Detecção do *Paracoccidioides* spp. em amostras ambientais e diferenciação do complexo *P. brasiliensis* da espécie *P. lutzii* por Nested PCR e hibridização *in situ*

THALES DOMINGOS ARANTES

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração *Biologia de Parasitas e Micro-organismos*.

Orientador: Prof. Titular Eduardo Bagagli

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RAQUEL CORDEIRO THEODORO
MARCUS DE MELLO TEIXEIRA
SANDRA DE MORAES GIMENEZ BOSCO
EDUARDO BAGAGLI

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"*A vida segue seu rumo como um rio, tudo passa, é carregado, seja leve ou pesado... mas na próxima curva tudo é renovado*".

RESUMO

O solo é o provável habitat do fungo patogênico *Paracoccidioides* spp., devido à detecção molecular deste patógeno neste tipo de amostra, associada à frequente infecção de trabalhadores rurais e isolamento em animais silvestres (*Dasypus novemcinctus* e *Cabassous centralis*). O presente estudo visou detectar e diferenciar as espécies *Paracoccidioides brasiliensis* (complexo) e *Paracoccidioides lutzii* no ambiente, pelas técnicas de Nested PCR, FISH, respectivamente, em amostras ambientais aerossóis e solo de tocas de tatus provenientes de áreas endêmicas para a Paracoccidioidomicose nas regiões Sudeste, Centro-Oeste e Norte brasileiras. Além da detecção ambiental de *Paracoccidioides* sp. por métodos moleculares, foi estudada a ocorrência de tatus infectados com *P. lutzii* no centro-oeste brasileiro, região de maior prevalência desta espécie, onde nenhum animal havia sido avaliado até o presente momento. Obtivemos a detecção positiva para ambas as espécies de *Paracoccidioides* por ambas as técnicas de detecção (Nested PCR e hibridização *in situ*), além da diferenciação por ITS (*Internal Transcribed Spacer*) pudemos visualizar os espécimes fúngicos em algumas amostras aerossóis. Acreditamos que os dados refletem a real ocorrência dos fungos em seu nicho, no entanto, o isolamento animal em tatus demonstrou-se negativo para 7 animais avaliados no trabalho, o que pode indicar que a relação da espécie *P. lutzii* com os tatus pode não ser a mesma com os casos de tatus infectados com *P. brasiliensis* em outras regiões brasileiras.

Palavras Chave: Paracoccidioidomicose, Amostrador Ciclônico, TSA-FISH, *Paracoccidioides* spp., Amostras Aerossóis.

ABSTRACT

Soil is probably the habitat of pathogenic fungi *Paracoccidioides* spp., due to the molecular detection of this pathogen in these samples, associated with the frequent of infection in rural workers and the isolation in wild animals (*Dasypus novemcinctus* and *Cabassous centralis*). This project aimed to detect and differentiate the species *P. brasiliensis* (complex) and *P. lutzii* in the environment, by the techniques of Nested PCR and FISH, respectively, in environmental aerosol samples and soil from armadillo's burrows, from endemic and non-endemic areas to Paracoccidioidomycosis in the Southeast, Midwest and North regions of Brazil. Besides the environmental detection of *Paracoccidioides* spp. by molecular methods, the occurrence of armadillos infected with *P. lutzii* the Brazilian center-west region with the highest prevalence of this kind, where no animals were evaluated at the present time will be studied. We achieved positive detection of both species of *Paracoccidioides* by both detection techniques (PCR and *in situ* hybridization), and further the differentiation by the ITS (Internal Transcribed Spacer) we could visualize fungal species in some aerosol samples also differentiating the two species. We believe of the data reflect the actual occurrence of fungi in their niche, however the animal isolation in armadillo's showed up negative for 7 animals evaluated at work, which indicated that the ratio of the species *P. lutzii* with armadillo may not be the same with cases of armadillo's infected with *P. brasiliensis* in other regions of Brazil.

Key words: Paracoccidioidomycosis, Cyclonic Sampler, TSA-FISH, *Paracoccidioides* spp., Aerosol Samples.

I. INTRODUÇÃO

O estudo dos aspectos biológicos e ecológicos de *Paracoccidioides brasiliensis* (SPLENDORE, 1910; ALMEIDA, 1930) e *P. lutzii* (TEIXEIRA et al., 2013) vem sendo desenvolvido por vários grupos de pesquisa nos últimos anos, em especial buscando isolar e/ou detectar estes organismos em amostras clínicas e ambientais, de forma a obter dados mais concretos sobre os fatores ecológicos que determinam a distribuição geográfica destes patógenos. Ambas as espécies, pertencentes ao Filo Ascomycota, Família Ajellomycetaceae, são causadoras da mais importante micose sistêmica da América Latina, a Paracoccidioidomicose (PCM) (BRUMMER et al., 1993; SHIKANAI-YASUDA et al., 2006). Trata-se de fungos termodimórficos, com apresentação da forma micelial (saprobiótica) à temperatura ambiente (25-28ºC) e leveduriforme (parasitária) à 37ºC (BAGAGLI et al., 2003). A forma micelial é produtora dos esporos infectantes, chamados artrósporos ou conídios, os quais devem ser aerossolizados antes de infectar hospedeiros susceptíveis pela via inalatória (ARANTES et al., 2012). Após a infecção, se transformam em células individualizadas (leveduras) com multibrotamentos, causando assim a micose (LACAZ et al., 2002).

A paracoccidioidomicose, uma micose classificada como sistêmica, é doença de reconhecido impacto socioeconômico, por acometer principalmente trabalhadores rurais do sexo masculino na faixa etária de 30 a 50 anos, faixa esta a mais produtiva no trabalhador, impossibilitando muitas vezes o retorno deste à suas funções normais de trabalho (MENDES et al., 2003; SHIKANAY-YASUDA et al., 2006). A manifestação da doença depende de fatores como imunidade do hospedeiro, predisposição por alcoolismo, tabagismo e doenças de base (tuberculose, aids, etc.), além da carga fúngica (inóculo) e o período de latência do fungo (MENDES, 1994). As principais formas clínicas da PCM são aguda/subaguda e crônica. A forma aguda/subaguda, também chamada juvenil, em geral compromete crianças, adolescentes e adultos jovens,

apresentando história clínica de curta duração (mediana de dois meses) (SHIKANAI-YASUDA et al., 2006). Esta forma é responsável por 20 a 25% dos casos, caracteriza-se por apresentar instalação mais rápida da doença, variando de algumas semanas a poucos meses, além de apresentar envolvimentos predominantes do sistema fagocítico mononuclear, sendo estes: baço, fígado, nódulos linfáticos e medula óssea. A forma crônica ocorre em 75% dos casos e apresenta história clínica de longa duração, em geral acima de seis meses. As manifestações pulmonares são muito frequentes, diferentemente da forma aguda onde estas são pouco comuns com elevada ocorrência de lesões em pele e mucosa. Com o acometimento pulmonar na forma crônica, outros órgãos tendem a ser comprometidos, assim como pele e adrenais (MENDES, 1994).

Segundo Restrepo et al., (1985) a manifestação clínica e o período de latência do fungo, associado às frequentes migrações das populações de áreas endêmicas, tornam praticamente impossível à identificação dos locais onde a infecção foi adquirida. Baseados nessa afirmação, podemos observar a real necessidade de se identificar o agente diretamente do seu habitat, conhecendo de forma mais clara sua atuação no micro nicho ao qual pertence e interage.

Sabe-se atualmente que o *Paracoccidioides* spp. tem seu habitat (local físico e geográfico de distribuição) localizado no solo, mas seu nicho ecológico (somatório de todas as interações do micro-organismo com os fatores bióticos e abióticos do meio) ainda não foi corretamente determinado, levando a necessidades de estudos a nível ambiental para este fungo (FRANCO et al., 2000).

O gênero *Paracoccidioides* apresenta características típicas de organismos com estratégias k de crescimento (IJDO et al., 2010), ou seja, com baixa taxa de reprodução e pouca produção de esporos, quando em saprobiose. Ainda, comparado com outros fungos de solo, este fungo apresenta crescimento lento em meios

laboratoriais, o que dificulta o seu isolamento ambiental, quer seja por cultivo direto, por extinção ou mesmo por recuperação após infecção animal experimental (GEZUELE, 1989; SHOME & BATISTA, 1963; SILVA-VERGARA et al., 1998).

Os estudos de distribuição do *Paracoccidioides* têm focalizado principalmente isolados provenientes de infecções humanas e alguns poucos isolados provenientes de animais silvestres e domésticos, uma vez que são raros os isolados obtidos diretamente de amostras ambientais. Os poucos casos de isolamento direto de amostras ambientais deste patógeno foram obtidos de solo, folhagem, ração de cachorro e de fezes de pinguim, quase que de forma casual, com pouca ou nenhuma repetitividade (FRANCO et al., 2000), limitando a capacidade de compreender tanto a ecologia como a real distribuição do gênero no ambiente de áreas endêmicas e não endêmicas.

Sendo parte e/ou a maioria dos isolados usados em estudos filogeográficos das espécies do gênero *Paracoccidioides* provenientes de amostras clínicas, fatores como a migração do hospedeiro e o período de latência da doença dificultam precisar o local da infecção e a ocorrência de cada espécie críptica na região endêmica de origem do paciente. A identificação das diferentes espécies em seu ambiente saprobiótico, além de demarcar locais de risco, também pode contribuir para uma melhor elucidação da filogeografia do gênero *Paracoccidioides* (BAGAGLI et al., 2003 e 2008).

Os estudos sobre a ecologia do *Paracoccidioides* spp. ganharam novas perspectivas com a descoberta de hospedeiros naturais silvestres (tatus), pelo desenvolvimento de metodologias de biologia molecular e também pela aplicação de métodos de geoprocessamento (NAIFF et al., 1986; BAGAGLI et al., 1998 e 2003; CORREDOR et al., 2005; THEODORO et al., 2008; BARROZO et al., 2009 e 2010). A ecologia do *Paracoccidioides* spp. ainda necessita de muitos dados experimentais para ser melhor fundamentada, em especial, dados de isolamento em amostras ambientais

que ao longo dos anos após a descrição do fungo, vem sendo apenas esporadicamente realizado (FRANCO et al., 2000). Tendo em vista a necessidade de compreender melhor os fatores ambientais do fungo, um maior número de grupos de pesquisa associados a várias frentes de trabalho (clínica, laboratório e ambiental) deveriam atuar juntos na pesquisa do *Paracoccidioides* spp., de modo a confluir dados de pesquisa para uma melhor compreensão da especiação, características de virulência e fatores ecológicos de cada espécie críptica do gênero.

O primeiro relato de isolamento do solo foi em 1962 e 1963, no Recife, onde pesquisadores descreveram o isolamento de *Paracoccidioides* spp. em amostras do solo em uma fazenda, no entanto uma posterior identificação micológica do mesmo isolado identificou este como sendo o fungo *Aspergillus penicillioides*, além da região de isolamento não ser reconhecida como área endêmica para a Paracoccidioidomicose (SHOME & BATISTA, 1963). Em 1967, outro trabalho relatou o isolamento de *Paracoccidioides* spp. do solo, na cidade de Chaco – Argentina, onde pesquisadores obtiveram 12 amostras do solo da zona rural, isolando o fungo nestas. Este isolado é aceito como verdadeiro, entretanto, seria necessário o envio da amostra para centros de referência para confirmação da identificação micológica do achado (NEGRONI, 1967).

O isolamento de *Paracoccidioides* spp. foi realizado também em 1971, na Venezuela, na cidade de Paracotos, onde pesquisadores isolaram o fungo dentre 87 amostras de solo obtidas em diferentes épocas do ano, sendo um dos primeiros trabalhos a relatar a infecção por via inalatória através da dispersão dos conídios do fungo (ALBORNOZ, 1971).

Em 1989 Gezuele, pesquisador uruguaio realizou o isolamento de um fungo com características semelhantes ao *Paracoccidioides* spp. em fezes de pinguim *(Pygoscelis adeliae)*, características estas comprovadas nos trabalhos sequentes

(CALEGARI et al., 1989, CAMARGO et al., 1992; GARCIA et al., 1993), por meio de provas imunoquímicas, produção de antígenos e aspectos micológicos.

Em 1990 Ferreira e colaboradores, realizaram o isolamento de *Paracoccidioides* spp. em ração de cachorro, provavelmente devido ao contato quase que direto do alimento com o solo, como fonte de contaminação (FERREIRA et al., 1990). No ano de 1993 Garcia et al., na sequência dos trabalhos de 1992, realizaram provas de cultivo, imunoquímicas, produção de antígenos, SDS-PAGE e imunoeletroforese, confirmando a identidade do fungo de Gezuele como sendo *Paracoccidioides* spp., descartando no entanto a proposta uruguaia de classificação desta como sendo uma nova espécie denominada *Paracoccidioides antarcticus*.

Em 1998, Silva-Vergara e colaboradores, isolaram um fungo a partir de amostras de solo de uma plantação de café em Ibiá-MG. O isolado apresentou dimorfismo térmico e com virulência frente inóculo animal por via intraperitoneal no município de Ibiá-MG, caracterizando-o como *Paracoccidioides* spp.

Além do isolamento em amostras ambientais, foram relatados dados de dois casos de PCM em cães (RICCI et al., 2004; FARIAS et al., 2005), onde foi obtida cultura fúngica positiva apenas para o segundo caso. O isolado foi caracterizado molecular e morfologicamente (BOSCO et al., 2005). Este achado é de grande importância para o estudo epidemiológico da PCM, pois representa o primeiro isolamento do *P. brasiliensis* de um animal doméstico, cujo contato com humanos é infinitamente superior se comparado com animais silvestres.

O tatu é um importante marcador para a detecção do *Paracoccidioides* spp., uma vez que é um animal sem hábitos migratórios e com uma área de vivência bem delimitada, o que facilita a demarcação do local de infecção do tatu pelo fungo, tornando assim possível caracterizar a área de captura como sendo uma área de

ocorrência do fungo. O isolamento em tatus (NAIFF et al., 1986; BAGAGLI et al., 1998 e 2003; CORREDOR et al., 2005; ARANTES et al., 2012) é uma boa metodologia, uma vez que torna possível identificar o isolado ambiental, caracterizando com precisão, assim como em isolados clínicos humanos a correta especiação do isolado fúngico.

No trabalho de Trejo-Cháves et al., 2011 além do tatu, outro animal silvestre foi descrito com isolamento positivo para o fungo *Paracoccidioides* spp., o bicho preguiça *Choloepus didactylus*, evidenciando a infecção em diferentes hospedeiros silvestres (TREJO-CHÁVES et al., 2011).

Enquanto os conhecimentos sobre a ecologia e em particular sobre o nicho ecológico e habitat do *Paracoccidioides* spp. tiveram poucos avanços nos últimos anos, os conhecimentos sobre o conceito de espécie do patógeno avançaram consideravelmente, principalmente pela aplicação de técnicas de biologia molecular e bioinformática. Matute et al., (2006) e Carrero et al., (2008), realizaram um estudo filogenético por genealogia de multi loci, no qual constatou-se um complexo de quatro espécies em *P. brasiliensis*. No trabalho de 2006, foram descritos 3 genótipos distintos do gênero (S1, PS2 e PS3), denominados de espécies crípticas, ou seja, espécies com diferenças morfológicas ausentes e/ou imperceptíveis, mas geneticamente distintas e reprodutivamente isoladas (MATUTE et al., 2006; CARRERO et al., 2008; TEIXEIRA et al., 2009).

O resultado do estudo realizado por Matute et al., (2006) demonstrou a existência de um grupo parafilético, denominado S1, e dois monofiléticos PS2 e PS3. A ocorrência destes achados fica restrita as áreas geográficas da Argentina, Brasil, Peru, Paraguai e Venezuela (S1 e PS2) e Colômbia (PS3).

Dois anos após o estudo de Matute e colaboradores, Carrero et al., (2008), ao analisarem 14 genes de um total de 21 isolados, observaram que o isolado Pb01

proveniente da região centro-oeste brasileira, não se agrupava com nenhuma das três espécies crípticas descritas por Matute et al., (2006). Utilizando um maior número de isolados (17) sendo 16 da região centro-oeste brasileira e um do Equador, Teixeira et al., (2009), determinou que estes novos isolados pertenciam a um grupo que não se agrupava as espécies S1, PS2 e PS3, mas que constituíam uma nova espécie, denominada inicialmente como Pb01-*like* e mais tarde como *P. lutzii*, em homenagem ao médico Adolfo Lutz, o descobridor da doença. (TEIXEIRA et al. 2014)

Após a descoberta das espécies crípticas, a distribuição geográfica das espécies do complexo *P. brasiliensis* e da espécie *P. lutzii* foi atualizada no trabalho de Theodoro et al., 2012, o qual apresenta um mapa da distribuição dos isolados analisados (Figura 01). No entanto, como já mencionado, esta distribuição foi baseada em sua grande maioria por isolados clínicos, o que reflete a problemática da migração dos hospedeiros e fatores ecológicos do fungo.
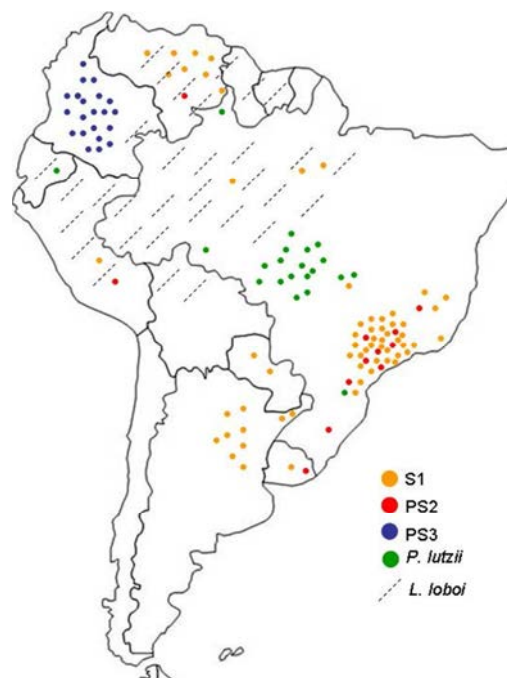

Figura 01. Mapa distribuição segundo Theodoro et al. 2012., demonstrando a disposição dos isolados das 4 espécies crípticas de *Paracoccidioides* sp. na América do Sul.

Ainda na figura 1, o mapa demonstra também as áreas de ocorrência de outro fungo, pertencente à família Ajellomycetaceae, o *Lacazia loboi*, fungo este filogeneticamente muito próximo ao Paracoccidioides spp., possuindo um ancestral comum, sugerindo uma divergência evolutiva que originou ambas as espécies, sendo o *L. loboi* um fungo incultivável até o momento e com afinidade pelo meio aquático.

Os estudos ecológicos do *Paracoccidioides* spp. desenvolvidos por nosso grupo de pesquisa, sempre foram formulados de modo a unir dados clínicos aos achados ambientais, gerando assim novos mapas de distribuição e um melhor entendimento da eco-epidemiologia do agente. Porém, esta é uma tarefa árdua, dada a vastidão do nosso território nacional e a difícil obtenção dos dados clínicos e laboratoriais desta doença que não é considerada de notificação compulsória.

Em um estudo recente, Vieira et al., (2014) analisaram a distribuição dos casos de PCM no estado de Rondônia, demonstrando um considerável percentual desta micose em pacientes daquela região, demonstrando assim a real situação epidemiológica da doença no estado, e deste modo, caracterizando o estado de Rondônia como uma importante área endêmica para a Paracoccidioidomicose (Figura 02).
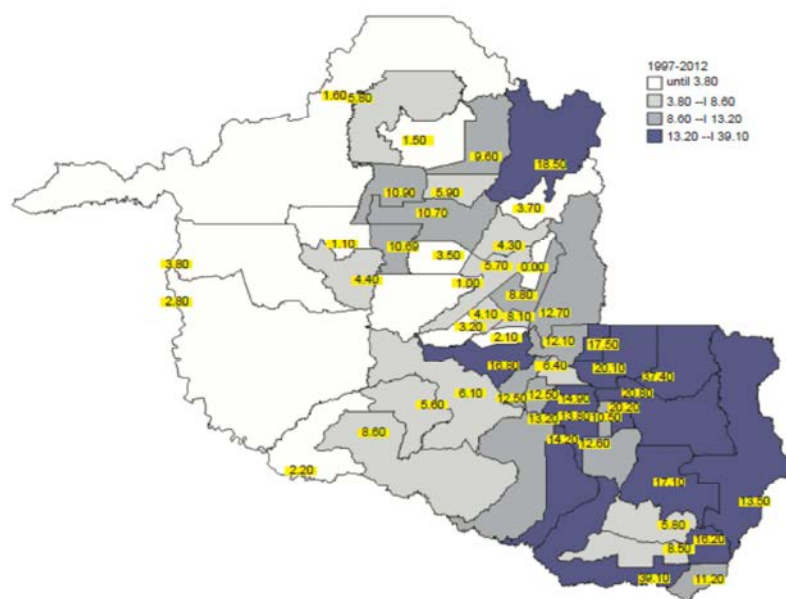
Figura 02. Mapa de incidência da PCM no estado de Rondônia, distribuição realizada por índice em cada município do estado nos anos de 1997-2012 (100.000 habitante/ano), Fonte SINAN e SINAN NET. Áreas em azul apresentam maior incidência de casos desta micose (Vieira et al. 2014).

No ano de 2014, Gegembauer e colaboradores, realizaram um estudo sorológico de pacientes dos estados de Mato Grosso e Mato Grosso do Sul no qual o diagnóstico foi positivo em maior frequência dos soros de pacientes testados da região centro-oeste para detecção com antígenos produzidos com cepas de *P. lutzii*, demonstrando a necessidade da criação de métodos de identificação ou diagnóstico que de fato sejam hábeis em detectar todas as espécies causadoras da Paracoccidioidomicose. O mapa da figura 03 demonstra os dados clínicos e de diagnóstico laboratorial da PCM nos estados da região Centro-Oeste, Sul e Sudeste neste estudo. Podemos observar que isolados ambientais, os quais não sofrem influência do fator migração, deveriam ser adicionados a este e a outros estudos a fim de atualizar e redefinir o mapa da distribuição das espécies de *Paracoccidioides*.

Figura 03. Mapa das regiões brasileiras de predominância do *Paracoccidioides brasiliensis* e do *P. lutzii* segundo Gegembauer et al., 2014.

Ainda quanto à relação diagnóstico sorológico versus a genotipagem do *Paracoccidioides*, Machado et al. 2013, realizaram um estudo sorológico de pacientes das regiões Sudeste e centro-oeste brasileiras, de modo que os soros foram cruzados com antígenos produzidos com cepas originalmente isoladas de cada região, de forma que soros de São Paulo fossem cruzados com antígenos da região Centro-Oeste e vice-versa. Após rápida avaliação sorológica constatou-se que para que a positividade aumentasse no diagnóstico sorológico é necessário produzir antígenos com cepas da região de procedência destes pacientes com doença ativa ou então com um preparado antigênico de várias cepas de todas as espécies crípticas de *Paracoccidioides*, de modo a evitar falsos negativos, contribuindo para a melhoria no diagnóstico da PCM em cada área endêmica, uma vez que a positividade nos cruzamentos antígenos e soros foram baixos para antígenos de regiões distintas dos soros.

Os dados atuais quanto ao diagnóstico da PCM podem refletir a melhoria no diagnóstico laboratorial desta micose e de outras patologias nos últimos 20 anos, no entanto, além da melhoria no diagnóstico laboratorial dessa micose, podemos ressaltar também a atuação do homem em áreas preservadas de vegetação nativa nestas áreas (cerrado, Amazônia e Mata Atlântica), tornando-as áreas de agronegócio. Esse avanço agrícola e a antropofização do solo pode levar ao surgimento de patógenos emergentes, e em especial ao fungo *Paracoccidioides* spp., levando a uma maior exposição dos trabalhadores rurais destas regiões ao fungo, devido à intensa atividade de manuseio do solo e facilitando assim a aerossolização das partículas fúngicas infectantes. Assim podemos lavar em consideração para taxarmos o grande número de casos de PCM nos estados do Centro-Oeste e Norte não somente por uma melhoria na qualidade diagnóstica, mas também a uma maior taxa de infecção em áreas até então nunca desbravadas pelo homem do campo.

Devido a toda problemática do isolamento ambiental deste fungo por técnicas clássicas (isolamento em cultivo e infecção experimental), a detecção por biologia molecular mostrou-se a mais promissora técnica de detecção deste agente nos últimos anos, tanto para amostras biológicas quanto para amostras ambientais. A literatura relata como a principal técnica a PCR (Polymerase Chain Reaction), na qual fragmentos do DNA fúngico, previamente extraído de amostras clínicas e ambientais têm sua amplificação delimitada pela complementariedade de *primers* espécie-específicos. Os fragmentos amplificados são então identificados segundo seu tamanho em pares de base, em um gel de Agarose ou Poliacrilamida pelo método de eletroforese. Isto vem sendo particularmente realizado utilizando-se do DNA ribossomal (MOTOYAMA et al., 2000; THEODORO et al., 2005a; TERÇARIOLI et al., 2007; ARANTES et al., 2012). Além do material genético ribossomal, o DNA genômico pode também ser

utilizado na detecção do *Paracoccidioides* spp. (RICHINI-PEREIRA et al., 2009), assim como elementos genéticos parasitas, denominados Inteins ou Inteínas, que podem ser utilizados para a caracterização dos isolados de *Paracoccidioides* spp. (THEODORO et al., 2008).

O material que é amplificado pela PCR pode ser também sequenciado para comparação e identificação das espécies. Existem bancos de dados on-line de genes de diversos fungos na internet, por exemplo, o GenBank disponível no site (www.ncbi.nlm.nih.gov/BLAST) e o Broad Institute (www.broadinstitute.org), ambos com a ferramenta de busca *Blastn* que se baseia em um algoritmo para identificar sequências de nucleotídeos semelhantes às indicadas pelo pesquisador, informando assim qual o organismo que possui maior identidade para aquela sequencia específica de DNA. Para o *Paracoccidioides* spp., uma boa técnica de detecção é a Nested PCR, uma variação da PCR original, na qual inicialmente são definidas e amplificadas regiões gênicas relativamente menos específicas, comuns em praticamente todos os fungos estudados atualmente e de maior tamanho, e em uma segunda reação, procede-se a amplificação de uma região menor, interna a primeira reação de amplificação, porém específica para o fungo de interesse (THEODORO et al., 2005a; TERÇARIOLI et al., 2007). Para a utilização desta técnica em fungos, os *primers* genéricos mais utilizados são ITS4 e ITS5, que são complementares as regiões 18S e 28S e amplificam as regiões *Internal Transcribed Spacer* (ITS) ITS1, 5.8S e ITS2, descritos por White et al., (1990), que é ideal para amplificação por ser multi cópias e por ser extremamente conservada, apresentando pequenas variações intraespecíficas (nas regiões ITS), possibilitando a correta identificação dos fungos estudados. Para a segunda PCR ou Nested PCR, *primers* internos específicos de *Paracoccidioides* spp. são utilizados (Pb-ITSE e Pb-ITSR) se anelando nas regiões ITS1 e ITS2. Estes *primers* vinham sendo usados para a

detecção do *Paracoccidioides* spp. em amostras ambientais e clínicas, em nosso laboratório no Instituto de Biociências de Botucatu (THEODORO et al., 2005a).

Contudo, devido à necessidade da tipagem e detecção das espécies crípticas no gênero *Paracoccidioides,* um novo *primer* antisense (Pb-ITS-T)*,* foi desenhado (Figura 04) a fim de se detectar qualquer uma das quatro espécies deste patógeno em amostras ambientais, demonstrado em gel de eletroforese com DNAs controle das 4 espécies crípticas do gênero *Paracoccidioides* (figura 05) (ARANTES et al., 2012).
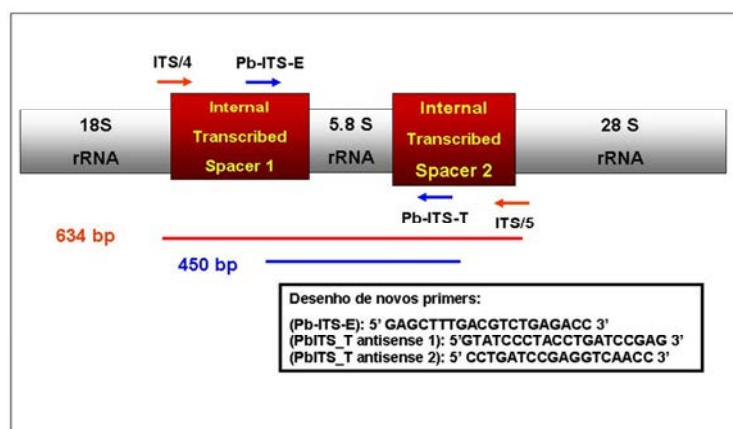

Figura 04. Esquema de região de rRNA fúngico para amplificação com novo desenho de primers específicos para o gênero *Paracoccidioides* (modificado de THEODORO et al., 2005a).

Figura 05. Reação de Nested PCR com novos primers Pb-ITSE e Pb-ITST para amplificação de DNAs controle das 4 espécies crípticas do gênero *Paracoccidioides* (ARANTES et al., 2012).

Na área da biologia molecular, além de técnicas já estabelecidas como a PCR e suas variantes, novas técnicas como as que enfocam o estudo de fungos não cultiváveis em amostras ambientais diversas (água, solo, plantas) vêm sendo desenvolvidas, tornando possível a identificação de fungos até então desconhecidos por meio de marcações fluoróforas in situ. Um exemplo é a técnica de FISH (Fluorescence *in situ* hybridization) que se baseia na formação *duplex*, sob condições bem definidas, de um fragmento de ácido nucléico de fita simples modificado (sonda/probe) e sua sequência complementar (sequência alvo) em um espécime biológico fixado. Trata-se de uma técnica de marcação de DNA, RNA ou proteínas. Inicialmente foi descrita na área de citogenética e imunohistoquímica, nas quais isótopos radioativos eram conjugados com as sondas para demarcar estruturas ou regiões gênicas e com o passar dos anos a técnica ganhou novas vertentes, incluindo opções alternativas a marcação radioativa, o que favoreceu sua execução por um número maior de centros de pesquisa, uma vez que ao não necessitar da conjugação de isótopos radioativos para emissão de fluorescência, a torna realizável em qualquer laboratório padrão com material necessário na análise molecular e/ou citogenética (ADAMS, 1992).

Na microbiologia e em especial na micologia, a referência no diagnóstico laboratorial é a demonstração dos patógenos em avaliação direta na amostra biológica e/ou seu isolamento em cultura, mas em alguns casos, devido à dificuldade de isolamento ou visualização nas amostras, o micro-organismo pode ser demonstrado por meio de técnicas moleculares, incluindo a de coloração *in situ* de DNA ou RNA alvo específicos do agente procurado. Esta abordagem *in situ* facilita sua correlação com a clínica, pois age diretamente na amostra biológica, e desta forma auxilia também a demonstração do agente em amostras ambientais, com a marcação das células fúngicas nesses tipos de amostras, delimitando a existência de fungos viáveis no local de coleta,

além de demarcar com precisão a área de ocorrência de possíveis patógenos humanos, como por exemplo, o *Paracoccidioides* spp. (LIEHR, et al., 2009).

De um modo geral e técnico, a detecção *in situ* de ácidos nucléicos exógenos é mais simples do que a dos ácidos nucléicos endógenos, principalmente porque as moléculas de ácidos nucléicos exógenos são mais acessíveis devido à diminuição de suas interações com as proteínas. Além disso, em muitas situações, o número de cópias dos ácidos nucléicos exógenos é maior do que para os ácidos nucléicos endógenos, simplificando questões de sensibilidade da aplicação desta técnica, no entanto, para ambos os materiais genéticos alvo podem ser feitas adequações da técnica (LIEHR, et al., 2009).

Com esta técnica é possível identificar alterações a nível genômico como, por exemplo, microdeleções ou quando um rearranjo cromossômico envolve uma região camuflada na leitura (rearranjo crítico) ou de difícil interpretação (rearranjos no interior do cromossomo), podendo também auxiliar na diferenciação de espécies fúngicas distintas em amostras ambientais (GEORGAKOPOULOS et al., 2009 in LIEHR, et al., 2009), que é uma de nossas propostas neste estudo na pesquisa do gênero *Paracoccidioides* sp. em amostras aerossóis.

O método de FISH se baseia na busca de fungos e outros micro-organismos através de sua marcação com sondas (óligos ou anticorpos) para vários genes e estruturas fúngicas, como por exemplo, o gene codificador da alpha-tubulina, marcado com um agente fluoróforo, esta marcação foi demonstrada em microscópio de fluorescência, o que permitiu o descobrimento de um novo filo dentro do reino Fungi, o *Cryptomycota* (JONES et al., 2011), estes dados são demonstrados na figura 06.
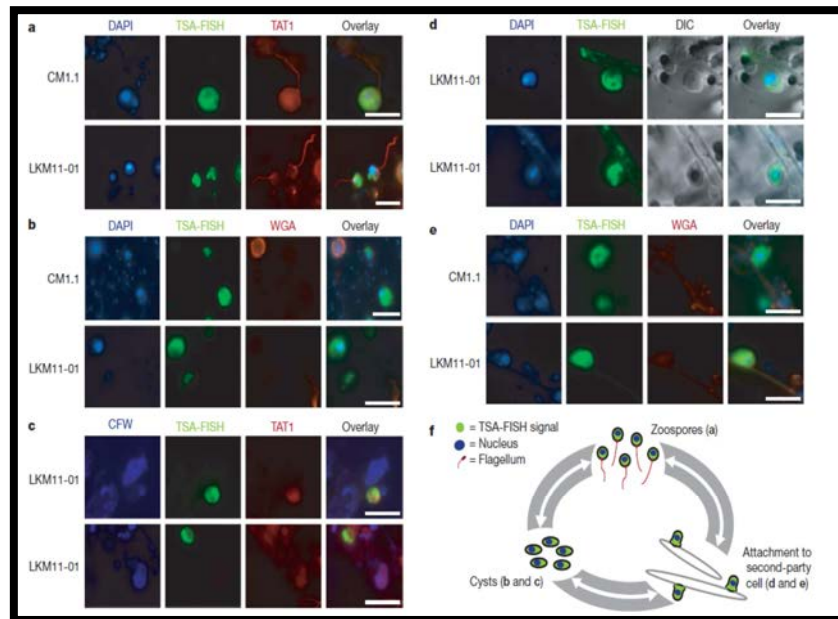
Figura 06. Marcação estrutural de células do filo *Cryptomicota* e suas diferenças durante o ciclo de vida. Marcações celulares com DAPI (núcleo) e marcações por TSA-FISH com Horseradish Peroxidase (JONES et al., 2011).

A maioria das sondas ou probes utilizadas na FISH são derivadas a partir de fragmentos de DNA genômico. Para a marcação em cromossomos, a presença de sequências de repetição centroméricas definidas permite a utilização de pequenos Oligo Desoxi Nucleotídeos (ODN) ou sondas sintéticas (MATERA et al. 1993 in LIEHR, et al., 2009). O uso de ODN sintéticos altamente específicos, os quais têm uma taxa de hibridização superior e menores custos de fabricação em comparação com sondas genômicas tradicionais, é uma abordagem que pode ser utilizada para atender aos requisitos da técnica de FISH. Os ODNs em geral tendem a hibridizar mais rapidamente com seu alvo, além de ser mais consistentes e mais baratos em sua síntese. (MATERA & WARD, 1992 in LIEHR et al., 2009).

Uma vantagem significativa das sondas de ODN é o seu pequeno tamanho e baixa complexidade, que é medida pelo número de combinações possíveis das sequências contidas na preparação de uma sonda. Estas características resultam em uma

cinética de hibridização mais rápida (em torno de 5 minutos) em comparação com sondas de alta complexidade, que exigem de 8 a 16 horas para completar o ciclo de hibridização.

No entanto, o pequeno tamanho das sondas ODNs limita o número de marcadores que podem ser incorporados a elas, e consequentemente sua sensibilidade. A fragmentação do DNA repetitivo elimina alguns problemas de sensibilidade, especialmente se a unidade de sequência repetida está presente em diversas de cópias (NAKAGOME et al. 1991 in LIEHR et al., 2009), como por exemplo, a região ITS (*Internal Transcribed Spacer*) do rRNA fúngico, que por ser uma região multi cópias, facilita sua marcação na célula fúngica. No entanto, para se obter uma taxa de sensibilidade maior, as sondas devem conter o máximo de moléculas fluorescentes possíveis (PINKEL et al., 1981 in LIEHR et al., 2009). As sondas podem receber marcações fluoróforas tanto na porção 5' quanto na 3', e em alguns casos mais de um fluoróforo é incorporado nas sondas.

Esta técnica de marcação de DNA alvo é uma abordagem que se mostra como uma nova vertente para pesquisas ambientais do gênero *Paracoccidioides* e outros fungos de difícil isolamento ambiental, como por exemplo, a espécie *Lacazia loboi* única espécie patogênica não cultivável da família Ajellomycetaceae (LACAZ et al., 2002). A pesquisa com sondas de DNA ou RNA fúngico, geralmente ribossomal, denominada de tecnologia de fluorescência por hibridização *in situ* – FISH (do inglês Fluorescence *in situ* Hybridization) associada à técnica de TSA (Tyramide Signal Amplification) (Figura 07) apresenta melhores resultados em amostras ambientais segundo relatos da literatura (SPEEL et al., 1999; JONES et al., 2011; KUBOTA et al., 2006).
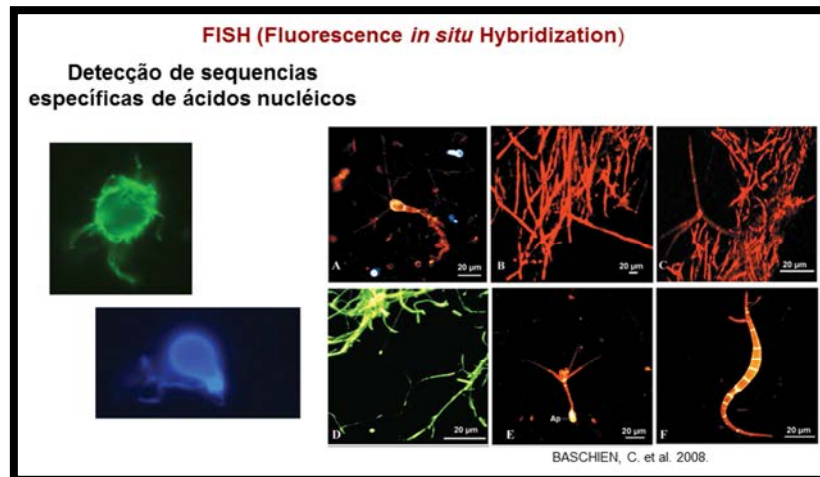
Figura 07. Detecção específica de ácidos nucleicos fúngicos em amostras ambientais (BASCHIEN et al., 2008).

Esta técnica também tornou possível a detecção de sequências específicas de ácidos nucléicos em cromossomos de células em tecidos morfologicamente preservados, tanto em amostras clínicas para diagnóstico laboratorial clínico quanto para a pesquisa ambiental de espécies fúngicas incultiváveis e/ou de difícil isolamento em laboratório (Figura 07) (MOTER et al., 2000; PERNTHALER et al., 2003; EICKHORST et al., 2008; BASCHIEN et al., 2008).

O uso da técnica de FISH associada à técnica de TSA (Tyramide Signal Amplification) é um reforço metodológico na detecção de genes alvo utilizando a união de um segundo fluoróforo que cliva a reação da sonda inicial, potencializando o sinal emitido pela sonda quando visualizada na microscopia de fluorescência. Na figura 08, podemos ver a sonda ligada com HRP (Horseradish Peroxidase), sendo ligada a fluoresceína-tiramida, potencializando o sinal inicial da sonda de Biotina. Desta forma, materiais escassos tendem a ser mais facilmente visualizados após a hibridização *in situ*.
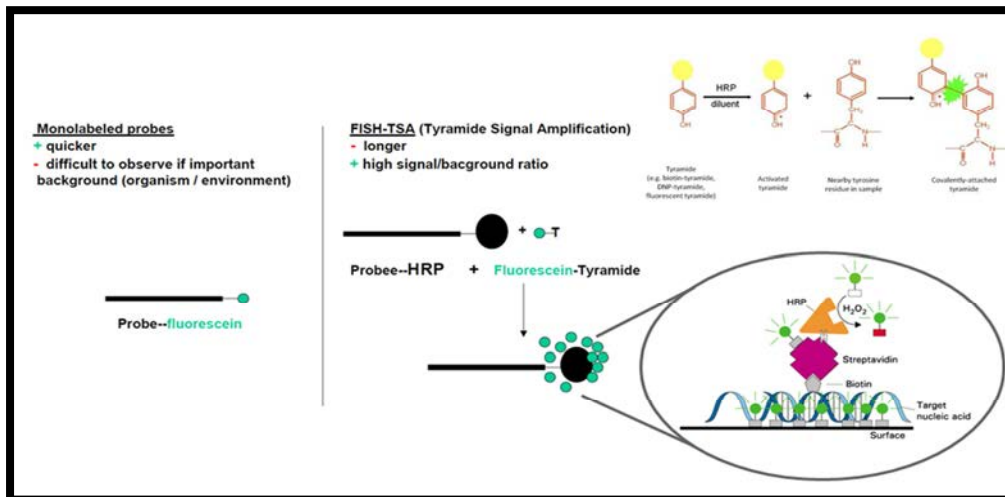
Figura 08. Esquema de marcação das sondas/probes na região do DNA alvo e a reação posterior de amplificação do sinal pela técnica de TSA (Tyramide Signal Amplification).

Os estudos filogenéticos prévios originados no trabalho desenvolvido por Arantes et al. (2012) indicaram a presença da espécie *P. lutzii* em amostras ambientais aerossóis e de solo coletadas na área endêmica da PCM em Botucatu-SP, segundo sequenciamento dos *amplicons* ambientais obtidos com os *primers* Pb-ITSE e Pb-ITST. Esta espécie só havia sido reportada, com alta prevalência, no centro-oeste brasileiro, sendo esta incidência baseada apenas em amostragens clínicas. Sendo assim, novos métodos de diferenciação espécie específica e de detecção e visualização deste agente diretamente em amostras ambientais podem auxiliar de forma significativa para uma melhor compreensão da distribuição geográfica das espécies do gênero *Paracoccidioides*, da relação patógeno-hospedeiro nas variadas formas clínicas da doença, bem como os padrões eco-epidemiológicos. O uso de sondas para a pesquisa ambiental do gênero *Paracoccidioides* é inédito, sendo apenas referenciada a técnica de ISH (*in situ* Hybridization) no trabalho de De Brito et al., 1999, que apresentou uma

baixa sensibilidade na marcação de células fúngicas de *Paracoccidioides* sp. (De Brito et al., 1999).

O isolamento e detecção ambiental das espécies de *Paracoccidioides* (*P. brasiliensis* e *P. lutzii*) têm grande importância na compreensão dos aspectos ecológicos destes agentes, porém têm sido pouco realizados, dada sua dificuldade metodológica. Neste estudo, visamos realizar a padronização e utilização de técnicas inovadoras para a pesquisa ambiental de *Paracoccidioides* por hibridização *in situ* com sondas de DNA espécie específicas, e também por técnicas moleculares já estabelecidas previamente, como a Nested PCR, associadas a amostragens ambientais diversas (solo, aerossol e animais silvestres), buscando assim contribuir tanto para o estudo ecológico como para a elucidação da biogeografia deste(s) fungo(s). Neste estudo foi dada continuidade à pesquisa ecológica deste fungo, com uma abordagem inovadora, ampliando a área até então estudada, incluindo as regiões Sudeste, Centro-Oeste e Norte brasileiras, em busca de novas informações ambientais sobre a distribuição deste fungo em áreas endêmicas e não endêmicas da PCM, gerando assim novos mapas da distribuição deste importante patógeno fúngico.

II. OBJETIVOS

O objetivo geral deste estudo foi a pesquisa da ocorrência do *Paracoccidioides* sp. em amostras ambientais em áreas endêmicas e não endêmicas nos estados de Minas Gerais, Goiás e Rondônia. Para tal, especificamente objetivou-se:

- Padronizar as metodologias de obtenção de amostras tanto aerossóis quanto para coleta de solo aplicadas as metodologia de detecção molecular utilizando as sondas de DNA;

- Avaliar a presença do *Paracoccidioides* spp. em amostras ambientais por métodos de hibridização com sondas de rRNA pelo método de TSA-FISH por detecção da região gênica ITS;

- Analisar filogeneticamente os dados de material amplificado na PCR e Nested PCR, visando à diferenciação do complexo *P. brasiliensis* da espécie *P. lutzii*;

- Isolar o *Paracoccidioides* spp. em tatus de nove bandas (*D. novemcinctus*) nas regiões avaliadas no estudo.

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IV. Artigo 1 - Use of florescent oligonucleotides probes for the detection and differentiation of *Paracoccidioides* species.

Title:

Use of florescent oligonucleotides probes for the detection and differentiation of

Paracoccidioides species.

Authors

Thales Domingos Arantes[1], Raquel Cordeiro Theodoro[2], Eduardo Bagagli[1]*

[1]- Departamento de Microbiologia e Imunologia, Instituto de Biociências de Botucatu, Universidade Estadual Paulista - UNESP

[2]- Departamento de Biologia Celular e Genética, Centro de Biociências, Universidade Federal do Rio Grande do Norte – UFRN

*Corresponding author:

Dept of Microbiology and Immunology

Institute of Biosciences, UNESP

Distrito de Rubião Júnior, Botucatu, SP, Brazil

Zip Code: 18618-970

Phone: +551438800418

Email address: bagagli@ibb.unesp.br

Abstract

FISH (Fluorescence *in situ* hybridization), associated with TSA (Tyramide Signal Amplification) using oligonucleotides labeled with non-radioactive fluorophores can be used to detect and differentiate fungal species in environmental and clinical samples, being suitable for those microorganisms whose isolation in culture is difficulty or even impossible. In this study, we standardized the differentiation of the two species complexes of *Paracoccidiodies* spp. using species-specific DNA probes, labeled with different fluorophores, for later application in environmental samples, assisting the studies on ecological aspects of this fungus. For differentiation of the species, we used tree isolates of *P. brasiliensis* and tree of *P. lutzii* cultured in Soil Extract Agar, in the two phases of *Paracoccidioides* (mycelial and yeast), and two different techniques, TSA-FISH for *P. brasiliensis* with HRP (Horseradish Peroxidase) linked to the 5' of the probe and FISH for *P. lutzii* with the fluorophore TEXAS RED-X® also linked to the 5' of the probe. After testing different protocols, we obtained the best procedure for both techniques in detection and differentiation of the two complexes of *Paracoccidioides*. For all protocols and samples tested, we used negative controls, such as the fungus *Histoplasma capsulatum*, which also belongs to the family Ajellomycetaceae and other fungi. We were able to detect and differentiate *P. brasiliensis* and *P. lutzii* by using labeled probes without cross-positivity index with controls. TSA-FISH and FISH showed suitable for molecular detection and identification of the *Paracoccidioides* spp., with high accuracy, sensitivity and specificity, pointing out your potential for a direct and fast PCM diagnosis as well as for molecular detection of this pathogen in environment.

Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis recognized for being a socioeconomic impacting disease that compromises mainly rural male workers around 30 to 50 years old, their most productive phase, incapacitating them to develop their normal functions [1, 2]. The manifestation of the disease depends on factors such as host immunity, predisposition for alcoholism and smoking, preexisting diseases (tuberculosis, AIDS, etc.), the fungal inoculum and the latency period of this fungus [3]. The main clinical forms of PCM are acute/subacute and chronic. Acute/subacute form, also called juvenile, generally undertakes children, teenagers and young adults [2]. This form is responsible for 20-25% of cases and it is characterized by a fast onset of the disease, ranging from a few weeks to a few months, and presenting a predominant involvement of the mononuclear phagocyte system, namely: spleen, liver, lymph nodes and bone marrow. The chronic form occurs in 75% of cases and it has a history of long lasting, generally over six months. Pulmonary manifestations are very common and other organs tend to be affected, like skin and adrenal glands [3].

The *Paracoccidioides* isolates, when compared with other soil fungi, present slowly growth in culture, which hinders their environmental isolation by direct cultivation, being necessary experimental animal infection procedures to isolate them [4, 5, 6, 7].

In the field of molecular biology, well established techniques such as PCR and its variants, have being largely used for studying non-cultivable fungi in several environmental samples (water, soil, and plants). Besides the amplification techniques (PCR, Nested PCR) the use of specific probes by Fluorescence *in situ* hybridization (FISH) has also, making the identification of new fungi species possible. One example is the FISH technique, which is based on duplex formation under well-defined

conditions, by a single strand fluorescent probe and its complementary (target) sequence into a biological specimen. This technique was initially described in the field of cytogenetic and immunohistochemistry, in which radioactive isotopes were combined with the probes to tag structures or gene regions, but over the years the technique, has been acquiring new dimensions, including alternative options, such as fluorophores instead of radioactive and non-radioactive isotopes. All these improvements make it achievable in any standard laboratory [8].

In microbiology and especially in mycology, the reference laboratory method for diagnosis is the demonstration of the pathogen in the biological sample and/or its isolation in culture, but in cases of few pathogen cells in the tissue or impossibility of culturing it, molecular techniques such as *in situ* staining of specific target DNA or RNA are the best choice. This *in situ* approach facilitates its correlation with clinical, because it acts directly in the biological sample, and thus it can also be applied for environmental detection of viable cells of the agent, mapping the risk areas [9, 10, 11].

This target DNA labeling technique is a new applied approach to environmental research of *Paracoccidioides* genus and other fungi known to be difficult or even impossible to isolate from the environment, such as *Lacazia loboi,* the only uncultivable pathogenic species of Ajellomycetaceae family [12].

The use of probes for environmental research of *Paracoccidioides* genus is unprecedented and it is only referenced in the work of De Brito et al [13] who used the ISH technique (*in situ* Hybridization) , which showed a low sensitivity for labeling *Paracoccidioides* spp. fungal cells. Research on fungi with probes generally is based on protein encoding genes or genes encoding ribosomal RNA, associated with fluorescence *in situ* hybridization - FISH (Fluorescence *in situ* Hybridization) technique associated with TSA (Tyramide Signal Amplification). The TSA technique is based on the ability

of peroxidase (HRP), in the presence of low concentrations of H_2O_2, to convert the labeled tyramide in a substrate containing an oxidized highly reactive free radicals that can covalently bind to tyrosine residues at or near to the HRP [8]. This technique gives better results in environmental samples [14, 15]. For instance, this technique was effective in the detection of a new phylum within the kingdom fungi, called cryptomicota [16].

In this study, we propose a new technique for research of *Paracoccidioides* by *in situ* hybridization with species-specific DNA probes. We believe this approach is suitable for clinical studies as well as for ecological studies on the distribution of the cryptic species of this genus across the Latin America.

Methods

Probes design for detection and differentiation of *P. brasiliensis* and *P. lutzii* species.

For probes design the chosen genomic region was the rRNA coding gene, more specifically the *Internal Transcribed Spacer*-1 (ITS-1) was used [17]. The rRNA sequences from *Paracoccidioides* species [18, 19] were aligned in order to select conserved regions within species but able to differentiate between them, so that one probe hybridize to the complex *P. brasiliensis* and the other to *P. lutzii*. In addition, sequences from other fungal species were also used to ensure probe specificity.

The alignment of the sequences was carried out in MEGA 6.0 program [20]. A total of thirty-eight ITS sequences from *P. brasiliensis,* nineteen from *P. lutzii*, 2 from *Histoplasma capsulatum* and 2 from *Emmonsia* sp. were used. The designed probes were submitted to the similarity analysis at NCBI site database by the blastn tool to check and confirm their specificity. Two probes were selected, one for *P. brasiliensis*

ITS (HRP-5'-CTCAAGCGCGGCTTGCGTGTTGGGCCCGCGT-3') and other for *P. lutzii* (Texas Red-5'-ATGGACGTGCCCGAAAAGCAGCGGCGGCGT-3').

Fungal isolates used in the standardization of *in situ* hybridization.

In this test, we used three isolates of *P. brasiliensis* (T16B1, Pb192 and T15LN1) and 3 isolates of *P. lutzii* (Pb01, Pb66 and PbEE) as positive controls in the standardization phase of hybridization. The isolates were obtained from our mycology collection (Biology Laboratory fungi), Department of Microbiology and Immunology, Biosciences Institute of Botucatu - UNESP and from the mycology collection of the Laboratory of Molecular Biology of fungi the University of Brasilia - UnB. These isolates are from both clinical and armadillo's samples previously identified as *P. brasiliensis* or *P. lutzii*. As negative controls other fungal species were used, such as *Histoplasma capsulatum*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Trichophyton mentagrophytes* [12].

Preparation of reagents for FISH and TSA-FISH methods

Reagents

The protocol for preparing the reagents was adapted from the TSA PerkinElmer® commercial kit and from the: Sampling-Protocol Analysis: Parasitic-Host Dynamics Study, Nautset Marsh (Salt Pond and Mill Pond).

The Cell fixation Solution (4% paraformaldehyde plus 0.1 M phosphate buffer) was used to kill the fungus and keep intact its structure and genetic material. A sequence of 50%, 80% and 100% ethanol solutions were used to remove the cell fixation solution and to dehydrate the cells so that they have the ability to absorb the probes to be used in the hybridization step. After dehydration, 10 mL of pre-

hybridization buffer [2,0 ml of ultra-pure H_2O; 4,0 ml of 40% Formamide; 1,8 ml of 5M NaCl; 200µl of 1M Tris (pH 7.5); 100µl of 1% SDS; 2 ml of 10% Buffer Blocking Agent], were added to the samples for stabilization and improvement of their permeability, by differences in osmotic pressure. After this first step, the cells were placed hybridized with probes which were prepared in a solution with hybridization buffer at a final concentration of 50ng/µl. After 16-17 hours of incubation at 42° C, the slides with fungal controls were washed with 50 ml of Washing Buffer [47,54 ml of ultra-pure H_2O; 460µl of 5M NaCl, 500µl of 0.5M EDTA, 500µl of 1% SDS and 1 ml of 1M Tris (pH 7.5)] for removal of non-specific binding probes. After washing, the slides were stabilized with 250 ml of TNT buffer [217,315 ml of ultra-pure H_2O; 25 ml of 1M Tris (pH 7.5); 7,5ml of 5M NaCl and 0,185 ml of Tween 20].

After equilibrating and washing the slides with TNT buffer, 30 µl of TSA solution of the commercial kit (TSA Plus PerkinElmer[®]) were added in each slide, for 30 minutes in a humid dark chamber at room temperature. Then, they were washed again, dried at room temperature, prepared with addition of DAPI and covered with a cover slip to be observed under a fluorescence microscope.

Standardization of FISH and TSA-FISH techniques in cultured samples of *P. brasiliensis* and *P. lutzii*.

The standardization of the FISH technique required several experiments for the improvement of cell attachment on microscope slides, fixation step, time for hybridization, and also for minimizing cross-reactions of the probes with other fungi species, in order to ensure maximum reliability of results.

The fixing solutions used were initially the methanol free formaldehyde at 4%, and 4% paraformaldehyde associated with 0.1 M phosphate buffer.

The protocol used for hybridization with the *P. brasiliensis* probe was performed according to the commercial TSA-Plus kit from Perkin Elmer®, using pure mycelia of T16B1 isolate. Unlike synthesized probe for the detection of *P. brasiliensis*, which consisted of a link with Horseradish Peroxidase (HRP), *P. lutzii* probe was synthesized with the 5' portion labeled with fluorophore with Texas Red, which has a signal emission at 615 nm and a maximum excitation of 596 nm. Since commercial synthesis of HRP-labeled probes no longer exists and also there is no TSA commercial kit for probes labeled with Texas Red-X, only the FISH steps were applied for *P. lutzii* detection.

Fifty slides with *P. brasiliensis* (25 slides) and *P. lutzii* (25 slides) mycelial fragments were prepared. To complement these experiments, tests with the yeast phase of *Paracoccidioides* (*P. brasiliensis* and *P. lutzii*) were also carried out, to ascertain the use of FISH and TSA-FISH in possible clinical diagnostic methods, using the same isolates previously cited. The probes were commercially synthesized by the GE LifeSciences®, both being individually diluted in TE (elution buffer) to final concentration at 50ng/µl. After the execution of the FISH and TSA-FISH protocols, the slides were prepared with Calcofluor or DAPI and observed under a fluorescence microscope (Olympus BX60 model) in WB, WU and WG filters.

Fungal cell attachment on microscope slides

For the standardization of hyphae attachment to microscope slides, conidia and chlamydospores of the mycelial phase of the *P. brasiliensis* (isolate T16B1) were used. The mycelial colony of five Soil Extract Agar (SEA) plates (0,2% Glicose; 0,1% Yeast Extract; 1,5% Agar and 50% v/v of Soil Extract) were scraped and fragmented. These fragments were maintained in 15 ml Falcon type tubes containing formaldehyde

solution (1%), paraformaldehyde Ultra-Pure (Sigma Aldrich[©]) at 36.5 to 38%, distilled Water and 100 ml of PBS (NaCl [10% w/v]; Na_2HPO_4 [0,15% w/v] and NaH_2PO_4 [0,023% w/v] at pH 7,2-7,4) at concentrations of 10, 5 and 1X were used for different incubation periods (12 and 24 hours) at -20°C, followed by vortex shaking for two minutes centrifugation at 13,000 G for 10 minutes for sedimentation. Then, 10 and 50 µl of the fragments deposited in the pellet were arranged in conventional microscope slides previously cleaned with alcohol-chloroform solution (1: 1). The layout of the fungal pellet on the slides followed the diagram in Figure 1.

Once prepared, the slides were placed in a dry box and incubated at 42° C for 30 minutes and then at 37° C overnight, for drying and fixing the fungal pellet on the slides to allow further hydration with hybridization solution, containing the fluorescent probes. After drying, the slides were washed with ethanol solution of 70 to 100%, for dehydrate the fungal structures.

For this test, 25µl of Calcofluor were added for binding to the fungal structures for fluorescence emission (560nm), and the slide were covered with a cover slip and observed under a microscope (Olympus model BX60) on fluorescence filter WU.

Sampling of aerosol mycelial phase of *P. brasiliensis*: positive control in flow chamber and subsequent labeling with dye Calcofluor.

The mycelial phase of *P. brasiliensis* is the infective form of the agent and therefore requires extreme caution in its implementation. For this procedure, all biosecurity protocols were followed in biological safety cabinet Class II to prevent contamination of the user and the environment. We sought in this experiment to mimic environmental aerosol collection of fungal spores in the armadillo burrow, so we developed an *in house* system, to capture the fungal spores by the cyclonic sampler

(NIOSH, model Cyclone 251 BC, developed by the Centers for Diseases Control - CDC [Morgentown, USA]) [21], using mycelial cultures on SEA medium of *Paracoccidioides* spp. (isolate T16B1). This experiment was conducted in a Styrofoam box, in which the cyclonic aerosol sampler was suspended inside, but without creating an atmosphere of vacuum.

To carry out this control experiment, 5 SEA plates containing the *P. brasiliensis* mycelial phase were attached to the bottom of the box and opened. A mechanical stirring was done to release fungal propagules (Figure 2). The cyclone sampler was placed about 10 cm above the plates and then turned on for 32 hours. The volume collected by the sampler within this period was approximately 6720 liters of air.

The stages of the sampler (Falcon and Eppendorf® tubes, 15 ml and 1.5 ml respectively), were then washed with 100µl of Calcofluor dye and each stage was evaluated separately. Later, 20 slides (10 for each stage) were prepared with 10 µl each and analyzed under a fluorescence microscope (Olympus BX60 model) in WU filter.

The Styrofoam boxes was sealed, sterilized and discharged safely.

To demonstrate and verify spores morphology in this culture medium, we performed a control test by scraping the surface of the colonies, staining the fragments with 25µL Calcofluor and analyzing them under a microscope

Results

Standardizations of FISH and TSA-FISH techniques in *Paracoccidioides* spp. mycelial cultures

Fungal cell attachment on microscope slides

The fungal structures were well fixed on slides even after washing and staining steps (Figure 3). In both provisions, 50 and 10μl, conidia and chlamydospores of *P. brasiliensis* (T16B1 isolate) of were visualized.

Fixation with formaldehyde, paraformaldehyde and labeling of genetic material with DAPI

The fixation and genetic staining with DAPI were effective, clearly demonstrating that the fungal structures were preserved by fixation by all solutions, but the paraformaldehyde showed the best results for fixation of *Paracoccidioides* spp., because the fungal cells were more preserved and with a higher resolution (Figure 4).

Aerosol mycelial phase of *P. brasiliensis* as positive control

For the test performed directly with the T16B1 mycelial culture, a large number of chlamydospores and/or yeast cells not translated into mycelium and few hyphae and conidia were observed (Figure 5). For the test with the aerosol sampling of fungal spores, three out of five slides presented mycelial fragments or conidia in the first and second stages of the cyclonic sampler (Figure 6).

FISH for *P. lutzii* culture

The probe for detection of *P. lutzii* successfully hybridized with cells of the Pb01 isolate, , whose genetic material was labeled with DAPI (Figure 7).

The best fixing solution was the 4% paraformaldehyde, because it best preserved cells after the incubation time.

Hybridizations with *P. lutzii* isolates Pb01, Pb66 and PbEE were positive, showing red points (Texas Red – X) together with the genetic material with DAPI in WU filter showed with help of Image J software (Figure 8). Similarly to the samples of the mycelial phase, the yeast phase tests showed positive hybridization cells with the Texas Red probe for *P. lutzii* (Figure 9).

TSA-FISH in culture samples of *P. brasiliensis*.

After reading the slides, fungal genetic materials were evidenced by DAPI dye. The mycelial structures can be seen in the views in Figure 10, which for the WB filter some cells indicate increased signal in the cytoplasm which can lead to a false positive for an indication of these structures due to the background of the fluorophore with TSA Plus Kit (PerkinElmer®), because showed no genetic material marking stained with DAPI when viewed with WB filters.

The HRP probe hybridized to the three *P. brasiliensis* isolates used in this experiment (T16B1, T15LN9 and Pb192) showing green points together with the genetic material labeled with DAPI in merged images with Image J software (Figure 11).

The yeast phase tests showed positive hybridization cells with the labeled probe HRP (green cells) and genetic material with DAPI (Figure 12).

Probes specificity

Figures 13 and 14 show the lack of cross-reaction of the Texas Red-probe (used in FISH for *P. lutzii* detection) and the HRP-probe (used in TSA-FISH for *P.*

brasiliensis) when tested against *P. brasiliensis* and *P. lutzii* isolates respectively. No cross-reaction was also observed for the test of both probes against *Histoplasma capsulatum*, (Figure 15) and other fungi, such as *Aspergillus flavus*, *A. fumigatus* and the dermatophyte *Trichophyton mentagrophytes*. A total of 24 slides were prepared for these test (six for each isolate), and after the observation on fluorescence microscope, no point of hybridized cells were visualized for both probes, that indicated the specificity of the probes for detection of *Paracoccidioides* spp. However, in just one slide of *A. flavus* the fungus showed retention of Texas Red probe within the hyphae (Figure 16).

Discussion

In this study, we modified existing protocols in order to use HRP- and Texas Red labeled oligonucleotides probes, for the identification of *Paracoccidioides* spp. cells (mycelial and yeast phase). The adaptation of these protocols required a great effort, because the commercial synthesis of these probes was not available in Brazil and for this reason, we opted for use two different fluorophores (HRP and Texas Red) for differentiation of the species complex of *P. brasiliensis* and *P. lutzii*, respectively.

During the optimization of FISH and TSA-FISH techniques, we adapted a final protocol joining several protocols available in the literature, from commercial protocols until those involved in similar researches. As a result, we obtained a satisfactory data for the differentiation of the species of *Paracoccidioides* spp. (complex *P. brasiliensis* and *P. lutzii* species), since both probes were able to perform hybridization to its target and showed no cross-reactivity within the genus. By the specificity test of the probes against other fungi, we could notice some false points of hybridized cells, because is no showed genetic material stained with DAPI when viewed

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with WB filters, due to the accumulation of material retained during hybridization step, in which some dried slides presented intracellular crystallization probes. Therefore, we believe that for both the tested probes, the specificity was very similar to that obtained *in silico*.

Evaluating the signal intensity of the probes issued by exposing the slides under a fluorescence microscope, we observed a significant difference between the signal emitted by HRP-labeled probe (*P. brasiliensis*) and the Texas Red-labeled probe (*P. lutzii*), since of the TSA-FISH technique significantly increases the signal emission of hybridized probes. Despite of this signal difference, only the application of FISH technique using a probe labeled with Texas Red, showed good results, mainly because its methodology is easier to apply and it exhibits good signal emission of the hybridized fungal structures. However, the fluorescence incidence tends to be difficult to measure and interpret, depending on the observer's experience, which requires time and patience to identify the hybridized target cells.

Aiming to use the *in situ* hybridization technique in our ecological studies of *Paracoccidioides*, an experiment control was conducted to confirm the ability of the cyclonic sampler to capture intact fungal structures in these samples, and so, reproducing the environmental collection methodology (in forests and armadillo's burrows), in which the molecular detection is possible by Nested PCR [19]. The results presented here reflect the positivity of the aerosol sample, since fungal structures of *Paracoccidioides* spp. were viewed on slides stained with Calcofluor.

The technique of *in situ* hybridization demonstrate to be an alternative for the molecular detection of *Paracoccidioides* spp. and also for the *in situ* differentiation between *P. brasiliensis* and *P. lutzii*.

In general, the display of fungal structures in biological samples and the ability to differentiate *P. brasiliensis* and *P. lutzii*, directly in the material analyzed, is a potential tool for clinical diagnosis or environmental research of these pathogens. Furthermore, compared with traditional molecular detection technique (Nested PCR), which is well outlined in our laboratory, the *in situ* hybridization technique shows promise for future environmental and clinical studies, given the ability to differentiate the fungus directly into the host tissue, while maintaining the classic diagnostic standard reference for any mycosis, which consists in displaying the fungal agent in the biological sample. This approach may also be applied to address the progress clinical studies of the manifestations of each of the cryptic species, assisting therefore to a better choice of antifungal treatment of paracoccidioidomycosis.

Acknowledgments

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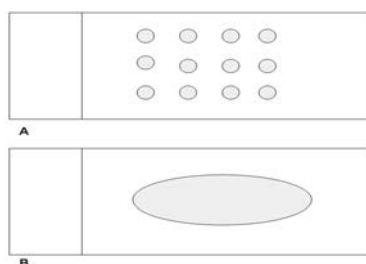
Annex

Figures

Figure 1. Fungal sediment disposal scheme. A: Slide with 10μL of fungal fragment per site. B: Slide with 50µL volume of fungal sediment.
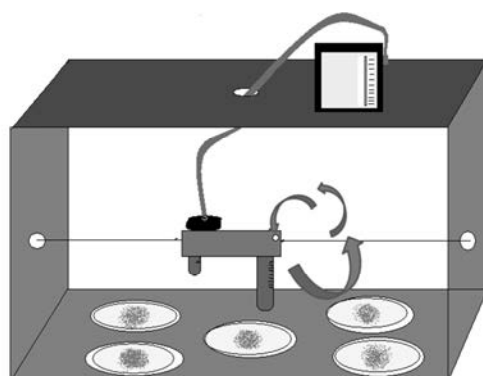

Figure 2. Collection system for cyclonic aerosol capture of spores (conidia and chlamydospores) of the mycelial phase of *Paracoccidioides.* sp. This set was maintained and operated within a Biosafety Board Class II (Forma Scientific Model 1200A).
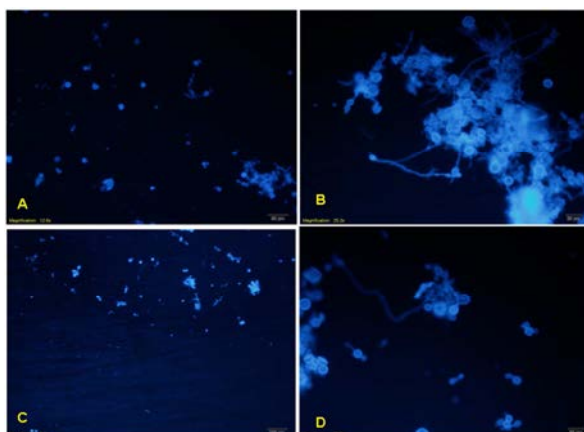
Figure 3. Fungal pellet (400x) fixed with 1% formaldehyde solution, stained with 0.5% Calcofluor and washed with Ethanol 70 and 100%. A and B: Slide with 50 µL volume of fungal sediment. C and D: Slide with 10 µL volume of fungal sediment 10µL per circle.
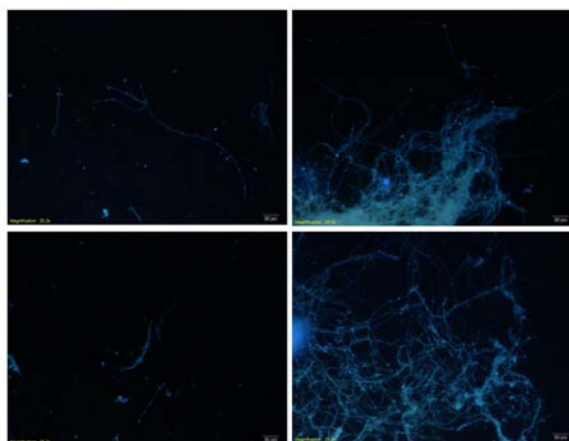

Figure 4. Fungal structures (hyphae, chlamydospores, conidia) (400x) in slides with *P. brasiliensis* (T16B1) sedimented, fixed with 4% formaldehyde (left) and 4% paraformaldehyde (right) and stained with DAPI (25µL each). Nuclear staining is more intense.
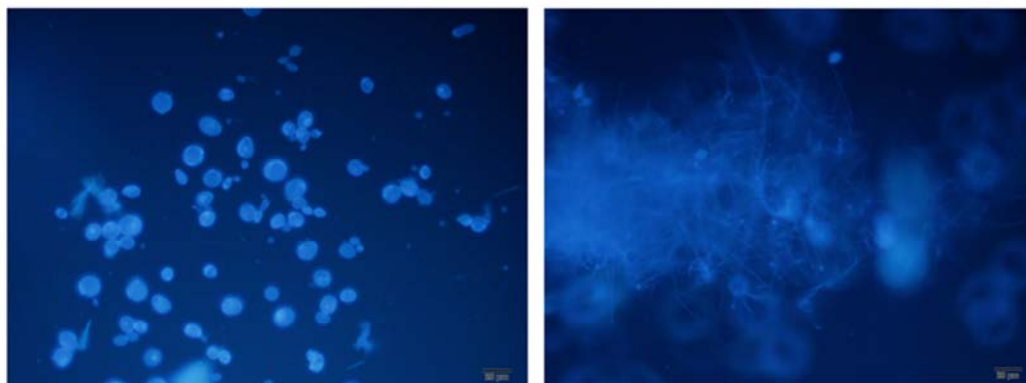
Figure 5. Chlamydospores of the mycelial phase of *P. brasiliensis* (isolate T16B1) (400x) scraped from the fungal culture and stained with 15μL Calcofluor solution (0.5%) observed under a fluorescence microscope in WU filter.
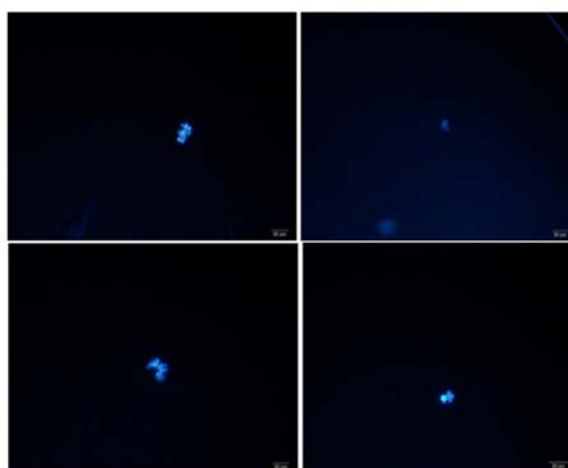

Figure 6. Mycelial structures from *P. brasiliensis* T16B1 isolate (400x) observed for the first (left) and second (right) stages of the cyclonic sampler after staining with 50µL of calcofluor solution (0.5%), under a epifluorescence microscope, using WU filter.
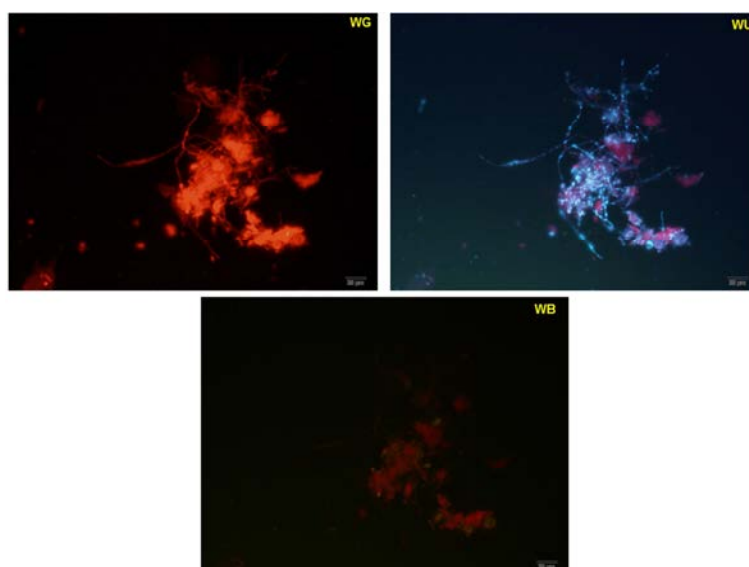
Figure 7. *P. lutzii* isolate Pb01 (400x) after hybridization of the ITS Texas Red probe by FISH technique (red). The cell genetic material visualized with DAPI (blue) under a fluorescence microscope in WG, WU and WB filters.
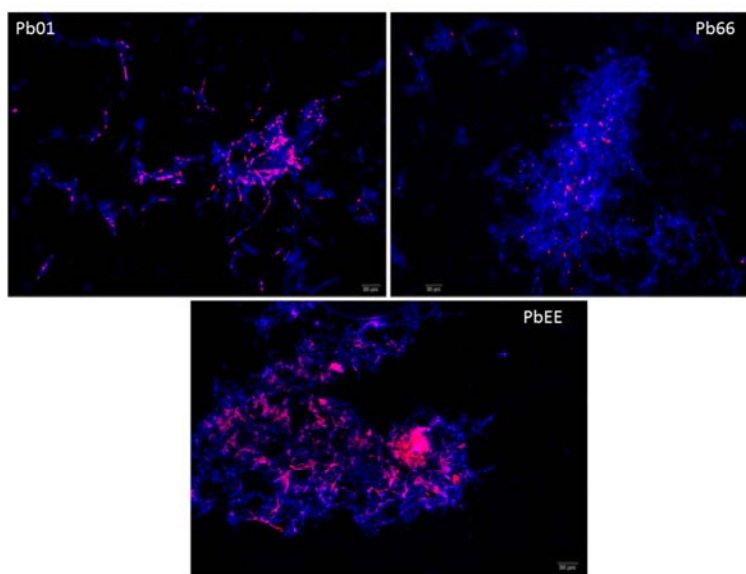

Figure 8. Merging images of *P. lutzii* mycelia from isolates Pb01, Pb66 and PbEE (400x) subjected to hybridization with the ITS Texas Red-X probe for *P. lutzii*, visualized in WG filter (red layer), and with DAPI (for cellular structures), visualized in WU filter (blue layer) under a fluorescence microscope.
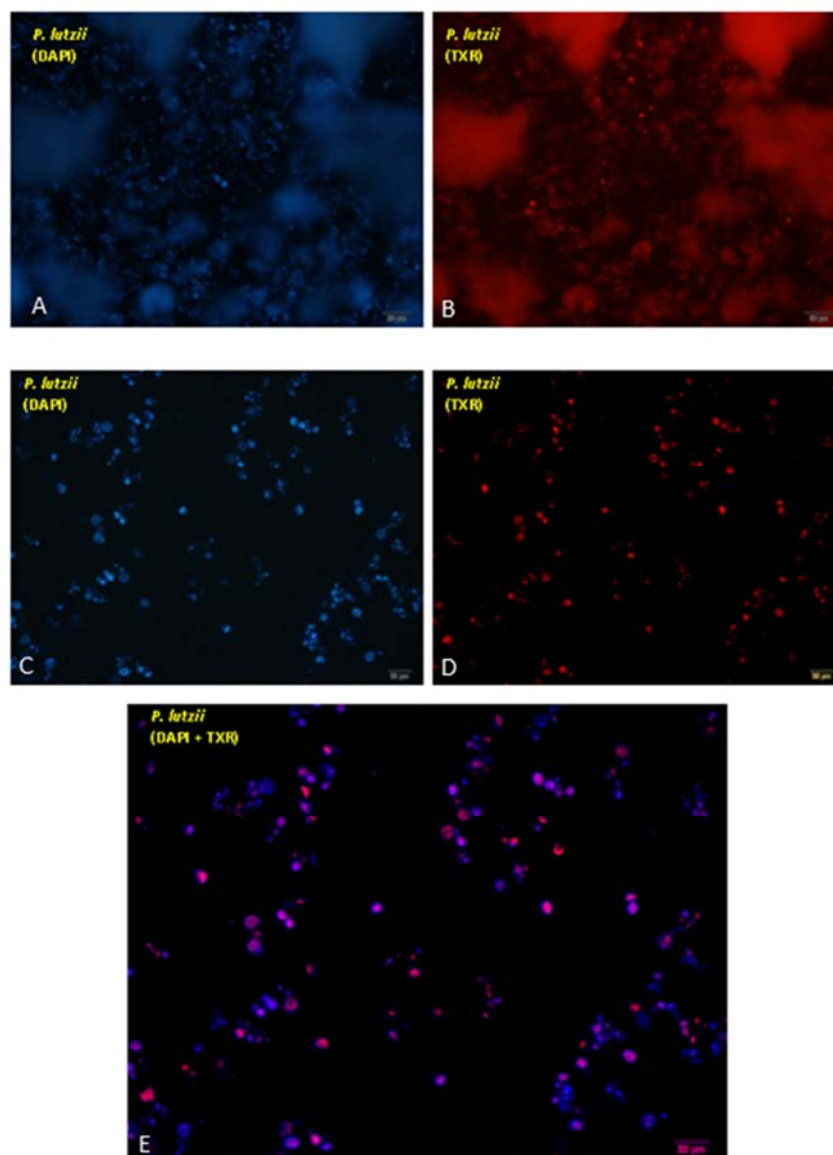
Figure 9.   Yeasts from isolate Pb01 (400x) hybridized with the ITS Texas Red-X probe for *P. lutzii* (observed in WG filter) (B and D) and stained with DAPI (genetic material - blue observed in WU filter) (A and C). Merged image in layers (E): Yeasts structure cell with DAPI (blue) and signal output of hybridized probes (red).

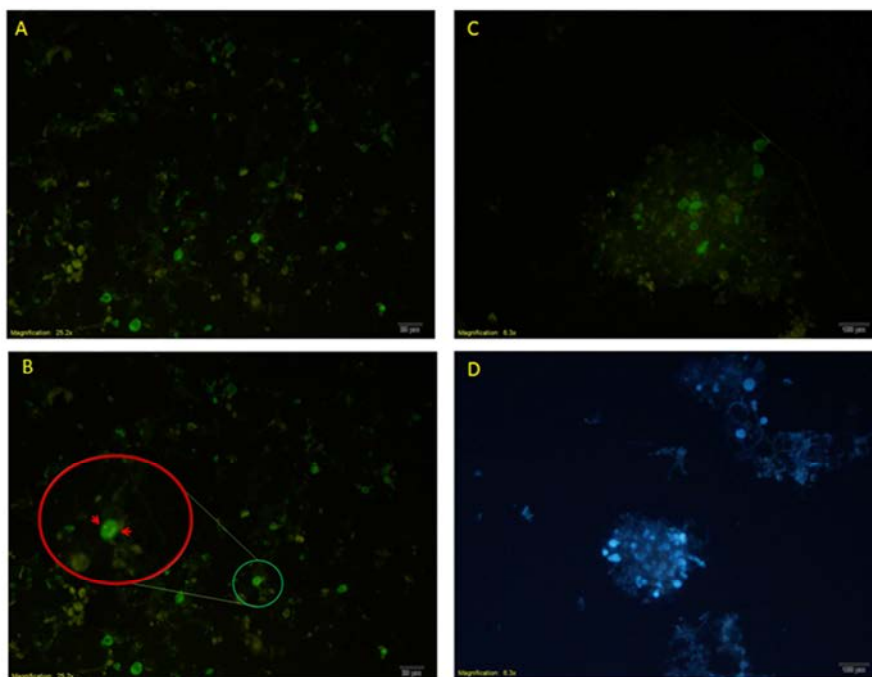**Figure 10**. *P. brasiliensis* isolate T16B1 (400x), subjected to hybridization with ITS HRP-probe by TSA-FISH technique. The hybridized cells are labeled with HRP and fluorescein (green) visualized in WB filter. The cell genetic material visualized with DAPI (blue) in WU filter (D) under a fluorescence microscope. The red circle is an amplification of fungi cell hybridized, indicated by the red arrows.
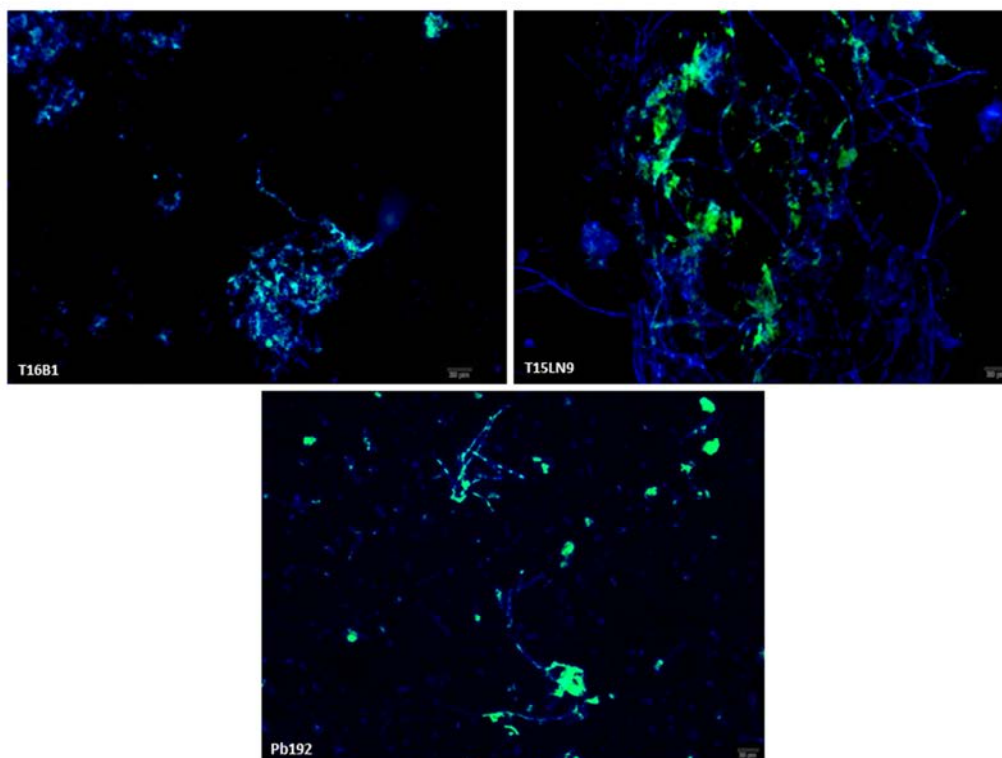
Figure 11. Merging images of slides with mycelia from *P. brasiliensis* (isolates T16B1, T15LN9 and Pb192) (400x) subjected to hybridization with the ITS HRP probe for *P. brasiliensis* visualized with DAPI (cellular structures) in WU filter (blue layer) and labeled with HRP in WB filter (green layer) under a fluorescence microscope.
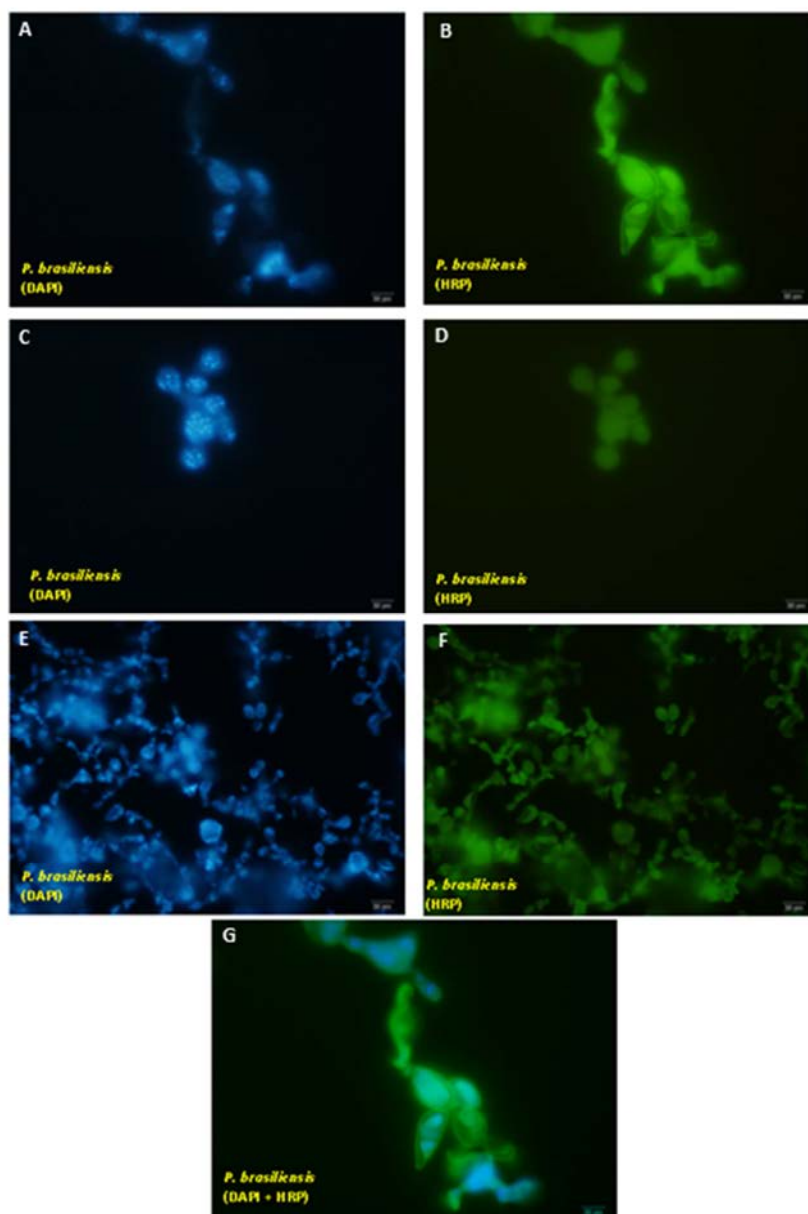
Figure 12. Yeast cells from isolate T16B1 (400x) hybridized with the ITS HRP probe for *P. brasiliensis* (B, D and F, observed in WB filter) and stained with DAPI (genetic material - blue observed in WU filter) (A, C and E) under a fluorescence microscope. Merged image (1000x) (G): Yeast cells hybridized with HRP probes (green) and genetic material visualized with DAPI (blue).

Figure 13. *P. lutzii* isolate Pb01 (400x), subjected to specificity test for hybridization with ITS HRP probe by TSA-FISH technique. No points of hybridized cells are present in the WB filter (HRP probe). The cell genetic material is visualized with DAPI (blue) under a fluorescence microscope in WU filter. Just points of non-hybridized cells are demonstrated in the WG filter image.
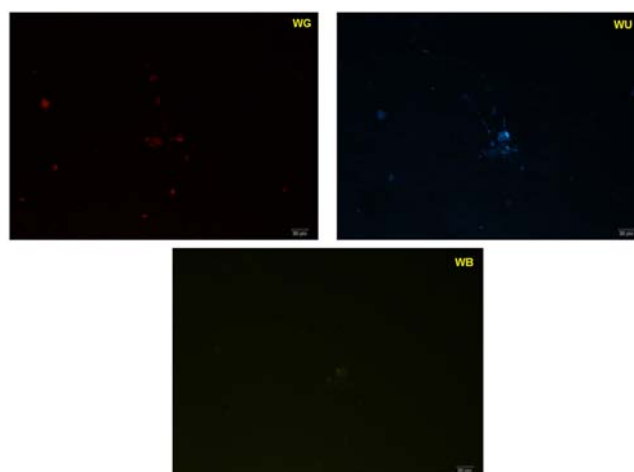

Figure 14. *P. brasiliensis* isolate T16B1 (400x), subjected to specificity test for hybridization with ITS Texas Red probe by FISH technique. No points of hybridized cells are present in the WG filter (Texas Red probe). The cell genetic material is visualized with DAPI (blue) under a fluorescence microscope in WU filter.

Figure 15. Specificity test of TSA-FISH technique (A, B, C and D) and FISH (E and F): slides with *H. capsulatum* subjected to hybridization with the ITS HRP probe for *P. brasiliensis* (B and D) (400x). The ITS Texas Red-probe for *P. lutzii* (E and F) (400x), and the cell genetic material was visualized with DAPI (blue) in WU filter (A, C and E), under a fluorescence microscope.

Figure 16. Specificity test of FISH (using ITS Texas Red-probe for *P. lutzii*) and TSA-FISH (using ITS HRP-probe for *P. brasiliensis*) technique against to *Aspergillus flavus; A. fumigatus* and *T. mentagrophytes* (400x). No points of hybridized cells are present in the WG filter (Texas Red probe) and in WB filter (HRP probe). The cell genetic material is visualized with DAPI (blue) in WU filter, on fluorescence microscope.

V. Artigo 2 – Environmental mapping of *Paracoccidioides* spp. by molecular detection in the Southeast, Midwest and North regions of Brazil.

Title:

Environmental mapping of *Paracoccidioides* spp. by molecular detection in the Southeast, Midwest and North regions of Brazil.

Authors

Thales Domingos Arantes[1], Raquel Cordeiro Theodoro[2], Marcus de Mello Teixeira[3], Sandra de Moraes Gimenez Bosco[1], Eduardo Bagagli[1]*

[1]- Departamento de Microbiologia e Imunologia, Instituto de Biociências de Botucatu, Universidade Estadual Paulista - UNESP

[2]- Departamento de Biologia Celular e Genética, Centro de Biociências, Universidade Federal do Rio Grande do Norte – UFRN

[3]- Departamento de Biologia Celular, Universidade de Brasília - UnB

*Corresponding author:

Dept of Microbiology and Immunology

Institute of Biosciences, UNESP

Distrito de Rubião Júnior, Botucatu, SP, Brazil

Zip Code: 18618-970

Phone: +551438800418

Email address: bagagli@ibb.unesp.br

Key-words: Paracoccidioidomycosis, Cyclonic Sampler, TSA-FISH, *Paracoccidioides* spp., Aerosol Samples.

Abstract

Soil is probably the habitat of the pathogenic fungus *Paracoccidioides* spp., due to its molecular detection in these samples, associated with the frequent infection of rural workers and its isolation from wild animals (*Dasypus novemcinctus*, *Cabassous centralis*). This work aimed to detect and differentiate the species complex *P. brasiliensis* and *P. lutzii* in the environment, by the techniques of Nested PCR and FISH, in aerosol and soil samples from armadillo's burrows, from endemic and not endemic areas of paracoccidioidomycosis in the Southeastern, Midwestern and Northern Brazil. Besides the environmental detection of *Paracoccidioides* spp. by molecular methods, the occurrence of armadillos infected with *P. lutzii* in the Center-Western Brazil, where this species is prevalent, had never been evaluated until the present time. We detected both species of *Paracoccidioides* in soil by PCR of ITS region and *in situ* hybridization. This updated data reflect the actual occurrence of the *Paracoccidioides* species in their niche, despite their absence/non detection, in seven armadillos evaluated in regions with high prevalence of PCM infection by *P. lutzii*. These results may indicate a possible ecological difference between *P. brasiliensis* and *P. lutzii* concerning their wild hosts.

Introduction

The study of biological and ecological aspects of *Paracoccidioides* spp. [1,2] and *P. lutzii* [3] has being developed by several research groups in recent years, in particular seeking to isolate and/or detect these pathogens in clinical and environmental samples, in order to obtain more concrete data on the ecological factors that determine their geographical distribution.

It is known that *Paracoccidioides* spp. has its habitat (physical and geographical distribution site) located in the soil, but its ecological niche (sum of all interactions of the microorganism with the biotic and abiotic factors of the environment) has not been properly determined, demanding more environmental studies for this fungus [4, 5, 6].

The studies of *Paracoccidioides* distribution have focused mainly in isolates from human infections and in a few isolates from wild and domestic animals, since there are rare isolates obtained directly from environmental samples. The few cases of direct pathogen isolation from environmental samples were obtained from soil, foliage, dog food and penguin feces, almost casually, with little or no repeatability [4, 7, 8, 9]. The isolation of *Paracoccidioides* spp. from armadillos represents an excellent source for environmental isolation and for mapping the areas of *Paracoccidioides* spp. occurrence, since this pathogen has been frequently isolated from this mammal host, which shows a very limited home range. The difficulties faced in these environmental studies, limits understanding about the ecology and real distribution of this genus in endemic and not endemic areas for PCM.

Since the majority of the isolates used in phylogenetic studies of the genus *Paracoccidioides* are from clinical specimens, which may have been influenced by factors such as the migration of host and latency period of this mycosis, it is very hard to specify the local of infection and the occurrence of each cryptic species in the

endemic and not endemic regions where the patients are from. The identification of risk areas, with the different species in can also contribute to a better understanding of the phylogeography of the *Paracoccidioides* genus and also indicate preventive methods [10, 11].

The environmental isolation or detection of *Paracoccidioides* (*P. brasiliensis* and *P. lutzii*) has a great importance to understanding the ecological aspects of these agents; however, little has been done, given its methodological difficulty. In this study, an environmental research and mapping of new areas of PCM occurrence was performed, by using a new approach based on a *in situ* hybridization technique with the species-specific DNA probes, as well as in the previously established molecular techniques, such as Nested PCR, in order to detect the pathogen in different environmental samples, such as soil, aerosol and wild animals (armadillo). In this study we continued previously ecological studies of our group, expanding the collection area, including the Southeastern, Midwestern and Northern Brazil in search for ecological biogeographic information of these fungi, thus generating a new map of the distribution of this important fungal pathogen.

Material and Methods

Selection of the study areas and environmental samples collection

For the collection of environmental samples, two states (Rondônia-RO and Goiás-GO), with a PCM incidence higher than São Paulo State [12, 13] were selected together with a third region with no apparent cases of PCM (Minas Gerais-MG). Samples were obtained by collecting aerosol and soil from armadillo's burrows and the animal specimen (*Dasypus novemcinctus*) in the same areas. During the study, approximately 12,000 kilometers were covered by road trip due to the need to take the

field materials for sample collection, since air travel would not be viable. The distribution of collection areas along the Brazilian map is showed in Figure 1, with demarcation of the assessed municipalities: Monte-Negro (RO), Santo Antônio de Goiás and Guarani de Goiás (GO) and Campina Verde (MG). The georeferenced sites of armadillo's burrows in the collection areas are listed on Table 1.

Collection of soil and armadillo's specimen's samples

The largest possible volume of soil samples was collected from the armadillo's burrows with the help of iron shovels avoiding destroy them. The soil was placed in 50 ml sterile universal bottles, sealed, identified and stored at room temperature until laboratory analysis. During the collection procedure, the collection instruments were decontaminated with 70% v/v ethanol solutions in order to avoid contamination of samples from one location to another [14, 15]. In addition to the samples collected during the field inspection, soil was also obtained from the Amazon region. This soil, called "Terra Preta", is an anthropogenic soil, rich in organic matter. These samples were kindly provided by the Center for Nuclear Energy Research Group in Agriculture (CENA) - USP, under the responsibility of PhD Professor Siu Mui Tsai.

For the capture of armadillos in the field, track traps were used and in some cases foresters and local hunters assisted us to identify areas with higher animal activity. These traps were placed next to the armadillo's burrows with recent animal activity. In addition to the provision of traps, active capture took place in the evening. The authorization of SISBIO-IBAMA was obtained to capture armadillo's in all Brazilian territory (IBAMA - 30585-1 and IBAMA - 37333-2), and the ethic committee from Biosciences Institute of Botucatu – UNESP (protocol number 528) also has approved these captures.

The animals were placed in tubes or drums with fresh and dry straw to better accommodate them during transportation and/or until euthanasia in the laboratory. The number of animals varied according to the season, as well as to the difficulties for finding specimens and transporting them to the laboratory. The euthanasia for all animals evaluated in this study was performed by subcutaneous administration of the anesthetic Zoletyl50 (0.2ml/kg/IV), followed by total cardiac puncture. The thorax was opened and the heart was clamped to ensure the animal's death. Spleen, liver and mesenteric lymph nodes were removed and placed in sterile plates and cleaned briefly in alcohol 70% v/v, and then in saline solution (0,9% w/v). Small fragments (1-2mm) of the organs were then plated in Mycosel® Agar culture medium with 50µg/ml gentamicin and incubated at 35°C during 45 days [10,15], after your evaluation, these plates were properly sterilized and discarded.

The blood obtained by heart puncture was centrifuged and the serum was separated into 1.5ml tubes for further immunological studies and/or detection of the agent in these samples. For the serum, some aliquots were shared with other research centers, as well as some organs fragments for the identification of other parasitic agents, genetic and anatomical studies, thus justifying the need for euthanasia of these animals.

Aerosol samples

Four air samplers by NIOSH, model Cyclone 251 BC, developed by the Centers for Diseases Control - CDC (Morgentown, USA) coupled to 4 vacuum pumps type 224-44XR Model SKC Universal Pumps were used [16]. The NIOSH sampler was placed next to the burrows and in the places where the armadillos had recently moved the soil in search for food. The vacuum pump has a rechargeable battery life up to 24 hours of operation at maximum flow, thereby facilitating the procedures for collection in remote

locations without any power sources. Sampling occurred in at least two seasons, dry/hot for Goiás and Minas Gerais and cold/rainy for Rondônia state, but in different regions for each period, given the difficulty of getting to the collection areas. Each aerosol collection was performed at a minimum period of 60 minutes with a flow rate of 3,500 ml/min. At least four samples were collected at each site. For soil samplings the minimum established number was 10 samples for burrows and trails at each site studied.

DNA extraction and PCR/Nested PCR reactions

Each soil sample was subjected to total DNA extraction in triplicate, using the commercial kit MOBio Extraction from Soil®. The total DNA obtained was resuspended in 100 µl of Nuclease free water and the DNA was quantified in NanoVue® equipment (GE Healthcare®). Fifty microliters of each one of the three replicates were mixed in a single 1.5 ml tube and concentrated to a final volume about 30µl in a concentrator (Eppendorf®) and then quantified again to confirm the new concentration of each sample. The aerosol samples were directly used for PCR, without any previous DNA extraction, by washing the tubes from cyclonic sampler with the PCR reaction mix.

The PCRs (Polymerase Chain Reaction) were carried out with ITS-4/5 primers for rRNA universal fungal region ITS1,5.8S,ITS2 (*Internal Transcribed spacer*) [17]. A Nested PCR was performed with the product of the first amplification using primers specific for the *Paracoccidioides* genus, annealing in the ITS-1 and ITS-2 regions, named PbITS-E (5'GAGCTTTGACGTCTGAGACC3') and Pb-ITST (5'GTATCCCTACCTGATCCGAG3') [15]. Both PCR mix were prepared using 12.5µl of Nuclease Free Water (Sigma®), 0.5µl of 0.2 mM dNTP mix, 5.0µl of 5X GC buffer, 2.5µl of 30% DMSO, 0.625µl of each primer at 20µM and 0.25µl of Taq

Phusion DNA Polymerase (ThermoFisher[®]) to 1000 units/μl for each reaction of 25.0μl of PCR for soil DNA sample and of 100μl for aerosol samples.

The thermal cycling conditions for the first PCR were the following: an initial cycle of denaturation at 98° C for 30 seconds and cycle denaturation at 98° C for 10 seconds, followed by an annealing step at 55° C for 45 seconds, and extension at 72° C for 45 seconds, these steps were repeated for 39 cycles, with a final extension at 72° C for 10 minutes. For aerosol samples the first step of denaturation was longer (5 minutes) than that one applied for soil DNA samples, in order to break the spores and other fungal structures in these samples, releasing the genetic material in the mix. For Nested PCR, the annealing step was adjusted for 58° C.

After the PCR and Nested PCR reactions, electrophoresis was held in agarose gel at 1.5% w/v. The bands around 450bp (*Paracoccidioides* spp.) or that best highlighted in the gel were cut out, purified with the commercial Kit (GE illustrates GFX PCR DNA and Gel Band Purification[®]), quantified and sent to the Laboratory for Molecular Diagnosis of the Department of Microbiology and Immunology (UNESP, Botucatu/SP-Brazil) for sequencing in an ABI 3500 DNA Analyzer (Applied Biosystems) equipment. For some samples, for which the gel bands showed low concentration of PCR product, a new amplification with the primers Pb-ITSE/T were performed (double PCR), followed by its purification and sequencing.

The obtained sequences were aligned to the reference sequences with the help of MEGA 6.0 program [18] and compared to online database (GenBank), to verify their identity, and phylogenetic comparison with other ITS deposited sequences from *P. brasiliensis* and *P. lutzii*.

Phylogenetic Analysis

The analysis was performed using the MEGA 6.0 program [18], in which all the sequences obtained were aligned with sequences of the same region (ITS1-5.8S-ITS2), deposited for the two species of the genus *Paracoccidioides*, *P. brasiliensis* and *P. lutzii* [19], whose access numbers are EU870314; EU870315; EU870316; AY631235; EU118561; EU118560; EU118548; EU118554; EU118553; EU118549; EU118546; EU118547; EU118545; EU118543; EU118542 for *P. brasiliensis* and EU870298; EU870303; EU870306; EU870309; EU870310; EU870311; AF092903; EU870299 for *P. lutzii*. Only the sequences, herein obtained from environmental samples, presenting ≥ 97% of similarity to *Paracoccidioides* spp. sequences in GenBank were considered for phylogenetic analysis. The alignment was carried out by ClustalW algorithm.

For the final analysis, in the MEGA 6.0 software a Neighbor Joining [20] tree was generated, based on the evolutionary model of Jukes-Cantor [21], according to the best model analysis, also performed in MEGA 6.0.

Detection and differentiation of *P. brasiliensis* complex and *P. lutzii* species by *in situ* hybridization in environmental aerosol samples

The probes used in this study were two commercially synthesized and labeled oligonucleotides, whose target is the rRNA region of *Paracoccidioides* spp., specifically the ITS-1,5.8S, ITS-2 region. For the probes designed ITS sequences from different *Paracoccidioides* isolates [11, 15, 19, 22, 23] were aligned in order to select conserved regions within species, so that the probes for the detection of *Paracoccidioides* spp., in order to select conserved regions within species, so that two probes were designed, one specific to *P. brasiliensis* and other to *P. lutzii*. A total of 60 sequences were evaluated for the probe design. After that, the probes were submitted to the similarity analysis at

NCBI site database by *blastn* tool to verify the specificity of each oligo sequence. Only the sequences that were 100% similar to *Paracoccidioides* spp. and not to other fungi were selected. Two probes were designed one for ITS of species complex *P. brasiliensis* and one probe for the species *P. lutzii* (Figure 2).

For labeling the probes with fluorophores, the Horseradish Peroxidase (HRP) was used for the *P. brasiliensis* probe, and the fluorophore Texas Red was used for *P. lutzii* probe, both linked in 5' end (Figure 2).

Aerosol samples were collected on the same regions that the soil samples. A total of 12 samples were collected in armadillo's burrows for *in situ* hybridization method. Overall, the samples with higher sediment were from drought areas (Goiás and Minas Gerais), where the dust generated in the burrows was easily aerosolized. For the state of Rondônia, which was facing an intense rainy season, the pellets were less visible to the naked eye.

The detection of *Paracoccidioides* spp. with DNA probes in the aerosol samples were first applied for samples with greater sediment volume in the tubes (stages) of the cyclone sampler. Twelve aerosol samples were selected: four from Goiás state, four from Minas Gerais and four from Rondônia. Regarding the Rondônia state, the samples presented pour pellets, due to weather conditions of high humidity in 2014. These samples were stored at room temperature for further fixation with 4% Paraformaldehyde plus 0.1 M Phosphate solution buffer in a volume of 1.5 ml for both stages of cyclone sampler.

The Cell fixation Solution (4% paraformaldehyde plus 0.1 M phosphate buffer) was used to kill the fungus and keep intact its structure and genetic material. A sequence of 50%, 80% and 100% ethanol solutions were used to remove the cell fixation solution and to dehydrate the cells so that they have the ability to absorb the

probes to be used in the hybridization step. After dehydration, 10 mL of pre-hybridization buffer [2,0 ml of ultra-pure H_2O; 4,0 ml of 40% Formamide; 1,8 ml of 5M NaCl; 200μl of 1M Tris (pH 7.5); 100μl of 1% SDS; 2 ml of 10% Buffer Blocking Agent], were added to the samples for stabilization and improvement of their permeability, by differences in osmotic pressure. After this first step, the cells were placed hybridized with probes which were prepared in a solution with hybridization buffer at a final concentration of 50ng/μl. After 16-17 hours of incubation at 42° C, the slides with fungal controls were washed with 50 ml of Washing Buffer [47,54 ml of ultra-pure H_2O; 460μl of 5M NaCl, 500μl of 0.5M EDTA, 500μl of 1% SDS and 1 ml of 1M Tris (pH 7.5)] for removal of non-specific binding probes. After washing, the slides were stabilized with 250 ml of TNT buffer [217,315 ml of ultra-pure H_2O; 25 ml of 1M Tris (pH 7.5); 7,5ml of 5M NaCl and 0,185 ml of Tween 20].

After equilibrating and washing the slides with TNT buffer, 30 μl of TSA solution of the commercial kit (TSA Plus PerkinElmer®) were added in each slide, for 30 minutes in a humid dark chamber at room temperature. Then, they were washed again, dried at room temperature, prepared with addition of DAPI and covered with a cover slip to be observed under a fluorescence microscope.

These slides were divided in two groups of four slides each: one was tested against *P. brasiliensis* probe and the other against *P. lutzii* probe. Two spare slides were used as controls during the hybridization phase for each of the two methods and probes used in this study.

Results

Animals evaluated in the collected areas

Seven armadillos were captured and evaluated, three from Goiás state, three from Rondônia and one from Minas Gerais. Information on gender, weight and cultivated organs fragments is listed on Table 2.

After 45 days of incubation, each plate was evaluated for fungal growth and/or colony similar to *Paracoccidioides* spp. by micro-morphological analysis. All the colonies presented morphological structures of bacteria, despite the addition of antibiotics to the culture medium. At the end of the 45 days incubation period, the plates were then considered negative for *Paracoccidioides* spp. growth and then properly sterilized and discarded.

Nested PCR and phylogenetic analysis of soil and aerosol samples

In all regions sampled in this study, a total of 44 soil samples were obtained, 12 of these were collected in armadillo's burrows in Goiás, 10 in Minas Gerais and 22 in the Rondônia state. For the aerosol samples from armadillo's burrows, 28 samples were collected in the three regions of work (10/GO, 10/MG and 08/RO), 16 of these samples were set aside for molecular detection by Nested PCR. The sampled burrows in the areas of Rondônia, Goiás and Minas Gerais states were mostly located in deforested areas of pastures and in some riparian forest sites. For evaluation of DNA obtained from Terra Preta, after observing the samples in electrophoresis gel, no amplicons were observed at 450bp, thus to Terra Preta, all DNA samples were considered negative for the detection of *Paracoccidioides* spp.

Around 52% and 94% of the soil and aerosol samples respectively were positive for *Paracoccidioides* spp. The *P. lutzii* was detected in all the positive soil samples in

Rondônia and Goiás. For aerosol samples, *P. brasiliensis* was detected in Goiás and *P. lutzii* in Minas Gerais (Table 3).

Phylogenetic analysis of the amplicons obtained from these samples was performed by comparing them (access numbers: KP636439-KP636474 [a total of 36 sequences]) with the sequences deposited on GenBank for *P. brasiliensis* and *P. lutzii*. Most samples (soil and aerosol) from Goiás, (9 of the 13 positive samples) were similar to *P. lutzii*, the other 4 samples showed similarity to the complex *P. brasiliensis* (Figure 4).

The nine sequences obtained from Minas Gerais, which are all from aerosol samples, clustered together with P. *lutzii* isolates in the phylogenetic tree (Figure 4). All the 14 positive samples obtained from Rondônia clustered together with *P. lutzii* isolates (Figure 4).

In the Figure 5 the areas circles sizes, reflect sampling effort in each collection site, which was based on displacement, number of soil samples, aerosol and animal samples taken in each area. The area of São Paulo state reflects previous studies [15].

Detection and differentiation of *P. brasiliensis* and *P. lutzii* in aerosol samples by *in situ* hybridization

The aerosol samples were collected in the same regions of soil samples. A total of 12 samples were collected in armadillo's burrows for *in situ* hybridization detection. Overall, the samples with higher sediment in the tubes were obtained in dry areas (Goiás and Minas Gerais) where the dust generated in the burrows was easily aerosolized when the soil of burrows was revolved. In the state of Rondônia, which was having an intense rainy season, the pellets were less visible to the naked eye.

A total of 48 slides were prepared for detection and differentiation of the two *Paracoccidioides* species with the specific probes herein designed. The remnant of each sample set was also used for making more 24 slides aiming to use most of the collected material.

From the 72 slides prepared, the probes detected *Paracoccidioides* spp. in 36 (50% of the slides). A total of 12 air samples collected in armadillo's burrows, eight or 66% were positive for *Paracoccidioides* spp. (Table 4). The images on Figures 6, 7 and 8 show fungal structures labeled with probes for hybridizations in aerosol samples. In Minas Gerais, the *in situ* hybridization was positive for *P. brasiliensis*, as well as in Northeastern of Goiás state. In the central regions of Goiás and Rondônia, the hybridization was positive for the labeling of both species (*P. brasiliensis* and *P. lutzii*).

In Figure 5, the sites in Brazilian territory where *P. brasiliensis* and *P. lutzii* were detected by Nested PCR and by *in situ* fluorescence hybridization are summarized.

Discussion

Soil and aerosols samples showed, as in previous studies of our group [15, 24], to be an excellent source for mapping endemic areas of paracoccidioidomycosis, since they are easy to obtain and handle in the laboratory. Both soil and aerosol samples were positive for the environmental detection of fungal DNA from *Paracoccidioides* genus. Aerosol samples were collected in smaller number compared with the soil samples, due to the methodological difficulties in the field and weather conditions of each sampled location. The soil collection methodology is faster and easier to perform than the aerosol, which requires more field effort to become representative. However, the aerosol sampling refers to the infection mechanism of rural workers dealing directly with the soil containing the fungus (mycelial particle aerosolization). The aerosol samples are

also methodologically simple to work in the laboratory, because DNA extraction is not required. Thus, the sampled areas, endemic or not endemic for PCM, may contain fungal cells with infectious potential for human and/or animal population, although the occurrence of infections can vary according to the type of land manipulation. It has been suggested that agricultural activities are the one that most favor the PCM infection in humans [25, 26].

Molecular detection in soil samples occurred in two of the sampled areas (RO and GO), but it was negative in MG. The soil molecular detection in Rondônia showed higher amplification rate as visualized in the gel, probably because of the weather conditions during the collection (rainy season), which according to work of Barrozo et al., 2009 and 2010, favor the maintenance of fungus in the soil, as well as dispersion, causing increased incidence of PCM. Positive samples were also obtained from northeast region of Goiás, considered an adverse local (warm and dry). However, the detection rate was lower than the ones in endemic areas of PCM. In most of the cases, the fungus was detected in locations whose air humidity and temperature were very similar those from the endemic areas of PCM.

In this study, new distribution nuances of *P. brasiliensis* and *P. lutzii,* including a remarkable resistance to adverse environmental conditions were revealed and the spatial distribution of the *Paracoccidioides* species seems to be larger than the one defined mostly based on clinical isolates [19, 22]. Both the growth and dispersion of this pathogen seems to be greatly influenced by the climate. While high levels of moisture increase fungal growth and maintenance in soil, dry weather makes it to disperse as aerosol easily. This explains the higher positive molecular detection of *Paracoccidioides* in soil samples than in aerosol samples from Rondônia, where the collection was carried out during the raining season.

In Rondônia and Goiás, the detection was positive mostly in deforested sites pastures and in some riparian forest sites. This is in concordance with the areas of greatest incidence of PCM cases in these states [12, 13], which are those of increased agricultural activity. Deforestation of preserved areas exposes the soil and alters its chemical conformation naturally or deliberately, which can favor the infection of rural workers or other people living in these areas, thus leading to emergent cases of PCM in these areas.

In this study, the detection of *Paracoccidioides* in aerosol samples in dry areas from the states of Goiás (*P. brasiliensis*) and Minas Gerais (*P. lutzii*), demonstrate the likely climate role in the dispersal of *Paracoccidioides* conidia, since the rainy period is short and the collection was carried out under a severe drought. The moisture induces growth and maintenance of the fungus in the soil but a brief period of drought and sun, dry the most superficial layer of the soil making the dispersion of aerosols (mycelia particles/conidia and other microorganisms) easy and intense. This could explain de negative detection of *Paracoccidioides* in aerosol samples from Rondônia, which faced one of the most severe rainy periods of the last few years. Therefore, different from soil samples, which directly demonstrate the presence of the fungus, aerosol samples also reflect the spread of the fungus in the environment and therefore its infective potential, being extremely important in the epidemiological study of *Paracoccidioides* spp.

Concerning the armadillo capture, considering the difficulty for capture and animal transportation, a reasonable number of animals for each sampled area were obtained. The area in Goiás where the armadillos were captured is not considered an endemic area of PCM, despite being in a state with a large number of cases. This could explain the negativity of *Paracoccidioides* isolation in evaluated animals. However, our environmental samples indicate the presence of *Paracoccidioides* DNA in these regions,

which expands may point out the relationship of environmental conditions and the possibility of infection and/or disease installation in these animals. In Minas Gerais, the collection area is also not considered an endemic area of PCM, and, the same way as for Goiás, the only animal evaluated in this state (MG) was negative for the isolation of *Paracoccidioides*. The isolation of *Paracoccidioides* spp. also was negative in the armadillos evaluated in Rondônia, but its detection was possible in soil samples, with high similarity to *P. lutzii*, which leads us to think that the relationship between *P. lutzii* and the armadillo *D. novemcinctus* may not follow the same pattern observed for *P. brasiliensis*.

Negativity isolation of *P. lutzii* in armadillo's in Minas Gerais, Rondônia and Goiás, and its detection in environmental samples may indicate a low sporulation of *Paracoccidioides* spp. this species in these regions. Obviously the number of animals may not reflect the large distribution and relationship of armadillos with the cryptic species of *Paracoccidioides* in the evaluated sites, however, if this pattern of *P. lutzii* absence in armadillo's continues in future searches, another possible hypothesis is that the close relationship with the species *D. novemcinctus* is a feature specific to the complex of *P. brasiliensis* species.

According to the phylogenetic analysis, there was a prevalence of sequences belonging to the *P. lutzii* species, in all areas evaluated, that reflect similar results to distribution from clinical isolates based in traditional methods of diagnosis observed in recent works in the same areas of our study [11, 12, 15].

Despite the absence of *P. lutzii* from armadillos, data from molecular analysis of other environmental samples (from soil and/or aerosols) obtained in this study may help to delineate the area of distribution of *Paracoccidioides* spp.

It is interesting to note that although the Central-Western Brazil presents a prevalence of PCM caused by *P. lutzii*, *P. brasiliensis* was also detected in these areas in this work and in a previous one [15]. This observation could indicate different patterns of sporulation depending on soil constitution and weather, so that the conidia production of *P. brasiliensis* and *P. lutzii* could be different in distinct areas, explaining the current distribution pattern of PCM caused by both species. Previous studies have already pointed out differences in sporulation (concerning conidia amount and morphology) among the different cryptic species [24, 27].

The ITS region is widely used in the characterization of fungal molecular biology, however, as evidenced by Teixeira et al [19], is not able to accurately distinguish cryptic species complex *P. brasiliensis*, but can clearly be used in this distinction between *P. brasiliensis* and *P. lutzii* [19]. Thus, our findings confirm, in fact, coexistence of the species *P. lutzii* and *P. brasiliensis* (S1 and PS2 species), although the power of infection in human patients seems to be different for different species, depending on the region.

This study presents important data regarding the eco-epidemiology of species of *Paracoccidioides* spp., as well as their actual distribution over Brazilian map. The results also pointed out for the possible differential wild host versus pathogen interaction of *Paracoccidioides* species, generating new hypotheses and new work fronts about this subject, which is still little studied by the scientific community. Thus, further studies based on interaction data with clinical and ecological aspects should be conducted in order to create a more reliable distribution map of this important systemic mycosis and its etiological agents.

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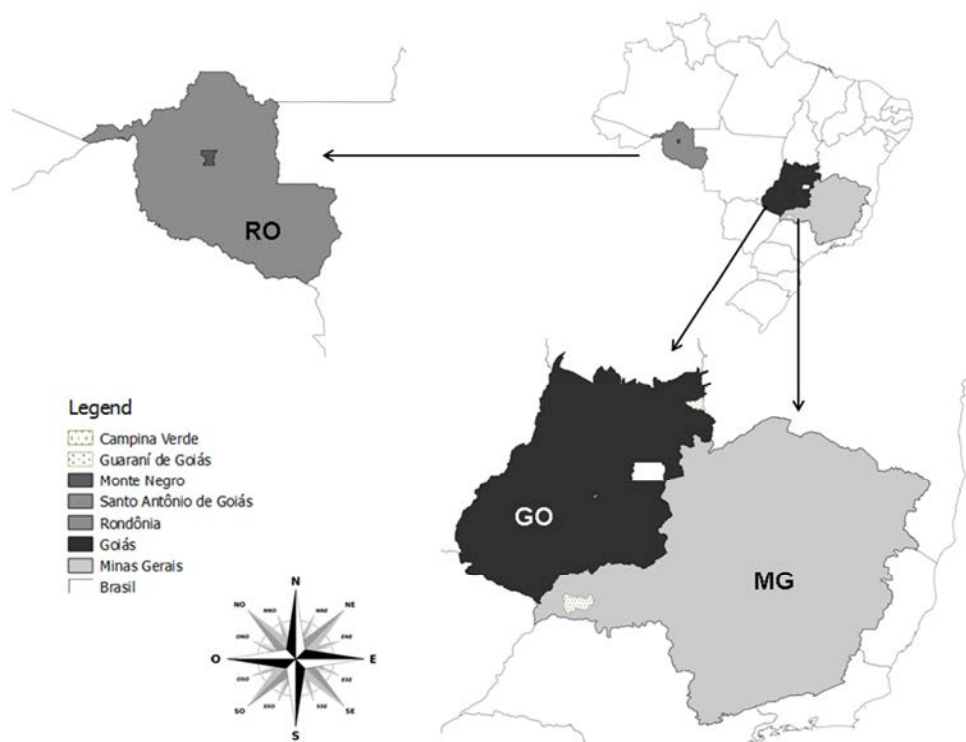
Annex

Figures

Figure 1. Collection areas of environmental samples (soil, aerosol and animals) in Brazil. The evaluated states (RO, MG and GO) are highlighted in different colors and the municipalities of sampling in different traces.
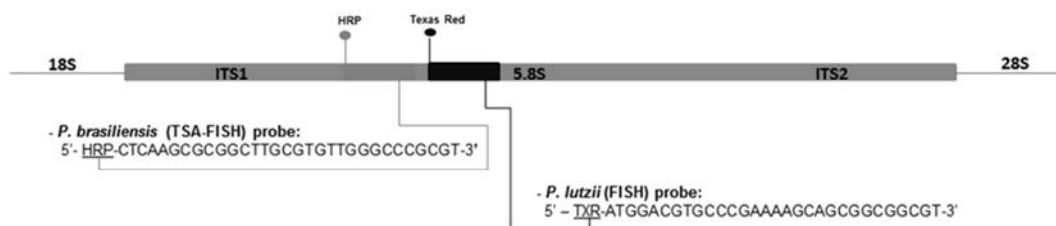

Figure 2. The ITS region with the location of oligo probes designed for the detection and differentiation of *P. brasiliensis* and *P. lutzii*, labeled with HRP and Texas Red, respectively.
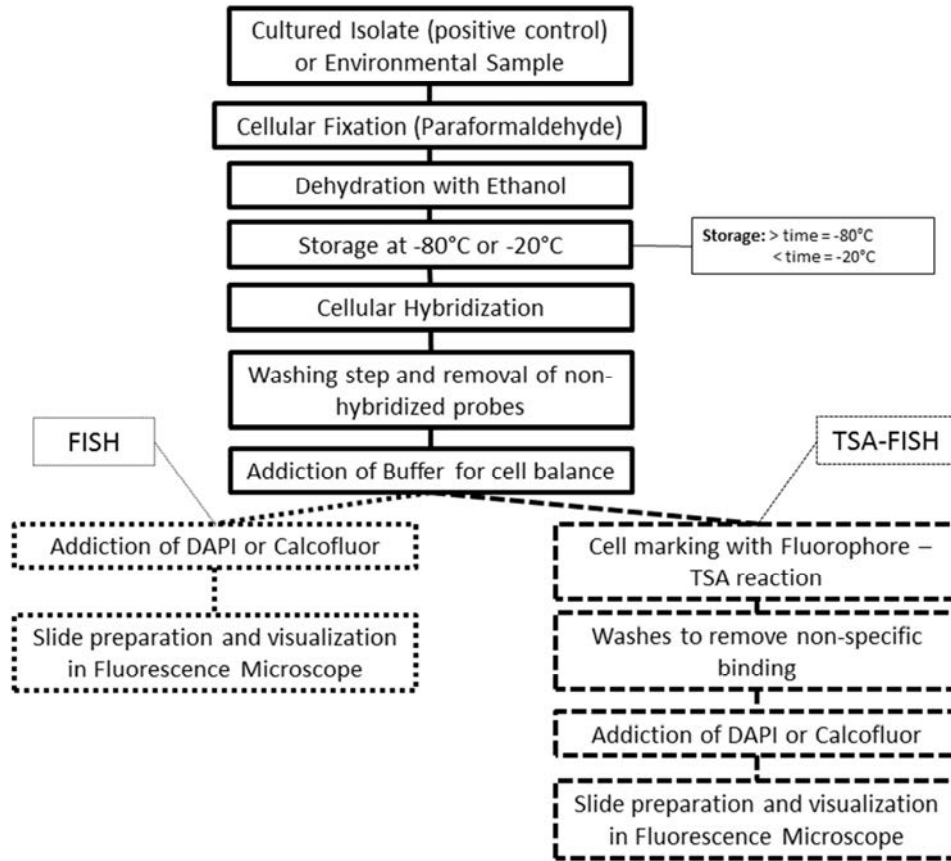
Figure 3. Work flow diagram for *Paracoccidioides* detection by the

techniques of FISH and TSA-FISH for positive controls, soil and aerosols samples.
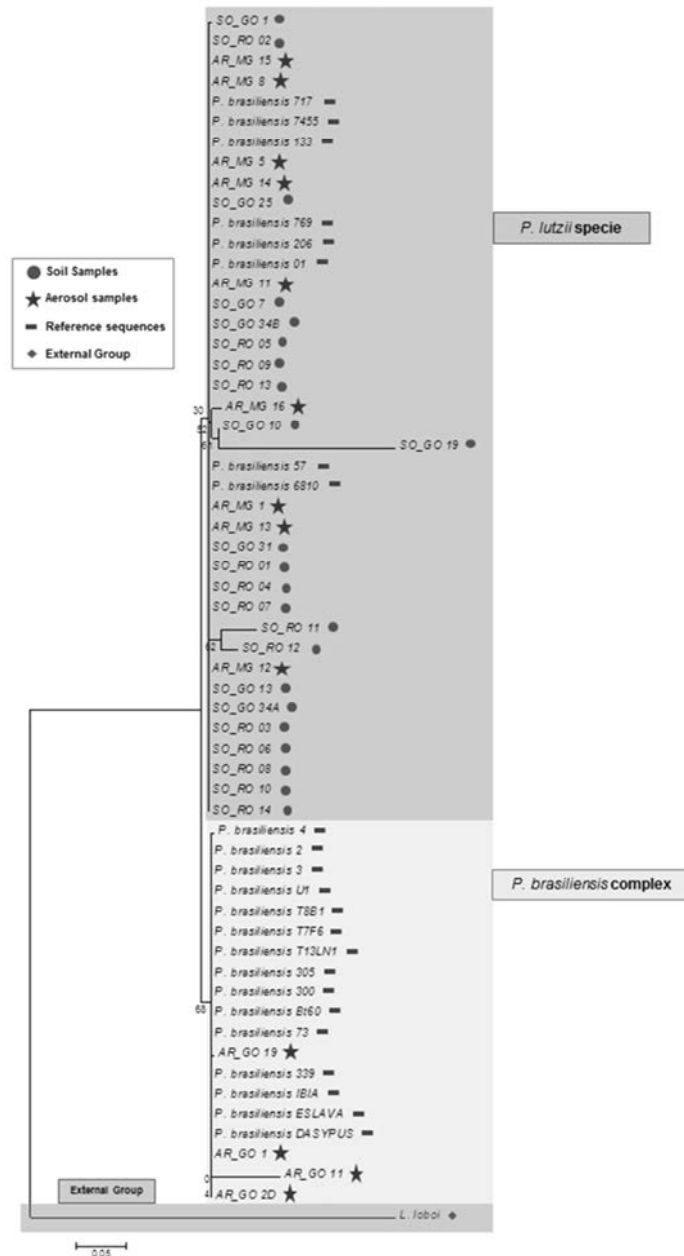
Figure 4. Molecular Phylogenetic Analysis by Neighbor Joining, using the Jukes-Cantor model parameters with range correction. Replication percentages on the tree are grouped in the bootstrap test (1000 replicates) and shown next to the branches. The analysis included 60 nucleotides sequences. All ambiguous positions were removed in each pair sequence. The total data set is presented at the end of 477 positions. The sequences related to environmental samples are identified by acronyms SO_GO (Soil of Goiás) and AR_GO (Aerosol Goiás), AR_MG (Aerosol Minas Gerais) and SO_RO (Soil of Rondônia).

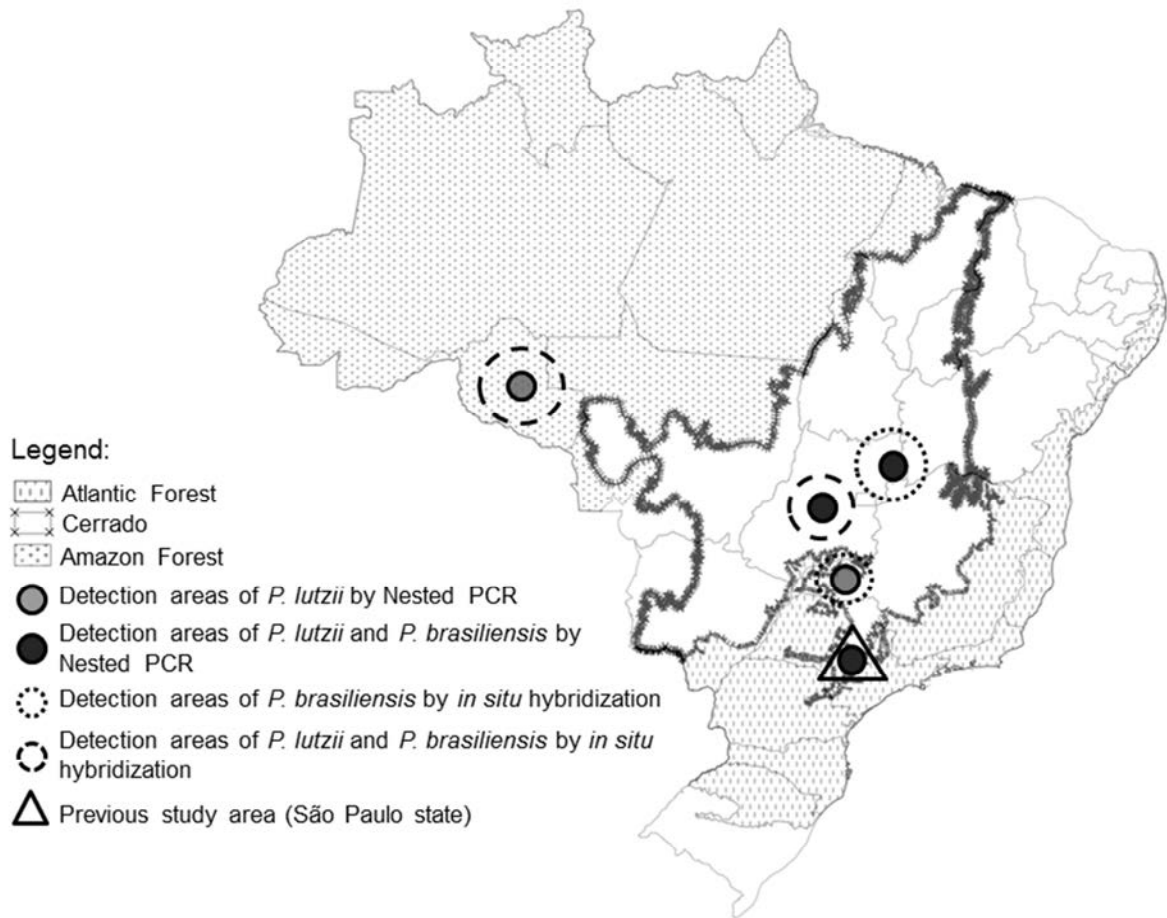**Figure 5.** Sites for the collection of environmental samples and the biomes of each region and sampling efforts are highlighted in the circles, respectively. The circles show the positive areas by Nested PCR and *in situ* hybridization methods.
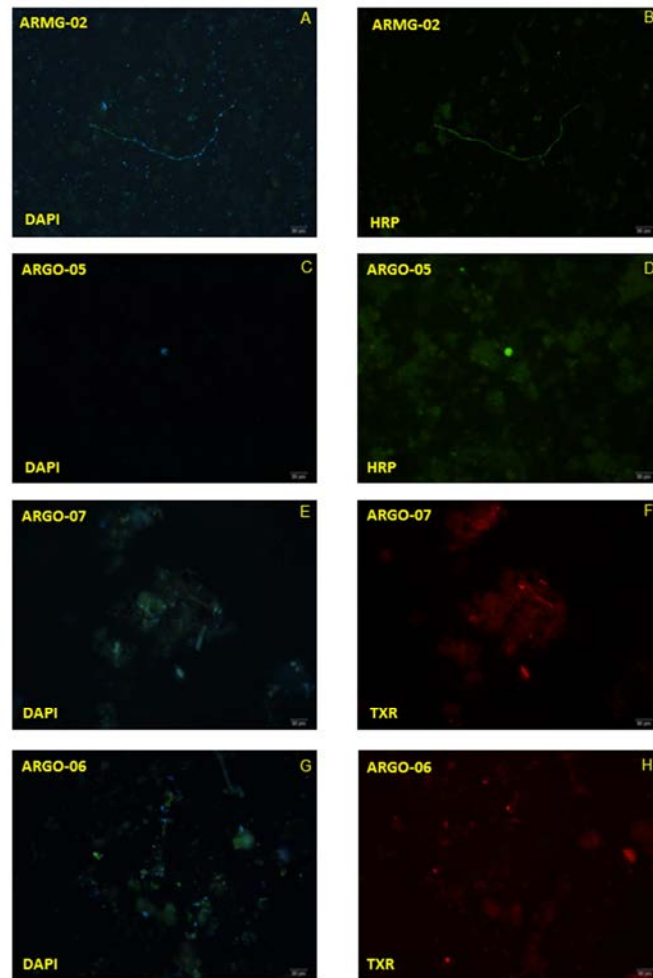
Figure 6. Fungal structures (400X) visualized by FISH and TSA-FISH techniques for aerosol samples from Minas Gerais and Goiás, using probes specific for *P. brasiliensis* (HRP – images B and D) and *P. lutzii* (Texas Red/TXR - images F and H), with genetic material labeled with DAPI (images A,C,E and G).

Figure 7. Fungal structures (400X) visualized by FISH and TSA-FISH techniques for aerosol samples from Rondônia, using probes specific for *P. brasiliensis* (HRP – images B, D and F) and *P. lutzii* (Texas Red/TXR – image H) with genetic material stained with DAPI (images A, C, E and G).

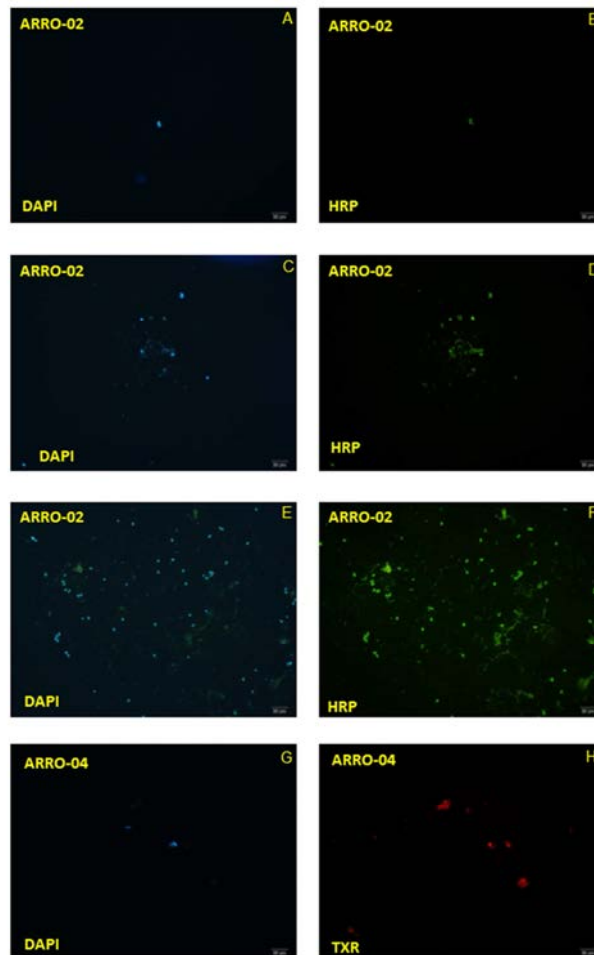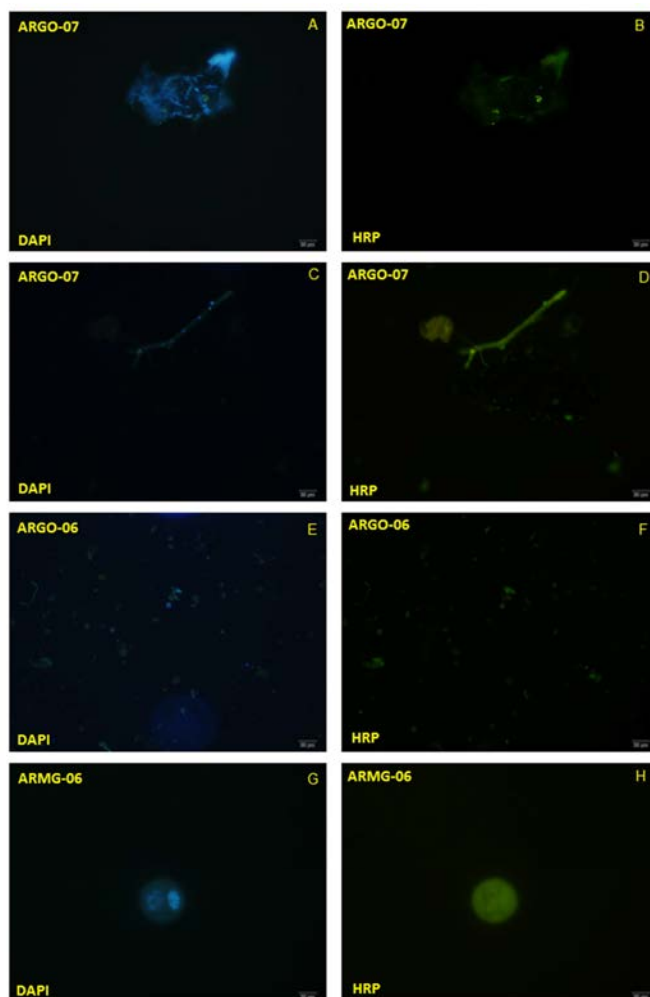**Figure 8.** *In situ* hybridization by TSA-FISH techniques for aerosol samples from Minas Gerais, using probes specific for *P. brasiliensis* (HRP – images B, D, F and H) with genetic material staining with DAPI (images A, C, E and G).

Tables

Table 1. Georeferenced sites for the field work (collecting soil samples, aerosols and armadillos).

ID	Description/Locality	Reference S	Reference WO
WP01	Trail/SAG	16°29'52,7''	49°17'25,0''
WP02	Burrow/SAG	16°29'53,4''	49°17'25,9''
WP03	Burrow/SAG	16°29'10,3''	49°17'44,1''
WP04	Burrow/SAG	16°29'09,7''	49°17'44,0''
WP05	Burrow/SAG	16°29'37,5''	49°17'27,3''
WP06	Burrow/GDG	13°56'53,9''	46°29'14,6''
WP07	Burrow/GDG	13°51'42,6''	46°31'05,2''
WP08	Burrow/GDG	13°51'43,1''	46°31'05,4''
WP09	Burrow/GDG	13°51'43,3''	46°31'05,2''
WP10	Burrow/GDG	13°51'43,9''	46°31'04,1''
WP11	Embrapa/SAG	16°30'18,1''	49°16'52,0''
WP12	House/Posse-GO	14°05'12,0''	46°21'47,2''
WP13	House/GDG	14°51'40,8''	46°48'10,5''
WP14	House/CV	19°39'48,9''	49°22'07,7''
WP15	Burrow/CV	19°40'00,6''	49°22'03,4''
WP16	Burrow/CV	19°39'59,8''	49°22'01,5''
WP17	Burrow/CV	19°39'59,1''	49°22'02,4''
WP18	Burrow/CV	19°39'59,5''	49°22'02,4''
WP19	Burrow/CV	19°39'54,1''	49°21'54,8''
WP20	Burrow/CV	19°39'57,9''	49°21'48,5''
WP21	Burrow/CV	19°39'57,4''	49°21'47,9''
WP22	Burrow/CV	19°39'57,5''	49°21'50,6''
WP23	Burrow/MN	10°18'46,3''	63°16'57,0''
WP24	Burrow/MN	10°18'56,2''	63°16'56,0''
WP25	Burrow/MN	10°17'23,3''	63°16'33,3''
WP26	Burrow/MN	10°17'19,5''	63°16'57,1''
WP27	Burrow/MN	10°14'41,6''	63°18'14,3''
WP28	Burrow/MN	10°14'05,9''	63°18'16,4''
WP29	Burrow/MN	10°09'09,6''	63°16'18,1''
WP30	Burrow/MN	10°09'09,1''	63°16'17,8''

Legend: Santo Antônio de Goiás (SAG); Guarani de Goiás (GDG); Campina Verde (CV) and Monte Negro (MN); S – South; WO- East-West.

Table 2. Armadillos (*D. novemcinctus*), and their respective organ fragments, evaluated in the states of Goiás, Minas Gerais and Rondônia.

ID Animal	Sex	Weight	Spleen (Plates)	Liver (Plates)	L. M. (Plates)	Total of Plates	Number of Fragments
AGO1	Male	5,5 kg	12	66	06	84	1.950
AGO2	Male	4,3kg	09	53	02	64	1.966
AGO3	Male	4,5kg	21	44	03	68	2.548
ARO1	Female	5,0kg	12	20	05	37	1.050
ARO2	Female	4,3kg	12	20	05	37	1.060
ARO3	Male	1,8kg	10	20	06	36	1.000
AMG1	Male	4,5kg	15	48	07	70	2.450
Total of Organ Fragments							**12.024**

Legend: AGO – Armadillo from Goiás; ARO – Armadillo from Rondônia; AMG – Armadillo from Minas Gerais; L.M. – mesenteric lymphnodes; Plates – Total number of culture plates assessed for each organ.

Table 3. Detection of *Paracoccidioides* spp. species by Nested PCR, both in soil and aerosol samples in Goiás, Minas Gerais and Rondônia.

Local	Detection from soil		Detection from aerosol		Total of positive samples
	P. brasiliensis	*P. lutzii*	*P. brasiliensis*	*P. lutzii*	
Goiás	0	9	4	0	13
Minas Gerais	0	0	0	9	9
Rondônia	0	14	0	0	14
Total	0	23	4	9	36

Table 4. Detection and differentiation of *Paracoccidioides* spp. in aerosol samples by *in situ* hybridization based in burrows with positivity.

ID Sample	Locality	HRP (*P. brasiliensis*)	Texas Red (*P. lutzii*)
ARGO-02	Goiás	(+)	(-)
ARGO-05	Goiás	(+)	(-)
ARGO-06	Goiás	(+)	(+)
ARGO-07	Goiás	(+)	(+)
ARMG-02	Minas Gerais	(+)	(-)
ARMG-06	Minas Gerais	(+)	(-)
ARRO-02	Rondônia	(+)	(-)
ARRO-04	Rondônia	(-)	(+)
Positive Burrows		07 (*87,5%)	03 (*37,5%)

Legend: HRP – Horseradish Peroxidase; ID – Identification; * Sensitivity rate front detection of *Paracoccidioides* spp.; Positive (+); Negative (-).