

Genetic Diversity of *Histoplasma Capsulatum* Isolates from Honduras

Carmen Galo¹, Ana L. Sanchez², Gustavo A Fontecha^{3*}

¹ Maestría en Enfermedades Infecciosas y Zoonóticas, Escuela de Microbiología, Universidad Nacional Autónoma de Honduras (UNAH). Ciudad Universitaria, J1 Edificio, 4to piso. UNAH. Tegucigalpa, Honduras.

² Department of Community Health Sciences, Brock University, 500 Glenridge Ave. St. Catharines, Ontario, Canada. L2S 3A1.

³ Instituto de Investigación en Microbiología, Escuela de Microbiología, Universidad Nacional Autónoma de Honduras (UNAH). Ciudad Universitaria, J1 Edificio, 4to piso. UNAH. Tegucigalpa, Honduras.

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ABSTRACT

Aim of the study: *Histoplasma capsulatum* is a thermo dimorphic fungus endemic to Honduras but the genetic diversity and phylogeography of circulating strains in the country have not been studied.

Materials and Methods: Twenty-six *Histoplasma capsulatum* isolates were analyzed; these were recovered from Honduran patients hospitalized with a diagnosis of locally acquired disseminated histoplasmosis. A multilocus approach was used through DNA sequencing of the ITS rRNA region and four independent protein-coding regions: *arf*, *ole*, *H-anti* and *tub*.

Results: Cluster analysis revealed all isolates in three congruent clades. A comparison of the sequences of native isolates with homologous sequences from North America and Latin America showed a geographic pattern, suggesting their inclusion into the LAmA group.

Conclusion: This investigation is the first to study the genetic diversity of *H. Capsulatum* in Honduras. More research to expand the understanding of the molecular epidemiology of this important pathogen is needed in the Central American region.

KEYWORDS: *Histoplasma capsulatum*; molecular phylogeny; Honduras.

INTRODUCTION

Histoplasma capsulatum is the causative agent of histoplasmosis, a pulmonary and systemic mycosis in humans. Most of the infections caused by *Histoplasma* are asymptomatic or produce mild clinical pulmonary manifestations. It has been estimated that more than 5% of cases progress to a severe disease, especially among immunocompromised individuals, such as AIDS patients [1, 2] and transplant patients receiving immunosuppressive therapy [3].

Histoplasma is a cosmopolitan fungus with worldwide distribution, but the endemicity of the infection is significantly higher in very well-known areas of the American Continent, such as Mid east regions of the USA [3], Mexico [4] and Brazil [5].

Despite the rising clinical relevance of histoplasmosis during recent decades, large knowledge gaps still remain. In the Americas in particular, the identification of transmission

patterns in different geographical zones as well as the distribution of fungal genotypes, natural reservoirs and infection risk factors deserve more investigation.

In recent years, *H. capsulatum* research has seen an increase of interest in regards to the fungus genetic variation and the potential role of genetic diversity in virulence as well as in geographical distribution [6, 7], while some studies have intended to clarify the phylogeography of this fungus on a regional or on a more extensive manner [8].

In the late 1980's, isozyme variation was the pioneer approach to analyze phenotype-genotype diversity [9] among *H. capsulatum* isolates. DNA-based techniques have also been used to discriminate isolates and fungal strains in a more sensitive way. Because of their technical advantages, random amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphisms (RFLP) have been widely implemented to establish correlations between genetic clusters and geographical location of isolated strains [10-13]. Some works succeeded in classifying the isolates based on a geographic pattern. In Brazil, for example, RAPD fingerprinting has been able to discriminate among *H. capsulatum* strains from different regions [10, 13]. Using RAPD, other authors have found a correlation between *Histoplasma* isolates from human patients with isolates from bat excreta [14]. However, a classification system for an organism based exclusively on RAPD fingerprints has a limited use because of concerns about reproducibility [15, 16].

More recently, sequencing of the internal transcribed spacer (ITS) region of rRNA gene has been used to classify *H. capsulatum* isolates from USA [17], Brazil [7] and other countries [18], because of its ability to differentiate genotypes based on nucleotide sequence variations. Gene sequencing is one of the most recent approaches to demonstrate the existence of genetically distinct phylogenetic species of *Histoplasma*. Using four protein-coding genes, Kasuga et al were able to propose eight geographically based clades to classify isolates from six continents [8]. This is the most extensive effort to establish a general classification of *H. capsulatum* based on a multilocus sequencing approach. However, worldwide

mapping of *H. capsulatum* genotype distribution is far from being complete. For example, isolates from Honduras or other Latin American countries have not been studied. Thus, through gene sequencing of five molecular markers (ITSrRNA, *arf*, *ole*, *H-anti*, *tub*), this study will provide additional information on the genetic diversity of this important pathogen.

MATERIALS AND METHODS

Ethical Statement

The present study was exempt from research ethics review. The investigation entailed secondary use of anonymized fungal cultures derived from biological materials collected for medical diagnosis with no initial intent to be used in research. Physicians had sent biological specimens to the clinical laboratories for fungal isolation and diagnosis, upon which, cultures were donated by the laboratory director to the researchers. Each isolate had an accompanying paper slip providing brief information as to the origin of the isolate but without personal identifiers or hospital record numbers. This anonymization process eliminated the risk for future re-identification of individuals. In absence of Honduran ethical guidelines, Canadian guidelines were adhered to as laid out in the TCPS 2-2nd edition of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (Article 2.4 and Chapter 12)[19]. In addition, an exemption letter was obtained from the Research Ethics Board of the School of Microbiology, National Autonomous University of Honduras (UNAH).

Isolates of *Histoplasma capsulatum*

Twenty-six anonymized *Histoplasma capsulatum* cultures were obtained from the same number of hospitalized Honduran patients between 2007 and 2011. Isolates had accompanying brief clinical reports. All patients had a clinical diagnosis of systemic histoplasmosis and fungal identification had been performed at the clinical laboratories of two major national hospitals (14 from the cardiopulmonar hospital and 12 from the university hospital), both located in Tegucigalpa, Honduras. Nine patients were female, 16 were male, and in one, sex was not reported. Age ranged from 4-66 years of age (median: 38) and there was only one child among the patients. In 14 cases, an AIDS diagnosis was reported; the child was suffering of severe malnutrition but no co-morbidities were stated for the remaining 11 patients. Specimens were collected from blood (n=14), cerebrospinal fluid (CSF) (n=4), lymph node biopsy (n=5), bone marrow (n=1), feces (n=1) and urine (n=1).

Cultures were transported as per guidelines for transportation of dangerous goods [20] a biosafety-level 2 laboratory at the National University of Honduras (UNAH), also in Tegucigalpa. Once at UNAH's laboratory, cultures were plated in Sabouraud's dextrose agar and Mycosel agar and incubated at 25 °C and 37 °C. Cultures were handled inside a biosafety cabinet (BSC) type II and any potentially bio hazardous waste was autoclaved according to

International guidelines [20]. Isolates were designated as HN1 to HN26.

DNA extraction

Genomic DNA was purified from mycelial-phase cultures of *Histoplasma*. Mycelium was grounded with a mortar and pestle in liquid Nitrogen. Nucleic acids were extracted by the DNeasy Blood & Tissue Kit (Qiagen® Hilden, Germany). DNA was quantified by Nanodrop technology (Nanodrop Technologies, Oxfordshire, UK) and its quality analyzed by ethidium bromide agarose electrophoresis. DNA was stored at -20 °C until further use.

Amplification of five genes of *H. Capsulatum*

Four *Histoplasma capsulatum* nuclear genes encoding delta-9 fatty acid desaturase (*ole*), ADP-ribosylation factor (*arf*), H antigen precursor (*H-anti*), and alpha-tubulin (*tub*) were amplified from all isolates according to Kasuga et al [21] with minor modifications. Briefly, primers sequences (5'-3') were as follows: *ole3* (tttaaacaagccccacgg) and *ole4* (caccacctcaacagcagca); *arf1* (agaatatggggcaaaaagga) and *arf2* (cgcaattcatcttcgttgag); *H-anti3* (cgcagtcacctccatactc) and *H-anti4* (gcgccgacattaacc); *tub1* (ggtggccaatcgcaactc) and *tub2* (ggcagcttccgttccctcagt).

The four reaction mixtures contained 100 ng of template DNA, 2X PCR Master Mix (Promega Corp., Madison, WI), 22.5 ng of each primer, in a total volume of 50µl. A "touchdown" PCR was performed as follows. A first step was a 2 min denaturation at 94°C followed by 12 cycles of 94°C for 15 s, 65°C - 0.7°C/cycle for 30 sand 72 °C for 1 min; and then 20 cycles of 94°C for 15 s, 56°C for 30 s and 72°C for 1 min. The final stage was a 5 min extension at 72°C. Negative controls were included in all cases.

The fifth sequence amplified was the ITS1-5.8S-ITS2 region according to Jiang [17]. Primers used were P3 (cggaaggatcattaccagccg) and 2R8 (cacgggtatccctactgatc). PCR Master Mix was similar to that described above but decreasing the primer concentration to 2 ng each. The amplification reactions were carried out under the following conditions: 94 °C for 10 min, 40 cycles of 94 °C/1 min, 47 °C/2 min, 72 °C/2 min and a final step of 72 °C for 10 min. All PCR products were electrophoresed on a 2% agarose gel to determine their sizes. Amplicons were purified by the Wizard® SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI).

Sequencing, analysis and Dendrogram construction

Purified PCR products were sent to MacroGen Facilities located in Maryland (US) and sequenced from both strands. Sequences were edited, aligned and compared with Chromas Pro (Technelysium) and Mega 5 software[22]. Edited sequences were deposited in GenBank (NCBI). Homologous sequences from isolates obtained in other geographic regions of the world were obtained from NCBI available databases. Phylogenetic trees were constructed for each gene using Mega 5 software [23] and the UPGMA method with a 500 replicates bootstrap test. Evolutionary distances were computed using the Maximum Composite Likelihood

method. Mesquite software was used to align sequences and establish phylogenies in order to be deposited in TreeBase (<http://treebase.org/>). Nucleotide diversity (π) was estimated by the Tajima's Neutrality Test [24]. NCBI sequences from ATCC strains for the five genes were used as outgroup for tree construction.

RESULTS AND DISCUSSION

Sequence analysis of five nuclear genes

DNA sequences of PCR products were determined for five nuclear genes. Generated sequences were visually edited to ensure good quality information and only those that were entirely satisfactory were deposited in the GenBank nucleotide sequence database (Accession numbers: JQ218335-JQ218359 (ITS1-5.8S-ITS2); JQ218399-JQ218420 (*ole*); JQ218421-JQ218436 (*arf*); JQ218360-JQ218378 (*H-anti*); and JQ218379-JQ218398 (*tub*)).

Nucleotide diversity ($\pi = 0.00753-0.01851$) was relatively low for all five *loci* due to evolutionary conservation of the selected genes. Additionally, the percentage of polymorphic sites ranged from 3.39% to 5.62% (Table 1), similar to data previously described [14, 21] for those *loci*. This result reveals that sequence variations do exist in these five genes

of *H. capsulatum*. Therefore, sequence analysis of these *loci* is a convenient tool to determine the genetic diversity of *Histoplasma* within a geographic region. Nucleotide diversity estimates revealed low heterogeneity among *H. capsulatum* isolates from Honduras. When the five markers were compared with each other, *arf* gene showed the lowest nucleotide diversity and *tub* gene the highest.

Phylogenetic analyses of Honduran *H. capsulatum* isolates

Sequences of five genes and UPGMA clustering method were used to analyze the phylogenetic structure of 26 *H. capsulatum* isolates from Honduras. Homologous sequences from *H. capsulatum* G217-B and ATCC38904 reference strains were incorporated in the analysis as outgroups (NCBI Accession Numbers: X85962; AF495651; AF495652; AF495654; AF322378). Trees and sequence alignments were deposited in TreeBase under the following Accession URL: <http://purl.org/phylo/treeBase/phyloWS/study/TB2:S14249>. Dendrograms based on the *H-anti* gene and the ITS1-5.8S-ITS2 region clustered the isolates into three separated clades, while the tree generated for *tub* gene grouped the isolates into two clades. A lower clustering capacity was revealed by *arf* and *ole* genes, which did not resolve internal groups of individuals (Figure 1).

Table 1. Lengths and sequence divergence of the analyzed genes

Locus	S	π	Sequence length (bp)	% Polymorphic sites
<i>arf</i> [28]	13	0.00753	384	3.39
ITS [29]	17	0.00831	529	3.21
<i>ole</i> [30]	19	0.00915	424	4.48
<i>H-anti</i> [31]	20	0.01214	394	5.08
<i>tub</i> [32]	10	0.01851	178	5.62

Abbreviations: S = Number of segregating sites, π = nucleotide diversity [33].

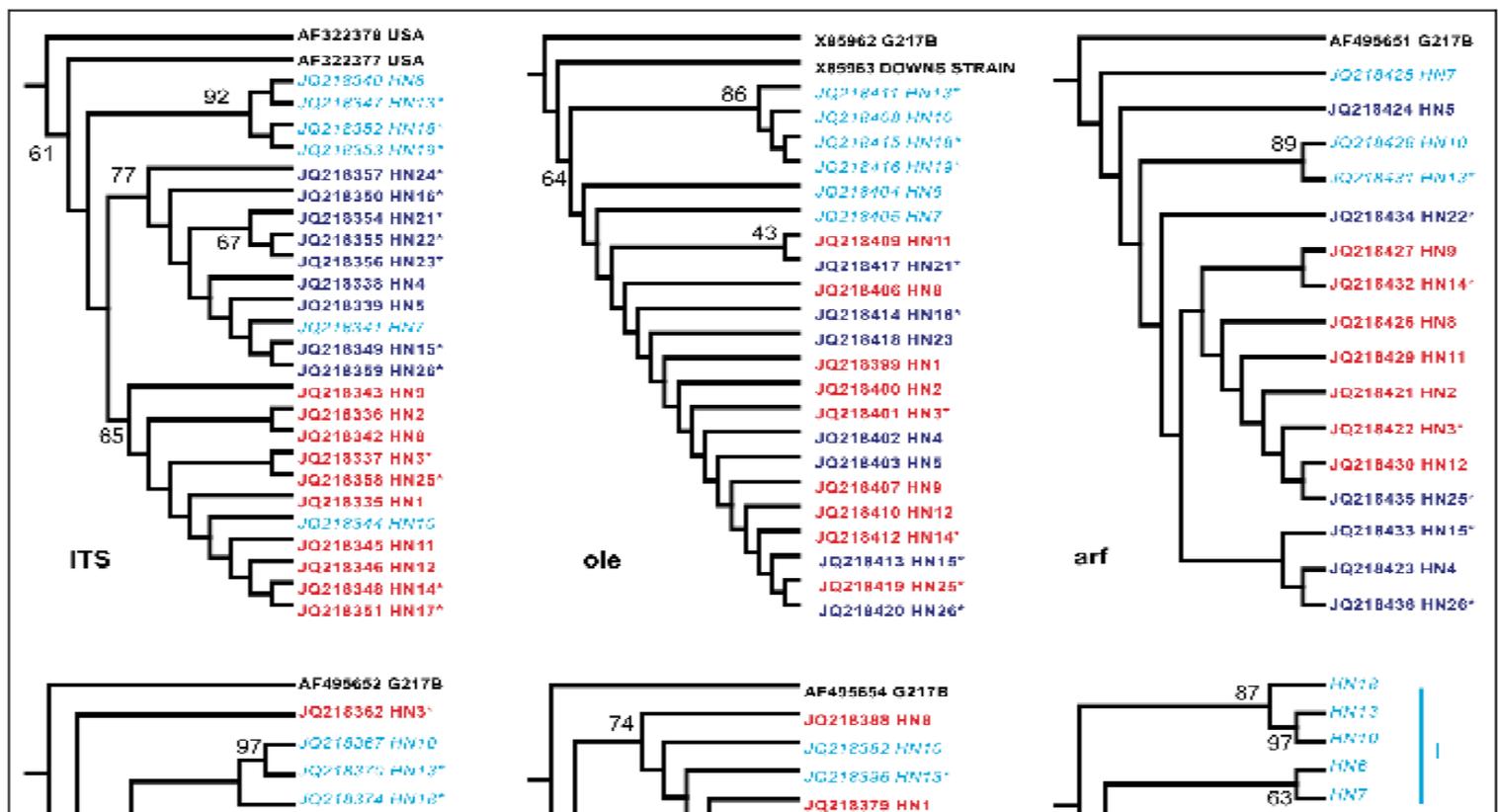


Figure 1. UPGMA trees of *Histoplasma capsulatum* isolates. Bootstrap = 500 replicates. Evolutionary distances were computed using the Maximum Composite Likelihood method. Positions containing gaps and missing data were eliminated. Three colors (blue, purple and red) have been used to indicate which isolates clustered into each clade. (*) Isolates recovered from HIV-infected patients.

By combining information for the five loci, a 2,081bp chimeric sequence was obtained that was used to construct a consensus dendrogram (Figure 1). According to this dendrogram, native *H. Capsulatum* isolates clustered into three clades, which were designated as HNI, HNII and HNIII. Outgroup sequences were always separated from the rest of Honduran sequences.

The consensus tree showed that most (n=9) sequences clustered in clade HNIII. Clade II was represented by seven sequences and clade I by five sequences.

A relative high concordance of clustering was observed among dendrograms obtained from each gene and the consensus dendrogram. *H-anti* gene seemed to separate individuals in a more similar pattern compared to the consensus tree.

Based on the congruency of how *Histoplasma* isolates clustered by partial sequences of five independent genes, a three-subclade classification could be proposed for Honduran strains. Unfortunately, the specific geographic provenance of each patient was unknown and therefore, correlation of phylogenetic clusters with a geographic origin is not possible at this time. However, given that Honduras comprises a relative small territory (112,492 Km²) without internal geological barriers, it is unlikely that geographical

patterns could be identified. Furthermore, since some systemic infections are the result of a secondary reactivation, it would be impossible to know where infection took place anyway [25].

It has been hypothesized that different *H. capsulatum* genotypes could be correlated with the severity of histoplasmosis and AIDS [26, 27]; however, isolates recovered from AIDS patients and the way those isolates clustered did not reveal any correlation, and all three sub clusters were composed of *Histoplasma* isolates cultured from AIDS patients (Figure 1). The lack of relatedness between AIDS and fungal genotype could indicate that the five *loci* used in this study are not appropriate markers to evaluate *Histoplasma* pathogenicity in HIV-infected patients.

Comparison of Honduran *H. capsulatum* isolates with homologous NCBI sequences from North America and Latin America

DNA sequences from native isolates of *Histoplasma* were compared to homologous sequences available from strains and isolates recovered both North America and Latin America (Table 2). Unfortunately, only three sequences belonging to Kasuga's Latin America B group [8] were available in the GenBank. For this reason Argentinean isolates could not be included in the present study.

Table 2. Gene sequences used in this study

Gene	GenBank Accession Numbers	Location
ITS	AF322377-78, AF322380, AF322382-83, EU048555-56, DQ239895-96, AF458085-86, AB071824, AB071832, AB055231	USA, Mexico, Colombia, Brazil, Argentina
<i>ole</i>	FJ435629-33, FJ435635, AF495629, AF495601, AF495645, AF495593, AF495609, AF495613, AF495617, AF495649, GU320994, GU320996, GU321003, GU321030, GU321032-34	Mexico, Brazil
<i>arf</i>	GU320852, GU320859, GU320864, GU320869, GU320835, GU320837, GU320841, GU320844, AF072367, AF495595, AF495591, AF495603, AF495619, AF495640, AF495644	Mexico, Argentina, Colombia, Brazil
<i>H-anti</i>	AF495652, AF495636, AF495628, AF495600, AF495643, AF495592, AF495604, AF495624, AF495639, GU320900, GU320934, GU320927, GU320896, GU320929, GU320907, GU320889, GU320902	Mexico, Brazil
<i>tub</i>	GU321067, GU321050, GU321070, GU321080, GU321038, GU321041, GU321056, GU321087, AF495602, AF495622, AF495606, AF495610, AF495626, AF495642, AF495650	Mexico, Brazil

Figure 2 shows the similarities among native and foreign *H. capsulatum* sequences through five dendrograms constructed by a UPGMA clustering approach. Reference strains (v.gr. G217B, Downs, ATCC60915) as well as homologous sequences from fungal related species

(*Paracoccidioides braziliensis*, *Lacazi aloboi*) and *H. capsulatum* var. *duboisii* were included as out group.

In each of the five trees the out group sequences were shifted away.

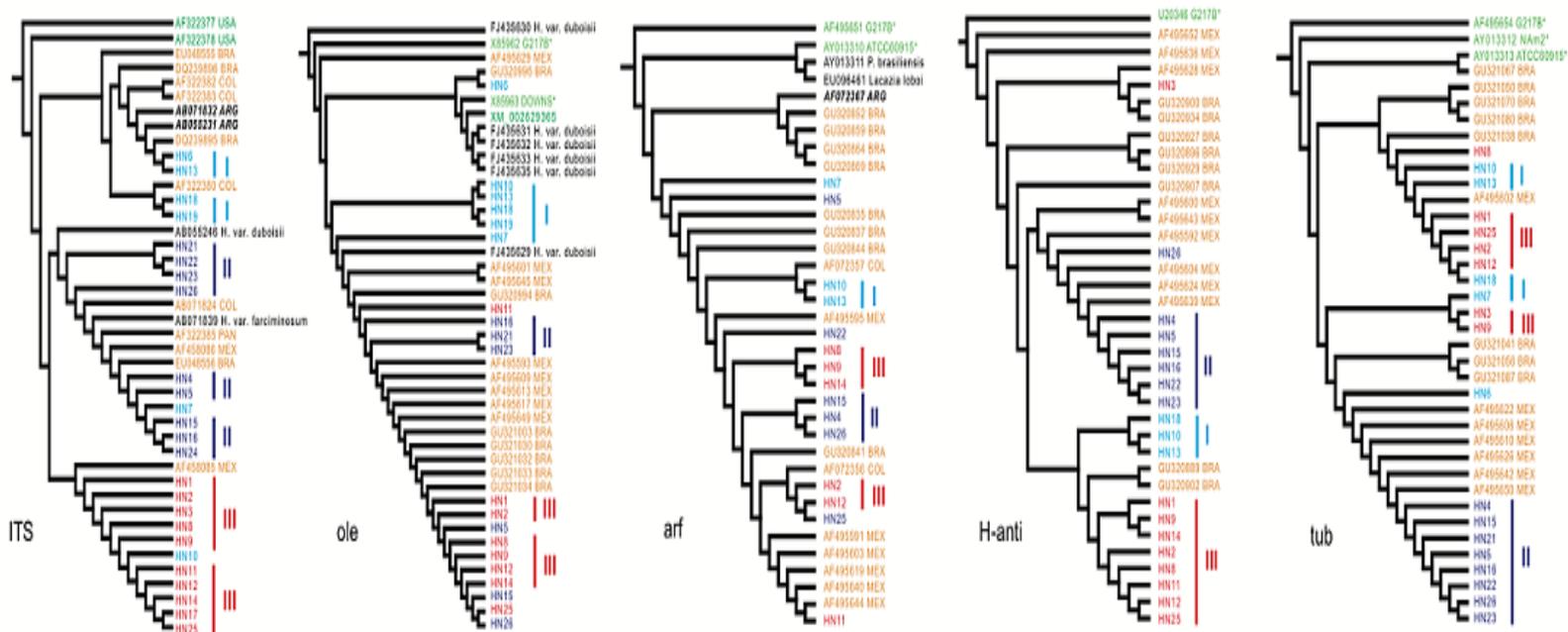


Figure 2. UPGMA trees of *H. capsulatum* isolates from Honduras and other geographic

Cluster analysis revealed no exclusive groups of Honduran isolates with any of their homologous sequences from Brazil, Mexico or Colombia. This result indicates that the present study's data set does not require proposing a new phylogenetic population for Honduran or Central American isolates but their inclusion in a preexistent and wider geographic group. It is interesting to note that North American isolates always remained separate from Latin American and Honduran isolates, which support the idea of genetically separated geographical populations in the American continent.

Several attempts to classify and define the genetic structure of *H. capsulatum* populations have been done. Until recently, this pathogen was classified into three varieties or subspecies based upon phenotype and pathogenicity: *H. capsulatum* (Hc) var. *capsulatum*, (Hc) var. *duboisii* and (Hc) var. *farcinosum*. However, the application of molecular techniques has modified this classic paradigm. In 1999 Kasuga et al proposed a new classification of *Histoplasma* in six classes based on the analysis of four partial protein-coding genes and isolates from the American Continent [21]. This proposal included two North American classes, two South American classes and one class from Central America with isolates only from Panama. Four years later, the same research team proposed a new classification into eight phylogenetic species by including a higher number of isolates coming from the six Continents and using the same four molecular markers [8]. In this work, Central American isolates were represented only by Panama and Guatemala, lacking representation from the remaining countries (Honduras, El Salvador, Nicaragua, Belize and Costa Rica). The new classification included two North American classes (NAm1 and NAm2); two Latin American groups (LAmA and LAmB), previously named "South American" classes; and four clades representing non-American classes.

According to those studies, the Latin American A group is formed by isolates from Brazil, Panama, Mexico, Guatemala and Colombia, excluding those from Argentina and some isolates from Colombia.

As observed in the present study, the sequences from Honduran isolates tend to group together with sequences from Brazil and Mexico, both belonging to the class LAmA, and reveal to be distant from NAm strains or sequences from *H. capsulatum* var. *duboisii*. Therefore, the results suggest that Honduran isolates should be included into the most extensive Latin American class A. This is not a surprising result since Honduras is strategically located in the middle of the American Continent and the movements of migratory animals, such as bats and birds, could contribute to spreading fungal spores throughout the region [6, 14]. Besides the insufficient number of sequences from the LAm B group in our analysis, only represented by two sequences for the ITS region and one for gene *arf*, LAm B isolates remained separated from Honduran sequences in those trees.

In conclusion, this is the first phylogenetic study of *H. capsulatum* isolates recovered from human patients from Honduras. The study's results suggest that Honduran isolates could be divided into three subclades and that they could be included in the LAm A class proposed by Kasuga et al [8].

RECOMMENDATION FOR FURTHER STUDIES

New studies including samples from the rest of Central American countries are needed in order to expand our current knowledge in regards the molecular epidemiology of *Histoplasma capsulatum* in Central America.

Summary Box

- All Honduran isolates of *Histoplasma capsulatum* were clustered in three congruent clades.
- A sequences analysis of conserved genes suggests the inclusion of Honduras isolates into the LAmA group.
- This is the first report of the genetic diversity of *H. capsulatum* in Honduras.

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