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Genetic diversity of *Histoplasma* and *Sporothrix* complexes based on sequences of their ITS1-5.8S-ITS2 regions from the BOLD System

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ABSTRACT

High sensitivity and specificity of molecular biology techniques have proven usefulness for the detection, identification and typing of different pathogens. The ITS (Internal Transcribed Spacer) regions of the ribosomal DNA are highly conserved non-coding regions, and have been widely used in different studies including the determination of the genetic diversity of human fungal pathogens. This article wants to contribute to the understanding of the intra- and interspecific genetic diversity of isolates of the *Histoplasma capsulatum* and *Sporothrix schenckii* species complexes by an analysis of the available sequences of the ITS regions from different sequence databases. ITS1-5.8S-ITS2 sequences of each fungus, either deposited in GenBank, or from our research groups (registered in the Fungi Barcode of Life Database), were analyzed using the maximum likelihood (ML) method. ML analysis of the ITS sequences discriminated isolates from distant geographic origins and particular wild hosts, depending on the fungal species analyzed.

This manuscript is part of the series of works presented at the "V International Workshop: Molecular genetic approaches to the study of human pathogenic fungi" (Oaxaca, Mexico, 2012).

Diversidad genética de los complejos *Histoplasma* y *Sporothrix* en función de las secuencias de sus regiones ITS1-5.8S-ITS2 del BOLD System

Palabras clave:
Histoplasma capsulatum
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Región ITS
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RESUMEN

Las técnicas de biología molecular han proporcionado instrumentos de alta sensibilidad y especificidad, útiles para la detección, identificación y tipificación de diferentes patógenos. Las regiones ITS (*Internal Transcribed Spacer*) del ADN ribosómico están altamente conservadas y no son codificantes. Estas regiones se han utilizado ampliamente en diferentes tipos de estudios, incluida la determinación de la diversidad genética de hongos patógenos del ser humano. La finalidad de este artículo es contribuir al conocimiento de la diversidad genética intra- e interespecífica de aislamientos de los complejos de *Histoplasma capsulatum* y *Sporothrix schenckii* a través del análisis de las secuencias disponibles de las regiones ITS en distintos bancos de secuencias. Las secuencias de las regiones ITS1-5.8S-ITS2, de cada hongo, depositadas en el GenBank, junto con las obtenidas por nuestros grupos de investigación (depositadas en la Fungal Barcoding of Life Database), se analizaron con el método de máxima probabilidad (ML, por sus

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[e] These authors participated in the design and coordination of the Mexican Thematic Net for Fungi Barcode of Life (MEXBOL) of the Consejo Nacional de Ciencia y Tecnología (CONACyT)-MEXICO and have equally contributed to this review.

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siglas en inglés). El análisis ML de las secuencias de las regiones ITS discriminó aislamientos de orígenes geográficos distantes y de huéspedes salvajes particulares, de acuerdo con la especie fúngica analizada.
Este artículo forma parte de una serie de estudios presentados en el «V International Workshop: Molecular genetic approaches to the study of human pathogenic fungi» (Oaxaca, México, 2012).

Reliable identification of pathogenic fungal species is fundamental to epidemiology in terms of biodiversity, geographical variation, and environmental changes. Species identification in fungi is particularly challenging because of their transient nature. Limitations to the studies of diversity in mammalian pathogenic fungi exist due to a lack of taxonomic specialists, and scarce and incomplete data for many taxonomic characters, which has been suggested by Suwannasai et al.[17] and Tantichareon.[19]

Pheno- and genotyping of fungal strains have been used as important tools for identifying environmental sources of outbreaks as well as confirming the existence of pathogens in natural habitats. These different typing methods have used both conventional and molecular techniques.

Although phenotyping has continuously been used to study fungi, sensitive and specific genotyping methods are being developed to characterize fungal species, but different criteria must be met to be accepted by specialists. In many cases, genotyping methods compare DNA polymorphisms and classify fungal organisms according to the principles of molecular systematic. For *Histoplasma capsulatum* (etiological agent of the systemic mycosis histoplasmosis) and *Sporothrix schenckii* (etiological agent of the subcutaneous mycosis sporotrichosis) typing and classification, different molecular techniques have been applied, among them, various PCR methods using genomic sequences.[7,8]

There is a wide array of molecular markers for microorganism identification and genotyping or molecular classification. Among them, the Internal Transcribed Spacer (ITS) regions stand out for the study of closely related taxa, due to genetic diversity associated with the high rate of evolutionary changes characteristic of these regions.[12] ITS consist of two variable non-coding regions (ITS1 and ITS2) inserted between the highly conserved small subunit 18S, the 5.8S, and the large subunit 28S of the rDNA gene cluster.[12]

ITS as a molecular target for fungal identification are supported by several unique characteristics: (i) The complete ITS region has a length between 600 and 800 bp and can be easily amplified, using universal primers that are complementary to rDNA sequences. (ii) The multicopy nature of the repeat regions of the rDNA allows for the amplification of the ITS regions from small, diluted or degraded DNA samples. (iii) Several studies have demonstrated that the ITS regions are highly variable among morphologically distinct fungal species.[12]

The usefulness of ITS markers has been documented in several studies of phylogeny and genotyping of *H. capsulatum*[1,5,6,11] and *S. schenckii*.[2–4,22]

The Mexican Barcode of Life project for the *H. capsulatum* and *S. schenckii* species complexes

The Mexican Barcode of Life (MEXBOL) resulted from the work of Mexican investigators as part of the international DNA barcoding (iBOL) project. MEXBOL is now part of a network with funding from the Consejo Nacional de Ciencia y Tecnología (CONACyT) and the Comisión Nacional para el Conocimiento y Uso de la Biodiversidad (CONABIO). The Natural Sciences and Engineering Research Council of Canada (NSERC) developed a Barcode of Life Database (BOLD) based on a specific informatics infrastructure. The cytochrome oxidase subunit 1 (COI), ribulose-bisphosphate carboxylase (rbcL), maturase K (matK), and ITS regions are among the Barcode sequences used. In addition to the assembly of barcode information and maintenance of these records by the BOLD system, a copy of all sequence and key specimen data is archived at the National Center for Biotechnology Information (NCBI) or its sister genomic repositories, the DNA Data Bank of Japan (DDBJ) and the European Molecular Biology Laboratory (EMBL), when results are ready for public release.[13]

The identification of *H. capsulatum* and *S. schenckii* isolates from different sources and origins by the sequences of the ITS regions started in 2010 as a project for the MEXBOL network for fungi. To date there are 19 ITS1-5.8S-ITS2 sequences of *H. capsulatum* from the Laboratorio de Inmunología de Hongos and 10 sequences of *Sporothrix* spp. from the Laboratorio de Micología Básica, Departamento de Microbiología y Parasitología, Facultad de Medicina, UNAM, deposited in the BOLD System. Sequences were obtained from isolates that were previously pheno- and genotypically well identified.[10,14,15]

Data regarding the natural hosts, sources, and samples of the 19 *H. capsulatum* and 10 *Sporothrix* spp. isolates are shown in Table 1. Fungal specimens are deposited in the Culture Collection of *H. capsulatum* from the Laboratorio de Inmunología de Hongos and the Culture Collection of Fungal Pathogens of the Laboratorio de Micología Básica, from the Departamento de Microbiología y Parasitología, Facultad de Medicina, UNAM. In addition, they are registered in the database of the World Federation for Culture Collection, with code number LIH-UNAM WDCM817 for *H. capsulatum* (http://www.wfcc.info/ccinfo/index.php/collection/by_id/817) and code number BMFM-UNAM WDCM834 for *Sporothrix* spp. (http://www.wfcc.info/ccinfo/index.php/collection/by_id/834).

Analysis of the genetic diversity of *H. capsulatum* and *S. schenckii* species complexes based on ITS sequences from MEXBOL project

Current data from our laboratory teams, using evolutionary and genetic distance analyses by maximum likelihood (ML) of ITS1-5.8S-ITS2 sequences of *H. capsulatum* or *Sporothrix* spp. from the BOLD System and GenBank datasets, produced robust results to aid in understanding the similarities and diversities among isolates either of *H. capsulatum* or *Sporothrix* spp. from different sources and geographic origins.

Sequences were generated by PCR assays with ITS5/ITS4 primers[9] for *H. capsulatum* and ITS1F/ITS4 primers[9] for *Sporothrix* spp. Fig. 1 shows the predicted products, 607 bp for *H. capsulatum* and 575 bp for *Sporothrix* spp, amplified by their respective primers. The ML trees generated are shown in Fig. 2.

Concerning *H. capsulatum*, Fig. 2A highlights the sequences of all isolates from different geographic origins and phylogenetic species that clustered together in a major group sustained by 99% of bootstrap values (BT). This finding confirms the high similarity of the isolates analyzed, separates a reference strain of *Ajellomyces dermatitidis* (nearby sister), and underlines the genetic distance from a heterologous pathogenic fungus, *Paracoccidioides brasiliensis*, used as an outgroup in the ML analysis. The ML tree topology of *H. capsulatum* sequences in Fig. 2A clearly confirms that inter-specific diversity among fungal pathogens that cause respiratory diseases