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New *Paracoccidioides brasiliensis* isolate reveals unexpected genomic variability in this human pathogen

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Abstract

By means of genealogical concordance phylogenetic species recognition (GCPSR), we have investigated coding and non-coding regions from various genes and the ITS sequences of 7 new and 14 known isolates of *Paracoccidioides brasiliensis*. Such isolates grouped within the three phylogenetic groups recently reported in the genus *Paracoccidioides*, with one single exception, i.e., Pb01, a strain that has been the subject of intense molecular studies for many years. This isolate clearly separates from all other *Paracoccidioides* isolates in phylogenetic analyses and greatly increases the genomic variation known in this genus.

Keywords: *Paracoccidioides brasiliensis*; Molecular phylogeny; Molecular variability; Genealogical concordance

1. Introduction

Molecular genetics and cladistic analyses provide a method to recognize species under the evolutionary species concept, using an operational method known as phylogenetic species recognition (PSR). One type of PSR, genealogical concordance PSR (GCPSR), is capable of detecting genetically isolated groups by comparing gene trees from a number of loci (Taylor et al., 2000). GCPSR has been useful in the recent split of *Coccidioides immitis*, a seemingly uniform species by phenotypic criteria, into two species: *C. immitis* (Californian isolates) and *C. posadasii* (non-Californian isolates) (Fisher et al., 2002; Taylor and Fisher, 2003), and the recognition of eight distinct phylogenetic species within the genus *Histoplasma* where, previously, three phenotypic varieties had been acknowledged (Kasuga et al., 2003).

The dimorphic fungus *Paracoccidioides brasiliensis*, causative agent of paracoccidioidomycosis, has been placed in the Ascomycota, order Onygenales, and family Onygenaceae, using large subunit rDNA and other gene sequences (Leclerc et al., 1994; Niño-Vega et al., 2007; review in San-Blas et al., 2002) and using the fine structure of cell wall galactomannans (San-Blas et al., 2005). This family comprises several fungal dimorphic pathogens that are involved in health-threatening systemic mycoses (San-Blas et al., 2002).

Previous RAPD analyses (Calcagno et al., 1998; Molinari-Madlum et al., 1999; Soares et al., 1995), RFLP (Niño-Vega et al., 2000), and partial sequences of some

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genes (Hebeler-Barbosa et al., 2003; Morais et al., 2000) from several *P. brasiliensis* isolates, revealed genetic variability and clusters correlated with geography (Calcagno et al., 1998; Niño-Vega et al., 2000) or virulence (Carvalho et al., 2005; Molinari-Madlum et al., 1999). Based on phylogenetic analysis of 65 *P. brasiliensis* isolates, Matute et al. (2006) proposed three distinct phylogenetic species within *P. brasiliensis*: S1 (a paraphyletic group containing 38 isolates of Argentinian, Brazilian, Peruvian and Venezuelan origins, plus an isolate from an Antarctic penguin), PS2 (a monophyletic group of six isolates, five of them of Brazilian origin, and one Venezuelan) and PS3 (a monophyletic group with 21 Colombian isolates). In this paper we present a phylogenetic study of coding and non-coding regions from various genes and the ITS region in 21 isolates of *P. brasiliensis*, seven of them new. This study showed that the majority of the sequences used by Matute et al. (2006) and those used in this study, grouped within two (S1 and PS3) of the three clades proposed by these investigators. However, one *P. brasiliensis* isolate, Pb01, was placed at the base of, and quite distant from, the three species reported by Matute et al. (2006). Interestingly, the transcriptome of this isolate has been the subject of intense research (Felipe et al., 2005). Pb01 raises two important questions; why is this genotype so unique among clinical isolates? and is there more genetic variation among *P. brasiliensis* to be found in nature?

2. Materials and methods

2.1. Organisms

Table 1 lists the *P. brasiliensis* isolates used in this study. They were cultured at 23 °C on PYG medium (peptone, 5 g; yeast extract, 5 g; and glucose, 15 g; per liter of distilled water, pH 7.0) for three days before DNA was extracted as previously described (Calcagno et al., 1998).

2.2. DNA analyses

Twenty-one loci from 14 *P. brasiliensis* genes were studied (Table 2).

2.3. PCR reactions

Sequences from coding regions in the following *P. brasiliensis* genes: *CHS4*, *Actin*, *ODC*, *URA3*, *CHS2*, *HSP70*, *FKS1*, *Hydrophobin*, *Kex1*, *Catalase A*, *Catalase P*, *Formamidase*, *Glyoxalase* (GenBank Accession No. in Table 3) and ITS region (NCBI Accession No. in Table 4), were selected to design primers that amplified the non-codifying regions in PCR reactions. Conditions were as follows: reaction mixtures were composed of 20 mM Tris–HCl pH 8.4 and 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each oligonucleotide (Gibco-BRL, England), 5 U Taq DNA polymerase (Invitrogen, Brazil), and 10 ng genomic DNA, for a final volume of 50 μl. A PTC-100™ thermocycler (MJ Research, Inc., USA) was used in two steps: (a) 12 cycles, 15 s/94 °C; 30 s/65 °C (−0.7 °C per cycle); extension 1 min/72 °C; (b) 20 cycles, 15 s/94 °C; 30 s/56 °C; extension 1 min/72 °C; final extension 5 min/72 °C. Exceptions to this protocol were: (a) Isolate Pb01, fragment E and isolate Pb300, fragment J, were amplified after initial denaturation of 3 min/94 °C, 35 cycles 1 min/94 °C, 1 min/50 °C and 1 min/72 °C, final extension 5 min/72 °C; (b) Isolate Pb01, fragments S, T, U and V were amplified after initial denaturation of 1.5 min/94 °C, 30 cycles 1 min/94 °C, 1 min/60 °C and 1 min/72 °C, final extension 5 min/72 °C; (c) Fragments R and W to Z for all the isolates were amplified after initial denaturation of 2 min/94 °C, 30 cycles 1 min/94 °C, 1 min/50 °C and 2 min/72 °C, final extension 7 min/72 °C. Fragments (Table 2) were purified with the Concert™ Rapid PCR Purification System (Gibco BRL, England) kit, and sequenced either at the Centro Nacional de Secuenciación y Análisis de Ácidos Nucleicos (CeSAAN; IVIC, Caracas) (fragments A to Q), Laboratório de Biologia Molecular-Universidade de Brasilia, Brazil (fragments S–V), or Laboratório de Biologia Molecular, Universidade Federal de Goiás, Brazil (fragments R and

Table 1
P. brasiliensis isolates used in this study

Isolate	Equivalent (S) in Matute et al. (2006)	Origin	Country
Pb 01[a]		Clinic	Brazil
Pb 18[b]	B17 (S1)	Clinic	Brazil
Pb 1684[a]		Clinic	Brazil
BT		Clinic	Brazil
Pb 135[a]		Clinic	Brazil
Pb 339[b]	B18 (S1)	Clinic	Brazil
BT 60 [c]	B14 (S1)	Clinic	Brazil
14-121	B16 (S1)	Clinic	Brazil
IBIA[c]	B12 (S1)	Soil	Brazil
Pb T7F6[c]	B4 (S1)	Armadillo	Brazil
Pb T1F1[c]	B1 (S1)	Armadillo	Brazil
Pb T8B1[c]	B5 (S1)	Armadillo	Brazil
T13LN1[c]	B9 (S1)	Armadillo	Brazil
ESLAVA [d]		Clinic	Colombia
HIGUITA[d]	C6 (S3)	Clinic	Colombia
Pb73[d] (ATTC 32071)	C20 (S3)	Clinic	Colombia
DASYPUS[d]	C21 (S3)	Armadillo	Colombia
Pb 305[e]	V3 (S1)	Clinic	Venezuela
Pb 309[e]		Clinic	Venezuela
Pb 307[e]		Clinic	Venezuela
Pb 300[e]	V1 (S1)	Soil	Venezuela

[a] Isolates belonging to M.S. Felipe's collection.
[b] Donated by Zoilo Pires de Camargo (Escola Paulista de Medicina, São Paulo, Brazil).
[c] Isolates donated by Eduardo Bagagli (Instituto de Biociências, UNESP, Botucatu, SP, Brazil).
[d] Isolates belonging to Angela Restrepo and Juan McEwen's collection (Corporación para Investigaciones Biológicas, Medellín, Colombia). Over the years, isolate Pb73 (ATCC 32071; originally C81) has been the reference isolate at IVIC's Mycology Lab (San-Blas' group).
[e] Donated by Mireya Mendoza (Instituto de Biomedicina, Caracas, Venezuela).

Table 2
Genes under study

Gene	NCBI Accession No.	Oligonucleotides			Size of PCR product (bp)
		Pairs	Identification	Sequence	
CHS4	AF107624	A	*CHS4*_Int_1S	GTTTGGGCGTGACCGTCCTT	428
			*CHS4*_Int_1AS	TTGTCCCGCTGGACGAGGAACA	
		B	*CHS4*_Int_2S	TCGACTCGGGCACGAGCATA	423
			*CHS4*_Int_2AS	TGCCCGTGAGGGTCAGAGTA	
		C	*CHS4*_Int_3S	GACTTGTGGCGAGTTGTGCG	410
			*CHS4*_Int_3AS	GGGAAATGCTTGGTCATGAG	
		E	7Down8	TGGCTGTTGTTTCTGGGTATGGAGG	498
			*CHS4*_Int_4AS3	CTCGAGACATAAAATGCCCTGGGTGA	
Actin	AY383732	H	ActInt5-S	GTTGGGTCTTGAAAGTGGCG	516
			ActInt5-AS	TACCACTCACACGCACAACC	
ODC	AF212867	J	Odc5′-S	TGATGTGTGTGTGCTTAGCCC	582
			Odc5′-AS	TCATCTGGATAATTTCGGGTC	
		L’	Ornitina3′-S	TGCTACCGAGGCTTACATGA	604
			Ornitina3′-AS	GGCTGCTACACCTCCACATG	
URA3	AJ133782	M	Ura5′-S	ACCTGCTTGACACTAAATCCG	520
			Ura5′-AS	GAGGGGTTGAGTTCTGTTTGA	
		N	Ura5′Int1-S	AACAGCCACATCATATCAACATC	616
			Ura5′Int1-AS	ATGTTTGCTTGCGAGGGATT	
CHS2	Y09231	P	Chs2Int1,2-S	CAAGCGAGAGGAGCAGTGGGTT	456
			Chs2Int1,2-AS	GACAGGGCACCAGCAATGAA	
		Q	Chs2Int2,3-S	GCGGACAGACCACACAATTA	575
			Chs2Int2,3-AS	GGAGACGATGATGTAGGTGAAAGT	
FKS1	AF148715	R	FKS1-S	TTCCTCTCTTATTCTTTTCCC	112
			FKS1-AS	CTGATCATTGTAGTATTCGTC	
HSP70	U91560	S	HSPMM1	AACCAACCCCCTCTGTCTTG	529
			HSPMM2	TACCCTGTTCGTTGGCAATG	
Hydrophobin	AF526275	T	5′Hyd	ATCATCAACAAGCATCAGTAC	512
			TAA-Xho	CCGCTCGAGGGTGTCGATTGGGCATTTAC	
		U	HydMMT1	GATAGCTACTCATGTTTTC	410
			3′Hyd	CTAAAGGAAAGTTAAGAAGC	
Kex	AF486805	V	K5	GAGTTGCCAGTCGATGAAC	387
			KX3	GTCACTAAGGAGCTGTTGG	
Catalase A	AY494834	W	CatA-S	GGACGAGTTCTTTCCACA	232
			Cat A-AS	CGGTTCCCTGAGTTATTTTG	
Catalase P	AF428076	X	Cat P-S	GTGCAGGAGCTTACGGTG	166
			Cat P-AS	GGAGAACCCTCTAGGATCGCG	
Formamidase	AY163575	Y	Fmd-S	TGCGAAGAATGGAGGCGGGTTTC	99
			Fmd-AS	TGCAAACGCATACGTAACTAAAG	
Glyoxalase	AY252117	Z	Gly-S	AAATATTGCCTTCGTCAAAG	254
			Gly-AS	GCGATGGATTGGATGAAGGATAC	
ITS		ITS	ITS4	TCCTCCGCTTATTGATATGC	634
			ITS5	GGAAGTAAAAGTCGTAACAAGG	

W–Z). Some sequences of the Pb01 isolate (gp43, CHS2), were fetched directly from the transcriptome project (https://www.biomol.unb.br/). ITS, 18S SSU rDNA and gp43 sequences other than those sequenced in this work, were downloaded from the National Center for Biotechnology Information (NCBI; Bethesda, MD) (Table 4).

2.4. Computational data analyses

Consensus sequences were built with the Contig Express v.1.0.0.0 program (Vector NTI Suite, Informax, Inc., USA, 1999). Sequences were aligned with the AlignX v.1.0.1.2 software (Vector NTI Suite, Informax, Inc., USA, 1999), based on the ClustalW algorithm (Thompson et al., 1994). The sequenced PCR fragments were then combined according to their gene origin, edited, and then visually inspected. Phylogenetic analyses were performed with the computational programs MEGA2 version 2.1 (Kumar et al., 2001) (Minimal Evolution, ME), using the Jukes–Cantor distances, Kimura 2, Transitions + transversions. Tests of internal branches with Phylogenetic Analysis Using Parsimony (PAUP version 4.0b.4a; D.L. Swofford,

Table 3
P. brasiliensis sequences used in this paper, GenBank accession numbers

Fragment	Accession No. (Range)
CHS2P	EF638867–EF638887
CHS2Q	EF642949–EF642969
CHS4A	EF642888–EF642908
CHS4B	EF642909–EF642928
CHS4C	EF638846–EF638866
CHS4E	EF645142–EF645162
ActinaH	EF642929–EF642948
ODCJ	EF645163–EF645183
ODCL	EF645184–EF645204
URA3M	EF645205–EF645224
URA3N	EF645225–EF645244
Glucan synthase	EF657122–EF657141
Glyoxalase	EF657142–EF657161
Formamidase	EF657162–EF657181
Catalase A	EF661665–EF661685
Catalase P	EF661686–EF661706
HSP21	EF672113–EF672133
hyd1_3UTR	EF672134–EF672154
hyd1_21	EF672155–EF672175
Hex21	EF672176–EF672196

Table 4
ITS sequences, NCBI access numbers

Species	Isolate, country of origin	Access No.
P. brasiliensis	Bt 4, Brazil	AB035710
P. brasiliensis	Pb 63255, Argentine	AB038164
P. brasiliensis	Ramos M 279, Argentine	AB038165
P. brasiliensis	UAMH 8037, Brazil	AF038360
P. brasiliensis	Pb 2, Brazil	AY374338
P. brasiliensis	Pb 4, Brazil	AY374336
P. brasiliensis	Pb 8, Brazil	AY374337
P. brasiliensis	Pb 10, Brazil	AY374339
H. capsulatum var. capsulatum	HP 13, Thailand	AB055241
H. capsulatum var. duboisii	H 147, Senegal	AB055247
H. capsulatum var. duboisii	IFM 5415	AB055248
H. capsulatum var. farciminosum	H 95	AB055249
Emmonsia parva	UAMH 130	AF038333
Blastomyces dermatitidis	ATCC 60915, USA	AF322388
Lacazia loboi	Brazil	AF322182

Illinois Natural History Survey, Champlain), and Maximum Parsimony (MP), used Felsenstein's Bootstrap test with 1000 resampled datasets. Neighbor-Joining analyses of the ITS, *18S SSU* and *gp43* sequences under investiga-

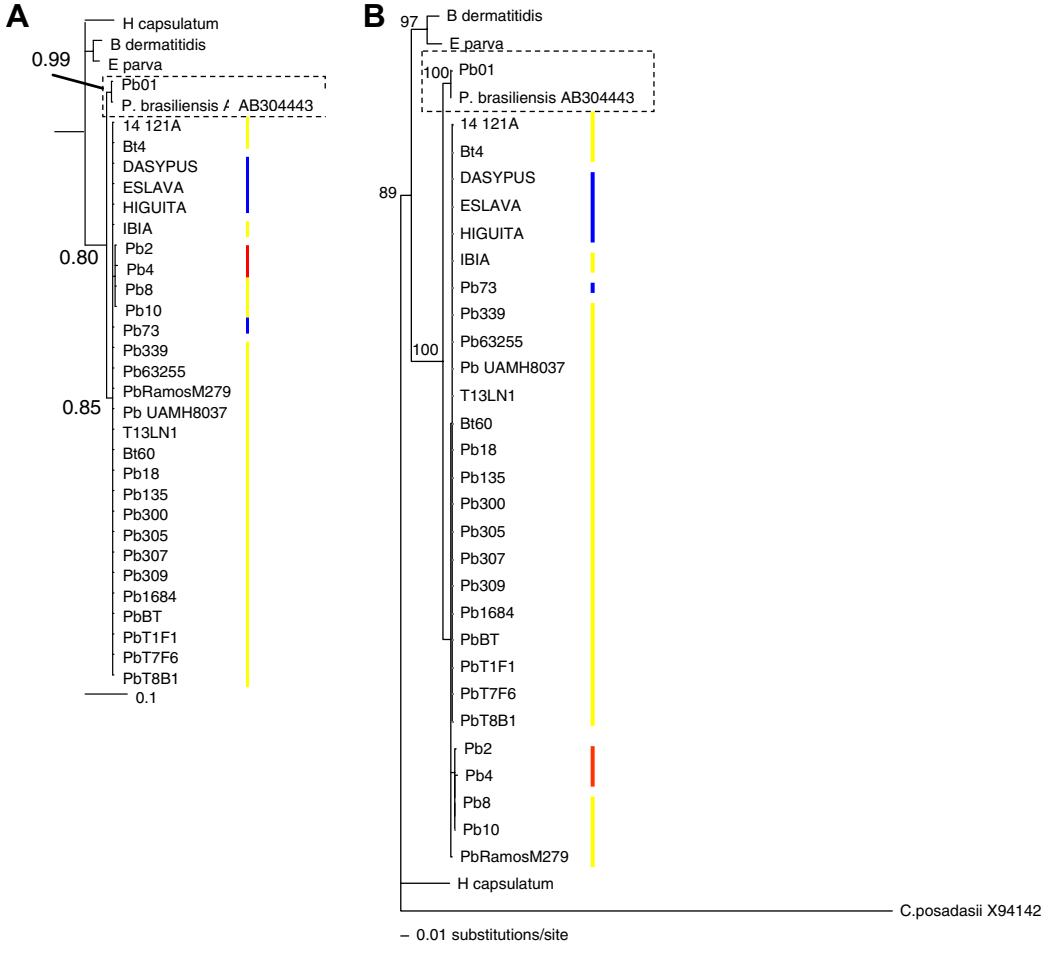

Fig. 1. ITS phylograms. (A) Bayesian; (B) neighbor joining (NJ); Yellow, red or blue lines group isolates from phylogenetic species 1, 2, and 3, respectively (Matute et al., 2006). For the Bayesian tree, *Coccidioides* was also included; its branch was removed and replaced by a root at the spot where *Coccidioides* branches. This solution includes all of the data but gets rid of the long *Coccidioides* branch.

tion used a maximum-likelihood multiple hit correction, Kimura 2, an empirical transition/transversion ratio, empirical base frequencies, and a gamma distribution of 0.5. Comparison of genetic distances for all pairs of taxa found very similar results whether corrections for multiple hits were used or not, as expected for sequences with few nucleotide substitutions. Bayesian analyses were also done, using evolutionary models selected by MrModeltest. For CHS2 using all taxa, the HKY + gamma distribution model was best and after removal of *Coccidioides*, the HKY + invariants model was best. For ITS with all taxa, or after removal of *Coccidioides*, the HKY + gamma distribution model was best. For each analysis, we made two MrBayes runs, each of which sampled 1 × 106 topologies and each of which used a cold and heated chain. The first 250,000 runs were discarded and branch support was calculated from topologies sampled from every 100th of the remaining 750,000 trees.

For the PCR fragment sequences coding proteins the conditions to generate the phylogentic trees were identical, except that the transition/transversion ratio used was 2/1. To assess internal branch support 1000 bootstrap resampled data sets were used by both neighbor-joining analysis and parsimony methods (heuristic). In some phylogenetic trees, sequences used as outgroups were selected from nearby dimorphic Onygenales using Basic Local Alignment Search Tool (BLAST). For phylogenetic analyses of the ITS fragments, additional orthologous sequences from diverse fungal species were obtained from the NCBI (Table 4). In doing Bayesian and Neighbor-Joining analyses, *ITS* and *CHS2* sequences deposited in GenBank, from other *P. brasiliensis* strains, were also included. These were: A2, B7, B10, B13, B15, B23, B26, C1, C13, C16, C17, C18, P1, P2, U1, V2 (Matute et al., 2006). Recently, sequences from an atypical *P. brasiliensis* strain (IFM 54648, Londrina, Brazil; reported by Sano et al., GenBank) were deposited in the GenBank under the accession numbers AB304443 (ITS) and AB304565 (*CHS2*). They were also included in our ITS and *CHS2* analyses.

3. Results and discussion

ITS phylograms (Fig. 1) were constructed, using *Coccidioides posadasii*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Emmonsia parva* as outgroup sequences. For *CHS2* trees, *C. posadasii* and *H. capsulatum* were selected as outgroups (Fig. 2). In both analyses, the exclusion of *C. posadasii* lead to similar phylograms, in which Pb01, and AB304443 (ITS) or AB304565 (*CHS2*) (sequences belonging to *P. brasiliensis* strain IFM 54648), clustered

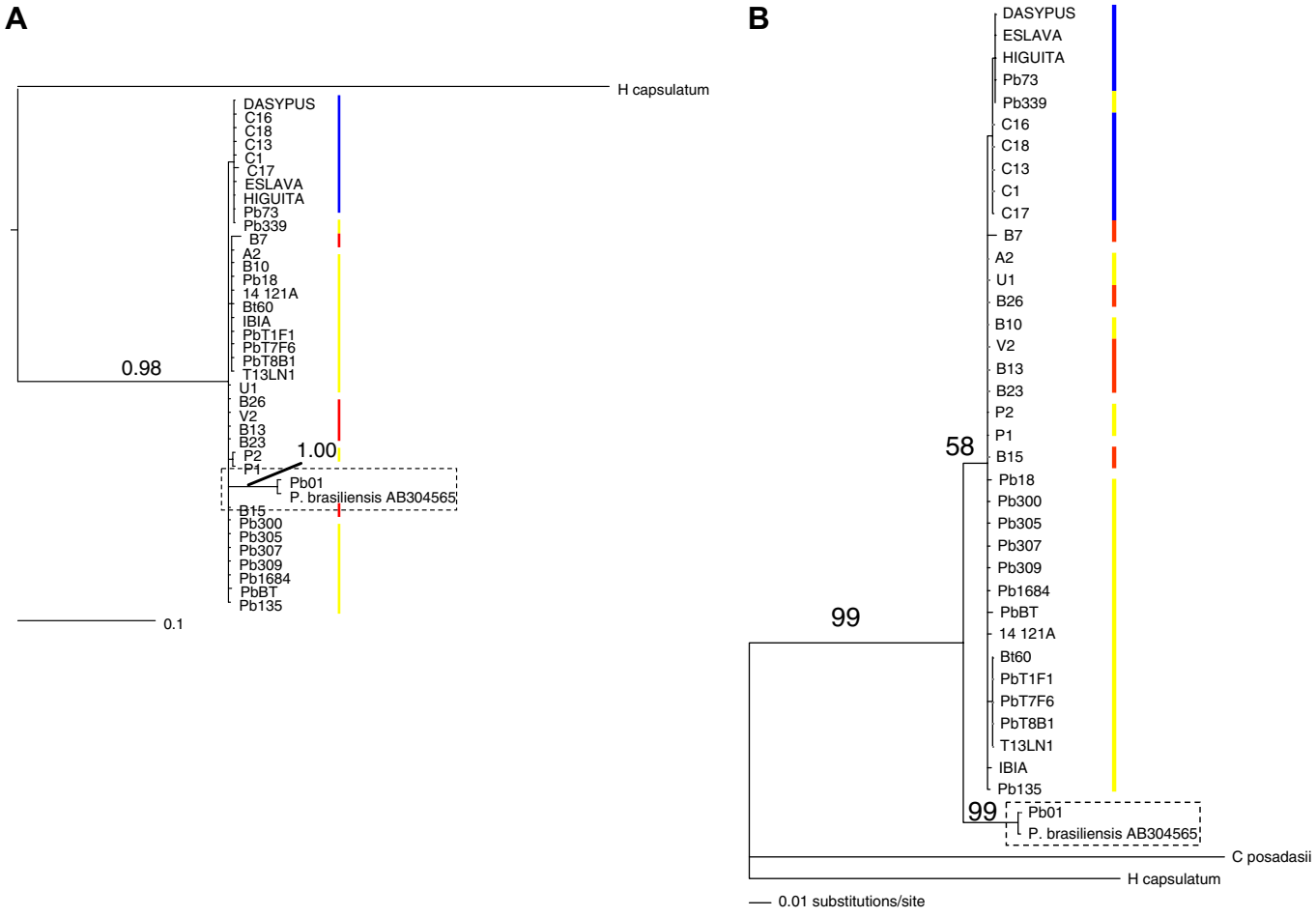

Fig. 2. *CHS2* phylograms. (A) Bayesian; (B) neighbor joining (NJ). Color lines as in Fig. 1. For the Bayesian tree, see note in Fig. 1.

apart from all other sequences (not shown). Individual trees constructed by Neighbor-Joining, with all other gene fragments (data not shown) were consistent with those of Figs. 1 and 2. Additionally, a phylogram based on sequences from the *CHS4*, *Actin*, *ODC*, *URA3*, *CHS2*, *HSP70*, *FKS1*, *Hydrophobin*, *Kex1*, *Catalase A*, *Catalase P*, *Formamidase*, and *Glyoxalase* genes was constructed (Fig. 3) (Huelsenbeck et al., 1996). The *gp43* gene also served to build a phylogram in which the exon 2 sequence of the isolate Pb01 was analyzed together with orthologous sequences downloaded from databases (NCBI accession number on each strain, see Fig. 4).

With the exception of *FSK1* and *Catalase A* (Fragments R and W, Table 2), *P. brasiliensis* isolate Pb01 separated from all other *P. brasiliensis* isolates used in this study, clustering together with strain IFM 54648, an atypical strain isolated from a patient in the southern Brazilian region of Paraná, under study by Sano A. et al. (see GenBank for source of information). The branch uniting the other *P. brasiliensis* isolates was well supported (>90%), whether the genes were highly conserved sequences, such as *HSP*, *Actin* or ribosomal genes, or more variable protein coding genes. In turn, other *P. brasiliensis* isolates grouped according to geographical location (Fig. 3). Using *gp43* sequences taken from Matute et al. (2006), to which isolate Pb01 was added (Fig. 4), a phylogenetic tree was derived in which, as expected, *P. brasiliensis* isolates formed two strongly supported groups, one formed by the Colombian strains (blue group), and the other formed by the Argentin-

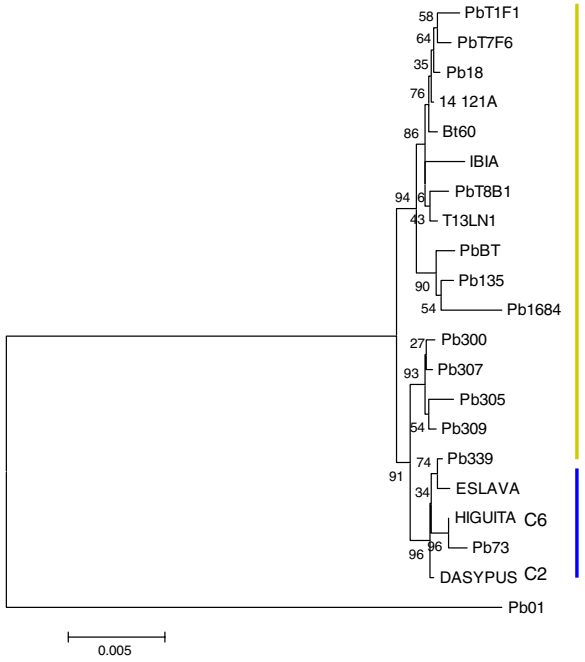

Fig. 3. Phylogram of total evidence, based on sequences from the *CHS4*, *Actin*, *ODC*, *URA3*, *CHS2*, *HSP70*, *FKS1*, *Hydrophobin*, *Kex1*, *Catalase A*, *Catalase P*, *Formamidase*, and *Glyoxalase* genes. Numbers correspond to the percentage of bootstrap resampled datasets containing the adjacent branch in ME phylograms. Color lines as in Fig. 1.

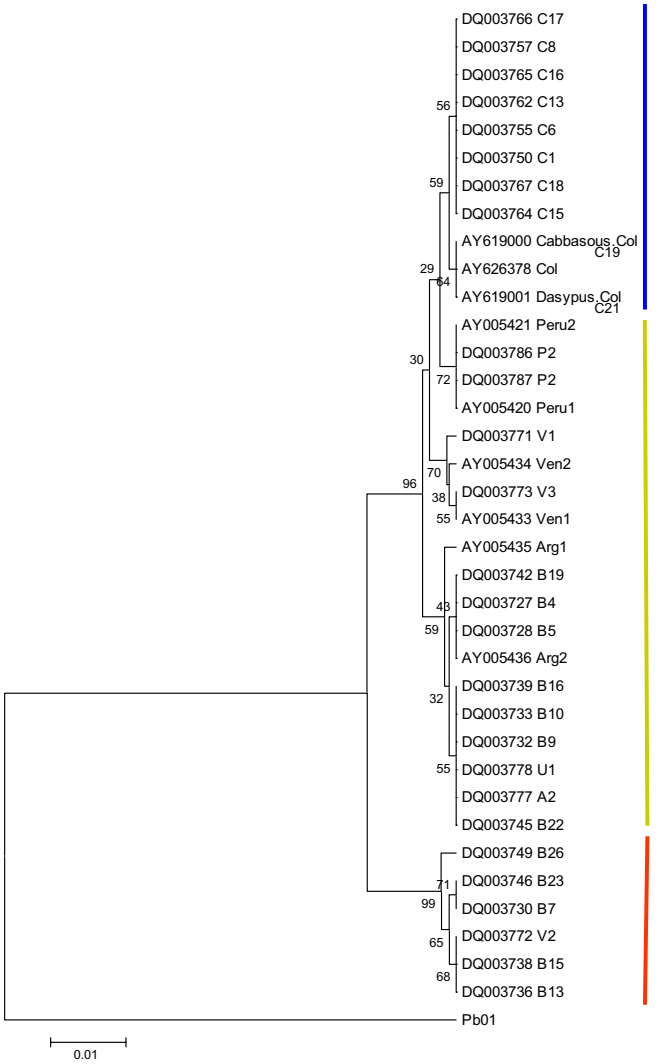

Fig. 4. Phylogram of gp43, sequences taken from Matute et al. (2006), with the exception of Pb01.

ean, Brazilian, Peruvian, and Venezuelans strains (yellow group), plus a third group formed by four Brazilian and one Venezuelan strains [red group; (Matute et al., 2006)]. However, Pb01 and IFM 54648 did not group with these phylogenetic species. In the protein coding gene trees, the branch leading to Pb01 is longer than that leading to any other isolate and the mean pairwise genetic distance of coding regions using all gene sequences among *P. brasiliensis* isolates increases from 2.83 to 6.81% when Pb01 is added to the analysis (Table 4). This degree of divergence increased to 2.93–13.15% (mean 6.81%) when the Pb01 sequences were included (Table 5). This finding suggests the possibility of more than three phylogenetic species in *P. brasiliensis*. This may also suggests that the isolate Pb01 and the newly reported IFM 54648 have been genetically isolated from the other *P. brasiliensis* strains for a considerable period.

Pb01 is of interest because this particular isolate has been the subject of intense molecular studies for many years. A thorough analysis of its transcriptome yielded

Table 5
Data of fragments under study

Fragment (nt)	Divergence degree		Length of MP tree	Consistency index	Homoplasy index	Consistency index re-scaled	Outgroup sequence
	With Pb01	Without Pb01					
A (295)	10.51%	0.68%	32	1.0000	0.0000	1.,0000	N.A.
B (289)	N.A.	1.38%	9	0.4444	0.5556	0.2963	N.A.
C (223)	5.38%	1.79%	175	1.0000	0.0000	1.0000	*Aspergillus oryzae* (AB081655)
E (412)	10.92%	0.97%	55	0.8182	0.1818	0.5361	N.A.
H (417)	4.61%	0.51%	131	0.8626	0.1374	0.0000	*Ajellomyces capsulatus* (U17498)
J (463)	5.11%	0.89%	166	1.0000	0.0000	1.0000	*C. immitis* (AF179245)
L′ (415)	2.93%	1.22%	167	0.9880	0.0120	0.8718	*C. immitis* (AF179245)
M (403)	N.A.	0.74%	3	1.0000	0.0000	1.0000	N.A.
N (471)	N.A.	0.85%	4	1.0000	0.0000	1.0000	N.A.
Q (433)	4.58%	0.69%	156	0.9423	0.0577	0.4123	*C. posadasii* (AF329357)
R (117)	2.56%	2.6%	N.E.	N.E.	N.E.	N.E.	N.A.
S (487)	4.72%	0.62%	**23**	**1.0000**	**0.0000**	**1.0000**	N.A.
T (206)	3.40%	0.97%	**7**	**1.0000**	**0.0000**	**1.0000**	N.A.
U (259)	11.20%	6.95%	**24..**	**1.0000**	**0.0000.**	**1.0000**	N.A.
V (163)	5.52%	0.61%	**9**	**1.0000**	**0.0000**	**1.0000**	N.A.
W (327)	13.15%	12.5%	N.E.	N.E.	N.E.	N.E.	N.A.
X (152)	11.84%	5.92%	N.E.	N.E.	N.E.	N.E.	N.A.
Y (124)	8.87%	8.06%	N.E.	N.E.	N.E.	N.E.	N.A.
Z (321)	9.97%	9.97%	N.E.	N.E.	N.E.	N.E.	N.A.
TOTAL sequences (ITS, B, M and N excluded) (5122)	6.81%	2.83%	423	0.8392	0.1608	0.5713	N.A.
ITS (536)	2.64%	0.88%	188	0.8777	0.1223	0.8048	See Table No. 3

N.E., not estimated; N.A., not available.

6022 assembled groups from mycelium and yeast phase expressed sequence tags, covering about 80% of the estimated *P. brasiliensis* Pb01 genome (Felipe et al., 2005). The results above may lead us to speculate that Pb01 might be a new species in the genus *Paracoccidioides*. However, establishing if Pb01 and IFM 54648 are phylogenetically a different species would require the finding of several new Pb01-like isolates to validate this hypothesis.

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