Major Article

Multilocus sequence typing (MLST) of clinical and environmental isolates of Cryptococcus neoformans and Cryptococcus gattii in six departments of Colombia reveals high genetic diversity

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Abstract

Introduction: The average annual incidence of cryptococcosis in Colombia is 0.23 cases per 100,000 inhabitants in the general population, and 1.1 cases per 1000 in inhabitants with Acquired Immune Deficiency Syndrome (AIDS). In addition, the causal fungus has been isolated from the environment, with serotypes A-B and C in different regions. This study aims to determine the genetic association between clinical and environmental isolates of C. neoformans/C. gattii in Colombia. Methods: Multilocus sequence typing (MLST) was used to identify possible clones, providing information about the epidemiology, ecology, and etiology of this pathogen in Colombia. Results: A total of 110 strains, both clinical (n=61) and environmental (n=49), with 21 MLST sequence types (ST) of C. neoformans (n=14STs) and C. gattii (n=7STs) were identified. The STs which shared clinical and environmental isolate sources were grouped in different geographical categories; for C. neoformans, ST93 was identified in six departments, ST77 in five departments; and for C. gattii, ST25 was identified in three departments and ST79 in two. Conclusions: High genetic diversity was found in isolates of C. neoformans/gattii by MLST, suggesting the presence of environmental sources harboring strains which may be sources of infection for humans, especially in immunocompromised patients; these data contribute to the information available in the country on the distribution and molecular variability of C. neoformans and C. gattii isolates recovered in Colombia.

Keywords: Cryptococcus neoformans. Cryptococcus gattii. Multilocus Sequence Typing. Incidence. Colombia.

INTRODUCTION

Cryptococcosis is a fungal disease of worldwide distribution. Patients acquire the infection by exposure and inhalation of fungal propagules present in environmental sources. This infection is considered potentially fatal, and affects the lungs and the central nervous system1-3 in both immunosuppressed individuals and in those with an apparently intact immune system4-5. In Colombia, the annual incidence for this infection is 0.23 cases per 100,000 inhabitants in the general population and 1.1 cases per 1000 inhabitants in Acquired Immune Deficiency Syndrome (AIDS) patients (period 1997-2013)3.

Although different taxonomic classifications have been proposed to categorize the etiological agent of the disease, Hagen et al. have suggested that different molecular types should be considered as independent species6. This suggestion has not been fully accepted by the scientific community7. In the present investigation, we refer to isolates as the C. neoformans species complex and the C. gattii species complex. Cryptococcosis is caused by the C. neoformans species complex and the C. gattii species complex7. The first species consists of two varieties, C. neoformans var. grubii (serotype A) and C. neoformans var. neoformans (serotype D). In addition to the hybrid AD serotype, the species has a worldwide distribution and preferentially affects immunocompromised individuals, mainly those infected with the human immunodeficiency virus (HIV)2-4. In the environment, it has been associated with bird excreta, especially from soils contaminated with pigeon (Columba livia) droppings8.
environment in decaying plant material (hollows, leaf, bark, flowers, soil, fruit) from different trees (Eucalyptus spp., acacias, Ficus spp., and Terminalia catappa) in various regions of the world. It is found mainly in tropical, subtropical, and temperate regions. Currently, several interspecies hybrids have been described between serotypes BD and AB.

Many molecular techniques have been applied in the epidemiological study of C. neoformans and C. gattii isolates. The most common techniques are PCR fingerprinting, restriction fragment length polymorphism (RFLP) of the PLB1 and URA5 genes, amplified fragment length polymorphism (AFLP), and the most recently developed technique of multilocus sequence typing (MLST). For genotyping Cryptococcus species using MLST, six conserved genes (CAP59, GPD1, LAC1, PLB1, SOD1 and URA5) and the intergenic region IGS1 are used. MLST has a high discriminatory power for the genotyping of isolates to determine clonality. It is also highly discriminatory for a large number of pathogens. MLST directly measures changes in the sequence of a series of conserved genes, characterizes isolates by allelic profiles, and is an excellent tool for taxonomic characterization at the molecular level. However, more robust techniques such as whole genome sequencing (WGS) allow for the detection of differences between the molecular types at the genomic level.

In Colombia, studies have been carried out describing the importance and potential relationship between clinical and environmental isolates using molecular typing techniques such as PCR fingerprinting and RFLP. More recently, molecular typing of Crypococcus species complex isolates in Colombia using MLST has been described by Casali A et al. (2003). Briefly, C. neoformans and C. gattii were plated on yeast extract-peptone-dextrose (YPD) agar for 48 hours at 27 °C; 10 μl of yeast cells was placed in an Eppendorf tube using an inoculation loop, and incubated at -20 °C for one hour. The cells were then suspended in 500 μl of lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0, and 1% SDS) and incubated at 65 °C for one hour; 500 μl of phenol:chloroform:isoamyl alcohol (25:24:1) was added, and the sample was centrifuged for 15 minutes at 13,000 rpm. The supernatant was transferred to a new tube, and an equal volume of isopropanol was added. The DNA was precipitated at -20 °C for one hour, and centrifuged for 15 minutes at 4 °C at 13,000 rpm. The DNA was then precipitated with 70% ethanol and centrifuged again for 15 minutes at 4 °C at 13,000 rpm, and subsequently dried at room temperature. The samples were resuspended in 5 μl Tris-EDTA (TE) buffer and stored at 4 °C.

Environmental data

Forty-nine environmental isolates were selected (C. neoformans: n=41; C. gattii: n=8); 73.5% (n=36) were recovered from 10 different types of trees, and 26.5% (n=13) were recovered from Columba livia droppings. Molecular Analysis

a) Genomic DNA extraction was performed as previously described by Casali A, et al. (2003). Briefly, C. neoformans and C. gattii were plated on yeast extract-peptone-dextrose (YPD) agar for 48 hours at 27 °C; 10 μl of yeast cells was placed in an Eppendorf tube using an inoculation loop, and incubated at -20 °C for one hour. PCR products were purified and sequenced commercially by the sequencing service provider Macrogen, Inc. Sequences were analyzed using the Sequencher Software 5.2 (Gene Codes Corporation, MI, USA). Six reference strains were used: WM148 (VNI-CBS10085), WM626 (VNI-CBS10086), WM179 (VGI-CBS10078), WM178 (VGII-CBS10082), WM175 (VGIII-CBS10081), and WM779 (VIVG-CBS10101).
individual locus sequences and the concatenated sequences\textsuperscript{23}. The evolutionary history was derived using the maximum likelihood method based on the Jukes-Cantor model, and bootstrap values were displayed for each branch (1000 repetitions). Allele types and combined sequence types were assigned using the ISHAM consensus database\textsuperscript{24}. The data were tabulated using Microsoft Excel\textregistered. Additionally, \textit{C. gattii} sequences reported previously by Lizarazo J, et al. in 2014\textsuperscript{21} were included to increase the robustness of the analysis.

Genetic diversity of isolates was determined by using the DnaSP v5 software; this variability was extracted from concatenated sequences associated with genes, department, molecular type, and origin (clinical or environmental)\textsuperscript{25}, to detect genetic polymorphism levels. The distribution was determined by calculating the haplotype (gene) diversity, nucleotide diversity (\(\pi\)) (the average number of nucleotide differences per site between two sequences), and \(\theta\) indexes (per site, as an indicator of mutation rate per nucleotide site per generation), calculated from \(\eta\) (the total number of mutations and “S”, the number of segregating/polymorphic sites). Each index was reported with the corresponding standard deviation. The \(\pi\) indexes for each set of data were compared to identify the category with the greatest diversity.

**RESULTS**

A total of 98 isolates were typed by MLST (\textit{C. neoformans}: \(n=88\); \textit{C. gattii}: \(n=10\)); additionally, 12 clinical isolates of \textit{C. gattii} sequences reported previously by Lizarazo J, et al. in 2014 were included\textsuperscript{21}. Twenty-one STs were identified, and 13 STs were assigned to the molecular type VNI and one ST to the \textit{C. neoformans} molecular type VNII; three STs were assigned to molecular type VGII, and two STs each were assigned to the VGI and VGIII molecular types (Table 1). The genetic associations among the

### TABLE 1: Sequence types of Cryptococcus neoformans and Cryptococcus gattii in clinical and environmental isolates from Colombia.

| Molecular type | ST | Antioquia | Atlántico | Bogotá | Cauca | Nte. Santander | Valle | Total |
|---------------|----|-----------|-----------|--------|-------|---------------|-------|-------|
| **Environmental** |    |           |           |        |       |               |       |       |
| VNI           | 15 | -         | -         | 1      | -     | 1             | -     | 2     |
|               | 23 | 4         | 4         | 1      | 1     | 1             | 2     | 13    |
|               | 56 | -         | -         | -      | 1     | -             | -     | 1     |
|               | 77 | 1         | -         | -      | 5     | 1             | 4     | 11    |
|               | 93 | 2         | 5         | 2      | 2     | 1             | 1     | 13    |
|               | 226| 1         | -         | -      | -     | -             | -     | 1     |
| VGII          | 25 | -         | -         | 1      | -     | -             | -     | 1     |
| VGIII         | 75 | -         | -         | 2      | -     | -             | -     | 2     |
|               | 79 | -         | -         | -      | 5     | -             | -     | 5     |
| **Clinical**  |    |           |           |        |       |               |       |       |
| VNI           | 2  | -         | 2         | 2      | 1     | -             | -     | 6     |
|               | 5  | 1         | 1         | -      | 1     | -             | 2     | 5     |
|               | 6  | -         | 1         | -      | -     | -             | -     | 1     |
|               | 63 | -         | -         | -      | -     | 1             | -     | 1     |
|               | 69 | 1         | -         | -      | -     | -             | 1     | 2     |
|               | 71 | -         | -         | -      | -     | 1             | -     | 1     |
|               | 77 | -         | 1         | -      | -     | -             | -     | 1     |
|               | 93 | 6         | 1         | 5      | 3     | 4             | 5     | 24    |
|               | 532| -         | -         | -      | 1     | -             | -     | 1     |
| VNII          | 40 | 1         | -         | 1      | -     | -             | -     | 2     |
| VGI           | 51 | 1         | -         | -      | -     | 1             | -     | 2     |
|               | 58 | -         | 1         | -      | -     | -             | 1     | 2     |
| VGII          | 25 | 2         | -         | 1      | -     | 4             | -     | 7     |
|               | 323| -         | -         | -      | -     | 1             | -     | 1     |
|               | 324| -         | -         | -      | -     | 1             | -     | 1     |
| VGIII         | 79 | -         | 1         | -      | -     | -             | -     | 1     |
| **Total**     | 20 | 16        | 17        | 16     | 22    | 19            |       | 110   |

ST: sequence type.
isolates for \textit{C. neoformans} and \textit{C. gattii} are shown in Figures 1 and 2, respectively. The MLST data of sequences of identified alleles were deposited in the GenBank database (Supplementary material 3).

In 88 clinical and environmental isolates of \textit{C. neoformans}, 14 different STs were identified, the most frequent of which was ST93 (42%), followed by ST23 (14.7%), ST77 (13.6%), ST2 (6.8%), ST5, ST6, ST15, ST40, ST56, ST63, ST69, ST71, and ST226. ST532, a novel \textit{C. neoformans} ST was identified, and this is the first report of this ST worldwide. Of the 22 isolates of \textit{C. gattii}, seven different STs were identified; the most frequent ST was ST25 (36.3%), followed by ST79 (27.2%) and, in lesser proportions, ST51, ST58, ST75, ST323, and ST324. Table 1 shows the STs found in both the clinical and environmental isolates.

Diversity indexes were also calculated per species, department, origin, and molecular type. It was found that the haplotype diversity in \textit{C. neoformans} was 14 and in \textit{C. gattii} it was 11; the nucleotide diversity index was higher in \textit{C. gattii} (Pi= 0.868) than in \textit{C. neoformans} (Pi= 0.779) (Supplementary material 3). The diversity index calculated by departments showed that Cauca and Valle presented with greater diversity of haplotypes for \textit{C. neoformans} (Hd=0.833 and 0.8443, respectively), and Bogotá and Norte de Santander for \textit{C. gattii} (Hd=0.8 and 0.818 respectively). The diversity by type of origin (clinical or environmental) did not vary between the two species. The haplotypic diversity for \textit{C. gattii} was higher in VGI and VGIII (Hd= 0.667 and 0.607 respectively), when compared to VGII (Hd= 0.378) (Supplementary material 4).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{phylogenetic_tree.pdf}
\caption{Phylogenetic analysis of 88 clinical and environmental isolates of \textit{C. neoformans}. The evolutionary history was derived using the maximum likelihood method based on the Jukes-Cantor model using concatenated nucleotide sequences of 7 loci and a representative for each multilocus sequence typing (MLST) sequence type. Bootstrap values are shown for each branch (1000 repetitions).}
\end{figure}
FIGURE 2: Phylogenetic analysis of 21 clinical and environmental isolates of C. gattii. The evolutionary history was derived using the maximum likelihood method based on the Jukes-Cantor model using concatenated nucleotide sequences of 7 loci, and a representative for each multilocus sequence typing (MLST) sequence type. Bootstrap values are shown for each branch (1000 repetitions).

Regarding the association between ST and geographical origin of the strain, C. neoformans ST93 was present in six departments in clinical and environmental samples, and ST77 in five departments (Antioquia, Atlántico, Cauca, Valle, and Nte. Santander); C. gattii ST25 was identified in three departments (Antioquia, Bogotá and N. Santander), and ST79 in two departments (Atlántico and Nte. Santander) (Figure 3).

DISCUSSION

We evaluated the genetic diversity of the C. neoformans and C. gattii clinical and environmental isolates recovered in six departments in Colombia by MLST typing, and found 14 and 7 sequence types for each species among 88 and 22 isolates, respectively. Furthermore, we identified four of the high frequency sequence types globally reported in clinical and environmental isolates, namely ST93 and ST77, for C. neoformans, and ST25 and ST79, for C. gattii. This study is similar to the investigation by Beale A, et al. (2015) who reported 50 different sequences types in 230 isolates of C. neoformans var. grubii in Cape Town and Pietermaritzburg, KwaZulu-Natal, revealing a high degree of genetic diversity and variability in the isolates26.

This study is the first to report sequence type 532 of C. neoformans in a Colombian clinical isolate. This species showed less genetic variability possibly because the majority of isolates were molecular type VNI (n=86), and were associated with 13 STs.

Our data are comparable to that reported in a study by Ferreira-Paim et al. (2017) conducted in Southeastern Brazil, which described low genetic diversity among the isolates of C. neoformans. The most frequent STs reported were ST93, ST77, and ST23, in agreement with this study. This correlation may be because the topological and climatic characteristics of these two countries are similar27.
ST93 was recovered from a majority of clinical and environmental isolates in this study, and it is one of the most widely reported STs in different countries such as China, India, Indonesia, South Africa, Uganda, Thailand, and Brazil, among others. Furthermore, it has also been associated with high mortality in Uganda.

Firacative et al., in 2019, used MLST analysis to show that in Cúcuta, a region with a significant number of cases in Colombia, isolates were highly clonal. The molecular type of all 13 isolates was VGII, with ST25 being the most common (n=11). In our study, diverse species of C. gattii were found, and the isolates were not clonal as reported by Firacative et al. This may be because we included isolates from five different cities and three molecular types (VGI, VGII and VGIII). Although the most common sequence type for C. gattii in the present investigation was ST25, it was identified in three cities: Cucutá (n=4), Bogotá, and Antioquia (n=2 in each city).

In 2016, this same author characterized the genetic structure of the molecular type VGIII by MLST, in 122 clinical, environmental, and veterinary isolates from Australia, Colombia, Guatemala, Mexico, New Zealand, Paraguay, United States of America (USA), and Venezuela. A total of 37 Colombian isolates were included, and ST79 was the most frequent (n=13) for the country. In the present investigation, the molecular type VGIII (n=8) was included, and ST79 was the most common (n=6). This may indicate that several STs of C. gattii are in circulation in the country.

The less frequent STs for C. neoformans were ST5, ST6, and ST56, and for C. gattii, ST51, ST58 and ST75. Some of these STs are prevalent in Europe, Asia, North America, and Oceania. C. neoformans ST5 has been previously found in China, Japan, South Korea, East Asia, and Thailand in clinical cases, environmental samples, and even in veterinary cases in cats. C. gattii ST51 has been found in Australia, China, India, Mexico, Papua New Guinea, and in the USA, in clinical, veterinary, and environmental samples.

Globally and in accordance with the results of various studies, genetic structures vary depending on geographic location. The species of yeast causing cryptococcosis in East Asian populations...
are genetically less diverse compared to those from Europe, Africa, and North and South America\textsuperscript{18,27,34-36}. One possible explanation for the diversity and distribution of sequence types observed in \textit{C. neoformans} in the environment may be bird migration. In South America, the origin of \textit{C. gattii} and possible global dispersion have been described, mainly in regions of Brazil where genetic diversity of this species has been found. The genetic diversity data were obtained using phylogenetic and recombination analyses based on AFLP and MLST\textsuperscript{36}.

**CONCLUSIONS**

The effort to increase knowledge about the genetics of populations of \textit{C. neoformans} and \textit{C. gattii} lies with the appearance of specific genotypes associated with disease and dispersal of genetic populations. MLST revealed significant genotypic variations in \textit{C. neoformans} and \textit{C. gattii} in six departments of Colombia; however, the frequently reported STs indicate that in the country, diverse disease-causing strains are circulating in the environment. Expanding the cohort to other departments has been suggested, to continue detecting circulating strains.

In this study, we used the MLST technique for the molecular typing of \textit{C. neoformans} and \textit{C. gattii} isolates in Colombia. The importance of combining clinical and environmental isolates together with molecular data for the study of cryptococcosis was demonstrated in this study, and this approach was essential to identify genetic associations between types of strains.

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**AUTHORS’ CONTRIBUTIONS**

PA: Writing the proposal for the major grant, experimental work, funding, and writing and critical review of the paper; NV: Experimental work, data analysis, and writing of the paper.

**CONFLICT OF INTERESTS**

The authors declare that there are no conflicts of interests.

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### Supplement 1. Sequence types of *Cryptococcus gattii* clinical isolates described by Lizarazo et al., in 2014 (20)

| Isolates       | Department   | Molecular type | CAP59 | GPD1 | IGS1 | LAC1 | PLB1 | SOD1 | URA5 | ST |
|----------------|--------------|----------------|-------|------|------|------|------|------|------|----|
| H0058-I-3096   | Antioquia    | VGII           | 2     | 6    | 25   | 4    | 18   | 12   | 10   | 25 |
| H0058-I-3286   |              | VGI            | 16    | 5    | 3    | 5    | 5    | 32   | 12   | 51 |
| H0058-I-3590   |              |                | 2     | 6    | 25   | 4    | 18   | 12   | 10   | 25 |
| H0058-I-3031   | Bogotá       |                | 16    | 11   | 13   | 19   | 15   | 34   | 14   | 58 |
| H0058-I-2792   |              |                | 2     | 6    | 25   | 4    | 18   | 12   | 10   | 25 |
| H0058-I-2858   |              |                | 2     | 6    | 25   | 4    | 18   | 12   | 10   | 25 |
| H0058-I-2877   | Norte Santander | VGII         | 2     | 6    | 95   | 4    | 18   | 12   | 10   | 323 |
| H0058-I-3146   |              |                | 16    | 5    | 3    | 5    | 5    | 32   | 12   | 51 |
| H0058-I-3151   |              |                | 2     | 6    | 25   | 4    | 18   | 12   | 10   | 25 |
| H0058-I-3266   |              |                | 2     | 21   | 25   | 4    | 41   | 12   | 2    | 324 |
| H0058-I-3407   |              |                | 2     | 6    | 25   | 4    | 18   | 12   | 10   | 25 |
| H0058-I-3172   | Valle        |                | 16    | 11   | 13   | 19   | 15   | 34   | 14   | 58 |
### Supplement 2A. Clinical manifestations, risk factors, and outcomes of cryptococcosis patients in Colombia, 2005-2014.

|                          | C. neoformans |       | C. gattii |       |              |       |
|--------------------------|---------------|-------|-----------|-------|--------------|-------|
|                          | n=47          | %     | n=14      | %     | Total        |       |
| **Demographic data**     |               |       |           |       |              |       |
| **Sex**                  |               |       |           |       |              |       |
| Male                     | 37            | 78.3  | 10        | 21.7  | 47           |       |
| Female                   | 10            | 21.7  | 4         | 8.7   | 14           |       |
| **Clinical features**    |               |       |           |       |              |       |
| Headache                 | 32            | 69.6  | 12        | 26.1  | 44           |       |
| Fever                    | 26            | 56.5  | 6         | 13    | 32           |       |
| Nausea and vomiting      | 26            | 56.5  | 5         | 10.9  | 31           |       |
| Seizures                 | 22            | 47.5  | 8         | 14.3  | 30           |       |
| Meningeal signs          | 10            | 21.7  | 3         | 6.5   | 13           |       |
| Visual alterations       | 8             | 17.4  | 3         | 6.5   | 11           |       |
| Cough                    | 7             | 15.2  | 1         | 2.2   | 8            |       |
| Loss weight              | 8             | 17.4  | 2         | 4.3   | 10           |       |
| **Risk factors**         |               |       |           |       |              |       |
| HIV/AIDS                 | 36            | 78.3  | 3         | 6.5   | 39           |       |
| Evans Syndrome           | 1             | 2.2   | 1         |       | 1            |       |
| Lupus                    | 2             | 4.3   | 2         |       | 2            |       |
| Arthritis                |               |       | 1         | 2.2   | 1            |       |
| **Outcome**              |               |       |           |       |              |       |
| Alive                    | 35            | 73.9  | 12        | 26.1  | 47           |       |
| Dead                     | 12            | 26.1  | 2         | 4.3   | 14           |       |

18 clinical cases do not report a risk factor

*C. neoformans, Cryptococcus neoformans; C. gattii, Cryptococcus gattii; HIV/AIDS, Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome;*
**Supplement 2B.** Environmental isolates of *Cryptococcus neoformans* and *Cryptococcus gattii* from Colombia, described by species of trees and birds (for isolates from bird droppings)

| Species                  | C. neoformans | C. gattii | Total (%) |
|--------------------------|---------------|-----------|-----------|
| Acacia mangium           | 2             | 2         | (4.1)     |
| Corymbia ficifolia       | 2             | 1         | 3 (6.1)   |
| Eucalyptus               | 7             | 4         | 11 (22.4) |
| Guaiacum officinale      | 1             | 1         | (2)       |
| Licania tomentosa        | 2             | 3         | 5 (10.2)  |
| Roystonea regia          | 1             | 1         | (2)       |
| Pinus sylvestris         | 1             | 1         | (2)       |
| Pithecellobium           | 1             | 1         | (2)       |
| Quercus robus            | 3             | 3         | (6.1)     |
| Terminalia catappa       | 8             | 8         | (16.3)    |
| Culumba livia            | 13            | 13        | (26.5)    |
| **Total**                | 41            | 8         | 49 (100)  |

*C. neoformans, Cryptococcus neoformans; C. gattii, Cryptococcus gattii*
### C. neoformans

| ID_strain/Allele | CAP59  | GPD1   | IGS1   | LAC1   | PBL1   | SOD1   | URA5   |
|------------------|--------|--------|--------|--------|--------|--------|--------|
| H0058-I-2371     | MT497126 | MT507884 | MT507982 | MT508080 | MT508276 | MT508178 | MT508374 |
| H0058-I-2287     | MT497127 | MT507885 | MT507983 | MT508081 | MT508277 | MT508179 | MT508375 |
| H0058-I-2676     | MT497128 | MT507886 | MT507984 | MT508082 | MT508278 | MT508180 | MT508376 |
| H0058-I-2890     | MT497129 | MT507887 | MT507985 | MT508083 | MT508279 | MT508181 | MT508377 |
| H0058-I-2668     | MT497130 | MT507888 | MT507986 | MT508084 | MT508280 | MT508182 | MT508378 |
| H0058-I-2624     | MT497131 | MT507889 | MT507987 | MT508085 | MT508281 | MT508183 | MT508379 |
| H0058-I-2817     | MT497132 | MT507890 | MT507988 | MT508086 | MT508282 | MT508184 | MT508380 |
| H0058-I-2688     | MT497133 | MT507891 | MT507989 | MT508087 | MT508283 | MT508185 | MT508381 |
| H0058-I-2503     | MT497134 | MT507892 | MT507990 | MT508088 | MT508284 | MT508186 | MT508382 |
| H0058-I-2705     | MT497135 | MT507893 | MT507991 | MT508089 | MT508285 | MT508187 | MT508383 |
| H0058-I-2881     | MT497136 | MT507894 | MT507992 | MT508090 | MT508286 | MT508188 | MT508384 |
| H0058-I-2573     | MT497137 | MT507895 | MT507993 | MT508091 | MT508287 | MT508189 | MT508385 |
| H0058-I-2405     | MT497138 | MT507896 | MT507994 | MT508092 | MT508288 | MT508190 | MT508386 |
| H0058-I-2799     | MT497139 | MT507897 | MT507995 | MT508093 | MT508289 | MT508191 | MT508387 |
| H0058-I-2925     | MT497140 | MT507898 | MT507996 | MT508094 | MT508290 | MT508192 | MT508388 |
| H0058-I-3489     | MT497141 | MT507899 | MT507997 | MT508095 | MT508291 | MT508193 | MT508389 |
| H0058-I-3116     | MT497142 | MT507900 | MT507998 | MT508096 | MT508292 | MT508194 | MT508390 |
| H0058-I-3215     | MT497143 | MT507901 | MT507999 | MT508097 | MT508293 | MT508195 | MT508391 |
| H0058-I-3746     | MT497144 | MT507902 | MT508000 | MT508098 | MT508294 | MT508196 | MT508392 |
| H0058-I-3463     | MT497145 | MT507903 | MT508001 | MT508099 | MT508295 | MT508197 | MT508393 |
| H0058-I-3227     | MT497146 | MT507904 | MT508002 | MT508100 | MT508296 | MT508198 | MT508394 |
| H0058-I-3589     | MT497147 | MT507905 | MT508003 | MT508101 | MT508297 | MT508199 | MT508395 |
| H0058-I-3460     | MT497148 | MT507906 | MT508004 | MT508102 | MT508298 | MT508200 | MT508396 |
| H0058-I-3845     | MT497149 | MT507907 | MT508005 | MT508103 | MT508299 | MT508201 | MT508397 |
| H0058-I-3485     | MT497150 | MT507908 | MT508006 | MT508104 | MT508300 | MT508202 | MT508398 |
| Code         | MT497180 | MT507938 | MT508036 | MT508134 | MT508330 | MT508232 | MT508428 |
|--------------|----------|----------|----------|----------|----------|----------|----------|
| H0058-I-4630|          |          |          |          |          |          |          |
| H0058-I-4677| MT497181 | MT507939 | MT508037 | MT508135 | MT508331 | MT508233 | MT508429 |
| H0058-I-4702| MT497182 | MT507940 | MT508038 | MT508136 | MT508332 | MT508234 | MT508430 |
| H0058-I-4711| MT497183 | MT507941 | MT508039 | MT508137 | MT508333 | MT508235 | MT508431 |
| H0058-I-4832| MT497184 | MT507942 | MT508040 | MT508138 | MT508334 | MT508236 | MT508432 |
| H0058-I-4837| MT497185 | MT507943 | MT508041 | MT508139 | MT508335 | MT508237 | MT508433 |
| H0058-I-5015| MT497186 | MT507944 | MT508042 | MT508140 | MT508336 | MT508238 | MT508434 |
| H0058-I-5240| MT497187 | MT507945 | MT508043 | MT508141 | MT508337 | MT508239 | MT508435 |
| H0058-I-5253| MT497188 | MT507946 | MT508044 | MT508142 | MT508338 | MT508240 | MT508436 |
| H0058-I-5353| MT497189 | MT507947 | MT508045 | MT508143 | MT508339 | MT508241 | MT508437 |
| H0058-I-5357| MT497190 | MT507948 | MT508046 | MT508144 | MT508340 | MT508242 | MT508438 |
| H0058-I-5407| MT497191 | MT507949 | MT508047 | MT508145 | MT508341 | MT508243 | MT508439 |
| H0058-I-4667| MT497192 | MT507950 | MT508048 | MT508146 | MT508342 | MT508244 | MT508440 |
| H0058-I-4872| MT497193 | MT507951 | MT508049 | MT508147 | MT508343 | MT508245 | MT508441 |
| H0058-I-4865| MT497194 | MT507952 | MT508050 | MT508148 | MT508344 | MT508246 | MT508442 |
| H0058-I-4521| MT497195 | MT507953 | MT508051 | MT508149 | MT508345 | MT508247 | MT508443 |
| H0058-I-3973| MT497196 | MT507954 | MT508052 | MT508150 | MT508346 | MT508248 | MT508444 |
| H0058-I-4706| MT497197 | MT507955 | MT508053 | MT508151 | MT508347 | MT508249 | MT508445 |
| H0058-I-4359| MT497198 | MT507956 | MT508054 | MT508152 | MT508348 | MT508250 | MT508446 |
| H0058-I-4070| MT497199 | MT507957 | MT508055 | MT508153 | MT508349 | MT508251 | MT508447 |
| H0058-I-4826| MT497200 | MT507958 | MT508056 | MT508154 | MT508350 | MT508252 | MT508448 |
| H0058-I-4611| MT497201 | MT507959 | MT508057 | MT508155 | MT508351 | MT508253 | MT508449 |
| H0058-I-4712| MT497202 | MT507960 | MT508058 | MT508156 | MT508352 | MT508254 | MT508450 |
| H0058-I-4411| MT497203 | MT507961 | MT508059 | MT508157 | MT508353 | MT508255 | MT508451 |
| H0058-I-4414| MT497204 | MT507962 | MT508060 | MT508158 | MT508354 | MT508256 | MT508452 |
| H0058-I-4435| MT497205 | MT507963 | MT508061 | MT508159 | MT508355 | MT508257 | MT508453 |
| H0058-I-4676| MT497206 | MT507964 | MT508062 | MT508160 | MT508356 | MT508258 | MT508454 |
| H0058-I-4697| MT497207 | MT507965 | MT508063 | MT508161 | MT508357 | MT508259 | MT508455 |
| H0058-I-4631| MT497208 | MT507966 | MT508064 | MT508162 | MT508358 | MT508260 | MT508456 |
| Strain Code | MT497209 | MT507967 | MT508065 | MT508163 | MT508359 | MT508261 | MT508457 |
|-------------|----------|----------|----------|----------|----------|----------|----------|
| H0058-I-4644 |          |          |          |          |          |          |          |
| H0058-I-4417 |          |          |          |          |          |          |          |
| H0058-I-3969 |          |          |          |          |          |          |          |
| H0058-I-5126 |          |          |          |          |          |          |          |
| H0058-I-3877 |          |          |          |          |          |          |          |
| H0058-I-3874 |          |          |          |          |          |          |          |
| H0058-I-3080 |          |          |          |          |          |          |          |
| H0058-I-3826 |          |          |          |          |          |          |          |
| H0058-I-5670 |          |          |          |          |          |          |          |
| H0058-I-2730 |          |          |          |          |          |          |          |
| H0058-I-3526 |          |          |          |          |          |          |          |
| H0058-I-3593 |          |          |          |          |          |          |          |
| C. gattii    |          |          |          |          |          |          |          |
| H0058-I-4064 |          |          |          |          |          |          |          |
| H0058-I-4066 |          |          |          |          |          |          |          |
| H0058-I-5531 |          |          |          |          |          |          |          |

*C. neoformans, Cryptococcus neoformans; C. gattii, Cryptococcus gattii*
### Supplement 4. Diversity index of *C. neoformans* and *C. gattii* in clinical and environmental isolates from Colombia.

| Genetic diversity | Number of sequences used | Number of polymorphic (segregating) sites (S) | Total number of mutations (Eta) | Number of haplotypes (h) | Haplotype diversity (Hd) | Nucleotide diversity (Pi) | Theta (per site) from Eta | Theta (per site) from S (ThetaW) |
|-------------------|--------------------------|---------------------------------------------|---------------------------------|--------------------------|--------------------------|--------------------------|---------------------------|-------------------------------|
| **C. neoformans** |                          |                                             |                                 |                          |                          |                          |                           |                               |
| Departament       |                          |                                             |                                 |                          |                          |                          |                           |                               |
| Antioquia         | 17                       | 67                                          | 67                              | 7                        | 0.75                     | 0.00355                  | 0.00497                    | 0.00497                       |
| Atlántico         | 15                       | 23                                          | 23                              | 6                        | 0.79                     | 0.00233                  | 0.00177                    | 0.00177                       |
| Bogotá            | 12                       | 63                                          | 63                              | 5                        | 0.667                    | 0.00374                  | 0.00524                    | 0.00524                       |
| Cauca             | 16                       | 51                                          | 51                              | 8                        | 0.833                    | 0.00303                  | 0.00385                    | 0.00385                       |
| Norte de Santander| 10                       | 25                                          | 25                              | 6                        | 0.778                    | 0.00246                  | 0.00221                    | 0.00221                       |
| Valle             | 18                       | 28                                          | 28                              | 7                        | 0.843                    | 0.00248                  | 0.00204                    | 0.00201                       |
| **Origin**        |                          |                                             |                                 |                          |                          |                          |                           |                               |
| Clinical          | 47                       | 84                                          | 84                              | 10                       | 0.711                    | 0.0033                   | 0.00478                    | 0.00478                       |
| Environmental     | 41                       | 21                                          | 21                              | 6                        | 0.741                    | 0.00216                  | 0.00123                    | 0.00123                       |
| **Molecular type**|                          |                                             |                                 |                          |                          |                          |                           |                               |
| VNI               | 86                       | 53                                          | 53                              | 13                       | 0.769                    | 0.0023                   | 0.00265                    | 0.00265                       |
| VNII              | 2                        | 0                                           | 0                               | 1                        | 0                        | 0                        | 0                          | 0                             |
| **C. gattii**     |                          |                                             |                                 |                          |                          |                          |                           |                               |
| Departament       |                          |                                             |                                 |                          |                          |                          |                           |                               |
| Antioquia         | 3                        | 827                                         | 827                             | 2                        | 0.314                    | 0.13573                  | 0.13573                    | 0.13573                       |
| Atlántico         | 1                        | 0                                           | 0                               | 0                        | 0                        | 0                        | 0                          | 0                             |
| Bogotá            | 5                        | 861                                         | 876                             | 3                        | 0.8                      | 0.12537                  | 0.10418                    | 0.1024                       |
| Norte de Santander| 12                       | 1125                                        | 1160                            | 6                        | 0.818                    | 0.11691                  | 0.09586                    | 0.09297                       |
| Valle             | 1                        | 0                                           | 0                               | 0                        | 0                        | 0                        | 0                          | 0                             |
| **Origin**        |                          |                                             |                                 |                          |                          |                          |                           |                               |
| Clinical          | 14                       | 1148                                        | 1183                            | 6                        | 0.747                    | 0.09843                  | 0.09279                    | 0.09005                       |
| Molecular type | Environmental | 8 | 811 | 824 | 4 | 0.75 | 0.06394 | 0.07843 | 0.07719 |
|---------------|---------------|---|-----|-----|---|------|----------|----------|----------|
| VGI           | 4             | 328 | 328 | 2   | 0.667 | 0.05324 | 0.04356 | 0.04356 |
| VGII          | 10            | 347 | 347 | 3   | 0.378 | 0.01697 | 0.03 | 0.03 |
| VGIII         | 8             | 306 | 310 | 3   | 0.607 | 0.01971 | 0.02913 | 0.02876 |