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Review article

Paracoccidioides brasiliensis and paracoccidioidomycosis: Molecular approaches to morphogenesis, diagnosis, epidemiology, taxonomy and genetics

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Paracoccidioides brasiliensis is an amenable model to study the molecular and biochemical events that lead to morphological transition in fungi, because temperature seems to be the only factor regulating this process. It is the causative agent of paracoccidioidomycosis, a systemic mycosis that affects humans and that is geographically confined to Latin America, where it constitutes one of the most prevalent deep mycoses. With the help of molecular tools, events leading to the morphological transition have been traced to genes that control cell wall glucan and chitin syntheses, and other metabolic processes such as production of heat shock proteins and ornithine decarboxylase activity. Molecular diagnosis and epidemiology of paracoccidioidomycosis are also the focus of intensive research, with several primers being proposed as specific probes for clinical and field uses. Although *P. brasiliensis* is refractory to cytogenetic analysis, electrophoretic methods have allowed an approximation of its genomic organization and ploidy. Finally, the recognition of *P. brasiliensis* as an anamorph in the phylum Ascomycota, order *Onygenales*, family *Onygenaceae*, has been accomplished by means of molecular tools. This phylogenetic placement has revised the taxonomic position of this fungus, which was traditionally included within now-abandoned higher anamorph taxa, the phylum Deuteromycota and the class Hyphomycetes.

Keywords *Paracoccidioides brasiliensis*, morphogenesis, dimorphism, virulence, phylogeny

Introduction

An important feature of several fungal pathogens is their inherent ability to change their morphology from a multicellular filamentous form to a unicellular form when they infect host tissues. Such processes are broadly referred to as dimorphism. This is an intrinsic genetic property of fungi such as *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Paracoccidioides brasiliensis*. Pathogenicity appears to be linked to morphogenesis since strains unable to undergo the morphologic transition are not virulent [1]. The morphological switch may not be necessary for the species to perpetuate itself, at least in the short term, since these fungi are able to live as soil saprobes and have the ability to grow in a mycelial form. It is only when they infect a susceptible host that the change in morphology occurs. Temperature, nutritional factors, or both, are usually the agents that activate this change in morphology [2]. The fact that temperature is the only factor triggering *P. brasiliensis* dimorphism [3] makes it an amenable model to study the molecular and biochemical events that lead to morphological transition, in contrast to other dimorphic fungi such as *Candida albicans* [4] or *H.*

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capsulatum [1], the phase transitions of which are linked not only to temperature but also to nutritional or environmental factors.

First described by Lutz in 1908 [5], *P. brasiliensis* is the causative agent of paracoccidioidomycosis, a human systemic mycosis geographically confined to Latin America, where it constitutes one of the most prevalent deep mycoses [6]. The frequency of mucocutaneous lesions suggests that inhalation of air-borne propagules is the main way in which infection is contracted [7]. After penetrating the host, the fungus must convert to its yeast form, a fundamental step for the successful establishment of the infection [3].

Most infected individuals develop only asymptomatic or subclinical paracoccidioidomycosis, which sometimes progresses into a disease with a diversity of clinical forms [6] depending on host factors, strain-level virulence differences, and environmental conditions [8]. High positive skin test reactivity (60-75%) in the adult population of endemic areas points to the relevance of this mycosis in South America, where figures suggest that around 10 million people may have been infected [7]. In this review we deal with molecular aspects of *P. brasiliensis* dimorphism, diagnosis, epidemiology, taxonomy and genetics. Molecular immunology of the fungus, though, was not incorporated here as this topic was covered in a previous review [8].

Molecular aspects of *P. brasiliensis* dimorphism

To dissect the mechanisms of morphogenetic control, researchers have studied the cell wall structure, metabolic processes, and signalling cascades, and have also used classical methods of enzyme detection and biochemical quantification of a wide range of metabolites. More recently, molecular tools have been used to discover genes with differential expression according to the fungal phase. In a simple model that can be extended to most pathogenic fungi, Ernst [4] proposed that hyphal induction of *C. albicans* proceeds in three phases. In the first phase, external signals are sensed by specific receptors on the cell surface, which in a second phase activate intracellular signal transduction pathways. In the third phase, the structural and regulatory components necessary for the formation of the hyphal form are produced. However, *Candida* is a difficult model to work with, since in this genus there are no clear-cut relationships between host colonization and pathogen morphology, so that budding yeasts, pseudohyphae, and hyphae may be all present simultaneously in lesions [9]. On the contrary, fungi such as *P. brasiliensis*, *H. capsulatum*, and *B. dermatitidis* are only present in lesions as yeast cells. This tends to make such species simpler models with which to study dimorphism.

Events leading to the synthesis and assembly of the fungal cell wall have been a major subject of research on morphogenesis. The interest in this topic has arisen because the wall is responsible for maintaining cellular shape and stability, and also because it is a likely candidate for the action of specific antifungal drugs. These drugs generally block the synthesis of wall glucan or chitin without interfering with any other metabolic process in the host. In pursuit of these goals, studies on biochemical details of glucan synthesis, and more recently, the sequencing of genes coding for glucan and chitin synthases, have been done for several pathogenic fungi. In fact, on January 2001 the U.S. Food and Drug Administration approved the use of Cancidas (caspofungin acetate), an echinocandin that blocks the synthesis of fungal glucan, as a new medication for patients who are unresponsive to or cannot tolerate standard therapies for the invasive form of aspergillosis.

In *P. brasiliensis*, the synthesis *in vitro* of β-glucan by β-1,3-glucan synthase requires UDP-glucose as the preferred nucleotide precursor [10], and this particulate enzyme is housed in the plasma membrane [11]. As occurs in some ascomycetes [12], the reaction is inhibited by GTP and other nucleotides [13], in sharp contrast to the general role played by these compounds as stimulators of fungal cell wall synthesis [14]. So far, only one related *P. brasiliensis* gene, *FKSPb1*, homologous to the β-glucan synthase genes *FKS1* and *FKS2* from *Saccharomyces cerevisiae* and *FKSa* from *Aspergillus nidulans*, has been cloned and sequenced [15]. This gene has an open reading frame of 5,942 bp; its complete sequence is interrupted by two putative introns. It also contains a promoter region with consensus sequences such as canonical TATA (-126) and CAAT (-244) boxes. At position −809 the sequence GCCAAG, which mediates pH-dependent gene expression in *A. nidulans* [16], was found. This was interpreted as being important in dimorphic transitions occurring during the establishment of host infection, while behaving as a possible complex mechanism that controls the expression of *P. brasiliensis* genes related to cell wall assembly. The deduced sequence of 1,926 amino acids (predicted MW 212 kDa) shows 85% similarity to FksAp from *A. nidulans*, and 71% to Fks1p and Fks2p from *S. cerevisiae*. Its molecular mass is similar to those of other Fks proteins [17,18]. Six potential N-glycosylation sites were found that may be the sites of post-translational modifications, which confer stability on the resulting protein [19]. Also, a PTS-Hpr (phosphotransferase system-phosphoryl carrier protein) element was found that may be involved in translocation across the cell membrane, assisting in the

vectorial synthesis and transport of cell wall components through the plasma membrane in a way that supports the orderly assembly of the wall [20]. Pereira *et al*. [15] also found two other DNA fragments that may correspond to distinct glucan synthase genes. This is in line with research on other fungal systems in which genes encoding glucan synthases appear to comprise multigene families [21]. While in *C. albicans* only one β-1,3-glucan synthase gene (*GSC1*) has been reported [22], in *S. cerevisiae* three highly homologous *FKS*-like genes have been described, two of which perform distinct functions. The *FKS1* gene predominates when yeast cells are grown on glucose. *FKS2*, in contrast, is essential for sporulation and is expressed in the absence of glucose [17]. A ubiquitous small GTP-binding protein, Rho1, functions as a regulatory component of β-1,3-glucan synthase holoenzymes in budding and fission yeasts, coupled to RHO effectors such as protein kinase C (PKC)-related molecules [23].

This knowledge sheds light on the cell wall as a crucial apparatus through which extracellular signals are received and integrated into intracellular processes [24]. The occurrence of more than one *FKS*-like sequence in *P. brasiliensis* may suggest differential gene regulation in the *FKS* family of this pathogen. However, it is also possible that one of these sequences is coding for α-1,3-glucan synthase, although no α-1,3-glucan synthase gene has been reported in *P. brasiliensis*. Such a gene (called *ags1*+ [25] or *mok1*+ [21]) has only been reported in the fission yeast *Schizosaccharomyces pombe*, where it is essential for cell viability and germination. Its predicted Ags1 or Mok1 protein (Mw 272 kDa) consists of two probable catalytic domains for α-glucan assembly, and a multipass transmembrane domain that might contribute to the transportation of the polysaccharide across the membrane. So far, the actual enzyme has remained biochemically elusive in this and other fungi, but it must exist, and be involved in the synthesis of α-1,3-glucan, an important component not only of the *S. pombe* cell wall, but also the major cell wall neutral polysaccharide constituent of the pathogenic yeast phase of several dimorphic fungi, namely, *P. brasiliensis* [26], *B. dermatitidis* [27], and *H. capsulatum* [28]. This polysaccharide is organized as a sort of outer capsule in the yeast phase of these three fungi. It replaces almost entirely the β-glucan that comprises the neutral polysaccharide of their vegetative mycelial phase, behaving like a virulence factor [26–28].

In all pathogenic fungi, chitin represents a major structural component of the cell wall [29] with functions in fungal morphogenesis [20], wall integrity [30,31], conidiophore development [30], or the anchoring and displaying of key molecules (such as the adhesin and virulence factor WI-1 on the surface of the yeast phase in *B. dermatitidis* [32]). In contrast to *S. cerevisiae*, where chitin constitutes a small percentage of the wall, this polysaccharide comprises a major fraction of the cell wall in filamentous ascomycetes and basidiomycetes [33]. Chitin synthesis in fungi is a rather complex process, and is regulated by multigene families encoding chitin synthase isoenzymes, some of them redundant, whose activities may be spatially ordered and otherwise strictly regulated to bring about the fulfillment of the several roles ascribed to them [34]. Based on differences in regions of high sequence conservation, chitin synthases have been organized into five classes [29,35,36] whose functional implications are not yet clear in all cases.

In *S. cerevisiae,* three chitin synthase isoenzymes without functional redundancy have been characterized (for a review see [29]). ScChs1p does not fall into any of the chitin synthase classes so far proposed [35], and is involved in cell wall repair after release of the daughter bud by chitinase action on the mother-bud neck, while ScChs2p (class II) synthesizes chitin of the primary septum [29]. Chitin synthase III activity, in contrast, is responsible for the synthesis of chitin during bud emergence and growth, mating and spore formation [29]. So far, five genes involved in CSIII activity have been identified [29,37–39]. The product of one of them (*CSD2/CHS3*) ScChs3p (class IV) is the catalytic subunit. The Csm-type (class V) and class III chitin synthases have been found so far only in filamentous fungi [36,40]. *A. nidulans* ChsB, *Aspegillus fumigatus* ChsC and ChsG, and *Neurospora crassa* Chs-1, all members of class III *CHS*s, play critical roles in normal hyphal growth and differentiation of conidiophores, while ChsA (class II) and ChsC (class I) of *A. nidulans* have important but redundant functions in hyphal cell wall integrity and differentiation [34]. These results are similar to those found in other fungal class I and class II chitin synthases, whose genes seem to be non-essential, as cells survive after gene disruption [41]. In turn, Csm-type are class V chitin synthases with a N-terminal myosin motor-like domain [40,42]. Disruption studies of *CsmA* of *A. nidulans*, and the selective expression (under the *alc*A promoter) of either the CHS domain or the whole *CsmA* gene in mutant strains, suggest that the gene could be important in the maintenance of hyphal wall integrity and polarized cell wall synthesis, for which the myosin motor-like domain would be indispensable [42].

Five chitin synthase genes, representing different classes of enzyme (*PbrCHS1* in class I , *PbrCHS2* in class II, *PbrCHS3* in class IV, and *PbrCHS4* and *PbrCHS5* in class V) are active in *P. brasiliensis* [43,44]; they help in the synthesis of chitin in amounts

that comprise 43% of the dry weight of the wall of the pathogenic yeast (Y) form and 13% of the mycelial (M) cell wall [3]. The nucleotide sequence of *PbrCHS2* contains an ORF 2,540 bp long, with three introns, each of which has the characteristic splicing signal (lariat formation) observed in such sequences. Two sequences resembling putative CAAT boxes (CAACT and CAAT) were identified 179 and 29 nucleotides upstream, respectively, of the proposed translation start site. Also, two sequences resembling putative TATA boxes (TATATTA and TATCAT) were identified 61 and 18 nucleotides upstream of the start codon. The deduced amino acid sequence is 1,043 residues long and predicts a 117 kDa protein that can be classified as a class II chitin synthase. The protein seems to have a three-domain structure that fits within the proposed model of vectorial synthesis and export of chitin from the cytoplasm to its final destination in the cell wall [20]. Phylogenetic trees of relatedness between PbrChs1 and PbrChs2, on the one hand, and PbrChs4 and PbrChs5, on the other, suggest that Chs1 and Chs2 group with similar enzymes of other fungi (Fig. 1A) while PbrChs4 clusters with the products of other class V genes, even though it is situated on another branch (Fig. 1B).

Expression of *P. brasiliensis* chitin synthase genes was explored by means of northern analysis for the temperature-induced dimorphic transitions from Y to M and back [44]. Transcripts of *PbrCHS3* were not detectable, perhaps due to the presence of a putative intron within the sequence of the probe, which may have reduced the sensitivity of detection for its transcript. *PbrCHS1* and *PbrCHS2* have similar transcript sizes at around 3.7 kb, while large transcripts of about 6.5 kb and 6.7 kb were observed for *PbrCHS4* and *PbrCHS5*, respectively. The structures of the last two *CHS*s are similar to *CmsA* from *A. nidulans* [43, Niño-Vega *et al.*, unpublished results], which has been proposed as a chitin synthase coupled to a myosin motor-like region. In association with cytoskeletal structures, the myosin region might translocate the newly synthesized chitin molecules to its site of deposition, in a fashion similar to that of the motor protein myosins that generate mechanical force to move the newly synthesized molecules along actin cables [40,42]. However, PbrChs4p does not possess the P-loop, switch I and switch II domains present in CsmA and conserved among the myosins [Niño-Vega *et al.*, unpublished results]. Also, the degree of its identity to the myosin-motor like domain of CsmA is lower than those of other class V chitin synthases, reinforcing our previous suggestion that PbrChs4 may be a subclass within class V chitin synthases [44], or perhaps, a new class on its own [Niño-Vega *et al.*, unpublished results]. Transcripts from these four *PbrCHS* genes were detected in both Y and M

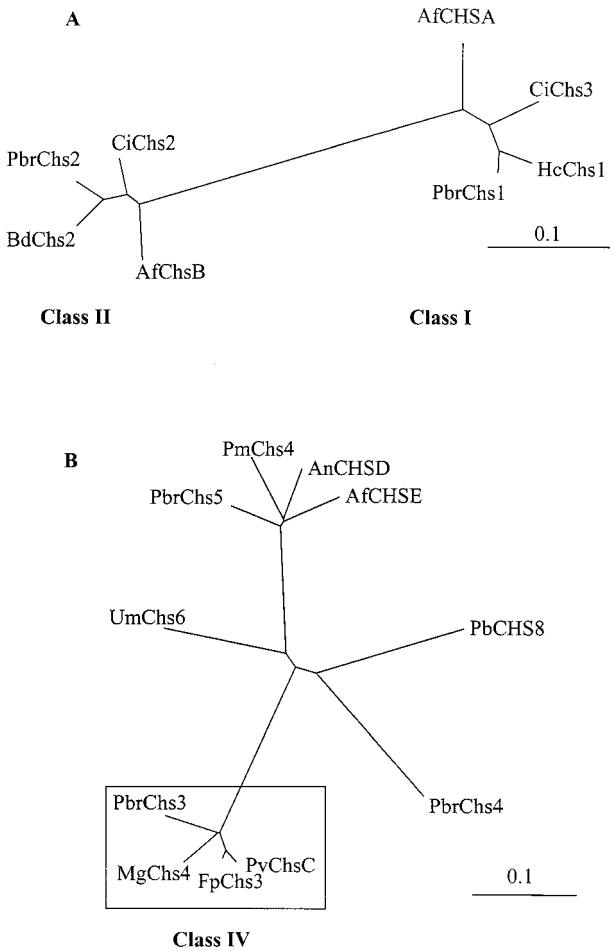

Fig. 1 Phylogenetic trees of relatedness between *P. brasiliensis* chitin synthase (PbrChs) enzymes constructed by the neighbour-joining method in the Clustal W program. Pv: *Phialophora verrucosa*; Fp: *Fonsecaea pedrosoi*; Mg: *Magnaporthe grisea*; Pb: *Phycomyces blakesleeanus*; Af: *Aspergillus fumigatus*; Pm: *Penicillium marneffei*; An: *Aspergillus nidulans*; Um: *Ustilago maydis*. (A) Shows phylogenetic relationships of PbrChs1 and PbrChs2 with other class I and class II Chs enzymes and (B) shows phylogenetic relationships of PbrChs3, PbrChs4 and PbrChs5 with other class IV and class V Chs enzymes [44]. Reproduced by permission.

cultures where all exhibited similar patterns of expression during the M to Y transition, with a preferential expression in the M form. This enhancement during M growth was unexpected, since the Y form of the organism has a higher chitin content than the M form [3]. Although higher levels of expression of *PbrCHS1*, *PbrCHS2*, *PbrCHS4* and *PbrCHS5* were observed in the M phase, it is possible that transcription of the genes is affected by changes in temperature or other environmental factors such as the production of metabolites by the fungus. Post-transcriptional modifications may also affect detected expression levels [45]. The absence of expression of some *PbrCHS* genes at 4 and 8 hours coincides with stage 1 heat-shock responses; hence, short

term changes in gene expression may be related to heat-shock rather than to morphogenesis per se. Also, heat shock elements within the promoters of *PbrCHS* genes may play a direct role in linking the expression of these genes to changes in temperature associated with colonization of the human body [44]. So far, there is no evidence of such elements in the promoter regions of the two genes (*PbrCHS2* and *PbrCHS4*) for which complete sequences are available [43; Niño-Vega *et al*., unpublished results]. To elucidate these questions and others related to the relevance of certain genes in the dimorphic process of *P. brasiliensis*, a gene transfer system designed to manipulate the genome and to permit identification of genetically defined mutants should be developed. This goal has so far been difficult to achieve in this fungus.

The search for factors involved in the expression of fungal dimorphism and morphogenesis has led several authors to explore the differential expression of a wide variety of proteins and genes other than the *PbrCHS*s [46,47]. By two-dimensional electrophoresis of cytosolic proteins from both fungal phases, Cunha *et al*. [46] were able to detect some differentially expressed proteins associated with either Y or M cells, particularly PbM46 (MW 46 kDa, present in higher amounts in the M phase) and PbY20 (MW 20kDa; present only in the Y phase). The first protein had a sequence similar to enolases from sources as varied as *S. cerevisiae* and human. The second had a high degree of similarity with: (a) two 22 kDa allergenic proteins from *Alternaria alternata* and *Cladosporium herbarum*, (b) a 26 kDa protein of unknown function related to a hypothetical gene located in chromosome III of *S. cerevisiae*, (c) a 25 kDa protein related to a thermoregulated gene of *S. pombe*, and (d) a 22.9 kDa protein related to another hypothetical gene from *S. cerevisiae* chromosome II.

The better studied proteins and genes related to fungal morphogenesis include the heat shock proteins (Hsp). When exposed to environmental stress such as temperature elevation, all living organisms respond by rapidly producing increased amounts of Hsp. These proteins presumably protect cells against the detrimental effects of the stress factor [48] as well as often being, coincidentally, immunodominant antigens in pathogenic organisms [49]. In the case of human pathogens, the organisms must undergo adaptation to a higher temperature upon infection of the host, an adaptation frequently correlating with cellular differentiation. This process runs parallel to the transient production of Hsps of which the most abundant is Hsp70. In *H. capsulatum*, the production of Hsp70 and Hsp82 is heat inducible. There is an overproduction of Hsps within the first hours of a 42° C heat shock. Both proteins are also constitutively expressed at low levels at all times [1]. In contrast, the *hsp70* gene isolated in *P. brasiliensis* is differentially expressed during transition from the M to the Y form and after heat shock of yeast cells at 42° C [47]. The gene encodes a 649 amino acid protein (predicted MW 70,461 kDa) of high identity with other members of the *hsp70* gene family (73.6% to *Blastocladiella emersonii*, 73.3% to *S. cerevisiae*, 89.2% to *H. capsulatum*), with six conserved sequence motifs characteristic of the Hsp70 family.

A metabolic process that appears to be involved in the dimorphic process is that concerned with the synthesis of polyamines. Polyamines are micromolecules necessary for cellular growth and differentiation. They originate in the decarboxylation of ornithine, giving rise to putrescine, the first polyamine in the metabolic pathway. This reaction is catalyzed by ornithine decarboxylase (ODC), one of the most highly regulated enzymes in eukaryotic systems [50]. ODC has been studied in several dimorphic fungi where its activity correlates with morphogenetic processes. In Mucorales [51] and *C. albicans* [52], among others, ODC activity is lower in yeasts (Y) and spores than in mycelia (M), increasing significantly during Y to M transition and spore germination. Contrary to other fungal systems, Y growth and M-to-Y transition in *P. brasiliensis* are accompanied by high ODC activity at the onset of budding, while ODC remains at a basal level during vegetative growth of both the M phase and the late stage of Y phase, and also through Y-to-M transition [53]. The *PbrODC* gene has been cloned and sequenced [Niño-Vega *et al*., unpublished results]. It encodes a putative 447 amino acid protein, with high homology to other fungal *ODC* gene products (74.8% to *Coccidioides immitis* CiODCP [54] and 54.7% to *N. crassa* NcODCP [55]). Expression of *PbrODC* has been found to be constant during growth of both the mycelial and yeast forms, as well as during the dimorphic transition either way [Niño-Vega *et al*., unpublished results]. These results suggest that, at least during the early stages of yeast growth and M-to-Y transition, regulation of ODC activity in *P. brasiliensis* might occur at a post-transcriptional level.

Molecular identification of *P. brasiliensis* for diagnostic purposes

Conclusive diagnosis of paracoccidioidomycosis has traditionally relied on the identification of *P. brasiliensis* from lesions found in patients, particularly upon the most characteristic feature of the yeast form, i.e., the pilot's wheel appearance of the mother cell surrounded by multiple peripheral daughter cells. Depending on the histopathological pattern, however, small forms of *P. brasiliensis* may be mistaken for other fungal infections

[56]. Alternatively, the diagnosis of paracoccidioidomycosis by indirect serological methods that rely on antibody detection is highly valuable. However, antibody levels may be absent in immunocompromised patients, or may remain present months after successful therapy. Antigens frequently lack enough sensitivity and specificity, leading to detectable cross-reactions with other fungi [57]. The most important and reliable *P. brasiliensis* antigen, gp43, disappears from circulation during treatment [58], while reports of false negatives [59], cross-reactivities with histoplasmosis [60] and lobomycosis [61], and strain variability in this antigen [62] have been made (for a review, see [8]). The cause of such interstrain variability may be the polymorphism recently reported for the gene coding for gp43 [63]. Diagnosis is further complicated by the fact that the disease presents itself in disseminated, mucocutaneous, or pulmonary forms which may be the result of host-related factors [6] as well as the characteristics of the infecting isolate [3].

Currently, rapid and efficient molecular methods to identify and distinguish fungal species are being applied for use as diagnostic tools. To date, several *P. brasiliensis* DNA sequences of potential diagnostic use have been reported. To this end, Goldani *et al*. [64] reported the cloning and sequencing of a species-specific 110 bp DNA fragment from *P. brasiliensis*. This fragment was generated by PCR using primers complementary to the rat β-actin gene at a low annealing temperature. Comparison of the fragment's nucleotide sequence with sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank) identified approximately 60% homology with an exon of a major surface glycoprotein gene from *Pneumocystis carinii* and a fragment of unknown function in *S. cerevisiae* chromosome VII. By Southern hybridization analysis, the fragment detected 1.0- and 1.9-kb restriction fragments within genomic DNA of *P. brasiliensis* digested with *Hin*dIII or *Pst*I, but failed to hybridize to DNAs from *C. albicans*, *B. dermatitidis*, *Cryptococcus neoformans*, *A. fumigatus*, *S. cerevisiae*, *P. carinii* and rat or human tissues. Additionally, the specific DNA fragment from three different isolates of *P. brasiliensis* was amplified by PCR with primers mostly complementary to non-actin sequences of the 110 bp DNA fragments. In contrast, there were no amplified products from other fungal genomic DNAs tested, including *H. capsulatum*. Using primers 1 and 2 derived from this specific sequence, a 62 bp fragment from *P. brasiliensis* DNA was detected in sera of five experimentally infected mice [65] (for sequences of these and other primers, refer to Table 1). The PCR method was able to detect as little as 10 pg of *P. brasiliensis* DNA in all five sera tested, and it was more sensitive than blood culture isolation, where only two cultures proved positive. There were no amplified fragments in sera from non-infected control mice. The sequence used for these tests was specific to *P. brasiliensis in vitro* [64]. A trial conducted using infected animals did not include infections provoked by agents other than *P. brasiliensis*, nor was the method tested in sera from patients, so that the efficiency of PCR diagnosis by this method remains to be evaluated.

Sequencing nearly 800 nucleotides from the 5′ terminus of the 28S ribosomal gene of *P. brasiliensis*, Sandhu *et al*. [66] chose a species-specific 14-base DNA probe (U1) that was able to identify *P. brasiliensis* ribosomal DNA (rDNA) in a panel of rDNAs representing a total of 48 species of fungi. This primer was later used for *in situ* hybridization to detect *P. brasiliensis* in lesions biopsied from the oral cavity of seven paracoccidioidomycosis patients and guinea pig testes inoculated with a culture of *P. brasiliensis* isolated from soil [67]. The probe did not hybridize with *H. capsulatum* or *Paracoccidioides (= Lacazia) loboi*, although it reacted with *Candida* species. The probe detected only 2-3% of *P. brasiliensis* yeast cells present in the tissues under examination, as judged by the results obtained with a combined Gridley stain. Hence, the technique at this stage proved unsuitable for routine diagnostic purposes. Other researchers [68] have looked at the region containing the 5.8S rDNA, and its flanking internal transcribed spacer regions (ITS). The ITS sequences have greater variability than does the 5.8S rDNA itself. ITS polymorphisms have been used for species identification of fungi [69; see next section]. After sequencing the 566 bp combined ITS and 5.85 region of *P. brasiliensis*, Imai *et al*. [68] designed two primers, sense PbITS1s and antisense PbITS3a, that were capable of identifying 29 strains of *P. brasiliensis* by means of a specific 418 bp fragment, but that did not amplify any fragment in several other pathogenic fungi. The complete sequence of *P. brasiliensis* 5.8S rDNA plus partial sequences of 28S and 18S rDNA and intergenic sequences were cloned and sequenced, yielding primers designated OL5 and OL3, for molecular identification of *P. brasiliensis* by PCR [70]. OL5, used in combination with primer ITS1 [71], and OL3 in combination with primer UNI-R [72] were recommended by the authors for double PCR, in view of the fact that the first pair alone produced a 496 bp fragment in *P. brasiliensis* samples that was hard to tell apart from a 500 bp fragment in *H. capsulatum* samples. This overlap was resolved in that the second pair of primers, used sequentially, generated a 203 bp fragment only in the former species.

A PCR assay based on primers derived from the sequence of the gene coding for the gp43 antigen was developed to detect the fungus in sputum [73]. To choose

Table 1 Primers used for detection of *P. brasiliensis*

Primers/Pairs	Abbrev.	Assay/Source of primers	Tested	Ref.
5′-TCG TTA TCC TCA TCG AA-3′/	Primer 1	PCR/β-actin	In vitro *and* murine model, serum*	64, 65
5′-AAG AGT CTT CCC TCG C-3′	Primer 2			
5′-ACT CCC CCG TGG TC-3′	U1	PCR/28S rDNA	*In vitro* and *in situ* hybridization of biopsies*	66, 67
5′-CCG CCG GGG ACA CCG TTG-3′/	Pb-ITS1s	PCR/5-8S rDNA	*In vitro*	68
5′-AAG GGT GTC GAT CGA GAG-3′	Pb-ITS3a			
5′-TGT GAC GAA GCC CCA TAC G-3′/	OL5	Double PCR/5-8S rDNA	*In vitro*	70, 71; 72
5′-TCC GTA GGT GAA CCT GCG G-3′	ITS1			
5′-CTC AGC GGG CAC TT-3′/	OL3			
5′-GGT CCG TGT TTC AAG ACG-3′	UNI-R			
5′-TCA TC*T* CAC G*T*C GCA TCT CAC ATT–3′	PC1	PCR/*gp43* geneA‡	Sputum from 11 chronic PCM patients	73
5′-*A*TA GAG GG*A* GAG CCA TAT GTA CAA GGT-3′	PC2			
5′-ATC AA**A** CAA ACC CTG **A**T*C* GGC AT-3′	PC3			
5′-AGC GCC *A*GA TGG TTT GCC C**G**C TAG GAA CGA A-3′	PC5			
5′-**G**GC TCC TCA AAG TCT G**C**C ATG AGG AAG-3′	PC6			
PC1/PC5; PC1/PC6; PC2/PC5; PC2/PC6; PC3/PC5				
Outer		Nested PCR/*gp43* gene‡	Murine model, lung	75
5′-AAC TAG AAT ATC TCA CTC CCA GTC C-3′	para I			
5′-**T**G**T** AGA CGT TC*T* TGT A*T*G **T**CT T**G**G G-3′	para II			
Inner				
5′-G**A**T *C*GC CAT CCA TAC TCT CGC AAT C-3′	para III			
5′-GGG CAG AGA AGC ATC CGA AAT TGC G-3′	para IV			
5′-TGC TGC GGC GGG GTT AAA CCA TGT C-3′	MAE	PCR/*gp43* gene‡	Armadillos	81
5′-GTT GTG GTA TGT GTC *G*A**T** G**T**A GAC G-3′	ATO			

‡Primers obtained from the *gp43* sequence were compared against the gp43 polymorphism work recently published [63], in which informative nucleotides (**ACGT**) and noninformative nucleotides (*ACGT*) , change according to strains.

oligonucleotide primers for PCR, the deduced amino acid sequence of the gp43 gene [74] was aligned with those of exo-1,3-β-D-glucanases from *S. cerevisiae* and *C. albicans*, with which gp43 had 56 and 58% homology, respectively. Five primers were derived from the regions that did not show amino acid homology, three of them sense primers (PC1, PC2, PC3) and two, antisense (PC5, PC6) (Table 1). Sputum samples from 11 patients with chronic paracoccidioidomycosis were subjected to PCR with all pairs of primers. In all cases, a single band of 0.6 kb specific for *P. brasiliensis* was produced, although the primer pair PC2-PC6 presented the highest sensitivity and specificity and gave a product clearly visible on the gel. Nested PCR was used for the detection of *P. brasiliensis* DNA in tissue samples from experimental animals, also using a sequence of the immunogenic *gp43* gene as a target [75]. The outer primers were: para I, and para II; the inner primers were: para III, and para IV (Table 1). The test was carried out on DNA extracts of lung homogenates from 23 experimentally *P. brasiliensis*-infected mice, 20 *H. capsulatum*-infected mice, and two uninfected animals. A detection limit of 0.5 fg of specific DNA was determined, with the production of a 196 bp fragment only in *P. brasiliensis* samples, and assay positivity in 21 out of 23 culture-positive lung homogenates. Some of the gp43 sequences used by Gomes *et al*. [73], and Bialek *et al*. [75] (Table 1), were taken from regions where later studies [63] showed the presence of nucleotide substitutions when sequences of the gp43 gene from 17 different strains were compared. The substitutions were grouped into 21 informative and 46 non-informative sites that all together helped to demonstrate a high polymorphism in this gene, and generated a phylogenetic tree [63]. On the other hand, some primer designs were based on regions where the *gp43* gene sequence originally reported [74] suffered corrections, as mistakes were later detected [63]. For example, primers PC5 [73], PC6 [73], and para II [75] were chosen from regions susceptible to change (Table 1). Therefore, it would be wise to test those primers [73,75] against many more strains to check on their universality as species-specific for *P. brasiliensis*. In designing diagnostic primers based on *gp43*, design them from the conserved regions, i.e., those in which no informative nucleotide substitutions were detected [63].

Molecular genetic variations in *P. brasiliensis* as a tool in epidemiology

Epidemiological, clinical and experimental data have substantiated the theory that paracoccidioidomycosis starts with primary lesions in the lungs by the inhalation of asexual propagules of the saprobic mycelial phase of the fungus in nature, followed by the generation of secondary systemic lesions arising through lymphatic and blood dissemination to various organs and systems. The pathogen apparently has its natural habitat in soil or in plants in endemic areas, and rural workers appear to become infected by inhaling dust containing the infecting propagules [76]. Paracoccidioidomycosis is characterized by long latency periods [77], an attribute that hinders the precise determination of the site where the infection was acquired, and impairs the chance of locating the exact ecological niche of *P. brasiliensis*. Seldom isolated from soil [76], the fungus has recently been found in Brazil in liver, spleen and lungs of armadillos (*Dasypus novemcinctus*) and has repeatedly been cultured *in vitro* [78,79]. The geographical distribution of both the vertebrate and the fungus superimpose very closely, from Southern Mexico to Argentina [77], thereby pointing to the armadillo as the main natural *P. brasiliensis* reservoir [78]. Sano *et al*. [80] compared 64 armadillo isolates with 19 clinical isolates by randomly amplified polymorphic DNA (RAPD) patterns, using the primer OPG-19 (Operon Technologies, Alameda, CA, USA). These 83 isolates were separated into clusters I and II (with two subclusters each), and cluster III (with three subclusters). Correlations between human and armadillo isolates were observed in clusters I and II, while cluster III consisted only of armadillo isolates. These results suggested that humans may acquire *P. brasiliensis* infection by contact with armadillos, and also that there may be genotypes peculiar to the animal. Further work [81] has suggested that a single armadillo may be susceptible to infection by multiple *P. brasiliensis* isolates simultaneously. The spleen isolate in one specimen was different from the liver and mesenteric lymph node isolates, according to a partial *gp43* sequence amplified by using primers MAE (5′-TGC TGC GGC GGG GTT AAA CCA TGT C-3′) and ATO (5′-GTT GTG GTA TGT GTC GAT GTA GAC G-3′) (Table 1). In this regard, it is worth recalling the note of caution about primers derived from gp43 (see preceding section), since modifications of the originally reported sequence [74] and a polymorphism within it were later published [63].

There is no evidence that paracoccidioidomycosis is contagious among humans [77] and it is accepted that *P. brasiliensis* isolates differ in their ability to cause human disease [82]. Therefore, distinction of different isolates may be useful in epidemiological surveys. Strain determination has been done in the past through laborious tests designed to identify virulence-related and biochemical characters of isolates. Currently, the molecular methods used for identifying *P. brasiliensis* are also used for epidemiological screening, and are also employed in molecular taxonomic studies. As with other fungal

species, randomly chosen primer sequences, the distribution of which tends to differ from genome to genome, are able to distinguish among isolates, including *A. fumigatus* [83] and *H. capsulatum* [84]. Molecular markers in the 28S ribosomal DNA region have also been described for other pathogenic fungi [72,85].

By means of RAPD analysis, Soares *et al*. [82] were able to distinguish between seven *P. brasiliensis* isolates, five from Goias State, Brazil, and two from Guayaquil, Ecuador. The amplification patterns obtained allowed clear differentiation of the isolates into two distinct groups with only 35% similarity in the pattern of bands seen. Interestingly, neither group was correlated either with the geographical origin of the strains or with the type of pathology seen in the corresponding cases. However, subsequent RAPD analysis of 33 strains of *P. brasiliensis*, using primers OPG 3 and OPG 14 [86], have indicated that DNA variation correlates with geographical areas throughout South America. A dendrogram showed a high degree of similarity, with clusters of genetically different isolates correlating with geographical regions but not with pathological findings. With a few exceptions, strains were sorted into five geographical groups, namely, group I, Venezuelan strains; group II, Brazilian strains (only from São Paulo State and nearby regions); group III, Peruvian isolates; group IV, Colombian isolates; and group V, Argentinian strains. The last group was the most genetically distant group. These results were later confirmed by means of RFLP analysis, using the restriction enzymes *Hinf*I and *Hinc*II (Fig. 2) [87]. Contrasting results between references [82] and [86] may have originated in the different set of primers used in each case and also in the origin and quantity of strains tested.

In contrast, Molinari-Madlum *et al*. [88] achieved discrimination that reflected degree of virulence. RAPD analyses separated 15 *P. brasiliensis* isolates, including 13 from Brazil and two from Ecuador, into two groups with only 17% genomic identity. The ability of these isolates to invade tissues in susceptible mice differed strongly, with group I isolates producing only localized infection while group II strains caused disseminated infection.

The above findings, taken together, suggest that *P. brasiliensis* may consist of several genetically distinct groups making up a single morphological species, as is the case in *C. immitis* [89], and *H. capsulatum* [90]. In *H. capsulatum*, different phylogenetic species exist and have different degrees of pathogenicity. If this were the case for *P. brasiliensis*, knowing which genetically distinct sibling species is involved in one or more cases might provide information about the potential virulence and epidemiological characters of the isolate or isolates. Establishing the relationships between morphological features, virulence, and DNA variation, would lead to a more realistic species differentiation concept in this organism.

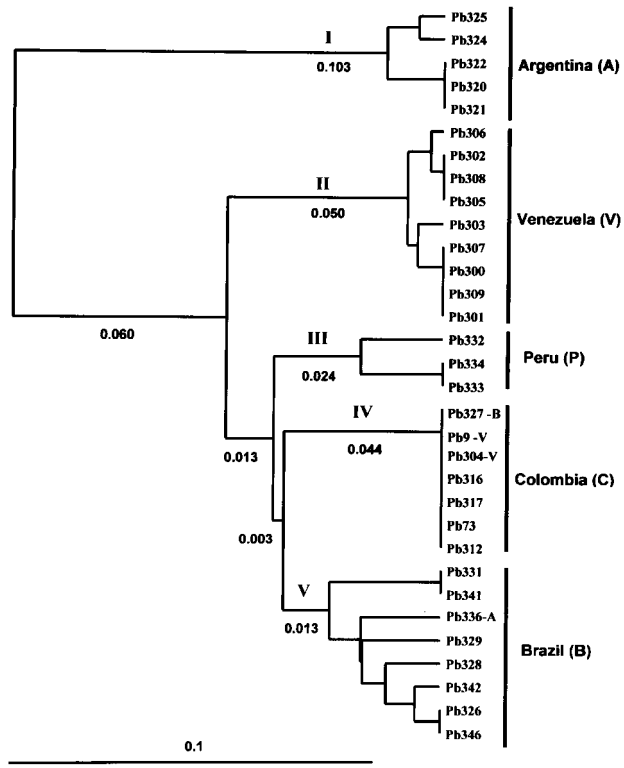

Fig. 2 Dendrogram of genetic relationships among 32 *P. brasiliensis* isolates after RFLP analysis. Roman numerals indicate the clusters. Capital letters refer to geographical origins of strains. Numbers under the branches represent evolutionary distances; the higher the number, the greater the genetic divergence between strains [87]. Reproduced by permission.

Do *P. brasiliensis* and all other pathogenic dimorphic fungi belong to the Onygenales?

In classic systematics, *P. brasiliensis* was classified as an imperfect fungus, in the broad artificial group of the anamorph phylum Deuteromycota, class Hyphomycetes. This was because no sexual structures were found that would allow a more precise classification. Recently, separate classification of dikaryomycetous anamorphs above the genus level has been formally abandoned as no longer necessary, and the former Deuteromycota are perceived simply as ''anamorphic'' or ''mitosporic'' fungi (in the latter case bearing in mind that some homothallic fungal ''sexual'' spores are also formed by mitosis). What has allowed this transition is the recent advance of molecular methodologies. In the medically important fungi, molecular methods used in epidemiological typing and population genetics have proved helpful in the

resolution of taxonomic problems. These methods include karyotype analysis, multilocus enzyme electrophoresis (isoenzyme typing), RAPD, RFLP, sequencing with arbitrary primers (SWAPP), sequence confirmed amplified region (SCAR), and single-strand conformational polymorphism [91].

Phylogenetic comparison of dermatophytes and dimorphic fungi based on large subunit (28S) ribosomal rDNA sequences [92], has more precisely placed *P. brasiliensis* as belonging in the order Onygenales, family Onygenaceae (phylum Ascomycota) together with *B. dermatitidis*, *H. capsulatum*, and *H. capsulatum* var. *duboisii*, the teleomorphs of which belong in the genus *Ajellomyces*. In a key study, Leclerc *et al*. [92] suggested that all pathogenic Onygenalean fungi could be placed in either the Onygenaceae, containing all the dimorphic-systemic pathogens in the group, or the Arthrodermataceae, containing the dermatophytes. Comparisons of *P. brasiliensis* ODC fragments [93] with genomic fragments from other dimorphic fungi, produced dendrograms in which *P. brasiliensis* fell into proximity with *C. immitis,* which had previously been confirmed a member of the order Onygenales [91] by molecular studies. Comparison of the complete ODC sequence [Niño-Vega *et al*., unpublished results] to other fungal ODC sequences reconfirmed this result.

Molecular phylogenetic studies in fungi have been particularly focused on ribosomal RNA (rRNA) and its corresponding template ribosomal DNA (rDNA). In general, the small and highly conserved 5.8S rDNA locus can provide evolutionary information among distantly related organisms, while the larger and more heterogeneous large subunit (LSU) and small subunit (SSU) rDNA loci allow distinctions among species within the same genus, and occasionally strain distinctions within the same species [94]. The sequences of the two ITS regions flanking the 5.8S rDNA have higher rates of divergence than SSU genes, and are therefore often useful for the differentiation of closely related species [95]. Sequences of the ITS1, ITS2, and 5.8S rDNA regions, as well as of the D1 and D2 domains of the LSU, have grouped members of the genus *Emmonsia* together with *B. dermatitidis* and *H. capsulatum*. Mating studies showed similar results, in that *Emmonsia crescens* formed a teleomorph in the genus *Ajellomyces*, which was named *A. crescens.* This teleomorph is very similar to *Ajellomyces dermatitidis,* the teleomorph of *B. dermatitidis,* and *Ajellomyces capsulatus*, the teleomorph of *H. capsulatum* [96]. In parsimony analysis of ribosomal sequences, *P. brasiliensis,* a non-mating species, fell in the vicinity of the *Ajellomyces* spp. Differences in the SSU [97] suggested that *P. brasiliensis*, *B. dermatitidis*, and *E. parva* were more closely related than *H. capsulatum* and *B. dermatitidis*. Together, these data confirm *P. brasiliensis* as a member of the family Onygenaceae.

The fact that several dimorphic systemic pathogens have been placed within the Onygenales has led some authors to postulate that all the dimorphic pathogenic fungi belong in this order [98]. This is neither true in the sense in which the word ''dimorphic'' has been traditionally used in medical mycology (thermally responsive, host-associated dimorphism), nor in the expanded sense of the word used in this review (including, for example, the dimorphism of *C. albicans*, readily observed *in vitro* at 25°C). *Sporothrix schenckii* and *Penicillium marneffei* must be included among the pathogens traditionally considered thermally dimorphic and, in the name of logical consistency, the agents of chromoblastomycosis should also be added to this traditional list. Additional non-Onygenalean pathogenic fungi must be included in a list of all pathogens showing dimorphism in the broader definition of the term. Table 2 shows these genera and species of the kingdom Fungi (''true Fungi'' or Eumycota), as well as the phyla, orders and families to which they belong.

All phyla from the kingdom Fungi share many morphological and biochemical characters. Ascomycota and Basidiomycota diverged from a relatively recent common ancestor. Phylogenetic relationships within the Ascomycota have been represented in a dendrogram based on SSU studies [99]. Saccharomycetales and filamentous ascomycetes are sister groups that diverged after the Archiascomycetes, containing *Pneumocystis carinii* and *Schizosaccharomyces pombe*, were separated [99]. The important dimorphic human pathogen, *C. albicans,* as well as the important opportunists *Candida kefyr, C. krusei, C. lusitaniae, C. parapsilosis,* and *C. tropicalis,* belong to the Saccharomycetaceae in the group of ascomycetous species traditionally considered to be yeasts (Table 2). The remaining dimorphic ascomycetes pathogenic to humans in Table 2 belong to the fungi conventionally classed as filamentous ascomycetes, which in their evolution diverged rapidly into species forming a vast array of different mycelial types, ascus structures, and ascocarp morphologies. One of these, the Onygenales, was traditionally organized into four distinct families [100], the Gymnoascaceae, the Arthrodermataceae, the Onygenaceae and the Myxotrichaceae. Recent molecular studies do not support this classification, since members of the family Onygenaceae, for example, appear to be divided into two different groups, both retained within the Onygenales [101,102]. The dimorphic Onygenales have a high degree of adaptation to human and animal pathogenicity compared to other fungi. Only a few members of this group

Table 2 Taxonomic position of some medically important dimorphic fungi

Phylum	Order	Family	Genus	Species	
				Anamorph	Teleomorph
Ascomycota	Onygenales	Onygenaceae	*Coccidioides*	*C. immitis*	unknown
			Emmonsia	*E. crescens*	*Ajellomyces crescens*
			Blastomyces	*B. dermatitidis*	*Ajellomyces dermatitidis*
			Histoplasma	*H. capsulatum*	*Ajellomyces capsulatus*
			Paracoccidioides	*P. brasiliensis*	unknown
	Eurotiales	Trichocomaceae	*Penicillium*	*P. marneffei*	*Talaromyces* sp. ?
	Chaetothyriales	Herpotrichiellaceae	*Cladophialophora*	*C. carrionii*	unknown
			Phialophora	*P. verrucosa*	unknown
			Exophiala	*E. dermatitidis*	*Capronia* sp. ?
	Ophiostomatales	Ophiostomataceae	*Sporothrix*	*S. schenckii*	*Ophiostoma* sp. ?
	Saccharomycetales	Saccharomycetaceae	*Candida*	*C. albicans*	unknown
Basidiomycota	Trichosporonales		*Trichosporon*	*T. asahii*	unknown

are involved; this may indicate a long history of adaptation and specialization [103]. In Arthrodermataceae, Gymnoascaceae, and Myxotrichaceae, no dimorphic pathogens are found, and only the Arthrodermataceae contains contagious dermatophytic skin pathogens. The Myxotrichaceae recently has been shown to be unrelated to the Onygenales [104]. Among the Onygenaceous pathogens, the genus *Coccidioides*, represented only by *C. immitis*, a monophyletic species complex [105] causing coccidioidomycosis, has no known teleomorph. Phylogenetically it is included in the Onygenaceae [106], though it resolves at considerably distance from *Histoplasma* and its allies [102]. The Onygenaceous genus *Emmonsia* has three pathogenic species, *E. crescens, E. parva* and *E. pasteuriana*. Of these, only *E. crescens*, an agent of adiaspiromycosis, has a known *Ajellomyces* teleomorph, *A. crescens* [107]. Another dimorphic Onygenaceous genus, *Histoplasma*, has a very complex structure. Kasuga *et al*. [90] studied 46 geographically diverse *H. capsulatum* isolates representing the three varieties, *H. capsulatum* var. *capsulatum, H. capsulatum* var. *duboisii* and *H. capsulatum* var. *farciminosum*, and were able to identify six clades, each genetically isolated from the others. Five clades included isolates considered *H. capsulatum* var. *capsulatum*, and one consisted of *H. capsulatum* var. *duboisii. H. capsulatum* var. *farciminosum* was found within one of the *H. capsulatum* var. *capsulatum* clades though it is distinct from it in pathology.

There are some non-Onygenalean dimorphic fungi that are as medically important as the Onygenalean pathogens. For example, *S. schenckii*, the agent of sporotrichosis, belongs to the order Ophiostomatales (Ascomycota), a group typically producing teleomorphs in the genus *Ophiostoma* (Ophiostomataceae). It is a mitosporic dimorphic fungus producing mycelium at room temperature, and yeasts at 35° C both *in vitro* and in host tissues. Another example of a non-Onygenalean dimorphic fungal pathogen lies within the order Chaetothyriales. This order is related to the Eurotiales and Onygenales [102]. Dimorphic agents of chromoblastomycosis (*Cladophialophora carrionii* and *Phialophora verrucosa*) have been shown to belong to the family Herpotrichiellaceae [108] within this order [102]. These fungi exist saprobically in nature as molds, and grow as mycelial colonies *in vitro*, but undergo a morphological change after animal host tissue infection into brown, thick-walled, globose, multiseptate, fungal forms known as muriform cells or sclerotic fission cells. Related as well to this group of ascomycetes is the genus *Exophiala* (teleomorph: *Capronia?*, Herpotrichiellaceae, Chaetothyriales) the main genus of black yeasts. *Exophiala dermatitidis* produces abundant yeast cells and hyphae bearing cylindrical phialides. *In vitro*, many isolates of *Exophiala* species form yeasts and mycelium simultaneously.

A significant human pathogen, *P. marneffei,* is the only dimorphic species in the genus *Penicillium*. The fungus manifests thermal dimorphism, growing in a yeast-like form at 37° C and as mycelial colonies at room temperature. *P. marneffei* is closely related to *Penicillium* species in the subgenus *Biverticillium,* as well as to sexual *Talaromyces* species (Ascomycota, Plectomycetes, Eurotiales, Trichocomaceae) that have a biverticillate penicillium anamorph with lanceolate phialides [69].

Other important human pathogens fall well apart from these ascomycetous fungi. Some are members of the phylum Basidiomycota. One such basidiomycetous fungus is *C. neoformans*, which is accounted as dimorphic in the broad sense because it is a yeast in its asexual state but mycelial in its mated state. Additional examples of potentially pathogenic, dimorphic Basidiomycota are found in the genus *Trichosporon*. With the exception of the nonpathogen *Trichosporon pullulans*, which falls within the Cystofilobasidiales, all *Trichosporon* spp. belong to a single Trichosporonales clade that is classified in the phylum Basidiomycota, class Hymenomycetes, sub-class Tremellomycetidae, order Trichosporonales [109]. Some species (e.g. *Trichosporon asahii* and *T. mucoides*) express a morphological transition between a yeast budding phase and mycelium giving rise to arthroconidia both *in vitro* and in host tissue. In a phylogenetic tree of basidiomycetous yeasts based on confidently aligned D1–D2 domains of the LSU [102], most *Trichosporon* species (including *T. asahii, T. asteroides, T. cutaneum, T. mucoides* and *T. ovoides)* and *Filobasidiella neoformans* (teleomorph of *C. neoformans*), were closely clustered with the order Tremellales (Basidiomycota, Hymenomycetes) [101]. These data suggest that if any *Trichosporon* species have teleomorphs, they may be *Tremella*-like.

In the phylum Zygomycota, order Mucorales, there are a few opportunistic pathogens such as *Mucor circinelloides* and, arguably, *Cokeromyces recurvatus* (pathogenic status unclear), that convert to a yeast phase under anaerobic conditions. These fungi are seen in human infection [102], but only *C. recurvatus* is generally seen in the yeast state in direct microscopy of lesions. There are no known dimorphic (or monomorphic) human pathogens belonging to the Chytridiomycota, a phylum of mostly aquatic, zoosporic organisms that is the fourth major fungal group along with the Zygomycota, Ascomycota and Basidiomycota. Sequence analysis has shown that certain other organisms once classified as fungi belong to other kingdoms: Oomycetes, for exam-

ple, belong to the kingdom Stramenopila, while the Myxomycetes belong to the polyphyletic kingdom Protozoa [110,111]. The recently proposed clade Mesomycetozoa [112] now includes *Rhinosporidium seeberi*, the agent of rhinosporidiosis, formerly considered to be a fungus. The Mesomycetozoans are close to the Choanoflagellates, a basal branch of the kingdom Animalia.

Like Bowman *et al*. [91], we conclude that the pathogens referred to as ''dimorphic'' in the broad sense are not a monophyletic group, but rather are interspersed among many different taxonomic groups that also contain non-pathogenic fungi. It is clear that both dimorphism and mammalian pathogenicity have arisen multiple times within the fungi.

Genetics

P. brasiliensis is multinucleate in its pathogenic yeastlike form, while a single nucleus is present in either conidia or individual mycelial cells [3,113]. As is the case with many other fungi, this microorganism has been refractory to the kind of classic cytogenetic analysis that could establish the chromosomal number and organization. In addition, classic genetics is not possible with this fungus because no sexual phase is known. The development of modern electrophoretic methods, however, has allowed the separation of intact chromosomal DNA molecules as large as 10 Mb, opening new possibilities for the analysis of genome organization. Pulsed-field gel electrophoresis (PFGE) has allowed the genomic characterization, chromosomal mapping, and molecular epidemiological biotyping of microorganisms otherwise refractory to genetic analysis. With it, the karyotype of several yeasts and fungi of medical importance have already been characterized, among them *C. albicans* [114], *C. neoformans* [115], *H. capsulatum* [116], and *C. immitis* [117]. *P. brasiliensis* has also been subjected to this analysis [118–120]. Depending on the samples and the techniques used for karyotyping, four to five chromosomes of variable molecular weights have been reported (Fig. 3). By far the largest study has been carried out by Montoya *et al*. [118,119] who analysed eight clinical and five environmental isolates (two from soil, one from armadillo, one from penguin faeces, and one from dog food). Seven of the clinical samples yielded five chromosomal-sized bands. Six isolates showed bands of MW $\geq$10.0, 8.8, 5.2, 4.1, and 3.2 Mbp (lane A, Fig. 3), while one had a different profile, namely, MW $\geq$10.0, 8.8, 7.2, 3.8, and 3.2 Mbp (lane B, Fig. 3). The remaining clinical isolates gave a completely different pattern, consisting of only four bands of MW $\geq$10.0, 6.7, 4.1, and 3.2 Mbp (lane C, Fig. 3). The five environmental isolates showed five bands of MW $\geq$10.0, 7.2, 5.2, 4.1, and 3.2 Mbp (lane D, Fig. 3), a profile very similar to that found in the majority of the clinical isolates (lane A, Fig. 3). In the latter, however, the 7.2 Mbp band was replaced by an 8.8 Mbp band. Cano *et al*. [120] also karyotyped two clinical isolates of *P. brasiliensis* and found four chromosomal bands in each of them. The band patterns differed between the isolates: strain 113 (lane E, Fig. 3) produced bands of

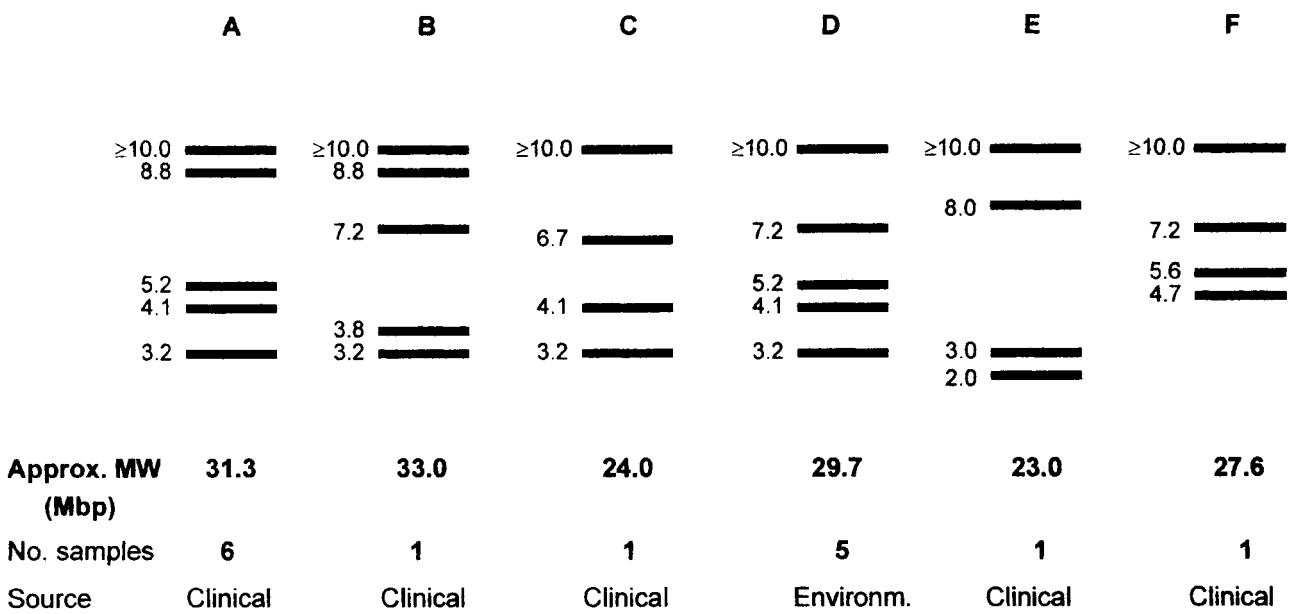

Fig. 3 Diagram representing the results obtained from CHEF gels of *P. brasiliensis* isolates. Lanes A-C are drawn from [118]; lane D, from [119]; lanes E and F, from [120].

MW ≥10.0, 8.0, 3.0, and 2.0 Mbp, while strain B-339 (the reference strain for production of gp43, *P. brasiliensis* reference antigen) had bands at MW ≥10.0, 7.2, 5.7, and 4.7 Mbp (lane F, Fig. 3). Some of strain B-339's bands correlated with those detected in clinical isolates analyzed by Montoya *et al.* [118]. Evidently, *P. brasiliensis* has chromosomal polymorphism, as has been reported in *C. albicans* [121], *C. immitis* [117], and *C. neoformans* [122], among other fungi. Polymorphisms may be attributable to genetic translocations or large-scale deletions, as in *C. albicans* and other pathogenic fungi [117,121]. The underlying mechanism may play an important role in promoting the genetic variability and accelerated evolution of different isolates. The plasticity inherent in these small genomes could have implications for the maintenance of genome functionality and for the control of gene expression in these organisms [123].

The approximate molecular size of the *P. brasiliensis* genome fluctuates between 23 and 31 Mbp, depending on the isolate. This range is close to that seen in the genomes of *C. immitis* (29 Mb) [117], and the *H. capsulatum* "Down" strain (30 Mb) [116]. Additional measurements based on combined PFGE and confocal fluorescence microscopy [120] suggest that the genome size of *P. brasiliensis* is in the order of 45.7–60.9 Mbp, two to three times that of *C. albicans* [123]. This size was twice the total size of chromosomal DNA molecules separated by PFGE (23.0–27.6 Mbp). For this reason, Cano *et al*. [120] reached the conclusion that the nuclei of *P. brasiliensis* yeast cells may be diploid.

Hybridization of selected gene probes (*gp43* and chitin synthase (*CHS*) genes) on chromoblots was used to further characterize *P. brasiliensis* karyotypes [120]. The *gp43* gene mapped onto chromosomal bands with different sizes in each isolate (10.0 Mbp for isolate 113 and 4.7 Mbp for isolate B-339). In contrast, Southern blot hybridization of megarestriction fragments with the gp43 probe gave very similar results for both isolates, each yielding two hybridizing *Sfi*I fragments of approximately 440 and 300 kbp and a single hybridizing *Pac*I fragment of 50 kbp. No restriction site for either enzyme exists within the *gp43* gene itself, which is present in a few copies per genome [74]. These results suggested at least two copies of the *gp43* gene on the same chromosomal band or, alternatively, two allelic forms of the gene, mapping onto two closely comigrating chromosomes [120]. With *CHS*, the probe hybridized with two chromosomal bands on each strain. For this test, Cano *et al*. [120] used a probe consisting of a 600 bp PCR amplification product obtained by using a set of primers designed by Bowen *et al*. [36,43]. The use of these primers on *P. brasiliensis* has been reported to yield a heterogeneous 600 bp product which actually is the result of the amplification of two different *CHS* gene fragments (from *CHS1* and *CHS2*) of about the same size [43,44]. In view of this fact, the results reported by Cano *et al.* [120] are probably due to these two different *CHS* genes hybridizing on different chromosomal bands.

Conclusions

In the last decade, research on paracoccidioidomycosis and its causal agent has increased exponentially in all fields. In particular, the introduction of molecular methods to study epidemiology and immunology (the latter has been recently reviewed in [8]) has provided new insights that will assist developments in the prevention, diagnosis and treatment of the disease. The quest to decipher the biology of the dimorphic transition in *P. brasiliensis*, a process pivotal in all aspects of its pathogenicity including the initial establishment of disease, has also been greatly stimulated. The last decade has witnessed a remarkable development in this area, as molecular methods have provided the tools for exploring fields that could not be approached with the biochemical methods used in the past. The synergy achieved by the joint use of molecular and biochemical approaches is providing new knowledge in various areas. We have learned much more about genes involved in the dimorphic transition, and in cell wall construction. The latter genes are of particular interest in attempts to develop highly selective antifungal drugs. We also have more understanding of the meaning of genetic and chromosomal polymorphism. Our knowledge of the ecological aspects of natural reservoirs and possible sources of contagion has improved significantly. Molecular taxonomy has revealed the correct classification of *P. brasiliensis*, and has also preliminarily suggested cryptic speciation hidden within this apparent unitary species. Understanding the diversity of *P. brasiliensis* types is of paramount importance in attempts to understand the wide spectrum of pathological manifestations of paracoccidioidomycosis and the different behaviours of strains. Without doubt, the years to come will see an intense increase in research focused on all these aspects of the most relevant systemic mycosis in Latin America.

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