

## ANALYSIS OF THE GENETIC POLYMORPHISM OF *Paracoccidioides brasiliensis* AND *Paracoccidioides cerebriformis* "MOORE" BY RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) AND 28S RIBOSOMAL DNA SEQUENCING - *Paracoccidioides cerebriformis* REVISITED

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### SUMMARY

Our purpose was to compare the genetic polymorphism of six samples of *P. brasiliensis* (113, 339, BAT, T1F1, T3B6, T5LN1), with four samples of *P. cerebriformis* (735, 741, 750, 361) from the Mycological Laboratory of the Instituto de Medicina Tropical de São Paulo, using Random Amplified Polymorphic DNA Analysis (RAPD). RAPD profiles clearly segregated *P. brasiliensis* and *P. cerebriformis* isolates. However, the variation on band patterns among *P. cerebriformis* isolates was high. Sequencing of the 28S rDNA gene showed nucleotide conservancy among *P. cerebriformis* isolates, providing basis for taxonomical grouping, and disclosing high divergence to *P. brasiliensis* supporting that they are in fact two distinct species. Moreover, DNA sequence suggests that *P. cerebriformis* belongs in fact to the *Aspergillus* genus.

**KEYWORDS:** *Paracoccidioides cerebriformis*; *Paracoccidioides brasiliensis*; *Aspergillus penicillioides*; RAPD, 28S rDNA sequence.

### INTRODUCTION

*Paracoccidioides brasiliensis*, a thermal dimorphic fungus is the etiological agent of paracoccidioidomycosis (PCM), isolated for the first time in 1908 by Adolfo Lutz. This human systemic mycosis (PCM) occurs mainly in rural laborers with high incidence in South America, especially in Brazil. It has been suggested that the frequency of pulmonary lesions is due to the inhalation of air-borne propagules. It is currently thought that the fungus metamorphosis from a multicellular filamentous form to the unicellular one, found in PCM lesions upon microscopic inspection, is driven by the shift of the environment temperature to that of the infected host (about 37 °C)<sup>6</sup>.

MOORE (1935) identified a new species of fungus, which he named *Paracoccidioides cerebriformis*, but did not grow well at 37 °C<sup>9</sup>. Yeast-like variants of *P. brasiliensis* obtained at room temperature have been reported, revealing that the dimorphism of the fungus does not depend exclusively on incubation temperature<sup>4,11,15</sup>. Lately, reports have suggested that nutritional factors may be also involved in the regulation of dimorphism in *P. brasiliensis*<sup>3,11</sup>.

LACAZ *et al.* (1997), studied *P. cerebriformis* strains (741 and 750) and showed that they preserved the characteristics described originally by MOORE<sup>9</sup>. The immunochemical analysis did not show the presence of 43-kDa glycoprotein (immunodominant antigen of *P.*

*brasiliensis*) in antigenic preparation from two *P. cerebriformis* strains (#741 and #750). They suggested that a molecular analysis would be necessary in order to better characterize and identify this fungus<sup>5</sup>. Recently, several papers have shown the use of molecular biology techniques to analyze the genomic variation of fungal different taxonomical levels<sup>1,2,7,8,10</sup>.

RAPD analysis has become a very popular tool for observation of genetic polymorphisms among microorganisms. It is based on the sensitive PCR technique, but with the advantage of requiring simpler procedures, due to the use of arbitrary primers and no "a priori" knowledge of target DNA sequences<sup>8,14,16</sup>. This technique has been useful to discriminate isolates of fungus, such as *Histoplasma capsulatum* and *Paracoccidioides brasiliensis*<sup>1,8,10,14</sup>. In the present investigation we used the RAPD technique to characterize the genetic affiliation between *Paracoccidioides brasiliensis* and *Paracoccidioides cerebriformis* strains. Moreover, we performed DNA sequencing of the 28S rDNA subunit gene in order to better characterize *P. cerebriformis* isolates.

### MATERIAL AND METHODS

**Isolates:** Six *P. brasiliensis* (113, 339, BAT, T1F1, T3B6, T5LN1), and four *P. cerebriformis* (735, 741, 750, 361) strains were used in the experiments, as depicted on Table 1. These strains are kept at the

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Mycological Laboratory of the Instituto de Medicina Tropical de São Paulo, and maintained on potato glucose agar slants (PDA), at room temperature, with subsequent and subcultures every three months. These cultures were grown in YPD liquid medium (peptone, 5 g; yeast extract, 5 g; dextrose, 15 g; per liter of distilled water) 30 mL of medium in 125-mL Erlenmeyer flasks, and were incubated for five days at 30 °C with continuous shaking on a gyratory shaker at 120 rpm.

**DNA extraction:** Fungal suspension from cultures were transferred to microtubes and suspended in 600 µL of sorbitol buffer (1 M sorbitol, 100 mM EDTA, 14 mM β-mercaptoethanol) with 3 mg/mL of Lysing Enzyme (SIGMA) and incubated for three hours at 37 °C. After incubation, DNA was extracted by a commercial kit (QIAamp DNA Mini Kit, QIAGEN – Valencia, CA, USA), following the manufacturer's instruction. Total DNA was eluted with 100 µL of AE-buffer and stored at -20 °C for further experiments. The concentration and purity of the DNA preparations was determined by spectrophotometry using absorbance at 260 and 280 nm. The  $A_{260}/A_{280}$  ratio of all samples was  $\geq 1.8$ .

**RAPD:** Three primers OPG05 (CTGAGACGGA), OPG14 (GGATGAGACC) and OPG18 (GGCTCATGTG) previously described by CALCAGÑO *et al.* 1998, were used in these experiments. RAPD reactions were carried out in a final volume of 25 µL, containing 1x PCR buffer (100 mM Tris-HCl, 500 mM KCl), 100 µM dNTPs, 2 mM MgCl<sub>2</sub>, 1 µM of each primer, 0.75 U *Taq* DNA polymerase (Invitrogen) and 100 ng of DNA. Amplification was performed in an MJ-Research (PTC-200) thermocycler, as follows: two minutes at 94 °C, followed by 40 cycles of 94 °C for one minute, 30 °C for two minutes, 72 °C for two minutes, and a final extension period of 72 °C for seven minutes. PCR products were analyzed by electrophoresis on a 1.5% agarose gel in TAE 1x buffer (400 mM Tris-acetate, 10 mM EDTA, [pH 8.3]), stained with ethidium bromide. All reactions were repeated for at least three times showing a good reproducibility. RAPD data was analyzed by visual inspection and assignment of the presence or absence of informative bands in between 200 and 2000 bp of length.

**PCR - 28S rDNA amplification and sequencing:** A portion of 799 bp spanning from coordinate 45 - 843 from the 28S ribosomal DNA (28S rDNA, *S. cerevisiae* GenBank accession number J01355) was selected for sequencing, due to its phylogenetically informative

content. 28S rDNA was amplified from each fungal strain (361, 735, 741, 750, and *P. brasiliensis* strains 339 and 265 as controls) by PCR using the universal fungal primers P1 and P2, as described by SANDHU *et al.*<sup>13</sup>. PCR reaction was carried out in a final volume of 100 µL, containing 1x PCR buffer (100 mM Tris-HCl, 500 mM KCl), 25 µM dNTPs, 2 mM MgCl<sub>2</sub>, 0.4 µM of each primer, 1 U *Taq* DNA polymerase (Invitrogen) and 100 ng of DNA. Amplification was performed in an MJ-Research (PTC-200) thermocycler, as follows: two minutes at 94 °C, followed by 40 cycles of 94 °C for thirty seconds, 45 °C for one minute, and 72 °C for two minutes and a final extension period of 72 °C for seven minutes. PCR products were purified using the QIAquick Spin Handbook (QIAGEN), the purified PCR products were used in sequencing reactions employing the same amplification primers and the BigDye Terminator Cycle Sequencing Standard 3.1 kit. Sequencing products were separated and analyzed on an ABI Prism 377 Genetic Analyzer. Sequences were submitted to GenBank and have the following Accession Numbers:

***P. brasiliensis***

Isolate	Accession Number
265	<b>AY821885</b>
339	<b>AY821884</b>

***P. cerebriiformis***

Isolate	Accession Number
361	<b>AY821886</b>
735	<b>AY753205</b>
741	<b>AY821888</b>
750	<b>AY821887</b>

**Sequence alignment and BLAST search** - DNA sequences were aligned by use of CLUSTAL W available through the web site <http://www.ebi.ac.uk/clustalw/>. Position numbered 1 in the alignment corresponds to coordinate 150 of a reference *S. cerevisiae* 28S gene (GenBank accession number J01355)

**RESULTS**

RAPD primers used produced informative DNA bands in between 500 bp and 2000 bp. OPG18 (Fig. 1) clearly discriminated *P. brasiliensis* from *P. cerebriiformis* isolates, with a monotonous two bands pattern

**Table 1**  
Description of strains used in this study

Strains	Year of isolation	Ref. fungi collection (IMTSP)	Localization or type of lesion in host/origin	Isolated by <sup>a</sup> :
<i>P. brasiliensis</i>	1971	113	lesion of mouth/human	CASTRILLÓN
<i>P. brasiliensis</i>	1992	339	PCM/human	RESTREPO
<i>P. brasiliensis</i>	1996	Bat	PCM/human	FREITAS
<i>P. brasiliensis</i>	1998	T1F1	liver/armadillo	BAGAGLI
<i>P. brasiliensis</i>	1998	T3B6	spleen/armadillo	BAGAGLI
<i>P. brasiliensis</i>	1998	T5LN1	lymph node/armadillo	BAGAGLI
<i>P. cerebriiformis</i>	1932	361	ganglion/human	Belo Horizonte (MG)
<i>P. cerebriiformis</i>	1931	735	skin lesion/human	MOORE
<i>P. cerebriiformis</i>	1927	741	ganglion/human	MOORE
<i>P. cerebriiformis</i>	1925	750	skin lesion/human	MOORE

<sup>a</sup>All *Paracoccidioides* cultures are from the Laboratory of Medical Mycology, Instituto de Medicina Tropical de São Paulo, University of São Paulo, São Paulo, Brazil.

on all *P. brasiliensis* and three distinct patterns on *P. cerebriformis* isolates, being 735 and 750 undistinguishable. OPG14 primer (Fig. 2) displayed similar results to OPG18 but providing more intensive and abundant bands. OPG05 primer (Fig. 3) reinforced the results obtained with both previous primers. In conclusion, each primer segregated *P. brasiliensis* and *P. cerebriformis* isolates and also showed *P. cerebriformis* strains 735 and 750 to be undistinguishable by RAPD analysis, and also a considerable degree of genetic identity in between *P. brasiliensis* strains.

DNA sequencing of both strands from the 799 bp fragment of the 28S rDNA gene of all four *P. cerebriformis* strains together with two *P. brasiliensis* strains allowed the identification of several nucleotide differences between both species. Moreover, *P. cerebriformis* isolates showed a high degree of nucleotide conservation among isolates and a few *P. cerebriformis* specific motifs such as a three nucleotide insertion (TCG) at position 586-588 (numbered as in Pc 750, Fig. 4) and several other minor substitutions when compared to *P. brasiliensis* sequences. Even so, a few nucleotide differences are noted in between *P. cerebriformis* isolates, ruling out the possibility of either PCR carryover or culture contamination.

Blast search with *P. cerebriformis* sequences as "query", retrieved several entries of *Aspergillus* genus with the strongest score for *Aspergillus penicillioides* followed by *Aspergillus conicus* and *gracilis*. Notably, after blast search, it came to our attention that isolates #741 and #750 were sequenced in the past by SANDHU, who deposited sequences in GenBank (Accession Numbers U81264 and U81265 respectively). In these entries the organism assigned was *Aspergillus penicillioides*. The homology of the sequences obtained in this paper and those deposited by SANDHU from the same original *P. cerebriformis* strains were 100% homologous.

## DISCUSSION

About 70 years ago, MOORE described what he called a new species of the *Paracoccidioides* genus (MOORE, 1935). He was then studying fungi from a collection at the Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, and described morphological aspects of the culture and clinical samples (pus) and also biochemical and nutritional characteristics. He noted that other specialists in other Brazilian cities had described "*Paracoccidioides cerebriformis*" already. In 1997 LACAZ and co-workers reanalyzed two isolates (#741 and #750) kept since then on Sabouraud agar in his mycology collection at the Instituto de Medicina Tropical de São Paulo, São Paulo, Brazil. They performed several immunological/antigenic tests and repeated morphological examination. Their conclusion was that *P. cerebriformis* does not belong to the genus *Paracoccidioides* and that further studies, including molecular biology methods, should be applied in the future, in order to better characterize the taxonomic status of this fungus.

In the present study, we first attempted to investigate by RAPD analysis the genetic relatedness of *P. cerebriformis* isolates and between them and *P. brasiliensis*. RAPD clearly segregated isolated belonging to the two "species" and also showed *P. brasiliensis* isolates to be more similar than *P. cerebriformis* ones. Although this data corroborate the conclusion of LACAZ *et al.*<sup>5</sup>, of two distinct genus, we went further into the molecular characterization of *P. cerebriformis* isolates by

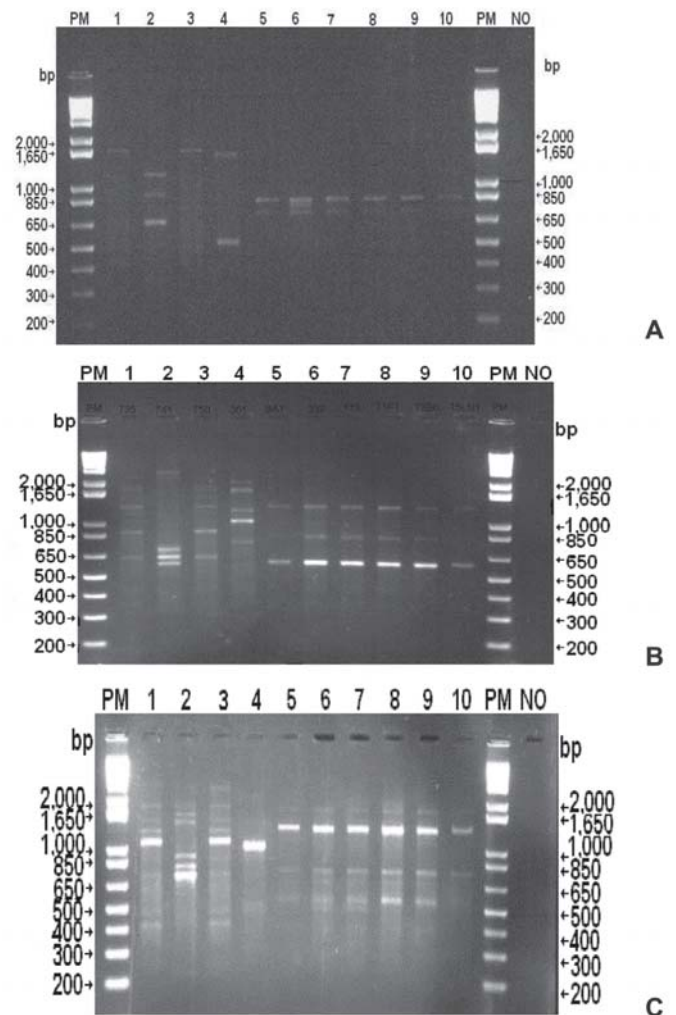


Fig. 1, 2, 3 – Comparison of RAPD profiles showing polymorphism between *P. brasiliensis* and *P. cerebriformis* strains with primers OPG18<sup>1</sup>, OPG05<sup>2</sup> and OPG14<sup>3</sup>. PM – Molecular weight marker 1 Kb plus DNA ladder (Invitrogen) 1 – 735, 2 – 741, 3 – 750, 4 – 361 (*P. cerebriformis* isolates), 5 – 113, 6 – 339, 7 – BAT, 8 – T1F1, 9 – T3B6, 10 – T5LN1 (*P. brasiliensis* isolates) – NO (negative control)

amplifying and sequencing a common target of molecular taxonomical studies, the ribosomal 28S gene. Ribosomal genes due to their conservancy are routinely used to assess phylogenetical relationship between organisms and also allow the placement of emerging agents in broad taxonomical groups. The assignment of *Pneumocystis carinii* to the Fungi kingdom is one of the more recent and notable examples of such application<sup>2</sup>.

Surprisingly, this approach has led to the observation that *P. cerebriformis* is in fact closely related to the *Aspergillus* genus, when considering DNA sequence homology at the 28S rDNA locus. SANDHU and co-workers didn't publish their *P. cerebriformis* sequence data, but made it available by depositing on GenBank and on their entries they assigned *Aspergillus penicillioides* as the source organism. Our data may sound biased since we sequenced the same two isolates



**Fig. 4** - Alignment of 28S rDNA sequences of *P. cerebriiformis* and *P. brasiliensis* strains as explained in material and methods. \* indicates that all strains are identical at that nucleotide position; - indicates that insertions exist in one or more strains, leading the software (Clustal W) to break one or more sequences in order to preserve the best possible alignment. U1/U2 indicates the nucleotide position of internal primers useful for fungal identification<sup>13</sup> and a probe used for *P. brasiliensis* identification on slot blot hybridization<sup>12</sup>.

as SANDHU, so it is obvious that a blast search would pick up these entries with the highest homology score. However it must be emphasized that we did sequence other two *P. cerebriiformis* isolates (#735 and #361), with identical results, and blast search retrieved not only SANDHU's entries but also many other from *Aspergillus* genus. The scores obtained when analyzing our four *P. cerebriiformis* entries do not allow the conclusion that these isolates are closer to *Aspergillus penicillioides* than other *Aspergillus* species, namely *conicus* and *gracilis*, since they are all very close.

In conclusion, we present molecular evidence that *P. cerebriiformis* do not belong to *Paracoccidioides* genus, as suggested by LACAZ *et al.*, 1997. We propose that would be more accurate to include it in the *Aspergillus* genus. Moreover, the sequences obtained allow the design of specific primers and probes for molecular detection of this agent, what could be useful in investigating the prevalence of infection by this agent on human and other host species.

## RESUMO

### Análise do polimorfismo genético do *Paracoccidioides brasiliensis* e *Paracoccidioides cerebriiformis* "Moore" pela técnica de amplificação aleatória do polimorfismo do DNA (RAPD) e sequenciamento do DNA ribossomal 28S - *Paracoccidioides cerebriiformis* revisitado

Nosso propósito foi comparar o polimorfismo genético de seis amostras de *P. brasiliensis* (113, 339, BAT, T1F1, T3B6, T5LN1), com quatro amostras de *P. cerebriiformis* (735, 741, 750, 361) do laboratório de micologia do Instituto de Medicina Tropical de São Paulo, utilizando a técnica de Amplificação Aleatória do Polimorfismo de DNA (RAPD). O perfil de bandas do RAPD diferenciou claramente os isolados de *P. brasiliensis* de *P. cerebriiformis*. Entretanto, ocorreu uma variação significativa no padrão de bandas das amostras de *P. cerebriiformis*. O sequenciamento do gene ribossomal 28S revelou seqüências de nucleotídeos bastante conservadas entre os isolados de *P. cerebriiformis*, fornecendo subsídio para o agrupamento taxonômico destas amostras, diferenciando estas de *P. brasiliensis* e mostrando que de fato são espécies distintas. A seqüência de DNA sugere que *P. cerebriiformis* pertence ao gênero *Aspergillus*.

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