Title

Single nucleotide polymorphisms and chromosomal copy number variation may impact the Sporothrix brasiliensis antifungal susceptibility and sporotrichosis clinical outcomes

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TITLE: Single nucleotide polymorphisms and chromosomal copy number variation may impact the *Sporothrix brasiliensis* antifungal susceptibility and sporotrichosis clinical outcomes

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HIGHLIGHTS

- *Sporothrix brasiliensis* isolates showed variable antifungal resistance phenotypes;
- Infections caused by this species also present varied clinical manifestations;
- Antifungal-resistant strains display significant copy number variation (CNVs);
- CNVs affect genes involved in lipid and isoprenoid metabolism;
- SNP`s in the *tac1* gene may also contribute to antifungal resistance;

KEYWORDS – *Sporothrix brasiliensis*, chromosomal variation, *tac1* gene, lipid metabolism, antifungal susceptibility.

ABSTRACT

Feline-transmitted sporotrichosis has garnered attention due to the recent high incidence and the lack of efficient control in the epicenter of the epidemic, Rio de Janeiro, Brazil. *Sporothrix brasiliensis* is the major pathogen involved in feline-to-human sporotrichosis in Brazil and displays more virulent genotypes than the closely related species *S. schenckii*. Over the last two decades, several reports of antifungal-resistant strains have emerged. Sequencing and comparison analysis of the outbreak strains allowed us to observe that the azole non-wild-type *S. brasiliensis* strain CFP 1054 had significant chromosomal variations compared to wild-type strains. This includes a region of 231 Kb containing 75 duplicated genes in the CFP 1054 genome compared to other *S. brasiliensis* strains, which were overrepresented for lipid and isoprenoid metabolism. We also identified an additional strain (CFP 1055) that was non-wild-type to itraconazole and amphotericin B, which had a single nucleotide polymorphism in the *tac1* gene. The patients infected with these two strains showed protracted clinical course and sequelae. These results suggest that specific point mutations and large chromosomal duplications may play an important role in antifungal resistance and clinical outcomes of sporotrichosis caused by this pathogen.

1. INTRODUCTION

Sporotrichosis is a globally distributed subacute or chronic subcutaneous fungal disease caused primarily by the thermodimorphic fungi *Sporothrix schenckii*, *Sporothrix brasiliensis*, and *Sporothrix globosa*. The disease is most common in tropical and sub-tropical countries but the *Sporothrix* genus has a global distribution (Chakrabarti et al., 2015; Orofino-Costa et al., 2017). While *S. schenckii* and *S. globosa* are cosmopolitan, *S. brasiliensis* is restricted to South America [reviewed in (Etchecopaz et al., 2021; Zhang et al., 2015b)]. The three species also seem to differ in the clinical manifestation of sporotrichosis. *Sporothrix schenckii* causes a benign chronic subcutaneous mycosis, *S. brasiliensis* frequently causes disseminated or disseminated cutaneous forms (Freitas et al., 2015), and *S. globosa* mainly causes fixed cutaneous lesions (Liu et al., 2021). Other *Sporothrix* species (e.g., *S. mexicana*) have not been commonly associated with disease (Bonifaz et al., 2020). Sporotrichosis transmission usually follows one of two routes. First, the disease is commonly caused by traumatic inoculation into the skin by soil and plant material (e.g., spines or thorns) harboring fungal cells; the second form involves a bite or scratch most commonly from a mammal (e.g., cats or dog) but in some cases by birds (Barros et al., 2011; Orofino-Costa et al., 2017). Other less common forms of transmission include inhalation of conidia and mucosal infection by aerosols containing *Sporothrix* cells (Arinelli et al., 2019; Aung et al., 2013). All *Sporothrix* spp. inhabit soils or are found in association with live and decaying plants as a filamentous mycelia producing infectious conidiogenous cells. After reaching the cutaneous or subcutaneous host tissues, mycelial fragments convert into the pathogenic yeast forms (Barros et al., 2011).

For almost a century, the disease was considered a classic implantation mycosis, mostly related to occupational activities, especially farmers (Govender et al., 2015; Hajjeh et al., 1997). The knowledge about sporotrichosis disease transmission changed in the late 90's due to the appearance of multiple sporotrichosis outbreaks related to feline-to-human or feline-to-feline transmission by either contact or bites or scratches caused by cats in Rio de Janeiro (RJ), Brazil (de Lima Barros et al., 2001; Orofino-Costa et al., 2017). The ongoing epidemic has affected at least 7,897 humans, 5,113 cats, and 244 dogs in RJ (Boechat et al., 2018; Gremiao et al., 2020). The cat-transmitted disease in the Southern states of Brazil is usually caused by the endemic species *S. brasiliensis* (Rodrigues et al., 2013; Rossow et al., 2020). More recently, different states from the Midwestern and Northeastern Brazil have also reported cat-transmitted sporotrichosis due to *S. brasiliensis* (de Oliveira Bento et al., 2021; Eudes Filho et al., 2020; Silva et al., 2021). The geographic range seems to extent out of Brazil, as *S. brasiliensis* has been isolated from soil samples from Argentina (Cordoba et al., 2018) and in a family from Paraguay (García Duarte et al., 2017), suggesting a broader occurrence of this pathogen in South America.

Antifungal resistance is arguably one of the main concerns among the medical mycology community (Wiederhold, 2017). Sporotrichosis is commonly diagnosed by isolation and identification of *Sporothrix* spp. on Sabouraud and/or Mycosel agar. Additionally, serological tests can be used for presumptive diagnosis (Orofino-Costa et al., 2017). Diverse therapeutic strategies are available to treat this fungal infection; topic or oral potassium iodide, azoles, in ~~special~~ particular itraconazole and posaconazole, as well as terbinafine, are frequently used to manage this disease. For disseminated sporotrichosis, intravenous amphotericin B is currently recommended (Kauffman et al., 2007).

Previous surveys have studied the extent of antifungal susceptibility in the *Sporothrix* genus and have found both inter and intra-specific variability (Waller et al., 2021). Whole genome surveys have also studied the magnitude of genetic variation in two different *Sporothrix* species with an emphasis on single nucleotide polymorphism [SNPs, (Eudes Filho et al., 2020)]. To date, no effort has characterized the extent of different genetic mechanisms of antifungal resistance in the genus *Sporothrix*. Resistance can occur through chromosomal aneuploidy (Yang et al., 2019) or SNPs (Billmyre et al., 2020; Sanglard, 2019) in *Candida* spp. or *Cryptococcus* spp. Nothing is known in the genome of *Sporothrix* about how much aneuploidy, or variation in genes known to cause antifungal drugs resistance.

Over more than 20 years of a zoonotic sporotrichosis epidemic due to *S. brasiliensis*, some therapeutic failures with different antifungal drugs have been reported in humans and felines (Almeida-Paes et al., 2017; Nakasu et al., 2021). Additionally, we found isolates with high minimum inhibitory concentration (MIC) for five different antifungals (non-wild type – NWT) among 335 *S. brasiliensis* isolates previously tested (Almeida-Paes et al., 2017). We hypothesized that NWT strains could have punctual-to-chromosome level mutations affecting genes involved in resistance to antifungals (Sanglard, 2019).

Sporothrix brasiliensis is a haploid fungus that displays a varied genome size ranging from 25.7 to 34.7 Mb (average 27.4 Mb), distributed among 5 to 7 chromosomes (Sasaki et al., 2014). Despite the designation of *S. brasiliensis* as a clonal species (Teixeira Mde et al., 2015), the ~~highly~~ variable genomic size ~~differences~~ indicate that ~~SNP`s,~~ partial or complete chromosomal duplication or losses are occurring (Sasaki et

al., 2014). The reference genome *S. brasiliensis* strain 5110 was sequenced to a 20X coverage using the 454 platform. The assembly resulted in a genome size of 33.2 Mb distributed in 13 scaffolds and 9,091 genes were predicted (Teixeira et al., 2014). Previous studies suggest that *S. brasiliensis* isolates display remarkable chromosomal variation, indicating that genomic polymorphism might impact clinically relevant phenotypic variation, such as virulence and antifungal resistance (Sasaki et al., 2014). In this study, we explored the link between genomic variation, antifungal resistance, and clinical phenotypes by identifying intraspecific point mutations, SNPs in well-known genes related to antifungal resistance and DNA copy number~~local ploidy shift~~ between *S. brasiliensis* "wild-type" (WT) and NWT strains against itraconazole, posaconazole, ketoconazole and amphotericin B, commonly used in the treatment of human sporotrichosis. Finally, we predict the impact of chromosomal polymorphisms on the *S. brasiliensis* biology based on gene ontology (GO) and functional characterization of affected genes.

2. MATERIAL AND METHODS

2.1. Strains and cultivation

We measured the susceptibility to antifungal drugs of four strains (Table 1). Initially, the strains were sub-cultured on potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD, USA) and kept at the Collection of Pathogenic Fungi of Fundação Oswaldo Cruz (Fiocruz, Brazil). The antifungal susceptibilities to itraconazole (ITR), posaconazole (POS), ketoconazole (KET), amphotericin B (AMB), and terbinafine (TRB) (all from Sigma-Aldrich, Co., St. Louis, MO, USA) were tested by using the Clinical Laboratory Standards Institute M38-A2 protocol (CLSI, 2008), and strains were classified as WT or

NWT using validated epidemiological cut-off values (ECV). Experiments were carried out in triplicates and using ten different concentrations of the drugs as reported previously ((Espinel-Ingroff et al., 2017)). These ECV for the WT/NWT differentiation were 2.0 mg/L for KET, ITR, and POS; 4.0 mg/L for AMB; and 0.12 mg/L for TRB.

2.2. Patient information

We retrieved the medical records and anonymously evaluated the clinical data of patients infected with the four strains included in this study: sex, age, clinical manifestation, the form of transmission, residence place, treatment and outcome. The research was approved by the Research Ethics Committee of the Evandro Chagas National Institute of Infectious Diseases (INI)/Fiocruz (CAAE 16160619.5.0000.5262).

2.3. Whole-genome sequencing (WGS) and chromosomal Copy Number Variation (CNV)

We sequenced the genomes from the four strains we had data on antifungal susceptibility (see above). DNA was extracted after collection of *S. brasiliensis* cells (0.3 g), disruption with liquid nitrogen, treatment with lysis buffer (1 M Tris pH 8.0, 50 mM EDTA, and 20% sucrose), and incubation at 65 ºC for five minutes. Equal volume of 24:1 mixture of chloroform - isoamyl alcohol (Sigma-Aldrich, St. Louis, MO) were applied to the suspension of DNA following protocols previously published (Woods et al., 1993). Next, the integrity of the extracted DNA was assessed using gel electrophoresis on 0.8% agarose gel stained with 0.5% ethidium bromide. DNA was quantified using the Nanovue Plus™ Spectrophotometer (GE Healthcare, Buckinghamshire, UK). Sequencing libraries using 1

µg of the purified material was prepared using the NEBNext® Ultra™ DNA Library Prep Kit. Paired-end genomic sequencing (2 X 150bp) was carried out on the NovaSeq 6000 platform using the S4 reagent kit (SRA accession number: SRR12483726-SRR12483729). Demultiplexed fastq reads were quality-controlled by removing of adapter sequence and base quality trimmed using Trimmomatic v 0.36 using default parameters (Bolger et al., 2014). The trimmed reads were mapped to the *S. brasiliensis* 5110 reference genome (GenBank: AWTV00000000.1) with the Burrows-Wheeler Aligner (BWA) v 0.7.7 (Li and Durbin, 2009) and converted to a BAM format with SAMtools (Danecek et al., 2021). Incompatible DNA intervals were purged by re-aligning the reads to the reference genome with the GATK v 3.3-0 RealignerTargetCreator and IndelRealigner tools (McKenna et al., 2010) and SNP's were retrieved. The reference genomes and re-aligned BAM files were uploaded to the Yeast Mapping Analysis Pipeline (YMAP - http://lovelace.cs.umn.edu/Ymap/) (Abbey et al., 2014) to detecting chromosomal polymorphisms between antifungal WT or NWT *S. brasiliensis* strains. This tool allows the screening of CNVs by 1) processing BAM files into the mpileup format containing relevant information about chromosome positions via SAMtools (Li et al., 2009); 2) correcting potential bias at the chromosomal telomeric regions; and 3) locally estimating copy numbers that are evened to the impact of high-frequency noise and multiplied by the *S. brasiliensis* ploidy [set to 1 - (Ferreira et al., 2019)]. We have excluded small CNVs with size lower than 10Kb and only those with unique profiles were considered for further analysis.

To identify SNPs, we ~~used~~ assembled each genome using the Automatic Assembly For The Fungi (AAFTF) pipeline version v 0.2.4 (Stajich and Palmer, 2019) which relies on

BBMap v 38.86 (https://sourceforge.net/projects/bbmap) and SPAdes v 3.13.1 (k: automatic selection based on read length, with enabled Repeat resolution, and MismatchCorrector (Bankevich et al., 2012). We also used coverage cutoff with an auto-detected threshold. Finally, we used Pilon v1.22 (Walker et al., 2014) for assembly correction, and vector contamination screening with NCBI BLAST. To verify potentially contaminated material or chimeric scaffolds, BlobTools v 1.0 (Laetsch and Blaxter, 2017) ~~(https://zenodo.org/record/845347#.YXlMvtbMKqA)~~ was carried out for taxonomic interrogation of the sequenced paired-end reads and purged any identified prokaryotic scaffolds. ~~(Basenko et al., 2018)~~

2.4. Functional annotation of duplicated loci.

Initially, we exported the segmental duplications found in different scaffolds defined by YMAP as tracks into the JBrowse server. Next, the gene models from the *S. brasiliensis* 5110 genome into JBrowse was imported to precisely investigate whether or not duplications involved protein-coding loci. Gene IDs were retrieved and used as queries for the functional characterization of the duplicated loci based on the *S. brasiliensis* 5110 annotation available at the FungiDB database (https://fungidb.org/fungidb/ - (Basenko et al., 2018)). The protein domains and structures were analyzed and categorized according to their GO groups and used the hypergeometric tests in expanded or contracted GO categories.

2.5. Characterization of point mutations and SNPs in *S. brasiliensis* in well-known antifungal-resistance genes for human fungal pathogens

We selected 28 genes with SNPs that are known to be important for antifungal resistance phenotype in human pathogenic fungi such as *Candida* albicans (Chen et al., 2019; Feng et al., 2020; Feng et al., 2017; Ramirez-Zavala et al., 2018; Whaley et al., 2016; Zhang et al., 2015a), *C. tropicalis* (Tan et al., 2015), *C. glabrata* (Ahmad et al., 2019; Arastehfar et al., 2019; Culakova et al., 2015; Delliere et al., 2016; Hou et al., 2019; Whaley et al., 2018), *C. parapsilosis* (Rybak et al., 2017), *C. dubliniensis* (Asadzadeh et al., 2017), *Saccharomyces cerevisiae* (Chen et al., 2014), *Cryptococcus* spp. (Billmyre et al., 2020; Son et al., 2018), *Aspergillus spp.* (Losada et al., 2015; Ukai et al., 2018; Wei et al., 2017) (55–57), and *Trichophyton interdigitale* (Rudramurthy et al., 2018) based on a literature search. These alleles of these genes are associated with resistance against several classes of antifungal drugs, such as azoles, polyenes, allylamines, echinocandins, and pyrimidine analogues (Supplementary Table 1).

The antifungal-resistance homologues present in the *S. brasiliensis* genome were identified using BLASTP, and individually extracted individual genes (Teixeira et al., 2014). The 28 antifungal-resistance genes were aligned across five *S. brasiliensis* genomes (1 reference and 4 tested) using MAFFT version 7 (Katoh and Standley, 2013) and retrieved all identified missense and nonsense mutations.

We also retrieved high confident SNP's deciphered by the GATK pipeline and those were characterized based on the genomic annotations available at the FungiDB repository (Basenko et al., 2018). We used the Integrative Genomics Viewer (IGV) to help in identifying the point mutations by uploading (i) the genomic scaffolds of the *S. brasiliensis* 5110 reference genome, (ii) annotation files (in .gff3 format) and individual variant call files (in .vcf format).

3. RESULTS

3.1. Antifungal susceptibility and sporotrichosis definition

~~Clinical manifestations~~The clinical outcomes of the four cases of sporotrichosis differed among them. A~~A~~ll patients presented with the lymphocutaneous form of the disease with lesions on the upper limbs that resulted from being injured by cats with sporotrichosis. Table 2 presents the clinical, demographic, therapeutic, and prognostic data of patients infected with these strains. Of the four cases, case 4 was the most serious as it also presented a contiguous osteoarticular involvement on her fourth metacarpophalangeal joint. Treatment of patients infected with the NWT strains was challenging, requiring a higher dose of antifungal, and changes in the regimen of application, as well as adjuvant non-pharmacologic therapeutics such as cryosurgery and curettage. Although all patients achieved clinical cure, two of them, infected with the CFP 1055 and CFP 1054 strains, developed permanent sequelae such as fibrous cord and amputation of the metacarpus respectively. We determined the MIC of each antifungal drug for the *S. brasiliensis* strains as presented in Table 1. Strain CFP 1056 displays a WT phenotype for all antifungal drugs tested, whereas strains CFP 1062 and CFP 1054 are NWT for all the azoles tested and strain CFP 1055 is NWT for itraconazole and amphotericin B.

3.2. Chromosomal variation within *S. brasiliensis*

It was observed low genomic variability in the 13 scaffolds surveyed within the four strains from Rio de Janeiro (CFP 1054, CFP 1055, CFP 1056, and CFP 1062). First, it was detected no major ~~ploidy~~ copy number variations in most lines. All but one rese-

quenced lines from ~~this population~~the *S. brasiliensis* RJ population, show a uniform distribution of read coverage along the 5110 reference genome. We did noted two significant copy number variation~~shifts to a local diploid~~ signature in the azole NWT strain CFP 1054. The two duplicated chromosome segments are located in scaffolds 4 and 10 of the *S. brasiliensis* reference strain 5110 (Figure 1). The gene content of these two segments were explored.

The duplicated loci localized on the scaffolds IV and X ~~corresponded~~ has ~~to~~ 161 Kb and 70 Kb, respectively, ~~totaling~~ totalizing 231 Kb (0.71% of the *S. brasiliensis* 5110 genome – Figure 2). To exclude the possibility of genome sequencing contamination, it was running BlobTools on this assembly and it was not find any significant bacterial or eukaryotic (non-*Sporothrix* DNA) contaminant contigs (Supplementary Figure 1)~~.~~. The duplicated genomic segments detected in the *S. brasiliensis* CFP 1054 strain were associated with 75 protein-coding genes as listed in the Supplementary Table 2. Among these duplicated loci, two GO slim terms were significantly overrepresented: lipid metabolism (GO:0006629, p=0.0231) and lipid binding (GO:0008289, $p=9.00 \times 10^{-5}$,) are affected by copy number variation~~local ploidy variation~~. Individual GO categories that are overrepresented in the duplicated loci are shown on Supplementary Table 3. Next, REVIGO was applied to summarize the lists of GO terms by discarding redundancy. The curated terms were visualized in semantic similarity-based scatterplots (Figure 3). Overrepresented GO slim terms are the metabolism and biosynthesis of lipids and isoprenoids and proton-transporting V-type and two-sector ATPases in the NWT strain CFP 1054. The genes related to metabolism and biosynthesis of lipids and isoprenoids are SPBR_05677,

SPBR_05940, SPBR_05903 and SPBR_05498 while the gene codified for a proton-transporting V-type protein is SPBR_06076. Genes related to the lipid binding function are SPBR_04543, SPBR_04589, SPBR_05377 and SPBR_05521 (see Supplementary Table 3)

3.3. Point mutations and Single nucleotide polymorphisms in resistance-related genes

The literature search retrieved 22 manuscript reporting 28 distinct genes containing SNP`s that impacts ~~associate with~~ amphotericin B (n=2), azoles (n=22), echinocandins (n=2), terbinafine (n=1), and flucytosine (n=2) resistance in several fungi, including *Aspergillus fumigatus* (n=5), *Aspergillus flavus* (n=1), *Candida albicans* (n=8), *Candida dubliniensis* (n=1), *Candida glabrata* (n=7), *Candida parapsilosis* (n=1), *Candida tropicalis* (n=1), *Cryptococcus neoformans* (n=2), *Saccharomyces cerevisiae* (n=1), and *Trichophyton interdigitale* (n=1). By searching these well-characterized antifungal resistance homologues in the *S. brasiliensis* genomes, ~~it was found all~~we identified all 28 ~~genes~~ homologues in all ~~4~~ 5 analyzed genomes ~~and~~ including the *S. brasiliensis* 5110 reference ~~5110~~ genome (Supplementary Table 1). A single non-synonymous mutation was observed in one of the 28 genes evaluated among the 4 analyzed *S. brasiliensis* strains: a nonsense mutation in the amino acid position 672 of the *Tac1* protein in strain CFP 1055 (Supplementary Figure 2). This strain is NWT to both itraconazole and amphotericin B.

In addition, we characterized 70 point mutations in the 4 *S. brasiliensis* isolates analyzed. It's worth nothing the most of the point mutations herein identified were observed in the NWT strains CFP 1054 and CFP 1055 that exhibit antifungal resistance phenotypes or are associated with poor clinical sporotrichosis outcomes. Moreover, we

observed that most non-synonymous point mutations were accumulated in those above-mentioned strains. We observed that 4 non-synonymous point mutations in the CFP 1055 (SPBR_03899, SPBR_00299, SPBR_00791, SPBR_06930) while 12 non-synonymous point mutations were identified in the CFP 1055 strain (SPBR_03648, SPBR_05529 SPBR_02188, SPBR_02191, SPBR_02295, SPBR_02181, SPBR_09104, SPBR_00274, SPBR_07141, SPBR_06450, SPBR_05046, SPBR_08716). The annotations and characteristics of all mutations are available at Supplementary Table 4. Particularly for the CFP 1055 strain, the SPBR_00299 gene codifies for a MATE (multidrug and toxic compound extrusion) efflux transporter and such non-synonymous mutation might impact in the antifungal resistant phenotype. Another mutation of particular interest was observed in the CFP 1054 strain at the SPBR_02295 gene. This gene codifies for a voltage-gated chloride channel protein and such mutation might alter the permeabilization of fungal cells. Overall, such finding needs to be further characterized using gain-and-loss of function studies to prove that such polymorphisms are related to poor disease outcomes or antifungal resistance phenotypes.

4. DISCUSSION

The evolution of antifungal-resistant phenotypes in fungal species is determined by the strength of selection on the trait (Fisher et al., 2020). In the past 24 years, zoonotic sporotrichosis, a disease initially observed in Rio de Janeiro, has spread over Brazil and has gained attention among health authorities. *Sporothrix brasiliensis*, the causal agent of the disease, is characterized by its high virulence, increased zoonotic transmissibility, and higher antifungal resistance, leading to a wide spectrum of severe clinical presentations (Almeida-Paes et al., 2017; Nakasu et al., 2021; Orofino-Costa et al., 2017). Genetic studies reveal that this species is haploid (Ferreira et al., 2019; Teixeira et al., 2014), a pattern confirmed by the analyses of the four strains in this report. Both MLST and whole-genome SNP suggest that the Rio de Janeiro isolates have low levels of nucleotide diversity (π) consistent with the possibility of a clonal outbreak (Eudes Filho et al., 2020; Teixeira Mde et al., 2015). Despite the low SNP genetic variability of *S. brasiliensis*, phenotypic variation in *in vitro* responses to antifungal drugs is extensive in *S. brasiliensis* (Almeida-Paes et al., 2017; Espinel-Ingroff et al., 2017; Sanchotene et al., 2017), including complete antifungal resistance (Almeida-Paes et al., 2017; Guterres et al., 2014; Nakasu et al., 2021). MLST suggest that strains with high itraconazole MIC seem to be genetically differentiated from susceptible strains (Rodrigues et al., 2014). Such differentiation was not observed for strains with differences in their susceptibility to posaconazole and amphotericin B (Rodrigues et al., 2014). Given the low genetic variability and the extent of phenotypic variation, the differences in susceptibility to itraconazole trait might be amenable to fine genetic mapping.

Two types of mutations have been associated with antifungal resistance, structural variation and SNPs. The first type, aneuploidy and copy number variation~~structural variation~~, seems to be pervasive in fungi (Yang et al., 2019). Karyotype profiling based on Pulsed-Field Gel Electrophoresis coupled with chromosomal blotting analysis suggested that chromosomal polymorphisms exist within *S. brasiliensis* strains (Sasaki et al., 2014). In this study, we find that the extent of genome structure variation is not only restricted to chromosome number. WGS indicates that the *S. brasiliensis* strain CFP 1054, isolated from a refractory case of sporotrichosis with poor prognosis and reduced susceptibility to antifungal drugs, shows copy number variation~~local ploidy variation~~ (Figure 1). Our results suggest that the duplicated segments in the *S. brasiliensis* NWT strain CFP 1054 genome are enriched for genes involved in the metabolism and biosynthesis of lipids and isoprenoids, genes encoding proton-transporting V-type and two-sector ATPases. Azoles, the family of compounds in several antifungals, directly affect membrane-bound enzymes and membrane lipid biosynthesis (Shafiei et al., 2020). For example, the modulation of isoprenoid and lipid biosynthesis impacts the plasma membrane lipid bilayer symmetry and fluidity, which increases virulence and multi-drug-resistant phenotypes in pathogenic bacteria and fungi (Rizzo et al., 2019). Moreover, the fungal plasma membrane and vesicles harbor a high number of proton ATPases that are associated with the maintenance of electrochemical proton gradients (Monk and Perlin, 1994). These enzymes are responsible for numerous processes of intracellular protein trafficking and translocation, and their overexpression might be related to azole resistance in *S. brasiliensis*. Lipid transporters have been recurrently associated with virulence and drug resistance in different species of pathogenic fungi (Mago and Khuller, 1989; Pan et al., 2018; Rizzo et al., 2019). The

results altogether suggest that strain CFP 1054 might have duplicated genes that directly affect the structure and organization of the plasma membrane. This association of copy number variations~~local ploidy variation~~ and resistance to antifungals warrants more investigation.

Structural genomic variation is unlikely to be the only source of increased resistance to antifungals. Strain CFP 1055, classified as NWT for multiple drugs, including itraconazole, did not show significant copy number variation~~local ploidy alterations~~. The strain does have a missense mutation on the gene that encodes the transcriptional activator *tac1*. In *C. albicans*, the TAC1 transcriptional activator regulates the expression of *cdr1* and *cdr2*, which encode transporters involved in azole resistance (Coste et al., 2004). In *C. haemulonii*, the overexpression of the CDR1 gene may confer triazole resistance (Zhang et al., 2019). The presence of extra copies of this gene on the isochromosome i (5L) of *C. albicans* also confers azole resistance (Selmecki et al., 2008)(. Similarly, variation at *tac1* has been associated with azole-resistant phenotypes in the emerging pathogen *C. auris* (Li et al., 2021). Our findings do not conclusively prove that mutations in *tac1* are the mechanism of resistance, but these analyses provide a working hypothesis for linking antifungal resistance and nucleotide variation in fungal pathogens. Lastly, we did not identify any DNA polymorphism in the candidate genes, or copy number variation ~~local ploidy shift~~ associated with the multi-azolic resistant strain CFP 1062. Other genes currently not associated with antifungal resistance might be responsible for azole resistance in this strain.

Our report opens at least two research lines to be pursued in *Sporothrix*. Even though the number of isolates included in our survey is low, our study serves as a springboard to broadly analyze the extent of genome plasticity in *Sporothrix*. More generally, antifungal resistance mechanisms in endemic fungal pathogens are poorly understood. Due to their dimorphic nature, the effect of antifungals might differ at distinct life stages; consequently the genetic architecture of the trait might also differ when measured at different stages. Additionally, population structure within species of *Spororothrix* might be of relevance to understand the spread of antifungal resistance syndromes. Recent work suggests that *S. brasiliensis* lineages from the epicenter of zoonotic sporotrichosis epidemic in Rio de Janeiro (Southeast) are genetically different from those recovered in Mid-Western Brazil (Eudes Filho et al., 2020). Such differences appear to have an impact on the *in vitro* susceptibility of this emerging pathogen and, more importantly, on the clinical outcome of sporotrichosis.

Genome plasticity is a common phenomenon in fungi [e.g., (Todd et al., 2019)] and has been often associated to emergent resistance to antifungals (Ford et al., 2015; Todd and Selmecki, 2020). WGS approaches are becoming cheaper and more accessible and, therefore, will be crucial to not only understand the extent of genome variability within *S. brasiliensis* strain typing, but also understand the drivers of virulence and antifungal resistance in the causal agents of sporotrichosis. The causes and phenotypic consequences of such genome variability need to be studied closely as they are likely to have relevance for the clinical manifestations of sporotrichosis and ultimately affect the epidemiology of the disease (Boechat et al., 2018; de Carvalho et al., 2020; Rodrigues et al., 2014; Rodrigues et al., 2013).

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AUTHOR CONTRIBUTIONS

Conceptualization: R.M.Z-O, R.A.P, A.R.B.E; Data curation: R.A.P, M.M.T, P.M.M, D.F.S.F; Formal analysis: M.M.T, R.A.P, A.R.B.E; Funding acquisition: R.M.Z-O; Investigation: M.C.G.G; P.M.M, D.F.S.F; A.C.F.V; Methodology: A.R.B.E, A.M.N, P.M.M; Project administration: R.M.Z-O, A.R.B.E; Resources: M.M.T, R.A.P, R.M.Z-O; Software: B.M.B, J.E.S.; Supervision: R.M.Z-O, B.M.B, J.E.S,; Validation: D.R.M, A.M.N; Visualization: M.M.T, R.A.P ; Roles/Writing - original draft: M.M.T, R.A.P; Writing - review & editing M.M.T, D.R.M, B.M.B, J.E.S, R.M.Z-O.

FIGURE CAPTIONS

Figure 1 – Copy number variation (CNV) analysis of *Sporothrix brasiliensis*. The vertical histograms represent local copy number variation ~~shifts in the ploidy~~ (changes in ploidy from n=1 to n=2~~1→ 2~~) of each analyzed strain based on read depth across the *S. brasiliensis* reference 5110 genome. Significant changes in the copy number variation~~ploidy~~ of the *S. brasiliensis* CFP1054 strain are highlighted, indicating the presence of CNVs in two scaffolds (Chr 4 and 10).

Figure 2 – Genomic representation of duplicated genes of the *S. brasiliensis* CFP1054 strain. Genes are represented according to their orientation on chromosomes 4 (AWTV01000004.1) and 10 (AWTV01000010.1).

Figure 3 – Enriched biological process (A) and molecular function (B) categories defined by the Gene Ontology (GO) of the *S. brasiliensis* CFP1054 strain. The GO terms were clustered using a neighbor-joining approach resulting in a two-level hierarchical structured data ~~are displayed in a two-dimensional space~~ according to their semantic similarities and closeness.

Supplementary Figure 1 – Taxonomical assignments of each scaffold of the *S. brasiliensis* CFP1054 strain. The coverage and the G and C base proportion were obtained by mapping the Illumina reads to its correspondent assembly and are displayed in a two-dimensional scatter plot. Genomic sequences are represented by dots and are colored based on taxonomic affiliation according to the sequence similarity search results.

Supplementary Figure 2 – Amino acid alignment of the Tac1 protein of all *S. brasiliensis* strains screened for missense and nonsense mutations of antifungal resistance genes. In this particular case, we observed a stop codon (*) in position 672 of the *S. brasiliensis* CFP 1055 strain.

Table 1: Minimum inhibitory concentrations of *Sporothrix brasiliensis* strains included in the study

Strain	Minimum inhibitory concentration (mg/L)				
	AMB	ITR	KET	POS	TRB
CFP 1056	0.12	0.5	0.03	0.25	<0.015
CFP 1062	1.0	4.0	8.0	>8.0	0.015
CFP 1055	>8.0	>8.0	4.0	0.25	0.015
CFP 1054	0.25	8.0	4.0	8.0	0.03

Table 2: Clinical, demographic, therapeutic, and prognostic information of patients with zoonotic sporotrichosis infected with the *Sporothrix brasiliensis* strains included in this study.

						Treatment		Outcome
Case	Sex	Age	Origin[1]	Strain	Year	Drugs and other methods	Months	
1	M	32	Anchieta	CFP1056	1999	ITZ 100mg/day KTZ 200mg/day	2	Clinical cure
2	F	66	Sepetiba	CFP1062	2005	ITZ 100mg/day Cryosurgery and curettage	14	Clinical cure
3	M	68	Nova Iguaçu	CFP1055	2007	TRB up to 500 mg/day ITZ up to 200 mg/day Cryosurgery	38	Clinical cure Cord of scarring fibrous tissue
4	F	73	Duque de Caxias	CFP1054	2014	ITZ up to 400 mg/day[2] TRB 250 mg/day	34	Clinical cure Amputation of 4th left finger

M: male, F: female. ITZ: itraconazole, KTZ: ketoconazole, TRB: terbinafine. [1]Anchieta and Sepetiba are neighborhoods in the city of Rio de Janeiro. Nova Iguaçu and Duque de Caxias are cities in the metropolitan region of Rio de Janeiro. [2]Due to bone sporotrichosis.

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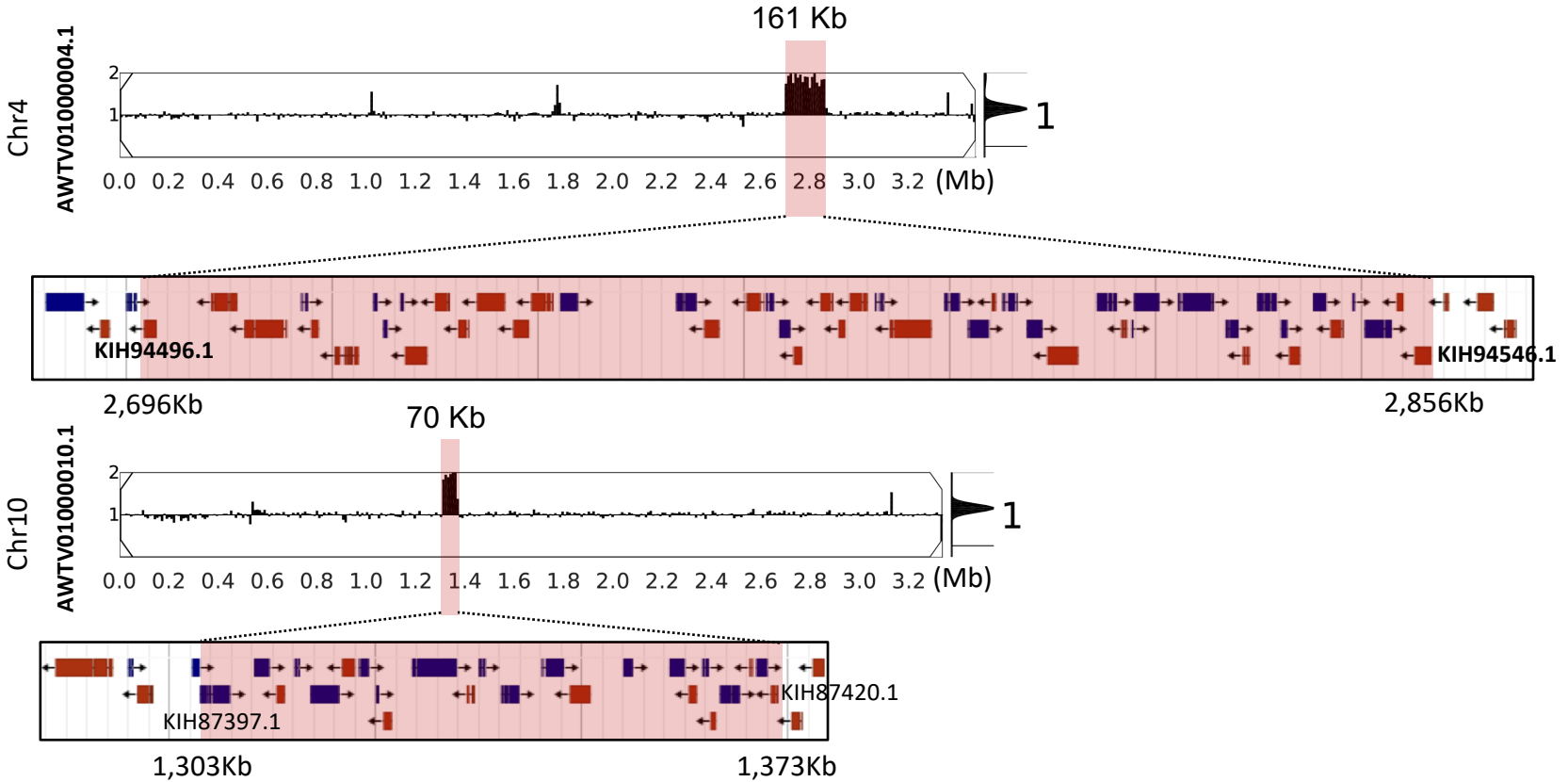
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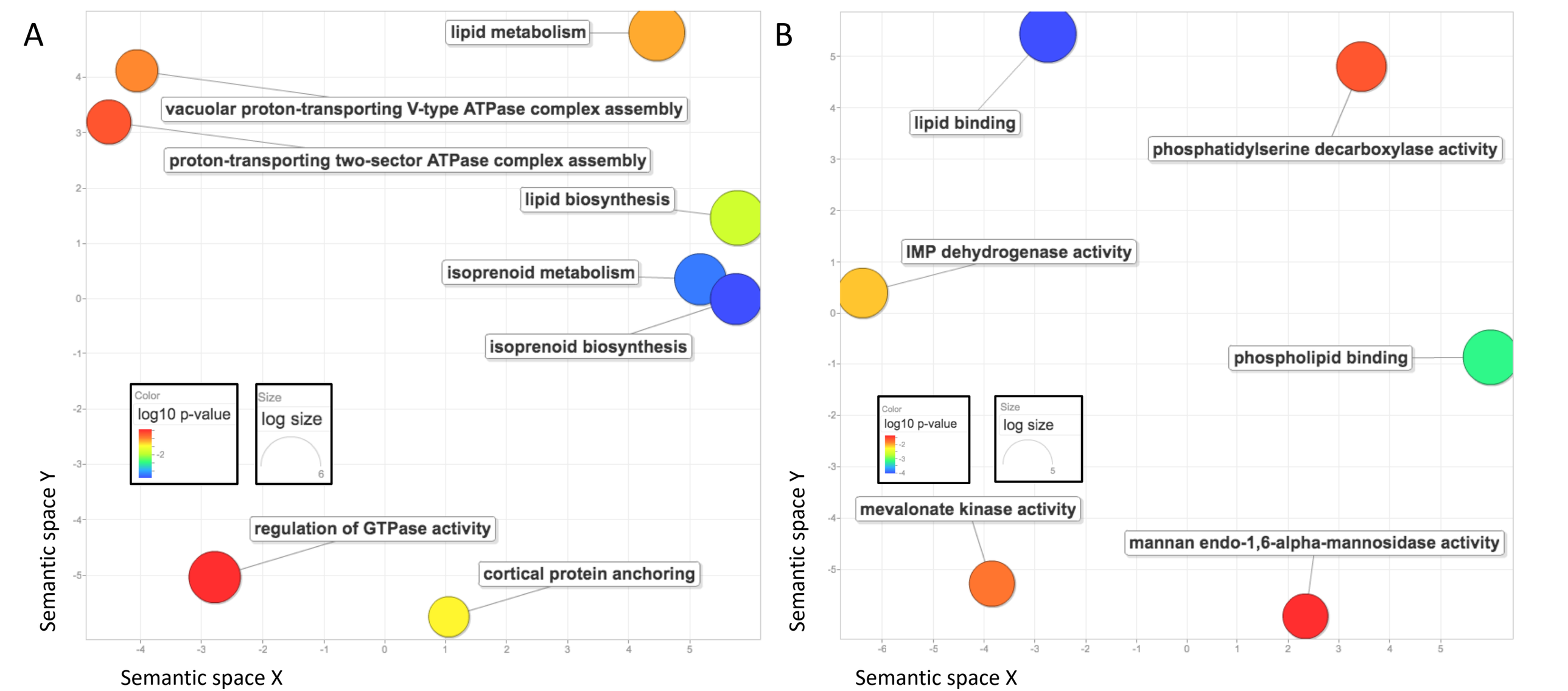
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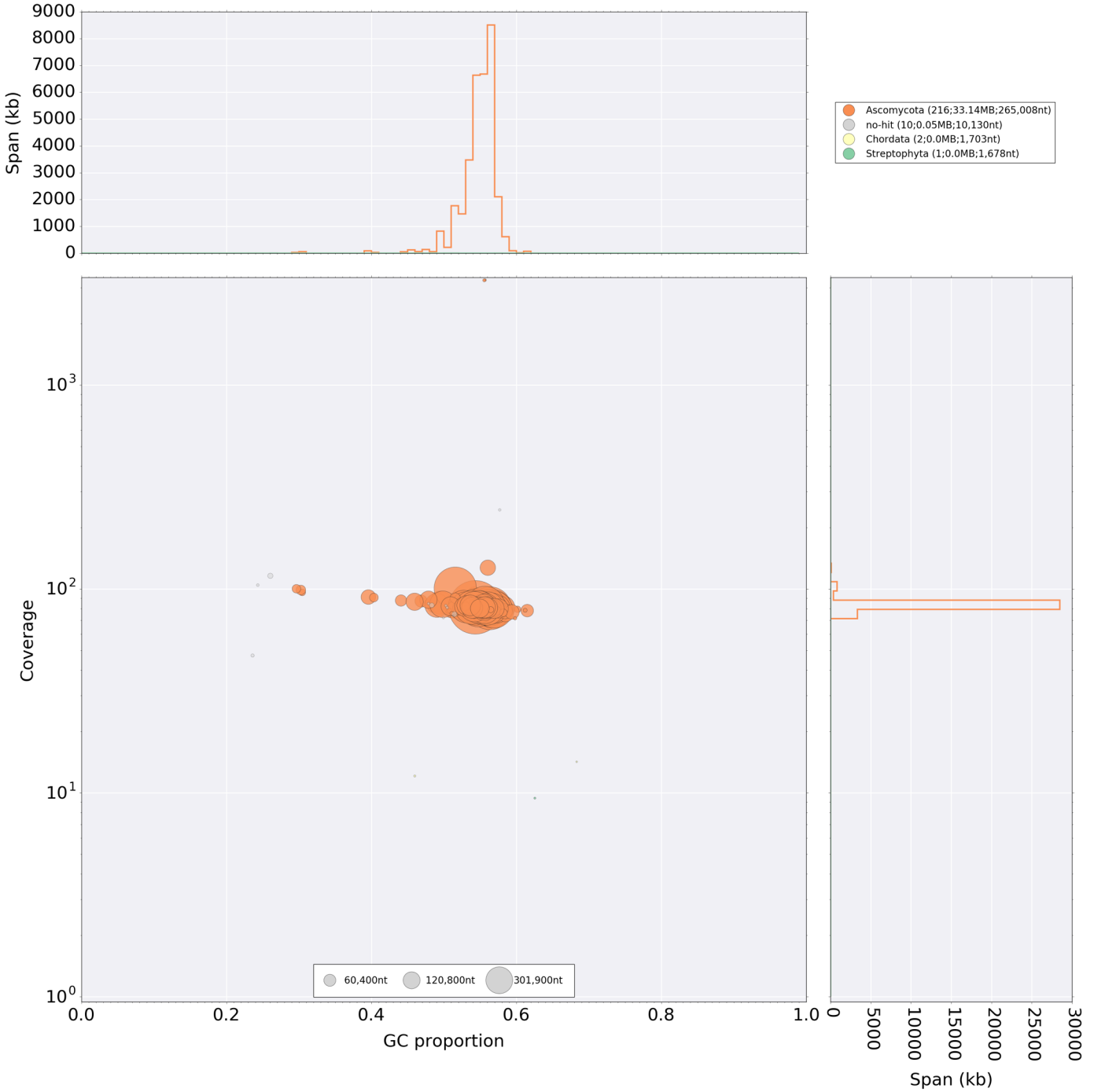
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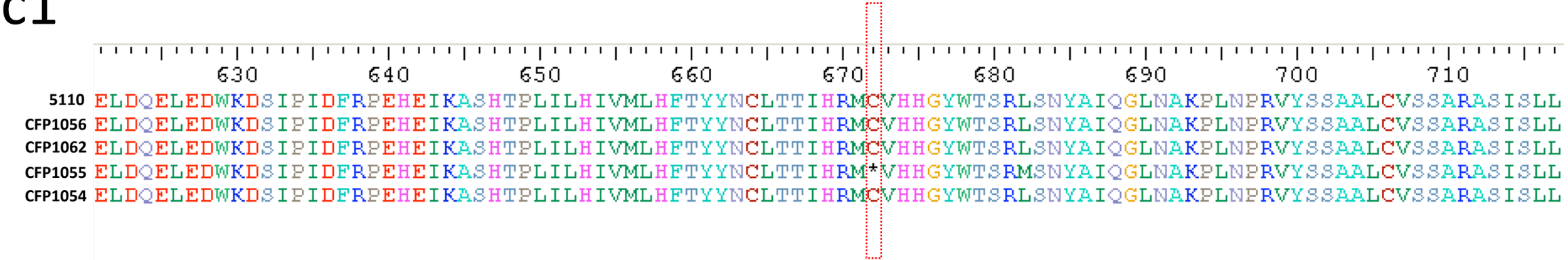
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