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Diversity in *Paracoccidioides brasiliensis*. The Pb*GP43* gene as a genetic marker

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Abstract *Paracoccidioides brasiliensis* is a temperature-dependent dimorphic fungus and the agent of paracoccidioidomycosis (PCM), which is prevalent in rural workers of Latin American countries. Until a decade ago, most of the studies involving *P. brasiliensis* used clinical isolates, since environmental samples from soil are difficult to obtain. More recently, *P. brasiliensis* has been isolated from infected wild and domestic animals, especially from the nine-banded armadillo *Dasypus novemcinctus* in Brazil. Over the years, diversity within the species has been observed at several phenotypic levels. The present review will discuss the reports focusing on genetic polymorphism, which culminated with the detection of *P. brasiliensis* phylogenetic species as a result of a multilocus study. Polymorphism in the Pb*GP43* gene is detailed. This gene encodes fungal glycoprotein gp43, a dominant *P. brasiliensis* antigen largely studied in the last two decades for its importance in diagnosis, immune protection, and adhesive properties to extracellular matrix-associated proteins. Fungal traits associated with genetic groups are discussed.

Keywords *Paracoccidioides brasiliensis* · Genetic polymorphism · Pb*GP43* gene · RAPD · Chromosomal polymorphism · Phylogenetic species

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Introduction

Paracoccidioides brasiliensis, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis* and *C. posadasii* constitute a group of thermal dimorphic fungi that cause endemic systemic mycosis. *P. brasiliensis* is the agent of paracoccidioidomycosis (PCM), which is prevalent in rural workers of Latin American countries. Infection is believed to occur by inhalation of conidia produced by the saprophytic phase of the fungus living in nature [1]. In the lungs, the infectious particles readily transform into the yeast pathogenic form [2] to cause an asymptomatic infection, or active disease in a small percentage of the infected individuals. Until a decade ago, most of the studies involving *P. brasiliensis* used clinical isolates, since environmental samples from soil have been difficult to obtain [3]. More recently, *P. brasiliensis* samples have been isolated from infected wild and domestic animals, especially from the nine-banded armadillo *Dasypus novemcinctus* in Brazil [1].

Over the years, diversity within *P. brasiliensis* has been observed in terms of microscopic [4, 5], macroscopic [6], growth and transition characteristics [7, 8], lipid composition [9, 10], cell wall components [11–13], isoenzyme profiles [14], antigen production [15–17], protein contents [18], and virulence degree [19–22]. The present review will focus on the genetic polymorphism observed in the species, which culminated with the detection of phylogenetic species as a result of a multilocus study [23]. Polymorphism in the Pb*GP43* gene [24] is detailed. This gene encodes fungal glycoprotein gp43, a dominant *P. brasiliensis* antigen largely studied in the last two decades since its characterization, purification, and production of monoclonal antibodies anti-gp43 [25, 26a, 26b, 27]. The antigen is mostly secreted to the extracellular environment, but it can be found within the cytoplasm and on the cell wall [28]. It bears only one *N*-linked chain [29]. The initial interest in gp43 was concentrated in its diagnostic value, since the protein moiety yields highly specific and sensitive reactions for diagnosis and prognosis of PCM [26a, 30, 31]. However, the importance of gp43 in the host–parasite relationship has later been recognized at the levels of its adhesive properties to extracellular matrix-associated proteins [32, 33] and immune protection. Towards this end, a major T-cell epitope has been mapped in peptide P-10, which is protective to mice and represents a vaccine candidate with immunotherapeutic potential use [34–37].

Chromosomal polymorphism and ploidy

Early evidences of polymorphism in the number and sizes of chromosomal bands were provided by Montoya et al. [38] and Cano et al. [39], who optimized conditions for separation of *P. brasiliensis* chromosome-sized DNA molecules from clinical isolates using pulsed field gel electrophoresis (PFGE). They found a haploid number of 4 or 5 chromosomal bands, ranging from approximately 2–10 Mb. These observations were later confirmed with an increased sample, including environmental isolates [40, 41]. Distinct karyotype profiles showed rather variable banding sizes, characterizing chromosomal length polymorphism. Differences in fluorescence intensity of the ethidium bromide-stained bands suggested that not all bands were present in equimolar amounts and that some could have resulted from co-migration of two or more chromosomes with similar sizes.

The existence of chromosomal polymorphisms made it difficult to compare the banding pattern among *P. brasiliensis* isolates. In order to determine chromosome identity, Feitosa et al. [41] used genetic markers corresponding to nine *P. brasiliensis* genes as probes to hybridize Southern blots of intact chromosomal bands from 12 isolates. Six distinct karyotype profiles and three synteny groups (genetic linkage groups) were identified containing two or three genes each. The adjacent genes Pb*LON* and Pb*MDJ1* [42] mapped to the same chromosomal band (9.5 Mb in most isolates, 8.7 Mb in Pb113 and 6.7 Mb in Pb339), and so did a putative manosyltransferase gene probe (clone 11), characterizing a synteny group. Another synteny group was *CHS1* and 28S*rRNA*. Adjacent Pb*GP43* and Pb*RanBP* also mapped to the same band (9.5 or 4.7), where the *NAG* probe localized to in 10 isolates. However, in PbSS and Pb339 there was a clear dissociation, with *NAG* being more distant from linked Pb*GP43* and Pb*RanBP*, suggesting chromosomal rearrangement. The patterns showing Southern blots cross-hybridization of the 10.0 and 3.5 Mb bands strongly suggested the existence of repetitive sequences in the genome of *P. brasiliensis*. No correlation could be established between the karyotype profile and the clinical-epidemiological characteristics of the isolates. Since the linkage groups were generally conserved, in spite of gross differences there is an underlying similarity in the genome organization of *P. brasiliensis* isolates.

The hybridization data [41] provided evidence that translocation and reciprocal translocation might contribute to chromosomal polymorphism in *P. brasiliensis* and that chromosomal rearrangements could provide a means for genetic variation in this organism, as proposed to occur in *Candida albicans*, and other fungi and eukaryotes [43–45]. The results on the molecular karyotypes of pathogenic fungi demonstrate the overwhelmingly fluidity of chromosome organization among eukaryotes with small genomes, which probably contributes to the maintenance of genome functionality and control of gene expression.

The size of the fungus genome was initially estimated to be in the range of 23–31 Mb by the summation of the molecular weights of chromosome-

sized DNA molecules resolved by PFGE (or CHEF) [38–41]. However, when the genome size of several isolates was calculated by microfluorometry of 4′,6–diamino-2-phenylindole (DAPI)-stained nuclei [39, 41] some values (60 Mb) reached twice those estimated by PGFE. Comparison of data generated by both methods indicated the possible existence of haploid, aneuploid and diploid isolates, being therefore inconclusive. On the other hand, both Southern blots of megarestriction fragments with a Pb*GP43* gene probe [24] and intra-specific polymorphism in the gene sequence [46] suggested diploidy.

Recently, Almeida et al. [47] applied a previously developed flow cytometry (FCM) protocol [48] to determine the genome size per uninucleated *P. brasiliensis* yeast cells and conidia of 10 representative isolates. The genome size calculated by FCM ranged from 26.3 ± 0.1 to 35.5 ± 0.2 Mb per uninucleated yeast cell. The DNA content of conidia from *P. brasiliensis* ATCC 60855 (30.2 ± 0.8 Mb) was similar, possibly excluding the occurrence of ploidy shift during morphogenesis. The ploidy of *P. brasiliensis* isolates was assessed by comparing genome sized by FCM with the previously described average haploid size obtained from electrophoretic karyotyping. The analysis of intra-individual variability of Pb*GP43* by sequencing cloned fragments of PCR fragments obtained with proof-reading Taq polymerase indicated that only one allele seems to be present. Overall, the results showed that all analyzed isolates presented a haploid, or at least aneuploid, DNA content and no association was detected between genome size/ploidy and the clinical-epidemiological features of the studied isolates.

Genetic variability in *P. brasiliensis* revealed by RAPD

In this session, we discuss the works that in the past 12 years have used random amplified polymorphic DNA (RAPD) to recognize *P. brasiliensis* genetic identities among isolates and correlate them with other overlapping fungal features. Details on the correlation with virulence are in a separate item below. RAPD became a popular technique in the 1990s for its simplicity to obtain information about genetic mapping, taxonomy and phylogeny. It is a modification of the polymerase chain reaction (PCR), where genomic fragments are amplified by a single arbitrarily chosen primer, providing a fingerprinting band pattern visible in agarose gels characteristic of similar template DNAs [49].

In *P. brasiliensis*, the first report suggesting the presence of genetically diverse subgroups in *P. brasiliensis* was released in 1995 [50]. It was a small-scale study with seven clinical isolates from the middle-west part of Brazil and from Equator, where the authors selected five commercially available random primers. They managed to distinguish between two major groups of isolates sharing 35% of genomic identity. Pb01 and Pb7455 composed a separate group II with all primers. The authors later broadened their investigation [51] with the inclusion of other eight isolates and 5 primers, and reached similar results: Group II was composed of Pb01, 7455 and 2514, which shared only 17% of genetic similarity with Group I, where subgroups could be identified. While the authors could not appoint any correlation between genetic groups and geographic origin in their studies, they found a relationship between the genetically distant Group II and increased virulence in B10.A mice.

Kurokawa et al. [52] could not find obvious correlation of *P. brasiliensis* virulence with genotypic groups. They analyzed 10 isolates, mostly from the city of Botucatu in São Paulo, Brazil, and used 17 commercial RAPD primers. They obtained a dendrogram with two major clades, where isolates Bt56 and Bt84 formed a separate group that shared only 56% of identity with the others. Pb18 and Pb18B6, recently recovered from mice organs, were not 100% identical, in contrast with the findings from Motta et al. [53]. These authors obtained RAPD profiles with 10 commercial primers and 25 isolates, mostly clinical from the state of São Paulo, Brazil. The resulting dendrogram had two distinct groups, where Bt56, Bt93 and Pb262 formed a small Group A.

Hahn et al. [54] tested 26 clinical, one armadillo and two soil isolates in their RAPD analysis with 5 primers, which individually generated similar phenograms with a subdivided group I and small group II composed almost exclusively of isolates from Mato Grosso, Brazil. The authors could not correlate genotypic groups with any of the patients' features. However, they observed that all the isolates in group II were from patients who responded well to therapy with trimethoprim-sulfamethoxazole, while other

three isolates from Mato Grosso, which were assembled in group I, derived from patients who had relapses or were refractory to the sulfa therapy.

Calcagno et al. [55] obtained RAPD profiles from 33 isolates from South American countries using 5 OPG primers. They managed to get full discrimination among isolates with all primers but OPG-18, while OPG-14 alone could discriminate isolates geographically. By using an SSI of 0.63, the isolates were generally grouped according to the country of origin, i.e., Argentina, Brazil, Colombia, Peru, and Venezuela. Geographic discrimination of the same samples was later confirmed using restriction fragment length polymorphism (RFLP), where the restriction enzymes *Hinf*I and *Hinc*II produced clear RFLP patterns [56]. Some authors [52, 54] have mentioned that they could not discriminate isolates by their geographical localization using similar primers as Calcagno et al. [55]. However, their results are difficult to compare, because in those investigations either the isolates analyzed were 80% from Brazil [54] or geographically restricted to São Paulo [52]. Hence, the intrinsic genetic diversity within each group prevailed.

Sano et al. [57] used random primer OPG-19 to study the genetic similarity among different armadillo clones isolated from the same animal, eventually from the same organ, and between armadillo and clinical samples. They studied a total of 83 samples, where 19 were clinical isolates. The resulting dendrogram showed clinical and armadillo samples clustered together in clades I and II, suggesting that the same environmental isolates can infect both humans and armadillos. In addition, they were able to conclude that one armadillo could be infected with genetically distinct isolates, which was later corroborated by Pb*GP43* partial sequencing of three samples [58]. Hebeler-Barbosa et al. [59] also found RAPD genetic similarity between *P. brasiliensis* isolates from 10 armadillos and two human PCM patients. In this work, isolates T10B1 (B7) and Bt84 (B25) formed a separate group I and were later found to belong to phylogenetic species 2 [23], as discussed later in this review.

RAPD studies have been useful in the past to determine the existence of genetically distinct *P. brasiliensis* groups, their relationship with geographic distribution and the similarity among clinical, animal and environmental samples; however the information obtained is limited and difficult to compare. Gene polymorphism and multilocus studies, as discussed below, result in more reproducible and informative data.

Genotypic variation in the Pb*GP43* gene

Pb*GP43* was the first gene fully characterized in *P. brasiliensis* [24]. In Pb339, the gene localized to a genomic *Eco*RI 3.8 kb cloned fragment, from which a 1,981-pb sequence was initially obtained (GenBank accession number U26160). This fragment contained the Pb*GP43* open reading frame (ORF) within 1,329 bp, and part of the 5′ and 3′ intergenic regions (326 bp of each). The Pb*GP43* ORF is composed of two exons separated by one 78-bp intron and the gene apparently has a single copy. ORF translation generates a precursor protein of 416 amino acids bearing a leader peptide of 35 residues, as deduced by *N*-terminal sequencing information of secreted gp43, which predicts alanine as the first amino acid of the extracellular, processed gp43.

We presently know the exact size of the Pb*GP43* transcript. The preferential transcription start point has been mapped in four isolates by primer extension at position –25 (CA), but two other and less intense sites at –33 (CA) and –35 (TA) have also been detected [60]. The Pb*GP43* transcription end-point was determined by 3′ RACE in 10 fungal samples, where 11 different poly-A sites, generally PyA, were mapped between positions 1,420 and 1,456 [Morais and Puccia, unpublished results]. Diversity within the poly-A site was detected both intra- and inter-isolates, considering that about ten 3′ RACE generated clones were analyzed for each isolate. Therefore, the Pb*GP43* transcript is between 1,367 and 1,413-bp long, which correlates well with the 1.5 kb originally found in Northern blot [24]. Full sequencing of the *Eco*RI 3.8-kb insert revealed that the Pb*GP43* 3′ neighbor is homologous to the gene encoding importin 11 or Ran binding protein 11 (RanBP11), involved in the nucleuscytoplasmatic transport of molecules. The 3′ ends of both genes converge to an intergenic region of about 550 bp. Pb*GP43* and Pb*RanBP*-like were found to be linked in 12 different isolates [41], as mentioned earlier.

The Pb*GP43* sequence revealed by Cisalpino et al. [24] was essential to map the gp43 murine T-cell epitope [34], and to investigate gene polymorphism

Table 1 Simplified presentation of the Pb*GP43* genotypes according to its substitution sites

nucleic acid		Genotypes[c]					Aminoacids[a]		
Substitution site[a]	consensus nucleotide[b]	A	B	C	D	E	consensus	position	substitution
-259	C	.	T	.	.	.			
-230	C	A	.	.	.	.			
-210	G	.	.	.	T	.			
-209	C	.	A	.	.	.			
-120	T	A	.	.	.	.			
-104	C	G	.	.	.	.			
-40	C	.	.	.	.	T			
27[d]	C	.	T	T	.	.	A	9	A
268[e]	G	A	.	.	.	.	D	90	N
578[e]	A	G/A	.	.	.	.	H	167	R
589[e]	A	.	.	.	G	.	I	171	V
617[e]	G	A	A	A	.	.	R	180	K
628[e]	A	G	.	.	.	.	I	184	V
751	G	A	.	.	.	.	E	225	K
763	C	G	.	.	.	.	H	229	D
799	G	.	.	.	T	T	A	241	S
821	C	.	T	.	.	.	P	248	L
830	C	T	.	.	.	.	T	251	I
852	C	.	G	.	.	.	P	258	P
856	A	G	.	.	.	.	T	260	A
872	A	T	.	.	.	.	Y	265	F
874	C	.	.	.	.	A	L	266	I
912	T	.	T/C	.	.	.	F	278	F
965	A	.	.	.	.	G	D	296	G
981	C	T/c	.	.	.	.	A	301	A
1082	C	G/A/c	.	.	.	.	P	335	R/H
1086	C	T	.	.	.	.	S	336	S
1143	C	.	.	.	.	C/T	S	355	S
1157	A	G	.	.	.	.	K	360	R
1166	T	G	.	.	.	.	L	363	R
1205	G	.	.	C	.	.	G	376	A

Characteristic substitutions for each genotype are colored

[a] Numbering according to Cisalpino et al. [24]

[b] Seen in more than half of the isolates [23, Supplementary Table 3]

[c] Adapted from Hebeler-Barbosa et al. [59]

[d] Not seen in Morais et al. [46]

[e] Not analyzed in Matute et al. [23]

[46]. It is worth mentioning that the original sequence, whose position numbering is followed here and in the related literature, has later been updated for mistakes in the GenBank.

The interest to study gene polymorphism originated from the existence of gp43 isoforms observed in the original work characterizing gp43 from Pb339 culture supernatant fluids [25]. Later, Moura-Campos et al. [17] showed that diversity in gp43 isoelectric points (pIs) ocurred both intra- and inter-isolates. The authors described four pI profiles in eight distinct isolates, i.e., A: 6.0, 6.2, 6.6 and 7.0; B: 6.4, 6.8 and 7.2; C: > 8.5; D: 5.8, 6.2, 6.6. Peculiar profile C was characteristic of only one sample (Pb1925 or Pb2 or V2). These pI differences were likely to be due to substitutions in the amino acid sequence, considering

the lack of ionic groups or acidic sugar moieties in the glycoprotein structure [29].

Sano et al. [58] made the first description of substitution sites in Pb*GP43*. They compared 539 bp from Pb*GP43* exon 2 of spleen (D3S1), liver (D3LIV1) and lymph nodes (D3LY1) isolates from the same armadillo and observed substitutions in sites 617, 799, 821 and 852 that distinguished the spleen isolate from the others. They also observed that D3S1 caused statistically significant more extensive lung lesions than the other isolates in ddY male mice inoculated intravenously (i.v.) with *P. brasiliensis*.

Morais et al. [46] characterized the Pb*GP43* polymorphism in a sample composed of one Brazilian armadillo, one Venezuelan soil and 15 clinical isolates from acute and chronic PCM patients mostly from Brazil, but also from Venezuela, Argentina and Peru. The authors compared two cloned PCR fragments from the whole gene (exon 1, intron and exon 2) and later from 326 bp of the promoter region [60]. They found 21 informative substitution sites in the ORF, mostly in exon 2, none in the intron (sites 464–541), and seven in the promoter region, which defined 5–6 genotypes. We are calling informative those sites found in at least two isolates. The maximum-likelihood phylogenetic tree generated by these sequences clearly reflected the presence of specific genetic groups in the species, as already suggested by RAPD. The sequences of isolates Pb2 (1925 or V2), Pb3 (608 or B26) and Pb4 (1017 or B23) were highly polymorphic (genotype A, Table 1) and phylogenetically distant from the other subgroups. The genetic peculiarity of Pb2, Pb3 and Pb4 was also suggested by Southern blot analysis with total DNA digested with *Bgl*II and hybridized with a Pb*LON* probe [42], which labeled a slower-migrating fragment in these isolates [46].

The existence of *P. brasiliensis* phylogenetic species, however, could only be confirmed later, through a multilocus study from Matute et al. [23], which included Pb*GP43* exon 2 and promoter region/leader sequence. The authors studied DNA extracted from 65 *P. brasiliensis* isolates from Brazil, Colombia, Paraguay, Peru, Uruguay and Venezuela. They analyzed PCR products directly by automated sequencing and considered both informative and non-informative sites in their analysis. The sample included 13 isolates from Morais et al. [23] and also 12 from Hebeler-Barbosa et al. [59], who used Pb*GP43* exon 2 sequence alignments and RAPD to genetically compare isolates from 10 Brazilian armadillos with clinical samples. Table 1 brings a simplified overview of the Pb*GP43* genotypic patterns (A–E) based on the informative sites reported by Morais et al. [46], Hebeler-Barbosa et al. [59] and by Matute et al. [23 Supplementary Table 3]. Consensus nucleotides are here defined as those present in more than half of the samples. Note that none of the existing ORFs is consensus, while promoter genotype C sequences (e.g. Pb339) are consensus. Generally, the substitutions resulted in non-synonymous amino acid changes. The characteristics associated with each Pb*GP43* genotype (Table 1) are listed below. Isoelectric points were calculated for the processed protein [46].

Genotype A (pIs 7.82–8.35): it is the most polymorphic, with up to 15 substitutions in the ORF and three in the promoter. At least 13 substitutions are characteristic of this genotype, specifically –230(A), -120(A), -104(G), 268(A), 628(G), 751(A), 763(G), 830(T), 856(G), 872(T), 1086(T), 1157(G), 1166(G). So far, six isolates presented this genotype, one from a chronic PCM Venezuelan patient (1925, Pb2 or V2) and five from Brazil, specifically São Paulo (608, Pb3 or B26; 1017, Pb4 or B23; Bt84 or B15; T10B1 or B7) and Minas Gerais (Uberlândia or B13). According to a multi-locus analysis discussed later in this review [23], these isolates apparently had an independent evolution and have been classified as phylogenetic species 2 (PS2). The PS2 genealogy was strongly supported by polymorphism in the genes encoding Pb*GP43* (both regions), α-tubulin and ADP-ribosylation factor.

Genotype B (pI 6,87): it has only been detected in five Venezuelan isolates and two samples from the liver and lymph nodes of same Brazilian armadillo [58], not included in the multi-locus study [23]. It can be distinguished from the others by sites –259(T), –209(A), 821(T) and 852(G). The Venezuelan group could not be considered an isolated phylogenetic species [23].

Genotype C (pI 6,87): it is characteristic of clinical and armadillo isolates from Colombia and of Pb339. It is characterized by site 1205(C) and by having consensus promoter. The Colombian isolates from this group form phylogenetic species 3 (PS3), a geographically restricted group that can be considered an independent lineage by the non-discordance

criterion [23], which is strongly supported by the α-tubulin genealogy.

Genotype D (pI 7,13): it has been detected in a small group of isolates from Peru, Paraguay, Argentina and two from Paraná, Brazil. It is characterized by –210(T) and 589(G).

Genotype E (pI 7,13)**:** it is characteristic of most Brazilian and Argentinean isolates, besides one from Uruguay. Sites –40(T), 874(A), and 965(G) can distinguish it. A subgroup of genotype E composed of one Argentinean and five Brazilian isolates is distinguished by site 1143(T).

Isolates that bear Pb*GP43* genotypes B, D and E are within the species S1, a major independent species [23].

While these results justify inter-individual variety in gp43 pI, the origin of intra-individual variation, as reported earlier [17, 25], remains debatable. Morais et al. [46] detected many unique substitution sites (non-informative) and four variations that alone were responsible for intra-individual gp43 pI change. Almeida et al. [48] obtained equal sequences in eight cloned exon 2 fragments from eight isolates. The use of proof-reading polymerase, the limited number of nucleotide analyzed, and the high number of PCR fragments tested by the latter authors are differences to be considered.

Impact of Pb*GP43* polymorphism at the protein level

At the protein level, the gp43 sequence belongs to the glycosyl hydrolases family 5 and shares about 50% of identity with fungal exo-β-1,3-glucanases, like that from *C. albicans* [24]. Purified gp43 and culture supernatants enriched for the glycoprotein were, however, negative for exo-glucanase activity when tested against laminarin or *p*-nitrophenyl-β-glucoside [24]. That has been attributed to the lack of a consensus NEP among β-1,3 as well as β-1, 6-glucanases [61], which is NKP in gp43 sequences from all isolates analyzed so far [23, 46]. While the involvement of the NEP site in catalysis has been proven in *C. albicans* [62, 63], in the closely related *H. capsulatum* and *B. dematitidis* databases (http://genome.wustl.edu) sequences almost 60% identical to gp43 have been found where NEP is also present. Hence, in these dimorphs the gp43 homologues possibly code for functional β-1,3-glucanases. Although the gp43 might be a non-functional glucanase, some traits characteristic of glucanase gene regulation are apparently maintained in the Pb*GP43* promoter. We are currently studying the Pb*GP43* gene regulation and mapping transcription elements in the 5′ untranslated region, from which we presently cloned and sequenced 1,805 bp. We observed that mRNA accumulation sharply decreased 30 min after addition of 1.5% glucose or cellobiose in the culture medium in three isolates tested [Rocha and Puccia, unpublished observations]. On the other hand, the gene seems to be strikingly regulated by NIT2 transcription elements, present at high numbers in the 5′ non-translated region, considering that mRNA accumulation greatly increased in 3 isolates when exogenously added ammonium sulfate was deprived from the culture medium. Addition of the salt had the opposite effect.

Souza et al. [64] tested purified gp43 isoforms from three isolates using a capture ELISA test and a number of sera from adult and juvenile PCM patients. They found that the reactivity was similar (100%) among isoforms when the capture monoclonal antibody was MAb8a, but there was a decrease in the number of positive sera from juvenile (56%) and adult (71%) cases reacting with the basic isoform when the capture antibody was MAb17c. This result suggests that some differences in epitope expression might occur depending on the isoform, especially in the most polymorphic basic form. It also indicates that the epitope recognized by MAb17c is common to all isoforms. We have expressed in *Pichia pastoris* soluble gp43 derived from Pb*GP43* genotypes A, D and E (Table 1). We verified that their reactivity in immunodifusion tests was comparable to that of native protein purified from Pb339 when 100 PCM sera were tested [Carvalho, Vallejo and Puccia, unpublished results]. We are currently testing more sensitive tests, where differences in reactivity could show using immobilized antigens.

At this point, it is difficult to predict if gp43 isoforms would have an impact in the outcome of PCM. The protective T-cell epitope and vaccine candidate P-10 [34] has a single polymorphic aminoacid at position 184 (Ile or Val), which is out of the epitope core and should not change T-cell recognition. There is one protective antibody detected so far (MAb32), which recognizes a conserved

epitope encoded in exon 1 [Bruissa-Filho et al., unpublished results]. Further investigation will be necessary to recognize other potentially relevant epitopes and their susceptibility to aminoacid substitution. This is also true for protein putative motifs, such as binding sites for extracellular-associated molecules. Mendes-Giannini et al. [33] have recently tested the capacity of 4 gp43 peptides to inhibit gp43 binding to fibronectin. They managed to inhibit binding to 57% with a conserved peptide 1 (NLGRDAKRHL), present in the conserved *N*-terminal. Other peptides (2 and 4) bear polymorphic amino acids, but were weak inhibitors.

Polymorphism in other genes, multilocus studies and phylogenetic species of *P. brasiliensis*

Several other *P. brasiliensis* genes have been studied for polymorphism, but Pb*GP43* exon 2 is the most polymorphic region seen so far. In a multilocus study undertaken by Matute et al. [23], eight regions from five nuclear genes were analyzed in 65 isolates representing 6 endemic areas: promotor-exon 1 and exon 2–4 from chitin synthase (*CHS2*), exons 2 and 3 from β-glucan synthase, exons 2–4 from α-tubulin, exons 2–3 from adenyl ribosylation factor (*FKS*), and promoter-exon 1 and exon 2 from Pb*GP43*. Among these gene regions, *FKS* exons 2 and 3 and *CHS2* promotor-exon1 were not relevant due to their low polymorphism. Based on these multilocus genealogies, it was possible to follow the evolutionary lineages and to identify phylogenetic species by means of the criteria of genealogical concordance and non-discordance [65]. This study indicates that *P. brasiliensis* consists of at least three different, previously unrecognized phylogenetic species as follows: S1 (species 1), formed by 38 isolates; PS2 (phylogenetic species 2) enclosing six isolates (five of them from Brazil and one from Venezuela); PS3 (phylogenetic species 3), composed solely by 21 Colombian isolates. The genealogies supporting these clades had both high bootstrap and posterior probabilities values above 70% and 0,95%, respectively. By means of a similar approach cryptic species have been found in other Onygenalean human pathogenic fungi such as *H. capsulatum* [66] and *Coccidioides immitis* [67]. The analyses by Matute et al. [23] also provided evidences supporting recombination in nature of at least one of the *P. brasiliensis* species, thus indicating the presence of sexual reproduction. This is reinforced by the presence of teleomorphs in close relatives of *P. brasiliensis*, such as *B. dermatitidis (Ajellomyces dermatitidis)* [68] and *H. capsulatum (Ajellomyces capsulatum)* [69].

Other regions from non-coding DNA have been used for classification purposes, such as the internal transcribed spacer (ITS) regions of the ribosomal DNA complex [59] and microsatellites [70, 71]. ITS1 and ITS2 presented low polymorphism, and most of the sequences were consensus. Microsatellites (simple sequence repeats) are segments of 1–6 nucleotides repeated in tandem. Variations in the number of repeated units result in length polymorphism that can be useful in fungal fingerprinting [72, 73]. In *P. brasiliensis*, microsatellite makers have been selected out of a series of identified repeated sequences and their length polymorphism profiles were originally used to unsuccessfully discriminate between clinical form and fungal genetic trait [70]. On the other hand, Matute et al. [71] were able to discriminate among the three *P. brasiliensis* phylogenetic species by using a set of microsatellite markers, which can be used as typing system. The approach was based on PCR amplification of three selected microsatellites. It is reproducible, easy to assay and acquiescent to high-throughput screening of a large sample of isolates. The system is especially useful for unambiguous discrimination of species S1 and PS2.

Further studies using the *CHS2* gene of chitin synthase II, for which the substitution rate was calculated from previous 18S DNA sequences, allowed us to calculate the divergence date of *P. brasiliensis* phylogenetic species. Using two calibration points, the divergence date for PS2 and PS3 was 8.04 mya (SD = 1.17) for the first point, and 8.37 mya (SD = 0.98) for the second point [74].

Ongoing work using partial mitochondrial sequences of five genes from 65 *P. brasiliensis* isolates have revealed that certain genes carry high polymorphism (*COB*, *RN1*, *RNS*), thus permitting to draw a more accurate phylogenetic connection between the related species and isolates. Genes such as *COX3* and *ATP6* have not shown significant variation, and appear highly conserved intraspecies, probably due to the region chosen for the analysis [Salgado and McEween, unpublished results].

Genetic groups of *P. brasiliensis* and disease

In this session, we present the associations made to date between genetic groups discussed earlier and virulence, as tested in animals, or clinical presentation of the disease. Few works have so far reported a positive correlation between genetic groups and disease [51, 60]. On the other hand, the relevance of conclusions and comparisons regarding virulence have to be critically evaluated by the readers, because the authors use different animal models, inoculum sizes, routes of infection, type of analysis, among other possible experimental differences that could lead to different results and conclusions for the same isolate. One important parameter generally disregarded by the researchers is the fungal status at the time of inoculation, i.e., if they have recently been recovered from organs, in-vivo adapted or adapted in vitro, which can lead to attenuation [75, 76]. A single animal passage might not be enough to re-establish full virulence. The use of only one route of infection might also be insufficient to label isolates as highly or weakly virulent. Pb18, a broadly studied isolate, is probably an exception, since it has repeatedly been proven to be highly virulent to different mice strains independently from the route of infection; however it is still susceptible to in vitro attenuation [76]. In regard to clinical PCM features, they are a product of the fungal–host interaction and greatly depend on the host's individual characteristics.

Molinari-Madlum et al. [51] selected six representative isolates from the two RAPD major groups I and II to inoculate sensitive B10.A male mice intraperitoneally (i.p.). Group I isolates showed regressive pattern of infection, with little tissue damage and dissemination, while infection with group II isolates (Pb01 and 7455) resulted in intense, progressive and disseminated peritonitis, with extensive tissue damage in the diaphragm and other organs caused by numerous non-organized granuloma. Other reports claimed unsuccessful correlation of RAPD genetic groups with virulence or PCM clinical features [53–55], however they generally relied on medical records and/or literature data about the isolate degrees of virulence. More recently, Kurokawa et al. [52] tested virulence of RAPD genetically distinct isolates and could not correlate the results; however they used outbred swiss mice.

Carvalho et al. [60] used the susceptible B10.A male mouse model to compare the degree of infection caused by *P. brasiliensis* bearing phylogenetically distant Pb*GP43* sequences (Pb2, Pb3, Pb4, phylogenetic group PS2) with others (Pb5, Pb8, Pb9, Pb12, Pb18, species S1). They compared the rate of fungal recovery from organs (c.f.u.) and histopathology (i) 21 days after i.p. inoculation of in vitro-adapted isolates, (ii) 30 and 60 days after intratraqueal (i.t) infection with organ-recovered isolates and (iii) survival after i.v. infection with organ-recovered and *in vivo*-adapted isolates. Pb2, Pb3 and Pb4 evoked fewer deaths (i.v. infection) and were recovered from the lungs (i.t. infection) at significantly lower c.f.u than the others. Anti-gp43 responses in animals infected with these isolates were richer in IgG2a, IgG2b and IgG3, suggesting a Th1 predominant type of immune response, in contrast with the other sera that were richer in IgG1 and IgA [Carvalho and Puccia, unpublished results]. Furher i.t. infection with *in vivo*-adapted Pb3, Pb5, Pb12 and Pb18 followed for 30, 60 and 120 days showed a similar pattern of anti-gp43 response. Mice infected with Pb3, whose c.f.u. in the lungs were initially high but declined after 120 days of infection, secreted increasing amounts of IFN-γ, while IL-10 could only be detected by 30 days of infection. The other animals had progressive infection, decreasing amounts of IFN-γ, and IL-10 was detected at all time points [Carvalho et al., unpublished results].

These results suggested that PS2 isolates elicit a distinct pattern of host response, which in the B10.A mouse model prevents disease progression. In contrast, PS2 isolate T10F1 (B7), which had been recently isolated from armadillo, was very aggressive in the hamster testicular model [59]. Isolate Bt84 (B15), also in PS2 group, was very virulent in outbred Swiss mice inculated i.v and analyzed 30 days later [52].

If it is true that genetically distinct groups of isolates might have differentially expressed molecules that determine the faith of infection upon contact with the host, some of the features might be related to differential transcription and/or translation regulation. Towards this end, Carvalho et al. [60] observed that the Pb*GP43* mRNA accumulation in yeast cells decreased during the first hour of heat shocked at 42°C and during the first hours of

temperature change to 36°C for mycelium-to-yeast transition, when compared to Pb18. The relationship between these differences with polymorphism in the promoter region is still under investigation. The Pb*MDJ1* and Pb*LON* genes, which share a 5′ intergenic region containing heat shock and oxidative stress-related transcription elements, respond slower to heat shock in Pb3 than in Pb18, while only in Pb18 does Pb*MDJ1* seem to be preferentially expressed in yeast [77]. Sequencing comparison of the intergenic region form Pb3, Pb18, Pb12, Pb5 and Pb339 revealed gaps, insertions and substitutions in Pb3 that might interfere with regulation.

Concluding remarks

Further work should be oriented to additional characterization of the different *P. brasiliensis* phylogenetic species, in order to look for previously undetected morphological and phenotypic differences, gene regulation, as well as for associated variation in virulence, as previously reported [60]. The possibility of genetic manipulation will be of great value in these studies [78].

Presently, there are two large expression sequence tag (ESTs) databases available for *P. brasiliensis*. One assembles 4,692 genes from the yeast phase of Pb18 (S1 species) recently recovered from the spleen of infected mice [79, http://143.107.203.68/pbver2/default.html]. The other one represents 6,022 expressed genes from the yeast and mycelial phases of in vitro-adapted Pb01 [80, 81, http://www.biomol.unb.br/Pb]. The complete genome of *P. brasiliensis* will be available in a near future, thanks to the efforts of the Broad Institute with support of the Dimorphic Fungal Genomes Consortium, which is currently developing a comparative genomics project on dimorphic fungal pathogens. The *P. brasiliensis* isolates included in this study are Pb18, representing major S1 group and virulence, Pb3 from phylogenetic species PS2, and Pb01 as a molecular model. Pb01 alone seems to belong to a phylogenetic group distinct from those previously identified [Carrero et al., unpublished data]. This study will allow the identification, among many other features, of shared and individual genetic determinants of pathogenicity and virulence in dimorphic fungi.

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