gel stain (Thermo Fisher Scientific) and 50 bp DNA ladder molecular marker (Sinapse) and compared according to the method described by Roberto et al. (2016).

**Sequencing of gp43 exon 2 loci**

To validate the tub1 gene PCR-RFLP method, gp43 exon 2 loci was sequenced using the primers Pb gp43-E2F: (5-CTA GAA TAT CTC ACT CCC AG-3) and Pb gp43-E2R: (5-GCC CCC TCC GTC TTC CAT GTC C-3) (Cisalpino et al., 1996; Hrycyk et al., 2018) at a concentration of 20 mM and annealing temperature of 58 °C. PCR product of gp43 exon 2 loci, approximately 722 bp, had nonspecific amplification and/or integrity verified in 2% agarose gel. Then PCR amplicons were purified using Wizard® SV Gel and PCR Clean-Up System kit (Promega), as instructed by the manufacturer. DNA sequences were determined with an ABI3730® DNA Analyzer (Applied Biosystems), using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Chromatograms were analyzed with ChromasPro® software (ChromasPro 2.6.5). The DNA sequences were compared to nucleotide database using the Basic Local Alignment Search Tool (blastn): https://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul et al., 1990). The sequences of gp43 exon 2 loci determined in this study were submitted to alignment and analysis of similar and conserved regions, using Clustal Omega software https://www.ebi.ac.uk/Tools/msa/clustalo/ (Sievers et al., 2011).

BAT clinical isolate sequence of gp43 exon 2 loci was deposited at GenBank: (https://www.ncbi.nlm.nih.gov/genbank/) under accession number MH484614.

**Results**

**BAT clinical isolate belongs to the genus *Paracoccidioides***

Genomic DNA from *Paracoccidioides* spp. reference strains and BAT isolate were subjected to standard PCR to partially amplify gp43 exon 2 loci. A PCR product of approximately 533 bp (Figure 1A) was observed, confirming that all study samples, including BAT clinical isolate, belong to the genus *Paracoccidioides*.

**Molecular characterization of BAT clinical isolate as *P. restrepiensis* (PS3)**

The tub1 gene of *Paracoccidioides* spp. reference strains and BAT isolate were PCR amplified, and 263 bp amplification products were observed (Figure 1B). BAT clinical isolate was identified as *P. restrepiensis* (PS3) (Figure 1C) since the 263 bp fragment of the tub1 gene does not have cleavage sites for BclI and MspI endonucleases; thus, it was maintained in its complete integrity (263 bp). The reference strains Pb 18, Pb dog–EPM194, T2 – EPM54, and Pb 01 had DNA fragment patterns produced by the endonucleases, as described by Roberto et al. (2016), validating the molecular identification of BAT isolate species by PCR-RFLP as *P. restrepiensis* (PS3) genotype (Figure 1C). The whole gp43 exon 2 loci of BAT clinical isolate was sequenced to confirm the result obtained by PCR-RFLP. The gp43 exon 2 loci DNA sequence showed 100% identity for nucleotide sequence of the reference strain T2-EPM54 *P. restrepiensis* (PS3). Alignment of nucleotide sequences of the gp43 exon 2 loci of BAT clinical isolate and the reference strains also showed genetic proximity with Pb 18–*P. brasiliensis sensu stricto* (S1b), but greater phylogenetic distance from Pb dog EPM194 – *P. americana* (PS2) and from Pb01 (*P. lutzii*); (Figure 2).

The geographical origin of BAT clinical isolate and of the other *Paracoccidioides* spp. isolates classified as *P. restrepiensis* (PS3 genotype) are shown in Figure 3.

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**Figure 1** (A) Partial amplification of the gp43 exon 2 loci of *Paracoccidioides* spp. by conventional PCR of BAT clinical isolate and reference isolates. M: 100 bp DNA ladder molecular marker, Ready-To-Use (Sinapse® Inc., United States). (B) Amplification of the tub1 gene by PCR-RFLP. M: 50 bp DNA ladder molecular marker (Sinapse® Inc., United States). (C) PCR-RFLP DNA fragment patterns obtained after cleavage with BclI and MspI endonucleases, showing similarity of BAT clinical isolate with T2-EPM54 (*P. restrepiensis* – PS3 reference strain). M: 50 bp DNA ladder molecular marker (Sinapse® Inc., United States). Pb 18: *P. brasiliensis sensu stricto* (S1b); Pb dog-EPM 194: *P. americana* (PS2); T2-EPM 54: *P. restrepiensis* (PS3); and Pb 01: *P. lutzii*; BAT: Clinical isolate under study.
Discussion

BAT clinical strain was isolated in 1985 and maintained at the Ribeirão Preto Medical School-USP, southeastern Brazil. It was classified as *P. restrepiensis* (PS3) by sequencing of *gp43* exon 2 loci and PCR–RFLP of *tub1* gene. This result was unexpected since phylogenetic species of PS3 (*P. restrepiensis*) have so far been isolated in Colombia and Venezuela, countries in northwestern South America. A sample of this strain was sent to Venezuela in the 1990s and included in two investigations on genetic diversity of *Paracoccidioides* spp. These studies showed a genotypic difference between BAT clinical isolate and other Brazilian isolates. First, BAT isolate (named Pb 327-B) was evaluated together with 32 *P. brasiliensis* clinical and environmental isolates from different South American countries. By employing randomly amplified polymorphic DNA (RAPD), BAT isolate was grouped near *P. brasiliensis* isolates from Colombia, which was later classified as PS3 genotype (Calcagno et al., 1998). All 33 *P. brasiliensis* isolates but one had their DNA analyzed by restriction fragment length polymorphism (RFLP), using *Hinfl* and *HincII* endonucleases. The dendrogram showed a great relationship of BAT clinical isolate with Colombian *P. brasiliensis* strains (Nino-Vega et al., 2000).

Some phenotypic characteristics of BAT isolate were evaluated. Micromorphological aspects, virulence for guinea pig, and serological reaction of BAT-isolate exoantigen with rabbit anti GP43 serum were typical traits of *P. brasiliensis* strains (Lacaz CS et al., 1999). Compared to other *P. brasiliensis* isolates, BAT isolate had high exoantigen production, which was recognized by sera from patients with PCM in southeastern Brazil (Silva-Vergara et al., 1998; Panunto-Castelo et al., 2003). BAT clinical isolate has been used as a source of antigens of *Paracoccidioides* spp. in serological tests for PCM diagnosis, since it, together with antigens of other strains, formed a pool with high reactivity against sera from PCM patients from São Paulo state, Brazil (Vidal et al., 2014). Sera from patients infected by *P. brasiliensis* S1 genotype reacted with BAT isolate exoantigen in immunodiffusion test (data not shown). Paracoccin, a 160...
kDa Glc-NAc-binding lectin of the BAT isolate yeast wall, adhered to laminin and induced TNFα and NO production by macrophage cells (Coltri et al., 2006).

To date, 31 *P. restrepiensis* (PS3) strains (clinical and environmental isolates) have been identified and reported in the literature. The studies encompassed phylogeny, molecular characterization, morphology, serology, and/or epidemiology (Table 1). The geographic distribution of isolates characterized as *P. restrepiensis* (PS3), including BAT clinical strain, is predominant in Colombia, totaling 87.2% of the 31 isolates, followed by 6.4% in Venezuela, and 6.4% in Brazil (Matute et al., 2006; Muñoz et al., 2016; Roberto et al., 2016). The occurrence of *P. restrepiensis* (PS3) in other South American countries (Brazil and Venezuela) suggests that its geographical distribution is not restricted to the Colombian territory as initially presumed. It is believed that the phylogeographic characteristics of *P. restrepiensis* (PS3) are due to a possible and relatively recent biogeographic expansion of *P. brasiliensis sensu stricto* (S1a and S1b) to Colombia, associated to events of geographic barriers represented by the uplifting of the Andes mountain range and submergence of the Colombian territory by formation of the Pebas-Solimões lake (Wesselingh and Salo, 2006; Teixeira et al., 2014). The emergence of *P. restrepiensis* (PS3) in Brazil was not clarified within the process of speciation of these phylogenies, due to absence of a geographical barrier in the territory (Teixeira et al., 2014).

Besides the geographical origin of *P. restrepiensis* (PS3), the respective clinical manifestation of PCM is one of the important aspects for understanding of the pathogenicity of this phylogenetic species. Among the 31 *P. restrepiensis* (PS3) isolates described in the literature, including BAT isolate, 25 (76%) strains were isolated from patients. The chronic form of PCM was more prevalent in patients infected with *P. restrepiensis* (PS3), representing 88% of the cases. Acute/subacute forms of PCM caused by *P. restrepiensis* (PS3) were only reported in two patients, including that with a disseminated disease from which the strain evaluated in this study was isolated (Matute et al., 2006; Muñoz et al., 2016; Roberto et al., 2016). In a comparative study of PCM cases caused by *P. brasiliensis sensu stricto* (S1a and S1b) and *P. americana* (PS2) in Rio de Janeiro state-Brazil, the prevalence of chronic form was observed for both species (de Macedo et al., 2019). The same was observed in a clinical and epidemiological study of PCM caused by *P. lutzii* in the state of Mato Grosso-Brazil, wherein all patients were diagnosed with the chronic form (Hahn et al., 2019). In general, chronic form predominance is common in PCM, so it does not distinguish *P. restrepiensis* (PS3) genotype in this regard.

Some of the *Paracoccidioides* spp. isolates characterized as *P. restrepiensis* (PS3) have already had their biology studied (Matute et al., 2006; Theodoro et al., 2008). Genotypic and phenotypic studies for *P. restrepiensis* (PS3) isolates were fungal antigenicity (Restrepo-Moreno and Schneidau, 1967), ketoconazole susceptibility (Hoyos et al., 1984), murine immune response to PCM, conidia morphol-

| Strain | Origin | Other ID | Source | Study Type | Identification method |
|---|---|---|---|---|---|
| C1 | Colombia | P149 | Chronic /PCM | MLST | Other IDC1 P149 Colombia Chronic /PCM MLST Phylogenetic (Matute et al., 2006) |
| C2 | Colombia | P159 | Chronic /PCM | MLST | C3 | Antioquia, Colombia Chronic /PCM MLST Phylogenetic (Matute et al., 2006) |
| C3 | Colombia | P163 | Chronic /PCM | MLST | C4 | Antioquia, Colombia Chronic /PCM MLST Phylogenetic (Matute et al., 2006) |
| C4 | Colombia | ATCC 60855 | Chronic /PCM | MLST | C5 | Antioquia, Colombia Chronic /PCM MLST Phylogenetic (Matute et al., 2006) |
| C5 | Colombia | C11 | Chronic /PCM | MLST | C6 | Antioquia, Colombia Chronic /PCM MLST Phylogenetic (Matute et al., 2006) |
| C6 | Colombia | P67 | Chronic /PCM | MLST | C7 | Antioquia, Colombia Chronic /PCM MLST Phylogenetic (Matute et al., 2006) |
| C7 | Colombia | P292 | Chronic /PCM | MLST | C8 | Antioquia, Colombia Chronic /PCM MLST Phylogenetic (Matute et al., 2006) |
| C8 | Colombia | P46 | Chronic /PCM | MLST | C9 | Antioquia, Colombia Chronic /PCM MLST Phylogenetic (Matute et al., 2006) |
| C9 | Colombia | P161 | Chronic /PCM | MLST | C10 | Antioquia, Colombia Chronic /PCM MLST Phylogenetic (Matute et al., 2006) |
| C10 | Colombia | 7653 | Chronic /PCM | MLST | C11 | Antioquia, Colombia Chronic /PCM MLST Phylogenetic (Matute et al., 2006) |
| C11 | Colombia | P41 | Chronic /PCM | MLST | C12 | Antioquia, Colombia Chronic /PCM MLST Phylogenetic (Matute et al., 2006) |
| C12 | Colombia | P120 | Chronic /PCM | MLST | C13 | Antioquia, Colombia Chronic /PCM MLST Phylogenetic (Matute et al., 2006) |

### Table 1 - *Paracoccidioides* spp. isolates genotypically characterized as *P. restrepiensis* (PS3): origin, source, molecular identification, and study type (1967 – 2019).
| ID | Location          | Clinical Form | Identification Method | Genotyping Method          |
|----|------------------|---------------|-----------------------|----------------------------|
| H31 | Boyaca, Colombia  | Chronic / PCM | MLST                  | Phylogenetic (Matute et al., 2006) |
| H45 | Cundinamarca, Colombia | Chronic / PCM | MLST                  | Phylogenetic (Matute et al., 2006) |
| H47 | Arauca, Colombia  | Chronic / PCM | MLST                  | Phylogenetic (Matute et al., 2006) |
| P206| Antioquia, Colombia | Chronic / PCM | MLST                  | Phylogenetic (Matute et al., 2006) |
| P151| Antioquia, Colombia | Chronic / PCM | MLST                  | Phylogenetic (Matute et al., 2006) |
| C1419 | Caldas, Colombia | Armadillo     | MLST                  | Molecular Identification (Corredor et al., 2005) |
| Pb73/ ATCC 32071 | Antioquia, Colombia | Unknown    | MLST                  | Serological tests (Restrepo-Moreno 1967) |
| C21 | Caldas, Colombia  | Armadillo     | MLST                  | Epidemiological (Corredor et al., 1999) |
| EPM54 | Caracas, Venezuela | Soil        | MLST, PCR – RFLP     | Phylogenetic (Roberto et al., 2016) |
| EPM62 | Caracas, Venezuela | Acute/PCM   | MLST, PCR – RFLP     | Phylogenetic (Roberto et al., 2016) |
| EPM77 | Medellin, Colombia | Unknown     | MLST, PCR – RFLP     | Phylogenetic (Roberto et al., 2016) |
| EPM81 | Medellin, Colombia | Chronic / PCM | MLST, PCR – RFLP | Phylogenetic (Roberto et al., 2016) |
| EPM83 | Bogotá, Colombia  | Chronic / PCM | MLST, PCR – RFLP     | Phylogenetic (Roberto et al., 2016) |
| Pb339 or Pb336 | Brazil | Unknown | MLST, PCR – RFLP | Serological tests (Restrepo-Moreno 1967) |
| ATCC 32069 | EPM01 or B339 or ATCC 200273 | Unknown | MLST, PCR – RFLP | Serological tests (Restrepo-Moreno 1967) |
| BAT Pb327 - B | Ribeirão Preto – São Paulo, Brazil | Subacute/PCM | MLST, PCR – RFLP | Phylogenetic (Muñoz et al., 2016) |

*Table 1: ID = Identification; Chronic/PCM - isolated from patients with chronic PCM; Acute/Subacute PCM - isolated from patients with PCM acute/subacute form; PCM – Paracoccidioides clinical isolate, Unknown - unknown clinical form. *B18 has divergences regarding its classification as P. restrepiensis (PS3).
ogy and sporulation at different culture media (Bustamante-Simon et al., 1985), dimorphism (Villar et al., 1988), morphological analysis and molecular identification of armadillo isolates (Corredor et al., 1999), and melanin production (Gomez et al., 2001). Other studies compared *P. restrepiensis* (PS3) isolates with different species belonging to *Paracoccidioides* spp complex (*P. brasiliensis sensu stricto* (S1), *P. americana* (PS2)) to evaluate genotypic and phenotypic differences. Corredor et al. (2005) studied polymorphic genes (gp43 exon 2 loci, ITS_1, and ITS_4) from clinical and armadillo strains isolated in the Colombian territory, comparing them with strains isolated in other South American countries. Polymorphic differences were found among genes when compared to strains identified as *P. brasiliensis sensu stricto* (S1); *P. restrepiensis* (PS3) showed high differentiation from other species (Corredor et al., 2005; Matute et al., 2006). PRP8 intein protein gene sequences from species of the *Paracoccidioides* spp. complex, including *P. restrepiensis* EPM83, were analyzed in a phylogenetic study. This gene can be used as a molecular marker since its polymorphism can separate species from the *P. brasiliensis* complex and *P. lutzii* (Theodore et al., 2008).

Some studies were directed to a phenotypic comparison of *P. restrepiensis* (PS3) isolates and other species belonging to the *Paracoccidioides* spp. (Table 2). Turissini et al. (2017) carried out a phenotypic study on yeast cells of cryptic species of *P. brasiliensis* complex (S1, PS2, PS3, and PS4) and *P. lutzii*. These authors observed that *P. restrepiensis* (PS3) has yeast cells larger than *P. brasiliensis sensu stricto* (S1) and *P. americana* (PS2) ones but no cell size differences with *P. venezuelensis* (PS4) and *P. lutzii* strains. A comparative study to evaluate PCM immunodiagnosis using species of the *P. brasiliensis* complex and *P. lutzii* showed higher GP43 production and best antigenic reactivity in an immunodiffusion assay with the EPM83 strain (*P. restrepiensis* – PS3) when tested with sera from patients living in a geographic area where *P. brasiliensis sensu stricto* (S1a and S1b) and *P. americana* (PS2) are prevalent (Machado et al., 2013). Another comparative study of proteomes by disrupting yeast cells of *Paracoccidioides* species representative isolates showed that EPM83 (*P. restrepiensis* – PS3) preferentially expressed 38 proteins, including heat shock proteins (HSP) and a higher level of GP43 production (Pigosso et al., 2013). An analysis of secretomes of two *Paracoccidioides* spp strains identified 95 extracellular proteins, 35 specific of *P. lutzii* and 36 specific of *P. restrepiensis* (PS3), including several ones related to fungal virulence factors and adhesion (de Oliveira et al., 2018).

B18 or Pb339 (ATCC32069) is a strain that has been reported in the literature to have divergent results regarding its classification as *P. restrepiensis* (PS3). It is originally from the state of São Paulo (Brazil) but from an unknown source. This strain was obtained from the National Communicable Disease Center (Atlanta, USA) and first studied by Restrepo-Moreno and Scheneidau JD in 1967. Matute et al. (2006) classified B18 as *P. brasiliensis sensu stricto* (S1b) by analyses of polymorphisms in nuclear genes, chitin syn-

| Yeast cell size | EPM83 production and antigenicity in the immunodiffusion test with PCM sera | Proteome | Secretome |
|-----------------|------------------------------------------|----------|-----------|
| P. restrepiensis | Higher number of BAT coagglutins recognized by patient sera | EPM83 presented higher expression of 38 proteins, including HSP and GP43 | EPM83 production of 36 specific proteins; 21 proteins shared with Pb01 |
| *P. brasiliensis* sensu stricto | HSP and GP43 production and antigenicity in the immunodiffusion test with PCM sera | Proteome | EPM83 |
| *P. americana* | EPM83 Pb339 – | EPM83 | Proteome |
| *P. venezuelensis* | EPM83 Pb339 – | EPM83 | Proteome |
| *P. lutzii* | EPM83 Pb339 – | EPM83 | Proteome |
| *P. restrepiensis* (S1) | EPM83 Pb01, Pb8334, Pb66 - *P. lutzii* | EPM83 | Proteome |
| *P. brasiliensis sensu stricto* | EPM83 Pb265 - | EPM83 | Proteome |
| *P. restrepiensis* (S1) | EPM83 Pb01, Pb8334, Pb66 - *P. lutzii* | EPM83 | Proteome |
| *P. brasiliensis sensu stricto* | EPM83 Pb265 - | EPM83 | Proteome |
| *P. restrepiensis* (S1) | EPM83 Pb01, Pb8334, Pb66 - *P. lutzii* | EPM83 | Proteome |
| *P. brasiliensis sensu stricto* | EPM83 Pb265 - | EPM83 | Proteome |
| *P. restrepiensis* (S1) | EPM83 Pb01, Pb8334, Pb66 - *P. lutzii* | EPM83 | Proteome |
| *P. brasiliensis sensu stricto* | EPM83 Pb265 - | EPM83 | Proteome |
| *P. restrepiensis* (S1) | EPM83 Pb01, Pb8334, Pb66 - *P. lutzii* | EPM83 | Proteome |
phased (CHS) 2, glucan synthase (FKS), tub1 (TUB), adenyl ribosylation factor (ARF), and gp43 exon 2 loci. On the other hand, Salgado-Salazar et al. (2010) studied polymorphism of five mitochondrial genes used as markers in molecular characterization of Paracoccidioides species. The findings enabled reclassification of B18 strain as *P. restrepiensis* (PS3) from *Paracoccidioides* spp. complex (Salgado-Salazar et al., 2010). Later, Roberto et al. (2016) evaluated B18 strain, termed in that study as B339 and /or EPM01 (ATCC200273) (Camargo et al., 2003), and classified B18 strain as *P. restrepiensis* (PS3) by PCR-RFLP of *tub1* gene and sequencing of CHS2, FKS, TUB, ARF, and GP43 nuclear genes, the same ones studied by Matute et al. (2016). A study conducted by Turissini et al. (2017) classified B18 strain as an independent genotype due to its phylogenetic and micromorphological differences already observed in previous studies, suggesting a hybrid species belonging to the genus Paracoccidioides.

Identification of an unusual genotypic variant in southeastern Brazil contributes to understanding speciation and propagation involving PCM agents and may help in knowing *P. restrepiensis* characteristics (PS3 genotype) such as morphology, virulence, and serological reactivity.

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Conflicts of interest

The authors declare no conflicts of interest.

Author Contributions

TAC conducted all experiments, analyzed the results, and contributed to manuscript writing and preparation; EN collaborated in carrying out experiments, analyzing the results, and provided suggestions for the manuscript; MRVZK and EB guided execution of some experiments, analysis of results, and gave suggestions for writing the manuscript; RM supervised study development and wrote the manuscript.

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