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Full Length Research Paper

Preservation methods of fungi in 35 years old stock culture storages: A comparative study

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The maintenance of fungi isolates and the development of methodologies were available from their phenotypic and genotypic characteristics. Alterations of *Histoplasma capsulatum* and *Candida* spp. Strains preserved by continuous subculturing on fungal collection and by lyophilization for 35 years were evaluated. *Candida* isolates maintained by lyophilized methodology were viable and preserved by the typical characteristics of each species. Only one lyophilized isolate from *H. capsulatum* was viable and demonstrated typical morphology. Both genus, which were preserved by continuous subculturing, revealed morphologic alterations and lost their sporulation capacity. The DNA from these isolates was sequenced (conserved 28S rDNA) in order to confirm their identity. Random amplified polymorphism DNA (RAPD)-based comparative analysis of the two preservation methods revealed alterations in the band profiles in 28 and 33% in *Candida* spp. and *Histoplasma capsulatum* strains respectively. The RAPD-based results confirm that the subculturing method alters phenotypic and genotypic characteristics by deleting or inserting nucleotides. Otherwise, the lyophilization was effective to yeasts, but it was not effective for dimorphic fungi.

Key words: Polymorphism, genotypic characteristics, lyophilization, random amplified polymorphism DNA.

INTRODUCTION

Culture collections containing fungi are important to biologists, microbiologists, epidemiologists and others involved in health and natural sciences. The medical importance of fungi and other microorganisms has led to the improvement of techniques and methods for their isolation and preservation. The continuous isolation of new strains and the need to maintain such strains in order to conduct pathological and taxonomical studies, as well as industrial applications, have motivated various scientific and industrial research centers to maintain large collections of living microorganisms. Consequently, the collection centers are responsible for repository reference strains and for the maintenance of these microorganisms (American Type Culture Collection, 1991).

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A fungus collection of the Institute of Tropical Medicine has been maintained by continuous subculturing and lyophilization, the latter was carried out in the 1970s. This collection is cited in the "World Data Center for Microorganisms" catalog under the number WCM 718. In these isolates preserved for approximately 35 years, we observed morphological alterations such as a reduction in capacity to produce blastoconidia and chlamydospores (*Candida* spp.) and microconidia and macroconidia tubercle (*Histoplasma capsulatum*), besides the typical hyphae morphology.

In this study *H. capsulatum*, as well as the genus *Candida* spp isolates, both fungi of medical importance were selected. The incidence of diseases caused by fungi has increased over the past decade (Rees et al., 1998; Armstrong et al., 1999; McNeil et al., 2001). The significant human pathogen *H. capsulatum*, in approximately 95% of histoplasmosis cases is subclinical or self-limiting. The invasive candidiasis is a persistent

Table 1. Preservation methods from Fungus Collection of the Sao Paulo Institute of Tropical Medicine maintenance by subculturing and lyophilized methods.

Number	Strain	FCol #*		Species	Genbank Nr
1	Ca99	99	Viable Lyo [a] FCol [b]	*Candida albicans*	DQ318806.1
2	Ca70	70	Viable Lyo [a] FCol [b]	*Candida pelliculosa*	DQ318803.1
3	Ca551	551	Viable Lyo [a] FCol [b]	*Candida albicans*	DQ318815.1
4	Ca407	407	Viable Lyo [a] FCol [b]	*Candida albicans*	DQ318812.1
5	Ca144	144	Viable Lyo [a] FCol [b]	*Candida albicans*	DQ318809.1
6	Ca168	168	Viable Lyo [a] FCol [b]	*Candida guilliermondii*	DQ318797.1
7	Ca103	103	Viable Lyo [a] FCol [b]	*Candida parapsilosis*	DQ318803.1
	Ca control	**ATCC 22019**	**Viable Lyo [a] FCol [b]**	***Candida albicans* (strain control)**	
1	Hc36	36	FCol [b]	*Histoplasma capsulatum*	DQ239891
2	Hc69	69	FCol [b]	*Histoplasma capsulatum*	DQ239892
3	Hc71	71[d]	Viable Lyo [a] FCol [b]	*Histoplasma capsulatum* *Histoplasma capsulatum*	DQ239889 DQ239889
4	Hc200 Hc Control Hc Control	200 ATCC A811 ATCC B923	Fcol [b] Fcol [b] Fcol [b]	*Histoplasma capsulatum* *H. capsulatum* (strain control) *H. capsulatum* (strain control)	DQ239887

[a]Viable Lyo: isolate growth after lyophilization; [b]FCol #: Fungus Collection of the Sao Paulo Institute of Tropical Medicine (continuous subculturing); [c]*all isolates were submitted to lyophilization in 1970; [d]*H. capsulatum* isolate that was viable from lyophilized.

public health problem; consequently a small percentage of chronic infections may turn into a serious progressive and often a fatal systemic disease, especially in immunocompromised individuals (Rees et al., 1998; McNeil et al., 2001; Nobre et al., 2003; Wheat and Kauffman, 2003; Pfaller and Diekema, 2007). The purpose of this research was to study two different methods of fungi preservation. These fungi were maintained by successive subculturing every three months at room temperature, since 1925 and lyophilization, 4°C (1970). Analyses were performed by means of studying the macro and micro morphology, as well as the molecular techniques at random amplified polymorphism DNA (RAPD) and by sequencing conserved region 28S rDNA.

MATERIALS AND METHODS

Strains and maintenance conditions

Seven isolates of different Candida spp: 4 C. albicans (99, 144, 407 and 551), 1 C. pelliculosa (70), 1 C. guillermondii (168), 1 C. parapsilosis (103) and four H. capsulatum isolates from the Fungus Collection (Table 1) Institute of Tropical Medicine were studied by maintaining them in continuous subculturing every three months at room temperature (25°C), and later on (beginning of the 70s) by lyophilization (4°C) (Table 1). Lyophilized isolates were inoculated

onto Sabouraud-dextrose agar and broth (25ºC), and the isolates that continued to grow thereon were designated as viable (25ºC) (Table. I). All cultures were verified for purity and then subcultured into tubes containing Sabouraud-dextrose agar, brain-heart infusion (Difco, Detroit, Michigan) and tryptone soya broth (Oxoid, London, England). Isolates that were preserved by continuous subculturing were designated as Fungus Collection (FCol), whereas those preserved by lyophilization were designated as lyophilized (Lyo). The isolates which were maintained by lyophilized were inoculated in culture media and after the fungi growth they were designated as viable (Viable Lyo). Isolates maintained in continuous subculture were used as quality control in all identification tests: C. albicans, ATCC 64548 and H. capsulatum ATCC A811 and ATCC B923. These strains were lyophilized to conduct the study.

Mycological characteristics

Micromorphology and biochemistry: Candida spp

Presumptive identification of isolates C. albicans was performed by germ tube test by incubation at 37ºC in sterile bovine fetal serum. All isolates Candida spp. were analyzed their micromorphological features by slide culture on rice/tween 80 agar. The genus Candida were identified on the basis of a limited number of morphological features and biochemical properties such as the ability to produce true hyphae, pseudohyphae or chlamydospores, and the ability to assimilate a range of compounds such as the sole source of carbon or nitrogen (Kwon-Chung & Bennett, 1992; de Hoog et al., 2000).

Micromorphology: Histoplasma capsulatum

Histoplasma capsulatum isolates maintained by FCol and viable Lyo were re-identified. The micromorphological features of these isolates were observed by slide culture on potato agar. In these isolates were evaluated through dimorphism at 37°C in brain-heart infusion broth supplemented with ovine erythrocytes (Kwon-Chung & Bennett, 1992; Lacaz et al., 2002; Kauffman, 2007).

DNA extraction

The **FCol , Lyo** and **Viable Lyo** isolates were grown from a single colony at 25°C in Sabouraud broth supplemented with chloramphenicol (100 mg/mL), up to the log phase corresponding to 5 in the McFarland scale (1.5×10^9 cells/mL), under constant agitation (40 rpm). The cells were washed with 0.5 M EDTA (pH 8.0). Cell wall lyses of *H. capsulatum* cells was incubated in 200 U of lysing enzyme (L1412; Sigma Chemical Co., St. Louis, MO, USA) at 37°C for 3 h in 1 M sorbitol, 100 mM EDTA and 14 mM βMe. The *Candida* spp. lyses were carried out using the same buffer, to which 200 U of lyticase (Sigma) was added. The isolates were then incubated for 30 min at 30°C and centrifuged (5000 × g; 7500 rpm) at room temperature. The precipitate was resuspended in 180 µL of ATL buffer (QIAamp DNA Mini Kit; QIAgen, Valencia, CA, USA). Cell membrane lysis of all isolates was carried out through incubation in proteinase K (100 mg/mL) at 56°C for 3 h. DNA was extracted using the QIAamp DNA MiniKit (QIAgen) and the DNA was quantified by spectrophotometry (determining the optical density ratio at 260/280 nm). The DNA integrity was evaluated by electrophoresis on a 1% agarose gel. The DNA was extracted from all strains, as well as the lyophilized, regardless of them being viable or not.

RAPD

The band profile of all strains was viewed on a 2% agarose gel stained with ethidium bromide (10 ng/mL). We used the following primers: 1281 - 5'-AACGCGCAAC-3', 1253 - 5'-GTTTCCGCCC-3', 1283 - 5'- GCGATCCCCA-3', 1247 - 5'-AAGAGCCCGT3' and P1 - 5'-GCAATCCCCA-3', for *H. capsulatum* and *Candida spp* (Kersulyte et al., 1992; Leung et al., 2000; Binelli et al., 2006). Similarities between band profiles were analyzed by means of Gelworks 1D Advanced V.4.01 software (Pearson's coefficient / algorithm: UPGMA), considering differences in the position and quantity of the bands. The experiments were performed using the primer which exhibited the best discriminatory capability. Therefore, this technique was repeated five times with each "primer" with the DNA of the same isolate. The reference isolates were not used in the dendogram analysis process; consequently, this study analyzed each isolate maintained by the different methods used in our Laboratory.

DNA sequencing

We used a concentration of 200 ng for *Candida* spp, and 50 ng for *H. capsulatum*, the reactions were developed with universal fungal primer (P1 5'-ATC AAT AAG CGG AGG AAA AG-3'; P2 5'-CTC TGG CTT CAC CCT ATT C- 3'), amplifying a 799-bp fragment. We followed the conditions for amplification recommended by Sandhu et al. (1995). The product of this (PCR) polymerase chain reaction (28S region) was purified by means of purelink PCR purification kit (Invitrogen, Carlsbad, CA, USA) and analyzed using electrophoresis on a 1% agarose gel with TAE buffer [1X] at 80 V, stained with ethidium bromide (10 ng/mL). Quantification was performed using the low DNA mass ladder (Invitrogen) on a 1% agarose gel. DNA sequencing was carried out with 10 µL of the PCR product (concentration = 20 ng/µL) using the DYEnamic ET Dye terminator cycle sequencing kit (Thermo Sequence™ II DNA Polymerase; Amersham Biosciences), by using the MegaBACE 1000 system (Amersham Biosciences, Piscataway, NJ, USA). We analyzed the sequences using the Sequence Analyzer/Base Caller Cimarron 3.12 program and identification was carried out using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Furthermore, multiple alignments were determined using Clustal W (http://www.ebi.ac.uk/clustaw) and all sequences were submitted to the GenBank with the following accession numbers (Table 1).

RESULTS

The aim of this research was to evaluate 11 pathogenic dimorphic fungi preserved by lyophilization and continuous subculturing. The preservation methods of these dimorphic fungi were evaluated from reactivated cultures and examined microscopically to establish micromorphological features from genus Candida and Histoplasma. H. capsulatum isolates maintained in continuous culturing and the Hc 71 isolate (Viable Lyo) were systematically examined microscopically to establish micro-morphological characters from *Candida* spp and *H. capsulatum*.

The micro-morphological aspects of *Candida* strains from the Viable Lyo were typical according to their respective species. However, the isolates preserved with continuous culturing revealed major differences due to the alterations in the morphological aspects and the isolates Ca99 and Ca70 presented typical morphology due to the presence of blastoconidia and chlamydo-spores of these species (Figure 1). No alteration was

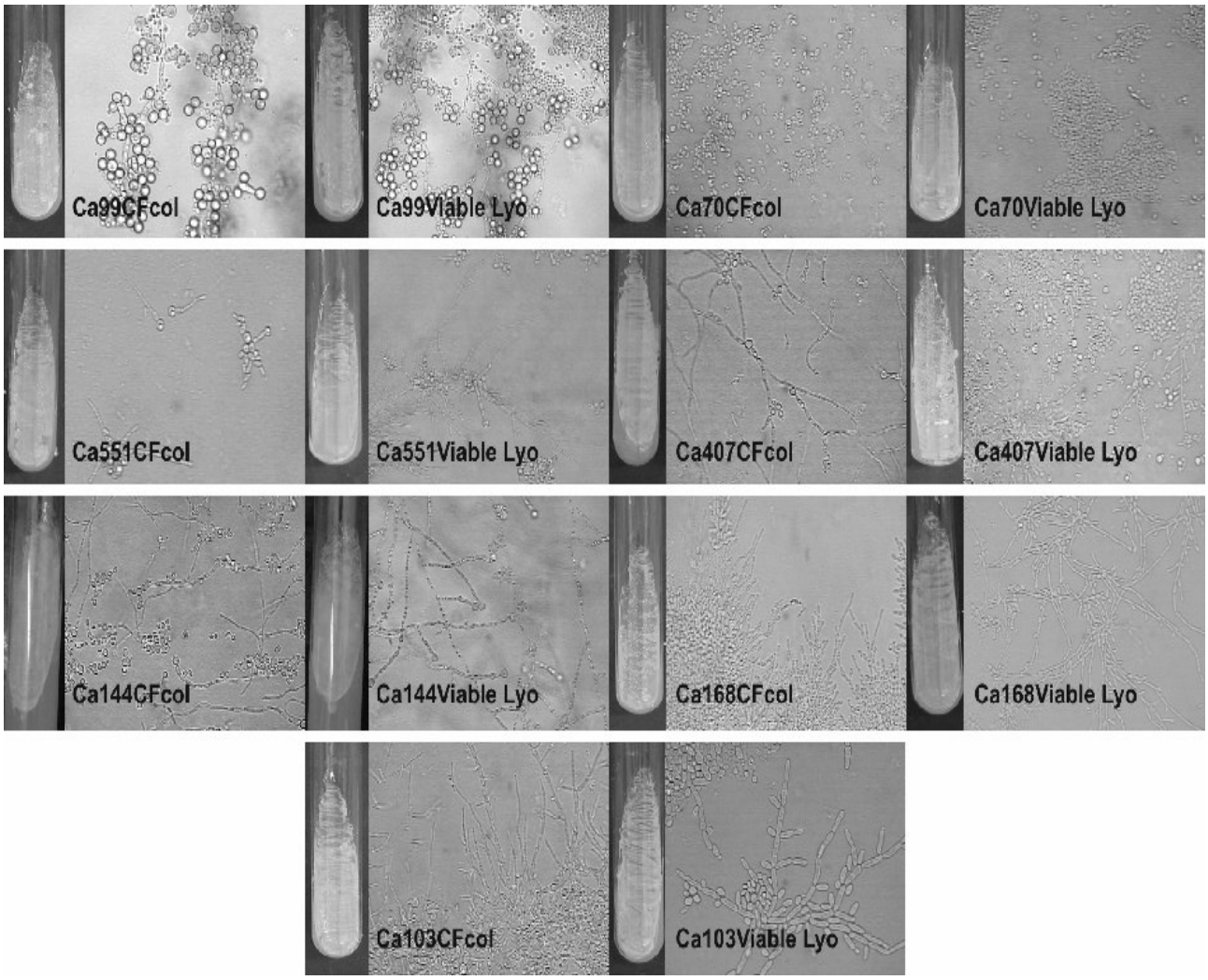

Figure 1. Micromorphological characters of the strains *Candida spp*. micromorphology obtain in corn meal agar (400X).

observed in the analyses of all isolates, furthermore the reference isolates were used for mycological and biochemical diagnosis (exclude this paragraph). Molecular analysis of each strain was performed by sequence (28S rDNA region conserved), as well as RAPD. The selection criteria which were used for analyses were strains grouped by similarity between the bands. The difficulties were found in the selection of the "primers" that represent the best discriminatory capacity for each isolate.

A 799-bp fragment from the 28S rDNA was amplified from all isolates and the DNA sequences obtained. An alignment sequence was generated by the CLUSTAL W and it revealed differences in nucleotides between FCol and Viable Lyo in these isolates, no alterations were present in the other 5 isolates. Strain 4 FCol (407 -*C. albicans*) showed the deletion of six nucleotides in three positions (346-352; 626-632; 691-697), while isolates 7 FCol (103 - *C. parapsilosis*) revealed the insertion of three nucleotides in the 10 -13 position with 13 nucleotides deleted from several different regions (Figure 2). RAPD analysis showed that isolates presented similarities in more than 90% of them, by using Gelworks 1D Advanced V.4.01 software; therefore the isolates 4 (Ca407 - *C. albicans*) and 7 (Ca103 - *C. parapsilosis*) presented significant alterations between FCol and Viable Lyo. *H. capsulatum* in continuous culturing and the Hc 71 isolate (Viable Lyo) were systematically examined microscopically to establish the presence of conidia and macroscopically to analyze the conservation of typical morphologies (Table 2). Alteration in micromorphology characteristics was observed from the hyphae of these dimorphic fungi maintained by continuous culturing of the isolates. RAPD analyses of the profiles are shown in (Figure 3). Analyses were carried out using the primer that presented the best discriminatory distinguishing power among the isolates of each species. *H. capsulatum* isolates were analyzed using the 1253

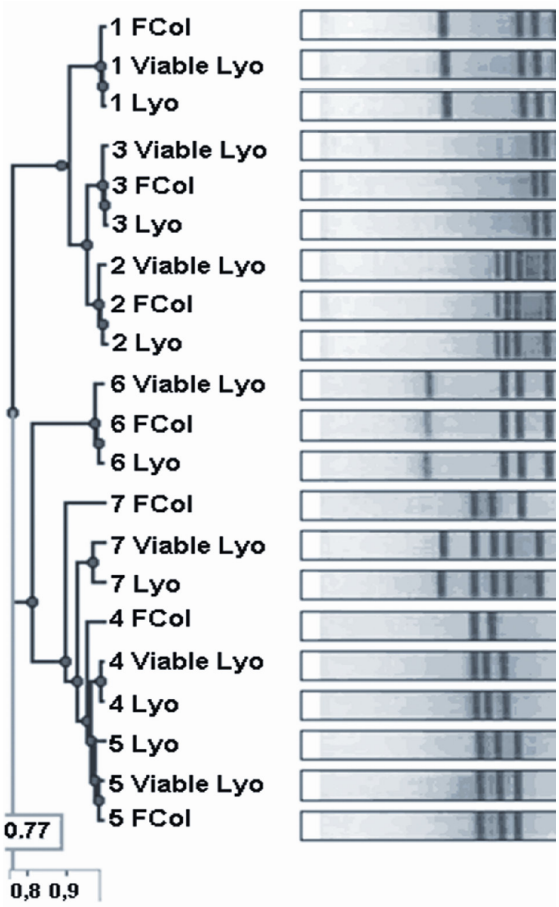

Figure 2. Similarity analysis between RAPD band profiles of the isolates of *Candida* spp. Gelworks 1D Advanced 4.01/Database v. 1.12. (Lyo) Lyophilized material was inoculated onto Sabouraud dextrose culture agar, (Viable Lyo) strains that presented growth and (FCol) fungal collection.

primer. The Hc71 was more than 85% similar to that conserved in culture and viable from the lyophilization; the similarity between the ones conserved in culture and which were viable from the lyophilization 1 (Hc36) and 2 (Hc69) was of more than 70%. The isolates Hc ATCC A811 and Hc ATCC B923 were used as profile patterns of isolate bands from *H. capsulatum*, but were not utilized for RAPD analysis. Sequencing of Hc71 isolate preserved by culturing, revealed alterations, such as nucleotide deletion, nucleotide insertion and in particular, nucleotide exchanges (C-T; T-G; G-A; A-G) at various positions.

DISCUSSION

The importance of the present study lies in the evaluation of two methodologies for the preservation of fungi isolates (fungus collection of the Institute Tropical Medicine), studying alterations of their phenotypic and especially genotypic characteristics over a period of 35 years. The alterations observed in the isolates preserved for prolonged periods of time are described in literature (McNeil et al., 2001; Cavalcante et al., 2007).

The process of lyophilization maintenance proved to be appropriate for the preservation of yeasts of the genus *Candida* spp. since 90% of the isolates had retained their viability and the phenotypic characteristics typical of their respective species. The genus *Candida* is an effective method as observed by Cavalcante et al. (2007), who emphasized the importance of selecting the appropriate preservation method. However, lyophilization was not effective for the *H. capsulatum,* since it interfered negatively with viability, probably in the mycelia form (dimorphic fungi), suggesting that water loss and protein denaturation resulted in loss of cell activity during the lyophilization procedure (Silva et al., 1994; Beattie et al., 1997; Borba and Rodrigues, 2000; Lima and Borba, 2001). We observed the absence or rare production of conidia in *H. capsulatum* isolates that were preserved in continuous culturing and Hc 71 isolate (Viable Lyo) isolate that was lyophilized. Borba et al. (2008) analyzed *P. brasiliensis* variant strains preserved under mineral oil for decades, by RAPD random amplified polymorphic DNA analysis (RAPD), but with the purpose of identifying a virulence fragment marker. The study showed a RAPD pattern that was reproducible and also discriminated between virulent strains with typical morphology and non virulent strains with a typical morphology. The data confirms what we found alterations in both aspects: molecular and mycological.

Mendes da Silva et al. (1994) in an effort to reestablish the dimorphim process in *P. brasiliensis* strains, used five strains in the transitional phase due to the long preservation time in mineral oil and two strains in the yeast-like phase were inoculated into male albino rats and as a result it was verified that the one in oil favored the alteration of cell wall contents, leading to differences in pathogenicity. In our study of the RAPD grouped by similarities of the profile pattern bands from the same strains in different preservation methods, only the *Candida* isolates presented ideal similarities.

The data obtained from the RAPD analysis of the band profiles are shown in (Figure 2). The strains 4 (Ca407 - *C. albicans*) and 7 (Ca103- *C. parapsilosis*) presented significant alterations in the RAPD reaction band profiles, but they demonstrated to have 95 and 90% similarities, respectively. Lyophilization is an effective method for storing *Candida* species strains, maintaining their viability for prolonged periods of time.

Molecular techniques have extraordinary tools for the evaluation of genotypic alterations, allowing the analyses of the genomic DNA of fungi for taxonomic and phylogenetic studies, as well as facilitating the identification of isolates. RAPD reaction amplifies random DNA sequences in order to determine genomic polymorphism, thereby allowing the comparative analysis of a single isolate preserved by two different methods.

Table 2. Morphology of *H. capsulatum* strains preserved under continuous subculture.

Strain		Growth time (Days)		Macromorphology	Micromorphology 25°C
		Sabouraud-dextrose 25°C	BHI- sheep blood (L) 37°C		
Hc 36	FCol[b] (presence of dimorphism)	14	17	Membranous	Thin, septate, branched hyaline hyphae and presence of conidia (tubercle macroconidia)
Hc 69	FCol[b] (presence of dimorphism)	14	17	Membranous	Thin, septa, hyaline hyphae absence of conidia.
Hc 71	Viable Lyo[a] (presence of dimorphism)	21	30	Membranous	Thin, septa, branched hyaline hyphae and presence of conidia (tubercle macroconidia)
Hc 71	FCol[b] (presence of dimorphism)	21	21	Membranous	Thin, septa, hyaline hyphae and presence of conidia (micro and tubercle macroconidia)
Hc 200	FCol[b] (presence of dimorphism)	14	21	Cotton	Thin, septate, branched hyaline hyphae and presence of conidia (micro and tubercle macroconidia)

[a] Viable Lyo: isolate growth after lyophilization; [b] FCol: fungus collection of the Sao Paulo Institute of Tropical Medicine (continuous subculturing); *all isolates were submitted to lyophilization in 1970; [c] *H. capsulatum* isolate that was viable from lyophilized.

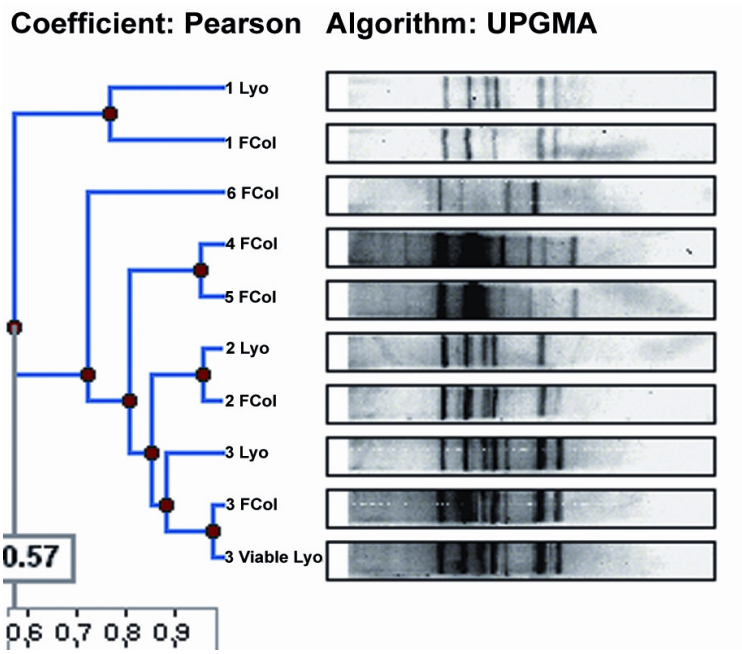

Figure 3. Similarity analysis between RAPD band profiles of the isolates of *H. capsulatum*. Gelworks 1D Advanced 4.01/Database v. 1.12. Lyo - Lyophilized material was inoculated onto Sabouraud dextrose culture agar, Viable Lyo - strains that presented growth and FCol- fungal collection.

This technique involves the primers that contain information for the identification of several polymorphic loci and the differences found can reveal mutations such as insertions or deletions of nucleotide sequences (Williams et al., 1990; Cavalcanti and Cavalcanti, 1994; Cavalcante et al., 2007).

According to Dobzhansky (1973), morphological alterations detected over a 35 years period may occur independently of genotypic alterations. Nevertheless, our findings demonstrate significant alterations as shown by the RAPD analyses that were confirmed by sequencing the conserved 28S rDNA region of the fungi. Such alterations are generally defined by variations in the DNA sequences that occur at frequencies of less than 1%, characterizing genomic polymorphism. The polymerphisms detected by the RAPD analyses are generated by mutations or rearrangements such as insertions or deletions between two different sites or within the primer hybridization site itself.

Plasticity characteristics in the strain may reflect an inherent ability of the *H. capsulatum* to adapt to environmental changes. Franzot et al. (1998) reported genotypic alterations and microevolution of a standard strain of *Cryptococcus neoformans* resulting in virulence alterations and other phenotypes. In this study the isolates preserved over a 35 years period revealed morphological and genetic alterations. There are no reports in literature on the genomes of fungi over such a prolonged period of time. Therefore, our data provides further evidence of the alterations in fungi genome, which might lead to potentially irreversible phenotypic variations. We believe that a study of the Hc 71 isolate (Viable Lyo) is necessary to understand the process of gene adaptation which allows the viability of this isolates.

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