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Investigating within-host evolution of antifungal resistance in *Candida (Lodderomyces) parapsilosis* using whole-genome sequencing

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ABSTRACT

Candida (Lodderomyces) parapsilosis is a fungal opportunistic pathogen and an important cause of different forms of candidiasis, including candidemia, especially in hospitalized and frail patients. The emergence of strains of this pathogen exhibiting resistance to main antifungal classes is a great challenge for treatment, and the increasing number of reported cases of resistance is currently a big concern for healthcare.

Recent advances in genomics have made whole-genome sequencing and bioinformatic analysis a widely used approach to investigate the genetic basis of drug resistance mechanisms in microbes. However, the genomic changes underlying *C. parapsilosis* adaptation to host environment and drug exposure during persistent infections remain incompletely understood. Analysis of sequential isolates from the same patient taken at different time spots of treatment can help to shed light on within-host antifungal resistance evolution and connect acquired genetic changes with changes in drug susceptibility.

In this study, we selected eight cases from a larger cohort of patients with candidemia treated at the IRCCS Fondazione Policlinico San Matteo, Pavia, Italy. These patients were selected because *C. parapsilosis* isolates, collected from blood cultures of these patients at different time points during treatment, exhibited changes in antifungal susceptibility over a short period. Antifungal susceptibility was assessed by measuring the Minimal Inhibitory Concentration (MIC). In total, 18 clinical isolates were recovered from the selected patients, and whole-genome sequencing of serial isolates was performed, allowing us to investigate genomic changes occurring during individual infections and under antifungal drug exposure. Sequencing reads were analyzed using ad-hoc bioinformatics tools to characterize genetic variation among isolates.

Genetic mutations were then identified in these isolates, and found to include variants affecting genes potentially involved in cellular and metabolic adaptation, as well as variants affecting transport proteins and potentially their expression, which can explain the different drug response during the treatment. Additional genomic changes, including changes in ploidy, were detected in one isolate pair.

Overall, within-host changes in antifungal susceptibility in *C. parapsilosis* appear to be complex and multifactorial. The relative contribution of many of these factors to the observed phenotypic changes remains unresolved, and further functional validation in larger longitudinal

datasets is needed to identify which changes actually contribute to the changes in antifungal susceptibility in clinical isolates.

RIASSUNTO

Candida (Lodderomyces) parapsilosis è un fungo patogeno opportunista e un'importante causa di diverse forme di candidosi, tra cui la candidemia, soprattutto nei pazienti ospedalizzati e fragili. La comparsa di ceppi di questo patogeno resistenti alle principali classi di antifungini rappresenta una grande sfida per il trattamento, e il numero crescente di casi di resistenza segnalati è attualmente motivo di grande preoccupazione per l'assistenza sanitaria.

I recenti progressi della genomica hanno reso il sequenziamento dell'intero genoma e l'analisi bioinformatica un approccio ampiamente utilizzato per indagare le basi genetiche dei meccanismi di resistenza ai farmaci nei microrganismi. Tuttavia, i cambiamenti genomici alla base dell'adattamento di *C. parapsilosis* all'ambiente dell'ospite e all'esposizione ai farmaci durante le infezioni persistenti rimangono incompletamente compresi. L'analisi di isolati sequenziali provenienti dallo stesso paziente, prelevati in momenti diversi del trattamento, può contribuire a fare luce sull'evoluzione della resistenza agli antifungini all'interno dell'ospite e a collegare i cambiamenti genetici acquisiti con le variazioni della sensibilità ai farmaci.

In questo studio, abbiamo selezionato otto casi da una coorte più ampia di pazienti con candidemia trattati presso l'IRCCS Fondazione Policlinico San Matteo, Pavia, Italia. Sono stati scelti perché gli isolati di *C. parapsilosis*, raccolti da emocolture di questi pazienti in momenti diversi durante il trattamento, hanno mostrato cambiamenti nella sensibilità agli antifungini in un breve periodo di tempo. La sensibilità agli antifungini è stata valutata mediante la misurazione della Concentrazione Minima Inibente (MIC). In totale sono stati recuperati 18 isolati clinici dai pazienti selezionati, ed è stato eseguito il sequenziamento dell'intero genoma degli isolati seriali, consentendoci di indagare i cambiamenti genomici che si verificano durante le infezioni individuali e l'esposizione ai farmaci antifungini. Le reads di sequenziamento sono state analizzate utilizzando strumenti bioinformatici ad hoc per caratterizzare la variabilità genetica tra gli isolati.

Sono state quindi identificate mutazioni genetiche in questi isolati, che includevano varianti a carico di geni potenzialmente coinvolti nell'adattamento cellulare e metabolico, nonché varianti a carico di proteine di trasporto e potenzialmente della loro espressione, in grado di spiegare la diversa risposta ai farmaci durante il trattamento. Ulteriori cambiamenti genomici, tra cui variazioni della ploidia, sono stati rilevati in una coppia di isolati.

Nel complesso, i cambiamenti della sensibilità agli antifungini all'interno dell'ospite in *C. parapsilosis* appaiono complessi e multifattoriali. Il contributo relativo di molti di questi fattori ai cambiamenti fenotipici osservati rimane irrisolto, e un'ulteriore validazione funzionale in

dataset longitudinali più ampi è necessaria per identificare quali cambiamenti contribuiscano effettivamente alle variazioni della sensibilità agli antifungini negli isolati clinici.

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1. INTRODUCTION

1.1. Fungal infections: an emerging global health concern

Fungal infections are historically considered to be a less common cause of clinical infections compared to other microorganisms, where bacteria and viruses are the main characters. However, in the last decades fungal diseases have become an increasing concern for global public health. In particular, this trend is being observed due to an increased number of immunocompromised patients which are specifically susceptible to these infections (Seagle et al., 2021). According to the latest estimates, which exclude influenza or COVID-19 outbreaks, over 6,55 million people are affected by life-threatening fungal infections, and over 3,75 million of the cases resulted in death, of which around 2,55 million are directly caused by that fungal disease (Denning, 2024). In the epidemiology of fungal infection diseases *Candida* spp. have one of the leading positions. For instance, a nationwide study in China on the etiology of invasive fungal infections revealed that *Candida* species accounted for over 90% (specifically 91.3%) of the causative pathogens (Qin et al., 2021).*Candida* spp. play a significant role in the development of bloodstream infections, endocarditis, and other systemic complications (Nguyen et al., 2025). A major clinical concern of Candida infections is the onset of invasive candidiasis (IC) which indicate both candidemia – the presence of *Candida* species in the blood – and deep-seated tissue with dissemination of the pathogen to normally sterile body sites such as bones, pleura, peritoneum etc. (Soriano, 2023). Invasive candidiasis is a life-threatening condition with reported mortality rates ranging from 20 to 45% (WHO, 2022). Within the genus, *Candida auris* has become the most prominent pathogen, showing increasing rates of outbreaks and multidrug-resistance phenotypes, accompanied by a relatively high mortality rate. It is ranked as a pathogen of critical significance level by the World Health Organization (WHO) (Kim et al., 2024).

The predominant *Candida* pathogens fall into two major categories: *C. albicans* and non-*albicans Candida* (NAC) species (Govrins, M., Lass-Flörl, 2024). In recent years, the importance of *Candida albicans* among these infections has been decreasing, while the incidence and antimycotic resistance of NAC has been increasing (Qin et al., 2021).

1.2. Epidemiological trends and clinical significance of *Candida parapsilosis*

In 2022, the WHO developed a Fungal Priority Pathogen List (WHO FPPL) as the first attempt to assess and prioritize the clinical relevance of individual fungal pathogens. The initiative was designed to emphasize research priorities and inform policy strategies aimed at improving the

global management of fungal infections and antifungal resistance. In this work, they ranked 19 fungal pathogens based on multiple criteria including antifungal resistance, mortality rate, evidence-based treatment, access to diagnostics, annual incidence and complications which together accounted for 95% of relative importance weight. In this list, *Candida parapsilosis* was included into the group of high risk together with *Nakaseomyces glabrata* (*Candida glabrata*), *Histoplasma spp.*, eumycetoma causative agents, Mucorales, *Fusarium spp.*, *Candida tropicalis*.

Due to the limited number of epidemiological studies, it is difficult to determine the precise annual incidence of *C. parapsilosis*-caused diseases. However, there is strong evidence indicating that the frequency of infections caused by *C. parapsilosis* has increased over the past decade (WHO, 2022). The ratio of *C. parapsilosis* among all *Candida* infections varies in different regions, having a high number in southern Europe (up to 41%), South America (up to 38,5%), Asia (up to 27,1%), and the Middle East (up to 34,7%) (Govrins, M., Lass-Flörl, 2024). The proportion of *C.parapsilosis* in invasive *Candida* infections worldwide is shown in Figure 1.

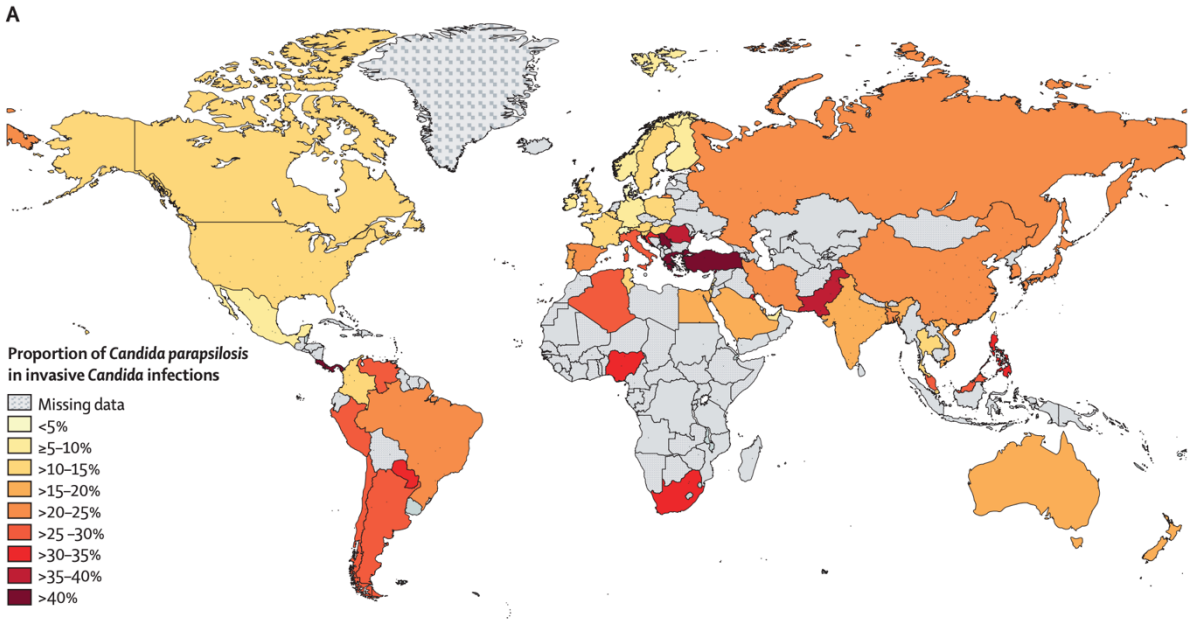


Figure 1. **Burden of *Candida parapsilosis* worldwide. The worldwide epidemiology of *C. parapsilosis*** (Daneshnia et al., 2023)

C. parapsilosis is a commensal of the human skin and gastrointestinal tract, yet it can act as an opportunistic pathogen in susceptible hosts. The principal risk groups include immunocompromised patients of various etiologies, such as those with malignancies, recipients of bone marrow or solid organ transplants, and individuals undergoing hemodialysis

(Uraguchi et al., 2022), as well as patients with prolonged hospitalization e.g. those patients hospitalized in ICU units (Zuo et al, 2021). One additional factor contributing to the increase of the incidence of diseases caused by *C. parapsilosis* is the spread of usage of life-support interventions like central venous catheter or parenteral nutrition (Horasan, 2010), as *C. parapsilosis* can form biofilms on the surface of intravascular devices. As a results, during the last decades there has been a notable increase in nosocomial infections caused by *C. parapsilosis*, particularly in intensive care units (ICUs), where these conditions – long lasting disease and hospitalization, immunosuppression in patient, the usage of life-support intervention are combined (Zuo et al, 2021).

Importantly, *C. parapsilosis* represents an important pathogen in neonatal populations, particularly among premature infants. In this group, *Candida* species often result in high mortality or severe neurodevelopmental disabilities. (Chow et al, 2012).

1.3. Antifungal resistance and its measurement

Besides its growing clinical and epidemiological relevance , *C. parapsilosis* represents an emerging challenge for clinicians and healthcare systems mainly due to the increasing prevalence of strains resistant to antifungal therapy. Figure 2 presents a world map showing the prevalence of fluconazole-resistant *C. parapsilosis* by country in 2023, showing the geographic heterogeneity of resistance. Notably, in Italy, the resistance rate reached 20–30%, highlighting the significance of resistance in this region.

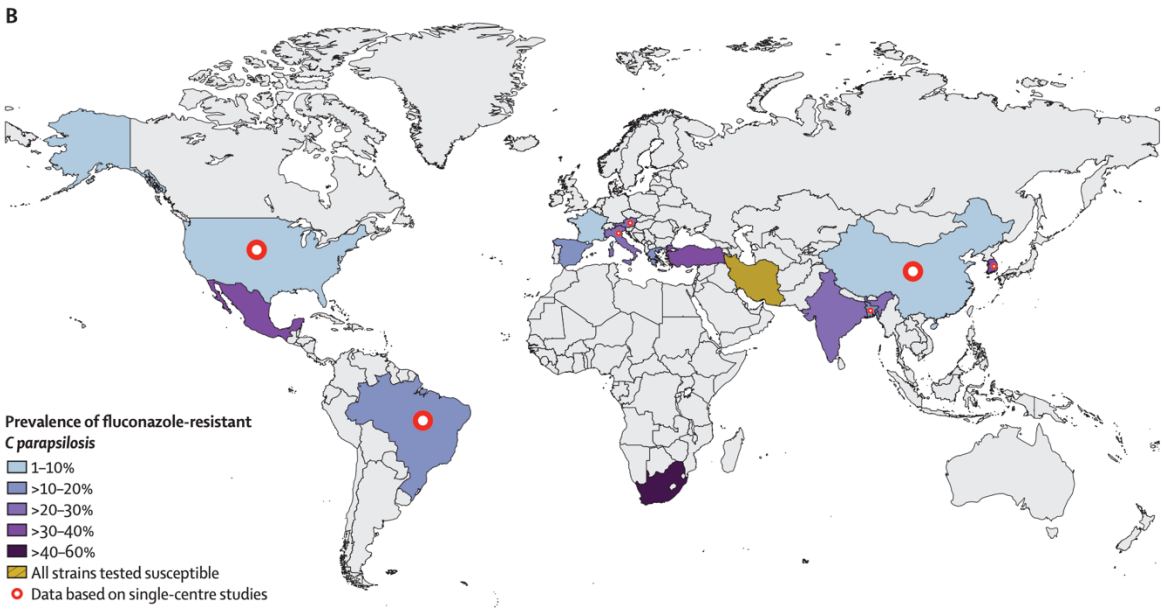


Figure 2. **Global prevalence of fluconazole-resistant *Candida parapsilosis* in 2023** (Daneshnia et al., 2023)

Antifungal resistance refers to the ability of fungal pathogens to survive in the presence of drugs at the concentrations that are ordinarily inhibitory or lethal to the majority of cells. Resistance to antifungal drugs leads to treatment failure, long-term infections, increased patient morbidity and mortality and increase of healthcare cost (Kadariswantiningsih et al., 2025). The presence of resistance to two or more drug classes of antifungal agents in a single isolate is called multidrug resistance (MDR) (Prasad et al., 2019).

A widely used method to assess the susceptibility of pathogens to drugs is a measurement of the Minimum Inhibitory Concentration (MIC) which is defined as the lowest concentration that effectively inhibits growth. The results of MIC determination are typically expressed as MIC_{50} or MIC_{80}, signifying the concentrations required for 50% or 80% growth inhibition, respectively (Xie et al, 2012; Singh-Babak et al., 2012). Antifungal susceptibility testing includes several standardized methodologies, including reference broth microdilution (CLSI/EUCAST), disk diffusion, and commercial automated platforms. However, no universally fixed breakpoints exist for precise MIC interpretation, as they vary considerably depending on the testing method, the specific organism–drug combination, and the interpretive criteria applied (Lass-Flörl et al., 2026).

Resistance mechanisms in fungi can be broadly classified into two categories: intrinsic and acquired. Intrinsic resistance is a constitutive, species-specific trait whereby antifungal agents are inherently ineffective due to pre-existing genetic determinants. For instance, intrinsic fluconazole resistance, occurring without prior drug exposure has been documented in *Aspergillus* spp., *Nakaseomyces glabratus* (formerly *C. glabrata*), and certain strains of *Candida auris*. In contrast, acquired resistance results from genetic mutations and adaptive evolutionary responses to environmental selective pressure, e.g. antifungal exposure. (Kadariswantiningsih et al., 2025).

It is important to distinguish between the terms "resistance" and "tolerance", which are frequently used in the literature as synonymous. Drug tolerance can be defined as the ability of drug-sensitive fungi to survive and maintain residual growth or be in the dormant state in the presence of antifungal agents at concentrations above the MIC (Berman and Krysan, 2020). Antifungal tolerance is reversible and is mostly attributed to epigenetic changes, whereas resistance is predominantly caused by genetic modifications and mutations. Antifungal tolerance differs from resistance, which is defined by an increase of MIC value rather than by the ability to survive at drug concentrations exceeding that value (Delarze and Sanglard, 2015).

1.4. Antifungal drugs classes

Only 3 main classes of antifungal drugs are known up to date as effective for systemic treatment of candidemia: the azoles, the echinocandins and the polyenes (Li et al., 2025).

1.4.1. Azoles

Azoles, particularly fluconazole, voriconazole, and itraconazole, represent the most widely used antifungal class. Their broad clinical application arises from their efficacy, availability, and favorable tolerability profile (Fekkar et al., 2026). The mechanism of action of azoles involves the inhibition of lanosterol 14-α-sterol demethylase, a key enzyme in the ergosterol biosynthesis pathway, and the subsequent violation of the fungal membrane's integrity. By inhibiting demethylase, azoles disrupt fungal growth, which is the key characteristic of their fungistatic effect (Pristov & Ghannoum, 2019). Besides reduced ergosterol biosynthesis itself, the action of azoles is accompanied by the accumulation of toxic precursor metabolites, also contributing to cell growth arrest (Li et al., 2025).

Although azoles have been in clinical use since the late 1960s (Maertens, 2004), the first reports of acquired azole resistance emerged in the 2000s, and since then, the documentation of azole-resistant isolates has steadily increased. Clusters of such resistant isolates frequently give rise to nosocomial outbreaks, particularly in intensive care units (Vumbaca et al., 2026; Fekkar et al., 2026; Li et al., 2025). In some healthcare settings, almost complete replacement of persistent susceptible strains by fully resistant ones has even been observed (Vumbaca et al., 2026; Franconi et al., 2023). Therapeutic efficacy in patients infected by resistance strains is quickly compromised also because treatment with fluconazole is the default empirical therapy, often initiated before susceptibility profile data become available, resulting in delayed targeted treatment (Guinea et al., 2025).

1.4.2. Echinocandins

Another widely used class of antifungal drugs is the echinocandins, in particular, caspofungin, micafungin, anidulafungin. Echinocandins carry out their effect by disrupting cell wall synthesis as well. More specifically, they noncompetitively inhibit (1,3)-β-D-glucan synthase, thus stopping the biosynthesis of 1,3-β-D-glucan, a key component of the *Candida* cell wall. This leads to disruption of osmotic homeostasis and cell lysis, exerting a fungicidal effect on *Candida*. Echinocandins contribute significantly to the treatment of candidiasis, including infections caused by azole-resistant strains, although their spectrum of actions remains narrow (Branco et al., 2023). Previously, resistance to echinocandins was rarely reported. However, in

recent years cases of acquired resistance have been documented in several countries, such as Greece, Iran, and China. Furthermore, cases of pan-echinocandin resistance have been reported, frequently in combination with multi-azole resistance (Guinea et al., 2025; Chen et al., 2023).

1.4.3. Polyenes

The last major class of antifungals is the polyenes, whose main representative is amphotericin B (AMB) - historically the first agent discovered within this group. AMB is used for particularly severe cases of candidemia due to its considerable toxicity. The mechanism of action involves binding to ergosterol in the fungal cell membrane, forming pores that disrupt membrane integrity, which consequently leads to osmotic imbalance and cell lysis (Silva et al., 2020). Resistance to amphotericin B remains relatively uncommon overall, and *C.parapsilosis* is usually considered to be sensitive to AMB (Branco et al., 2023). Nevertheless, certain *Candida* species, such as *C. auris*, have exhibited reduced susceptibility to this drug (Rybak et al., 2022).

1.5. Antifungal resistance mechanisms

Resistance to the previously discussed antifungal classes is acquired through a wide variety of distinct mechanisms. A comprehensive understanding of these resistance pathways is of fundamental importance for the development of novel therapeutic approaches. (Li et al., 2025). Antifungal resistance mechanisms in *C. parapsilosis* include classical mechanisms, such as point mutations in target genes and transcriptional upregulation of the genes and efflux pumps, as well as additional mechanisms that are less studied, including epigenetic regulation, heteroresistance, chromosomal copy number variations, in particular aneuploidy, and biofilm formation. Each of these will be discussed in detail below and summarized schematically in Figure 3.

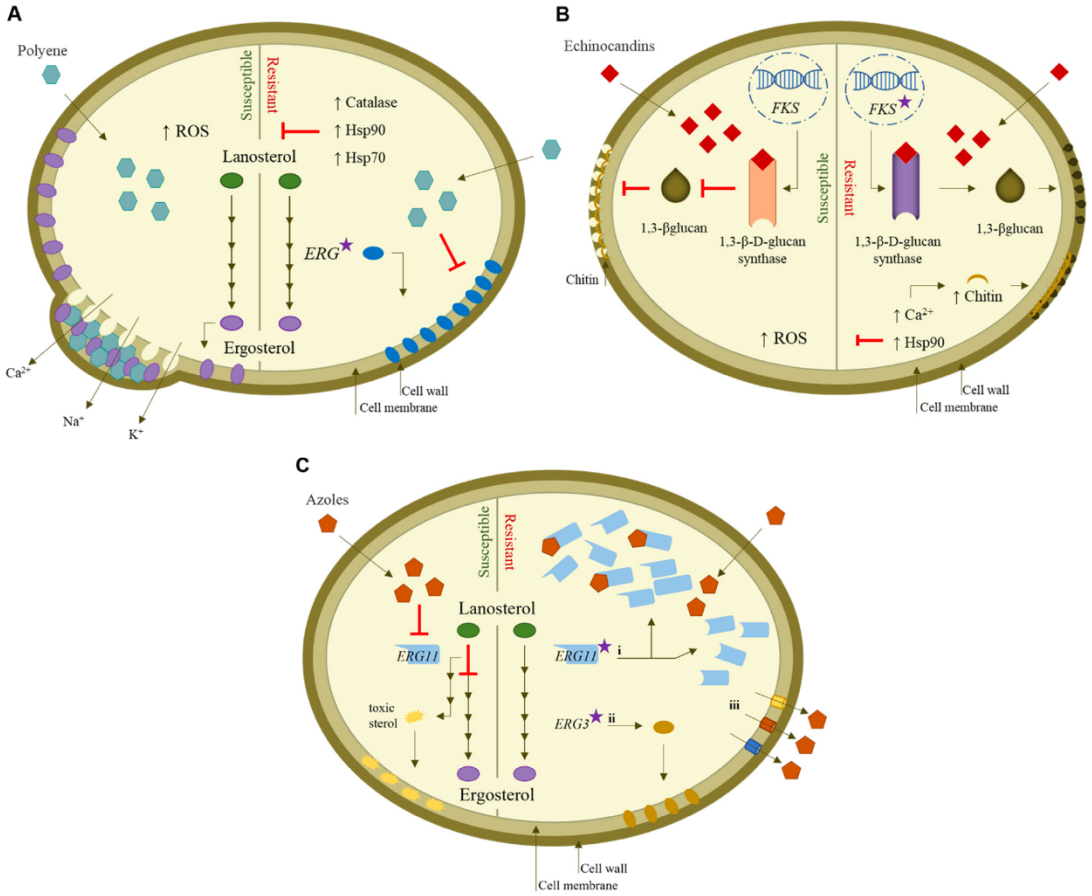


Figure 3. **Summary of the main mechanisms of action of resistance to polyenes, echinocandins, and azoles in *Candida parapsilosis***. (A) Polyenes form polyene–ergosterol aggregates in the fungal membrane, thereby destabilizing it. Resistance may be mediated by mutations in ergosterol biosynthesis genes that alter sterol composition and by activation of stress-response pathways, such as catalase and Hsp. (B) Echinocandins act as noncompetitive inhibitors of 1,3-β-D-glucan synthase, encoded by FKS genes, causing depletion of cell-wall 1,3-β-glucan. Echinocandin resistance in Candida is associated with mutations in FKS genes and activation of cell-wall stress-response mediator pathways, including Hsp90 and calcineurin (Ca^{2+}), which increase chitin content. (C) Azoles inhibit the enzyme lanosterol 14α-demethylase, encoded by ERG11, leading to the accumulation of toxic sterols. Azole resistance involves: (i) point mutations in ERG11, which may cause its overexpression and/or reduced azole–target binding affinity; (ii) mutations in ERG genes involved in the ergosterol biosynthesis pathway, particularly ERG3; and (iii) increased efflux of azole drugs from the fungal cell through overexpression of multidrug efflux pumps. Red T-shaped bars indicate inhibition; stars indicate gene mutations (Branco et al., 2023).

1.5.1. Azoles resistance

1.5.1.1. Alteration of the drug target and its expression

Resistance mutations in the coding region of the *ERG11* gene can lead to structural changes in its protein product, Erg11p, which in turn reduces its binding affinity for azole drugs, thereby their efficacy. Among the mutations associated with resistance, the most extensively studied are those in the related organism *Candida albicans*, for which over 140 distinct amino acid

substitutions have been described. It has been demonstrated that the impact of these substitutions on resistance varies for each specific mutation, and that the presence of two substitutions confers stronger resistance than a single mutation alone (Li et al., 2025). According to the data obtained for non-*albicans Candida* (NAC) species, the genetic sequence of ERG11 exhibits a considerably lower degree of variability. Specifically, among azole-resistant isolates of *C. parapsilosis*, the Y132F substitution has been identified, detected both independently and in combination with the R398I substitution (Berkow et al., 2017). However, the latter substitution R398I has also been described in fluconazole-susceptible isolates, suggesting that it does not contribute to resistance alone (Sharma et al., 2025).

Another mechanism affecting the increase in MIC in *C.parapsilosis* involves increased ERG11 expression. As a result, the intracellular concentration of Erg11p increases, and susceptibility to azoles declines, because target binding cannot be longer complete and ergosterol synthesis can continue. Nevertheless, ERG11 overexpression in NAC azole-resistant isolates, including *C. parapsilosis*, is rarely reported independently of other mutations; thus, this mechanism appears to function more as a contributor to MIC increase rather than as an independent resistance mechanism (Li et al., 2025). At the molecular level, it has been shown that ERG11 overexpression is frequently associated with gain-of-function (GOF) mutations in the UPC2 gene, whose product Upc2p is a zinc cluster transcription factor induced upon ergosterol depletion. Gain-of-function mutations in Upc2p render this transcription factor constitutively active, subsequently driving the upregulation of Erg11p synthesis and its accumulation in the cell (Berkow et al., 2017). Among the variants reported in *C. parapsilosis*, the N455D substitution in UPC2 has been identified. However, its direct causal role in azole resistance has not yet been experimentally confirmed (El Hady et al., 2025; García-Hernández et al., 2026).

1.5.1.2. Efflux of the drug

Efflux pump activity constitutes a key mechanism of intrinsic resistance to antifungals. To exert their therapeutic effects, drugs must reach a certain concentration at their intracellular compartment. By increasing efflux pump activity, *Candida parapsilosis* can actively displace antifungal agents from the cell, thereby lowering intracellular drug accumulation and compromising their therapeutic efficacy (Kadariswantiningsih et al., 2025). Two families of transporters are known to be linked to azole resistance: ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters (Czajka et al., 2023). In particular, fluconazole-resistant clinical isolates of *C.parapsilosis* rely on the overexpression

of the specific efflux pumps CDR1 (Candida Drug Resistance) of ABC-transporters and MDR1 (Multidrug resistance) of MFS-transporters. Although a direct role for these genes themselves in fluconazole susceptibility has not been established, their overexpression is known to be driven by gain-of-function mutations in their respective regulators - TAC1 (encoding the transcription factor Tac1) for CDR1 and MRR1 (encoding the regulator Mrr1) for MDR1, with specific mutations including G650E in TAC1 and A854V, R479K, I283R, K873N and G583R in MRR1 (Doorley et al., 2022, 2023; Hartuis et al., 2024; Branco et al., 2017).

Additionally, upregulation of *Ndt80* – another transcriptional factor – was observed in resistant isolates. This overexpression led to increased expression of 13 involved in ergosterol biosynthesis genes, as well as overexpression of the CDR1 efflux pump. In *C. albicans*, *Ndt80p* has been shown to bind to the promoters of ergosterol biosynthesis genes, including the azole target ERG11, and to regulate azole tolerance by controlling CDR1 expression (Silva et al., 2011; García-Hernández et al., 2026).

1.5.2. Echinocandins resistance

Decreased sensitivity to echinocandins in *C. parapsilosis*, according to the literature, is mainly due to point mutations in the FKS1 gene, which encodes one of the subunits of β-1,3-D-glucan synthase—the target enzyme of echinocandins. Compared to other fungal pathogens, *C. parapsilosis* exhibits intrinsically higher minimum inhibitory concentrations (MICs) toward echinocandins, which are attributed to a naturally occurring single-nucleotide polymorphism in hotspot 1 (HS1) of FKS1, resulting in a P600A substitution. In addition to this intrinsic reduced susceptibility, *C. parapsilosis* isolates can acquire additional mutations in HS1 (amino acids 652–660) and HS2 (1369–1376) of FKS1 upon exposure to echinocandins (Ünal et al., 2024; Guinea et al., 2025; Pristov and Ghannoum, 2019). These mutations directly affect the target structure and its binding affinity for the drug, leading to resistance; they are among the most frequently observed mutations in echinocandin-resistant clinical isolates (Kadariswantiningsih et al., 2025).

1.5.3. Sterol biosynthesis pathway

Beyond the previously described mechanisms, alterations in other ergosterol biosynthesis genes contribute to the development of resistance to azoles and echinocandins. Among these, ERG3 is reported as one of the most significant players. It encodes an enzyme responsible for the conversion of nontoxic 14α-methylated sterol intermediates into the toxic sterol 14α-methyl-ergosta-8,24(28)-dien-3,6-diol. Reduced activity or complete loss of this enzyme leads

to the accumulation of nontoxic ergosta-7,22-dienol and, consequently, an increase in azole resistance (Rybak et al., 2017). An example of Erg3 effect on drug resistance was presented by Branco et al., in 2017, when they showed that the R135I mutation in ERG3 completely inactivates the Erg3 enzyme. This inactivation prevents the conversion of nontoxic sterol intermediate into toxic one. Thus, when azoles inhibit ERG11, toxic sterols do not accumulate, allowing the cell to survive despite antifungal treatment. The upregulation of UPC2, *Ndt80*, and other ERG genes represents a compensatory response to sterol depletion, but it does not restore susceptibility because Erg3 remains nonfunctional. Specific mutations, in particular G111R amino acid substitution, in Erg3 was demonstrated to be responsible for a MDR phenotype, including resistance to azoles and echinocandins (Rybak et al., 2017; Ünal at al., 2024).

Resistance to AMB is reported quite rarely, but there are mechanisms that contribute to development of resistance. For example, other ERG genes can play a role in the appearance of resistant phenotype. Loss-of-function mutations in ERG2, ERG3, ERG6 can lead to reduction of ergosterol – the target molecule of AMB - content in the fungal membrane, therefore conferring resistance to this drug. In addition, upregulation of ERG5, ERG6, and ERG25 drives the accumulation of sterols other than ergosterol, also associated with amphotericin B resistance. Furthermore, since transcriptional factors UPC2 and *Ndt80* regulate multiple ERG genes—including ERG11, ERG2, ERG3, ERG4, ERG6, and ERG25—their overexpression leads to increased expression of these genes and have been observed in fluconazole-, voriconazole-, and posaconazole-resistant *C. parapsilosis* isolates (Franconi et al., 2023, Rybak et al., 2017).

1.5.4. CNV and polyploidy

Another mechanism of acquired resistance is occurrence of copy number variations (CNVs), which can influence the expression of multiple genes and provide an ability to adapt rapidly to environmental changes, including antifungal drug exposure (Selmecki, 2023). When selective pressures are not exerting e.g. drug removal, CNV can be lost due to the fitness cost associated with maintaining additional genetic material.

Recent studies evidenced that amplification of ERG11 gene – ranging from partial aneuploidy of chromosome 3, carrying this gene, to smaller amplifications - was observed in azole-resistant strains of *C. parapsilosis.* In addition, it is assumed that there is a link between CNVs at CDR1B locus and decreased sensitivity to azoles in some *C. parapsilosis* isolates (Bergin et al., 2024; Li et al., 2025).

Sun et al. (2023) demonstrated that *C. parapsilosis* can acquire tolerance to echinocandin drugs by trisomy for chromosome 5, while mutations in FKS gene in these isolates were rare. This phenomenon has been explained as a result of increased copy number and subsequent overexpression of CHS7, a gene located on the chromosome and encoding a protein essential for chitin synthesis, therefore elevating chitin content in the cell wall which compensate the decreased 1,3-β-D-glucan content caused by echinocandins action. Notably, this effect was not stable and *C.parapsilosis* reversed to diploidy in the absence of the drug.

Overall, this mechanism of adaptation in *C. parapsilosis* remains poorly understood and requires further investigation.

1.5.5. Biofilm formation

Biofilms are communities of microorganisms that adhere to surfaces and embedded in an extracellular matrix that they produce, and they are considered to be an important virulence factor, as they can restrict the penetration of substances, including drugs, through the matrix and provide protection against the host immune response (Li et al., 2025). *C.parapsilosis* biofilm structure varies among strains but generally includes yeast and pseudohyphal morphologies, forming either compact multilayers or non-contigous aggregates. The extracellular matrix is primarily composed of carbohydrates, with low protein levels, and generally, extracellular matrix proportion in *C.parapsilosis* biofilms is less compared to other NAC species (Cavalheiro and Teixeira, 2018).

As discussed before, the use of medical devices, such as catheters, is a significant risk factor associated with *C.parapsilosis* infections, as these surfaces provide a niche for biofilm formation. This process is accompanied by differential expression of the range of adhesin molecules. Biofilms formed on medical devices show significantly reduced susceptibility to azoles (Gómez-Molero, 2021). The fungal cells forming biofilms are characterized by lower rates of growth and higher resistance to antimicrobial drugs. Specifically in *C.parapsilosis,* biofilms formation can decrease susceptibility to fluconazole, voriconazole, ravuconazole, amphotericin B, nystatin, chlorhexidine, and terbinafine (Cavalheiro and Teixeira, 2018; Bizerra et al., 2011).

1.5.6. Compensatory stress response

Along with resistance mechanisms described before, *Candida* species have developed stress responses pathways that ensure protection from antifungal-induced stress. One of the key components of this stress response net that is related to drug resistance is Hsp90. This protein

is a highly conserved chaperone, regulating an activity of a wide range of proteins (Li et al., 2025) via regulation of post-translational modifications, including acetylation and phosphorylation (di Silva et al., 2024). As a result, through activation of calcineurin signaling pathway this can enhance synthesis of cell wall chitin, providing protection against antifungal drugs (Branco et al., 2023).

Thus, Hsp90 has been reported to function as a molecular trigger that enhances resistance across the entire spectrum of antifungal agents available against *C.parapsilosis*, including azoles, echinocandins, and polyenes (Branco et al., 2023; di Silva et al., 2024; Kim et al., 2019). Also it was demonstrated by di Silva et al. (2024) that MIC of micafungin drops significantly *in vitro* (from 1–2 μg/ml down to just 0.015 μg/ml) when combined with Hsp90 inhibitors, and, therefore, these inhibitors emerge as a promising class of potential drugs for combination antifungal therapy.

1.5.7. Epigenetics

Although this field remains only partially explored, emerging evidence suggests epigenetic regulation involvement in antifungal drug resistance (Patra et al., 2022; Tarek et al., 2024). Epigenetic modifications are defined as stable, heritable cellular changes that arise independently of DNA sequence alterations, and usually include post-translational modifications of histones, RNA-based mechanisms, DNA methylation (Patra et al., 2022; Zhang et al., 2025). Via these mechanisms of epigenetic modifications, the expression of key resistance genes can be modulated, including multidrug efflux pumps (CDR1, MDR1), enzymes of ergosterol biosynthesis pathways (ERG11) and cell wall integrity pathways.

Although the studies focused on epigenetic modifications in *C.parapsilosis* are still limited, recent works on the other emerging fungal pathogens like *Candida auris* have underscored the central role of the histone acetyltransferase Gcn5, which governs resistance to azoles, polyenes, and echinocandins by regulating drug efflux, calcineurin signaling, and the transcription factor Cas5 - with its pharmacological inhibition showing promising synergy with caspofungin (Zhang et al., 2025).

Despite the research advances in this field, epigenetics in fungi remains predominantly characterized by studies restricted to a limited number of species and laboratory-adapted strains, while the precise interplay between distinct histone marks, the molecular basis of phenotypic resistance, and the target specificity of pharmacological inhibitors remain largely undefined and required further investigation.

1.5.8. Heteroresistance

Heteroresistance is not a mechanism of resistance itself, but must be discussed in the context of fungal drug resistance because it facilitates pathogen survival.

Heteroresistance can be defined as a phenomenon in which a clonal fungal (or microbial) population includes small subpopulations exhibiting a different phenotype, whose prevalence can be as rare as one in one million cells. The MIC of this subpopulation for a given antifungal drug can be higher than the average value in the main population and, in particular, may correspond to a resistant phenotype. Therefore, these resistant cells can survive and multiply under treatment at drug concentrations that normally would kill susceptible cells (Zhai et al., 2024). Furthermore, the phenotype of resistant cells can reverse after removal of the selective drug pressure (Guinea et al., 2025). As this resistant cell population is small, heteroresistance is usually undetected by standard antifungal susceptibility testing, resulting in clinical difficulties due to ineffective treatment, as resistant subpopulation can withstand under its exposure.

C. parapsilosis demonstrates capability to develop heteroresistance through formation of trimeras - three-lobed cells with two nuclei. Aberrant chromosome segregation in trimeras leads to progeny with doubled chromosome numbers, whose unequal segregation drives aneuploidy in the population. Although this mechanism of aneuploidy and heteroresistance formation does occur under azole exposure, the development of stable resistance is more common in C. parapsilosis (Li et al., 2025; Harrison et al., 2014).

For instance, Zhai et al. (2024) have demonstrated clinical significance of this phenomenon by identifying micafungin-heteroresistant strains of *C.parapsilosis*. The study revealed that heteroresistance to micafungin contributes to the emergence of breakthrough *C. parapsilosis* infections in patients undergoing allo-HCT (Allogeneic Hematopoietic Cell Transplantation) and receiving micafungin prophylaxis. Importantly, no classical, stable resistance mechanisms to micafungin were identified in this study. These findings indicate that the prophylaxis failure was caused by heteroresistant strains rather than by classical resistance.

1.6. Fungal typing

Given the rising threat of drug-resistant fungal pathogens, the ability to accurately identify the species and resolve them at the sub-species level is an essential requirement for successful epidemiological surveillance to improve public health monitoring strategies. First attempts to distinguish fungal species were based on morphological and physiological characteristics,

which is limited due to minimal morphological differences among these taxa. Discriminating isolates within the same species using these methods remains challenging due to their phenotypic similarity. To overcome these limitations, molecular approaches have been developed allowing accurate identification of clinical isolates (Neppelenbroek at al., 2014).

The first-line molecular typing approach for species identification is the sequencing of the Internal Transcribed Spacer (ITS) regions of the ribosomal DNA, which is the standard fungal identification region**.** ITS is highly effective for distinguishing between different species, but it often lacks the discriminatory power required to differentiate between genetically closely related isolates (within a single species), for instance during outbreak investigations. To tackle this issue, more targeted genotyping methods, such as microsatellite length polymorphism (MLP) and Multi-Locus Sequence Type (MLST), have been developed (Kauserud, 2023). MLP utilizes the amplification of short tandem DNA repeats (STRs) at multiple loci throughout the eukaryotic genome. The high diversity and mutation rates of these STRs in certain species provide enough discriminatory power to distinguish between closely related strains, and for *Candida* spp. this method has been used for strain typing, populational structure analysis and epidemiological studies, being recognized as a reproducible technique for *C. parapsilosis* molecular genotyping.

MLST is based on the sequencing of a set of multiple housekeeping genes, where the combination of allelic profiles of the loci is associated with a specific sequence type, allowing strain discrimination (Alanio et al., 2017; Spampinato and Leonardi, 2013). Although primarily developed for bacterial pathogens, MLST has been also adopted for fungal genotyping. While MLST contributed significantly to phylogenetics studies, and, for instance, allowed to isolate *C. orthopsilosis* and *C. metapsilosis* of the *C. parapsilosis* complex, its application to outbreak studies remains limited due to relatively low mutation rate in housekeeping genes (Ryan et al., 2025). Despite this issue, attempts to develop an MLST strategy for *C.parapsilosis* have been made (Brassington et al., 2025).

Nowadays, although ITS typing is still broadly used for *C. parapsilosis*, the usage of whole genome sequencing (WGS) is increasing significantly, as it offers improved resolution compared to the other typing methods. Isolates can be differentiated if they have a minimal genetic difference of several single nucleotide polymorphisms (SNPs), and WGS additionally enables the identification of underlying drug-resistance mutations, which makes WGS ideal in the context of genomic epidemiology e.g. spot disease outbreaks. However, the usage of WGS can be limited by several factors, including the associated costs and timescales, especially in

settings with limited resources, including computational and analytical ones. (Spruijtenburg et al., 2024).

1.7. NGS

Next-Generation Sequencing (NGS) technologies was introduced between 2004 and 2006 and resulted in significant increase in sequencing data output as, introducing massive parallel sequencing, it allowed to overcome the limitations associated with traditional first-generation Sanger method, which was restricted to sequencing a single DNA fragment at a time (Hu et al., 2021; Lu et al., 2025).

Second-generation sequencing technologies are mainly represented by Illumina and Ion Torrent platforms, and characterized as “short-read” sequencing methods that typically generate fragments of 250-800 base pairs length. These platforms are based on a sequencing-by-synthesis (SBS) principle, which involves DNA fragmentation, end-repair, adapter ligation, surface attachment, and in-situ amplification, enabling millions of individual sequencing reactions to occur at the same time. The advantage of short-read sequencing is its high accuracy, cost-effectiveness for specific use cases (e.g. clonal complex assignment), and the extensive availability of bioinformatic tools specifically designed for short-read data analysis. However, because these technologies sequence only short fragments, downstream bioinformatics analyses may be limited e.g. genome assembly could fail to reconstruct complete genomes, presenting significant challenges when resolving structural variations, repetitive regions, and low-complexity sequences (Hu et al., 2021).

In contrast, third-generation sequencing technologies, such as Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT), are defined as "long-read" sequencing methods and capable of generating contiguous sequences exceeding 10 kb, and in some cases reaching unfragmented DNA molecules, for instance, complete viral DNA. PacBio's Single Molecule Real-Time (SMRT) sequencing monitors the incorporation of fluorescently labeled nucleotides by individual DNA polymerase in real-time without template amplification, while ONT sequencing passes a single strand of nucleic acid through a nanoscale protein pore, identifying nucleotides by measuring characteristic disruptions in ionic current. The key advantages of third-generation technologies include minimal library preparation steps, the ability to handle repetitive genomic regions and improved resolution of structural variants (Hu et al., 2021; Lu et al., 2025). Moreover, these platforms can generate data more rapidly than second-generation approaches, making them particularly valuable for rapid pathogen identification and real-time outbreak surveillance (Lu et al., 2025). However, early limitations of long-read sequencing

included lower base-call accuracy compared to short-read technologies, although this has been improved through ongoing advancements (Hu et al., 2021).

1.8. Whole-Genome Sequencing and Its Applications in Phylogenomics and Genomic Epidemiology

Currently, the integration of second- and third-generation sequencing approaches in whole-genome sequencing (WGS), combines the high accuracy and cost-effectiveness of short-read sequencing with the long-range contiguity and structural resolution of long-read technologies. Whole-genome sequencing (WGS) is a comprehensive sequencing methodology that resolves the complete nucleotide sequence of an organism's genome, covering both protein-coding genes and non-coding sequences. By enabling high-throughput data covering all genetic alterations - from single-nucleotide polymorphisms to complex copy-number changes and large structural rearrangements - WGS serves as the primary empirical instrument underlying the broader discipline of genomics (Lu et al., 2025).

WGS, as it provides sufficient resolution to detect even minimal genetic differences in fungal genomes, enables the inference of phylogenetic relationships - the evolutionary history and relatedness among organisms. In the context of fungal genomics, this phylogenomic approach is essential to define population structure, reconstruct spread and transmission, and explain the evolution of antifungal resistance (Schikora-Tamarit, Gabaldón, T., 2022; Chow et al., 2020).

Calculation of SNPs distance is essential for inferring these phylogenetic relationships and understanding pathogen epidemiology and evolution, and WGS data enables precise calculation of pairwise SNPs distances between isolates. Usually SNP distance is determined by mapping sequence data from individual isolates against a closely related genome. Subsequently, the pairwise SNP distance can be used to infer phylogenetic relationship between strains through various methods such as Maximum parsimony, Maximum likelihood and Bayesian approach (Uelze et al., 2020; Kulik et al., 2022; Ludden et al., 2021). Defined SNP distances can identify closely related clusters and reveal their evolutionary relationships, which is useful for outbreak surveillance and for understanding population-level genomic diversity (Uelze et al., 2020).

The importance of phylogenomic approaches can be illustrated by their influence on fungal taxonomy. Recent phylogenomic studies have led to changes in the taxonomy of the *Candida spp*. and, as a result, *Candida parapsilosis* has been reclassified as *Lodderomyces parapsilosis* (Liu et al., 2026). As the reclassification is relatively recent, the name *Candida parapsilosis* is

still predominantly used in literature. Therefore, in this thesis the name *Candida (Lodderomyces) parapsilosis* is used for consistency.

NGS technologies together with associated bioinformatics tools have led to the rise of the field of genomic epidemiology, where WGS approaches along with epidemiological data are used to provide the highest possible resolution for outbreak characterization. Genomic epidemiology can offer practical insights into transmission chains, particularly in the context of healthcare-associated infections (HAIs). For instance, besides identification and characterization of the causative agent, when epidemiological linkages are missing or unclear at first, WGS data can help to generate hypotheses regarding the source of an outbreak and help to distinguish between cases that are related or unrelated to the outbreak event. Therefore, it makes it possible to improve the understanding of transmission networks and guide public health responses, for instance, to develop new procedures and policies aimed at preventing future incidents, such as decisions to close contaminated rooms or wards or implement advanced infection control measures (Tang et al., 2017).

1.9. Adaptive microevolution and within-host evolution

Antimicrobial resistance can emerge under drug exposure as a response to selective pressure (Fisher et al., 2022). They experimentally demonstrated that fungal pathogens can rapidly acquire drug resistance under antifungal treatment, but also lose this resistance upon subsequent removal of the drug, as the acquisition of drug resistance often carries a fitness cost (Beekman et al., 2020). The rapid enrichment of resistant variants under selective pressure is referred to as adaptive microevolution. Previously discussed mechanisms of drug resistance, such as mutations in key genes, accumulation of mutations across multiple genes, alteration to the gene expression and development of aneuploidy were registered as the genetic basis of this phenomenon due to recent advances in NGS (Boyce, 2023). Microevolution is particularly important for pathogen adaptation within the host during infection and treatment. Environmental factors that favor some traits over others, determining which organisms have better reproductive success, are more diverse inside the host than those in vitro. For instance, they include immune response, fluctuating pH, variable nutrient supplies, microbial competition. Together with genomic plasticity - the capacity to undergo rapid structural, ploidy, and copy-number rearrangements under stress - of fungal pathogens (Zande et al., 2023) it creates a wider range of possible genetic changes and enables rapid increase in virulence and antifungal resistance, also leading to life-threatening conditions. Within-host emergence of

antifungal resistance has been reported during individual infections caused by *C. albicans*, *A. fumigatus*, and *Cryptococcus* spp. (Beekman et al., 2020). NGS and comparative genomics can help to track the genetic changes responsible for changes in resistance during this process. For example, Ford et al., 2015, used these approaches to track *C.albicans* isolates from a single patient over the course of antifungal treatment, revealing that the acquired resistance was associated with recurrent loss of heterozygosity (LOH) and SNPs in 166 genes. For *C.parapsilosis* the emergence of antifungal resistance after prolonged treatment was also reported in previous studies (Zhang et al., 2025).

2. AIM OF THE WORK

C. parapsilosis is an emerging fungal pathogen with an increasing incidence of infection and growing number of reported cases of antifungal resistance. During treatment exposure, resistance can arise due to selective pressure, hampering patient management and hospitalization. Understanding the process and factors that contribute to the development of resistance is pivotal for improving treatment strategies and development of new antifungal drugs.

The aim of this Master's thesis was to identify genomic drivers of rapid changes in *C. parapsilosis* in relation to antifungal resistance exposure and following phenotypic changes using whole-genome sequencing and comparative genomics. By comparing genetically closely related isolates with different antifungal susceptibility profiles, the study aimed to identify genomic variants and larger-scale genomic changes that could potentially contribute to the observed phenotypic differences. In addition, the study aimed to link observed genomic and phenotypic changes to antifungal therapy received by the patients.

3. MATERIALS AND METHODS

3.1. Clinical Samples and Antifungal Susceptibility Data

3.1.1. Data collection

The study included a total of 18 *C. parapsilosis* clinical isolates obtained from blood cultures of eight patients with candidemia between 2018 and 2024 at the IRCCS Hospital San Matteo, Pavia, Italy. The samples were collected at different time points of the treatment. The assessment of MIC to different antifungal drugs was performed in the laboratory using Sensititre YeastOne ITAMYUCC, commercialized, colorimetric broth microdilution (BMD) platform for antifungal susceptibility testing.

3.1.2. Illumina sequencing

Genomic DNA was extracted from *C. parapsilosis* isolates using the DNeasy ® Blood & Tissue Kit (QIAGEN). The extracted DNA was sequenced using Illumina MiSeq, then raw paired-end Illumina FASTQ files were obtained and quality check was performed for subsequent bioinformatic analysis.

3.1.3. Reference genomes

Two *C. parapsilosis* reference genomes were used for different purposes in the analysis. The publicly available reference genome retrieved from the NCBI database (genome assembly ASM3628897v1) was used to identify and locate genes previously associated with antifungal resistance and to investigate previously reported resistance-associated variants.

The internal *C. parapsilosis* reference genome provided by the hospital was used as the reference for pairwise genomic comparisons (Vumbaca et al., 2024).

Variant calling and annotation were performed using the same workflow for analysis with both genomes and described below.

3.2. Quality Control and Preprocessing

Raw paired-end sequencing reads were subjected to initial quality control analysis using FastQC v0.11.9, and the results were aggregated and summarized using MultiQC v1.28. Subsequently, to prevent false-positive variant calls and mapping artifacts, trimming of adapters and low-quality reads (with a minimum Phred quality threshold of Q15) were performed using fastp v0.23.4, and quality of the resulting reads was reassessed using FastQC.

3.3. Variant Calling

Variant calling from reads is the gold standard in fungal genomics to outline population structure and infer evolutionary relationships among isolates, because reconstructing complete genomes from short-read data alone remains challenging. In order to perform this analysis, first the reads were aligned to the *C. parapsilosis* reference genome using Bowtie2 v2.5.2 with default settings, and the resulting SAM files were converted into BAM format using Picard AddOrReplaceReadGroups v3.4.0, with read-group information assigned to each sample. Then, they were sorted using Picard SortSam v3.4.0, which enables efficient processing and duplicate marking. Duplicates from PCR were identified and marked using Picard MarkDuplicates v3.4.0, so that they are not counted as independent evidence during variant calling.

To perform variant calling (SNPs and indels) Genome Analysis Toolkit (GATK) v4.2.2.0 HaplotypeCaller was applied with default setting, while specifying samples diploidy (--sample-ploidy 2, because *C.parapsilosis* is a diploid organism), and using -ERC GVCF option to produce GVCFs that includes both variant and non-variant sites for subsequent joint genotyping. The obtained GVCFs were further loaded into a GATK GenomicsDB to merge multiple sample GVCFs into a combined VCF using the GenotypeGVCFs GATK function, which performs joint genotyping across isolates to improve accuracy. Subsequently, the variants were filtered with VariantFiltration GATK function to retain high-confidence variants specifying following parameters: DP<=20 || QD<2.0 || MQ <40.0 || FS>60.0 || MQRankSum < -12.5 || ReadPosRankSum<-8.0. These filters remove variants with low depth, low quality, poor mapping quality, strand bias, or biased position in reads. A filter that identifies heterozygous sites (isHet ==1) was applied to mark heterozygous genotypes and then use them in downstream analysis. Variant clustering was assessed using the --cluster-size 5 and --cluster-window-size 20 parameters, so the variants that appear too close together and may represent misalignments are filtered out.

Next, only SNPs were selected using GATK SelectVariants with –select-type-to-include SNP option. Low-quality SNPs were then filtered out using VcfFilter v0.2 with the parameters -minGenomeQuality 50 and -minSampleDepth 30.

3.4. Phylogenetic analysis

The obtained multi-samples VCF was converted into a FASTA alignment using vcf2phylip which takes the genotype information from the VCF and converts it into an alignment that can be used for phylogenetic inference. Heterozygous positions were retained and represented using the corresponding IUPAC ambiguity codes. Invariant sites were removed using snp-sites v2.5.1. Based on resulting alignment a maximum-likelihood phylogenetic tree was inferred with IQTREE v2.0.7 using MFP-GTR model and 1000 bootstrap replicates. In order to contextualize analyzed samples into broader background of San Matteo Hospital, phylogenetic analysis was performed including additional 32 genomes of *C. parapsilosis*, also sampled and sequenced in the hospital between 2020 and 2024.

Pairwise SNPs distances between isolates were calculated using the dist.dna function from the R package "ape", and then the obtained distance matrix was visualized as a heatmap using the "pheatmap" package.

3.5. Variant annotation

In order to predict functional impact of genomic variants of *C. parapsilosis,* the merged multi-sample VCF was annotated using SnpEff v5.3a with a custom database generated from the reference genome. SnpEff predicts variant effects by comparing each variant's position to known gene models and translating the resulting codon change into a functional impact class, which include:

- High impact (e.g. frameshift, stop gained)
- Moderate impact (missense mutations)
- Modifier impact (mutations in non-coding regions)
- Low impact (synonymous) mutations.

Gene prediction for database building on the reference genome was performed de novo using Augustus v3.3.3 with prediction based on the *C.albicans* model, being the closest species to our case study organisms.

3.6. Identification of Antifungal Resistance-associated Variants

To identify variants located within genes previously reported to be involved in antifungal resistance (Berkow et al., 2017; El Hady et al., 2025; Doorley et al., 2022, 2023; Hartuis et al., 2024; Branco et al., 2017; Ünal et al., 2024; Guinea et al., 2025; Pristov and Ghannoum, 2019)

the sequences of the genes were retrieved from public databases (NCBI and Candida Genome Database) and used as queries in a BLAST search against the reference genome using the NCBI BLAST+ suite v2.16.0+**.** The obtained coordinates were subsequently used to select variants of interest. The analysis focused on genes previously associated with antifungal resistance in *C. parapsilosis*, including ERG11, ERG3, ERG4, ERG6, FKS1, UPC2, TAC1, MRR1, NDT80, CDR1, CDR1B and MDR1B**.**

3.7. Longitudinal comparison of isolate variants from the same patient

Identification of variants that differed between sequential isolates obtained from the same patient was performed using BCFtools v1.19 with "isec" mode. The identified isolate-specific variants were further classified according to genotype (homozygous or heterozygous state) and their predicted functional impact (HIGH, MODERATE, LOW of MODIFIER) of the protein structure based on previous SnpEff annotation.

Genes carrying homozygous mutations and mutations predicted to have a HIGH impact of the variants were further investigated using the NCBI BLASTp web interface to confirm gene identity. Literature research was subsequently conducted to determine whether these identified genes were previously noted to be associated with antifungal resistance.

For proteins carrying predicted heterozygous mutations of MODERATE and MODIFIER impact of the variants, the corresponding protein sequences were extracted and blasted against a *Candida* (*Lodderomyces) parapsilosis* protein database downloaded from UniProtKB on 10 July 2026 using BLASTp. Matches with >90% sequence identity were retained, and the corresponding UniProt protein identifiers were extracted. These identifiers, together with previously reported antifungal resistance-associated proteins (ERG11, ERG3, ERG4, ERG6, FKS1, UPC2, TAC1, MRR1, NDT80, CDR1, CDR1B, and MDR1B), were subsequently analysed using STRING v12.0 to investigate potential protein–protein interactions. A combined score threshold of 0.7 was used to define high-confidence functional association.

3.8. Mutational Hotspots analysis

SNP density was calculated using VCFtools v0.1.16 was used with --SNPdensity 1500 option, which calculated the number of variants located within every 1500 nucleotides. Afterwards, custom script in R was used to plot the resulting SNP-density and identify hotspots of mutations.

3.9. CNV and aneuploidy

Copy number variation (CNVs) and aneuploidy were assessed using Delly v1.7.2 from the processed BAM files. The results were subsequently plotted using the rd.R script provided by the Delly tool.

4. RESULTS

4.1. Clinical Samples and Antifungal Susceptibility Data

A total of 18 *C. parapsilosis* clinical isolates obtained from eight patients with candidemia between 2018 and 2024 were included in the study. For each patient, sequential isolates were available at different time points during treatment. The clinical and sampling metadata of the isolates are summarized in Table 1. Hereinafter, isolate IDs consist of a sample number and patient initials; random identical initials referring to the same patient (e.g., 1AG and 2AG are two samples from patient AG, where 1AG was obtained prior to 2AG) were set for anonymisation.

All isolates were susceptible to echinocandins (anidulafungin, caspofungin, micafungin) and amphotericin B. Fluconazole resistance (MIC ≥ 64 μg/mL) was detected in the majority of isolates.

Changes in MIC values and in susceptibility phenotype were observed between sequential isolates obtained from the same patient. Fluconazole susceptibility changes were observed in half of the patients (AG, BA, TM, BAL), showing significant MIC values difference (from 0.5 to 256 μg/mL) ranging from ten days up to several weeks, with isolates belonging to patient BA showing a return to resistance after a transient susceptible period. Changes in fluconazole MIC values were also accompanied by changes in voriconazole MIC values in several sequential isolates. All MIC values are reported in Table 2.

Since MIC changes were observed across sequential isolates from the same patients, we performed sequencing to verify if the isolates were the same strain showing phenotypical changes, and it was not the case of co-occurrence of multiple strains in the same patient. Afterwards, we could assess whether any mutations arose that could explain this shift.

4.2. Sequencing Quality Control

Quality check was performed before and after adapters trimming. The final metrics (after trimming) for samples are summarized in Table 1.

Table 1. **Quality control metrics of raw sequencing reads across all studied isolates**. Columns: percentage of duplicate reads (Dups), GC content (GC), median read length in base pairs (Median len), and total number of sequences in millions (Seqs).

Sample	Dups	GC	Median len	Seqs
1AG_S7_L001_R1_001	15.310	38	247	1.528

1AG_S7_L001_R2_001	10.607	38	247	1.528
1BAL_S2_R1	22.313	40	251	1.303
1BAL_S2_R2	20.905	40	251	1.303
1BA_S8_L001_R1_001	15.126	38	251	1.670
1BA_S8_L001_R2_001	11.142	38	251	1.670
1CS_S3_L001_R1_001	15.354	38	247	1.583
1CS_S3_L001_R2_001	8.706	38	247	1.583
1FA_S5_L001_R1_001	15.313	37	251	1.753
1FA_S5_L001_R2_001	11.481	37	251	1.753
1TM_S6_R1	36.723	42	251	1.631
1TM_S6_R2	35.494	42	251	1.631
2AG_S1_R1	24.696	41	251	1.035
2AG_S1_R2	21.082	41	251	1.035
2AL_S1_L001_R1_001	12.852	38	251	1.375
2AL_S1_L001_R2_001	7.4106	38	251	1.375
2BAL_S3_R1	23.923	41	251	1.163
2BAL_S3_R2	22.086	41	251	1.163
2BA_S4_R1	35.923	44	251	1.521
2BA_S4_R2	34.066	43	251	1.521
2CS_S4_L001_R1_001	12.858	38	251	1.318
2CS_S4_L001_R2_001	8.431	38	251	1.318
2FA_S6_L001_R1_001	17.248	38	247	2.066
2FA_S6_L001_R2_001	12.852	38	247	2.066
3AL_S2_L001_R1_001	18.938	38	167	3.069

3AL_S2_L001_R2_001	15.105	38	167	3.069
3BA_S5_R1	29.867	42	251	2.110
3BA_S5_R2	26.918	41	247	2.110
3TM_S7_R1	25.021	40	251	2.415
3TM_S7_R2	23.444	40	251	2.415

The mean quality of the reads across all the samples is shown in Figure 4. The forward reads passed the control, while reversed reads showed lower quality. Moreover, the quality of reads drops by the end of the sequences. Both patterns are well documented across Illumina paired-end datasets and are generally treated as expected technical behavior. Thus, overall quality of the reads was significant enough to proceed the analysis.

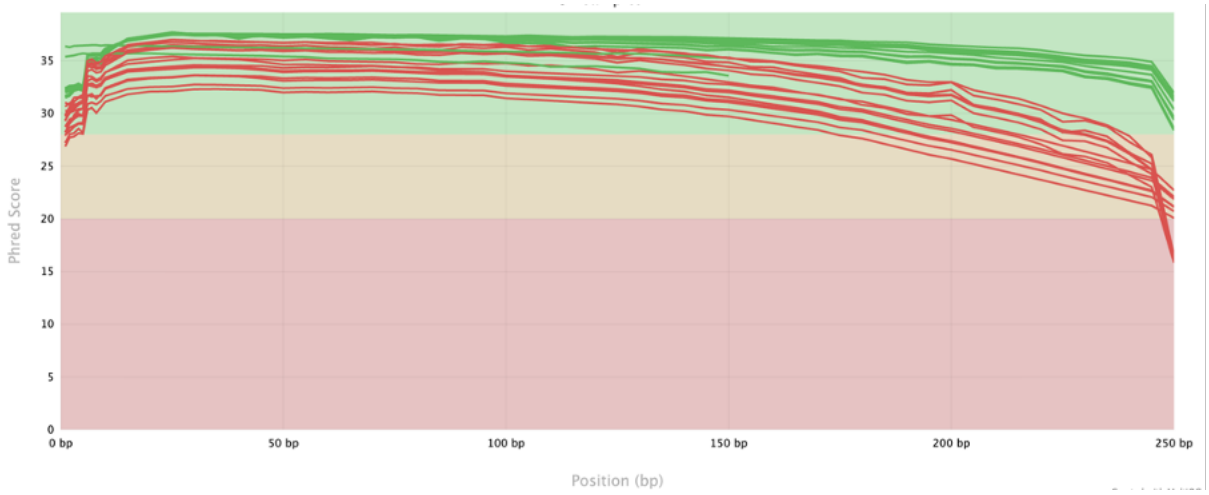


Figure 4. **Mean Quality Score across all the samples**. Green — forward reads (passed quality check); red — reverse reads (failed quality check).

During subsequent reads alignment to the reference genomes, one sample (1FA) showed a markedly low alignment rate; therefore, we performed taxonomic classification using Kraken2, which identified the sample as *Candida metapsilosis*. This isolate was excluded from the downstream analysis.

4.3. Phylogenetic analysis and SNP distance

To evaluate the relatedness of isolate pairs from single hosts, and thus evaluate whether changes in MIC were related to within-host evolution rather than co-infection by multiple

isolates, a phylogenetic analysis was performed. To provide phylogenetic context for the isolates characterized in the present study, 32 additional *C. parapsilosis* isolates, also sampled and sequenced in the the IRCCS Hospital San Matteo between 2020 and 2024, were included in the phylogenetic analysis. These isolates had been previously characterized (Vumbaca et al., 2026) and found to belong to three groups: monophyletic groups A and B, and sporadic isolates. Group B was characterized as a persistent fluconazole-resistant cluster in the previous study. All the isolates belonging to group A showed sensitivity to all azole drugs. The phylogenetic tree obtained including these isolates is shown in Figure 5.

After defining the epidemiological relationships between isolates, we restricted downstream analyses to pair of isolates that clustered together in the phylogeny (i.e., those that were closely genetically related), for a total of 3 pairs.

Phylogenetic analysis identified a cluster containing isolates from the present study together with isolates belonging to Group B (highlighted in red). The analysis revealed that 11 of the studied isolates belong to the persistent *C. parapsilosis* cluster, in which we identified 5 pairs of sequential isolates recovered from the same patient: 2AL and 3AL, 1CS and 2CS, 1BA and 2BA, 1BAL and 2BAL, 2TM and 3TM.

The AL, CS, as well as FV, isolate pairs clustered together, but did not show a change in fluconazole MIC (the difference of one fold double dilution is not considered significant) or susceptibility phenotype and were therefore excluded from subsequent comparative analysis. The isolates retrieved from AG patients didn't cluster together, thus, they were also excluded from further within-patient analysis. Additional isolates from patients BA and TM (3BA and 1TM, respectively) clustered separately from the corresponding sequential isolate pairs and were therefore excluded from the comparative analysis. Consequently, further genomic analysis focused on the three sequential isolate pairs from patients BA, BAL, and TM: 1BA-2BA, 1BAL-2BAL, and 2TM - 3TM.

The information regarding the treatment of BA, BAL and TM patients was requested from clinicians of IRCCS Hospital San Matteo. The treatment was following:

- BA was treated with caspofungin in the period of 21-31 of July, 2022.
- BAL was treated with caspofungin starting from the 5th of July, 2022. The date of the end of the treatment is unknown.
- TM was treated with a combination of caspofungin and isavuconazole prior to isolation of the 2TM strain. Isavuconazole was then withdrawn, and caspofungin monotherapy was continued until isolation of the 3TM strain. The exact dates of therapy are not available. The dosage and the frequency of use are not available.

ID	Collection date (dd/mm/yy)	**Amfotericina B**		**Anidulafungina**		**Caspofungina**		**Fluconazolo**		**Itraconazolo**		**Micafungina**		**Posaconazolo**		**Voriconazolo**	
1AG	13/08/2021	0,25	S	1	S	1	S	0,5	S	0,03	S	2	S	0,015	S	<0,008	S
2AG	31/08/2021	0,25	S	0,5	S	1	S	256	R	0,12	S	2	S	0,06	S	2	R
2AL	15/01/2021	0.25	S	1	S	1	S	64	R	0,12	S	1	S	0.03	S	0.5	I
3AL	24/01/2021	0.5	S	1	S	1	S	64	R	0.12	S	2	S	0.06	S	0.5	I
1BAL	04/07/2022	0,5	S	1	S	1	S	64	R	0,12	S	1	S	0,06	S	0,5	I
2BAL	18/07/2022	0,12	S	1	S	1	S	8	R	0,03	S	1	S	0,015	S	0,12	S
1BA	20/07/2024	0.5	S	1	S	1	S	128	R	0,12	S	1	S	0,06	S	1	R
2BA	31/07/2024	0.25	S	1	S	2	S	1	S	0.12	S	2	S	0,03	S	0.015	S
3BA	18/09/2024	0.5	S	1	S	1	S	64	R	0.06	S	2	S	0.03	S	0.5	I
1CS	21/08/2022	0.5	S	1	S	1	S	64	R	0.25	SDD	2	S	0.06	S	1	R
2CS	14/10/2022	0.5	S	1	S	1	S	64	R	0.25	SDD	2	S	0.06	S	1	R
1FA	20/05/2023	0.5	S	0.5	S	0.5	S	2	S	0.25	SDD	0.25	S	0.12	S	0.06	S
2FA	08/06/2023	0.25	S	1	S	1	S	1	S	0.12	S	1	S	0.06	S	0.03	S
1TM	19/10/2018	0,25	S	1	S	1	S	0,5	S	0,12	S	1	S	0,03	S	<0,008	S
2TM	31/10/2018	0.5	S	1	S	1	S	128	R	0.12	S	1	S	0.06	S	0.5	I
3TM	24/11/2018	0.25	S	1	S	1	S	8	R	0,25	SDD	1	S	0.03	S	0,015	S
1FV	05/04/2019	0.5	S	2	S	0.5	S	0.5	S	0.06	S	2	S	0.06	S	0.015	S
2FV	12/01/2020	0.5	S	2	S	0.5	S	2	S	0.06	S	2	S	0.06	S	0.03	S

Table 2. **Clinical and sampling metadata**. MIC – minimum inhibitory concentration (μg/mL). S – susceptible, I – intermediate, R – resistant, SDD – susceptible dose-dependent.

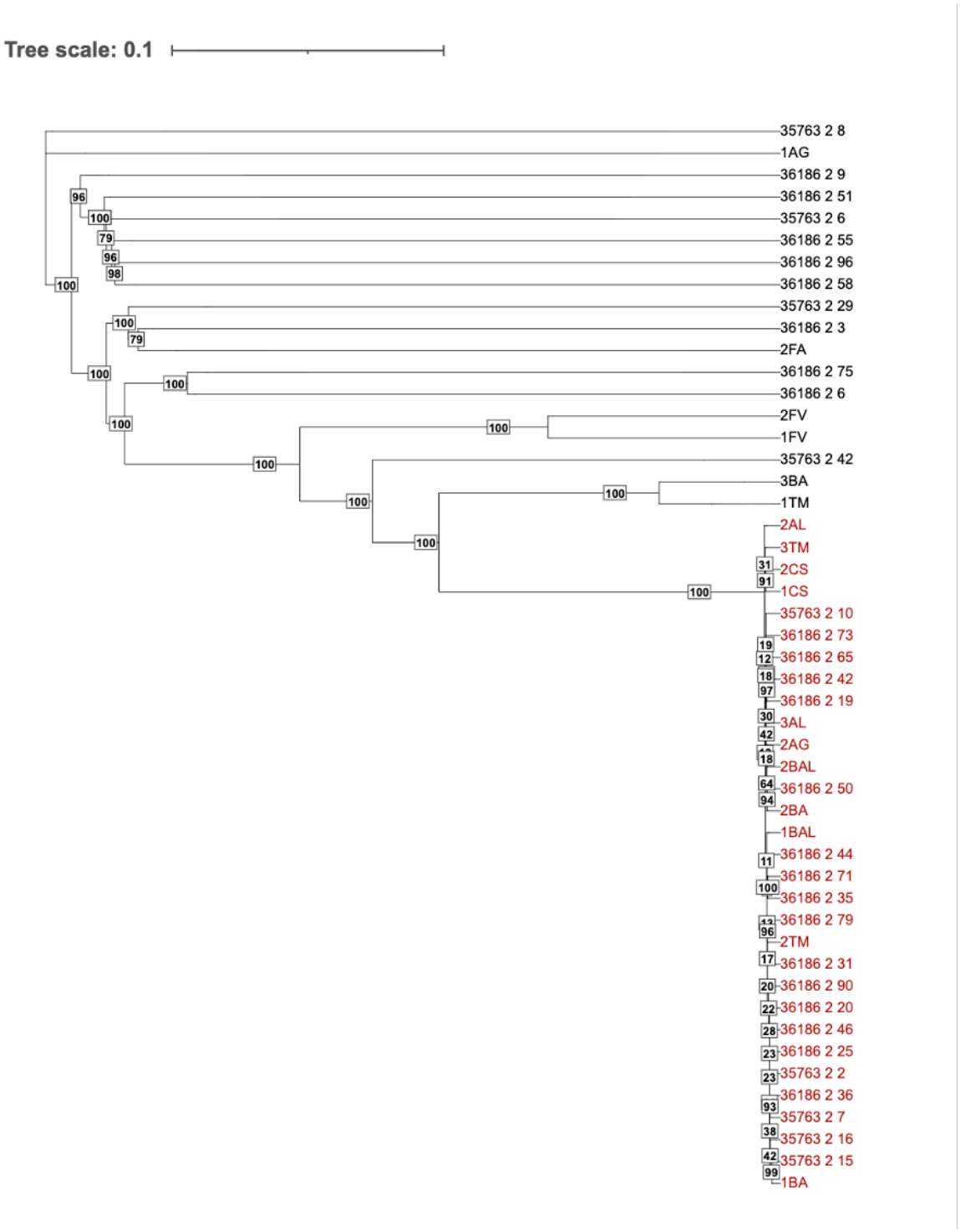


Figure 5. **Phylogenetic relationships among *Candida parapsilosis* clinical isolates**. The phylogenetic tree includes the 17 isolates analyzed in the present study together with additional isolates included for genomic contextualization. The cluster consisting of isolates belonging to Group B, previously described as a persistent fluconazole-resistant cluster, as well as isolates analyzed in the present study, is highlighted in red.

Pairwise SNP distance (Figure 6) analysis confirmed clustering among the isolates. Isolates within the same cluster showed low pairwise SNP distances, whereas substantially higher distances were observed between not epidemiologically linked clusters, as expected. In

particular, the cluster including the isolates analyzed in the present study showed high genomic similarity with the previously characterized Group B (pairwise distances varying from 1 to 29 SNPs), which was consistent with phylogenetic analysis that showed these isolates belonging to the same monophyletic group.

Among the isolates selected for further analysis, the pairwise SNP distance was 7 SNPs between 1BA and 2BA, 1 SNP between 2TM and 3TM, and 2 SNPs between 1BAL and 2BAL. These distances were calculated considering only homozygous variants.

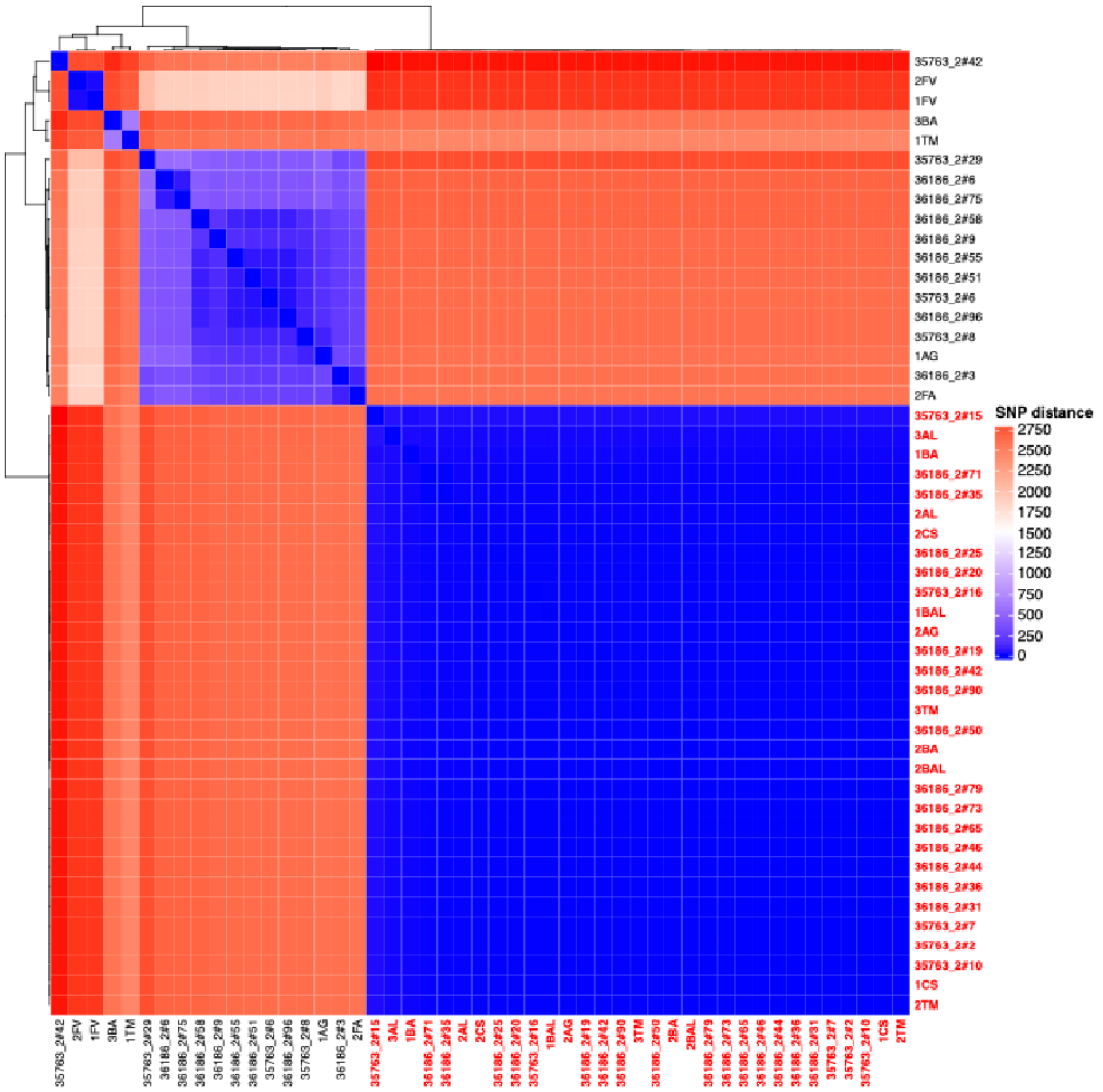


Figure 6. **SNP distance heatmap.** The cluster consisting of isolates belonging to Group B, previously described as a persistent fluconazole-resistant cluster, as well as isolates analyzed in the present study, is highlighted in red.

4.4. Mutations in genes known to be involved in resistance

The variants in the selected isolates were then analyzed for their occurrence in genes previously known to be involved in antifungal resistance. The identification of these variants was performed using external reference retrieved from the NCBI database. The observed mutations are summarized in Table 3.

Table 3. **Variants identified in genes previously associated with antifungal resistance.** Patient IDs are presented in rows, the genes analyzed are presented in columns. Protein-level amino acid substitutions are reported using standard nomenclature, with the original amino acid, its position in the protein sequence, and the substituted amino acid indicated (e.g., Y132F denotes a substitution of tyrosine (Y) by phenylalanine (F) at position 132).

	ERG11	UPC2	TAC1	MRR1	FKS1	ERG3	Ndt80	ERG4	CDR1	ERG6	MDR1B	CDR1B
1BA	Y132F	N455D	-	S1907C	-	-	-	P63S	I1287V	S208G, S304G	-	-
2BA	Y132F	N455D	-	S1907C	-	-	-	P63S	I1287V	S208G, S304G	-	-
1BAL	Y132F	N455D	-	S1907C	-	-	-	P63S	I1287V	S304G, S208G	-	-
2BAL	Y132F	N455D	-	-	-	-	-	P63S	I1287V	S304G, S208G	-	-
2TM	Y132F	N455D	-	S1907C	-	-	-	P63S	I1287V	S304G, S208G	-	-
3TM	Y132F	N455D	-	-	-	-	-	P63S	I1287V	S304G, S208G	-	-

Analysis revealed several amino acid substitutions in genes associated with antifungal resistance across the isolates. Specifically, we identified Y132F in ERG11, N455D in UPC2, S1907C in MRR1, I1287V in CDR1, P63S in ERG4, S304G and S208G in ERG6.

Most variants were detected across all isolates, indicating that they represent common characteristics of the strain, and not unique features of single isolates potentially linked to variations in MIC. The only exception was the S1907C substitution, which was present in only one isolate among the two sequential isolates recovered from the BAL and TM patients.

4.5. Pairwise mutation comparison

Further, we defined which mutation and in which genes differed among sequential isolates and can possibly contribute to MIC changes. Firstly, we discriminated unique (present in only one isolate among two recovered from the same patient) and shared (present in both isolates) variants. Then, the unique ones were also divided into heterozygotes and homozygotes. The number of observed mutations is summarized in Table 4.

Table 1. **Pairwise comparison of unique and shared variants among sequential *Candida parapsilosis* isolates from individual patients.** Unique variants are defined as variants present in only one isolate; shared variants are present in both isolates. Homozygous and heterozygous unique variants are shown separately.

	1BA	2BA
Unique variants in total	103	91
Unique variants (homozygous)	16	4
Unique variants (heterozygous)	87	87
shared	446	

	2TM	3TM
Unique variants in total	137	59
Unique variants (homozygous)	2	4
Unique variants (heterozygous)	135	55
shared	434	

	1BAL	2BAL
Unique variants in total	134	215
Unique variants (homozygous)	5	3
Unique variants (heterozygous)	129	212
shared	384	

Across the analyzed pairs, 384–446 variants were shared between sequential isolates, and the number of unique variants ranged from 59 to 215. Most unique variants were heterozygous, whereas homozygous unique variants were considerably less frequent, ranging from 2 to 16 variants per isolate pair, which is consistent with the SNP distance data.

These variants were subsequently analyzed separately according to their zygosity to identify genomic changes potentially relevant to the observed differences in antifungal susceptibility.

4.6. Detailed analysis of candidate variants

4.6.1. Homozygous mutations

BA patient

Among homozygous variants belonging to **1BA,** we identified variants mapped to the following genes (the identifiers used for genes hereinafter are identifiers assigned using AUGUSTUS software for gene prediction). Synonymous mutations were not annotated, as their contribution to phenotypic change is expected to be negligible. The identified variant are follows:

- 8 variants were located within g5533, annotated as an OPT2 (Oligopeptide transporter) gene. Seven of these variants were synonymous having low impact on protein structure and one classified as a missense variant, having moderate impact.
- 3 upstream gene variants with modifier impact were located to g2907 gene. Annotated as a hypothetical protein in *Candida (Lodderomyces) parapsilosis*.
- 1 upstream gene variant with modifier impact was located to g2974 gene, which codes for protein with homology to Git1p, which has been associated with glycerophosphoinositol and metabolite transport in *Candida orthopsilosis*.
- 3 upstream gene variants with modifier impact were located to g5421 gene, the product of which was annotated as an unnamed protein in *Candida parapsilosis*, with homology to Sef1p, a transcription factor involved in iron regulation in *Candida metapsilosis*.
- 1 upstream variant was also identified in g5535, annotated as Gzf3p in *C. parapsilosis*.

In isolate **2BA**, two synonymous variants were identified in g4942, one synonymous variant in g3955, and one upstream variant in g2537. The last was annotated as a transcriptional activator of glycolytic enzymes family protein.

BAL patient

For **1BAL** isolate we identified five following homozygous variants:

- 2 synonymous variants were identified in g5533 and g1259.
- 2 upstream gene variants having modifier impact in g3440 and g3914. Both identified as proteins, belonging to MFS (Major Facilitator Superfamily proteins)
- 1 missense variant with moderate impact in g3139, whose predicted protein product was annotated as an uncharacterized protein in *Lodderomyces orthopsilosis*. The protein showed homology to Vma21p in *Saccharomyces cerevisiae* which is described as a component of the vacuolar proton-transporting V-type ATPase complex and is localized to the endoplasmic reticulum membrane.

In isolate **2BAL**, 3 homozygous unique variants were identified. Two variants were classified as missense variants with moderate predicted impact and were located in g3155 and g4942, respectively. The predicted protein encoded by g3155 showed homology to the uncharacterized protein CPAR2_405740 from *Candida parapsilosis* and contained a predicted domain associated with nucleic acid binding. The g4942 gene was annotated as encoding a PT repeat family protein. An additional homozygous variant was identified in the upstream region of g1163 and was classified as having a modifier impact variant. The predicted product of g1163 showed homology to ATP synthase subunit in *Candida margitis*.

TM patient

Among the homozygous unique variants identified between the sequential TM isolates, two variants were detected in isolate 2TM and four in isolate 3TM.

2TM

- 1 variant was classified as a splice acceptor variant with high predicted impact and was located in g4942, annotated as a PT repeat family protein.
- 1 upstream variant with modifier predicted impact was identified in g5099. The predicted protein product showed homology to Ecm21p in *Candida parapsilosis*, an adapter protein involved in the endocytic pathway and the regulation of cell wall stress responses.

3TM

- 1 upstream variant with modifier predicted impact was identified in g2974, the product of which showed homology to Git1p in *Candida orthopsilosis*, a protein associated with the transport of glycerophosphoinositol and its metabolites.
- 2 synonymous variants with low predicted impact were identified in g4703.
- 1 synonymous variant with low predicted impact was identified in g4942.

4.6.2. Heterozygous mutations

Unique heterozygous mutations were classified according to their impact on protein structure into variants having HIGH, MODERATE, MODIFIER or LOW impact. The number of observed variants is summarized in Table 5.

Table 5. **Counts of unique heterozygous variants categorized by predicted impact (high, moderate, modifier, low) in each isolate.**

	BA patient		BAL patient		TM patient	
The isolate	1BA	2BA	1BAL	2BAL	2TM	3TM
Total number	87	87	129	212	135	55
High	0	1	2	4	0	0
Moderate	22	26	30	68	37	8
Modifier	36	40	68	75	47	29
Low	29	20	29	65	51	18

High impact mutations

The proteins contained variants having high predicted impact were also manually analyzed and following structures were affected:

- In **2BA** isolate we identified stop-gained mutation in g2189, annotated us unnamed protein product in *C.parapsilosis*, which showed homology with ubiquitin-binding ESCRT-I (endosomal sorting complexes required for transport) subunit protein in *C.orthopsilosis*.
- In **1BAL** stop-gained mutation in g4721, which refers to tetratricopeptide repeat family protein of *C.parapsilosis,* and stop gained mutation in g1705, which was

annotated as member of the MDR family of major facilitator transporter superfamily.

- All four high impact variants found in **2BAL** were classified as stop-gained. Among them the variant in g3387 showed homology to the cell wall protein Iff3 in *C. parapsilosis*; variant in g4287 was annotated as an MFS transporter in *C.parapsilosis*; one variant was identified in g894,with predicted protein annotated as an uncharacterized protein in *C.metapsilosis*. In some fungal species, homologous proteins have been annotated as the transcriptional repressor Tup1 or as a chromatin-silencing transcriptional regulator; a variant was identified in g2189, annotated as an unnamed protein product in *C.parapsilosis*. According to the available orthology-based annotation, homologous proteins have been associated with ubiquitin binding, ATP export, late endosome-to-vacuole transport, negative regulation of protein polyubiquitination, protein targeting to membranes and vacuoles, and reticulophagy.

Annotation of moderate and modifier heterozygous variants.

The number of MODERATE and MODIFIER mutations was relatively high, ranging from 55 to 212 across the studied isolates. Therefore, detailed curation of every variant is challenging and resource-intensive. To prioritize variants, we submitted the protein product IDs of genes containing variants to STRING (using *C. parapsilosis* as reference organism), together with genes previously associated with antifungal drug resistance, in order to identify potential protein–protein interactions. The resulting interaction network was filtered to retain only high-confidence interactions (combined score > 0.7). As expected, the network contained numerous interactions among the known resistance genes, but no significant interactions involving resistance genes and genes carrying MODERATE and MODIFIER heterozygous mutations.

Furthermore, we performed functional enrichment analysis of genes carrying variants. No significantly enriched networks were identified for isolates 1BA, 2BA, 2BAL, or 2TM. The enrichment results for 1BAL and 3TM are summarized in Table 6.

Table 6. **Functional enrichment analysis of genes carrying heterozygous variants having MODERATE or MODIFIER predicted impact in 1BAL and 3TM**. *Category* — source database or ontology of the enriched term. *Observed gene count* — number of variant-carrying genes annotated with the term, presented as observed/background (e.g., "4 of 5" indicates 4 observed genes out of 5 background genes); *Strength* — Log10 (observed / expected); how large the enrichment effect is. It's the ratio between i) the number of proteins in the network that are annotated with a term and ii) the number of proteins that we expect to be annotated with this term in a random network of the same size.; *Signal* — enrichment signal score reported by the STRING, where higher values indicate stronger enrichment support.

Category	Term ID	Term description	Observed gene count	Strength	Signal	False discovery rate
1BAL						
GO Process	GO:0042183	Formate catabolic process	4 of 5	1.89	0.78	0.0043
GO Function	GO:0008863	Formate dehydrogenase (NAD+) activity	4 of 5	1.89	0.91	0.0017
GO Component	GO:0009326	Formate dehydrogenase complex	4 of 5	1.89	0.98	0.0010
KEGG	map00680	Methane metabolism	4 of 26	1.17	0.46	0.0322
Pfam	PF02826	D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain	4 of 9	1.63	0.74	0.0051
3TM						
GO Process	GO:0042183	Formate catabolic process	4 of 5	2.27	1.63	0.00012
GO Function	GO:0008863	Formate dehydrogenase (NAD+) activity	4 of 5	2.27	1.8	4.54e-05
GO Function	GO:0051287	NAD binding	4 of 30	1.49	0.9	0.0039
GO Component	GO:0009326	Formate dehydrogenase complex	4 of 5	2.27	1.88	2.84e-05
STRING clusters	CL:7709	Mixed, incl. Fructose biosynthesis, and Formate catabolic process	4 of 28	1.52	0.83	0.0067
STRING clusters	CL:7715	NAD-dependent formate dehydrogenase, and D-xylose metabolic process	3 of 6	2.06	0.91	0.0067

STRING clusters	CL:7565	Metabolism of carbohydrates, and Nucleotide-sugar metabolic process	5 of 100	1.06	0.57	0.0200
STRING clusters	CL:5133	ABC-type transporter activity, and Pleiotropic drug resistance protein PDR/CDR	3 of 23	1.48	0.56	0.0392
KEGG	map00680	Methane metabolism	4 of 26	1.55	1.12	0.00097
KEGG	map00630	Glyoxylate and dicarboxylate metabolism	4 of 38	1.39	0.96	0.0019
Reactome	MAP-389661	Glyoxylate metabolism and glycine degradation	4 of 33	1.45	0.71	0.0142
Pfam	PF02826	D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain	4 of 9	2.01	1.55	0.00014
InterPro	IPR006139	D-isomer specific 2-hydroxyacid dehydrogenase, catalytic domain	4 of 9	2.01	1.37	0.00043
InterPro	IPR006140	D-isomer specific 2-hydroxyacid dehydrogenase, NAD-binding domain	4 of 9	2.01	1.37	0.00043
InterPro	IPR033689	NAD-dependent formate dehydrogenase	3 of 4	2.24	1.14	0.0019

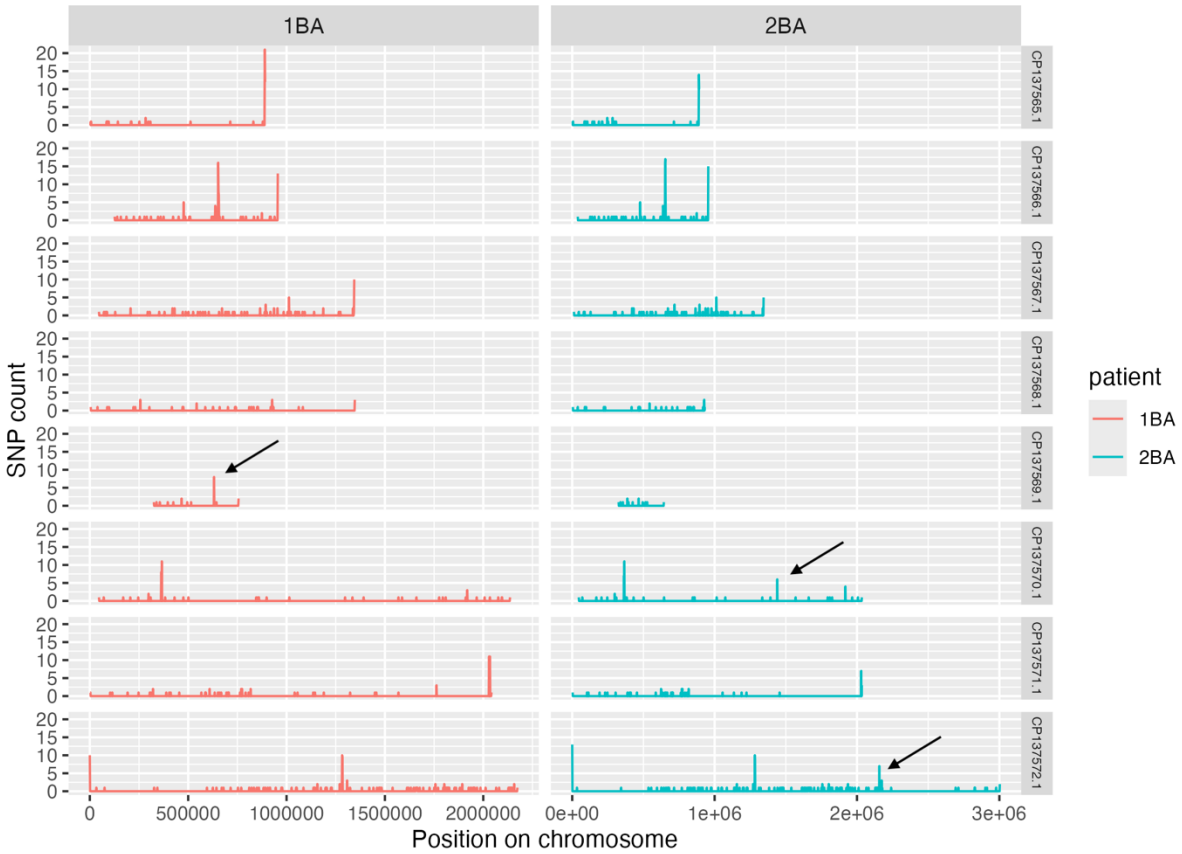
In 1BAL, five terms were enriched, all linked to formate metabolism and formate dehydrogenase. These included the formate dehydrogenase complex, its NAD^+-dependent activity, and formate catabolism, along with a Pfam domain and the methane metabolism pathway. In 3TM, a set of fifteen terms was enriched, again with a formate dehydrogenase appearance. In addition to these core terms, enrichment was observed for NAD binding, glyoxylate and dicarboxylate metabolism, and several STRING clusters related to carbohydrate metabolism and ABC-type transporter activity, and pleiotropic drug resistance protein PDR/CDR.

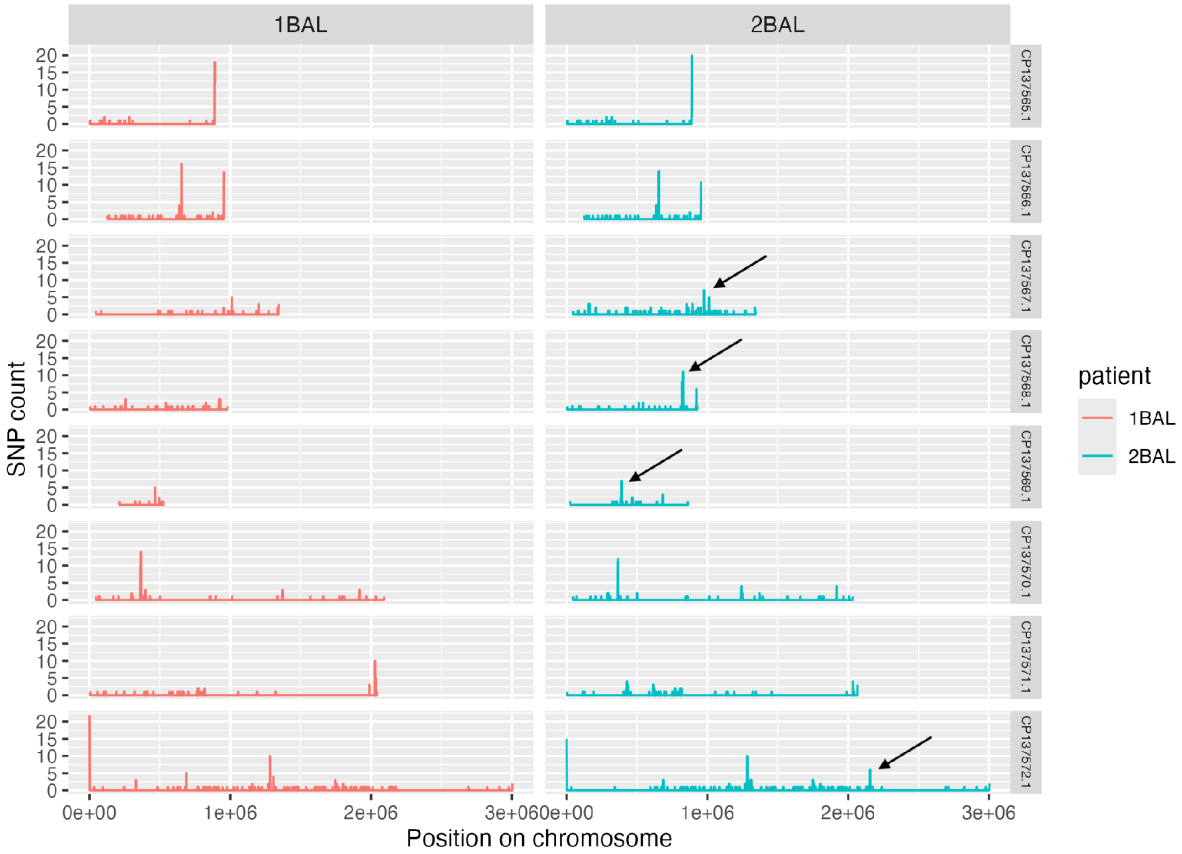
4.7. Mutational hotspots analysis

To identify genomic regions showing increased sequence variation between the sequential isolates, the distribution of SNPs across the chromosomes was examined using SNP density plots. The results of SNP density distribution analysis are shown in Figure 7.

Particular attention was given to genomic regions showing differences in SNP density between two sequential isolates. The peaks occurring in only one of two isolates could potentially indicate regions containing variants that can be associated with changes in MIC in the sequential isolates. If a peak is observed in the isolate with higher MIC, these could involve the variants potentially associated with increased antifungal resistance. Conversely, if a peak is located in the isolate with lower MIC, given that the reference genome we used was itself resistant, these can indicate a reversion to wild type gene. Therefore, regions with isolate-specific SNP density peaks were subsequently examined for the presence of unique variants and their genomic context, including the genes located within these regions. The observed variants are summarized in Table 7. Regions showing similarly high SNP density in both isolates were considered less informative for distinguishing isolate-specific genomic changes, although we did not perform any statistical testing to check for significant variation between samples.

Peaks localized at the ends of the contigs (within 2000 bp from the end) were excluded from downstream analysis, as these regions are frequently enriched for repetitive elements and are prone to assembly and read-mapping artifacts, which can result in false-positive SNP density peaks.


1BA
2BA
SNP count
Position on chromosome
patient
1BA
2BA
CP137565.1
CP137566.1
CP137567.1
CP137568.1
CP137569.1
CP137570.1
CP137571.1
CP137572.1


1BAL
2BAL
SNP count
Position on chromosome
patient
1BAL
2BAL
CP137565.1
CP137566.1
CP137567.1
CP137568.1
CP137569.1
CP137570.1
CP137571.1
CP137572.1

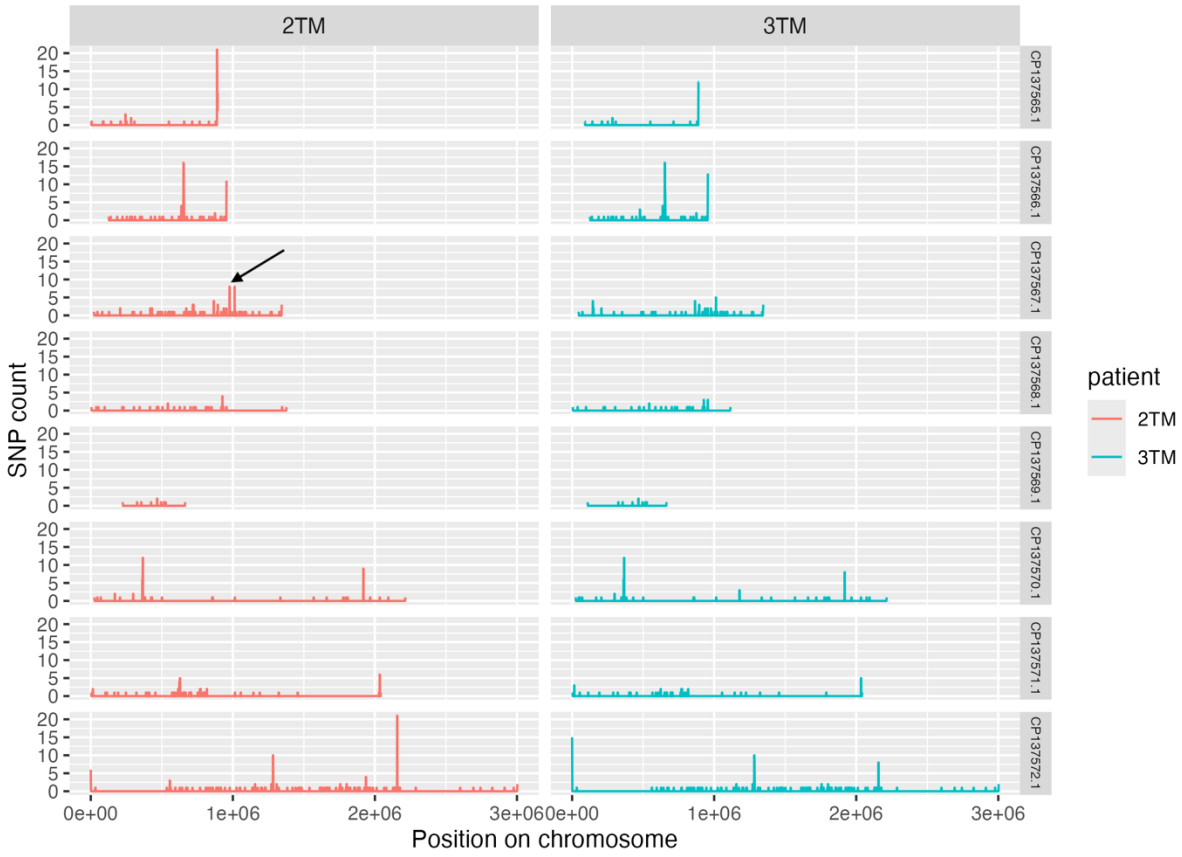


Figure 7. **Mutational hotspots analysis.** Distribution of SNP counts across genomic positions for different patient groups. The x-axis represents the position on the chromosome, and the y-axis represents the SNP count. The black arrows indicate the positions studied in more detail.

Table 7. **The number and annotation of variants found in isolate-specific SNP density peaks.**

Sample	The number and annotation of variants
1BA	8 variants mapped to g4557. Among them six were annotated as having low impact (synonymous) and two as moderate impact (missense mutations). Annotated as uncharacterized protein CPAR2_702970 (*Candida (Lodderomyces) parapsilosis*), containing domains with predicted transmembrane transporter activity and role in transmembrane transport.
2BA	1: 6 variants mapped to g628. Among them five annotated with low impact (synonymous) and one as moderate impact (missense mutations). Annotated as dicarboxylic amino acid permease (*Candida (Lodderomyces) parapsilosis*).

	2: 7 variants mapped to g2954. Among them three are annotated with low impact (synonymous) and four as moderate impact (missense mutations). Annotated as Mtr2p (*Candida (Lodderomyces) parapsilosis*).
1BAL	-
2BAL	1: 10 variants mapped to g3356, which was annotated as CPAR2_502600 in *C.parapsilosis*, a protein of unknown function. Among the variants two are low impact, six moderate impact (missense mutations) and two having modifier impact (upstream gene variants). 2: 11 variants mapped to g2694, ten of which are predicted to have low impact (synonymous mutations) and one with moderate (missense variant). Annotated as CPAR2_403710 (*Candida (Lodderomyces) parapsilosis*), which has domain(s) with predicted role in amino acid transport, transmembrane transport and membrane localization. 6 variants mapped to g2730, all of them had predicted modifier impact (downstream gene variant). Annotated as inositol pentakisphosphate 2-kinase in *Candida* (*Lodderomyces) parapsilosis*. 3: 7 variants mapped to g4451, with five of which having predicted low impact (synonymous mutations) and two moderate (missense variants). The product of the gene is annotated as CPAR2_701880, which has domain(s) with predicted aspartic-type endopeptidase activity and role in proteolysis. 6 variants mapped to g1954, two of which have low (synonymous) impact and four have moderate (missense mutation). The product of g1954 is annotated as an unknown protein of *Candida (Lodderomyces) .parapsilosis*.
2TM	12 variants mapped to g3356, including two low impact (synonymous and splice region/intron variant), two modifiers (upstream gene variants) and eight moderate missense variants). The product of g3356 is annotated as hypothetical protein K4G61_g606 in *Candida (Lodderomyces) .parapsilosis*

	8 variants mapped to g3372, which includes five low (synonymous) and three having moderate impact (missense variants). Annotated as an unknown protein product [*Candida parapsilosis*].
3TM	-

4.8. CNV and aneuploidy

CNVs and chromosome-level copy number alterations were examined in the sequential isolates from patients BA, BAL, and TM. No notable CNV or aneuploidy was detected between the sequential isolates within patients BA and BAL. In contrast, isolate 2TM showed evidence of trisomy involving chromosome CP137568.1 (chromosome 3), whereas no corresponding trisomy was detected in the sequential isolate 3TM. These isolates exhibited a decrease of MIC from 128 μg/mL in 2TM to 8 μg/mL in 3TM. The results of the DELLY analysis for the TM patient are presented in Figure 8. Chromosome CP137568.1 contains the *ERG11* and *TAC1* genes, both of which have been previously associated in azole resistance in *C. parapsilosis*.

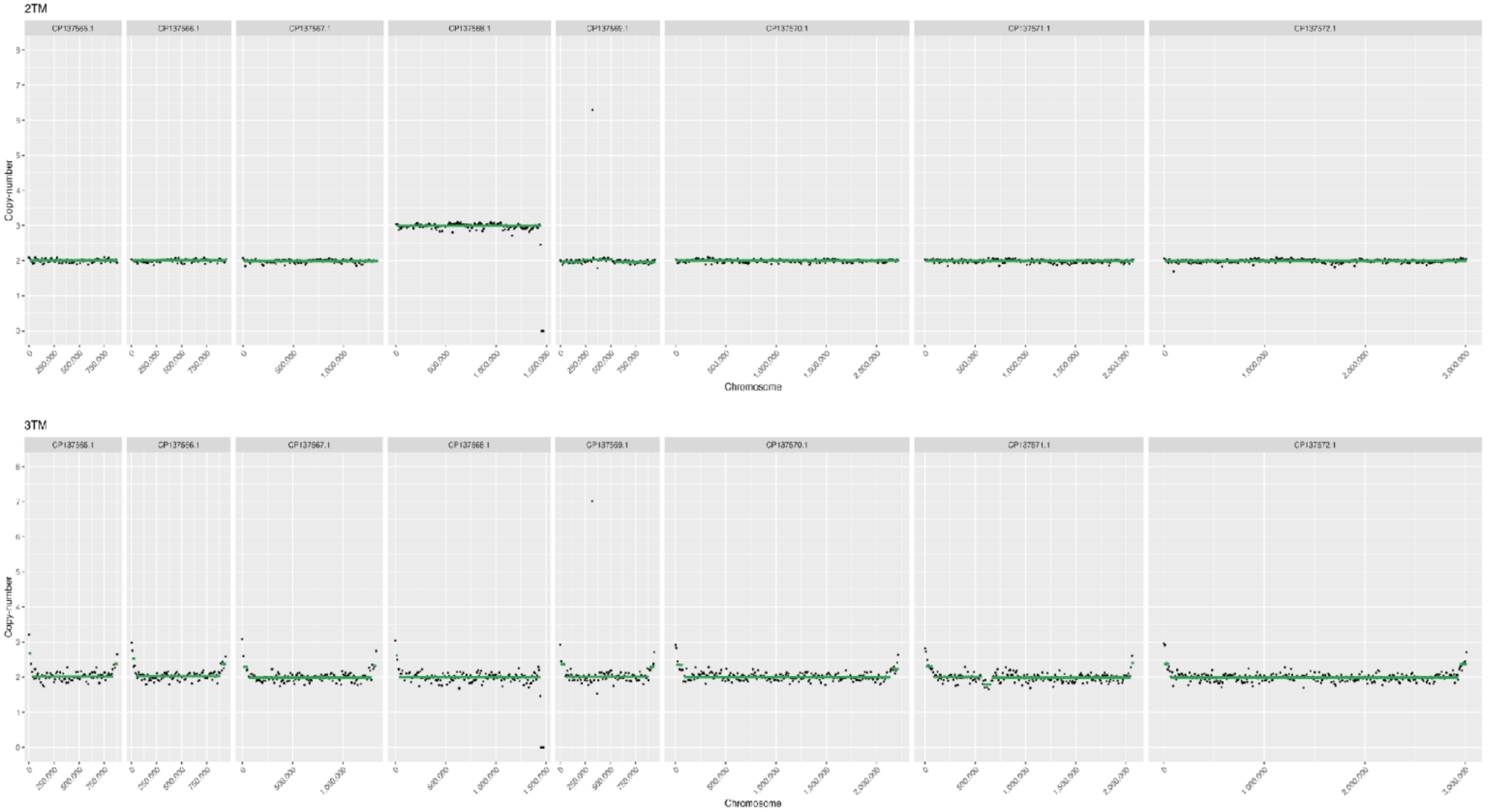


Figure 8. **Copy-number variation (CNV) and aneuploidy analysis using DELLY for TM patient**. The y-axis shows estimated copy number and the x-axis shows chromosomal coordinates. In the 2TM isolate, CP137568.1 (chr3) exhibits triploidy, with the median copy number shifted to approximately 3. 3TM isolate, in contrast, remains diploid.

5. DISCUSSION

The emergence of antifungal resistance in *Candida parapsilosis* represents an increasing clinical concern, partly due to changes in antifungal susceptibility that could occur during prolonged or repeated exposure to antifungal therapy. However, the genomic changes underlying the within-host shift in antifungal susceptibility remain incompletely understood.
In the present study, through a comparative genomic approach, we investigated sequential clinical isolates of *C. parapsilosis* obtained from patients with candidemia to characterize genomic variation associated with changes in antifungal susceptibility.

Phylogenetic analysis revealed three out of eight patients (BA, BAL and TM) whose paired isolates were epidemiologically related then they were selected for downstream analyses. This initial analysis was fundamental to ensure that the MIC changes observed in the initial dataset would be due to genetic changes from the same clone occurring in the genome during infection and under treatment, rather than originating from co-infections of two different strains. In addition to phylogenetic analysis, the cluster analysis based on SNPs distance calculation confirmed the genetic relatedness of the isolates within the pairs, ranging from 1 to 7 SNPs.
All three pairs of longitudinal isolates showed a decrease in Minimum Inhibitory Concentration (MIC) over time. In one case, namely, patient BA, this reduction was particularly notable, indeed other than the numerical decrease in MIC from 128 μg/mL to 1 μg/mL, it also exhibited a shift from a resistant to a sensitive phenotype. The sequential isolates sampled from patients BAL and TM remained resistant to fluconazole, but exhibited a decrease in MIC from 64 μg/mL to 8 μg/mL and from 128 μg/mL to 8 μg/mL, respectively.

Several previously reported substitutions associated with antifungal resistance were identified across all isolates, including Y132F in ERG11 (Doorley et al., 2023; Berkow et al., 2015), N455D in UPC2 (Vumbaca et al., 2026; De Lourdes García-Hernández et al., 2026), S1907C in MRR1 (Vumbaca et al., 2026), I1287V in CDR1 (De Lourdes García-Hernández et al., 2026). Among them, the mutation previously identified and strongly associated with resistance is Y132F in ERG11, while N455D (UPC2) and I1287V (CDR1) are observed, however, their association with resistance was not experimentally validated, and S1907C (MRR1) has only recently been described.
As the listed above mutations were found in all the studied isolates, their presence alone was insufficient to explain the observed within-patient changes in fluconazole susceptibility. The

only substitution which was unique for one out of two subsequent isolates was S1907C in 1BAL and 2TM, which exhibited higher MICs compared to the following isolates. MRR1 encodes a transcription factor that regulates the expression of multidrug resistance (MDR) efflux pumps (Grossman et al., 2014; Morschhäuser et al. 2007). Therefore, the S1907C substitution could potentially act as an activating mechanism, leading to the overexpression of these pumps and consequently contributing to the observed higher MICs. However, its impact requires further experimental validation.
In addition, several novel mutations were observed in all isolates: P63S (ERG4), S208G (ERG6), and S304G (ERG6). As these variants have not been reported in previous surveys, they could represent novel uncharacterized mutations contributing to the resistant phenotype or lineage-specific polymorphisms. These variants could be prioritized for further research.

Furthermore, we performed a more detailed examination of variants found in only one of the two sequential isolates (hereinafter called as "unique") collected from the same patient, with the aim of identifying genomic differences that could contribute to differences in susceptibility to antifungal drugs. Firstly, all unique mutations were classified as homozygous or heterozygous, since the effect of variants can differ depending on zygosity. For instance, gain-of-function mutations in transcriptional regulators of drug efflux pumps can act dominantly, so even a heterozygous state may lead to increased pump expression and, consequently, reduced antifungal susceptibility. Conversely, a loss-of-function variant, such as a stop-gain mutation in the same transcriptional regulator, might not necessarily lead to reduced pump production, as the remaining wild-type allele could be upregulated and compensate for the lost function.

Analysis of unique homozygous variants revealed two upstream gene variants in proteins annotated as homologous to the Major Facilitator Superfamily (MFS) in the isolate from patient BAL, which exhibited the higher MIC within the pair. These mutations could be located in promoter regions and increase MFS protein transcription, potentially leading to increased production of efflux pumps and enhanced drug efflux, thereby contributing to reduced drug susceptibility. However, because the in silico analysis we performed determines upstream gene variants primarily through physical distance to the transcription start site rather than empirical regulatory networks, these annotations represent potential rather than validated regulatory disruptions (Cingolani et al., 2012).
In the same isolate (BAL), a missense mutation was found in a gene coding for a protein with homology to the Vma21 protein of another fungal species. Vma21 is an assembly factor for

fungal V-ATPase, which localizes to vacuolar membranes and plays a role in various cellular processes, such as pH and ion homeostasis. Impaired V-ATPase function has been shown to increase susceptibility to azoles and amphotericin B in *C. glabrata* (Minematsu et al., 2019). Furthermore, Xu et al. (2021) demonstrated that V-ATPase activity is an important factor required for azole tolerance in *C. albicans*, highlighting that the mechanisms of tolerance and resistance to azoles in fungal species are complex and not fully addressed. Thus, the identified mutation in a gene crucial for vacuolar ATPase assembly can contribute to the increased tolerance or resistance of the studied isolate. However, this hypothesis also requires further investigation through in vitro validation e.g. gene knockdown.

The BA-related isolate showing the higher MIC in the pair carried eight variants in the OPT2 (oligopeptide transporter) gene. Although seven of them were synonymous, one missense variant remains a candidate contributor to the resistant phenotype. OPT2 is an oligopeptide transporter that mediates uptake of the antifungal peptides or a related metabolite (Schielmann et al., 2017), hence a moderate-impact missense change could reduce drug influx, lower intracellular drug concentration, and thereby increase MIC. However, as reflected in the name of the transporter, its main function is the uptake of peptide molecules, whereas azoles and polyenes are non-peptidic, so their direct transport by OPT2 is unlikely. On the other hand, if the antifungal type are echinocandins (a cyclic lipopeptides), peptide-transporter-mediated uptake or efflux becomes more plausible.
The same isolate also carried one upstream variant in a gene whose product is annotated as the Gzf3 protein in *C. parapsilosis*. Experimental genomic screens show that this putative transcription factor Gzf3 acts as a positive regulator of fluconazole tolerance in *C. albicans* (Delarze et al., 2020). As the mutation is an upstream modifier variant, alterations in its promoter could influence GZF3 expression levels, potentially altering azole tolerance phenotypes. Nevertheless, such results strictly rely on variant impact annotation.

When heterozygous high-impact variants were examined, stop-gained mutation in a protein which was annotated as an MFS transporter was found in 2BAL isolate, having lower MIC in the pair. Interestingly, a stop-gain mutation was also found in 1BAL in another gene, whose product was also annotated as a member of MFS transporters. Because these transporters often play a role in drug efflux (Costa et al., 2014; Banerjee et al., 2021), loss of function would be expected to reduce efflux and lower MIC, which is consistent with the lower MIC of 2BAL compared with 1BAL. However, the nature of this mechanism is challenged by the presence

of a stop-gain in another MDR-family MFS transporter in 1BAL, which had the higher MIC. This suggests that the two transporter variants are not equivalent, and they can differ in substrate specificity, expression level or relative contribution to drug efflux. Moreover, because the mutations are heterozygous, compensatory upregulation of the non-defective allele could maintain sufficient transporter expression and mask the effect of the stop-gain in 2BAL. Alternatively, the 1BAL stop-gain may be a passenger mutation, and the higher MIC can be explained by other resistance mechanisms, whereas the 2BAL stop-gain may contribute to its lower MIC. The fact that similar MFS stop-gain mutations appeared in isolates with different MICs highlights the idea that antifungal resistance mechanisms in fungi are complex and involve multiple genes and pathways. Therefore, these variants should be treated as candidates, and further studies, e.g. expression analysis, are needed to determine whether they causally affect MIC.

In addition, in 2BAL stop-gained mutation was found in a gene coding for Tup1 homologous. In *C. albicans* Tup1 is a transcriptional repressor of filamentation, and loss of TUP1 drives constitutive filamentous growth (Braun and Johnson, 1997). As biofilms and hyphae are associated with higher antifungal resistance or tolerance, disrupting a function of Tup1-like regulator could affect drug response indirectly through morphology and biofilm formation. Consistent with that, several antifungal and biofilm studies report that increasing Tup1 expression suppresses hyphae and can increase drug sensitivity (Hamdy et al., 2020).

In 2BA, we found a stop-gain mutation in a protein homologous to an ESCRT-I (endosomal sorting complexes required for transport) subunit. ESCRT components play a pivotal role in extracellular vesicle (EV) transport in a variety of eukaryotes, particularly it performs its function in biofilms. Zarnowski et al. (2018) showed that ESCRT-defective *C. albicans* mutants exhibited reduced biofilm EV production, reduced matrix polysaccharide levels, and greatly increased sensitivity to fluconazole. Therefore, this stop-gain mutation in one ESCRT subunit could contribute to the increased sensitivity of 2BA compared with 1BA in biofilms. However, since the mutation is heterozygous, compensation through overexpression of the unaffected allele remains possible, which could partially or fully rescue the phenotype.

Overall, individual unique variants investigation gave some candidate variants which could contribute to changes in MIC. However, for *C.parapsilosis*, the functional contribution of these variants has not been sufficiently studied and requires experimental validation.

We also sought to identify any possible protein-protein interactions between proteins carrying heterozygous variants having MODERATE or MODIFIER impact on protein structure and products of genes previously associated with resistance, but no significant results were observed. This absence of significant interactions does not exclude the possibility that the affected proteins interact with known resistance genes, as our analysis is restricted to previously reported and predicted interactions and cannot detect novel associations.

Functional enrichment analysis of variant-carrying genes revealed no significant hits for isolates 1BA, 2BA, 2BAL, 2TM1BA, 2BA, 2BAL, or 2TM. In 1BAL and 3TM isolated alterations in metabolic processes, including formate catabolism, NAD-dependent dehydrogenase activity and glyoxylate/dicarboxylate metabolism were identified. These pathways are not established mechanisms of antifungal resistance in *C. parapsilosis*, but they could represent mechanisms of adaptation to the host selective environment. For instance, they can promote survival under stress by detoxifying formate and maintaining redox balance, and thus likely reflect adaptation to the selective host environment (Du 2022; Partipilo 2021). Li et al. (2015), in a study of the proteome of *C. albicans* biofilm persisters displaying tolerance to antifungal drugs, demonstrated that glyoxylate and dicarboxylate metabolism was selectively upregulated, maintaining survival in multidrug-tolerant persisters.

In the 3TM isolate, three genes were annotated as being involved in ABC-type transporter activity and pleiotropic drug resistance protein PDR/CDR. As previously shown, ABC-type transporter activity and PDR/CDR proteins directly contribute to clinical azole resistance across Candida species, particularly *C. parapsilosis*, through multidrug efflux pumps (Branco et al., 2023). Thus, disruption of the expression or structure of these pumps could partly explain the decreased MIC in this isolate compared with the 2TM isolate.

Fungal genomics and proteomics are still evolving fields, and the proteome of *C. parapsilosis* has not yet been comprehensively characterized. Many of its proteins remain unannotated and require further investigation. This incomplete annotation considerably limits the sensitivity of interaction prediction or functional enrichment analysis, leaving room for more sophisticated methods like AlphaPullDown (Yu et al., 2023) to test protein-protein interactions.

In addition to the comparison of previously described resistance-associated genes and unique variants, we performed SNP-density analysis to identify genomic regions containing clusters of isolate-specific variation. These regions contained variants in several genes, most of which were predicted transporters or permeases. However, the majority of these variants were synonymous or modifier changes, and the affected genes remain largely uncharacterized in *C.*

parapsilosis. These variants should therefore be considered candidates rather than confirmed determinants of the observed phenotypic differences.

Further, in patient TM, trisomy of chromosome 3 was detected in the first isolate, which had a higher MIC. This chromosome carries ERG11 and TAC1 genes. Because additional copies of these genes are generated, target overproduction suppresses the action of fluconazole on lanosterol 14α-demethylase, which fluconazole selectively inhibits (Hartuis et al., 2024; Bergen et al., 2024). Therefore the presence of chromosome 3 trisomy in 2TM provides a plausible explanation for the higher fluconazole MIC observed in this isolate. This phenomenon has been documented in previous studies, including full extra copy of chromosome 3 (Bergen et al., 2024) and extra copy of chromosome 3 long arm (Ning et al., 2023), which also led to increased ERG11 copy number in clinical outbreak isolates. In addition, because TAC1 encodes a transcription factor that regulates efflux pump CDR1, its extra copy may further contribute to resistance by increasing drug efflux. Together, aneuploidy can amplify both drug target and efflux pathways, providing a complex but plausible genetic basis for the elevated MIC in this isolate.

In summary, aiming to find the genomic drivers of the observed MIC changes we prioritize the following findings:

For patient BA, the shift from a resistant to a sensitive phenotype can be partially explained by the acquisition of a stop-gain mutation in an ESCRT-I subunit in the later isolate (2BA), which has been associated with increased fluconazole sensitivity. In addition, the earlier resistant isolate (1BA) carried variants in OPT2 and GZF3 that could contribute to its reduced susceptibility.

For patient BAL, the higher MIC of 1BAL may be partly influenced by S1907C substitution in MRR1 and upstream variants in MFS transporters; the lower MIC of 2BAL could be associated with a heterozygous stop-gain in an MFS transporter, although compensatory overexpression mechanism complicate this interpretation.

For patient TM, the higher MIC of 2TM is likely explained by trisomy of chromosome 3, which increases copies of ERG11 and TAC1, while the subsequent decrease in MIC in 3TM may be related to the loss of this aneuploidy and the presence of variants in ABC-type transporters and PDR/CDR genes.

Overall, although in the study we identified several variants possibly linked to MIC changes, most of them were not validated experimentally and were based on prediction and information from the literature. The annotation of variant effects depends on how well the genome is

annotated, and many genes in *C. parapsilosis* are still uncharacterized or described only by homologous genes in other fungal species. Therefore, we can only suggest that these variants might affect antifungal susceptibility.

Besides genetic changes, the variation in drug susceptibility can be modified by other factors, such as differential gene expression, epigenetic regulation, the phenomenon of heteroresistance (Li et al., 2025).

The limitation of this study is based merely in silico analysis, which we were not able to validate experimentally. Transcriptional analysis requires RNA to be isolated at the moment under the specific conditions that influence the phenotype, e.g. under drug exposure, but the isolates used here were collected and stored several years ago, making it not feasible to infer such analysis. Finally, heteroresistance – co-existence of multiple subpopulation within the same clone having different phenotypes – is hard to evaluate in our dataset, as multiple isolations from the same colony are required to test this condition.

Another limitation of the study is that as we analyzed only a small number of sequential isolate pairs, our findings cannot be easily generalized. We could not test statistically whether individual variants are linked to MIC changes, because more samples would be needed.

To summarise, all three studied cases of *C. parapsilosis* evolution exhibited a decrease in fluconazole resistance over time. Combined with clinical data showing that the patients were treated with echinocandins in the interval between the sequential isolates collection, this pattern is consistent with the concept of a fitness cost. This means that acquired traits, whether genetic or regulatory, can severily impact on the metabolic costs in the absence of the drug (Popp et al., 2017; Sasse et al., 2012). For example, maintaining a resistant phenotype can occur through decreased membrane permeability, which reduces drug influx, but could also limit nutrient supply and impair fungal growth. Similarly, the production of additional efflux pumps requires cells to consume more resources. Therefore, the reduced MIC to fluconazole is consistent with the fact that treatment was performed with an antifungal drug of a different class, meaning the absence of selective pressure. This phenomenon was shown previously in several works, for instance, Nunez-Rodríguez et al. in 2026 demonstrated that fluconazole resistance in *C. parapsilosis* decreases after switch to anidulafungin and interprets it as fitness-cost-driven resistance loss. Thus, the restriction of azole use could reduce the proportion of fluconazole-resistant *C. parapsilosis* during epidemic outbreaks (Daneshnia et al., 2023).

6. CONCLUSION

The aim of this work was to highlight the genomic drivers of *Candida (Lodderomyces) parapsilosis* isolates during within-host infection that could explain the phenotypic changes in susceptibility to antifungal drugs. For this purpose, we used a comparative genomics approach to analyze three pairs of subsequent isolates taken from the same patients and representing the same strain of *C.parapsilosis.*

All three pairs of isolates showed a decrease in Minimum Inhibitory Concentration (MIC) to fluconazole in a period of time from 10 to 25 days. Two of them exhibited pronounced reduction in fold-dilution from 128 to 8 μg/mL and from 64 μg/mL to 8 μg/mL, albeit maintaining the resistant phenotype. Conversely, the other isolates pair showed a phenotypical switch from 128 μg/mL (resistant) to 1 μg/mL (sensitive). The SNP distance, accounting for homozygous positions, was low and ranged between 1 and 7 SNPs for each pair, underlying their epidemiological relation.

This observation suggested that the phenotypic changes could have resulted from relatively few homozygous genomic differences, could involve heterozygous variants or reflect mechanisms that were not analyzed in this study, such as changes in gene expression, epigenetic regulation or the phenomenon of heteroresistance.

The differences in phenotypes could not be fully explained by known mechanisms contributing to the resistance in *C. parapsilosis*. Thus, together with these previously observed mechanisms, such as substitutions in ERG11 – the main target of azoles class, UPC2 – its transcriptional regulator, or CDR1 (Candida Drug Resistance), which codes for a drug efflux pump (Silva et al., 2011; Doorley et al., 2023; Hartius et al., 2024; Branco et al., 2022), we identified a set of novel variants, which could contribute to the shift in antifungal susceptibility. These variants differ in their zygosity state and potential impact, including missense variants, potentially belonging to promoters of genes, whose expression could modify drug susceptibility, as well as stop-gained mutations disrupting the structure of proteins e.g., MDR (multidrug resistance) superfamily transporters. As fungal genomics is still an evolving field, the actual functional impact of several variants remains unknown, and further research is needed for experimental validation. For instance, checking the effect of promoter mutations on gene expression, generating knockout mutants for stop-gained mutations, and performing genome editing using CRISPR-Cas9 for missense mutations. Moreover, a larger cohort of genomic isolates is needed to infer statistical associations between presence/absence of the observed variants and their contribution to the resistant phenotype, such as the case of Genome Wide Association Study (GWAS).

In addition, aneuploidy was detected as another mechanism contributing to differences in antifungal susceptibility. In one isolates pair, the triploidy for the chromosome carrying ERG11 and TAC1 genes was found in the one exhibiting higher MIC. This mechanism was reported in previous studies (Bergen et al., 2024) and increases antifungal resistance by having multiple copies of ERG11 and TAC1, thereby elevating the levels of their gene products.

All of the three pairs of isolates showed decreasing MIC to fluconazole during treatment with echinocandins. The observed changes in susceptibility reflect a trade-off between resistance and fitness during adaptation to the host environment. When exposed to the drug, resistance-associated traits provide a fitness advantage, as far as selective pressure is exerted. But after drug removal (in our case, the treatment with echinocandins and not fluconazole), these resistance-associated traits can impose a fitness cost, e.g., a need for greater requirements of cellular resources for efflux pump production. These findings suggest that reduced fluconazole exposure, together with routine clonal resistance profiling in hospital settings, could inform more targeted antifungal management strategies and thereby help reduce the global burden of fluconazole resistance.

To conclude, antifungal susceptibility variation can have a multifactorial genomic basis rather than being determined by a single resistance-associated mutation. Our data illustrate this through the combined contribution of established resistance mechanisms, novel variants of differing zygosity and impact (missense, promoter, and stop-gained), and chromosome triploidy increasing ERG11 and TAC1 dosage. Further studies investigating gene expression, epigenetic regulation, heteroresistance, and genome-wide associations are needed to better understand the diverse factors contributing to antifungal resistance in fungal pathogens.

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