WILEY-BLACKWELL

Cladistics 26 (2010) 613–624

Cladistics

10.1111/j.1096-0031.2010.00307.x

The human fungal pathogen *Paracoccidioides brasiliensis* (Onygenales: Ajellomycetaceae) is a complex of two species: phylogenetic evidence from five mitochondrial markers

Catalina Salgado-Salazar[a,b,*†], Leandro R. Jones[c,d], Ángela Restrepo[a] and Juan G. McEwen[a,b]

[a]*Cell Biology and Immunogenetics Unit, Corporación Para Investigaciones Biológicas, Medellín, Colombia;* [b]*Instituto de Investigaciones Médicas, Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia;* [c]*Division of Molecular Biology, Estación de Fotobiología Playa Unión, CC 15 (9103), Rawson, Chubut, Argentina;* [d]*Nacional AIDS Reference Center, Microbiology Department, Faculty of Medicine, University of Buenos Aires, C1121ABG, Buenos Aires, Argentina*

Accepted 6 January 2010

Abstract

Paracoccidioides brasiliensis is the aetiological agent of paracoccidioidomycosis, the most important systemic mycosis in Latin America. In order to study the diversity of *P. brasiliensis* mitochondrial genes, to evaluate previous taxonomic proposals, and to explore the hypothesis that the previously described "divergent isolate" B30 (also called Pb01) could represent a new *P. brasiliensis* species, we undertook a molecular phylogenetic analysis based on five mitochondrial markers. Mitochondrial sequences of 59 *P. brasiliensis* isolates obtained from clinical and environmental samples, and the orthologous genes from outgroup species, are reported and analysed using parsimony and Bayesian methods. The combined data set comprised 2364 characters, of which 426 were informative. One of the studied strains presented a 376-nt insertion at the apocytochrome *b* (*cob*) gene. The corresponding sequence had a high similarity (79%) with an intron found in the *Neurospora crassa cob* gene. Interestingly, this intron is absent in the previously published sequence of the *P. brasiliensis* mitochondrial genome. Our trees were moderately congruent with the previous *P. brasiliensis* taxonomic proposals. Furthermore, we identified a new monophyletic group of strains within *P. brasiliensis*. Nevertheless, the phylogenetic species recognition (PSR) analyses described here suggested that these groups of strains could represent geographical variants rather than different *Paracoccidioides* cryptic species. In addition, and as previously proposed by other authors, these analyses supported the existence of a new specie of *Paracoccidioides*, which includes the previously described, divergent isolate B30/Pb01. This is the first report providing evidence, independent of nuclear markers, for the split of this important human pathogen into two species. We support the formal description of the B30/Pb01 as new specie.

Worldwide, systemic fungal diseases are a major cause of human death. *Paracoccidioides brasiliensis* (Splend.) F.P. Almeida 1930, a thermodimorphic haploid fungus belonging to the family Ajellomycetaceae (Leclerc et al., 1994; Untereiner et al., 2002; San-Blas et al., 2005; Nino-Vega et al., 2007), is the aetiological agent of paracoccidioidomycosis (PCM), one of the most prevalent systemic mycoses in Latin America (Brummer et al., 1993; Borges-Walmsley et al., 2002; Restrepo-Moreno, 2003; Restrepo and Tobon, 2005). This disease, affecting mainly adult males, progresses chronically by haematogenous and lymphatic dissemination. The infection, which is associated with extensive sequelae, initially causes lesions in the lungs and subsequently disseminates to other organs and tissues (Carvalho et al., 2005). At environmental temperatures (18–26 °C), *P. brasiliensis* grows as a mould producing

*Corresponding author:
E-mail address: salgadoc@umd.edu
†Present address: Department of Plant Sciences and Landscape Architecture, University of Maryland, College Park, MD 20742, USA.

conidia. Mycelial fragments and/or conidia produced by saprobe mycelia are thought to be the infectious propagules that, once inhaled into the lungs and due to the temperature of the human body, differentiate into the distinctive pathogenic yeast form (McEwen et al., 1987; Brummer et al., 1993; Borges-Walmsley et al., 2002). The yeast form can be parenterally transmitted (Zacharias et al., 1986), although there are no reports of transmission by this route in nature. PCM is endemic in an area extending from Mexico to Argentina and, to date, it is estimated that over 10 million individuals are infected with the fungus across its entire area of endemicity (Brummer et al., 1993), with 2% of this population developing the disease (McEwen et al., 1995). Countries such as Brazil have an annual incidence of 10–30 per million inhabitants, and the mean mortality rate is 1.4 per million per year (Restrepo-Moreno, 2003).

Due to the importance of this fungal disease, several studies have been conducted with the aims of developing new diagnostic methods (Nascimento et al., 2004; Correa et al., 2006), identifying drug targets (Albuquerque et al., 2004), understanding the pathogenesis of PCM (Molinari-Madlum et al., 1999; Carvalho et al., 2005; Kurokawa et al., 2005; Batista et al., 2007; Theodoro et al., 2008a,b; Teixeira et al., 2009), and studying molecular aspects of the pathogen (Morais et al., 2000; Goldman et al., 2003; Printzen and Stefan, 2003; Bonfim et al., 2006; Marques-da-Silva et al., 2006). Nevertheless, few analyses have attempted to untangle the evolutionary history of the fungus. Matute et al. (2006a), based on an analysis of several nuclear sequences, proposed the existence of three groups of species within *P. brasiliensis*: S1 (species 1), PS2 (phylogenetic species 2) and PS3 (phylogenetic species 3). That proposal was based on a phylogenetic species recognition (PSR) procedure (Taylor et al., 2000; as described by Dettman et al., 2003). No outgroups were included in the study of Matute et al. (2006a), and the proposed taxonomy was grounded on minimum span networks (Matute et al., 2006a). Carrero et al. (2008) also claimed to recover S1, PS2 and PS3 based on nuclear sequences; however, these groups were poly- or paraphyletic in some of their trees. In addition, some of the trees, including the one resulting from the total evidence analysis, were unrooted due to the lack of outgroup sequences (Carrero et al., 2008).

The heterogeneity in virulence and heat shock proteins observed for different *P. brasiliensis* isolates suggests that there could be significant disparities in the biological properties of different *P. brasiliensis* strains (Carvalho et al., 2005; Kurokawa et al., 2005; Batista et al., 2007; Theodoro et al., 2008a,b). Carrero et al. (2008) observed that the B30/Pb01 strain has significant genetic differences from other *P. brasiliensis* isolates. As suggested elsewhere (Carrero et al., 2008; Theodoro et al., 2008a,b; Teixeira et al., in press), we hypothesize that B30/Pb01 should be assigned to a new specie. Here we investigate this hypothesis using data from five mitochondrial markers of 59 *P. brasiliensis* isolates and outgroup species.

Materials and methods

Fungal isolates and DNA extraction

Fifty-nine fungal isolates were included in this study. These isolates are part of the *P. brasiliensis* living culture collection kept at the CIB's Cell Biology and Immunogenetics Unit (Corporacion para Investigaciones Biologicas-CIB, Medellin, Colombia). This collection includes strains isolated from different sources and locations, according to the geographical prevalence of PCM (restricted to Central and Southern America) (Table 1). The corresponding accession numbers of the sequences reported here for each isolate are also included in Table 1. Culture and storage of the isolates were performed according to Diez et al. (1999). Total DNA was extracted from yeast-form cultures using glass beads (Van Burik et al., 1998) or by maceration of frozen cells (Morais et al., 2000).

DNA amplification and sequencing

Five PCR primer pairs were used to amplify coding sequences in the mitochondrial genes *cob*, *cox3*, *rns*, *rnl* and *atp6* (Table 2). The *cob*, *cox3* and *rnl* primers were designed using OLIGO 4 (National Biosystems, Plymouth, MN, USA), with the complete mitochondrial genome of *P. brasiliensis* (AY955840) used as a template. The *rns* and *atp6* primers were taken from Untereiner et al. (2002) and Rekab et al. (2004), respectively. PCR (100 μL) contained 10 mM Tris–HCl pH 8.5, 50 mM KCl, 0.2 mM dNTPs, 0.25 mM of each primer, 4 U TucanTaq (Corpogen, Bogotá, Colombia) and approximately 100 ng DNA. The $MgCl_2$ concentrations were 2.5 mM for ATP6-F and ATP6-R; 2 mM for PBcox3-F, PBcox3-R, PBcob-F and PBcob-R; and 1.5 mM for primers PBrnl-F, PBrnl-R, RNS-F and RNS-R. PCR involved an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, required annealing temperature ("AT" in Table 2) for 45 s and extension 72 °C for 2 min. The final extension step consisted of 72 °C for 10 min. Nucleotide sequences were determined on both strands of PCR amplification products at the Macrogen sequencing facility using Big Dye terminator v. 3.1 sequencing kit on a ABI 3730XL DNA analyzer (Macrogen Inc., Seoul, Korea). Sequence data were examined with Sequence Navigator v. 1.0.1 (Applied Biosystems) and consensus sequences were compared against sequences from GenBank using Blast (Altschul et al., 1990).

Table 1
New sequences reported in this study

Strain	Geographical location*	Source	GenBank accession number				
			atp6	*cob*	*cox3*	*rnl*	*rns*
B2	Botucatu, Brazil	Armadillo	EF579391	EF579445	EF579340	EF579500	EF579558
B3	Botucatu, Brazil	Armadillo	–	–	–	EF579501	EF579559
B5	Botucatu, Brazil	Armadillo	EF579393	EF579447	EF579342	EF579503	EF579561
B6	Botucatu, Brazil	Armadillo	EF579394	EF579448	EF579343	EF579504	EF579562
B7	Botucatu, Brazil	Armadillo	EF579395	EF579449	–	EF579505	EF579563
B9	Botucatu, Brazil	Armadillo	EF579396	EF579450	EF579344	EF579506	EF579564
B10	Botucatu, Brazil	Armadillo	EF579397	EF579451	EF579345	EF579507	EF579565
B12	Minas Gerais, Brazil	Soil	EF579398	EF579452	EF579346	EF579508	EF579566
B13	Uberlandia, Brazil	Dog food	EF579399	–	EF579347	EF579509	–
B14	Botucatu, Brazil	PCM	EF579400	EF579453	EF579348	EF579510	EF579567
B15	Botucatu, Brazil	PCM	EF579401	EF579454	–	EF579511	EF579568
B16	Rio de Janeiro, Brazil	PCM	EF579402	EF579455	EF579349	EF579512	EF579569
B17	Sao Paulo, Brazil	Chronic PCM	EF579403	EF579456	EF579350	EF579513	EF579570
B18	Sao Paulo, Brazil	Chronic PCM	EF579404	EF579457	EF579351	EF579514	EF579571
B19	Goias, Brazil	Acute PCM	EF579405	EF579458	EF579352	EF579515	EF579572
B20	Sao Paulo, Brazil	Chronic PCM	EF579406	EF579459	EF579353	EF579516	EF579573
B21	Parana, Brazil	Acute PCM	EF579407	EF579460	EF579354	EF579517	EF579574
B23	Sao Paulo, Brazil	Chronic PCM	EF579408	EF579461	–	EF579518	EF579575
B25	Sao Paulo, Brazil	Chronic PCM	EF579409	EF579462	EF579355	EF579519	EF579576
B26	Sao Paulo, Brazil	Chronic PCM	EF579410	EF579463	–	EF579520	EF579577
B30	Goias, Brazil	Chronic PCM	EF579411	EF579464	EF579356	EF579521	EF579578
V1	Miranda, Venezuela	Soil	EF579433	EF579487	EF579379	EF579545	EF579601
V2	Caracas, Venezuela	Chronic PCM	EF579434	EF579488	–	EF579546	EF579602
V4	Barinas, Venezuela	Chronic PCM	EF579435	EF579489	EF579380	EF579547	EF579603
V5	Valencia, Venezuela	Unknown	EF579436	EF579490	EF579381	EF579548	EF579604
V6	Caracas, Venezuela	Acute PCM	EF579437	EF579491	EF579382	EF579549	EF579605
U1	Montevideo, Uruguay	Faeces (*Pygoscelis* sp.)	EF579432	EF579486	EF579378	EF579544	EF579600
PE1	Lima, Peru	Acute PCM	EF579431	EF579485	–	EF579543	EF579599
A1	Chaco, Argentina	Acute PCM	EF579383	EF579438	EF579332	EF579492	EF579550
A2	Chaco, Argentina	Chronic PCM	EF579384	EF579439	EF579333	EF579493	EF579551
A3	Chaco, Argentina	Chronic PCM	EF579385	EF579440	–	EF579494	EF579552
A4	Chaco, Argentina	Chronic PCM	–	–	EF579334	–	–
A5	Chaco, Argentina	Chronic PCM	EF579386	EF579441	EF579335	EF579495	EF579553
A6	Misiones, Argentina	Chronic PCM	EF579387	EF579442	EF579336	EF579496	EF579554
A7	Misiones, Argentina	Chronic PCM	EF579388	EF579443	EF579337	EF579497	EF579555
A8	Buenos Aires, Argentina	Acute PCM	EF579389	EF579444	EF579338	EF579498	EF579556
P1	Asuncion, Paraguay	Chronic PCM	EF579430	EF579484	EF579376	EF579542	EF579598
P2	Asuncion, Paraguay	Chronic PCM	–	–	EF579377	–	–
C1	Antioquia, Colombia	Chronic PCM	EF579413	EF579466	EF579357	EF579523	EF579580
C2	Antioquia, Colombia	Chronic PCM	EF579414	EF579467	EF579358	EF579524	EF579581
C3	Antioquia, Colombia	Chronic PCM	EF579415	EF579468	EF579359	EF579525	EF579582
C4	Antioquia, Colombia	Chronic PCM	EF579416	EF579469	EF579360	EF579526	EF579583
C5	Antioquia, Colombia	Chronic PCM	EF579417	EF579470	EF579361	EF579527	EF579584
C6	Antioquia, Colombia	Chronic PCM	EF579418	EF579471	EF579362	EF579528	EF579585
C7	Antioquia, Colombia	Chronic PCM	EF579419	EF579472	EF579363	EF579529	EF579586
C8	Antioquia, Colombia	Chronic PCM	–	–	–	EF579530	–
C9	Antioquia, Colombia	Chronic PCM	EF579420	EF579473	EF579364	EF579531	EF579587
C10	Cordoba, Colombia	Chronic PCM	EF579421	EF579474	EF579365	EF579532	EF579588
C11	Antioquia, Colombia	Chronic PCM	EF579422	EF579475	EF579366	EF579533	EF579589
C12	Antioquia, Colombia	Chronic PCM	EF579423	EF579476	EF579367	EF579534	EF579590
C13	Antioquia, Colombia	Chronic PCM	EF579424	EF579477	EF579368	EF579535	EF579591
C15	Cundinamarca, Colombia	Chronic PCM	–	EF579478	EF579369	–	EF579592
C16	Arauca, Colombia	Chronic PCM	EF579425	EF579479	EF579370	EF579536	EF579593
C17	Antioquia, Colombia	Chronic PCM	–	–	EF579371	EF579537	–
C18	Antioquia, Colombia	Chronic PCM	EF579426	EF579480	EF579372	EF579538	EF579594
C19	Caldas, Colombia	Armadillo	EF579427	EF579481	EF579373	EF579539	EF579595
C20	Antioquia, Colombia	Unknown	EF579428	EF579482	EF579374	EF579540	EF579596
C21	Caldas, Colombia	Armadillo	EF579429	EF579483	EF579375	EF579541	EF579597
BL1	La Paz, Bolivia	Unknown	EF579412	EF579465	–	EF579522	EF579579

*Geographical location of the primary isolate.

Table 2
Primers used in this study

Target DNA	Primer	Primer sequence and orientation	Primer position*	AT† (°C)	Size (bp)	Reference
ATP synthase F0 subunit 6	*atp6*	F: 5′ AGTCCWYTWGMYCAATTTGAAA 3′ R: 5′ CATGTGACCWSWTAAWATRTTWGC 3′	19–40 543–567	48	548	Rekab et al., 2004
Small subunit ribosomal RNA	*rns*	F: 5′ GCAGTGAGGAATATTGGTCAATGG 3′ R: 5′ CACTACTGGTTTCAGAAACGGTC 3′	315–338 834–856	64	541	Untereiner et al., 2002
Cytochrome *c* oxidase subunit 3	PBcox3	F: 5′ ATTTAGAGATATTATTTCTGAAAG 3′ R: 5′ ATAAAATATTCCTGATTCTAATC 3′	195–218 725–747	52	552	This study
Large subunit ribosomal RNA	PBrnl	F: 5′ AGCAGGTACAGAATTAAGATCTC 3′ R: 5′ TAACACTTGATAAAGGTTTTGTACT 3′	525–546 1110–1133	60	608	This study
Apocytochrome *b*	PBcob	F: 5′ CATATTATGCGTGATGTTAATAATG 3′ R: 5′ TGCAAATGGTAATCTATCATAATTAC 3′	1447–1469 1890–1915	62.3	468	This study

*Relative position in the complete mitochondrial sequence of *P. brasiliensis* published in GenBank (accession number AY955840).

†Annealing temperature.

Phylogenetic analyses

The species *Epidermophyton floccosum*, *Penicillium marneffei* and *Aspergillus niger* were chosen as outgroups because of the availability of their mitochondrial genomes (AY916130, NC_005256 and NC_007445, respectively), the similarity of their mitochondrial genomes with that of *P. brasiliensis* (Cardoso et al., 2007), and the relatedness of the Trichocomaceae and Ajellomycetaceae families (Barbosa et al., 2004; Nino-Vega et al., 2007). *Penicillium marneffei* was chosen to root the phylogenetic trees.

All the sequences were aligned with ClustalW v. 1.83 (Thompson et al., 1994), using default parameters. The Genetic Data Environment (GDE) software v. 2.2 (Smith et al., 1994) was used to inspect the primary homology assignments. Manual editing was performed only in the *cob* sequence alignment. This was necessary because the B30/Pb01 strain presented a disparate length in its mtDNA region (see following sections), a fact that resulted in conspicuously misaligned regions.

Parsimony analyses were performed with the program TNT (Goloboff et al., 2003a,b, 2008) following the criteria of Goloboff (1999) and Goloboff and Farris (2001): hitting the shortest-length trees many times and ensuring the consensus tree did not change on addition of RAS + TBR cycles (Table 4). Obtaining a stable consensus tree (one that is no longer changed by the addition of further RAS + TBR cycles) indicates that every possible topology that could be supported by the data is represented among the trees found (Goloboff, 1999; Goloboff and Farris, 2001). These analyses were performed with a small TNT script that is available on request from L.R.J. Gaps were treated as a fifth state in the phylogenetic analyses. Ambiguously supported branches were automatically collapsed during tree searches. Clade support was evaluated by symmetrical jackknifing (Goloboff et al., 2003a,b) using 1000 resampled matrices with 100 RAS + TBR, holding one tree while swapping.

Bayesian analyses were performed with the program MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001, 2005). MrBayes was run remotely on the computer cluster of the Research Information Technology group (Harvard Medical School). MrAIC.pl (Nylander, 2004) was executed to choose the appropriate model of nucleotide substitution. A general time-reversible (GTR) model of nucleotide substitution with gamma best explained the entire data set. Eight Markov chain–Monte Carlo (MCMC) chains were run for 10×10^7 generations, sampling every 1000 generations. These conditions ensured adequate mixing and convergence in all cases, giving effective sample sizes (EES) > 1000, as assessed by the program Tracer v. 1.4.1 (Rambaut and Drummond, 2007). For each analysis, posterior probabilities were calculated and reported on a 50% majority rule consensus tree of the post-burnin (burnin = 500) sample.

Genealogical concordance has been evaluated by checking whether a clade of interest is present in the majority of single-locus genealogies (Dettman et al., 2003). With this aim, Dettman et al. (2003) used the majority rule consensus tree of the single locus trees. A caveat of this procedure is that consensus methods present several limitations (Steel et al., 2000). Furthermore, Dettman et al. (2003) defined the ''genealogical nondiscordance'', which assures that the group is ''well'' supported by bootstrap (≥ 70) and posterior probabilities (≥ 0.95) in at least one of the single locus topologies and is not contradicted in any other single-locus genealogy at the same level of support. Thus, if the group is contradicted in the optimal topology/ies obtained from a data set but with low bootstrap/posterior probability, this information is not allowed to influence the outcome of the PSR procedure (Goloboff, 2007). Weakly supported groups are still supported by

the evidence and we see no reason to exclude them. Furthermore, the cut-offs of 70% in the resampled analyses or 0.95 in the Bayesian are not justified and are sometimes difficult to apply (for example, which branches of the tree have to be ''well'' supported?). Dettman et al. (2003) did not consider the case of polytomic branches (what rule is followed if the group of interest is included in a more inclusive polytomy; see Fig. S1a in Supporting Information). Thus in this study we used two indices that consider polytomic nodes and every optimal topology. To indicate whether the tree or trees derived from a given partition contain or contradict a group of interest, we used an index that we have called *single-locus phylogenetic support* (SLPS). The SLPS takes a value of 0 if the group of interest is compatible with the single-locus tree (the group would be supported in some resolutions of the tree but contradicted in others; Fig. S1a), and values of 1 and −1 if the group is supported (the group is monophyletic in the optimal single-gene tree; Fig. S1b) or contradicted (the group is para- or polyphyletic in the single-locus tree; Fig. S1c), respectively. Then, to summarize the contribution of all the single-locus trees, we used the *average phylogenetic support* (APS), which is defined as follows:

$$\mathrm{APS} = \frac{\sum_{i=1}^{n} \mathrm{SLPS}_i}{n}$$

where n is the number of loci and SLPS_i is the SLPS of locus i. The APS can take values between −1 (neither of the single locus trees contains the group of interest) and 1 (the group of interest is monophyletic in all the single locus trees). Thus APS constitutes a summary index of both ''genealogical concordance'' and ''genealogical non-discordance''.

Statistic analyses were performed with R software (http://www.R-project.org). The Ape package v. 1.8-4 (Paradis et al., 2004) was used for statistical analyses based on branch lengths (B, S, b_i/B, s_i/S).

Results

In order to examine the presence of pseudogenes and contaminant sequences in our data set, we performed comparisons with sequences in GenBank. All the sequences showed a high identity (*e*-value 0.0, max. ident. 100%) with the *P. brasiliensis* mitochondrial genome (AY955840.1). This strongly suggests the mitochondrial nature of our amplicons.

PCR amplification of the *cob* gene from the B30 strain resulted in a product of a larger size compared with the rest of the sequences. Sequence analyses revealed that this strain has a 376-nt insertion at the *cob* gene (positions 257–633 in EF579464). The corresponding sequence had similarity (79%) with an intron (intron 2) described in the *Neurospora crassa cob* gene (Burke et al., 1984; accession number K01881.1). Other studies have shown that the Pb01 strain (here B30) can have substitutions and/or deletions in several nuclear regions and genes, such as intein regions and conservative heat shock proteins (Theodoro et al., 2008a,b), suggesting that this strain might have diverged considerably from the remaining phylogenetic species.

Once aligned, the combined data set comprised 2364 characters, of which 426 were phylogenetically informative. First, in order to perform a preliminary exploration of the data set, we analysed these sequences using the program TNT and obtained a Bremer consensus tree (Bremer, 1990) using the whole data set. This total evidence (TE) tree was used to evaluate each single-locus data set and tree (Table 3). The informative sites were distributed more or less heterogeneously across the individual loci (20.6% in *atp6*, 12.6% in *cob*, 17.4% in *cox3*, 26% in *rnl* and 23.4% in *rns*; Table 3). Nevertheless, the values for consistency index (CI; Kluge and Farris, 1969) and retention index (RI; Farris, 1989), calculated on the total evidence tree for each marker (Table 3), did not depend on the number of informative sites ($P = 0.23$ and $P = 1$, respectively; Spearman's test; Fig. S2); consequently we can infer that all the genes used here had a similar impact on the total evidence tree.

Four floating taxa, all presenting an important proportion of missing data, were identified by manual inspection of the preliminary parsimony trees: strains A4, C8, P2 and B13 (hereafter, ''floating taxa'': 80% of missing data for strains A4, C8 and P2; 60% of missing data for strain B13). Keeping either any one of these taxa, or any combination of them, resulted in highly unresolved consensus trees. Incomplete taxa can be problematic because their presence can result in many equally parsimonious trees with a poorly resolved consensus tree (Philippe et al., 2004; Wiens, 2006), thus we decided to exclude A4, C8, P2 and B13 from the subsequent analyses. The B13 strain, which was assigned to PS2 by Matute et al. (2006a), could not be confidently assigned to any group based on our trees (not shown).

Table 3
Single-locus statistics (floating taxa A4, C8, P2 and B13 active)

Gene	*atp6*	*cob*	*cox3*	*rnl*	*rns*
Informative sites	88	54	74	110	100
CI	0.92	0.87	0.89	0.86	0.90
te-CI*	0.88	0.90	0.66	0.64	0.78
RI	0.87	0.92	0.88	0.87	0.95
te-RI†	0.84	0.85	0.51	0.54	0.87

*TE tree CI on the single gene data set.

†TE tree RI on the single gene data set.

Table 4
Parsimony search summary (floating taxa A4, C8, P2 and B13 inactive)

RAS*	10	20	30	40	50	60	70	80	90	100
Trees	100	110	120	130	140	143	143	143	143	143
Nodes†	18	18	18	18	18	18	18	18	18	18

*Number of random addition sequences.

†Number of nodes in the strict consensus tree.

When the floating taxa were inactive during tree searches, we found 143 trees of 1204 steps (Table 4). The corresponding strict consensus tree is shown in Fig. 1. The Bayesian analyses supported most of the groups found by the parsimony analyses (Fig. 2). Following Dettman et al. (2003), the groups recovered in a single analysis were dismissed.

Our results suggested that both PS2 and PS3, proposed by Matute et al. (2006a), are monophyletic. Nevertheless, in our analyses the strain C10 (Colombian isolate), which was previously included in the PS3 group, was not included in that group. The B18 strain (Brazilian isolate), which has been previously included in the S1 group, was clearly related to PS3 in our phylogenetic trees. Even though our data support the PS2 and PS3 groups, here they are redefined as indicated in Figs 2 and 3. The S1 group (Matute et al., 2006a) was not supported in any of our analyses: these isolates were broadly distributed across the tree and did not cluster

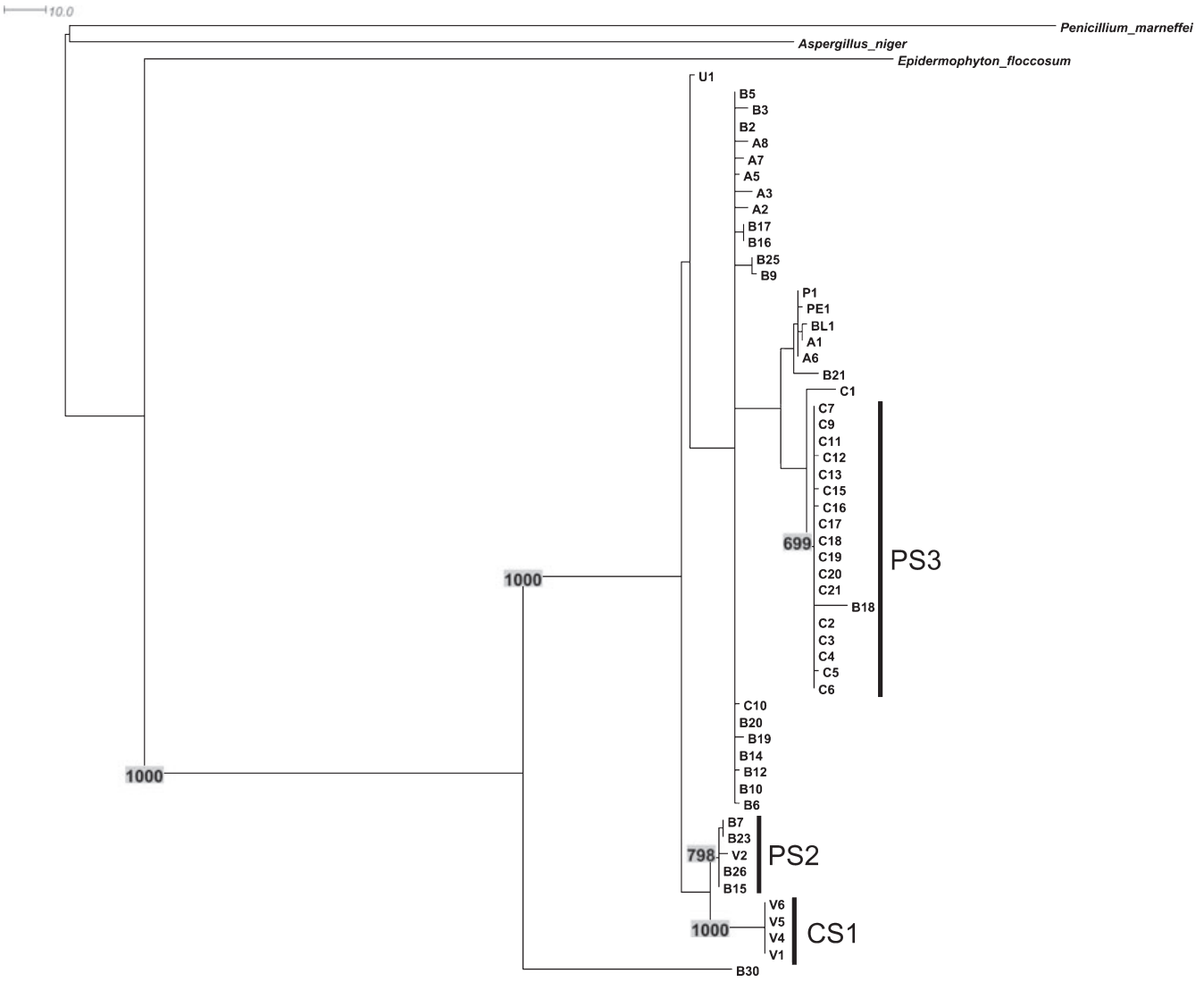

Fig. 1. Strict consensus tree of the 143 shortest trees (1204 steps; CI = 0.74, RI = 0.72) obtained for the combined dataset. The single locus sequence alignments (*atp6*, *cob*, *cox3*, *rnl* and *rns*) were concatenated and analyzed. Grey boxes on branches indicate the number of jackknife pseudo-replicates (out of 1000) in which the clades of interest were recovered. The clades corresponding to the groups described previously (PS2, PS3) and the new lineage identified here inside the *P. brasiliensis* complex (CS1) are indicated by vertical lines. Branch lengths are proportional to the number of substitutions (bar = 10 substitutions).

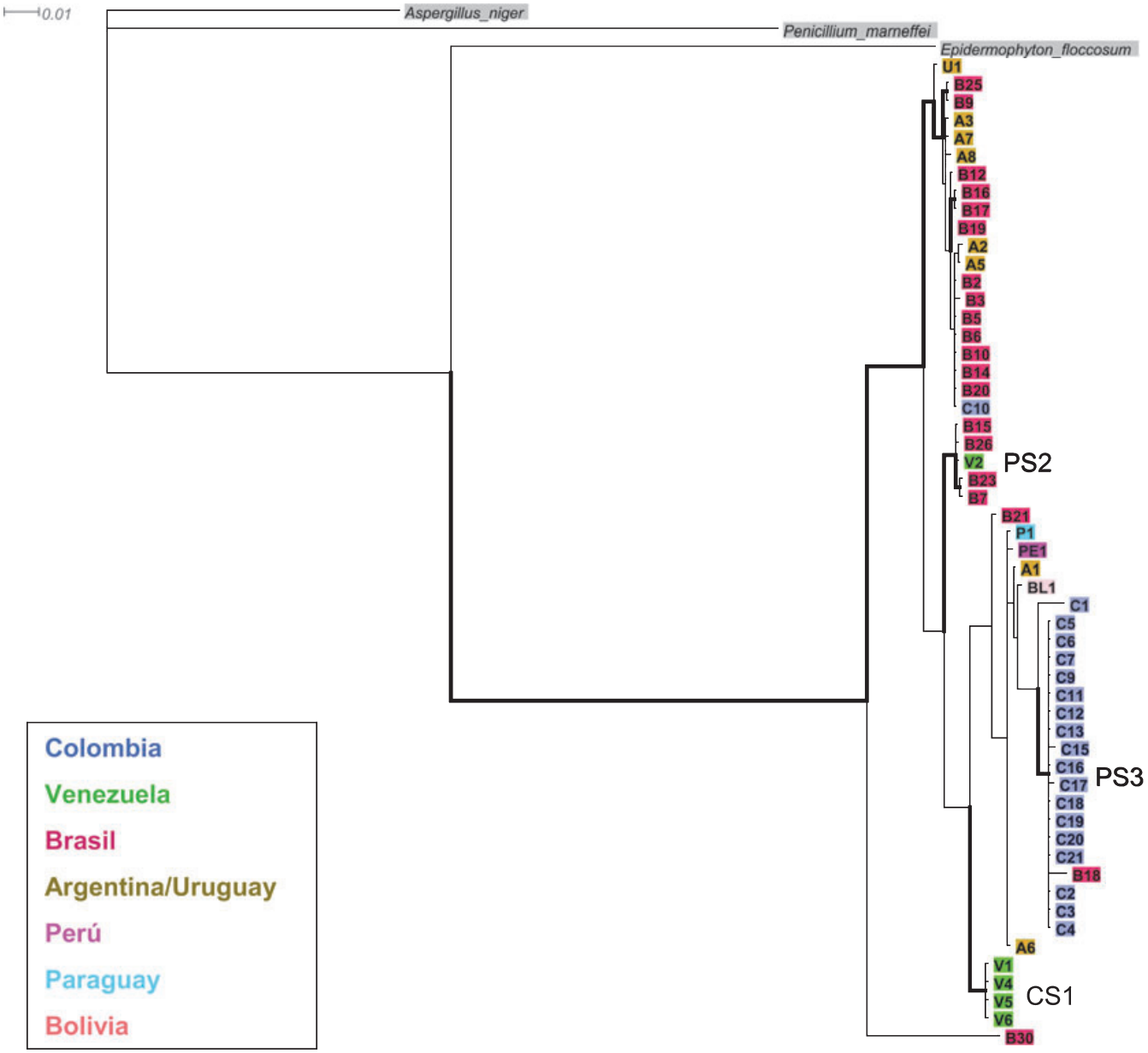

Fig. 2. Fifty per cent majority rule consensus of the posterior sample of trees obtained for the concatenated dataset. Thicker branches indicate posterior probabilities higher than 0.9. The clades corresponding to the groups described previously (PS2, PS3) and the new lineage identified here inside the *P. brasiliensis* complex (CS1) are indicated. The tree leaves are coloured according to the strain's geographic origin. Branch lengths are proportional to the number of substitutions per site (bar = 0.01).

with any one subgroup of strains proposed here, although there is a slight tendency of the isolates to group according to geographical origin (Figs 2 and 3). A conspicuous clade, which we named CS1 (cryptic subspecies 1), was present in both the parsimony and Bayesian trees (Figs 2 and 3).

It is evident from both parsimony and Bayesian analyses that B30/Pb01 is very different from the remaining strains studied here. These results confirm the observations of Carrero et al. (2008), Theodoro et al. (2008a,b) and Teixeira et al. (in press), who performed phylogenetic analyses with several nuclear encoding regions, showing significant genetic distance between B30/Pb01 and the remaining phylogenetic groups. They support the idea that this strain represents a different *Paracoccidioides* species. We used PSR analyses to investigate further the validity of the PS2, PS3, CS1 and B30/Pb01 clusters. As mentioned above, Matute et al. (2006a) used the PSR approach, described by Dettman et al. (2003), to discover S1, PS2 and PS3.

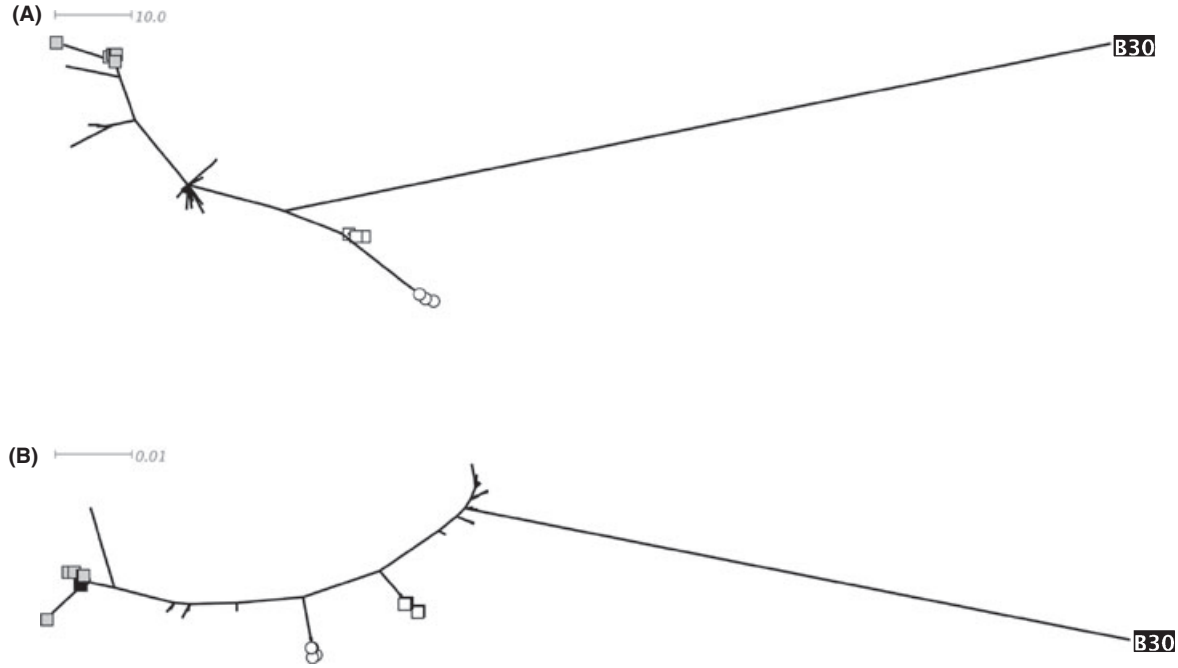

Fig. 3. Unrooted Parsimony (A) and Bayesian (B) trees displaying the distribution of genetic variation among different groups inside the ingroup (see also Table 5). The groups PS2, PS3 and CS1 are indicated by open squares, grey squares and open circles, respectively. The B30/Pb01 strain is shown in a black box.

Dettman et al. (2003) established that a group can be recognized as a phylogenetic species if "… it satisfied either of two criteria: (1) Genealogical concordance: the clade was present in the majority (3/4) of the single-locus genealogies … (2) Genealogical nondiscordance: the clade was well supported in at least one single-locus genealogy, as judged by both MP bootstrap proportions … and Bayesian posterior probabilities … and was not contradicted in any other single-locus genealogy at the same level of support". Furthermore, they applied two criteria to be satisfied in the combined data analyses: "… (1) Genetic differentiation … phylogenetic species had to be relatively distinct and well differentiated from other species. (2) Exhaustive subdivision: all individuals had to be placed within a phylogenetic species."

The "exhaustive subdivision" criterion was grounded on Dettman et al.'s (2003) particular objectives and therefore cannot be applied in the present study. Here we use APS as a summary index of both "genealogical concordance" and "genealogical non-discordance". Although convincing, the "genetic differentiation" criterion is quite difficult to apply, as Dettman et al. (2003) did not provide a procedure establishing how differentiated a group should be in order to be considered a phylogenetic species. One possibility is to plot unrooted trees and compare the branch lengths of the groups of interest in the context of the tree (if the branch corresponding to group is much larger than other branches along the tree, then the graph support this group as a separate entity). We used the following procedure as an approximation to Dettman et al.'s "genetic differentiation" criterion. Let s_i be the number of substitutions assigned to a test split i in the parsimony tree, and let b_i be the length of i in the Bayesian one. In addition, we define S and B as the median of the number of branch substitutions in the total evidence parsimony tree and the median of the branch lengths in the Bayesian tree, respectively. Then b_i/B and s_i/S give a measure of how much of the total variation in the data set is given by i. These statistics must be calculated from unrooted trees.

Table 5 and Fig. 3 compare PS2, PS3, CS1 and B30/Pb01 clades using the approaches described above. The B30/Pb01 group conforms well to all the PSR procedure requirements, forming a clade independent of the three currently recognized phylogenetic species. Although PS2, PS3 and CS1 do not comply with all of

Table 5
Phylogenetic species recognition (PSR) analyses

Clade	PS2*	PS3*	CS1	B30/Pb01
s_i/S	1	2	13	110
b_i/B	6.65	4.32	6.65	99.7
Jackknife support	789	699	1000	1000
Posterior probability	0.98	0.98	1.00	1.00
Average phylogenetic support (APS)	0.25	0.2	−0.2	1

*As hereafter redefined.

Dettman et al.'s criteria, these groups were monophyletic and supported in both the parsimony and Bayesian analyses. Although Matute et al. (2006a,b) proposed that those geographical groups were indeed phylogenetic species, other studies have shown that *P. brasiliensis* isolates clustered according to the geographical location of the primary isolate (Calcagno et al., 1998; Nino-Vega et al., 2000; Carrero et al., 2008), which suggests that the 'phylogenetic species' could, in reality, be geographical variants. When we analysed the geographical origin of our strains (Table 1) in light of our trees, it became apparent that some degree of regionalization was present (Fig. 2). Thus we think that PS2, PS3 and CS1 could represent geographical variants inside *P. brasiliensis*, as also suggested by Teixeira et al. (in press).

Discussion

The present report constitutes the first mitochondrial multi-gene-based phylogenetic study of the *P. brasiliensis* species complex. It demonstrates that the B30/Pb01 strain has an intron in the *cob* gene. Although other introns are present in the *Paracoccidioides* mitochondrial genome (Cardoso et al., 2007), this particular intron of the *cob* gene has not been reported previously for the *P. brasiliensis* species complex, but is known to occur in other fungi, which can contain one or several introns in their *cob* genes (Lang et al., 1985; Seraphin et al., 1987; Biswas et al., 2001). Others have shown that the presence of organellar introns (introns from groups 1 and 2) in fungal mitochondrial genomes can account for intra- and interspecies size variation in fungal mtDNA (Gray, 1989; Kouvelis et al., 2004; Tambor et al., 2006).

Others (Matute et al., 2006a) have suggested previously that *P. brasiliensis* could be a complex of cryptic species, and proposed the existence of at least three species: S1, PS2 and PS3. Our analyses suggested that PS2 and PS3, as well as a third group identified here, CS1, are monophyletic (Figs 2 and 3). Although present in our analyses, these groups do not conform to some of the PSR criteria used here (Fig. 3; Table 5), a fact that raised concerns about whether the groups correspond to different species, or should be considered geographical variants of *P. brasiliensis*, as also suggested by Teixeira et al. (in press). Within species groups, there is considerable controversy as to which variants represent distinct species and which represent population variation (Andrew et al., 2009). Furthermore, recognizing PS2, PS3 and CS1 within *P. brasiliensis* leaves the remaining specimens as a para- or polyphyletic assemblage. Such delimitation of groups is therefore questionable in terms of proper taxonomic actions, as well as the importance of this classification for medical impact regarding treatment and detection.

Based on evidence published elsewhere (Carrero et al., 2008; Theodoro et al., 2008a,b; Teixeira et al., in press), and the analyses performed here (Fig. 3; Table 5), we support that B30/Pb01, a strain widely used as a model isolate in most of the previous medical studies, should be assigned to a new specie within the *Paracoccidioides* genus. This indicates the need to update the current *Paracoccidioides* classification, and we expect that future studies—including more extensive B30/Pb01 taxon sampling, selection of target genes, and collection of enough data to obtain a robust estimate, as well as additional analyses of the biological properties of the members of this new species—will help to support this proposal. Following Teixeira et al. (in press), we recommend the formal description of B30/Pb01 as the new specie *Paracoccidioides lutzii*, a tribute to Adolpho Lutz, discoverer of *P. brasiliensis* in 1908.

Some authors have shown that important biological differences can be present between different *P. brasiliensis* isolates (Carvalho et al., 2005; Kurokawa et al., 2005; Batista et al., 2007; Theodoro et al., 2008a,b), and it has been shown that the virulence of *P. brasiliensis* isolates can be correlated with genetic variability (Molinari-Madlum et al., 1999). According to the data released by the Broad Institute Genome Initiative, the *P. brasiliensis* B30/Pb01 strain nuclear and mitochondrial genomes have differences in size and gene content regarding the other *P. brasiliensis* strains (B17 and B26) (http://www.broadinstitute.org). The implications of the existence of different *Paracoccidioides* species for prevention and treatment of PCM are crucial and remain to be studied.

The increasing interest in *P. brasiliensis* has resulted in significant advances in the study of the molecular and evolutionary biology of this fungus (Goldman et al., 2003; Bonfim et al., 2006; Marques-da-Silva et al., 2006; Matute et al., 2006b, 2007, 2008; Almeida et al., 2007; Batista et al., 2007; Theodoro et al., 2008a,b), and in the discovery of molecular markers useful for clinical and epidemiological studies (Morais et al., 2000). Well supported phylogenies will provide the basis for distinguishing ancestral versus derived character states, and can reveal the details regarding the evolutionary history of *P. brasiliensis*. We hope that the results described here and elsewhere (Matute et al., 2006a; Carrero et al., 2008; Theodoro et al., 2008a,b; Teixeira et al., in press) will constitute the beginning of much-needed studies for a better understanding of the natural history of this important human pathogen.

Acknowledgements

The authors wish to thank the Universidad de Antioquia-Proyecto Sostenibilidad 2005–2006 and the Fundación Para la Promoción de la Investigación y la

Tecnología-Banco de la República, Colombia, for financial support. The Corporacion para Investigaciones Biológicas and Colciencias provided travel funds to C.S.-S. The technical assistance of I. Torres, and D. R. Matute's advice in the initial part of this work, are gratefully acknowledged. Continuous support from CONICET (Argentina) to L.R.J. is deeply appreciated. The authors are grateful to Manuel Gomez Carrillo and Horacio Salomón for providing office space to C.S.-S. at the Centro Nacional de Referencia para el SIDA (AIDS National Reference Center; Faculty of Medicine, University of Buenos Aires, Argentina). During part of this work, L.R.J. was a Research Scholar at the Department of Microbiology and Molecular Genetics, New England Primate Research Center, Harvard Medical School. L.R.J. thanks the Research Information Technology group (Harvard Medical School) for granting access to the Orchestra cluster facility. The authors are in debt to A. Cresce for manuscript proofreading, and wish to thank the reviewers for their constructive criticism and comments.

References

Albuquerque, P., Kyaw, C.M., Saldanha, R.R., Brigido, M.M., Felipe, M.S., Silva-Pereira, I., 2004. *Pbhyd1* and *Pbhyd2*: two mycelium-specific hydrophobin genes from the dimorphic fungus *Paracoccidioides brasiliensis*. Fungal Genet. Biol. 41, 510–520.

Almeida, A.J., Matute, D.R., Carmona, J.A., Martins, M., Torres, I., McEwen, J.G., Restrepo, A., Leao, C., Ludovico, P., Rodrigues, F., 2007. Genome size and ploidy of *Paracoccidioides brasiliensis* reveals a haploid DNA content: flow cytometry and GP43 sequence analysis. Fungal Genet. Biol. 44, 25–31.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Andrew, M., Peever, T.I., Pryor, B.M., 2009. An expanded multilocus phylogeny does not resolve morphological species within the small-spored *Alternaria* species complex. Mycologia 101, 95–109.

Barbosa, M.S., Cunha Passos, D.A., Felipe, M.S., Jesuíno, R.S., Pereira, M., de Almeida Soares, C.M., 2004. The glyceraldehyde-3-phosphate dehydrogenase homologue is differentially regulated in phases of *Paracoccidioides brasiliensis*: molecular and phylogenetic analysis. Fungal Genet. Biol. 41, 667–675.

Batista, W.L., Barros, T.F., Goldman, G.H., Morais, F.V., Puccia, R., 2007. Identification of transcription elements in the 5′ intergenic region shared by LON and MDJ1 heat shock genes from the human pathogen *Paracoccidioides brasiliensis*. Evaluation of gene expression. Fungal Genet. Biol. 44, 47–56.

Biswas, S.K., Yokoyama, K., Nishimura, K., Miyaji, M., 2001. Molecular phylogenetics of the genus *Rhodotorula* and related basidiomycetous yeast inferred from the mitochondrial cytochrome *b* gene. Int. J. Syst. Evol. Microbiol. 51, 1191–1199.

Bonfim, S.M., Cruz, A.H., Jesuino, R.S., Ulhoa, C.J., Molinari-Madlum, E.E., Soares, C.M., Pereira, M., 2006. Chitinase from *Paracoccidioides brasiliensis*: molecular cloning, structural, phylogenetic, expression and activity analysis. FEMS Immunol. Med. Microbiol. 46, 269–283.

Borges-Walmsley, M.I., Chen, D., Shu, X., Walmsley, A.R., 2002. The pathobiology of *Paracoccidioides brasiliensis*. Trends Microbiol. 10, 80–87.

Bremer, K., 1990. Combinable component consensus. Cladistics 6, 369–372.

Brummer, E., Castañeda, E., Restrepo, A., 1993. Paracoccidioidomycosis: an update. Clin. Microbiol. Rev. 6, 89–117.

Burke, J.M., Breitenberger, C., Heckman, J.E., Dujon, B., Rajbhandary, U.L., 1984. Cytochrome *b* gene of *Neurospora crassa* mitochondria. Partial sequence and location of introns at sites different from those in *Saccharomyces cerevisiae* and *Aspergillus nidulans*. J. Biol. Chem. 259, 504–511.

Calcagno, A.M., Nino-Vega, G., San-Blas, F., San-Blas, G., 1998. Geographic discrimination of *Paracoccidioides brasiliensis* strains by randomly amplified polymorphic DNA analysis. J. Clin. Microbiol. 36, 1733–1736.

Cardoso, M.A., Tambor, J.H., Nobrega, F.G., 2007. The mitochondrial genome from the thermal dimorphic fungus *Paracoccidioides brasiliensis*. Yeast 24, 607–616.

Carrero, L.L., Niño-Vega, G., Teixeira, M.M., Carvalho, M.J.A., Soares, C.M.A., Pereira, M., Jesuino, R.S.A., McEwen, J.G., Mendosa, L., Taylor, J.W., Felipe, M.S., San-Blas, G., 2008. New *Paracoccidioides brasiliensis* isolate reveals unexpected genomic variability in this human pathogen. Fungal Genet. Biol. 45, 605–612.

Carvalho, K.C., Ganiko, L., Batista, W., Morais, F.V., Marques, E.R., Goldman, G.H., Franco, M.F., Puccia, R., 2005. Virulence of *Paracoccidioides brasiliensis* and *gp43* expression in isolates bearing known PbGP43 genotype. Microbes Infect. 7, 55–65.

Correa, M.M., Bedoya, A.M., Guerrero, M.P., Mendez, J., Restrepo, A., McEwen, J.G., 2006. Diagnosis of paracoccidioidomycosis by a dot blot assay using a recombinant *Paracoccidioides brasiliensis* p27 protein. Mycoses 50, 41–47.

Dettman, J.R., Jacobson, D.J., Taylor, J.W., 2003. A multilocus genealogical approach to phylogenetic species recognition in the model eukaryote *Neurospora*. Evolution 57, 2703–2720.

Diez, S., Garcia, E.A., Pino, P.A., Botero, S., Corredor, G.G., Peralta, L.A., Castaño, J.H., Restrepo, A., McEwen, J.G., 1999. PCR with *Paracoccidioides brasiliensis* specific primers: potential use in ecological studies. Rev. Inst. Med. Trop. Sao Paulo 41, 351–358.

Farris, J.S., 1989. The retention index and homoplasy excess. Syst. Biol. 38, 406–407.

Goldman, G.H., dos Reis Marques, E., Duarte Ribeiro, D.C., de Souza Bernardes, L.A., Quiapin, A.C., Vitorelli, P.M., Savoldi, M., Semighini, C.P., de Oliveira, R.C., Nunes, L.R., Travassos, L.R., Puccia, R., Batista, W.L., Ferreira, L.E., Moreira, J.C., Bogossian, A.P., Tekaia, F., Nobrega, M.P., Nobrega, F.G., Goldman, M.H., 2003. Expressed sequence tag analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: identification of putative homologues of *Candida albicans* virulence and pathogenicity genes. Eukaryot. Cell 2, 34–48.

Goloboff, P.A., 1999. Analyzing large data sets in reasonable times: solutions for composite optima. Cladistics 15, 415–428.

Goloboff, P., 2007. Calculating SPR distances between trees. Cladistics 23, 1–7.

Goloboff, P.A., Farris, J.S., 2001. Methods for quick consensus estimations. Cladistics 17, S26–S34.

Goloboff, P., Farris, J.S., Nixon, K.. 2003a. T.N.T.: Tree Analysis Using New Technology. Program and documentation available from the authors and at http://www.zmuc.dk/public/phylogeny.

Goloboff, P.A., Farris, J.S., Källersjo, M., Oxelman, B., Ramirez, M., Szumik, C., 2003b. Improvements to resampling measures of group support. Cladistics 19, 324–332.

Goloboff, P.A., Farris, J.S., Nixon, K.C., 2008. TNT, a free program for phylogenetic analysis. Cladistics 24, 774–786.

Gray, M.W., 1989. Origin and evolution of mitochondrial DNA. Annu. Rev. Cell Biol. 5, 25–50.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Huelsenbeck, J.P., Ronquist, F., 2005. Bayesian analysis of molecular evolution using MrBayes. In: Nielsen, R. (Ed.), Statistical Methods in Molecular Evolution. Springer, New York, pp. 183–226.

Kluge, A., Farris, J., 1969. Quantitative phyletics and the evolution of Anurans. Syst. Zool. 18, 1–32.

Kouvelis, V.N., Ghikas, D.V., Typas, M.A., 2004. The analysis of the complete mitochondrial genome of *Lecanicillium muscarium* (synonym *Verticillium lecanii*) suggests a minimum common gene organization in mtDNAs of Sordariomycetes: phylogenetic implications. Fungal Genet. Biol. 41, 930–940.

Kurokawa, C.S., Lopes, C.R., Sugizaki, M.F., Kuramae, E.E., Franco, M.F., Peraçoli, M.T., 2005. Virulence profile of ten *Paracoccidioides brasiliensis* isolates: association with morphologic and genetic patterns. Rev. Inst. Med. Trop. Sao Paulo 47, 257–262.

Lang, B.F., Ahne, F., Bonen, L., 1985. The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe*. The cytochrome b gene has an intron closely related to the first two introns in the *Saccharomyces cerevisiae* cox1 gene. J. Mol. Biol. 184, 353–366.

Leclerc, M.C., Philippe, H., Gueho, E., 1994. Phylogeny of dermatophytes and dimorphic fungi based on large subunit ribosomal RNA sequence comparisons. J. Med. Vet. Mycol. 32, 331–341.

Marques-da-Silva, S.H., Colombo, A.L., Blotta, M.H., Queiroz-Telles, F., Balthazar, A.B., Lopes, J.D., de Camargo, Z.P., 2006. Diagnosis of Paracoccidioidomycosis by detection of antigen and antibody in bronchoalveolar lavage fluids. Clin. Vaccine Immunol. 13, 1363–1366.

Matute, D.R., McEwen, J.G., Puccia, R., Montes, B.A., San-Blas, G., Bagagli, E., Rauscher, J.T., Restrepo, A., Morais, F., Nino-Vega, G., Taylor, J.W., 2006a. Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies. Mol. Biol. Evol. 23, 65–73.

Matute, D.R., Sepulveda, V.E., Quesada, L.M., Goldman, G.H., Taylor, J.W., Restrepo, A., McEwen, J.G., 2006b. Microsatellite analysis of three phylogenetic species of *Paracoccidioides brasiliensis*. J. Clin. Microbiol. 44, 2153–2157.

Matute, D.R., Torres, I.P., Salgado-Salazar, C., Restrepo, A., McEwen, J.G., 2007. Background selection at the chitin synthase II (chs2) locus in *Paracoccidioides brasiliensis* species complex. Fungal Genet. Biol. 44, 357–367.

Matute, D.R., Quesada-Ocampo, L.M., Rauscher, J.T., McEwen, J.G., 2008. Evidence for positive selection in putative virulence factors within the *Paracoccidioides brasiliensis* species complex. PLoS Negl. Trop. Dis. 2, e296.

McEwen, J.G., Bedoya, V., Patiño, M.M., Salazar, M.E., Restrepo, A., 1987. Experimental murine paracoccidioidomycosis induced by the inhalation of conidia. J. Med. Vet. Mycol. 25, 165–175.

McEwen, J.G., Garcia, A.M., Ortiz, B.L., Botero, S., Restrepo, A., 1995. In search of the natural habitat of *Paracoccidioides brasiliensis*. Arch. Med. Res. 26, 305–306.

Molinari-Madlum, E.E., Felipe, M.S., Soares, C.M., 1999. Virulence of *Paracoccidioides brasiliensis* isolates can be correlated to groups defined by random amplified polymorphic DNA analysis. Med. Mycol. 37, 269–276.

Morais, F.V., Barros, T.F., Fukada, M.K., Cisalpino, P.S., Puccia, R., 2000. Polymorphism in the gene coding for the immunodominant antigen gp43 from the pathogenic fungus *Paracoccidioides brasiliensis*. J. Clin. Microbiol. 38, 3960–3986.

Nascimento, E., Martinez, R., Lopes, A.R., de Souza, L.A., Barco, C.P., Goldman, M.H., Taylor, J.W., McEwen, J.G., Nobrega, M.P., Nobrega, F.G., Goldman, G.H., 2004. Detection and selection of microsatellites in the genome of *Paracoccidioides brasiliensis* as molecular markers for clinical and epidemiological studies. J. Clin. Microbiol. 42, 5007–5014.

Nino-Vega, G., Calcagno, A.M., San-Blas, G., San-Blas, F., Gooday, G.W., Gow, N.A.R., 2000. RFLP analysis reveals marked geographical isolation between strains of *Paracoccidioides brasiliensis*. Med. Mycol. 38, 437–441.

Nino-Vega, G., Perez-Silva, C., San-Blas, G., 2007. The actin gene in *P. brasiliensis*: organization, expression and phylogenetic analyses. Mycol. Res. 111, 363–369.

Nylander, J.A., 2004. MrAIC.pl. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Paradis, E., Claude, J., Strimmer, K., 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20, 289–290.

Philippe, H., Snell, E., Bapteste, E., Lopez, P., Holland, P., Casane, D., 2004. Phylogenomics of eukaryotes: impact of missing data on large alignments. Mol. Biol. Evol. 21, 1740–1752.

Printzen, C.E., Stefan, E., 2003. Local population subdivision in the lichen *Cladonia subcervicornis* as revealed by mitochondrial cytochrome oxidase subunit 1 intron sequences. Mycologia 95, 399–406.

Rambaut, A., Drummond, A.J., 2007. Tracer v1.4. Available from http://beast.bio.ed.ac.uk/Tracer.

Rekab, D., del Sorbo, G., Reggio, C., Zoina, A., Firrao, G., 2004. Polymorphisms in nuclear rDNA and mtDNA reveal the polyphyletic nature of isolates of *Phomopsis* pathogenic to sunflower and a tight monophyletic clade of defined geographic origin. Mycol. Res. 108, 393–402.

Restrepo, A., Tobon, A., 2005. *Paracoccidioides brasiliensis*. In: Mandell, G.L., Bennett, J.E., Dollin, R. (Eds.), Principles and Practice of Infectious Diseases. Elsevier, Philadelphia, pp. 3062–3068.

Restrepo-Moreno, A., 2003. Paracoccidioidomycosis. In: Dismukes, W.E., Pappas, P.G., Sobel, J.D. (Eds.), Clinical Mycology. Oxford University Press, Oxford, pp. 328–345.

San-Blas, G., Prieto, A., Bernabé, M., Ahrazem, O., Moreno, B., Leal, J.A., 2005. alpha-galf I → 6-alpha-mannopyranoside side chains in *Paracoccidioides brasiliensis* cell wall are shared by members of the Onygenales, but not by galactomannans of other fungal genera. Med. Mycol. 43, 153–159.

Seraphin, B., Boulet, A., Simon, M., Faye, G., 1987. Construction of a yeast strain devoid of mitochondrial introns and its use to screen nuclear genes involved in mitochondrial splicing. Proc. Natl Acad. Sci. USA 84, 6810–6814.

Smith, S.W., Overbeek, R., Woese, C.R., Gilbert, W., Gillevet, P.M., 1994. The genetic data environment an expandable GUI for multiple sequence analysis. Comput. Appl. Biosci. 10, 671–675.

Steel, M., Dress, A.W.M., Bocker, S., 2000. Simple but fundamental limitations on supertree and consensus tree methods. Syst. Biol. 49, 363–368.

Tambor, J.H.M., Guedes, R.F., Nobrega, M.P., Nobrega, F.G., 2006. The complete DNA sequence of the mitochondrial genome of the dermatophyte fungus *Epidermophyton floccosum*. Curr. Genet. 49, 302–308.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., Fisher, M.C., 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet. Biol. 31, 21–32.

Teixeira, M.M., Theodoro, R.C., de Carvalho, M.J.A., Fernandes, L., Paes, H.C., Hahn, R.C., Mendoza, L., Bagagli, E., San-Blas, G., Felipe, M.S.S., 2009. Phylogenetic analysis reveals a high level of speciation in the *Paracoccidioides* genus. Mol. Phylogenet. Evol. 52, 273–283.

Theodoro, R.C., Bagagli, E., Oliveira, C., 2008a. Phylogenetic analysis of PRP8 intein in *Paracoccidioides brasiliensis* species complex. Fungal Genet. Biol. 45, 1284–1291.

Theodoro, R.C., de Moraes Gimenes, B.S., Araújo, J.P., Candeias, J.M., da Graça Macoris, S.A., Trinca, L.A., Bagagli, E., 2008b. Dimorphism, thermal tolerance, virulence and heat shock protein 70 transcription in different isolates of *Paracoccidioides brasiliensis*. Mycopathologia 165, 355–365.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap

penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Untereiner, W.A., Scott, J.A., Naveau, F.A., Currah, R.S., Bachewich, J., 2002. Phylogeny of *Ajellomyces*, *Polytolypa* and *Spiromastix* (Onygenaceae) inferred from the analysis of rDNA sequence and non-molecular data. Stud. Mycol. 47, 25–35.
Van Burik, J.A., Schreckhise, R.W., White, T.C., Bowden, R.A., Myerson, D., 1998. Comparison of six extraction techniques for isolation of DNA from filamentous fungi. Med. Mycol. 36, 299–303.
Wiens, J.J., 2006. Missing data and the design of phylogenetic analyses. J. Biomed. Inform. 39, 34–42.
Zacharias, D., Ueda, A., Moscardi-Bacchi, M., Franco, M., San-Blas, G., 1986. A comparative histopathological, immunological, and biochemical study of experimental intravenous paracoccidioidomycosis induced in mice by three *Paracoccidioides brasiliensis* isolates. J. Med. Vet. Mycol. 24, 445–454.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Genealogical concordance between three data sets (a–c) with respect to a group of interest: {1, 2, 3, 4}. The strict consensus trees obtained from each data set are depicted in panels (a–c), with the group of interest indicated in red. In (a) the group is neither supported nor contradicted, as in different resolutions of the polytomy the group could be supported [e.g. ((1,2,3,4)(5,6))] or contradicted [e.g. ((1,2),(3,(4,(5,6))))]; thus SLPS takes a value of 0. In (b) the group is monophyletic, so it is supported and the SLPS index takes a value of 1; in (c) the group is polyphyletic, so SLPS takes a value of −1. The individual supports are summarized by the APS index (d), which correspond to the average of the SLPSs.

Figure S2. Fit to the single locus (CI, RI) or total evidence (te-CI, te-RI) trees of the data set analysed here. The *x* axis indicates the number of parsimony-informative sites.

Appendix S1. Alignment of mitochondrial sequences (total evidence analysis) ''salgado.et.al.data set.nex''

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.