Genetic characterization of morphologically variant strains of *Paracoccidioides brasiliensis*

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Molecular characterization of Paracoccidioides brasiliensis *variant strains that had been preserved under mineral oil for decades was carried out by random amplified polymorphic DNA analysis (RAPD). On* P. brasiliensis *variants in the transitional phase and strains with typical morphology, RAPD produced reproducible polymorphic amplification products that differentiated them. A dendrogram based on the generated RAPD patterns placed the 14* P. brasiliensis *strains into five groups with similarity coefficients of 72%. A high correlation between the genotypic and phenotypic characteristics of the strains was observed. A 750 bp-RAPD fragment found only in the wild-type phenotype strains was cloned and sequenced. Genetic similarity analysis using BLASTx suggested that this RAPD marker represents a putative domain of a hypothetical flavin-binding monooxygenase (FMO)-like protein of* Neurospora crassa.

Key words: FMO - *Paracoccidioides brasiliensis* - RAPD-PCR

Paracoccidioides brasiliensis (Splendore) Almeida, 1930 is an anamorphic and dimorphic fungus (23°C - filamentous phase; 36°C - yeast-like phase) that causes paracoccidioidomycosis, a serious disease in humans that is prevalent in South America and particularly Brazil (Lacaz 1982). It is believed that the phase transition from mold to yeast is essential for activating pathogenicity, and the mechanism that regulates this switch is still unknown (Nemecek et al. 2006). Factors such as successive subculturing and storage in vitro (Brummer et al. 1990) result in changes to morphology and fungal cell wall content, with concomitant attenuation or complete loss of virulence (Hamdan & Ferrari 1995, Tanaka et al. 2001, Macoris et al. 2006). In a previous study, we showed that only 18 out of 70 *P. brasiliensis* strains that had been isolated from individual patients and preserved under mineral oil for periods of time up to 45 years were viable (Mendes da Silva et al. 1994). However, these strains (except Pb IOC 3698) remained morphologically in the transitional phase (i.e., thick filaments, yeast-like cells and buds, instead of thin spidery filaments) at 23°C. They were also found to be nonvirulent, as seen from studies using rat and mice models (Mendes da Silva et al. 1996, Borba & Schäffer 2002). By detecting the presence of either the GP43 protein or the *gp*43 gene, we confirmed the identity of the morphologically variant *P. brasiliensis*, that remained in the transitional phase (Borba et al. 2005).

The genetic diversity of *P. brasiliensis* has been assessed by random amplified polymorphic DNA (RAPD) analysis (Molinari-Madlum et al. 1999, Kurokawa et al. 2005), restriction fragment length polymorphism (RFLP) (Niño-Vega et al. 2000) and polymorphic microsatellites located on noncoding and coding sequences (Nascimento et al. 2004, Matute et al. 2006). RAPD technology has also been successfully used to distinguish pathogenic and nonpathogenic strains of other fungi (Aufauvre et al. 1992, Guthrie et al. 1992, Woods et al. 1993, Mondon et al. 1995, Lasker 2002). In the present study, we used RAPD analysis to identify molecular markers related to morphological alterations and/or pathogenic impairment of morphologically variant *P. brasiliensis* strains.

Fourteen *P. brasiliensis* strains were grown on PYG agar (Mendes da Silva et al. 1994). The strains Pb IOC 949, 950, 1059, 1099, 1124, 1148, 1163, 1205, 1208, 1210, 1479 and 3698 were obtained from the Culture Collection of the Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro, Brazil. Two strains, Pb 18 and 339, were kindly provided by Dr. Rosely Zancopé (Laboratório de Micologia, Hospital Evandro Chagas-Fiocruz, Rio de Janeiro, Brazil). All the strains were incubated at 23°C and 36°C, and after 13 days the strains were examined under an optical microscope (Axiophot model, Zeiss, Germany). The fungal cells were stained using Amann's lactophenol cotton blue. DNA was extracted, quantified and assessed as described by Borba et al. (2005). RAPD-polymerase chain reaction (PCR) was performed using six different primers named OPF-01 (ACGGATCCTG), OPF-08 (GGGATATCGG), OPF-11 (TTGGTACCCC), OPF-12 (ACGGTACCAG), OPF-13 (GGCTGCAGAA) and OPF-14 (TGCTGCAGGT) (Operon Technologies, Inc., USA). The reaction mixture was set up consisting of 1x buffer without $MgCl_2$ (Gibco BRL, USA), 1.5 mM $MgCl_2$, 16 µM dNTP, 9% glycerol, 50 pmol of primer, 1 U of Taq polymerase (Gibco) and 30 ng of DNA template,

in a final volume of 25 μl. This was then overlaid with 30 μl of mineral oil. The amplification program consisted of 30 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, using a DNA thermal cycler (Perkin-Elmer Corporation, USA). The resulting amplified fragments were analyzed by electrophoresis in a 2% agarose gel/TAE buffer. The gels were stained for 15 min with 0.05 μg/ml ethidium bromide/TAE and photographed under UV light using Polaroid film. The electrophoretic DNA profiles were used to determine the similarity of DNA fingerprint patterns by calculating a simple matching coefficient. An agglomerate method for clustering accessions (unweighted pair group method with arithmetic mean, UPGMA) was used. The DNA profiles produced were analyzed using the NTSYS-pc software, version 1.70. A 750 bp DNA fragment was isolated from the virulent strains, Pb IOC 3698 and Pb 339, and ligatingthe DNA fragment was ligated into a TA vector (PinPoint™ Xa-1 T-Vector, Promega) and then transformed into *Escherichia coli* DH5-α cells. The identity of the cloned insert was confirmed by PCR using vector-specific primers. The reaction mixture was set up consisting of 1x buffer without MgCl$_2$, 1.5 mM MgCl$_2$, 2 mM dNTP, 25 pmol of the primers TAF (CGAAGGTCGCGAAGCTTCAGC) and TAR (GATCGCGGCCGCCCCGGGAG), 1 U of Taq polymerase and 5 μl of recombinant clone suspensions from Pb IOC 3698 and Pb 339. This was then overlaid with 30 μl of mineral oil. The amplification program consisted of 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 2 min, performed in a Perkin-Elmer DNA thermal cycler. An amplicon of 850 bp in size was viewed by electrophoresis on 1% agarose/TAE gel, as described earlier. The amplicon was isolated and purified by using the Concert gel extraction system (Gibco), in accordance with the manufacturer's instructions. The nucleotide sequence of the 850 bp fragment was determined by using the Thermo Sequenase Cy5 dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, USA) in an ALFexpress II sequencer (Amersham). The sequences obtained were analyzed using the BLAST software. The 850 bp fragment was named ceja-1. Specific primers for the ceja-1 sequence were designed using the Oligos 4.0 software. Their specificity was first examined by PCR using gDNA of the morphologically typical pathogenic strains (Pb 18, Pb 339 and Pb IOC 3698) and morphologically variant non-pathogenic strains (Pb IOC 1059, Pb IOC 1124, Pb IOC 1208 and Pb IOC 1210). The reaction mixture was set up consisting of 1x buffer, 1.5 mM MgCl$_2$, 0.2 mM dNTP, 25 pmol of the primers PBEXF (5'GCGGGTCCCCAAAAGGATCTGGC) and PBEXR (5'AAAAGATTGCTCTCCTGGACGCGTCG), 1 U of Taq polymerase and 25 ng of gDNA. This was then overlaid with 30 μl of mineral oil. The amplification program consisted of 30 cycles of 94°C for 30 s, 68°C for 1 min and 72°C for 1 min. The ceja-1 amplified fragment was 400 bp in length and was analyzed by electrophoresis in a 1.5% agarose/TAE gel, as described earlier. RNA from 10 mg of dry *P. brasiliensis* strains was extracted by the Trizol reagent (Invitrogen, São Paulo, Brazil) and was resuspended in 20 μl of DEPC water. cDNA was synthe-

sized from 5 μl of RNA using random primers with Superscript RT II, as suggested by the supplier (Invitrogen), prior to amplification of the ceja-1 fragment as described above. Amplification of the constitutive glyceraldehyde 3-phosphate dehydrogenase gene was used as a positive PCR quality control. The resulting fragment was subcloned into a set of pGEX expression vectors covering all three possible open reading frames, and this was used to transform the *E. coli* BL21 strain. The clones were first screened using specific ceja-amplification by PCR, and then a positive clone was grown in Lurian-Bertani medium supplemented with ampicillin overnight at 37°C. A 10% inoculum was grown in fresh medium for 3 h, and then production of the fused protein GST-CEJA1 was induced with 0.1 mM of isopropyl-6-D-thiogalactoside. The proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane and probed with mouse serum infected with the wild-type *P. brasiliensis* strain.

The morphological differences and preservation lengths among the *P. brasiliensis* strains studied here are presented in Table. The morphological analysis and strain categorization was done prior to the molecular typing analysis, in order to avoid selection bias. The strains Pb 18 and 339 were maintained on agar slants, showed typical morphology and are described as virulent (Kashino et al. 1990, Vaz et al. 1994). Pb IOC 3698 was the only strain with typical morphology that had been kept under mineral oil, which was for a time period of 16 years, and it is virulent (Borba & Schäffer 2002). The Pb IOC variant strains were classified into three morphological groups on the basis of: (I) large quantities of thin and thick filaments with dilatations and rare yeast-like cells; (II) thick filaments with terminal and intercalary dilatations and yeast-like cells; (III) few thick filaments and large quantities of budding yeast-like cells (Fig. 3). The primer OPF-08 was able to amplify all of the isolates, and the isolates Pb 18, 339 and Pb IOC 3698 presented very similar RAPD fingerprinting (Fig. 1) with a fragment of 750 bp that was not present in the morphologically variant non-pathogenic strains. The RAPD-PCR reactions were repeated on at least three different occasions to ensure reproducibility. The 14 *P. brasiliensis* strains showed significant genetic diversity, but by taking a threshold similarity coefficient of 72%, it was possible to divide them into five groups (Fig. 2). The strains in the RAPD-a group presented morphological pattern III. The RAPD-b group correlated with morphological group II. The strains in morphological group I were clustered in the RAPD-c group, with the exception of the strain Pb IOC 1205. This did not show any correlation with the DNA fingerprinting pattern of the RAPD-c group, and thus formed a separate group, RAPD-d, despite presenting the same phenotypic characteristics as group I. Strains with typical morphology (group RAPD-e) formed a single group with similarities to each other of 85 to 95%. The ceja-1 fragment was amplified from the gDNA and cDNA of a morphologically typical pathogenic strain of *P. brasiliensis*, thus suggesting that ceja-1 is a partial sequence of an expressed gene. BLAST-n analysis showed no similarity to any other sequence that has been deposited in GenBank. The ORF

TABLE

Morphology of *Paracoccidioides brasiliensis* strains preserved under mineral oil for various periods of time
at room temperature that were used for RAPD genotyping

Pb IOC strains	Preservation (years)	Morphology
949	33	
950	46	
1099	u	
1148	37	Thin and thick filaments with dilatations
1163	42	and rare yeastlike cells
1205	46	
1210	37	
1124	42	Thick filaments with terminal and intercalary
1208	46	dilatations. Presence of yeastlike cells
1059	42	Thick filaments and large quantities of
1479	41	budding yeastlike cells
3698	16	Delicate hyphae with chlamydospores

u: unknown.

Finder software identified several potential ORFs ranging from 114 to 237 bp. BLAST-x analysis revealed homology with a domain of a hypothetical flavin-binding monooxygenase-like protein of *Neurospora crassa*. The results from the polypeptide sequence alignment using BLAST-p showed low homology with a value of e^{-10}. The GST-CEJA1 recombinant protein was recognized by mouse serum infected with a morphologically typical virulent strain of *P. brasiliensis*.

In the present study, we showed that dimorphism impairment and virulent behavior were associated with genetic polymorphisms of *P. brasiliensis* strains. The phenomenon of in vitro attenuation of virulence has been described for *P. brasiliensis* (Kurokawa et al. 2005), and the strain virulence can be recovered after several passages through animals (Carvalho et al. 2005). However, the morphologically variant strains Pb IOC 1210, 1208, 1124 and 1059 used here continued to be nonvirulent after inoculation in rats and mice by the intraperitoneal route (Mendes da Silva et al. 1996, Borba & Schäffer 2002), thus suggesting the existence of stable non-virulence even after passage through animals. We showed that the OPF-08
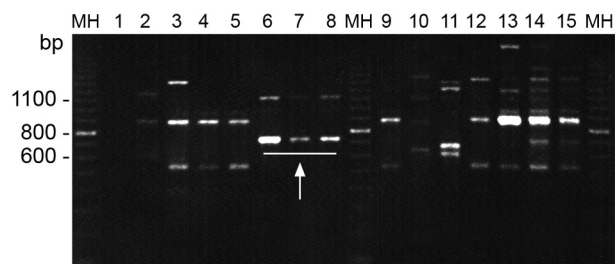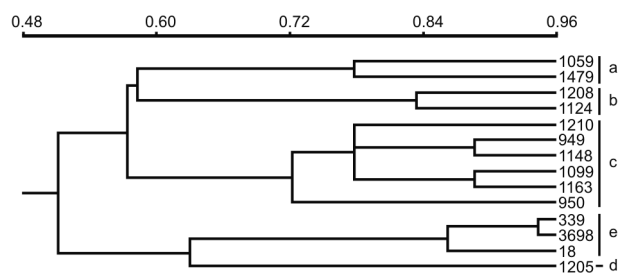

Fig. 2: dendrogram of *P. brasiliensis* strains based on genetic similarity coefficients derived from RAPD-PCR using the OPF-08 primer, showing five groups with similarity of at least 72%.
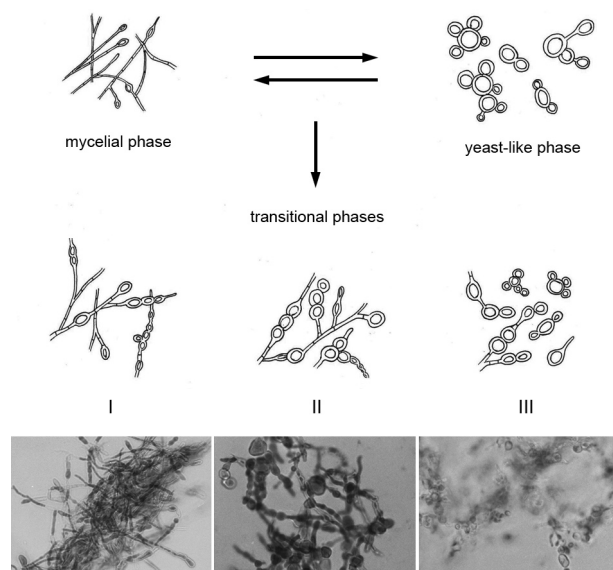

Fig. 3: diagram and microphotographs (original magnification, 400X) of the morphologies observed for *P. brasiliensis* strains based on Salazar & Restrepo (1984). Strains were grown on PYG agar. I: large quantities of thin and thick filaments with dilatations and rare yeast-like cells; II: thick filaments with terminal and intercalary dilatations and yeast-like cells; III: few thick filaments and large quantities of budding yeast-like cells.

Fig. 1: comparison of RAPD-PCR genetic patterns with DNA prepared from 14 strains of *P. brasiliensis* using the OPF-08 primer and 1.5 mM MgCl₂. The amplification reactions are described in the text. Lanes - 1: negative control; 2: Pb IOC 1059; 3: Pb IOC 1208; 4: Pb IOC 1210; 5: Pb IOC 1099; 6: Pb 339; 7: Pb 18; 8: Pb IOC 3698; 9: Pb IOC 1163; 10: Pb IOC 1205; 11: Pb IOC 1479; 12: Pb IOC 1124; 13: Pb IOC 950; 14: Pb IOC 949; and 15: Pb IOC 1148. Molecular weight (MH) markers: 100 bp DNA ladder is shown. The arrow indicates the 750 bp fragment that was present in the virulent strains.

primer provided a RAPD pattern that was reproducible and discriminated between virulent strains with typical morphology and nonvirulent strains with atypical morphology. The technique of RAPD analysis used in association with virulence, morphological and immunological characteristics might prove adequate for detecting differences between *P. brasiliensis* isolates (Kurokawa et al. 2005). Additionally, we identified one expressed sequence (ceja-1) that was selectively present in morphologically typical virulent strains. The function of the ceja-1 sequence remains to be determined. In conclusion, RAPD methodology proved to be simple, inexpensive and appropriate for genetic discrimination of virulent and nonvirulent strains.

Nucleotide sequence accession number - The nucleotide sequence of the ceja-1 fragment from *P. brasiliensis* was submitted to GenBank. Accession number: AY665294 (Pb IOC 3698).

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