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2012

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JOURNAL OF MEDICAL MICROBIOLOGY, READING, v. 61, n. 12, pp. 1688-1695, DEC, 2012
http://www.producao.usp.br/handle/BDPI/42676

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DOI 10.1099/jmm.0.044073-0

Evaluation of the genetic diversity of *Histoplasma capsulatum* var. *capsulatum* isolates from north-eastern Brazil

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Since the beginning of the HIV epidemic, there has been a significant increase in the number of histoplasmosis cases in Ceará, a state in north-east Brazil. The lack of epidemiological data on the genotypes circulating in the north-east region shows the importance of more detailed studies on the molecular epidemiology of *Histoplasma capsulatum* var. *capsulatum* in this region. Different molecular techniques have been used to better characterize the genetic profile of *H. capsulatum* var. *capsulatum* strains. The aim of this study was to analyse the genetic diversity of *H. capsulatum* var. *capsulatum* isolates in Fortaleza, the capital of Ceará, through the sequencing of the internal transcribed spacer (ITS)1-5.8S-ITS2 region, and establish the molecular profile of these isolates, along with strains from south-east Brazil, by RAPD analysis, featuring the different clusters in those regions. The isolates were grouped into two clusters. Cluster 1 included strains from the south-east and north-east regions with separation of isolates into three distinct subgroups (subgroups 1a, 1b and 1c). Cluster 2 included only samples from north-east Brazil. Sequencing of the ITS1–5.8S–ITS2 region allowed the detection of two major clades, which showed geographical correlation between them and their subgroups. Therefore, it can be concluded that the *H. capsulatum* var. *capsulatum* isolates from Ceará have a high degree of genetic polymorphism. The molecular data also confirm that populations of this fungus are composed of different genotypes in Brazil and worldwide.

Received 22 March 2012
Accepted 6 September 2012

INTRODUCTION

Histoplasmosis is an important systemic mycosis, whose aetiological agent is the dimorphic fungus *Histoplasma capsulatum*. The natural habitat of this fungus is soil, where it grows as a filamentous saprophyte (Kauffman, 2009). This mycosis is widely distributed in the Americas, with a predominance of cases in some areas of the United States, such as along the valleys of the Mississippi and Ohio rivers, as well as in countries of Central and South America (Rossini & Goulart, 2006; Ferreira & Borges, 2009).

In Brazil, the occurrence of histoplasmosis is relatively common, as demonstrated by the observation of autochthonous clinical cases, either in isolated cases or in the

Abbreviations: ITS, internal transcribed spacer; UPGMA, unweighted pair group analysis with arithmetic mean.

The GenBank/EMBL/DDBJ accession numbers for the ITS1–5.8S–ITS2 region sequences of strains used in this study are given in Table 1.

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form of outbreaks (Chang *et al.*, 2007). With the advent of AIDS in the 1980s and 90s, several cases of histoplasmosis were observed among patients with this syndrome. Currently, histoplasmosis is the most common systemic mycosis among HIV-infected individuals in endemic areas (de Francesco Daher *et al.*, 2006).

In Ceará, the incidence of histoplasmosis has more than doubled among patients with AIDS in the past decade. There were 191 cases of histoplasmosis in AIDS patients in the period between 1999 and 2005 (Pontes *et al.*, 2010). In 2011, a descriptive analysis was conducted of histoplasmosis cases in AIDS patients in Ceará in the period from 2006 to 2010 (Brilhante *et al.*, 2012); this study evaluated 208 cases of individuals diagnosed with histoplasmosis and AIDS. Due to the increase in histoplasmosis cases in the world, many authors have attempted to trace a molecular and epidemiological profile of this disease through molecular techniques in order to better understand its distribution and pathogenicity (Taylor *et al.*, 2000; Karimi *et al.*, 2002; Kasuga *et al.*, 2003).

Some studies have investigated the molecular profiles of *H. capsulatum* var. *capsulatum* strains in the southern and south-eastern regions of Brazil (Zancopé-Oliveira *et al.*, 2005; Muniz *et al.*, 2010). However, there is a scarcity of data regarding genotypes circulating in other regions of the country, including the north-east. Thus, the aim of the present study was to evaluate the genetic diversity of human and animal strains of *H. capsulatum* var. *capsulatum*, isolated in Fortaleza, north-eastern Brazil, through sequencing of the internal transcribed spacer (ITS)1–5.8S–ITS2 regions, and establish the molecular profile of these isolates, along with strains from south-eastern Brazil, by RAPD analysis, featuring the different clusters in those regions.

METHODS

Micro-organisms. In this study, we used 31 strains of *H. capsulatum* var. *capsulatum*. Twenty-six isolates were from the state of Ceará, in north-eastern Brazil, 24 of which were recovered from AIDS patients with histoplasmosis from the municipalities of Fortaleza (n=18, CEMM-05-1-097, CEMM-05-1-100, CEMM-03-1-052, CEMM-05-1-069, CEMM-05-2-002, CEMM-05-2-052, CEMM-05-2-034, CEMM-05-1-098, CEMM-05-1-099, CEMM-05-2-001, CEMM-05-2-035, CEMM-05-1-070, CEMM-05-2-074, CEMM-05-2-049, CEMM-05-2-038, CEMM-05-2-019, CEMM-05-1-066 and CEMM-05-2-039), Paracuru (n=2, CEMM-05-2-043 and CEMM-05-2-072), Itaitinga (CEMM-05-2-042), Trairi (CEMM-05-2-037), Cascavel (CEMM-05-1-096) and Horizonte (CEMM-05-2-021); with the two remaining isolates from Ceará obtained from cats from Fortaleza and Baturité (CEMM-03-3-055 and CEMM-03-6-059, respectively). Five of the 31 clinical strains were isolated from the south-eastern region of Brazil (CEMM-03-6-024, CEMM-03-6-020, CEMM-03-4-081, CEMM-03-4-036 and CEMM-05-4-015). These strains are stored in the culture collection of the Specialized Medical Mycology Center (CEMM), Federal University of Ceará, Brazil. All procedures involving the manipulation of the strains were performed in a biosafety level 3 laboratory (NB-3).

Extraction of genomic DNA. Genomic DNA was extracted from yeast-phase cultures of the micro-organism grown for 5 days at 35 °C in Sabouraud agar (Himedia) supplemented with 10 % sheep's blood. DNA extraction was performed as described by Ausubel *et al.* (2002). Genomic DNA was extracted by mechanical lysis with glass beads and chemistry lysis using a breaking buffer. The nucleic acids were precipitated with ethanol after exposure to a solution of phenol/chloroform/isoamyl alcohol to separate the cellular debris. The DNA pellet was then resuspended in TE buffer and stored at 4 °C. DNA quantification was performed by spectrophotometry and its integrity was analysed by 1 % agarose gel electrophoresis and ethidium bromide staining.

RAPD-PCR assay. Genetic polymorphisms of the *H. capsulatum* var. *capsulatum* isolates were assessed by the RAPD analysis. RAPD reactions were performed with the 31 strains of *H. capsulatum* using four primers: primer 2 (5′-GTTTCGCTCC-3′), primer 3 (5′-GTAGACCCGT-3′), primer 4 (5′-AAGAGCCCGT-3′) and primer 5 (5′-AACGCGCAAC-3′), as described by Zancopé-Oliveira *et al.* (2005), with modifications. The RAPD reaction was performed in a total volume of 10 µl, containing 50 ng *H. capsulatum* DNA, 1× buffer, 1 mM $MgCl_2$, 2 pmol primer, 0.5 mM each dNTPs and 1 U Hot Start *Taq* polymerase (Promega). Negative controls, without the presence of DNA, were subjected to amplification simultaneously. The amplification was performed in a Mastercycler thermocycler (Eppendorf), involving initial denaturation at 95 °C for 5 min followed by 45 cycles of denaturation at 95 °C for 1 min, annealing at 36 °C for 1 min and extension at 72 °C for 2 min, and finally, one cycle of final extension at 72 °C for 10 min. The visualization of the amplified products was performed by electrophoresis on a 2 % agarose gel containing ethidium bromide, followed by exposure to UV light through a transilluminator.

RAPD-PCR data analysis. The RAPD profiles were defined by bands. The size of all bands seen on agarose gel was calculated using the analysis software E-Capt (Vilber Lourmat). The matrix of data was determined by the presence (1) or absence (0) of amplification products. The construction of the dendrogram was based on the similarity of the matrix data, using character transformation and distance transformation analyses, through the unweighted pair group method with arithmetic mean (UPGMA) (Huson & Bryant, 2006).

Sequencing of the ITS1–5.8S–ITS2 region. Considering that strains within the same group present similar genotypic profiles, at least one isolate from each RAPD cluster was selected to be further analysed by ITS–5.8S–ITS2 sequencing. A total of 15 randomly chosen strains of *H. capsulatum* var. *capsulatum* were used in the sequencing reactions: 12 from the north-eastern region of Brazil [10 isolates from HIV-infected patients with histoplasmosis (CEMM-05-2-001, CEMM-05-1-070, CEMM-05-1-096, CEMM-05-1-098, CEMM-05-2-034, CEMM-05-2-039, CEMM-05-2-002, CEMM-05-2-072, CEMM-05-2-049 and CEMM-05-2-037) and two from animals (CEMM- 03-3-055 and CEMM-03-6-059)] and three clinical strains from the south-eastern region of Brazil (CEMM-03-6-020, CEMM-03-4-036 and CEMM-05-4-015). Sequencing reactions were performed at the Genetics Laboratory of the Federal University of Ceará, Brazil.

The ITS1–5.8S–ITS2 region was amplified using the universal primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) as described by White *et al.* (1990). The amplification reactions were performed in a final volume of 25 µl, containing 200 ng DNA, 1× Green GoTaq Buffer (Promega), 1.5 mmol l^{-1} $MgCl_2$, 12.5 pmol each primer, 10 mmol l^{-1} each dNTP and 1 U GoTaq Hot Start DNA polymerase (Promega). Negative controls, without the presence of DNA, were subjected to amplification simultaneously. The amplification was performed in a Mastercycler thermocycler (Eppendorf), involving initial denaturation at 95 °C for 2 min followed by 33 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 90 s and extension at 72 °C for

Table 1. Details of the *H. capsulatum* strains whose ITS1–5.8S–ITS2 sequences were used in the study and their places of origin

Locations in Brazil were abbreviated as follows: CE, Ceará; PE, Pernambuco; SP, São Paulo; RJ, Rio de Janeiro; RS, Rio Grande do Sul; GO, Goiás; MS, Mato Grosso do Sul; ES, Espírito Santo.

Strain(s)	Accession number	Origin	Group code
H. capsulatum CEMM 05-2-072 (1)	JX051637	CE-Brazil	A
H. capsulatum CEMM 05-2-039 (2)	JX051639	CE-Brazil	A
H. capsulatum CEMM 05-1-098 (3)	JX051642	CE-Brazil	A
H. capsulatum CEMM 05-1-096 (4)	JX051643	CE-Brazil	A
H. capsulatum CEMM 05-1-070 (5)	JX051644	CE-Brazil	A
H. capsulatum (6)	GU320956.1	CE-Brazil	A
H. capsulatum (1)	AB055241.2	Thailand	B
H. capsulatum (2)	AB055238.2	Thailand	B
H. capsulatum (1)	AB055230.2	United States	C
H. capsulatum (2)	AB071821.1	United States	C
H. capsulatum (3)	AB071822.1	United States	C
H. capsulatum (4)	AB055229.2	United States	C
H. capsulatum (3)	AB055243.2	Thailand	D
H. capsulatum (4)	AB055240.2	Thailand	D
H. capsulatum (1)	AB055236.2	UK	D
H. capsulatum (8)	EU048556.1	Brazil	E
H. capsulatum (16)	GU320957.1	PE-Brazil	E
H. capsulatum (17)	GU320958.1	PE-Brazil	E
H. capsulatum (18)	GU320952.1	SP-Brazil	E
H. capsulatum CEMM 03-4-036 (19)	JX051636	SP-Brazil	E
H. capsulatum (20)	GU320984.1	RJ-Brazil	E
H. capsulatum (21)	GU320983.1	RJ-Brazil	E
H. capsulatum (22)	GU320939.1	RJ-Brazil	E
H. capsulatum (2)	AB071843.1	Australia	E
H. capsulatum (1)	AB071840.1	China	E
H. capsulatum (2)	AB055237.2	China	E
H. capsulatum (1)	AB071827.1	Ecuador	E
H. capsulatum (1)	AB071824.1	Colombia	E
H. capsulatum (2)	AB071823.1	Colombia	E
H. capsulatum (1)	AB055235.2	Indonesia	E
H. capsulatum (3)	AY623792.1	Japan	E
H. capsulatum CEMM 05-2-001 (9)	JX051647	CE-Brazil	E
H. capsulatum CEMM 05-2-049 (10)	JX051635	CE-Brazil	E
H. capsulatum CEMM 05-2-034 (11)	JX051641	CE-Brazil	E
H. capsulatum CEMM 05-2-037 (12)	JX051634	CE-Brazil	E
H. capsulatum CEMM 05-2-002 (13)	JX051638	CE-Brazil	E
H. capsulatum CEMM 03-3-055 (14)	JX051648	CE-Brazil	E
H. capsulatum CEMM 03-6-059 (15)	JX051640	CE-Brazil	E
H. capsulatum (6)	AF129547.1	United States	F
H, capsulatum (7)	AF129546.1	United States	F
H. capsulatum (9)	AF129539.1	United States	G
H. capsulatum (10)	AF129538.1	United States	G
H. capsulatum (11)	AF129540.1	United States	G
H. capsulatum (14)	AB071770.1	United States	H
H. capsulatum (15)	AB055228.2	United States	H
H. capsulatum (1)	AB071832.1	Argentina	I
H. capsulatum (2)	AB055231.2	Argentina	I
H. capsulatum (24)	GU320985.1	RS-Brazil	I
H. capsulatum (25)	GU320936.1	RS-Brazil	I
H. capsulatum (26)	GU320951.1	SP-Brazil	I
H. capsulatum (27)	GU320955.1	GO-Brazil	I
H. capsulatum (28)	GU320954.1	GO-Brazil	I
H. capsulatum (29)	GU320981.1	MS-Brazil	I

Table 1. cont.

Strain(s)	Accession number	Origin	Group code
H. capsulatum (30)	GU320953.1	MS-Brazil	I
H. capsulatum (31)	GU320941.1	RJ-Brazil	I
H. capsulatum CEMM 03-6-020 (23)	JX051646	ES-Brazil	I
H. capsulatum (36)	GU320938.1	RS-Brazil	J
H. capsulatum (37)	GU320937.1	RS-Brazil	J
H. capsulatum (38)	GU320986.1	RS-Brazil	J
H. capsulatum (33)	GU320950.1	ES-Brazil	J
H. capsulatum (34)	GU320948.1	ES-Brazil	J
H. capsulatum (35)	GU320949.1	ES-Brazil	J
H. capsulatum (3)	AB055232.2	Colombia	J
H. capsulatum (40)	AB055234.2	Brazil	K
H. capsulatum CEMM 05-4-015 (41)	JX051645	SP-Brazil	K
H. capsulatum (42)	GU320982.1	RJ-Brazil	K
H. capsulatum (43)	GU320975.1	RJ-Brazil	K
H. capsulatum (44)	GU320960.1	RJ-Brazil	K
H. capsulatum (45)	GU320946.1	RJ-Brazil	K
H. capsulatum (46)	GU320968.1	RJ-Brazil	K
H. capsulatum (47)	GU320980.1	RJ-Brazil	K
H. capsulatum (48)	GU320978.1	RJ-Brazil	K
H. capsulatum (49)	GU320943.1	RJ-Brazil	K
H. capsulatum (50)	GU320971.1	RJ-Brazil	K
H. capsulatum (51)	GU320970.1	RJ-Brazil	K
H. capsulatum (52)	GU320942.1	RJ-Brazil	K
H. capsulatum (53)	GU320963.1	RJ-Brazil	K
H. capsulatum (54)	GU320944.1	RJ-Brazil	K
H. capsulatum (55)	GU320972.1	RJ-Brazil	K
H. capsulatum (56)	GU320974.1	RJ-Brazil	K
H. capsulatum (57)	GU320962.1	RJ-Brazil	K
H. capsulatum (58)	GU320976.1	RJ-Brazil	K
H. capsulatum (59)	GU320961.1	RJ-Brazil	K
H. capsulatum (60)	GU320940.1	RJ-Brazil	K
H. capsulatum (61)	GU320979.1	RJ-Brazil	K
H. capsulatum (62)	GU320977.1	RJ-Brazil	K
H. capsulatum (63)	GU320947.1	ES-Brazil	K
H. capsulatum (65)	GU320959.1	RJ-Brazil	L
H. capsulatum (66)	GU320966.1	RJ-Brazil	L
H. capsulatum (67)	GU320965.1	RJ-Brazil	L
H. capsulatum (68)	GU320969.1	RJ-Brazil	L
H. capsulatum (69)	GU320964.1	RJ-Brazil	L
H. capsulatum (70)	GU320967.1	RJ-Brazil	L
H. capsulatum (1)	AF458086.1	Mexico	
H. capsulatum (1)	AB071830	Guatemala	
H. capsulatum (32)	EU048555.1	Brazil	
H. capsulatum (64)	GU320974.1	RJ-Brazil	
H. capsulatum (16)	AF129545.1	United States	
H. capsulatum (13)	AF129544.1	United States	
H. capsulatum (5)	AF129543.1	United States	
H. capsulatum (12)	AF129542.1	United States	
H. capsulatum (8)	AF129541.1	United States	
H. capsulatum (1)	DQ980237.1	Costa Rica	
H. capsulatum (1)	AB071844.1	Australia	
H. capsulatum (39)	AB071826.1	Brazil	
H. capsulatum (7)	AB071825.1	SP-Brazil	
H. capsulatum (1)	AB055245.2	Japan	
H. capsulatum (2)	AB055244.2	Japan	
H. capsulatum (5)	AB055242.2	Thailand	
H. capsulatum (6)	AB055239.2	Thailand	

3 min, and one cycle of final extension at 72 °C for 8 min. The visualization of the amplified products was performed by electrophoresis on a 1 % agarose gel containing ethidium bromide, followed by exposure to UV light. Subsequently, the PCR products were purified using the GFX PCR DNA and gel band purification kit (GE Healthcare Life Sciences). The ITS1–5.8S–ITS2 region was sequenced using primers ITS4 and ITS5, as described above. The sequencing reactions were then analysed in a MegaBACE 1000 automated sequencer (GE Healthcare Life Sciences). The program CodonCode Aligner 3.03 was used to generate the consensus sequences. The determined sequences were compared to those already deposited in the GenBank database using the BLAST program (www.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1990). The program MEGA4 (version 4.1) (Tamura *et al.*, 2007) was used for phylogenetic analysis.

Phylogenetic analysis. The phylogenetic relationships between the ITS1–5.8S–ITS2 sequences from the clinical *H. capsulatum* var. *capsulatum* isolates used in this study and corresponding sequences from other *H. capsulatum* var. *capsulatum* strains circulating in the world, was determined using the MEGA4 program (version 4.1) (Tamura *et al.*, 2007). A total of 96 ITS1–5.8S–ITS2 sequences of strains from different geographical locations were retrieved from the GenBank database and used for analysis (Table 1). The evolutionary history was inferred by using the maximum-parsimony method (Eck & Dayhoff, 1966) and the close-neighbour-interchange algorithm (Nei & Kumar, 2000). Branches corresponding to partitions reproduced in <50 % bootstrap replicates were collapsed. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analysed. There were a total of 405 positions in the final dataset, 12 of which were parsimony informative.

RESULTS

RAPD profile analysis revealed a total of 68 fragments with sizes ranging from 143 to 1291 bp. Primer 2 (5′-GTTTCGCTCC-3′) was the most polymorphic of the primers tested, producing a total of 21 different bands. A representative RAPD pattern obtained by primer 2 is shown in Fig. 1, in which 25 different genotypic patterns were observed.

The dendrogram reconstructed using the UPGMA cluster analysis method grouped all 31 samples into two major clusters (Fig. 2). Cluster 1 included 26 strains, originating from the north-east and south-east regions of Brazil. Within this cluster there was a clear separation into three distinct subgroups (subgroups 1a, 1b and 1c). The first subgroup included 10 samples from both the north-east and south-east regions. Subgroup 1b included four samples from the north-east region and subgroup 1c included three samples, two of which were from animal sources. The rest of the samples showed similar differences between them, allowing no inference of close evolutionary relationships. The second cluster included five samples, all of them from the city of Fortaleza, north-east Brazil.

The ITS1–5.8S–ITS2 sequences determined in this work were submitted to similarity searches on the GenBank database and the results confirmed the identification of all strains as *H. capsulatum* var. *capsulatum*.

In the phylogenetic tree based on the ITS1–5.8S–ITS2 sequences (Fig. 3), the isolates were classified into two distinct clades. The clusters formed by *H. capsulatum* var. *capsulatum* isolates correlated with their geographical origin. Clade 1 comprised the majority of strains and included isolates from different geographical locations. Within this clade, there were three baseline subclades. The first subclade grouped strains from different parts of the world, such as Asia, the Americas and Oceania. It is of note that all isolates from north-eastern Brazil, including the 13 strains from Ceará and two strains from the neighbouring state of

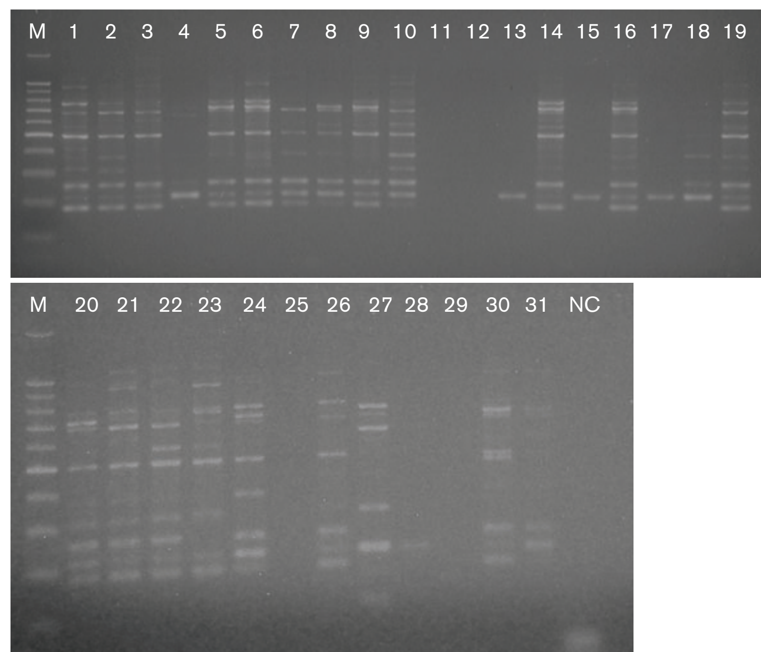

Fig. 1. Electrophoresis on 2 % agarose gels showing representative RAPD patterns of *H. capsulatum* strains obtained with primer 2. M, 100 bp molecular mass marker; lanes 1–31, *H. capsulatum* strains tested. Lanes: 1, CEMM-05-1-069; 2, CEMM-05-1-096; 3, CEMM-05-2-072; 4, CEMM-05-1-099; 5, CEMM-05-1-066; 6, CEMM-05-2-034; 7, CEMM-05-2-052; 8: CEMM-05-2-037; 9, CEMM-05-1-070; 10, CEMM-05-1-098; 11, CEMM-05-1-100; 12, CEMM-05-2-021; 13, CEMM-03-1-052; 14, CEMM-05-2-074; 15, CEMM-05-2-038; 16, CEMM-05-2-019; 17, CEMM-05-2-001; 18, CEMM-05-2-042; 19, CEMM-05-1-97. 20, CEMM-05-2-035; 21, CEMM-05-2-002; 22, CEMM-05-2-039; 23, CEMM-05-2-049; 24, CEMM-05-2-043; 25, CEMM-05-4-015; 26, CEMM-03-4-036; 27, CEMM-03-6-024; 28, CEMM-03-6-020; 29, CEMM-03-4-081; 30, CEMM-03-6-059; 31, CEMM-03-3-055; NC, negative control.

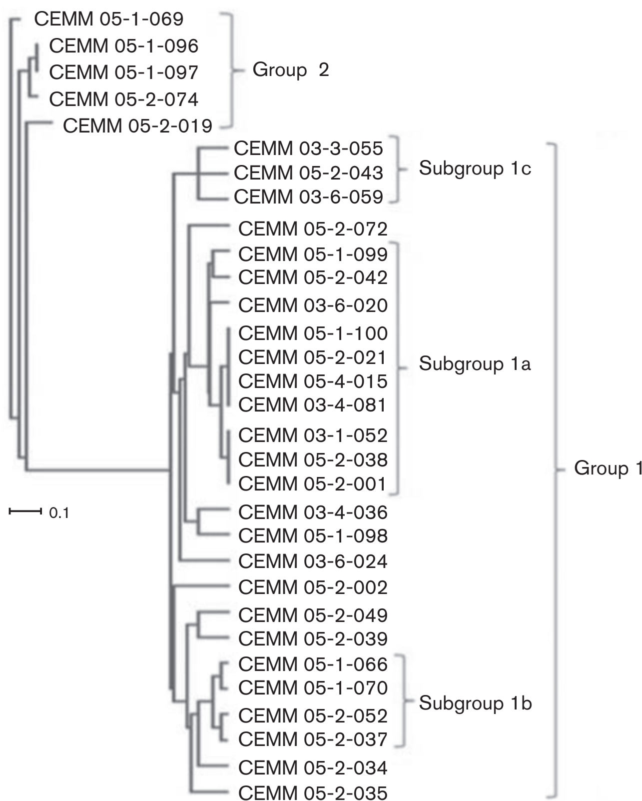

Fig. 2. Dendrogram (unrooted) showing the UPGMA distances among 31 taxa using the primer 2 (5′-GTTTCGCTCC-3′). Uncorrected p distances were used to infer the evolutionary reconstruction with RAPD data transformed to binary characters. The tree was inferred using the program SplitsTree version 4 (http://www.splitstree.org/).

Pernambuco, were grouped in this subclade. The second subclade consisted of isolates from the United States together with an isolate from Costa Rica. The third subclade grouped Brazilian isolates from the midwest, south-east and south regions together with two isolates from Argentina and one isolate from Colombia. The second clade was composed exclusively of isolates from Rio de Janeiro state.

DISCUSSION

The increasing number of histoplasmosis cases, together with the lack of data on the epidemiology and genotypes circulating in north-east Brazil demonstrates the importance of further studies of *H. capsulatum* var. *capsulatum* in this region. In the present study, we observed a high degree of genetic variability among *H. capsulatum* var. *capsulatum* isolates analysed by the RAPD assay, allowing characterization of the molecular profile of the isolates and detection of different clusters circulating in the north-east and south-east of Brazil. Moreover, the analysis of polymorphisms in the ITS1–5.8S–ITS2 region revealed that *H. capsulatum* var. *capsulatum* presents different molecular types that are distributed throughout the world. This information is particularly useful for a better understanding of the genotypes of *H. capsulatum* var. *capsulatum* circulating in Latin America.

Regarding the RAPD analysis, the results allowed the characterization of two main clusters. The first cluster represented a large proportion of the genotypes detected, constituting 84 % of the strains (n=26) of human and animal origin from the north-east and south-east regions of Brazil. On the other hand, cluster 2 was restricted to 16 % of the samples (n=5), which, being composed of strains of human origin exclusively from the north-east region of Brazil, had a high rate of genetic similarity between them. More detailed studies are needed to verify if the strains belonging to this group (cluster 2) show any differences in virulence or pathogenicity that separate them from the larger group (cluster 1). Previous studies have shown that *H. capsulatum* var. *capsulatum* strains can be grouped according to their geographical origin. According to the study by Zancopé-Oliveira *et al.* (2005), there are three clusters, relating to the north-east (cluster I; n=3), south and south-east (cluster II; n=17) and midwest regions (cluster III; n=2) of Brazil. In this study, the cluster of strains in the north-east region was composed of only three strains from this region, two from Pernambuco and one from Ceará. In contrast, this study provided a more accurate analysis of the genetic variability and the existence of different clusters among *H. capsulatum* var. *capsulatum* strains in the north-east region. We analysed a larger number of isolates (n=31), assessing the most appropriate way of forming a general overview of histoplasmosis in Brazil. As observed in other studies, *H. capsulatum* var. *capsulatum* isolates tend to cluster according to geographical origin and different genotypes can be found within the same cluster (Kasuga *et al.*, 2003; Muniz *et al.*, 2010).

As observed in the present study, the ITS1–5.8S–ITS2 region proved to be adequate for the detection of different clades, showing the geographical correlation among the isolates. The analysis of the relationship between the ITS1–5.8S–ITS2 region sequences of *H. capsulatum* var. *capsulatum* isolates from Brazil and other countries revealed that all the isolates from north-east Brazil were grouped in the same subclade and showed genetic similarities with strains originating from countries on several other continents. A group of isolates consisting exclusively of strains from Rio de Janeiro state were grouped into a distinct clade. This result corroborates the findings of Muniz *et al.* (2010), who successfully typed *H. capsulatum* using the same genetic region and observed a single genetic population in the microenvironment of Rio de Janeiro state.

The present study is a pioneer in the analysis of genetic variability of *H. capsulatum* var. *capsulatum* strains circulating in the north-east region of Brazil and will contribute greatly to the characterization of populations of this microorganism in this region.

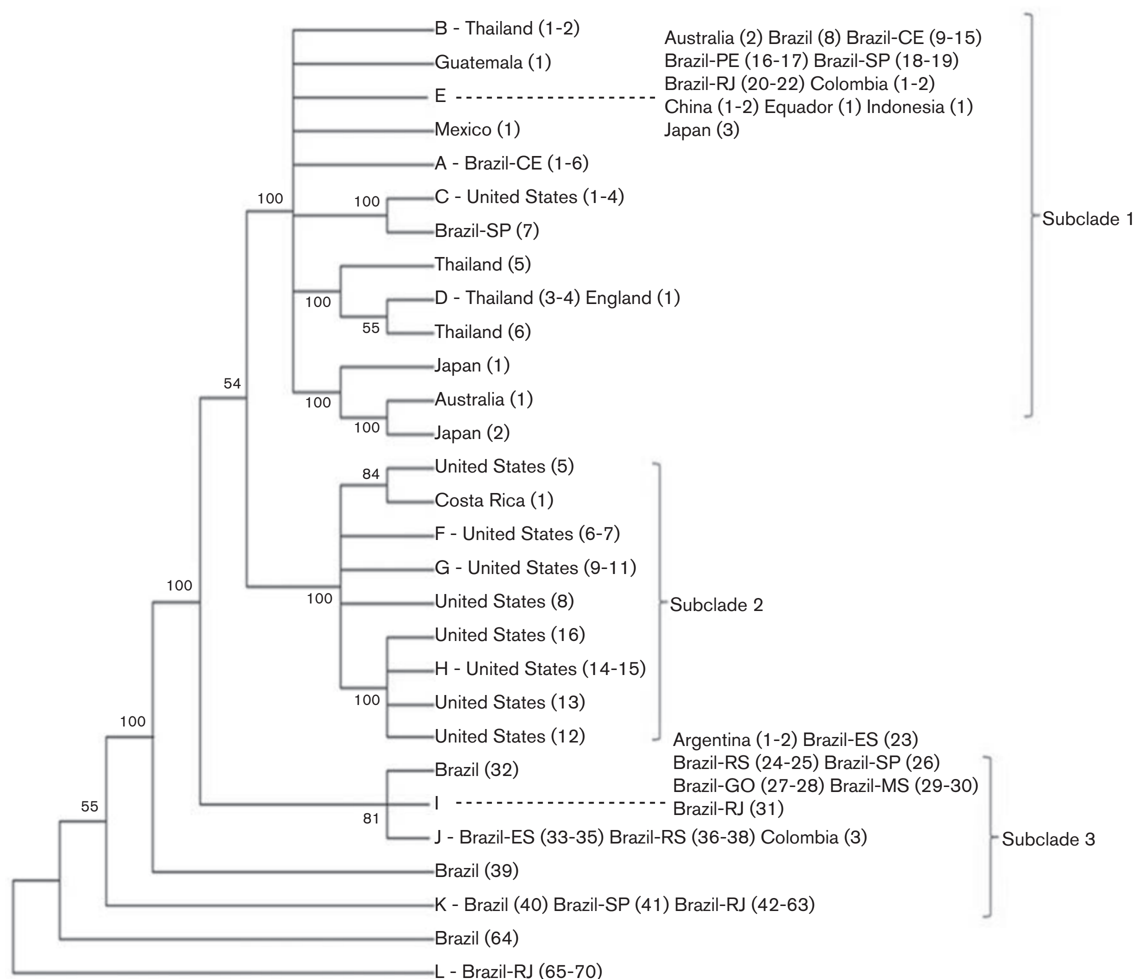

Fig. 3. Representative phylogenetic tree based on the sequences of the ITS1–5.8S–ITS2 region. The strict consensus tree topology was obtained by the maximum-parsimony method using 111 taxa, including *H. capsulatum* strains circulating in north-eastern Brazil and *H. capsulatum* strains circulating in the world. Bootstrap values $>50\%$ (based on 500 replications) are shown at tree nodes. All the sequences are deposited in the GenBank database. Strains that presented sequences with 100 % identity were grouped (letters A to L) and the numbers in parentheses identify the different strains in each group (see Table 1). Locations in Brazil were abbreviated as follows: CE, Ceará; PE, Pernambuco; SP, São Paulo; RJ, Rio de Janeiro; RS, Rio Grande do Sul; GO, Goiás; MS, Mato Grosso do Sul; ES, Espírito Santo.

ACKNOWLEDGEMENTS

This work was funded by the National Council for Scientific and Technological Development (CNPq; Brazil; PROTAX process: 562296/2010-7, CNPq processes 552161/2011-0, 304779/2011-3 and 473025/2012-4) and by the Brazilian Federal Agency for the Support and Evaluation of Graduate Education (CAPES; Brazil; PNPD process: 2103/2009).

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