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Molecular typing of pathogenic fungi

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In this Round Table, the application of several methods of molecular typing were discussed in reference to four important pathogenic fungi: *Coccidioides immitis*, *Histoplasma capsulatum*, *Candida albicans* and *Paracoccidioides brasiliensis*. Among the different methods the following were discussed: restriction fragment length polymorphisms (RFLP), single nucleotide polymorphisms, random amplified polymorphic DNA (RAPD), polymerase chain reaction (PCR)-RFLP and microsatellites. By means of these methods, several important biological questions related to speciation, mode of reproduction and population genetics could be approached. The basic information obtained from this approach has implications in the understanding of these pathogenic fungi in relation to their behavior and the development of pathogenic features, such as resistance to antimicrobials and virulence factors used for colonization of mammalian hosts. The knowledge obtained from these studies could also be used for the development of innovative diagnostic methods, as well as for novel therapeutic approaches and production of vaccines.

Keywords microsatellites, PCR, RAPD, RFLP

Phylogenetic species, recombination and molecular typing

Identification of pathogenic fungi has changed dramatically over the past decade through direct examination of the tremendous variation present in DNA. Recent studies of a number of fungi have identified three key points necessary to get the most information from strain typing [1,2]: (i) independent genetic markers for which every allele can be scored (independent, co-dominant polymorphic loci) provide the most information and the most opportunities for analysis; (ii) sampling isolates from throughout the range of the pathogen is essential because of the surprisingly narrow geographic range of pathogen populations and the mobility of human hosts; (iii) applying phylogenetic analysis to the data enables the identification of genetically isolated groups (phylogenetic species) and the reproductive mode (clonal or recombining). When these points are addressed, molecular typing provides biologically relevant information in addition to laboratory identification. With this information, treatments specific to phylogenetic species should emerge.

Typing

The methods for typing pathogenic microorganisms have been developed for, and are most often applied to, bacteria and virus. These microbes are often spread from host to host and their reproduction is not coupled to recombination, as is the case in eukaryotes. Application of the traditional typing methods to fungi responsible for the mycoses may not be appropriate in cases where the fungus is not transferred from host to host and where

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reproduction is coupled to recombination. Typing methods that take the biology of the fungus into account may be the most useful, and the advent of molecular typing methods provide a seemingly infinite array of markers to implement these methods. To illustrate this approach, this report refers to research conducted with the human pathogenic fungus, *Coccidioides immitis*, the agent of coccidioidomycosis.

Species

The first aim of typing is to identify the species of fungus responsible for the disease. Identification to species level is linked to the definition of a species. The broad availability of nucleic acid sequence and the analytical tools of phylogenetics has spawned the Phylogenetic Species Concept [3] which converges with the Biological Species Concept [4]. A Phylogenetic Species Concept based on concordance of gene genealogies has been applied to *C. immitis* with the result that there are two phylogenetic species, one found in California and the other outside of California [5,6]. A typing method for *C. immitis* must be able to discriminate between the two species. Due to their genetic isolation for approximately 11 million years [5,6], almost any polymorphic marker will accomplish this task. These markers include the restriction fragment length polymorphism (RFLP) variation found by Zimmermann *et al*. [7], the single nucleotide polymorphisms (SNP) developed by Burt *et al*. [8], the variable nucleotide positions found in five genes by Koufopanou *et al*. [5,6], the variable positions found in the proline rich antigen by Peng *et al*. [9], or the SNP developed by Fisher *et al*. [10,11].

Reproduction

Once species are defined and the means of typing individuals to species is at hand, the next aim of typing is to identify individuals within species. To accomplish this task, the method of reproduction in each species must be determined because typing strategies for clonal organisms are different from those for recombining organisms. For a completely clonal organism, every part of the genome is inherited in the same way from one parent and the only source of variation among individuals is mutation. For a recombining organism, different parts of the genome have different histories of inheritance from parents, grandparents, etc., and both mutation and recombination provide variation among individuals. In *C. immitis*, the variation observed between species in gene fragments was not sufficient to distinguish between strict clonality and recombination, so anonymous SNP had to be developed in both the non-California species [8] and the California species [12]. The multilocus genotypes composed of 14 loci in each case were then analyzed by a phylogenetic test for similar evolutionary history of each locus and a population genetic test for association of alleles at the different loci. For both species, the analyses were consistent with recombination. It had been clear that *C. immitis* can reproduce clonally due to its mitosporic arthroconidia, but it is now also clear that *C. immitis* recombines in nature. Due to recombination, it will not be possible to type *C. immitis* individuals with a single polymorphic locus, instead several polymorphic loci will be needed to type individuals.

Populations

Having diagnosed the species of *C. immitis*, and determined that both species have a history of recombination as well as the capability for clonal reproduction, the next goal for typing is to search for genetically differentiated populations within the species. Burt *et al*. [13] provided the first evidence for barriers to gene flow and the resulting genetically differentiated populations in the non-California species by characterizing SNP in individuals from Texas and Arizona. Their study required 11 SNP, all of which had only two alleles (nucleotide bases), as have all the SNP and all of the variable nucleotide positions in gene sequences found so far in *C. immitis*. To simplify the search for genetically differentiated populations, loci with more than two alleles were sought, leading to a search for microsatellite loci.

Microsatellites

Microsatellites are regions of the genome having short repeats (two to four nucleotides) that are found in tandem arrays of from 10 to 20 or more repeats. DNA polymerase has difficulty replicating the microsatellite faithfully, leading to mutations in the form of more or fewer repeats. Whereas the SNP and the variable nucleotide positions in genes of *C. immitis* were all biallelic among individuals of both species, microsatellites in *C. immitis* have as many as seven alleles in a single species, and between six and 10 alleles in the two species [11,12, M.C. Fisher & J.W. Taylor, unpublished results]. A key concern about the use of microsatellites to type fungi is the possibility that their mutability might cause alleles to be identical by chance instead of by descent, which could mislead those using them to diagnose species or populations. Through comparisons of phylogenetic trees based on SNP, DNA sequence variation in regions flanking the microsatellite loci, and length variation of the microsatel-

lites, it was found that a collection of seven microsatellites arrived at the same evolutionary conclusions as the single nucleotide polymorphisms and the flanking regions [M.C. Fisher & J.W. Taylor, unpublished results]. That is, microsatellites show that there are two species of *C. immitis*, that within the non-California species there is differentiation between individuals in Texas and Arizona, and that within the California species there is differentiation between individuals in Bakersfield, California and those in San Diego, California [M.C. Fisher & J.W. Taylor, unpublished results].

Future typing method

We are now characterizing nine microsatellites in over 150 isolates of *C. immitis* obtained from throughout the Americas range of the fungus from California to Brazil and Argentina, to learn as much as possible about populations of both species. We hope that a typing scheme based on microsatellites can be developed that will be robust enough to be used in clinical labs, and that will provide physicians and researchers with information about the species, population and individual genotype of *C. immitis* isolates causing disease in their patients. With this information, it will become possible to correlate features of disease with pathogen genotype.

Histoplasma and molecular typing

Studies on the genetic diversity and epidemiology of *Histoplasma capsulatum* have used a variety of different molecular markers to type isolates of this fungus. These have included nuclear and mitochondrial RFLP, random amplification of polymorphic DNA (RAPD), SNP and DNA sequencing. These analyses have found *H. capsulatum* to be a genetically diverse organism, with a number of 'classes' defined within the *H. capsulatum* species [14].

We are interested in extending current work to allow rapid strain typing *H. capsulatum* and enable large-scale epidemiological studies to be undertaken. The above techniques all suffer from limitations when applied to large epidemiological studies: RFLP and DNA sequencing are laborious and expensive to perform; RAPD, while simple and inexpensive, is almost impossible to standardize between different laboratories; and SNP frequently cannot be used in all populations of a genetically diverse species. To overcome these limitations we have begin to develop microsatellite markers from the genome of *H. capsulatum*.

Microsatellites are tandem repeats of short nucleotide motifs, embedded in otherwise unique sequences of DNA, for example, …CACACACACACACAC…. These are distributed throughout the genomes of all eukaryotic organisms and do not appear to be associated with any function. As their repetitive nature leads to errors during replication, microsatellites frequently differ in length between closely related organisms. This feature makes them ideal for strain typing, as a relatively limited set of microsatellites may be expected to identify organisms to the level of the individual or clone [15]. To date, there have been relatively few studies using microsatellites to study the epidemiology of medically important eukaryotic microorganisms. Here we report on the development and application of four different microsatellite markers to type strains of *H. capsulatum*.

The *H. capsulatum* clinical isolates studies were obtained from Indiana, IN, Birmingham, AL, Colombia and Australia. Four North American 'class 1' isolates were also included, as well as 12 isolates of unknown origin.

Microsatellite development

Microsatellites were isolated using two methods: (i) locus 610.2-CA: arbitrarily amplified DNA fragments were screened using a $(CA)_n$ microsatellite probe, hybridizing bands were isolated and sequenced, and specific primers were designed around the microsatellite locus [16]; (ii) loci HSP-TC, ATPase-AT and ATPase-CT: these microsatellites were all found by querying the GenBank database with microsatellite motifs. HSP-TC is a $(TC)_n$ microsatellite and is upstream of the heat shock protein 60 gene; ATPase-AT and ATPase-CT are both downstream from the *H. capsulatum* plasma membrane ATPase gene, and are $(AT)_n$ and $(CT)_n$ microsatellites, respectively.

Microsatellite analysis

Microsatellite amplification primers were labeled with fluorescent dyes and amplicons were electrophoresed in an ABI373 automated sequencer (Applied Biosystems, Foster City, CA, USA). Fragment sizes were assigned using Genescan software. A radial tree showing the relationship of isolates was produced using treedraw, based on a similarity matrix produced using the proportion shared alleles option in Microsat 5.1 (E. Minch, Stanford University, CA, USA).

Microsatellites are polymorphic in genetically diverse isolates of H. capsulatum

Microsatellites HSP-TC and 610.2-CA were successfully amplified from all isolates, regardless of class or geo-

graphic origin. The ATPase-CT microsatellite was reliably amplified from North American and Colombian isolates but not from the Australian isolates. Neither ATPase microsatellite could be amplified from the class 1 isolates. Where amplification was successful, microsatellites were polymorphic in all populations with two exceptions: HSP-TC was monomorphic in the Australian population, and 610.2-CA was monomorphic in all isolates of the Colombian population except isolate H69 (Fig. 1).

Four microsatellites allow discrimination of most isolates

A radial tree produced by combining the results of the four microsatellites shows that the majority of isolates could be distinguished from one another (Fig. 1). Exceptions are H30/H32/U110, H60/H62, H64/H65/H113, U114/U119, U115/U131 and H124/H133/H134. Isolates H124/H133/H134 were obtained from a single patient over different days and have previously been found to be indistinguishable. H30/H32 and H60/H62 have been discriminated using other markers, however [17]. The relatedness of the unknown (U) isolates with one another and with other isolates included in this study has not been determined previously.

Microsatellites group isolates according to class or geographic location

The majority of isolates of *H. capsulatum* grouped on the radial tree according to their geographic location or Class. All of the Colombian isolates (bold, underlined) clustered together, except H69 which was the only Colombian isolate that differed at the 610.2-CA locus. The Australian isolates (prefixed with 'A') also formed a closely related group, although H126, a class 1 isolate, appeared to group with these. Class 1 isolates (bold) could only be typed with two of the four microsatellites, and it is likely that when more microsatellites are analyzed this isolate will fall into the class 1 group, as other molecular markers have clearly placed it in this class. All other class 1 isolates formed a distinct cluster. The majority of unknown isolates (prefixed with 'U') grouped with the North American isolates from Indianapolis (prefixed with 'H', normal text) and Birmingham (italicized), indicating that infection by these isolates was most likely acquired in the USA. Isolates U115 and H131 grouped with the class 1 isolates, and isolate U113 grouped with Colombian isolates. Other molecular markers have confirmed that these isolates belong in these groups.

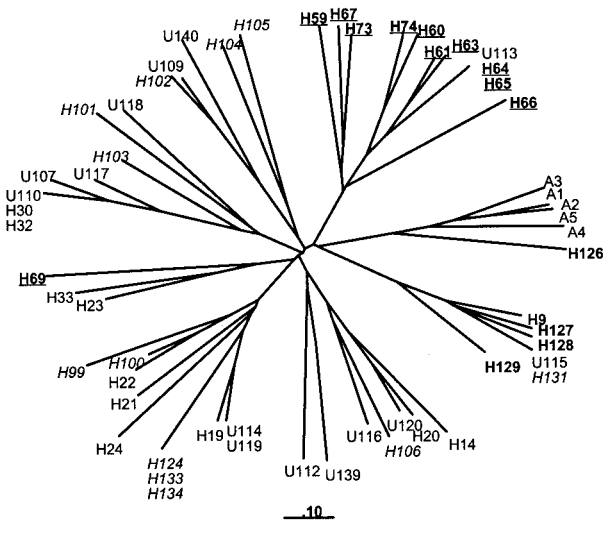

Fig. 1 Radial tree showing placement of *H. capsulatum* isolates based on microsatellite alleles.

In conclusion, microsatellites offer a rapid and powerful means of distinguishing between isolates of *H. capsulatum*. Of the four microsatellites developed, HSP-TC and 610.2-CA could be amplified from the DNA of any isolate, regardless of class or geographic origin, and were polymorphic in most populations. The two ATPase microsatellites failed to be amplified from the class 1 isolates, which are known to be genetically very different to all other *H. capsulatum* classes. Likewise, ATPase-CT could not be amplified from isolates from Australia. To date, only limited attempts have been made to optimize amplification primers and conditions, and it is likely that with more effort primers will be developed that can amplify these microsatellites from all isolates.

As with other molecular markers, microsatellites indicated that the genetic diversity of *H. capsulatum* is very high. The majority of isolates could be distinguished using only four different microsatellites, and adding two or three more polymorphic microsatellite loci should be sufficient to distinguish between all isolates or clones with a high degree of certainty. In addition, the four microsatellites were able to group most isolates according to geographic origin or class and to place isolates of unknown origin into these groups. These markers can therefore be used to determine the probable origin of an infection by *H. capsulatum*. In our future studies we will use microsatellites to conduct a large-scale study of the epidemiology of *H. capsulatum*, and to examine the global population structure of this fungal pathogen.

Molecular population genetics of *Candida albicans*

Unlike many pathogenic fungi, *Candida albicans* is diploid. Consequently, the development of genetic markers and analytical tools for studying the population genetics and epidemiology of *C. albicans* differ from those for haploid species. This report outlines the development of a set of co-dominant polymerase chain reaction (PCR)-RFLP markers and the application of these markers to understanding the population genetics of *C. albicans*.

Development of PCR-RFLP markers

In diploid organisms, co-dominant genetic markers provide much more information than dominant/recessive markers (e.g. RAPD, PCR-fingerprinting, Southern hybridization of repetitive elements and amplified FLP [AFLP] markers) and are better able to infer the reproductive mode and population structure of natural samples of isolates. To develop co-dominant markers for analyzing *C. albicans*, 10 bp primers were first used to generate random PCR fragments by RAPD. Amplified DNA fragments were excised from low-melting agarose, cloned into plasmid vectors and used to transform competent *Escherichia coli* cells. Both termini of the cloned DNA fragments were sequenced using forward and reverse M13 primers. From these sequences, locus-specific PCR primers of 19–26 nucleotides were designed for further screening. Restriction polymorphisms were detected by initially screening a test panel of 10–20 random strains with four-base cutting restriction enzymes (e.g. *Dde*I, *Hin*fI, *Alu*I, *Cfo*I and *Msp*I). Only primer and restriction enzyme combinations that generated polymorphisms were used for further analysis of large samples [18]. Typical restriction enzyme reactions consisted of 7 μl PCR product, 2–3 Units of enzyme, 1·5 μl 10× reaction buffer, and sterile H_2O to a total volume of 15 μl. Because of the availability of much of the *C. albicans* genomic sequences in the database, we are also developing PCR primers directly from the database and screening for genetic variations among isolates.

Analytical methods

Reproductive mode

Because there are two alleles at each locus in each strain, the associations of alleles within a locus in natural samples can provide information regarding the mode of reproduction in populations of *C. albicans*. This analysis involves a simple test to determine whether observed genotypic counts deviate significantly from those expected under the assumptions of random mating, large population size, and the neutrality of genetic markers under consideration (the Hardy–Weinberg equilibrium test, or HWE) [19]. If the observed genotype counts do not significantly differ from the expected counts, the results are consistent with recombination in nature.

The tests of associations among alleles between loci in diploids are more complicated than those in haploids. Currently, there are two approaches to analyze diploid data in the interlocus association tests. The first is to treat each unique diploid genotype at an individual locus as an allele, thus separating the within locus allelic association from between locus associations. The second method is to examine allelic associations at all levels, treating each allele individually [20]. This latter method combines both HWE and linkage disequilibrium in the same analysis. A large sample size is required for the second analysis, and preferably the first as well, to yield statistically meaningful results.

Population genetic relationships

The relationships among populations are usually described according to one of three measures. The first is Nei's standard genetic distance, D, which is calculated based on allele frequencies between pairs of populations [21]. In the second, the amount of genetic differentiation is usually quantified by Wright's F_{ST} [22,23]. In the third test, the statistical significance of genetic differentiation is calculated based on gene frequency differences through the Chi-square test, or Fisher's exact test, when the expected allelic counts are small [24,25]. Procedures for individual tests and calculations can be found in various books dealing with biostatistics and population genetics [2,7].

Results and comments

Samples of *C. albicans* from different host groups within a geographic region as well as samples of the same host group from different geographic areas were compared using the approaches described above. The results suggest several emerging trends.

First, in the analysis of samples of *C. albicans* from three different host groups from Durham, North Carolina, the PCR-RFLP method produced genotypes for 204 *C. albicans* isolates from three populations in the Duke University community: two from clinical sources (one from patients infected with human immunodeficiency virus [HIV] and the other from patients without HIV infection) and the third from healthy student volunteers [18]. The results indicated: (i) extensive evidence for clonality within and between populations of *C. albicans*;

and (ii) more genotypic and gene diversities in the non-clinical population than clinical populations, regardless of HIV status. The two clinical populations were genetically more similar to each other than either was to the population consisting of isolates from healthy people. Within each population sample, there was a general lack of heterozygotes and random associations of alleles within and between loci were found in less than 50% of the loci or pairs of loci. These findings are consistent between the two sets of samples analyzed: those including all isolates and those including only clone-corrected isolates where only one strain of each multilocus genotype was used [18].

To study the effects of geographic separation on the pattern of genetic variation in *C. albicans*, one additional sample including 64 strains from patients with HIV infection from Vitória, Brazil, was compared with the sample from HIV-infected patients from Durham, North Carolina, USA [26]. Consistent with previous studies on the population structure of *C. albicans*, the results showed evidence for extensive clonality within the sample from Vitória, Brazil. Furthermore, these two geographic populations from HIV-infected patients had similar gene and genotypic diversities, with little evidence for genetic differentiation. Among the 45 unique multilocus genotypes, six were shared between the two populations and these six genotypes accounted for 81 of the 126 isolates.

Current studies continue the genetic analysis of geographic samples from various regions around the world. The preliminary results indicate (i) lower levels of genetic differentiation among geographic samples originating from the Americas compared with those from Europe, Africa, Asia, (ii) different patterns of allelic association among different geographic samples, and (iii) significant differentiation among global geographic populations of *C. albicans*.

Molecular biology of *Paracoccidioides brasiliensis:* typing and differential gene expression

The dimorphic fungus *Paracoccidioides brasiliensis* is the etiological agent of human paracoccidioidomycosis (PCM), a systemic disease [27]. A high incidence of PCM is found in Latin America, specially in Brazil in its tropical and subtropical areas [28]. *P. brasiliensis* reaches the human host usually through the respiratory route. Airborne fungal propagules are thought to initiate infection in the lung by conversion to the yeast fungus phase, giving rise to different clinical forms of PCM [27]. Variations in intensity, extension and other characteristics of lesions in PCM patients, are shown on dependence of the fungal virulence, environmental aspects and the host defense mechanisms [29].

We contemplated several objectives when undertaking these studies. Initially and due to the presumptive correlation between isolates and ability to cause disease, genetic characterization was considered an important task. Evolutionary data concerning species isolation aspects appeared helpful in establishing diagnostic and therapeutic procedures related to *P. brasiliensis* infections.

In addition to the distinct ability of isolates to cause disease, establishment of the infection is strictly dependent on the transition of this dimorphic fungus. However, the host immune response also plays a crucial role in the ability of *P. brasiliensis*, as well as of other pathogenic microorganisms, to cause infection. We are engaged in programs related to cloning and characterization of genes with a putative role in the dimorphic transition and as such, potentially involved in the host–parasite interactions in this mycosis.

In order to study genetic diversity among *P. brasiliensis*, different isolates were used for DNA extraction. The random primers from Operon Technologies Inc. (Alameda, CA, USA) and others synthesized at Brasilia University, Brasília-Brazil were used in PCR reactions. The randomly amplified fragments were analyzed by agarose gel electrophoresis. The DNA fragment profiles were analyzed by calculating the simple matching (Jaccard's and Dice's) coefficient. Data were scored for the presence or absence of amplification products. The virulence of six isolates from two defined RAPD groups was investigated in an experimental model of PCM. Mice were infected intraperitoneally (IP) with yeast cells and a qualitative–quantitative method was developed to determine, by histopathological analysis, the damage caused by each isolate.

For cloning and characterization of differentially expressed genes different approaches have been used. (i) Total *P. brasiliensis* proteins from both mycelium and yeast were analyzed by two-dimensional electrophoresis and differentially expressed proteins were identified. The proteins were submitted to automated protein sequencing of their N-terminal regions. In order to obtain the PCR amplification of a DNA fragment corresponding to the protein, we designed primers by alignment of cDNAs with proteins deposited in GenBank, that show high similarity with the differentially expressed protein in *P. brasiliensis*. (ii) For *hsp70* and *hsp60* genes, differential expression was detected by Western-blot of a two-dimensional isoelectric focusing (IEF)/sodium dodecyl sulfate (SDS-PAGE) containing total protein extracts from *P. brasiliensis* mycelium and yeast cells. The genes were identified using heterologous probes coding for *Blasto-*

cladiella emersonii hsp70 or for *H. capsulatum hsp60* genes. (iii) For identification of differentially expressed genes by differential display reverse transcriptase (DDRT)-PCR we used total RNA isolated from mycelium, yeast cells and cells during the transition from mycelium to yeast in *P. brasiliensis*. The total RNA was used for reverse transcription and the PCR product was analyzed by sequencing gels. The differential pattern expression was confirmed by Northern blotting.

In recent years, work has been carried out in order to distinguish and characterize *P. brasiliensis* isolates. It has been established that randomly selected primers are adequate to distinguish and characterize genetic diversity among *P. brasiliensis* isolates [30]. We initially used 25 different random primers in order to test their ability to generate RAPD fragments from *P. brasiliensis*. The DNA amplification patterns allowed clear differentiation of isolates into two distinct groups with only 17% genomic identity. The ability of *P. brasiliensis* isolates to invade tissues was studied in an experimental model using susceptible B10.A mice [31]. The analysis was performed according to the severity of the lesions including the number and size of the granuloma, the number of fungi disseminating to different organs. The isolates from two RAPD groups demonstrated a marked difference in their virulence patterns for B10-A mice. In the early post-infection period, isolates Pb S, 662, Bt and 166 (group I) elicited localized infection restricted to the liver and showed compact epithelioid granuloma with few fungi (slightly virulent). On the other hand, isolates Pb 01 and 7455 (group II) elicited a disseminated infection, showed extensive areas of necrosis and large numbers of viable fungal cells (highly virulent). It was shown that two groups of isolates defined by RAPD analysis were distinct according to their ability to cause disease in an experimental model using B10.A susceptible mice. Some attempts to establish a correlation between groups and geographical distribution had also been performed for *P. brasiliensis* isolates [32].

This work has enabled the identification of differentially expressed proteins in *P. brasiliensis* morphological phases [32–35]. The *in vitro* differentiation process is reversible and dependent on temperature shift. Differentially expressed proteins were identified by two-dimensional electrophoresis; using this technique we identified the protein PBY20 which is expressed only in the yeast form. The sequence of the 34 N-terminal amino acid residues has shown high similarity with specific allergens, such as the protein Alta7 (*Alternaria alternata*) and Clah5 (*Cladosporium herbarum*). To obtain the amplification of a DNA fragment corresponding to PbY20 by PCR, we designed three primers by alignment of cDNA sequences with proteins Alta7, Clah5 and the *obr1* gene from *Schizosaccharomyces pombe*. The PCR resulted in a main fragment of 550 bp. The results show that this DNA fragment probably corresponds to the gene that encodes the PbY20 protein. Similarity analysis suggests that the protein PbY20 can probably perform an allergen function in paracoccidioidomycosis, as with the proteins Alta7 and Clah5, or may act in maintenance of the yeast chromatin structure, similar to the P25 protein or in brefeldin A resistance, as the protein codified by the *obr1* gene. In addition, genes coding for the hsp70 and hsp60 proteins preferentially expressed in *P. brasiliensis* yeast phase were characterized [36, S.M. Salem Izacc *et al.*, unpublished results]. Both genes are regulated in response to temperature stress during the transition from mycelium to yeast in *P. brasiliensis*.

We have isolated and characterized cDNA and genomic clones that encode a 70 kDa heat shock protein (hsp70). The gene encodes a 649-amino acid protein showing high identity with other members of the *HSP70* gene family. The *HSP70* gene is induced during both heat shock of yeast cells at 42 °C and the mycelial-to-yeast transition. A differential expression of this gene can be observed between mycelial and yeast forms, with a much higher level of expression in the yeast. We found two introns of 178 and 72 nucleotides in the *P. brasiliensis HSP70* gene. Splicing of these introns is regulated during the heat shock process and possibly during infection. The mycelial-to-yeast differentiation will exert the adaptational effect of increasing the organism's resistance to environmental stress, which may be necessary for parasite survival in the mammalian host. A gene encoding hsp60 was cloned and characterized. The deduced amino acid sequence of the *P. brasiliensis HSP60* gene and the respective cloned cDNA consists of 592 residues highly homologous to other fungal HSP60 proteins. The *HSP60* gene is present as a single copy in the genome and a transcript of 2·1 kb was detected in yeast cells. The complete coding region of *P. brasiliensis HSP60* was expressed in *E. coli* and a Western immunoblot experiment demonstrated that the recombinant protein as well as the native hsp60 was recognized by serum from patients with PCM. The immunoscreening of a *P. brasiliensis* library with sera from these patients provided a cDNA that was strongly expressed in the yeast phase but poorly in mycelium fungus phase. In addition, we were able to detect genes differentially expressed in one form of this fungus using differential display [37]. Studies concerning the function of those genes will clarify if they are involved in the differentiation process and in the *P. brasiliensis* infectious process.

In summary, it has been shown that RAPD analysis proved to be an adequate tool for analyzing and comparing different genomes in *P. brasiliensis* isolates. The correlation between genetic groups and virulence of isolates was also established. Additionally, we were able to detect differentially expressed genes in stage-specific cells and also during the transition from mycelium to yeast in *P. brasiliensis*. The function of those genes can be readily known once gene disruption experiments can be carried out.

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