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Research Article

Molecular Identification, Diversity, and Antifungal Susceptibility of Clinical Yeasts Associated with Human Skin Spots

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ABSTRACT

Fungi associated with different parts of human body and cause approximately a million deaths and a billion superficial infections annually. That need to identify their causes and the optimum antifungal treatments. In this work, biodiversity, morphology and molecular identification using rDNA ITS, and antifungal susceptibility of isolated fungal yeasts associated with collected samples from 30 patients' skin spots from different regions in Iraq have been investigated. Phenotypic and molecular identification, and a phylogenetic tree were utilized to assess yeasts to species level and their biodiversity. Furthermore, the efficacy of three certain antifungal agents for detected dermophyte fungi was tested. The results in this study revealed the association of the ascomycetous yeasts such as *Candida intermedia* (12 isolates) and *Candida* sp. (9 isolates) and basidiomycetous yeasts; *Naganishia diffluens* (25 isolates), *Rhodotorula glutinis* (4 isolates), *Rhodotorula diobovata* (one isolates), *Rhodotorula mucilaginosa* (one isolates), and *Filobasidium magnum* (3 isolates) with skin spots. Based on available data, this is the first record of all detected yeast species associated with skin spots in Iraq except *R. mucilaginosa*. Biodiversity of yeasts revealed that *Naganishia diffluens* and *Candida intermedia* were predominant species and the highest diversity across the patients based on diversity indices. Antifungal susceptibility test showed that *R. mucilaginosa* was resistant to two of three tested antifungals. It is concluded most yeast species were recorded for the first time from the skin spots in Iraq with variant Antifungal susceptibility of three yeast species. These findings could provide insight to fungi associated with skin spots and selecting the optimum antifungals.

1. Introduction

Fungi, as eukaryotic microorganisms, play significant impact on Earth, contributing to extensive crop loss, and even association and destruction of various life forms (Fisher *et al.* 2020; Alshuwaili *et al.* 2021). Besides their prevalent presence in the environment, numerous fungal species form part of the normal flora across diverse anatomical sites, including oral cavity, skin, lungs, genitourinary, and gastrointestinal tracts, where they play essential roles in human health and diseases (Rolling *et al.* 2020; Al Anbagi *et al.* 2025). In instances of the compromised immune systems, commensal fungal species can transform into invasive pathogens and systematically translocate leading to the development of invasive fungal infections and affect multiple organs and organ systems (Zhai *et al.* 2020). The impacts of fungi on human health extend beyond acute and chronic infections, with emerging studies linking certain fungal species' colonization to the progression of conditions like pancreatic cancer (Aykut *et al.* 2019) and alcoholic cirrhosis (Yang *et al.* 2017).

Despite being underestimated as a cause of infection in humans, fungi are associated with approximately

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1.5 million deaths and 1.7 billion superficial infections annually, resulting in a substantial economic burden. Fungal species belonging to *Candida*, *Aspergillus*, *Cryptococcus*, and *Pneumocystis* genera represent the most clinically relevant pathogens causing invasive fungal infections (Brown *et al.* 2012). However, other emerging yeast infections have been reported and present challenges in terms of the difficulties of species identification, at risk population, certain virulence features, and antifungal sensitivity profiles (Sharma and Chakrabarti 2023). The main focus is on ascomycetous yeasts such *Candida* species associated with skin infection in Iraq (Fadhel and AL-Khazali 2025). However, basidiomycetous yeasts have very significant species that need be identified and investigated.

Unlike the numerous classes of antibiotics used for bacterial infections, antifungals are limited and fall into three main classes, including azoles, echinocandins and polyenes. Azoles inhibit Erg11 in *Candida* and Cyp51A in *Aspergillus* species, disrupting the production of ergosterol, a critical sterol component of the fungal cell membrane. Echinocandins target the catalytic subunit of β-1,3-D- glucan synthase encoded by FKS genes, interfering with β-1,3-D- glucan production, a major cell wall structural component. Lastly, polyenes bind to ergosterol in the cell membrane, causing cell death through the formation of large pores and interrupting osmotic pressure (Berman and Krysan 2020).

Considering host- and drug-related factors influencing antifungal therapeutic failure (Costa-de-Oliveira and Rodrigues 2020), the emergence of resistance to a specific antifungal agent can be devastating, severely limiting the available antifungals for treating IFIs (Cowen *et al.* 2014). Before the widespread use of antifungals in clinical settings, epidemiological studies indicated the prevalence of fungal species highly susceptible to all classes of antifungals. However, the extensive use of antifungals has altered the epidemiological landscape of IFIs. Fungal species exhibiting resistance to one or multiple classes of antifungals are increasingly identified in clinical settings, correlating with therapeutic failure (Romero *et al.* 2019; Latge and Chamilos 2019). Misidentification and using wrong antifungals could led to worst outcomes of infection treatments. Therefore, this study aimed to isolation of yeast species associated with skin spots, morphological and molecular identification of these yeasts, and testing some antifungals on the most common isolated yeasts.

2. Materials and Methods

2.1. Sample Collection, Fungal Isolation, and Morphological Characterization

Samples of specimens were collected from 30 individuals (males) who have skin spots within the age range of 9-50 years old from various regions in Iraq following clinical diagnosis by a specialist physician based on ethical protocol. Swap samples, using swaps wetted with distilled sterile water, were taken from the lesions on the skin of the affected individuals and cultured on plates with Sabouraud Dextrose agar medium (Himedia, India). Then, inoculated plated were incubated at 35°C for 72 hours. Fungal colonies were accurately counted, sub-cultured, purified. The fungal isolates were examined macroscopically and microscopically using lactophenol cotton blue as a mounting material under a light microscope with an objective lens magnification power of 40X.

2.2. DNA Extraction, Amplification, and Sequencing

The extraction of the whole genomic DNA from cultures of fungal isolates was performed using FavorPrep™ Fungi/Yeast Genomic DNA Extraction Mini Kit following the manufacturer's instructions (Favorgen Biotech Corp., South Korea). The extracted DNA of samples was stored at -20°C for subsequent processes.

For the molecular identification of the presented species, the ITS rDNA regions have amplified using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers related to the conserved nucleotides. The PCR-prepared mixtures, programs, and visualized products were performed as being described in Al Anbagi *et al.* (2025). The PCR products (500-540 bp) were loaded into the wells of the electrophoresis chamber and run on 1% agarose gel in a loading buffer under 100 voltages for 45 min. The PCR amplicons were visualized under UV light after being stained the agarose gel with RedSafeTM nucleic acid stain (iNtRON Biotechnology, Korea). The PCR products of the successful amplified DNA were sequenced using ABI BigDye Terminator v.3.1 (Macrogen Inc., Seoul, Korea).

2.3. Bioinformatics and Phylogenetic Analysis

The cleaned and trimmed ITS sequences using Geneious software (V 9.1.8) (Biomatters Ltd., Newark, NJ) were uploaded and compared using the BLASTn

tool (Altschul *et al.* 1990) in the National Center for Biotechnology Information (NCBI) to gain initial identifications. The sequences of queries and references were aligned, compared and identify based on the high percent similarities. Later, the identified gene sequences of the Iraqi representative isolates were deposited in GenBank under the accession number PX854062- PX854069. The Iraqi sequences were used to construct a phylogenetic tree after multiple sequence alignments were created with MAFFT (v7.309) (Katoh and Standley 2013). The maximum likelihood analyses were performed in Geneious (v9.1.8) using PhyML v7.2.820 (Guindon *et al.* 2010) and quick bootstrapping with the GTR model of nucleotide replacement for 1000 replicates. The constructed phylogenetic tree was visualized in Geneious (v 9.1.8).

2.4. Antifungal Susceptibility Testing

The species of *Candida intermedia* (C10), *Naganishia diffluens* (C3), and *Rhodotorula mucilaginosa* (C9) were selected for *in vitro* antifungal susceptibility profiles. Three antifungals including Fluconozole, Nystatin and Ketoconazole (Sigma-Aldrich, MO, USA) were performed on the Muller-Hinton culture medium (HiMedia Laboratories Private Limited, Mumbai, India). The yeast suspensions were prepared independently at a final cell concentration ranging from 1×10^4 to 1×10^6 CFU/mL and 250 µL of each isolate were spread on Muller-Hinton plates with three replicates. Then, the antifungal discs were added on the inoculated plate surfaces and plates were incubated at 35 °C for 48 hours. Later, the dimeters of inhibition zones were measured by observation with naked eye (CLSI 2009).

2.5. Statistical Analysis

The data analysis of the diversity and two-way cluster dendrogram were achieved for yeast community composition associated with the different patients in PC-ORD v.6. (McCune and Mefford 2011). The two-way ANOVA Analysis was performed in JMP® 18 (SAS Institute Inc., Cary, NC, USA) to test the effect of three antifungals on three yeast species. The Tukey's honestly was used to determine the significant differences between treatment means using a significance level of 0.05.

3. Results

3.1. Molecular Identification, Species Diversity and Phylogenetic Relations

The symptoms of thirty patients included skin patches and skin irritation, characterized by severe itching and redness. Some patients exhibited severe ulcerations and skin erosion, particularly between the toes. Scalp flakiness was also observed in certain patients, while others presented with pustules (Figure 1).

The swabs collected from the lesions on the patients' skins, successfully isolated and identified several distinct fungal yeast species from human skin samples with ranging from 1 to 3 species in each patient. These yeast isolates exhibited various colors of colonies, with white being the most common, followed by yellowish-white, and orange being the least common color. The result of morphological features and molecular identification revealed that all isolated yeast strains recovered from skin spots were assigned into two fungal phylae, Basidiomycota and Ascomycota and four genera *Candida, Filobasidium, Naganishia,* and *Rhodotorula*, representing a diverse assemblage of yeast species, which were identified for the first time associated with the human skin spots in this study exceptss *R. mucilaginosa*. These included five basidiomycetous species (*Naganishia diffluens, Filobasidium magnum* and three species of *Rhodotorula* genus), and two ascomycetous species (*Candida* sp. and *C. intermedia*). The total number of isolates were 55 isolates for all yeast species. (Figures 1 and 2).

Sequence analysis of the ITS- rDNA region confirmed species identification with ≥97% similarities to reference sequences from the NCBI GenBank database. Furthermore, phylogenetic analysis of Maximum Likelihood for yeast isolates showed that all sequenced yeasts of Iraqi isolates noticeably clustered as distinct clades with type materials of known specific specimens and clearly separated from other species (Figure 3) except one. The sequence of *Candida* sp. failed to be identified

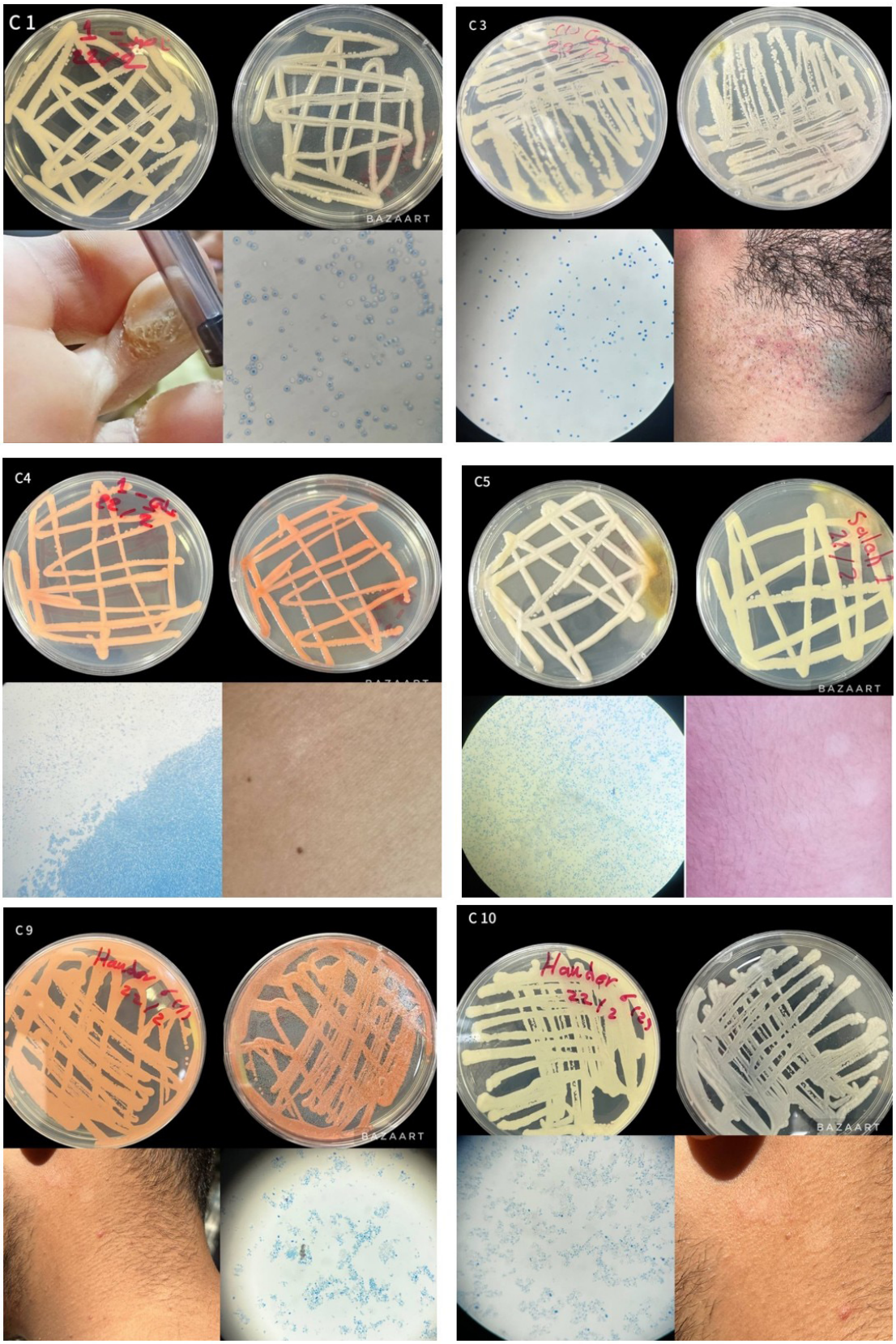


Figure 1. Colony features, clinical presentations, and yeasts cells (asexual stage) of some isolates from human skin. The isolates C1 and C3 are related to *Naganishia diffluens*, C4, C5, C9, and C10 are related to *Rhodotorula diobovata, Filobasidium magnum, Rhodotorula mucilaginosa* and *Candida intermedia* respectively, which were confirmed via molecular identification

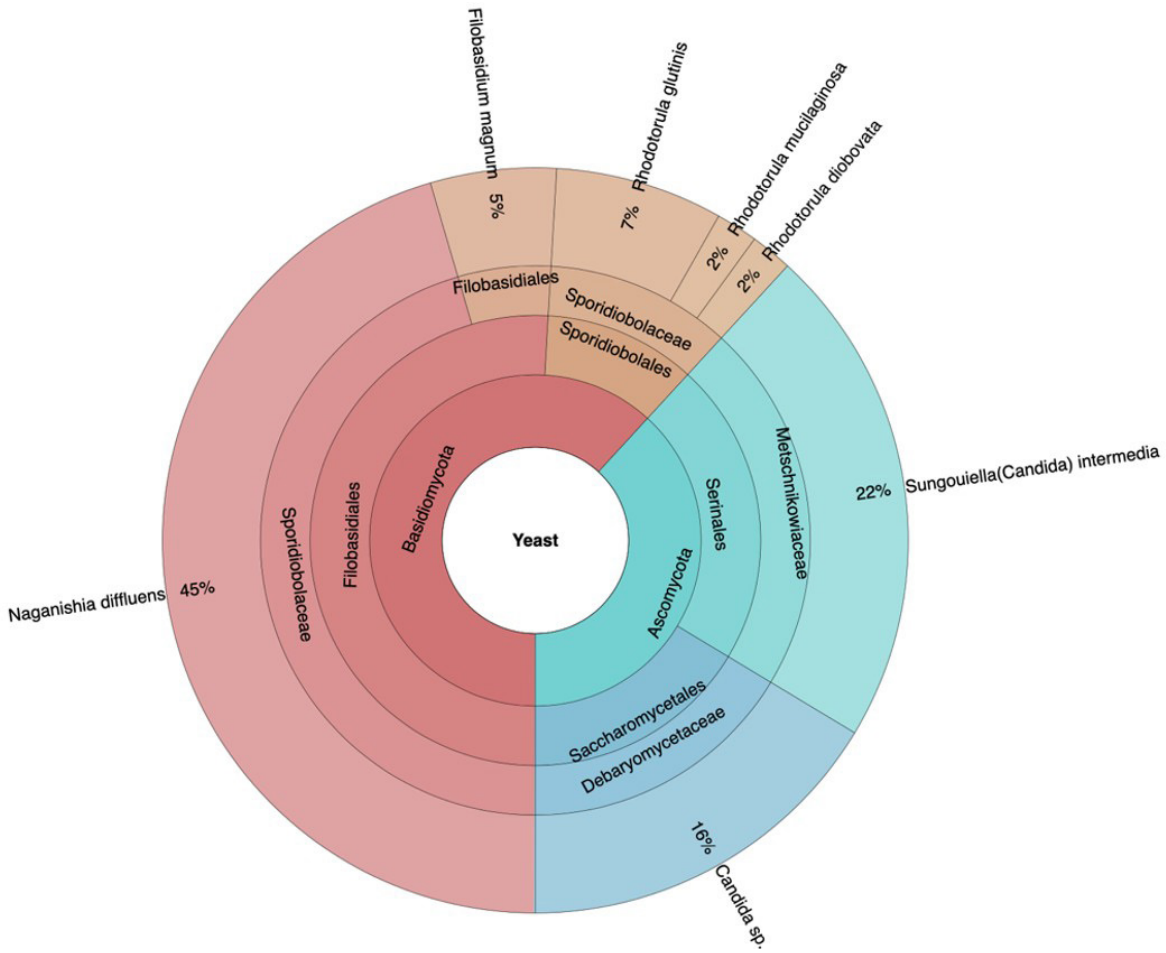


Figure 2. Diagrams of frequency percentiles of human pathogenic yeasts in the total patients. The data represented the relative frequency of isolated yeasts

into the species level due to the quality of the received sequences.

The yeast form of identified species using both morphological and molecular tools was represented by 25 isolates of *Naganishia diffluens*. These were symbolized by four sequences in the phylogenetic tree accounting for approximately 45% of the total Isolates, which formed a tight monophyletic cluster. Additionally, 12 isolates of *Candida intermedia* were exemplified via three sequences in the phylogenetic tree estimated to account for approximately 22% of the total Isolates. Three *Rhodotorula* species were also recorded in this study. These involved four isolates of *Rhodotorula glutinis* and one isolates each of *Rhodotorula diobovata* and *Rhodotorula mucilaginosa*, each of which was represented via a single sequence in the phylogenetic tree. Likewise, the tree isolates of *Filobasidium magnum* were represented by one sequence in the phylogenetic tree (Figure 3).

The presented species showed a variety of morphological traits and diversity indexes. Typical colony morphologies of *Naganishia diffluens* isolates on SDA were cream to pale white while color of *Candida intermedia* colonies was creamy, smooth and opaque. The *Rhodotorula* species formed distinguishing pink to coral-colored colonies, with *R. mucilaginosa* showing more concentrated pigmentation compared to *R. diobovata* and *R. glutinis*. Furthermore, *Filobasidium magnum* exhibited white, glossy colonies with a slightly filamentous margin (Figure 1). These species distributed across 30 patients and most of the sampled patients had an average of two yeast species (Figure 4). Although the species abundance, richness, and evenness values indicated moderate fungal diversity within the sampled skin microhabitats, the Shannon–Wiener diversity index ($H' = 1.33$) and Simpson's index ($D = 0.56$) proposed that *Naganishia diffluens* and *Candida intermedia*

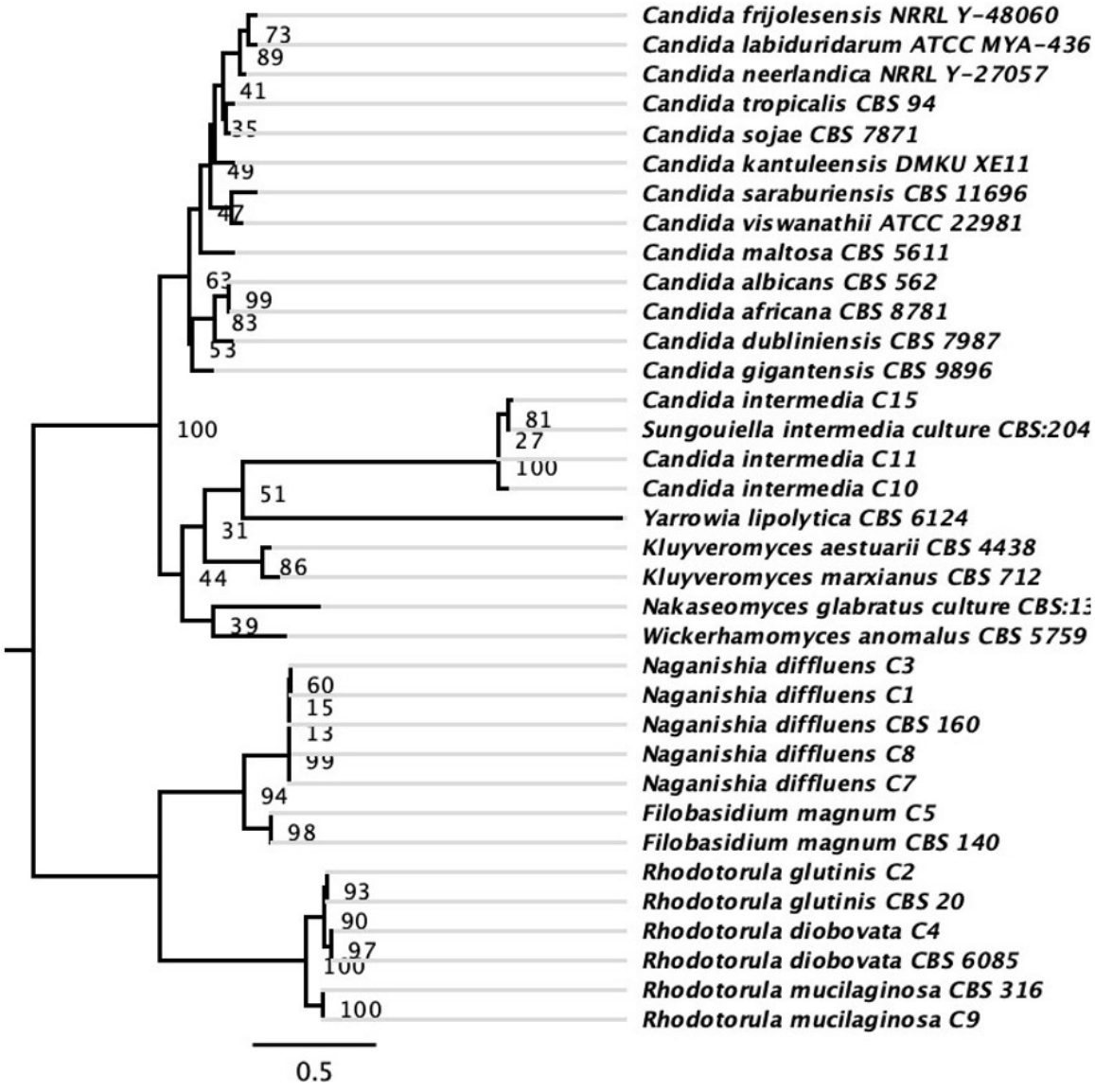


Figure 3. Phylogenetic tree of ITS phylogeny of fungal isolates, *Naganishia diffluens* (C1, C3, C7& C8), *Rhodotorula glutinis* (C2), *Rhodotorula diobovata* (C4), *Filobasidium magnum* (C5), *Rhodotorula mucilaginosa* (C9), *Candida intermedia* (C10, C11, & C15) with their related species using the Maximum Likelihood phylogenetic analysis. The nodes display Bootstrap support values. All other sequences use beside the sequences of this study are recovered from type materials

were dominant. However, the presence of several *Rhodotorula* species contributed to overall yeast community heterogeneity that were recorded with low occurrence and abundance (Table 1).

3.2. Antifungal Susceptibility Profiles

The results of *in vitro* susceptibility testing of identified yeast species to Fluconazole, Ketoconazole and Nystatin are varied based on the specific fungus and the antifungal type (Figure 5). Of the tested drugs, the fluconazole demonstrated exceptionally was high efficacy against all tested fungi, completely preventing development of *Naganishia diffluens, Candida intermedia* and *Rhodotorula mucilaginosa*. Conversely, the isolate of Rhodotorula mucilaginosa exhibited complete resistance to both nystatin and ketoconazole compared to other tested species. The inhibition zones of ketoconozole against *Candida intermedia* and *Naganishia diffluens* were 26.33 and 30 mm respectively. In another hand, nystatin exhibited an inhibitory zone diameter of 11.87 mm against *Naganishia diffluens* and 31.33 mm against *Candida intermedia* (Figure 5).

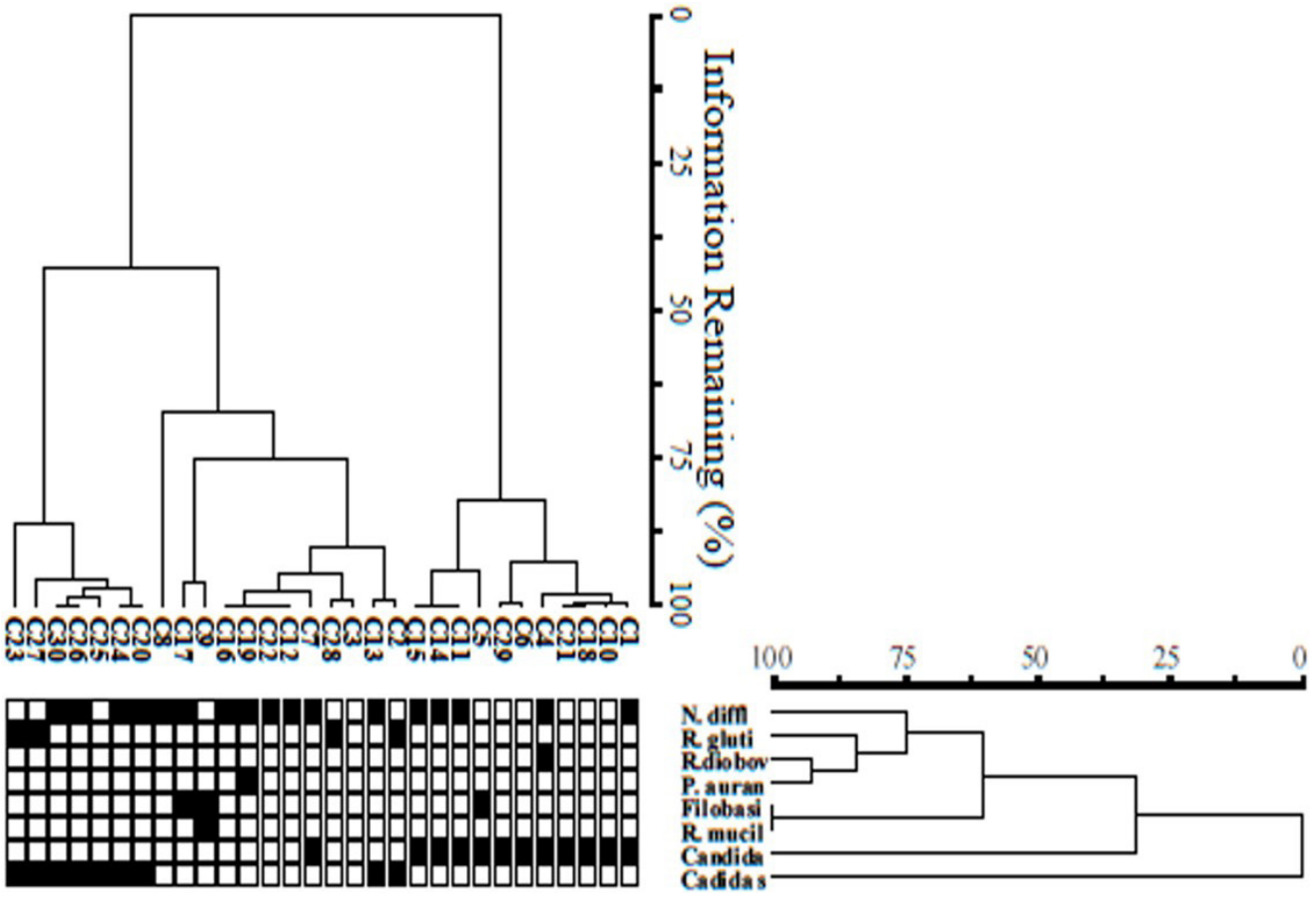


Figure 4. Species distribution of fungal species clustered on the horizontal matrix axis, across 30 investigated infected patients (C1-C30) grouped on the vertical axis using two-way cluster analysis. The species coded the first letters of genus and few letters for species

Table 1. Biodiversity indices of yeast species associated with patients' skin in the study samples

Name	Mean	S.D.	Sum	S	E	H	D	Skewness	Kurtosis
N. diffluens	9.9	14.7	298	17	0.9	2.6	0.89	14.3	3.3
R. glutinis	3.1	9.7	92	4	0.8	1.2	0.64	17.9	4.0
R. diobovata	0.4	2.4	13	1	0.0	0.0	0.00	30.0	5.5
R. mucilaginosa	0.6	3.5	19	1	0.0	0.0	0.00	30.0	5.5
F. magnum	3.7	12.1	112	3	0.9	1.0	0.62	10.5	3.3
C. intermedia	19.1	26.8	574	12	0.9	2.4	0.90	-0.5	1.0
Candida sp.	15.0	24.6	450	9	0.9	2.1	0.88	-0.2	1.2
Averages	7.4	13.4	222	6.7	0.62	1.33	0.56	14.6	3.4

S: Richness, E: Evenness, H: Shannon`s diversity index, D': Simpson`s diversity index. SD: Standard division

4. Discussion

The human skin microbiota has been widely investigated, particularly the complex bacterial community. However, limited articles deal with skin mycobiota compared to the bacterial community have been performed (Zhang *et al.* 2011). That may be mutually related to bacteria and/or other skin microorganisms in maintaining hemostasis. In this study, most of the species presented have been reported as emerging yeasts cause human infections (Sharma and Chakrabarti 2023) in other countries. The current findings demonstrated *Naganishia diffluens* and *Candida intermedia* have a high accidence and distribution in sampled patients. The species have been recorded frequently colonizing the skin of patients with atopic dermatitis as well as neonates (Morales-López and Garcia-Effron 2021; Khozani *et al.* 2024). *C. intermedia* have been considered as a part of skin microbiome with ability to become pathogenic (Espinosa-Hernández *et al.* 2020).

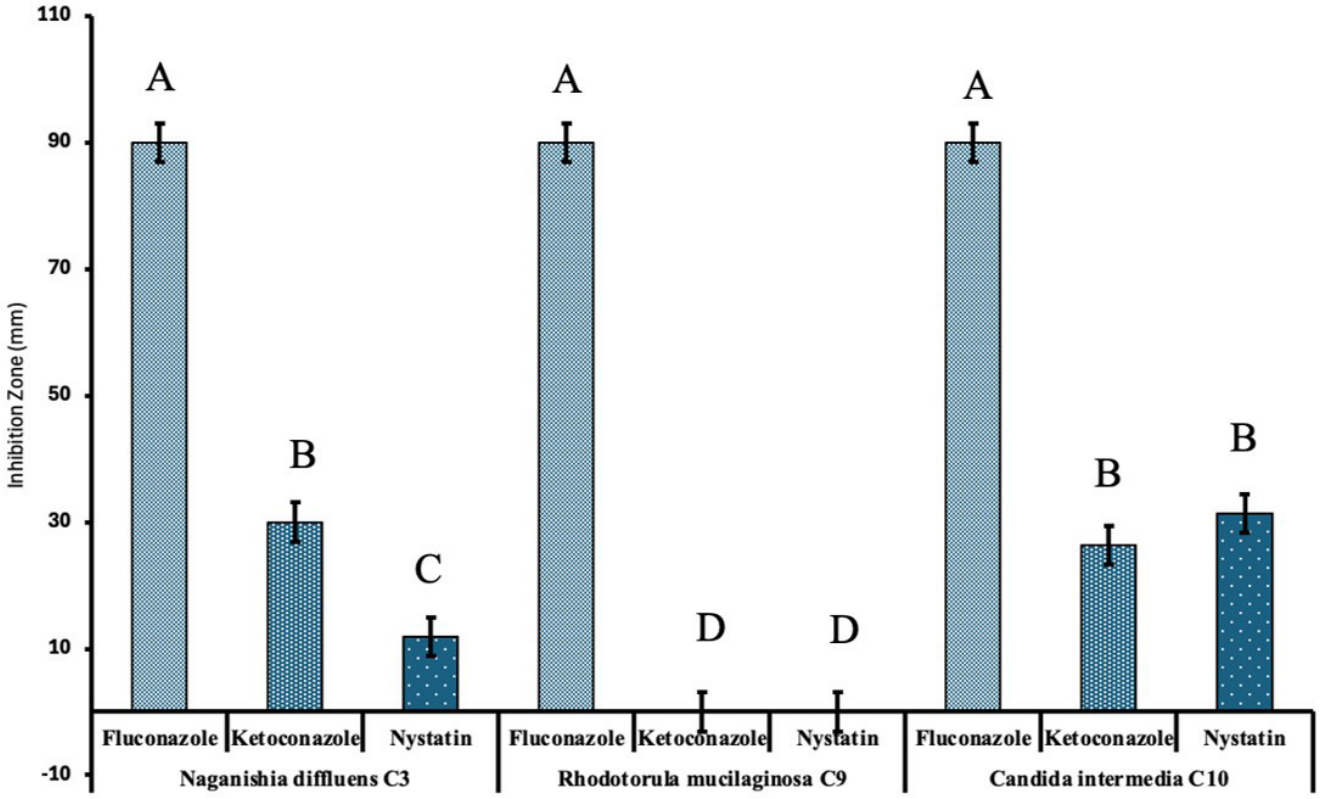


Figure 5. Effect of three investigated antifungals against *Naganishia diffluens, Candida intermedia* and *Rhodotorula mucilaginosa*

The yeast species are usually overlooked or have not been isolated in some studies associated with infected human skins especially in Iraq. Therefore, this is the first record of detected yeast species; *Naganishia diffluens, Rhodotorula glutinis, Rhodotorula diobovata, Filobasidium magnum,* and *Candida intermedia*. There are limited studies focus on yeast species associated skin spots in Iraq and have not recovered species represented in this study. For instance, Fadhel and AL-Khazali (2025) isolated ascomycetous yeasts such *Candida* species associated with skin infection in Iraq and have not identified any species isolated in this study. Furthermore, Ali *et al.* (2017) reported that eight species of the *Tinea* genus linked with dermatophyte fungal infections. Another study revealed that *Candida vaginitis* and *Tinea capitis* were more common infections associated dermatophyte infections (Mohammad *et al.* 2024). However, other findings showed that *Tinea corporis* and *Tinea capitis* caused by Microsporum were the most common infections linked with skin infection (Al-Kayalli *et al.* 2011). Another work found that yeast genera such as *Malassezia* species are the predominant colonizers of infected human skins in Japan. Furthermore, Zhang *et al.* (2011) detected *N. (Cryptococcus) diffluens*, with low accidence, which was the most common species in our study. The fungus *N. diffluens*, a rare species of non-neoformans *Cryptococcus*, formerly known as *Cryptococcus diffluens*. This basidiomycetous yeast has been documented as a causative agent of skin mycoses, including tinea cruris, a common fungal infection affecting the buttocks, groin, perineum, and perianal area, predominantly caused by parasitic fungi. The findings are consistent with previous studies (Yang *et al.* 2023). The second common species in this study was *C. intermedia*. Previous research found that *Candida* spp. are common species associated with human body and cause human diseases (Al-Dabbagh *et al.* 2023). *Candida* sp. was diagnosed, known to affect human skin, as corroborated by previous studies (Kantarcıoğlu *et al.* 2015). The three *Rhodotorula* species in addition to *Filobasidium magnum* that was detected in this effort have not noted in the mentioned previous studies in Iraq, but they have been increasing worldwide. Rhodotorula, which also affects humans, predominantly in cases of infection due to *Rhodotorula* in humans, was associated with bloodstream fungal infections related to the use of central venous catheters (CVC). In the last decay, several studies have pointed to increased human infections including the moist areas of skin by

species of *Rhodotorula* although the genus had been considered nonpathogenic (Ioannou *et al.* 2018). The most common underlying conditions included solid and hematological malignancies, with other localized infections caused by *Rhodotorula*, including meningitis, dermatitis, ocular, and peritoneal infections, as reported in previous studies (Nunes *et al.* 2013; Ioannou *et al.* 2018). Its prevalence among total isolates was 5 and 20%. Besides their pervasive presence in the environment, numerous fungal species form part of the normal flora across diverse anatomical sites, including the skin, lungs, genitourinary, oral, and gastrointestinal tracts, where they play crucial roles in human health (Rolling *et al.* 2020). In instances of compromised immune systems, commensal fungal species can transform into invasive pathogens, systematically translocating and leading to the development of invasive fungal infections that affect multiple organs and organ systems (Zhai *et al.* 2020). Although this study determined many significant yeast species for the first time associated with skin spots in Iraq, using Next Generation Sequencing (NGS) metagenomic amplicon sequencing could recover many missed or difficult to recovered yeast species (Alshuwaili *et al.* 2021).

Distinguished variable susceptibility of tested yeast species to antifungals were noted in this research. Fluconozole completely inhibited the growth of all tested isolates. However, *R. mucilaginosa* exhibited complete resistance to both nystatin and ketoconazole. The current study was disagreed with findings of a study that revealed the isolates of *R. mucilaginosa* were resistance to azoles including fluconazole and voriconazole while being sensitive to polyenes including amphotericin B and nystatin, with the ability to adhere and form biofilm on the abiotic surface of polystyrene (Jarros *et al.* 2020). Furthermore, the present study disagreed with the results of a study from Iraq that revealed *Rhodotorula mucilaginosa* was resistant to fluconazole and highly sensitive to ketoconazole and nystatin (Abed and Hussein 2017). The differences in response and resistance to antifungals can be attributed to various factors related to the host, the drug, and the fungus itself (Perlin *et al.* 2017). Similar patterns of antifungal sensitivity of the current study have been reported for *C. intermedia* and other species isolates in Iran (Razzaghi-Abyaneh *et al.* 2014). The antifungal susceptibility profile of the clinical isolates provides a pre-significant insight to recommend a specific antifungal to a specific fungal species after robust molecular identification of causal agents.

In conclusion, the findings of the current study conclude that molecular and morphological identification based on ITS sequencing are necessary to identify yeast species linked to human skin infection. Significant yeast including *N. diffluens, C. intermedia*, two species of *Rhodotorula* and *F. magnum* are detected species associated with human skin for the first time in Iraq based on available data. *N. diffluens* and *C. intermedia* were predominant species associated with skin spots in Iraq. Fungal susceptibility and Antifungal resistance of the tested fungi were variable based on fungal species and type of antifungals. These results could provide understanding the common yeast associated with human skin and the risks of antifungal resistance.

Ethical Approval

This study was approved by the ethical committee, the Al-Qasim Green University /College of Biotechnology with a protocol certificate No.M7-2024.

Conflict of Interest

The authors declare no conflict of interest to the present study.

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