

MOLECULAR IDENTIFICATION, ANTIFUNGAL  
RESISTANCE AND DISTRIBUTION  
OF PATHOGENIC YEASTS IN BULGARIA

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**Abstract**

A total of 111 yeast isolates were obtained from clinical samples collected at University Multipurpose Hospital for Active Treatment “Sveti Georgi” in Plovdiv, Bulgaria. Species identification was performed using DNA barcoding of the internal transcribed spacer region (ITS1+2). Antifungal susceptibility testing against six drugs revealed that 25 isolates (26.6%) were multidrug-resistant, including strains of *Candida albicans*, *C. parapsilosis*, and *C. tropicalis*. Resistance was observed against amphotericin B, itraconazole, and posaconazole. These findings underscore the need for continuous local epidemiological surveillance of antifungal resistance in pathogenic yeasts to establish effective treatment strategies. To evaluate the influence of patient demographics on yeast species distribution, Principal Component Analysis (PCA) was employed to explore correlations between yeast species and patient characteristics such as age and gender. The analysis revealed distinct clustering patterns, suggesting that demographic factors may influence the distribution of pathogenic yeasts.

**Key words:** pathogenic yeasts, DNA barcoding, antifungal susceptibility, principal component analysis (PCA)

**Introduction.** Opportunistic yeast pathogens are commonly present on human skin, the mouth, the gastrointestinal tract, the female reproductive system,

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and organs such as the lungs, kidneys, bloodstream, and central nervous system, typically without causing harm. However, under certain conditions, they can proliferate rapidly, leading to superficial infections or severe, life-threatening invasive diseases. Yeast infections affect approximately 1.57 million people worldwide each year, resulting in nearly one million deaths, with a staggering mortality rate of 63.6% [1].

The most frequently isolated ascomycetous yeast species from clinical samples globally include *Candida albicans*, *Nakaseomyces glabratus* (formerly *C. glabrata*), *C. parapsilosis*, *C. tropicalis*, and *Pichia kudriavzevii* (formerly *C. krusei*). Less commonly isolated pathogens include *Clavispora lusitaniae*, *Meyerozyma guilliermondii* (formerly *Pichia guilliermondii*), and *Pichia inconspicua*. Among basidiomycetous yeasts, the top-ranked pathogenic species are *Cryptococcus neoformans* and *Trichosporon asahii*.

Pathogenic yeasts exhibit variable susceptibility to antifungal drugs, with some species displaying intrinsic or acquired resistance. Notably, *N. glabratus* and the recently emerged *Candidozyma auris* (formerly *C. auris*) demonstrate multidrug resistance, posing significant treatment challenges [2,3].

Effective treatment of fungal infections depends on rapid and accurate identification of yeast pathogens, which necessitates applying DNA barcoding of rDNA markers [4]. Epidemiological studies from Europe and the USA have reported an increasing incidence of yeast infections, notably those caused by non-*albicans* *Candida* species [5,6]. A recent pan-European study involving 17 countries demonstrated considerable variation in yeast species distribution across 41 participating centres [7]. However, this study did not include data from Bulgaria, resulting in a gap in regional epidemiological data.

To help address this gap, we conducted molecular identification of yeast pathogens isolated from clinical samples at “Sveti Georgi” University Hospital in Plovdiv, Bulgaria, using DNA barcoding of the ITS region. Additionally, we determined antifungal resistance patterns to detect potential multidrug-resistant yeast strains. We applied Principal Component Analysis (PCA) [8] to explore correlations between isolated pathogenic yeasts and patient demographics (e.g., gender, age).

**Materials and methods. Collection of clinical samples and isolation of yeast.** A total of 111 clinical samples were collected from patients of “Sveti Georgi” University Hospital in Plovdiv. Our analysis included a broader range of clinical samples (blood and urine cultures, vaginal wound and throat swabs, central venous catheters, feces, and skin) originating from various departments of the hospital (gastroenterology, gynaecology, paediatrics, urology, haematology, cardiology, toxicology, nephrology, oncology, anaesthesiology, and intensive care unit).

**Isolation and identification of yeast strains.** The clinical samples were inoculated onto YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone,

and 2% glucose) in the Microbiology Department of Medical University, Plovdiv. To ensure purity, the yeast isolates were subcultured three times. Genomic DNA was extracted from the yeast isolates using the Bacterial & Yeast Genomic DNA Purification Kit (GeneMATRIX, Poland), according to the manufacturer's protocol. The ITS region was amplified and sequenced following previously described protocols [9, 10]. Consensus DNA sequences were generated from the forward and reverse sequencing reads using the CAP3 program (<https://doua.prabi.fr/software/cap3>). The nucleotide sequences of the ITS regions were compared with reference sequences from the NCBI GenBank database (<http://ncbi.nlm.nih.gov>) for identification using BLAST v.2.16.0. All sequences obtained in this study were deposited to GenBank database under accession numbers – JQ026352.1; ON016539-ON016585; PV482838-PV482901.

**Antifungal susceptibility testing.** Due to incomplete demographic data, 94 yeast isolates were included in the analysis. The susceptibility of yeast isolates to six antifungal drugs – caspofungin, amphotericin B, itraconazole, fluconazole, posaconazole, and voriconazole – was determined using the Ezy MIC<sup>TM</sup> Strips system (HiMedia, India), according to the manufacturer's instructions. *Candida parapsilosis* ATCC 22019 and *Pichia kudriavzevii* ATCC 6258 were used as control strains.

**Principal component analysis.** PCA was applied initially to reduce the dimensionality of the dataset, retaining the most significant variance in yeast pathogen distribution [8]. This analysis was conducted using Orange, an interactive data visualization and data mining software [11].

**Results and discussion.** A total of 111 yeast isolates were obtained from clinical samples at “Sveti Georgi” University Hospital. Molecular identification of these strains revealed *Nakaseomyces glabratus* as the most dominant species, accounting for 42% of the isolates, followed by *Candida albicans* (21%), *C. parapsilosis* and *C. tropicalis* (8% each), and *Trichosporon asahii* (6%). Several less common species were also identified, each represented by one to three isolates. These species included *Kluyveromyces marxianus*, *Yarrowia lipolytica*, *Pichia inconspicua*, *Pichia norvegensis*, *Meyerozyma guilliermondii*, *Candida zeylanoides*, *Pichia kudriavzevii*, and *Magnusiomyces capitatus* (Table 1).

Two strains show low sequence similarity of 97%, and 98%, respectively, with *Clavispora lusitaniae* CBS 5094 and CBS 6936T. The sequence similarity between two *Clavispora* strains is 97%. Since *Clavispora lusitaniae* has highly divergent ribosomal markers, conclusions regarding the identity of these two strains should be drawn based on the analysis of additional markers [12].

It is important to acknowledge that this study was conducted in a single hospital, which may limit the extent to which our findings can be applied to the broader Bulgarian population. Studies across different regions highlight significant variability in the distribution of pathogenic yeasts and their antifungal resistance patterns. For instance, a 15-year Swiss study reported a decrease in *C. albicans*

T a b l e   1  
The identity of yeast isolates

| Yeast species identity   | Number of yeast isolates | The range of ITS sequence similarity to the closest matching sequence in GenBank |
|--------------------------|--------------------------|----------------------------------------------------------------------------------|
| <i>N. glabratus</i>      | 47                       | 98.3–99.6%                                                                       |
| <i>C. albicans</i>       | 23                       | 99.3–100%                                                                        |
| <i>C. tropicalis</i>     | 9                        | 99.8–100%                                                                        |
| <i>C. parapsilosis</i>   | 9                        | 99.9–100%                                                                        |
| <i>T. asahii</i>         | 7                        | 99–100%                                                                          |
| <i>P. inconspicua</i>    | 2                        | 99.7%                                                                            |
| <i>Y. lipolytica</i>     | 2                        | 99.4%                                                                            |
| <i>K. marxianus</i>      | 2                        | 100%                                                                             |
| <i>C. metapsilosis</i>   | 1                        | 99.8%                                                                            |
| <i>P. norvegensis</i>    | 2                        | 99.3%                                                                            |
| <i>M. guilliermondii</i> | 1                        | 100%                                                                             |
| <i>P. kudriavzevii</i>   | 1                        | 100%                                                                             |
| <i>M. capitatus</i>      | 1                        | 100%                                                                             |
| <i>C. zeylanoides</i>    | 1                        | 99%                                                                              |
| <i>C. lusitaniae</i>     | 1                        | 100%                                                                             |
| <i>Clavispora</i> sp.    | 1                        | 97%                                                                              |
| <i>Clavispora</i> sp.    | 1                        | 98%                                                                              |

cases (from 60% to 53%) and an increase in *N. glabratus* infections (from 18% to 27%) [13]. In Denmark, *C. albicans* (42.1%) and *N. glabratus* (32%) were the dominant species [14]. A recent study of European Confederation of Medical Mycology (ECMM) also revealed regional variations, with *C. albicans* being most prevalent in Austria (77%), while higher proportions of *N. glabratus* infections were observed in the Czech Republic, France, and the United Kingdom, ranging from 25% to 33% [7].

In Russia, the prevalence of *C. albicans* dropped from 52.38% to 32.7%, while non-*albicans* *Candida* species increased from 47.62% to 67.21% [15]. In India, *C. tropicalis* (40.1%) was the most frequent species, followed by *C. albicans* (15.2%) and *Wickerhamomyces anomalus* (13.1%), with a rising resistance to azoles [16]. In China, *C. albicans* remained dominant (64.25%), followed by *C. tropicalis* (12.61%) and *N. glabratus* (10.79%), with increased resistance to echinocandins [17]. In the United States, *C. albicans* remains the most common cause of invasive candidiasis followed by *N. glabratus* [18].

**Antifungal susceptibility and resistance of yeast isolates.** Susceptibility and resistance of yeast isolates to six antifungal agents were assessed through determination of minimum inhibitory concentrations (MICs), interpreted in ac-

cordance with the latest EUCAST clinical breakpoints (version 11.00, 02.12.2024; [https://www.eucast.org/ast\\_of\\_fungi](https://www.eucast.org/ast_of_fungi); last accessed 20 May 2025). *Candida albicans*, *C. parapsilosis*, and *C. tropicalis* isolates were classified as resistant (R) when MICs exceeded drug-specific breakpoints. Breakpoints were unavailable for other species, including *N. glabratus*.

All isolates were susceptible to fluconazole and voriconazole. Resistance to amphotericin B was detected in 9.6% of *C. albicans*, 5.3% of *C. parapsilosis*, and 5.3% of *C. tropicalis* isolates. Itraconazole resistance rates were 16% for *C. albicans*, 6.4% for *C. tropicalis*, and 5.3% for *C. parapsilosis*. For posaconazole, resistance was observed in 12.8% of *C. albicans*, 6.4% of *C. tropicalis*, and 4.3% of *C. parapsilosis* isolates. Multidrug resistance was recorded in 13.8% of *C. albicans*, 7.5% of *C. tropicalis*, and 5.3% of *C. parapsilosis* (Table 2).

In contrast, a recent European study [7] reported that all analyzed yeast isolates were susceptible to amphotericin B. Additionally, in that study, fluconazole resistance was detected in 4% of *C. tropicalis* and 17% of *C. parapsilosis* isolates. These findings highlight the importance of conducting country-specific surveillance to monitor emerging antifungal resistance trends among yeast pathogens.

**Principal component analysis.** The dataset was arranged as a matrix with 94 yeast isolates (columns), each characterized by 81 variables (rows) representing patient- and isolate-specific attributes. These data points occupy 81- and 94-dimensional spaces, respectively, and can be conceptualized as a cloud of vectors. PCA centres this cloud at its centroid and performs an orthogonal rotation of the coordinate system to generate new axes – principal components – that represent directions of maximal variance and capture the principal patterns of variation within the dataset. Components explaining more than 90% of the total variance were retained.

Applying PCA to this multidimensional dataset – including yeast species, isolate sources, antifungal susceptibility profiles, and patient factors such as age, gender, and diagnosis – enabled dimensionality reduction while preserving the majority of variance, facilitating the identification of biologically relevant correlations. The first two principal components (PC1 and PC2) accounted for 91.9% of the total variance as presented in Fig. 1.

**Scatter plot analysis.** The distribution of yeast pathogens by patient gender and age group is presented in Fig. 2. Data points are colour-coded by gender (blue for females, red for males), and each yeast species is represented by a distinct symbol.

The plot shows distinct clustering patterns:

**Children (1–11 years):** *Candida parapsilosis* and *Nakaseomyces glabratus* were the predominant isolates. All patients in this group were female.

**Young Adults (18–45 years):** *N. glabratus* was dominant, followed by *C. albicans*. This group consisted exclusively of female patients.

T a b l e 2

MIC ranges (µg/µl) of six antifungal agents against yeast isolates. Resistant isolates of *C. albicans*, *C. parapsilosis*, and *C. tropicalis* were identified based on MIC values according to EUCAST v11.0 (2024) breakpoints: amphotericin B (MIC > 1 for all species), itraconazole (*C. albicans* > 0.06; *C. parapsilosis* and *C. tropicalis* > 0.125), fluconazole and posaconazole (MIC > 0.06 for all species), and voriconazole (MIC > 0.25 for all species)

| Yeast species<br>(No of isolates)   | Caspofungin  | R | Amphotericin | R | Itraconazole | R  | Fluconazole | R | Voriconazole | R | Posaconazole | R  |
|-------------------------------------|--------------|---|--------------|---|--------------|----|-------------|---|--------------|---|--------------|----|
| 40 strains<br><i>N. glabratus</i>   | 0.002–0.64   | – | 0.12–32      | – | 0.125–32     | –  | 0.25–4      | – | 0.016–0.94   | – | 0.002–2      | –  |
| 19 strains<br><i>C. albicans</i>    | 0.002–0.064  | – | 0.5–8        | 9 | 0.047–0.5    | 14 | 0.064–1     | – | 0.008–0.047  | – | 0.047–0.125  | 12 |
| 9 strains<br><i>C. parapsilosis</i> | 0.002–0.094  | – | 0.5–3        | 5 | 0.047–0.5    | 5  | 0.19–1      | – | 0.008–0.32   | – | 0.032–0.064  | 4  |
| 8 strains<br><i>C. tropicalis</i>   | 0.002–0.125  | – | 1–8          | 6 | 0.094–0.38   | 6  | 0.25–1.5    | – | 0.023–0.125  | – | 0.032–0.125  | 6  |
| 4 strains<br><i>T. asahii</i>       | 32           | – | 1–4          | – | 1–32         | –  | 1           | – | 0.047–0.19   | – | 0.019–0.125  | –  |
| 3 strains<br><i>C. inconspicua</i>  | 0.002–0.047  | – | 0.5–6        | – | 0.125–1      | –  | 4–6         | – | 0.016–0.094  | – | 0.032–0.25   | –  |
| 2 strains<br><i>Y. lipolitica</i>   | 0.008–1      | – | 2–8          | – | 0.25–1       | –  | 0.25–0.5    | – | 0.016–0.032  | – | 0.0094–0.19  | –  |
| 2 strains<br><i>P. norvegiensis</i> | 0.002; 0.047 | – | 0.032; 1     | – | 0.25; 0.5    | –  | 2; 8        | – | 0.064        | – | 0.0032; 32   | –  |
| <i>K. marxianus</i>                 | 0.002        | – | 8            | – | 0.5          | –  | 3           | – | 0.064        | – | 0.125        | –  |
| <i>C. metapsilosis</i>              | 0.064        | – | 1            | – | 0.125        | –  | 1           | – | 0.023        | – | 0.064        | –  |
| <i>M. quillermontii</i>             | 0.002        | – | 3            | – | 6            | –  | 2           | – | 0.064        | – | 0.094        | –  |
| <i>M. capitatus</i>                 | 32           | – | 32           | – | 0.5          | –  | 3           | – | 0.094        | – | 0.064        | –  |
| <i>C. zeylanoides</i>               | 0.38         | – | 0.002        | – | 0.19         | –  | 0.5         | – | 0.002        | – | 0.004        | –  |
| <i>P. kudriavzevii</i>              | 0.23         | – | 2            | 1 | 1            | 1  | 6           | 1 | 0.25         | – | 0.064        | –  |
| <i>C. lusitanae</i>                 | 0.19         | – | 1            | – | 0.064        | –  | 0.002       | – | 0.006        | – | 0.032        | –  |

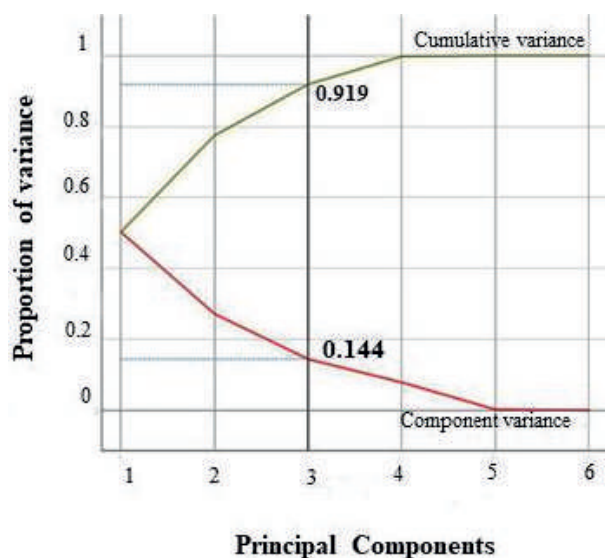


Fig. 1. Principal Component Analysis (PCA) of the dataset

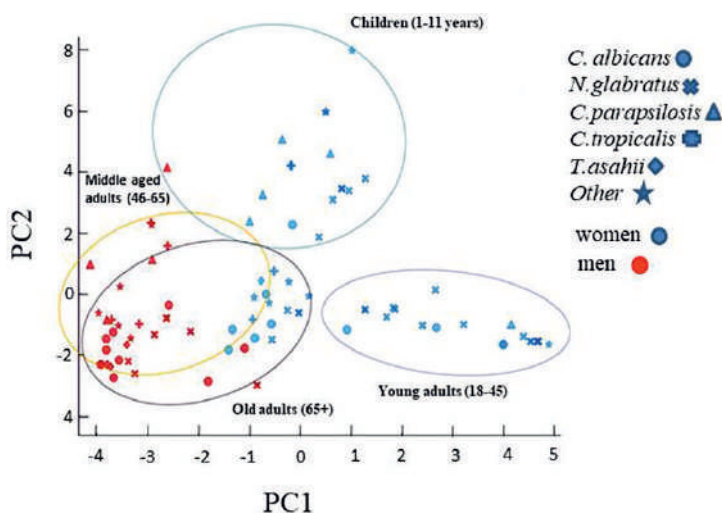


Fig. 2. Scatter plot of fungal pathogens by gender and age group

**Middle-Aged Adults (46–65 years)** Males predominated. *C. albicans* was the most frequent isolate, followed by *N. glabratus* and *C. parapsilosis*.

**Old Adults (65+ years):** Male patients slightly outnumbered females. The most prevalent species were *C. albicans* and *N. glabratus*, both with near-equal gender distribution.

These observations suggest age-and-gender-associated clustering of yeast species. The predominance of *C. parapsilosis* in children aligns with prior report [19].

Similarly, the association of *C. albicans* with middle-aged and older adults is consistent with its established role as a leading cause of candidemia in these populations [20]. The elevated prevalence of *N. glabratus* among young adults may reflect regional variation or be influenced by the relatively small, single-centre sample size. Future large-scale studies encompassing all regions of the country will be crucial to provide a comprehensive and representative overview of yeast pathogen epidemiology in Bulgaria.

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