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Evaluation of Genetic Variability of *Cryptococcus neoformans* VNB Isolates

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A B S T R A C T

Phylogenetic analysis of pathogenic microorganisms contributes to better understanding the distribution of genotypes that may be specific of certain regions and maybe associated with an increased virulence or antifungal resistance. This study performed phylogenetic analysis of 105 Sequence Types (ST) described for the VNB genotype of *Cryptococcus neoformans* available on the MLST database in order to better understand this population structure. We found three main clusters, being Cluster 1 the largest with 83 STs and Cluster 2 the smallest with three STs. In general, the isolates presented high genetic variability with haplotype diversity (HD) of 0.998 and nucleotide diversity (π)of 0.00372.Results also demonstrated recombination events, although PHY test showed no significance (p=5.5511). These findings are important since they illustrate that a genotype previously restricted to Africa and now distributed worldwide presents high genetic variability, with potential impact in the development of increased virulence, antifungal resistance, among other factors.

Introduction

Cryptococcosis is a worldwide distributed fungal infection, and the second most prevalent in HIV/AIDS patients after candidiasis. It may be a localized or disseminated infection, especially in immunocompromised patients (not only due to HIV infection, but also due to diabetes, transplants, hematological neoplasms, and other immunosuppressing factors) and its most severe and prevalent manifestation is acute meningoencephalitis. Immunocompetent patients, however, can also be affected (Mitchell and Perfect 1995, Kurtzman and Fell 2011).The *Cryptococcus* genus includes several species; however, only *Cryptococcus neoformans* and *Cryptococcus gattii* are

recognized as pathogenic (Kwon-Chung, Polacheck *et al.,* 1982). *Cryptococcus neoformans* was the first basidiomycete to have its genome thoroughly sequenced (Kavanagh 2006). This species has five main genotypes: VNI, VNII, VNIII, VNIV and VNB. VNB genotype was previously described as a group of serotype A *C. neoformans*, genetically diverse haploid and geographically restricted to Botswana (Chen, Litvintseva *et al.,* 2015). Later on, VNB isolates were described in different parts of the world, such as Sequence Types (ST) restricted to Brazil (Andrade-Silva, Ferreira-Paim *et al.,* 2018).

Different molecular techniques have been used for molecular characterization of *Cryptococcus spp*. Nowadays, the Multilocus Sequence Typing technique (MLST) is often used in studies of *C. neoformans* and *C. gattii*: this method utilizes sequences of different *loci* to characterize genetic variability of pathogenic microorganisms, being attractive due to its high reproducibility and discriminatory power. The International Society for Human and Animal Mycology (ISHAM) standardized a MLST protocol that is the most widely adopted worldwide. This protocol allows for better comparisons of results to assess the real genetic variability between isolates from different regions of the world (Meyer, Aanensen *et al.,* 2009). Furthermore, its data can be used to determine several factors such as: species recognition, recognition of different populations within species, determining whether a microorganism is purely clonal or has undergone recombination, associating genetic and phenotypic characteristics and assigning the origin of an unknown individual. Therefore, phylogenetic analysis of pathogenic microorganisms contributes to better understanding the distribution of genotypes that may be specific of certain regions and may, sometimes, be associated with an increased virulence or antifungal resistance (Taylor and Fisher 2003).

This study aimed to perform phylogenetic analysis of the described STs for the VNB genotype, obtained according to the MLST consensus for *C. neoformans*, in order to better understanding of the population structure of this genotype.

Materials and Methods

Fungal sequences evaluated

We have evaluated all the 103 STs type VNB available at the *C. neoformans* MLST database (http://mlst.mycologylab.org/Biolomics.aspx?Table=Sequence%20types%20C.%20neoformans), along with two VNB STs described by the research group from the Mycology Lab at the TrianguloMineiro Federal University (UFTM), Uberaba, Brazil (Ferreira-Paim, Andrade-Silva *et al.,* 2017, Andrade-Silva, Ferreira-Paim *et al.,* 2018), also currently available at MLST database. Multiple alignment was performed in the Clustal W software (https://www.ebi.ac.uk/Tools/msa/clustalw2/)(Thompson, Higgins *et al.,* 1994, Thompson, Albert *et al.,* 2014). The phylogenetic analysis was performed in MEGA 6.0 software using the Neighbour Joining (NJ) method with 1,000 bootstrap replicates (Tamura, Stecher *et al.,* 2013). The extent of DNA polymorphisms along with its relevant description parameters, such as the number of polymorphic sites (S), nucleotide diversity (p), number of haplotypes (h), haplotype diversity (Hd), and average number of nucleotide differences (k), were calculated using DNAsp 5.10 (Librado and Rozas 2009). Furthermore, Tajima's D, Fu & Li's F*, and Fu's Fs tests for neutrality were performed. Recombination degree was calculated using the Watterson estimator (theta) method (also available on DNAsp

5.10) and by the Splits Tree software, v 4.13.1 (http://www.splitstree.org/) (Barreto de Oliveira, Boekhout *et al.,* 2004, Huson and Bryant 2006). The Pairwise Homoplasy Index (PHY) test was used to infer whether there was statistical significance for recombination.

Results and Discussion

Among the 105 VNB genotype isolates analyzed, we found three main clusters that were differentiated both via Neighbour Joining (Fig.1) and Split Decomposition (Fig 2) methods. Cluster 1 was the largest with 83 STs. Cluster 2 was composed by 3 STs (ST26, ST393, and ST527) and Cluster 3 by 19 STs. The two VNB isolates from UFTM (STs 527 and ST504) were allocated in Clusters 2 and 3, respectively.

Phylogenetic analysis by the Maximum Likelihood method of the STs available at MLST database for VNB *Cryptococcus neoformans*

The analysis involved 103 VNB *C. neoformans* isolates from MLST database (mlst.mycologylab.org) and two VNB STs from Uberaba, Brazil (highlighted in red). The phylogenetic tree is drawn to scale, with each branch lengths measuring the number of substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. Final dataset presented a total de 4014 positions. Numbers at each branch indicate bootstrap values > 50% based on 1,000 replicates by the Maximum Likelihood algorithm. The isolates identification is described by their Sequence Type number (ST).

Split decomposition analysis of the 105 VNB *Cryptococcus neoformans* evaluated

This analysis occurred applying the Neighbour Net algorithm using the uncorrect-p parameter model and evidencing the diversity and branching ambiguities due to recombination events. Isolates are linked to each other via multiple pathways and forming and interconnected network instead of a single forked tree, which suggests the occurrence of recombination; the phi test for recombination implemented in the Split Tree software, however, didn't show significant evidence (p = 5,5511).

The STs from the three main clusters identified in the phylogenetic analysis (Fig. 1) were also discriminated via split decomposition as follows: Cluster 1: red, Cluster 2: blue, and Cluster 3: green. The two VNB STs from Uberaba are highlighted in red.

Analysis of the DNA polymorphisms is displayed on Table.1. As presented by our data, the isolates showed high genetic variability with a haplotype diversity (HD) of 0.998 and a nucleotide diversity (π) of 0.00372. The results also demonstrated that the isolates had undergone at least 15 recombination events.

In addition, split decomposition analysis demonstrated occurrence of recombination, visualized by the formation of parallelograms between neighbors using the Neighbor Net algorithm (Fig 2). However, PHY test showed no statistically significance for recombination (p = 5.5511). The Tajima's D, Fu & Li's D, Fu & Li's F, and Fu's F neutrality tests demonstrated evidence of purifying selection or population expansion.

The present study found that the 105 VNB isolates can be sorted in three main clusters (as showed in Fig. 1); this finding corroborates a previous study, performed by Litvintseva *et al.,* (2006), on this serotype, although the clusters were nominated differently (VNBa, VNBb, and VNBc) (Litvintseva, Thakur *et al.*, 2006).

Fig.1 Phylogenetic analysis by the Maximum Likelihood method of the STs available at MLST database for VNB *Cryptococcus neoformans*

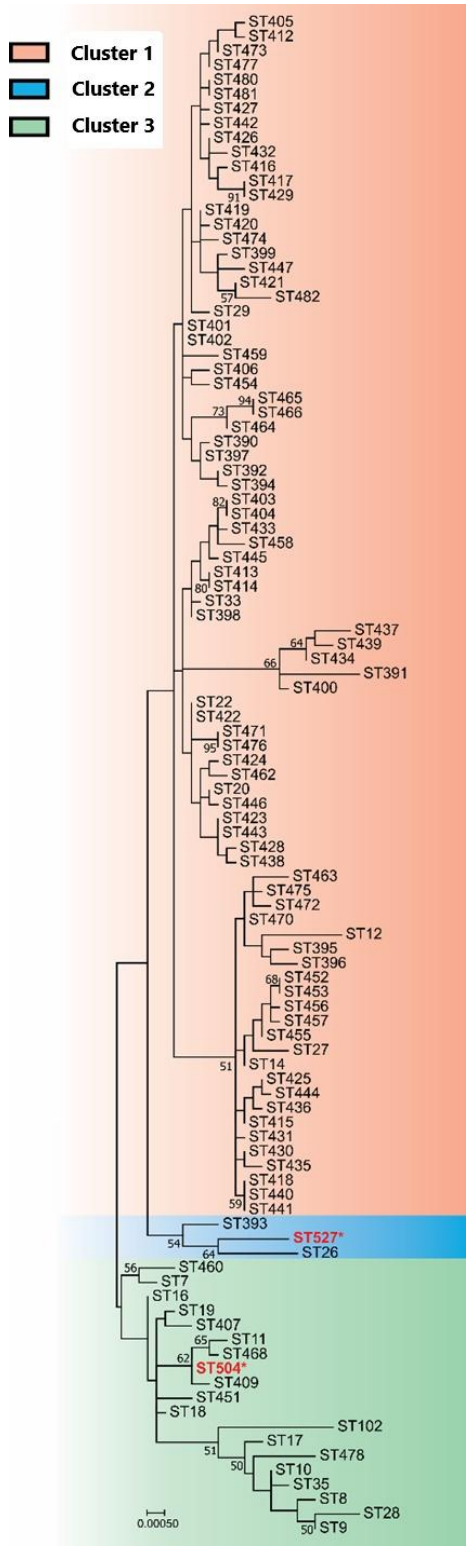

Fig.2 Split decomposition analysis of the 105 VNB *Cryptococcus neoformans* evaluated

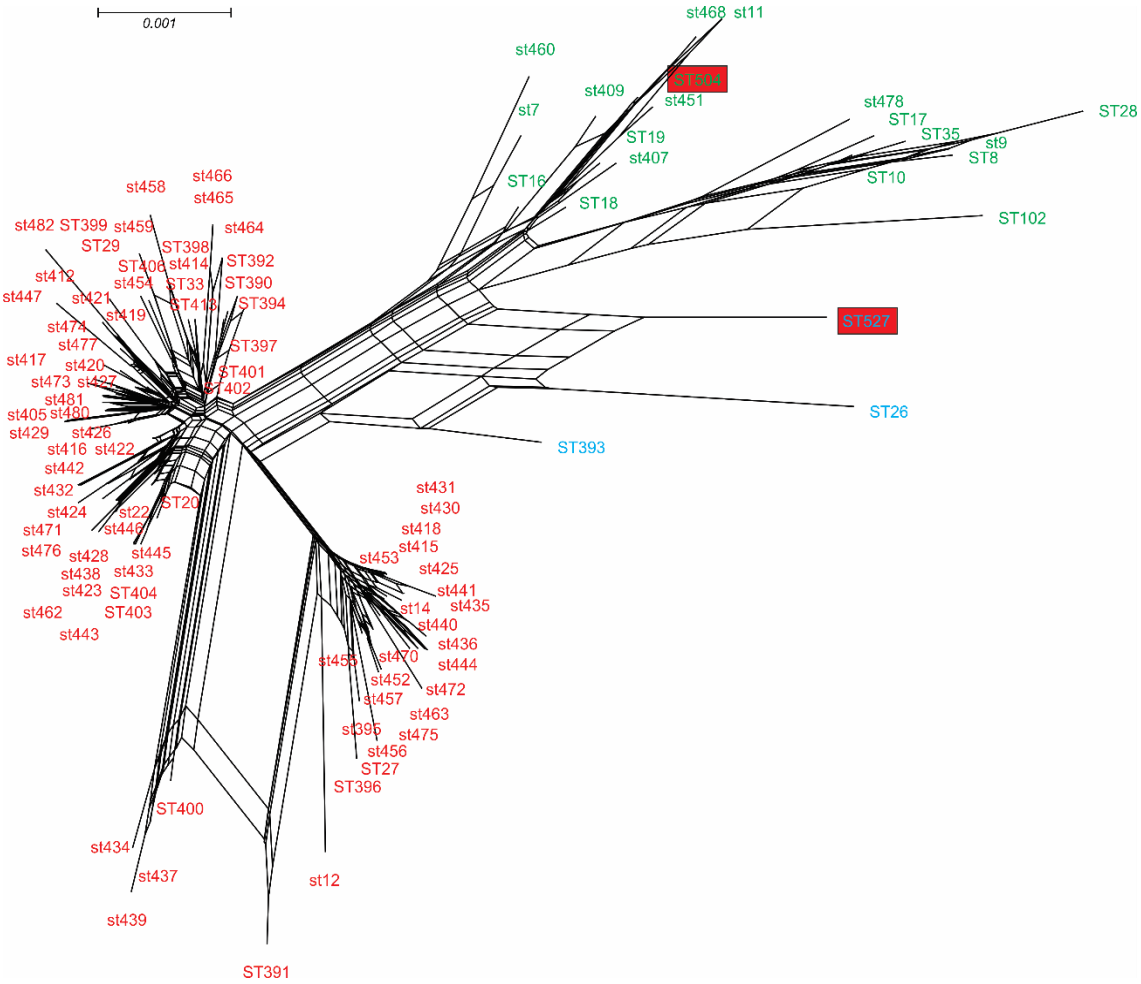

Table.1 DNA polymorphisms in the 105 VNB genotype *Cryptococcus neoformans* isolates analyzed in this study

Number of isolates	Length	S	*Π*	k	h	Hd	D	FD	FF	FS	Theta-w	Rm	PHY
105	4014	95	0.00372	14.752	105	0.998	- 0.6417	- 0.37831	- 0.57431	- 94.559	18.777	15	5.5511

Caption: S – number of polymorphic sites; π – nucleotide diversity, Pi; k – average number of nucleotide differences; h – number of haplotypes; Hd – haplotype diversity; D – Tajima's D; FD – Fu and Li's D; FF – Fu and Li's F; FS – Fu's Fs; Theta-w – Theta (per sequence) from S; Rm – Minimum number of recombination events.; PHY – Pairwise Homoplasy Index.

The two isolates from Uberaba (ST527 and ST504) were grouped in Clusters 2 and 3, respectively. In a previous study from the research group of the Mycology Lab, they had been clustered in a same group (VNBA and/or VNBII). However, that study analysed significatively less isolates (21 STs) from the VNB genotype (Andrade-Silva, Ferreira-Paim *et al.*, 2018). Thus, the segregation of the isolates in two different clusters probably occurred due to the incorporation, in the present study, of a larger number of isolates with higher genetic variability among them.

As for the genetic variability, our findings were higher than described for VNI genotype *C. neoformans*, for which the HDs varied between 0.50-0.65, and πbetween 0.0012-0.0033 for isolates from South America; worldwide isolates showed a HD of 0.89 with π = 0.0023(Litvintseva, Thakur *et al.*, 2006, Chen, Litvintseva *et al.*, 2015, Ferreira-Paim, Andrade-Silva *et al.*, 2017).

Taken together, this study's results demonstrate that the VNB isolates of *C. neoformans* present high genetic variability and occurrence of recombination events. These findings are important since they illustrate that a genotype previously restricted to Africa and now distributed worldwide presents high genetic variability; it potentially results in phenotypical changes that may increase virulence and adaptation to other geographic niches, shift antifungal susceptibility, among other relevant factors.

The use of more data from the isolates such as their clinical and geographic origin, denomination of subgenotypes, susceptibility profile, clinical data from patients can help improving current findings and demonstrating possible correlations between molecular and phenotypic features.

The VNB isolates analyzed in this study were distributed into three main clusters. They displayed high genetic variability and had possibly undergone recombination events.

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