Medical Mycology, 2025, **63**, myaf064
https://doi.org/10.1093/mmy/myaf064
Original Article
Advance access publication date: 21 July 2025

A notable azole-nonsusceptible *Candida orthopsilosis* in the *Candida parapsilosis* complex isolated from onychomycosis in Hue City, Central Vietnam

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Abstract

The *Candida parapsilosis* complex, consisting of *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis*, is a major cause of *Candida* onychomycosis. Increasing reports of high levels of resistance to antifungal drugs, particularly fluconazole and echinocandin, have raised concerns about *C. parapsilosis* complex. This study investigates antifungal resistance and hydrolytic enzyme activity in these species. Species were identified using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) and internal transcribed spacer (ITS) 1-4 sequencing. Antifungal susceptibility was assessed using Sensititre™ YeastOne™. Hydrolytic enzyme production was assessed by agar plate culture. Among 43 isolates, *C. parapsilosis sensu stricto* was most prevalent (48.8%, $n = 21/43$), followed by *C. orthopsilosis* (39.6%, $n = 17/43$) and *C. metapsilosis* (11.6%, $n = 5/43$). All *C. parapsilosis sensu stricto* isolates were susceptible to antifungal agents, except 4.8% ($n = 1/21$) showing dose-dependent susceptibility to fluconazole and 4.8% ($n = 1/21$) resistance to amphotericin B. *Candida orthopsilosis* showed significant resistance to fluconazole and voriconazole (52.9% each, $n = 9/17$), posaconazole (23.5%, $n = 4/17$), and low resistance to amphotericin B (5.9%, $n = 1/17$). One *C. metapsilosis* isolate (20%) showed cross-resistance to fluconazole and voriconazole, and another (20%) was resistant to 5-flucytosine. Enzymatic assays showed higher protease and lipase activity in *C. parapsilosis sensu stricto* and *C. orthopsilosis* compared to *C. metapsilosis*, with *C. parapsilosis sensu stricto* showing the highest protease activity. Comprehensive research into antifungal susceptibility and virulence factors of the *C. parapsilosis* species complex is essential to monitor the growing threat of antifungal resistance and to better understand its role in onychomycosis pathogenesis.

Lay summary

This study reported a high prevalence of resistance to azole agents, including fluconazole, voriconazole, and posaconazole, in *Candida orthopsilosis* among *C. parapsilosis* complex isolated from onychomycosis in Central Vietnam. Additionally, *C. parapsilosis sensu stricto* and *C. orthopsilosis* exhibited higher protease and lipase activity than *C. metapsilosis*.

Key words: *Candida parapsilosis* complex, *Candida parapsilosis sensu stricto, Candida orthopsilosis, Candida metapsilosis,* antifungal susceptibility testing, onychomysosis.

Introduction

Onychomycosis is the most common nail disease worldwide and is caused by fungal infections with dermatophytes, yeasts, and non-dermatophyte molds.[1–3] Approximately 20% of onychomycosis cases are caused by yeasts, with *Candida* species being the predominant pathogens.[2,4] The most common clinical manifestation of *Candida* onychomycosis is chronic paronychia, with frequent hand contact with water being a significant predisposing factor.[2] Although *Candida albicans* (*C. albicans*) was previously considered the most prevalent species causing nail infections,[1,5] an increasing number of scientific reports now highlight *C. parapsilosis* as the most commonly isolated species in Candida onychomycosis.[6–9]

The *C. parapsilosis* complex has been divided into three species: *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis*.[10] More recently, *C. theae* has been added to this species complex and has also been described as a causative agent.[11] Identification of cryptic species within the *C. parapsilosis* complex relies primarily on molecular techniques, including amplified fragment length polymorphism, restriction fragment length polymorphism, polymerase chain reaction (PCR) amplification and internal transcribed spacer (ITS) region sequencing.[12] In addition, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been shown to be a reliable and accurate method for species identification.[13]

The *Candida parapsilosis* species complex is associated with a wide range of mycoses including fungemia, endocarditis, endophthalmitis, arthritis, peritonitis, vulvovaginitis, and skin and nail infections. It is particularly associated with a high prevalence of disseminated mycoses in premature infants and has been implicated in hospital outbreaks.[14,15] Among

Received: May 6, 2025. **Revised:** June 27, 2025. **Accepted:** July 18, 2025

the species of this complex, *C. parapsilosis sensu stricto* is the most frequently isolated pathogen in clinical infections, compared to *C. metapsilosis* and *C. orthopsilosis*.[16,17] Notably, antifungal susceptibility testing has raised concerns regarding emerging resistance, particularly to fluconazole and echinocandin, mainly among isolates from invasive candidiasis cases.[18–20] However, resistance rates vary across studies, with some reporting no resistant isolates within this species complex.[18,21,22]

The *C. parapsilosis* complex is increasingly recognized for its role in *Candida* onychomycosis. However, there is limited information on resistance patterns of these cryptic species isolated from this disease. In addition, routine biochemical methods are insufficient for reliably differentiating cryptic species such as *C. orthopsilosis* and *C. metapsilosis* from *C. parapsilosis*.[14] As a result, the prevalence of *C. orthopsilosis* and *C. metapsilosis* among fungal pathogens associated with human disease may be underestimated, contributing to a gap in the understanding of their antifungal resistance profiles. This study aims to determine the prevalence of subspecies within the *Candida parapsilosis* complex, evaluating their antifungal susceptibility profiles, and evaluate enzyme activities associated with the pathogenicity.

Material and methods

Ethical approval

This study was approved by the Ethics Committee of Hue University of Medicine and Pharmacy (code DHH2024/547).

Study population

This study was conducted from April 2024 to April 2025 at Hue University of Medicine and Pharmacy Hospital and Hue City Hospital of Dermatology and Venereology. Nail samples were collected from patients presenting with nail disorders.

Direct microscopic examination was performed using a 20% potassium hydroxide solution. Samples that tested positive results for yeast fungi had characteristic morphologies, including round to oval cells, budding cells, or pseudo hyphae. Initial culture was grown on Sabouraud dextrose agar supplemented with chloramphenicol (HiMedia, India). Microscopic examination of the cultured colonies revealed round to oval yeast cells, budding cells, and hyphal structures (2–4 μm in size), including both pseudo hyphae and true hyphae. All isolates were further subcultured on Brilliance Candida medium (Oxoid, England), where they were identified as non-*albicans Candida* species.

Phenotypic identification of yeast

Species identification of all yeast isolates was initially conducted by API 20C AUX test (BioMerieux, France) following the procedure, and the results were as *Candida parapsilosis* with 93%–99.9% accuracy.

Identification of *C. parapsilosis* complex by PCR-RFLP

Fungal DNA was extracted using a MasterPure™ Yeast DNA Purification Kit (Lucigen, USA) following manufacturer's instructions. PCR amplification targeting the nuclear ribosomal ITS1-2 region of the *Canida parapsilosis* complex was performed using universal fungal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), which produced a fragment of 520 bp. Each 50 μl PCR reaction contained 0.2 mmol/l dNTPs, 1.5 mmol/l $MgCl_2$, 0.2 μmol/l of primers, and 1.0 IU Taq polymerase (Invitrogen, Waltham, USA) using a SureCycler 8800 thermal cycler (Agilent Technologies, USA). PCR products were visualized on 1% agarose gel stained with GelRed™ (Biotium, Fremont, USA) and visualized under a UV transilluminator.

A PCR-restriction fragment length polymorphism (PCR-RFLP) analysis was employed to differentiate the *Candida parapsilosis* complex from other *Candida* species, following the protocol of Mirhendi et al.[23] A 20 μl aliquot of the ITS1-2 PCR product was digested with 10 U of *Msp*I (Thermo Fisher Scientific, USA) in FastDigest Green buffer and incubated at 37°C for 30 min. The digested fragments were resolved on a 1.8% agarose gel in TBE buffer, stained with GelRed™ (Biotium, Fremont, USA), and visualized under UV light. The *C. parapsilosis* complex was identified by its characteristic undigested band of ~ 520 bp.[24] Subspecies identification of *C. parapsilosis sensu stricto*, *C. metapsilosis*, and *C. orthopsilosis* was conducted using double enzymatic digestion with *Sau96I* and *HhaI* (Thermo Fisher Scientific, USA) as described by Barbedo et al. [25] A 25 μl PCR product was incubated with 10 U of Sau96I and 20 U of HhaI at 37 °C for 30 minutes. Digested fragments were separated on a 3% agarose gel in 1 × TBE buffer containing GelRed™, run at 100 V for 120 minutes, and visualized under UV illumination. Fragment patterns differentiated the subspecies: *C. parapsilosis sensu stricto* (117, 178, 225 bp), *C. orthopsilosis* (102, 183, 225 bp), and *C. metapsilosis* (114, 187, 228 bp).[25] Reference strains *C. parapsilosis* ATCC 22019, *C. metapsilosis* ATCC 96143, and *C. orthopsilosis* ATCC 96141 were used as positive controls.

Sanger sequencing and phylogenetic analysis

To confirm species-level identification, PCR amplicons from selected isolates were purified and subjected to Sanger sequencing via the Apical Scientific service (https://base-asia.com/services/sanger-sequencing-services/). The resulting sequences were analyzed using Basic Local Alignment Search Tool (BLAST) against the GenBank database. Species identity was further validated through ITS-based phylogenetic analysis. Sequence alignment was performed in BioEdit v7.2.6 using 24 reference sequences representing various *Candida* species, as described by Hassanpour et al.[26] A phylogenetic tree was constructed using the maximum likelihood method with the Tamura 3-parameter model and 1000 bootstrap replicates in MEGA 11 (https://megasoftware.net/). Branches with bootstrap values > 80% were considered strongly supported.

Antifungal susceptibility testing

Antifungal susceptibility testing was performed using the Thermo Scientific™ Sensititre™ YeastOne™ YO10 AST (SYO) plate. Each isolate was subcultured on Sabouraud dextrose agar and incubated at 35°C for 24 h. A standardized inoculum equivalent to a 0.5 McFarland turbidity standard was prepared in sterile 0.85% saline. SYO panel contained serial twofold dilutions of antifungals in the following concentration ranges: anidulafungin (0.015–8 μg/ml), micafungin, caspofungin, and voriconazole, posaconazole (0.008–8 μg/ml), itraconazole (0.015–16 μg/ml); fluconazole (0.12–256 μg/ml), 5-flucytosine (0.06–64 μg/ml), and amphotericin

B (0.12–8 μg/ml). Plates were prepared according to the manufacturer's instructions. Each well was inoculated with 100 µl of the yeast suspension using a multichannel pipette. Plates were sealed and incubated at 35 °C for 24 h in a CO_2-free incubator. Minimum inhibitory concentrations (MICs) were determined based on a colorimetric change from blue (no growth) to red (growth) after 24 h of incubation. MICs for fluconazole, voriconazole, anidulafungin, caspofungin, and micafungin against *C. parapsilosis* were interpreted according to Clinical and Laboratory Standards Institute (CLSI) M60-Ed2 guidelines,[27] for itraconazole, posaconazole, and amphotericin B against *C. parapsilosis*, and all antifungals tested against *C. metapsilosis* and *C. orthopsilosis*, MICs were interpreted using epidemiological cutoff values (ECVs) as defined in CLSI M59-Ed3.[28] Due to the absence of established breakpoints or ECVs for 5-flucytosine, MICs were interpreted using the ECV proposed by Pfaller et al., with a suggested threshold of 0.5 µg/ml for *C. parapsilosis*.[29] Antifungal susceptibility testing result was classified as "resistant (R)" if the isolate had the MIC value that belonged to resistant MIC breakpoint or MIC value was above ECVs. *Candida albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 were used as quality control strains.

The essential agreement between the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference methods and the SYO method was evaluated in a study by Garzón et al. with 22 reference strains representing 14 *Candida* species, including *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*. The essential agreement rates between SYO and the EUCAST and CLSI methods were 91% and 89%, respectively.[30] These findings were consistent with previous studies.[31–33] The SYO method demonstrated good performance and was considered a reliable approach for antifungal susceptibility testing of *Candida* species.

Enzymatic capacity testing

The phospholipase activity of *C. parapsilosis* complex isolates was measured using the egg yolk plate medium, following the methodology described by Neji S. et al.[34] The medium consisted of 65 g Sabouraud dextrose agar (HiMedia, India), 55.3 g NaCl, 5.5 g $CaCl_2$, and 10% sterile egg yolk. Protease production by all isolates was assessed on agar plates containing bovine serum albumin (BSA), according to the method described by Kantarcioglu et al.[35] The test medium consisted of 2% glucose, 0.25% potassium dihydrogen phosphate, 0.1% magnesium phosphate, 0.5% sodium chloride, 2% agar, 0.1% yeast extract, and 0.25% BSA (HiMedia, India). Lipase activity was assessed using the Tween (Sigma-Aldrich) agar plates.[36] The medium consisted of 1 g peptone, 0.5 g NaCl, 0.01 g $CaCl_2$, 1.5 g agar, and 100 ml distilled water, adjusted to pH 7.0. After autoclaving, the medium was cooled to ~ 50 °C, after which 0.5 ml of Tween was added. A 10 μl fungal inoculum, standardized to a 0.5 McFarland turbidity after 48 hours of cultivation, was inoculated at three equidistant points per plate. Plates were incubated at 37°C for 10 days. *Candida albicans* ATCC 10028 was used as a control strain for phospholipase and protease activity experiments, and *Malassezia furfur* ATCC 14521 was used as a control strain for lipase activity assessments. A precipitation zone around the colonies indicated phospholipase activity on the egg yolk medium. The formation of a clearance zone around the colonies signified protease activity. Lipase activity was indicated by the formation of a precipitation zone around the colonies, resulting from the hydrolysis of Tween and subsequent binding of the released fatty acids with calcium.

Enzyme activity was assessed by measuring the zone of enzyme activity for each colony according to the method described by Price et al.[37] The enzyme index (EI) for each isolate was calculated as the ratio of the colony diameter to the sum of the colony diameter and the surrounding zone of activity (measured in millimeters), which included a slight opacity zone for phospholipase activity, a clear zone for protease activity, and a precipitation zone for lipase activity. Enzyme activity was classified based on the EI as follows: no activity (EI = 1.0); very low activity (+, EI = 0.9 - <1.0); low activity (++, EI = 0.8 - 0.89); high activity (+++, EI = 0.70 - 0.79); and very high activity (++++, EI ≤ 0.69).

Data analysis

Data were analyzed using SPSS version 20.0. Differences in geometric mean MIC values between species were assessed using one-way analysis of variance (one-way ANOVA) on log-transformed data. A P-value < .05 was considered statistically significant. The Fisher's exact test was used to compare the enzyme production capabilities of three fungal species based on their percentage proportions, and a statistically significant difference was considered when P < .05.

Results

Clinical manifestations and associated factors

During the study period, a total of 43 fungal isolates belonging to the *Candida parapsilosis* complex were collected from 86 patients diagnosed with *Candida* onychomycosis at two hospitals in Hue City, Vietnam. *Candida parapsilosis* was the predominant species, accounting for 50% of the *Candida* isolates identified in the onychomycosis cases. Of the patients, 67.4% were female and 32.6% were male. The age of the patients ranged from 27 to 79 years, with a mean age of 45.5 ± 14.2 years. Demographic data, clinical manifestations, and occupational status are shown in Table 1.

Identification of *C. parapsilosis* complex species

PCR targeting the fungal ITS1-2 region with ITS1 and ITS4 primers revealed a band of ~ 520 bp for all 43 isolates. The result of the PCR-RFLP product digested by *Msp I* showed that this restriction enzyme did not digest all amplicons (Fig. 1A). Next, the analysis of PCR-RFLP results with double digest by *Sau96I* (10 U/µl) and *HhaI* (20 U/µl), the distribution of species was as follows: *C. parapsilosis sensu stricto* accounted for 48.8% (n = 21), *C. orthopsilosis* for 39.6% (n = 17), and *C. metapsilosis* for 11.6% (n = 5). The bands corresponding to each species are shown in Figure 1B.

Two isolates from each species within the *Candida parapsilosis* complex, identified by PCR-RFLP, were selected for Sanger sequencing to confirm species-level identification. The selected isolates included *C. metapsilosis* (#C15, #C65), *C. orthopsilosis* (#C36, #C45), and *C. parapsilosis sensu stricto* (#C92, #C99). Sequencing results were consistent with the PCR-RFLP results, and a phylogenetic tree illustrating the relationships between the isolates is presented in Figure 2. The corresponding GenBank accession numbers are provided in Table 2.

Table 1. Demographic data and clinical manifestation of *Candida parapsilosis* complex patients.

Patients' characteristics		
Age range	27–79 years	
Mean age	45.5 ± 14.2 years	
Male/female ratio	14/29	
Occupations	**Number (%)**	
Housewife	8 (18.6%)	
Farmer	7 (16.3%)	
Jobs involving prolonged submersion of hands in water	22 (51.2%)	
Bartender	*2*	
Food service	*7*	
Fish and seafood trade	*9*	
Car wash worker	*2*	
Construction worker	*2*	
Other jobs	6 (13.9%)	
Nail involved	**Number (%)**	
Finger nail	42 (97.7%)	
Single	13 (31%)	
Multiple	30 (69%)	
Toenail	1 (2.3%)	
Clinical aspects	**Number (%)**	***Candida* species (number of isolate;%)**
Paronychia with trachyonychia	10 (23.3%)	*C. parapsilosis* (3; 30%) *C. orthopsilosis* (6; 60%) *C. metapsilosis* (1; 10%)
Paronychia with distal and lateral subungual onychomycosis	8 (18.6%)	*C. parapsilosis* (3; 37.5%) *C. orthopsilosis* (3; 37.5%) *C. metapsilosis* (2; 25%)
Total dystrophic onychomycosis	25 (58.1%)	*C. parapsilosis* (15; 60%) *C. orthopsilosis* (8; 32%) *C. metapsilosis* (2; 18%)

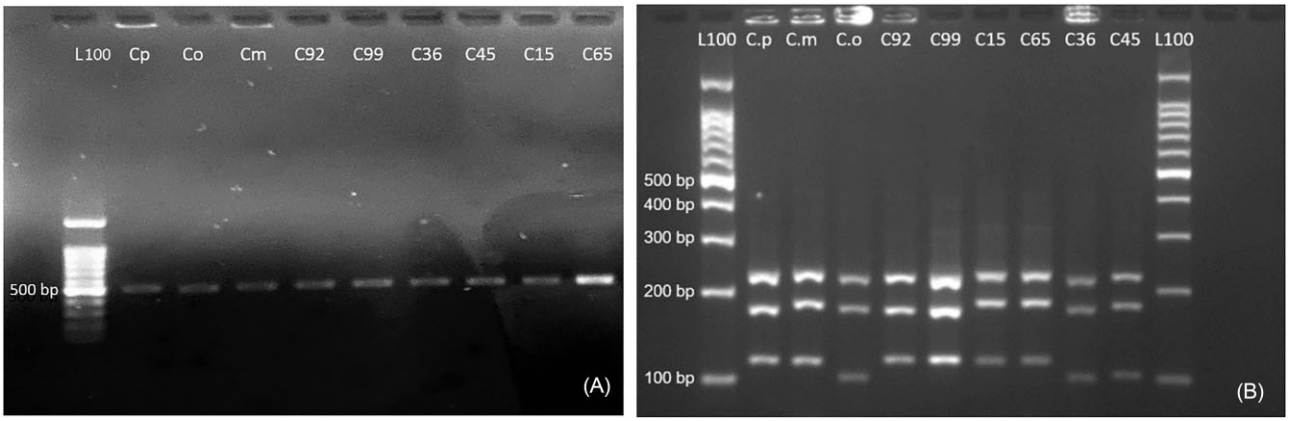

Figure 1. Gel electrophoresis. (A) PCR product of *Candida parapsilosis* complex with ladder 100 bp; (B) PCR-RFLP products of *Candida parapsilosis* complex with ladder 100 bp; Cp: *C. parapsilosis* ATCC 22019, Co: *C. orthopsilosis* ATCC 96141, and Cm: *C. metapsilosis* ATCC 96143; C92, C99, C36, C45, C15, and C65: isolates from this study.

Antifungal susceptibility testing results

In this study, all 43 isolates were susceptible to itraconazole, caspofungin, anidulafungin, and micafungin. Resistance rates within the *Candida parapsilosis* complex were 25.6% ($n = 11/43$) for fluconazole, 23.3% ($n = 10/43$) for voriconazole, and 9.3% ($n = 4/43$) for posaconazole. Low resistance rates were observed for amphotericin B (4.7%, $n = 2/43$) and 5-flucytosine (2.3%, $n = 1/43$).

Antifungal susceptibility testing revealed different resistance profiles among the three species of the *Candida parapsilosis* complex (Table 3). All *C. parapsilosis sensu stricto* isolates were susceptible to the antifungal agents tested, with the exception of 4.8% ($n = 1/21$) showing a susceptible dose-dependent (SDD) response to fluconazole (4 μg/ml), and a further 4.8% ($n = 1/21$) showing resistance to amphotericin B. No echinocandin resistance (caspofungin, anidulafungin, micafungin) was observed in this group. However, significantly higher geometric mean MIC values for echinocandins were recorded compared to the other two species ($P < .01$). In contrast, *C. orthopsilosis* demonstrated notable resistance to azoles, with 52.9% ($n = 9/17$) of isolates resistant to fluconazole and voriconazole, and 23.5% ($n = 4/17$) resistant

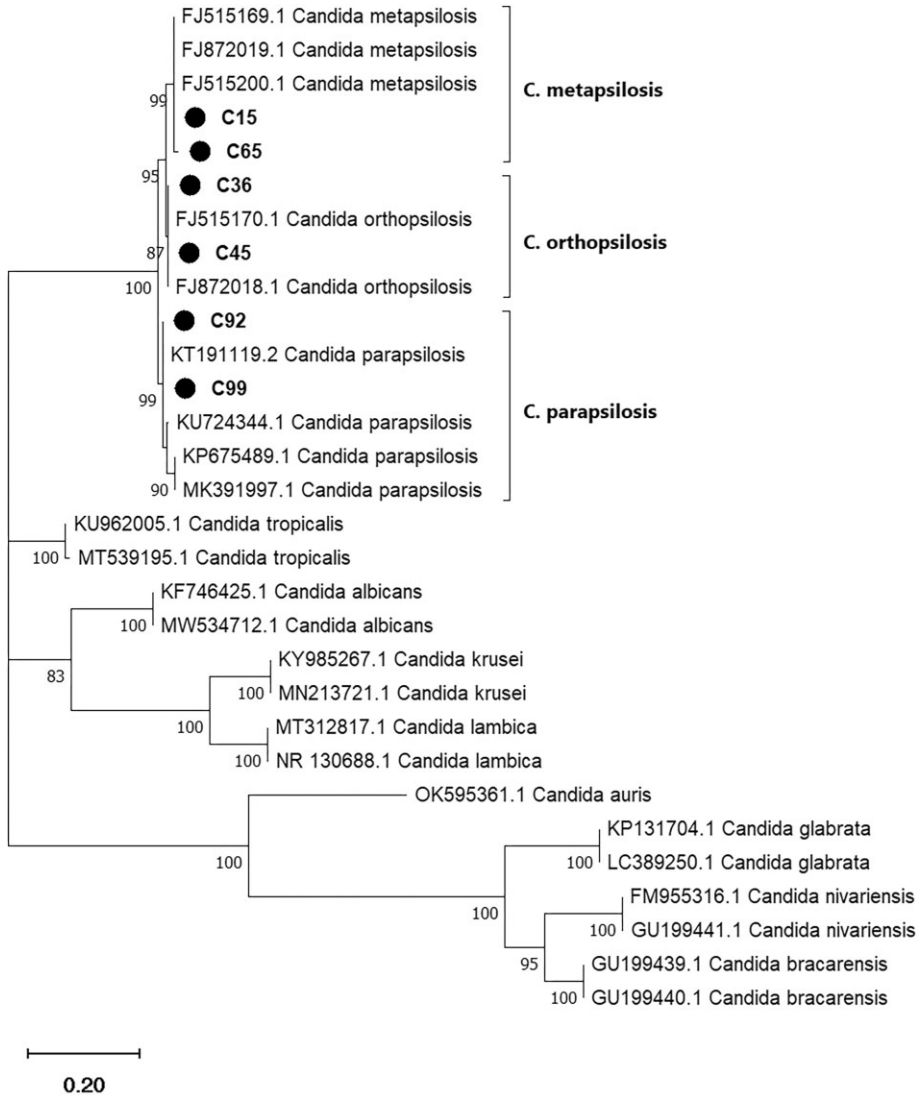

Figure 2. Phylogenetic tree of *Candida* species based on the ITS1-2 region, constructed using the Maximum Likelihood method with the Tamura 3-parameter model. Bootstrap values > 80 are shown. The six isolates from this study are indicated with filled circles.

Table 2. GenBank accession number of *Candida parapsilosis* complex selected isolate.

No.	Isolate ID	PCR-RFLP identification	GenBank accession number
1	C15	*C. metapsilosis*	**PV565575**
2	C36	*C. orthopsilosis*	**PV565576**
3	C45	*C. orthopsilosis*	**PV565577**
4	C65	*C. metapsilosis*	**PV565574**
5	C92	*C. parapsilosis sensu stricto*	**PV565579**
6	C99	*C. parapsilosis sensu stricto*	**PV565578**

to posaconazole. All fluconazole-resistant isolates also exhibited cross-resistance to voriconazole, and 23.5% ($n = 4/17$) showed co-resistance to all three azoles. In addition, 5.9% ($n = 1/17$) of *C. orthopsilosis* isolates were resistant to amphotericin B. Among *C. metapsilosis* isolates, one (20%) exhibited cross-resistance to fluconazole and voriconazole, while another was resistant to 5-flucytosine (MIC = 1 μg/ml). In addition, two isolates (40%) were classified as non-susceptible to caspofungin, each with an MIC value of 0.5 μg/ml.

Virulence enzymes activity

Protease activity was detected in 95.2% of *C. parapsilosis sensu stricto* isolates ($n = 20/21$) and 82.4% of *C. orthopsilosis* isolates ($n = 14/17$), whereas only 40% of *C. metapsilosis* isolates were protease positive ($n = 2/5$). A statistically significant difference in protease activity was observed between *C. parapsilosis sensu stricto* and *C. metapsilosis* ($P < .01$). Lipase activity was observed in 33.3% of *C. parapsilosis sensu stricto*, 47.1% of *C. orthopsilosis*, and 20% of *C. metapsilosis* isolates. Phospholipase activity was detected in 47.6% of

Table 3. Antifungal susceptibility testing profile of *Candida parapsilosis* complex.

Antifungal Agents	MIC (μg/ml)											
	C. parapsilosis sensu stricto ($n = 21$)				*C. orthopsilosis* ($n = 17$)				*C. metapsilosis* ($n = 5$)			
	Range	GM MIC	MIC_{50} -MIC_{90}	R (%)	Range	GM MIC ± SD	MIC_{50} - MIC_{90}	R (%)	Range	GM MIC	MIC_{50}	R (%)
Fluconazole	0.5–4	1.22 ± 0.8	1 - 2	4.8	1–256	11.1 ± 79	16 - 256	52.9	2–8	4 ± 2.18	4	20
Itraconazole	0.03–0.25	0.081 ± 0.077	0.06 - 0.25	0	0.12–0.5	0.28 ± 0.16	0.25 - 0.5	0	0.12–0.25	0.14 ± 0.05	0.12	0
Voriconazole	0.015–0.06	0.025 ± 0.023	0.03 - 0.06	0	0.03–8	0.35 ± 2.2	0.5 - 4.8	52.9	0.03–0.12	0.03 ± 0.02	0.06	20
Posaconazole	0.03–0.12	0.064 ± 0.026	0.06 - 0.12	0	0.06–0.5	0.19 ± 0.16	0.25 - 0.5	23.5	0.03–0.12	0.07 ± 0.02	0.06	0
Caspofungin*	0.5–2	0.97 ± 0.27	1 - 1	0	0.25–1	0.39 ± 0.28	0.5 - 1	0	0.12–0.25	0.22 ± 0.05	0.25	0
	0.5–2	1.39 ± 0.57	2 - 2	0	0.25–1	0.48 ± 0.27	0.5 - 1	0	0.25–0.5	0.38 ± 0.12	0.5	0
Anidulafungin*												
Micafungin*	0.5–2	1.44 ± 0.53	2- 2	0	0.25-0.6	0.39 ± 0.19	0.5 - 0.5	0	0.25–0.5	0.38 ± 0.12	0.5	0
Amphotericin B	0.25–2	0.59 ± 0.38	0.5 -1	4.8	0.12–4	0.42 ± 0.88	0.5 - 2.4	5.9	0.5–1	0.71 ± 0.25	0.75	0
5-flucytocine	<0.06–0.25	0.14 ± 0.11	0.12 - 0.25	0	<0.06–0.12	0.07 ± 0.03	<0.06 - 0.12	0	0.06–1	0.28 ± 0.37	0.5	20

MIC: Minimum Inhibitory Concentration; GM: Geometric Mean; SD: Standard deviation; MIC_{50}: MIC in which 50% of isolates were inhibited; MIC_{90}: MIC in which 90% of isolates were inhibited; R: Resistant; *: $P < .01$; differences in geometric mean MIC values for *C. parapsilosis sensu stricto* compared to other species were determined using one-way ANOVA on log-transformed data.

C. parapsilosis sensu stricto, 35.3% of *C. orthopsilosis*, and 40% of *C. metapsilosis* isolates. No significant differences in lipase and phospholipase production were found among the three species ($P > .05$). The enzymatic activity profiles of each species are summarized in Table 4.

Discussions

In this study, *Candida parapsilosis sensu stricto* was the most frequently identified species within the *C. parapsilosis* complex, followed by *C. orthopsilosis* and *C. metapsilosis*. This distribution is consistent with previous reports, where *C. parapsilosis sensu stricto* predominates in both candidemia and other clinical infections.[17,38,34,39] The relative distribution of *C. orthopsilosis* and *C. metapsilosis* varies between geographical regions and study populations. A higher prevalence of *C. metapsilosis* has been reported in Spain, Tunisia, and Japan,[22,34,21] whereas an equal distribution of both species was observed in Taiwan.[40] In contrast, *C. orthopsilosis* was more common in Iran, representing 5.3% of isolates within the *C. parapsilosis* complex, compared to 0.17% for *C. metapsilosis*.[39] Data on the distribution of subspecies within the *C. parapsilosis* complex in onychomycosis remain limited. In a study from Hue City, Vietnam, *C. parapsilosis sensu stricto* was the most common isolate (71.4%), followed by *C. orthopsilosis* and *C. metapsilosis* (both 14.3%).[41] In Brazil, an analysis of isolates from candidemia and onychomycosis showed *C. parapsilosis sensu stricto* in 89.7% of cases, with *C. orthopsilosis* and *C. metapsilosis* accounting for 5.7% and 4.6%, respectively.[42] Similarly, a study by Pakshir et al. identified *C. parapsilosis sensu stricto* in 91.5% and *C. orthopsilosis* in 8.5% of isolates from nail and oral lesions, with no *C. metapsilosis* detected.[43]

Candida onychomycosis presents with a wide range of clinical patterns, including paronychia with trachyonychia, paronychia with distal and lateral subungual onychomycosis, distal and lateral subungual onychomycosis, total dystrophic onychomycosis (TDO), and erosion of distal and lateral nail plate.[44] In this study, TDO was the most common clinical presentation, observed in 58.1% of cases, predominantly affecting fingernails (97.3%). Consistent with previous findings, infections caused by the *C. parapsilosis* complex were more common in females.[7,45] This complex has also been isolated from various environmental sources, including plants, soil, seawater, insects, and groundwater, as well as human skin.[14] The increasing incidence of *C. parapsilosis* complex in onychomycosis may be attributed to both endogenous sources and exogenous risk factors, such as frequent exposure of the hands to water. Notably, 94% of those affected in this study worked in humid environments. Furthermore, the ability of these species to thrive in glucose-rich media may help to explain the association of *Candida* onychomycosis with occupations involving frequent hand immersion or domestic work.[15,46]

Azole resistance in the *Candida parapsilosis* complex has been increasingly reported, with fluconazole resistance increasing from 11.6% before 2016 to 36.7% between 2016 and 2022.[18,47] In Asia, resistance remains comparatively lower, with an estimated fluconazole resistance rate of 6%.[47] In this study, 23.3% of isolates were resistant to fluconazole and voriconazole, and 9.3% were resistant to posaconazole. Previous studies in Vietnam (2012–2016) reported no resistance among *C. parapsilosis* complex isolates from nail

Table 4. Enzymatic activities characteristics of *Candida parapsilosis* complex isolates.

Enzyme index		*Candida parapsilosis* complex *n* (%)		
		C. parapsilosis sensu stricto (*n* = 21)	*C. orthopsilosis* (*n* = 17)	*C. metapsilosis* (*n* = 5)
Protease	No activity	1 (4.8%)	3 (17.6%)	3 (60%)
	Very low activity	0	5 (29.4%)	0
	Low activity	0	1 (5.9%)	0
	High activity	0	0	2 (40%)
	Very high activity	20 (95.2%)	8 (47,1%)	0
Lipase	No activity	14 (66.7%)	9 (52.9%)	4 (80%)
	Very low activity	0	0	0
	Low activity	0	6 (35.3%)	0
	High activity	4 (19%)	2 (11.8%)	1 (20%)
	Very high activity	3 (14.3%)	0	0
Phospholipase	No activity	11 (52.4%)	11 (64.7%)	2 (40%)
	Very low activity	0	0	0
	Low activity	2 (9.5%)	0	1 (20%)
	High activity	8 (38.1%)	6 (35.3%)	1 (20%)
	Very high activity	0	0	0

samples,[41] and blood samples,[48] while more recent data from burn patients (2017–2019) showed a fluconazole resistance rate of 14%.[49] Our findings support the global trend of increasing azole resistance in the *C. parapsilosis* complex.[47] Interestingly, while most reports do not distinguish between subspecies, our study identified higher resistance in *C. orthopsilosis* and *C. metapsilosis*, compared to *C. parapsilosis sensu stricto*, of which only one isolate classified as fluconazole SDD manner. However, due to the limited number of *C. metapsilosis* isolates in this study, the observed resistance rate of this species warrants further investigation with a larger number of isolates to determine whether this apparent resistance trend is representative. The resistance rates of *C. orthopsilosis* to fluconazole and voriconazole observed in this study are higher than those previously reported in Japan, Spain, and Italy.[21,22,50] Although voriconazole is less commonly used in Vietnam, resistance may be due to shared mechanisms with fluconazole and from prolonged or inappropriate use of fluconazole, potentially promoting cross-resistance.[14] Furthermore, all fluconazole-resistant *C. orthopsilosis* isolates in our study were also resistant to voriconazole, consistent with previous reports.[50,51] In contrast, itraconazole showed potent in vitro activity against all three species of the *C. parapsilosis* complex in our study, with low MIC values. All patients in our study received pulse itraconazole therapy at a dose of 200 mg twice daily for one week per month, administered during the first and fifth weeks of treatment. However, a limitation of our study is the absence of post-treatment follow-up to evaluate patient outcomes. Itraconazole resistance in *C. orthopsilosis* has been reported in Iran, with a prevalence of 12.5%, along with 3.12% of isolates classified as fluconazole SDD and 6.25% resistant to voriconazole.[39] Although *C. parapsilosis sensu stricto* showed high susceptibility to azoles in our study, resistance to fluconazole and voriconazole has been reported in several regions. In Brazil, resistance rates of *C. parapsilosis sensu stricto* to fluconazole and voriconazole reached ∼ 50%.[51] High fluconazole resistance has also been documented in Italy (22%), Turkey (26%), Mexico (54%), and South Africa (78%).[52–54] In addition, Spain reported resistance rates of 69% to fluconazole and 55% to voriconazole among *C. parapsilosis sensu stricto* isolates.[55]

Compared to other *Candida* species, the *C. parapsilosis* complex has naturally elevated MICs to echinocandins due to an intrinsic FKS1 polymorphism, although true resistance remains rare.[14] In our study, all isolates were susceptible to echinocandins. However, *C. parapsilosis sensu stricto* showed higher MICs for caspofungin, micafungin, and anidulafungin than *C. orthopsilosis* and *C. metapsilosis*, aligning with previous findings.[22,56] Echinocandin resistance has been reported primarily in *C. parapsilosis sensu stricto* from invasive candidiasis cases, while cryptic species typically remain susceptible.[40,42,57] Amphotericin B resistance in the *C. parapsilosis* complex is rare (∼1%), with typical MICs of 0.125–1 μg/ml.[14,18] In the present study, amphotericin B resistance was observed in one *C. parapsilosis sensu stricto* isolate (MIC = 2 μg/ml) and one *C. orthopsilosis* isolate (MIC = 4 μg/ml), which is consistent with previous studies.[42,47] All isolates had low MICs to 5-flucytosine, except for one *C. metapsilosis* isolate (MIC = 1 μg/ml), which was classified as resistant, in agreement with reports from China.[58,59]

Protease, lipase, and phospholipase are important virulence factors of *C. parapsilosis*, contributing to host cell adhesion, tissue damage, and inflammation.[60,61] The results of this study on protease production in the *C. parapsilosis* complex are consistent with previous reports,[36,43,62] indicating that *C. parapsilosis sensu stricto* and *C. orthopsilosis* produce higher levels of protease than *C. metapsilosis*. This increased protease activity probably enhances the ability of these species to degrade host proteins, thereby facilitating tissue invasion. No significant differences in phospholipase and lipase activity were observed among the three species, and none showed strong enzymatic activity overall. However, previous studies have reported considerable variability in hydrolytic enzyme production among *Candida* species.[34,63] Notably, lipase production in this species varies widely, with global prevalence ranging from 5% to 80%.[63–65] While *C. metapsilosis* is generally considered to have lower enzymatic activity than its sister species, Ge et al.[65] reported similar levels of phospholipase and protease activity in *C. parapsilosis sensu stricto* and *C. metapsilosis*, with ∼ 90% of isolates exhibiting phospholipase activity, 80% positive for protease, and lipase detected in 5%

and 18.2% of isolates, respectively. Although the number of fungal isolates collected in this study was limited, our results suggest that differences in protease and lipase activity among the subspecies may contribute to variations in nail pathogenicity and colonization. This finding may also explain why *C. parapsilosis* sensu stricto was the most frequently identified subspecies associated with TDO (Table 1). Although *C. parapsilosis* is generally considered to be less virulent than *C. albicans*, its pathogenic potential may increase under certain conditions, such as high glucose environments or hyperalimentation.[15,46]

This study highlights distinct antifungal resistance patterns and enzymatic profiles among *C. parapsilosis* complex subspecies influenced by geographic and clinical factors. Based on our findings, itraconazole is recommended as a first-line treatment for onychomycosis in Vietnam, especially when species identification is not possible. Variability in hydrolytic enzyme activity may also contribute to pathogenicity. Further research is needed to monitor resistance trends and to better understand the virulence of this species complex.

Acknowledgement

We would like to thank all the patients who agreed to participate in this study. We also express our sincere appreciation for the support provided by Hue University of Medicine and Pharmacy Hospital, Hue City Hospital of Dermatology and Venereology, Hue University of Medicine and Pharmacy, and Hue University.

Author contributions

Thi Minh Chau Ngo (Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing), Dong Duong Ton That (Conceptualization, Investigation, Methodology), Phuong Anh Ton Nu (Conceptualization, Investigation, Methodology), Cao Le Chi (Formal Analysis, Investigation, Software, Writing – original draft, Writing – review & editing), Giang Tran Thi (Formal Analysis, Investigation, Software), Thi Bich Thao Do (Investigation), Thi Ngoc Thuy Ha (Investigation), Tiep Vo Minh (Investigation), Phuoc Vinh Nguyen (Investigation), Ba Hoang Anh Mai (Investigation), My Nguyen Thi Tra (Investigation), Dac Hanh Nguyen (Investigation), Thanh Huy Nguyen (Investigation).

Funding

This research was funded by Hue University (Grant number: DHH-2024-04-221).

Declaration of interest

The authors have no relevant financial or non-financial interests to disclose..

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Received: May 6, 2025. **Revised:** June 27, 2025. **Accepted:** July 18, 2025