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Molecular characterization of Pathogenic Genital *Candida* spp. Isolated from Women Attending Antenatal Clinics in Southern Highland Regions, Tanzania

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Abstract

Fungal vaginal infections, especially those caused by *Candida* species, are common in tropical regions including sub-Saharan Africa. This study aimed to isolate and genetically characterize *Candida* species from symptomatic and asymptomatic women attending antenatal clinics in the Southern Highlands of Tanzania between 31st January 2020 and 30th June 2020. A total of 28 *Candida* isolates were obtained from 280 high vaginal swab samples and identified using chromogenic agar, API test strips, and PCR of the ITS1-5.8S-ITS2 region. Sequencing and phylogenetic analysis revealed the predominant species were *C. albicans* (41%), *C. glabrata* (18.3%), *C. tropicalis* (7.1%), *C. parapsilosis* (1.8%), and *C. krusei* (31%). Comparative phylogenetic analysis of the ITS sequences showed intraspecific genetic diversity within *C. albicans* and *C. glabrata* isolates. This study provides insight into the diversity of pathogenic *Candida* species colonising women in Southern Tanzania, with *C. albicans* being most prevalent. Molecular methods enabled precise identification and revealed cryptic genetic variability within medically important *Candida* species.

Keywords: *Candida,* antenatal, vagina, infections and virulence

Introduction

The genus *Candida,* especially *Candida albican*s, is known to be the normal body flora in the buccal cavity and women's genitalia. More than 90% of invasive infections caused by *Candida* species have been linked merely to a few spp.; *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* among all other spp. (Papon et al. 2013), where 70% out of which are associated with *C. albicans* (Almirante et al. 2006) being habitant of various locations of the human body like the oral cavity, digestive tract, vagina and skin, species of *Candida* constitute normal flora microbiome's components with opportunistic pathogenicity mostly occurring in cases of immune-compromised states of individuals health. Because of host debilitation or change in the local environment that promotes *Candida* growth, opportunistic infections by such normal flora become viable (Gow et al. 2011) and therefore resulting in symptomatic candidiasis like mucosal and cutaneous candidiasis as well as systemic candidiasis. Clinical incidences of candidiasis have been increasing due to various factors which lead to compromised immunity, such as HIV/AIDS pandemic, increased use of antibiotics, organ transplantation, and the use of invasive devices (Rokas 2022). The transit from a harmless commensal to disease-causing pathogens and vice versa by *Candida*

is based on the compromised immune system of the host relative to *Candida* virulence (Yang 2003)

The virulence factors that contribute to the transformation from harmless commensals to pathogenic forms under the conditions of a dysfunctional host defence system include adhesion proteins, germ tube and mycelia formation, as well as secreted proteolytic and hydrolytic enzymes. Indicators of *Candida* virulence have therefore been their ability to adhere to the host tissue and secretion of those virulent extracellular enzymes, which promotes both dimorphic transition (Colombo et al. 2003) and penetration of *Candida* to host cells. Adherence to the host tissue, which paves to colonisation and subsequent infection, is considered an early stage of infection by *Candida* (Haynes, 2001). Secreted virulent enzymes, mainly proteinases and phospholipases, are believed to play an important role in both *Candida* overgrowth as they facilitate adherence as well as their penetration into host tissue (Tsang et al. 2007). Some of these enzymes are capable of hydrolysing even the ester bonds in glycerides (Naglik et al. 2003). Recently, there have been numerous reports on differences in virulence among the genotypes distinct *Candida spp.* (Naglik *et* al. 2003), which correlates with expressions of virulence factors. The study proposed for this study aimed at characterising *Candida spp*. amid many other microorganisms in the genital microbiomes of symptomatic and asymptomatic women who attended antenatal clinics in hospitals in the Southern Highland Regions of Tanzania between January 2020 and June 2020.

Materials and Methods

Sample collection

A total of 280 high vaginal swab (HVSs) samples were collected from both symptomatic (n=200) and asymptomatic (n=80) women who attended antenatal clinics in government and private hospitals in the Southern Highland Regions of Tanzania between January 2020 and June 2020. Ethical clearance with REG. no. 2016-06-00248 was obtained through the Ministry of Health, Community Development, Gender Elderly and Children. All participated women, aged 15 to 45 years, granted their consent to provide samples for this study. For women below 15 years of age, her mother's consent was obtained to allow her daughter to participate in this study. High vaginal swab (HVS) samples were collected aseptically by a qualified physician using sterile cotton swabs. All collected samples were transported aseptically to Mbeya Zonal Referral Hospital (MZRH) at UDSM - MCHAS laboratory (Department of Microbiology and Immunology). Samples were later analysed microscopically as wet mount to know the presence of yeast cells. The samples were then stored at 4ºC for further identification.

Study design

This was a descriptive-analytical study, where participants consented to participate in this study

Extraction of DNA

Yeast cells were harvested from an aliquot of broth culture (malt extract broth 1 ml) by centrifugation (14,000 rpm, 4 ºC 5 min). Cells pellets were suspended in DNA extraction buffer [2% Triton X-100, 1% SDS, NaCl (100 mM), Tris (pH 8, 10 mM), EDTA (1 mM)] (75 µl,) and grinded to homogeneity. The obtained mixture was then incubated in a water bath at 60 ºC for 20 minutes. Potassium acetate (7.5 mM, 40 µl) was added before the mixture was incubated on ice for 20 minutes, followed by centrifugation (14,000 rpm, 4 ºC, 4 min). The obtained supernatant was mixed with an equal volume (400 µl) of chloroform, vortexed (10 sec), and centrifuged (14,000 rpm, 4 ºC, 4 min). The upper phase was mixed with pre-chilled isopropanol (700 µl) and incubated at -20 ºC for 15 minutes. DNA was collected as pellets by centrifugation (16,000 rpm, 4 °C, 10 min) and left to dry in the air for 10 minutes before being washed (75 % cold ethanol, 100 µl) re-centrifuged (14,000 rpm, 4 ºC,5 min) and left to dry in the air again for 10 minutes. Dried DNA was mixed with TE buffer (Tris-HCl pH 7.0 – 8.0, 10 Mm) and EDTA (pH 8.0, 1 mM)] (30 µl) and stored at -20 ºC. DNA quantitation was

performed by spectrophotometric measurements of optical density (OD) at 260 nm according to standard methods (Sambrook et al. 1989).

Amplification of the ITS 1-5.8-ITS 2 region of the rDNA

Primers for amplification of DNA

PCR amplification of ITS 1, 5.8S and ITS 2 region was done by using ITS-1 and ITS-4 oligonucleotides primer sequences (Table 1). PCR was carried out in a total volume of 25µl reaction mixture, where the mixture consisted of 50 mM KCl, 10 Mm Tris HCl, (pH 9), 1.5 mM MgCl2, 200 µM of each dinucleotide triphosphate (dATP, dCTP, dTTP, dGTP), 2.5 units of Taq DNA polymerase, 0.8 µM of ITS 1 and 4 primers and 1µl of genomic DNA of filamentous fungi. A negative control, which included all requirements for amplification except the template, and a positive control using a known culture of yeast were used. Veriti-Applied Bio-systems PCR (9902) machine was used for amplification of the fungal DNA with the following profile: initial denaturation step at 94 ºC for 5 minutes was followed by 30 cycles of denaturation at 94 ºC for 30 seconds, annealing at 56 ºC for 45 seconds and extension at 72 ºC for 1 minute, with a final extension step of 72 ºC for 7 minutes. Amplified products were visualised using 1.5% agarose gel electrophoresis in TBE buffer (0.09 M Tris, 0.09 M Boric acid and 2 mM EDTA, pH 8.3), stained with 5-mg/ml Ethidium bromide. UV transilluminator was then used to visualise the band patterns.

Sequencing of ITS 1-5.8-ITS 2 region of the ribosomal DNA

After amplification, the PCR products were labelled packed in Eppendorf tubes and sent to a commercial facility at Inqaba Biotechnical Industries, South Africa, for sequencing. The procedure was done using the Big Dye chain termination method using an ABI 3730 Genetic analyser. Each sample was sequenced using either a forward primer or reverse primer.

Processing sequence of PCR products and phylogenetic analysis

Sanger sequences of PCR products were received as raw data, where quality control was done by using BioEdit (v.7.2). To gain insight into the possible taxonomic group position of each sequence, nucleotide basic local alignment search tool (BLASTn) was used to match the DNA sequence of PCR products with those from redundant database of nucleotides from the National Centre for Biotechnology (NCBI). All multiple alignments were done using MAFFT (v.7.0) and refined with AliView (v.3.0) (Larsson 2014). Thereafter, 15 top hits were downloaded and chosen based on nucleotide identity (90-100%) as representative species or strains for phylogenetic analysis. Phylogenetic analysis was accomplished using Molecular Evolutionary Genetics Analysis X (MEGA-X) (Kumar et al. 2018) based on the Maximum Likelihood method. Substitution models were calculated using algorithms within the MEGA-X programme and are shown in their respective phylogenetic results parts.

Results

A total of 28 *Candida spp.* from 280 high vaginal swab samples were cultured and isolated in Saboraud dextrose agar, which was previously identified by CHROMagar and API 20AUX systems. Moreover, all 28 were confirmed as true positive by PCR using OPAX-20 primers. On the basis of ITS and ITS 1, 5.8S and ITS 2 sequences, the isolates were most likely linked to the genera *Candida* (Figures 1). Among the isolates studied, all 28 confirmed *Candida* spp. were from both symptomatic and asymptomatic patients.

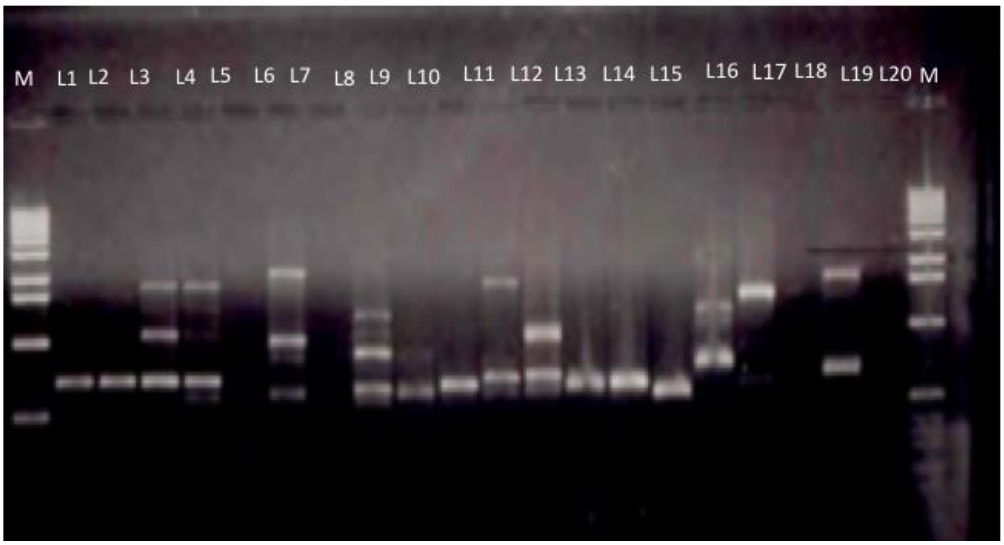

Figure 1. 1kb ladder size marker (lane1 and 22), *C. albicans* ATCC 32354 (lane2), *C. albicans* isolated from women (lanes 3-5, 8, 9, 13, 19 and 20), *C. famata* (lane 6), *C. glabrata* (lane 12, 14, 15 and 18), *C. krusei* (lane 10 and 21), *C. tropicalis* (lane 7 and 11), *C. parapsilosis* (lane 17) and *C. lusitaniae/ C. guilliermondii* (lane 16).

Phylogenetic and taxonomic insights into each isolate

To predict the possible relationship among 28 isolates from symptomatic women with *Candida* positive, a comparative phylogenetic analysis (Maximum Likelihood method) was done. As shown in Figure 2, the strains assume seven clusters. Cluster 1 contains C1, C2, C3, C6, C10, C11, C13, C14, C15, C18 and C19. Cluster 2 contains C21, while cluster 3 covers C7, C8, C9, C12 and C28. Cluster 4 is formed by C16 as a distinct single, while cluster 5 is composed of C4 and C22. Cluster 6 is composed of C20 while cluster 7 is composed of C5, C17, 23 C24, C25, C26 and C27. These clusters represent putative evolutionary clades, underlying genetic diversity within the fungal isolates. As clearly substantiated (Merseguel et al. 2015), ITS sequence is critically important in differentiating medically important fungal species of the genus *Candida* and the differences are correlated with strain diversity, virulence and possible epidemiological diversity among various *Candida* species.

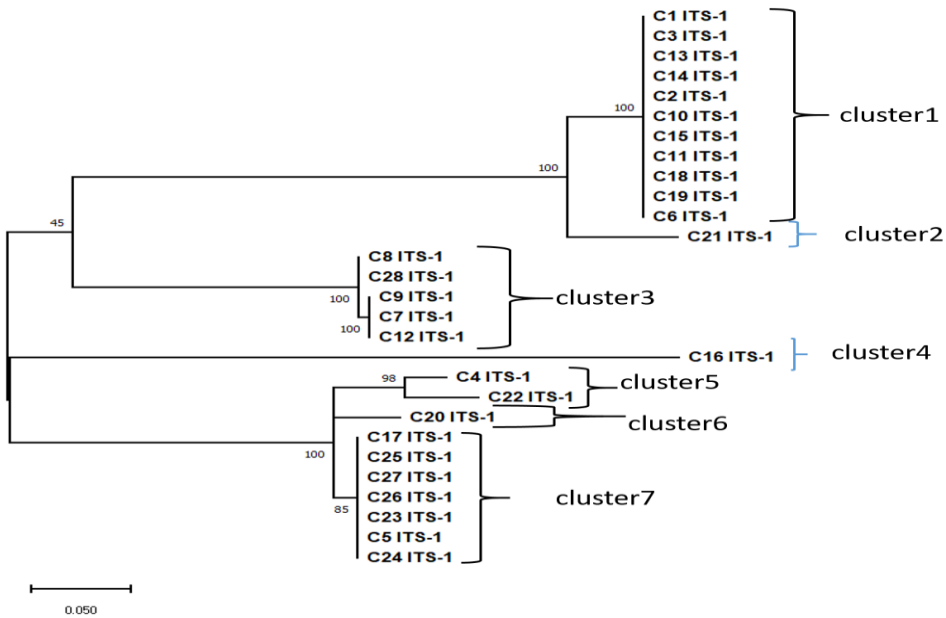

Figure 2: Comparative phylogeny of all 28-sample nucleotides.

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura (2001) 3-parameter model [14]. The tree with the highest log likelihood is shown. The *bootstrap* values, generated from 1000 iterations, are indicated beneath the tree branches. The scale bar represents residue substitution per site. The putative clades were labeled by the cluster number against each cluster**.**

Given the seven phylogenetic distinct clusters, it follows that each isolate belongs to one of the species, i.e., *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* (also *Pichia kudriavzevii*). To rule out any ambiguity, nucleotides sequence alignments by BLAST was done for each ITS sequences. The proportion was contributed by each were *Candida krusei* (10 samples), followed by *Candida albicans* (10 samples*), C. glabrata* (7 samples) and the last was *Rhodotorula mucilaginosa* (1 sample).

Sequences that matched with *Candida albicans* (Table 1) were selected for alignment with at least the top 15 matched strains. From phylogenetic analysis, two clusters A, B, were distinguishable (Figure 3).

Table 1. BLAST hits and percentage identities for cluster 1, identified as *Candida albicans* sequence matches

Sample ID	Size (bp)	Top hit species	Hit accession	Hit Size	Percentage ID
C4_ITS-1	496	*C.albicans* strain H251B	KP674932.1	512	85.71
C5_ITS-1	806	*C. albicans* strain M235A	KP675409.1	485	99.80
C17_ITS-1	586	*C. albicans* isolate BAL	MT539315.1	515	99.80
C20_ITS-1	470	*C. albicans* strain H106A	KP674800.1	510	90.26
C22_ITS-1	483	*C.albicans* strain	KP674540.1	509	86.38

		d1b			
C23_ITS-1	507	*C.albicans* strain B144A	KP674496.1	501	99.02
C24_ITS-1	558	*C.albicans* 16A5147	LC612890.1	863	98.42
C25_ITS-1	891	*C. albicans* isolate C26	MK153067.1	779	94.09
C26_ITS-1	863	*C. albicans* isolate ZB088	JN606311.1	546	99.61
C27_ITS-1	505	*C. albicans* strain M219B	KP675389.1	511	100

The phylogenetic pattern of *Candida albicans* in Figures 3 shows that the isolates fall under two major intraspecific clusters, which branch into four sub-clusters. Although the isolates conform to a single clade, the sprouting into such sub-clades is highly suggestive of intraspecific diversity. On the other hand, 10 samples matched with *Pichia* and were all correlated with *P. kudriavzevii* strains, 11 with a percentage identity of above 99% and one with 83%. This is equivalent to 40% of all the samples.

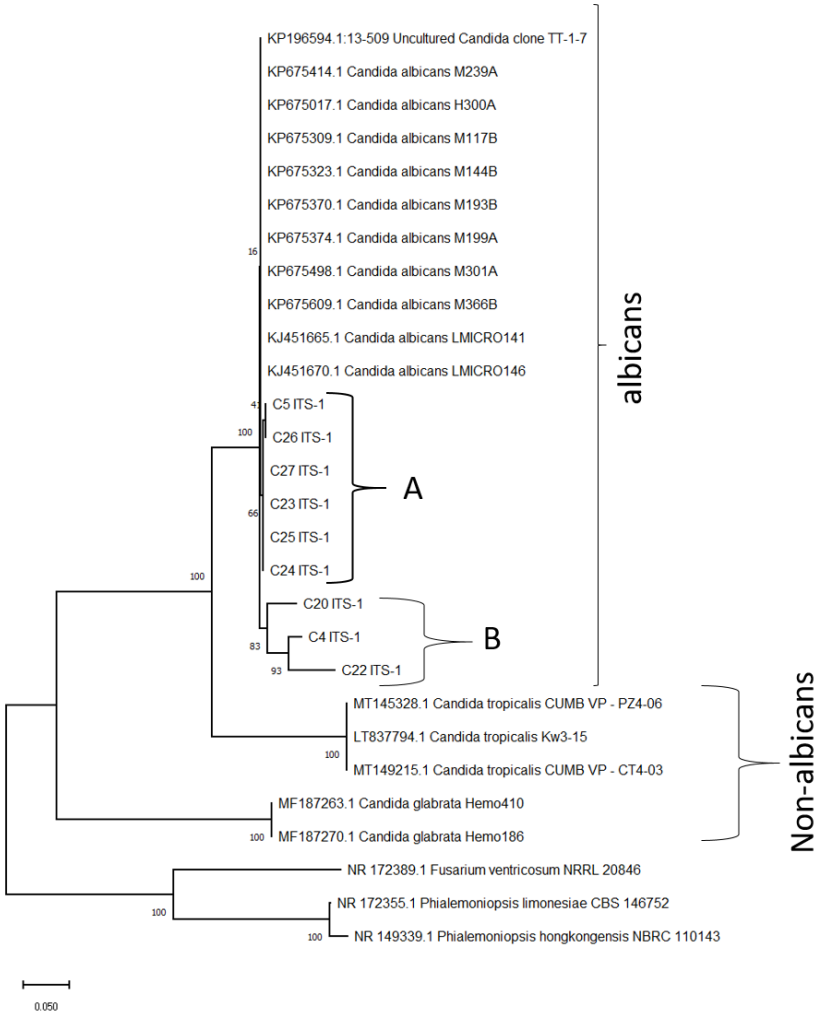

Figure 3. Phylogenetic position of each strain within the *Candida albicans* group.

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-*parameter* model. The cluster to which each strain belongs is represented by a letter A or B against each putative cluster. Candida tropicalis and Candida glabrata species were randomly included as comparative species, whereas Fusarium and Phialemoniopsis spp are presented as out-groups

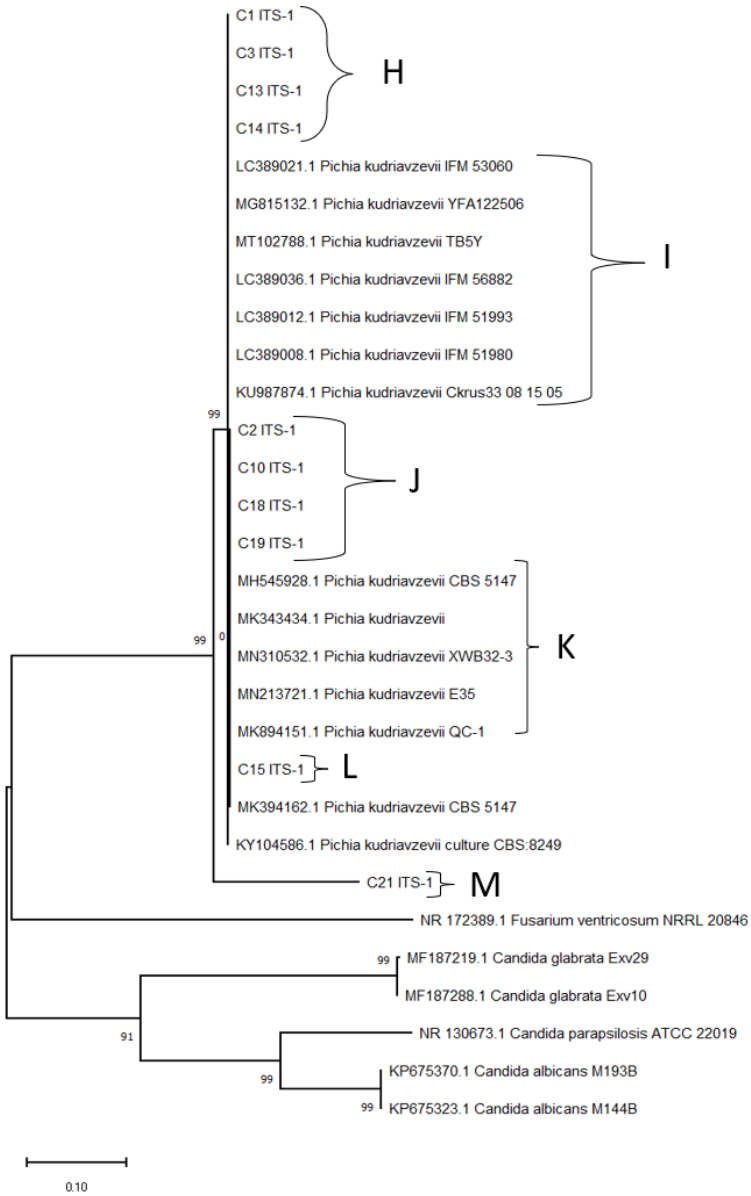

Figure 4: Phylogenetic position of each of the strains within the Pichia clade.

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter mode. Clusters resulting from ML analysis are shown by letters H-M to indicate the position of each isolate. *Candida* spp. were included for comparison while *Fusarium ventricosum* NRRL 20846 is presented as an out-group.

Most sequences (Table 2) exhibit high identity (99-100%) with reference strains from NCBI GenBank, suggesting a close similarity within the clade of *Pichia kudriavzevii.* Phylogenetically, the isolates present at least two features of intraspecific diversity, with H, J and L being close but potentially diverse clusters. Cluster M, comprised of the isolate C21 exhibit by far less similarity (i.e. 83.03%). Combining this

BLAST identity with phylogenetics, the isolate C21 might belong to a different species.

Table 2. BLAST hits and percentage identity matches for *Pichia kudriavzevii* (*Candida kruzei*) sequences

Sample ID	Size (bp)	Top hit species	Hit accession	Hit size (bp)	Percentage ID
C1_ITS-1	476	*P.kudriavzevii* strain TB5Y	MT102788.1	539	99.58
C2_ITS-1	569	*P.kudriavzevii* isolate JGF26	MZ089543.1	485	99.58
C3_ITS-1	481	*P.kudriavzevii* strain TB5Y		539	99.79
C6_ITS-1	481	*P.kudriavzevii* strain TB5Y		539	99.79
C10_ITS-1	469	*P.kudriavzevii* strain CBS 5147	MH545928.1	2746	99.9
C11_ITS-1	479	*P.kudriavzevii strain CBS 5147*	MH545928.1	2746	99.79
C13_ITS-1	476	*P.kudriavzevii* strain TB5Y	MT102788.1	539	99.79
C14_ITS-1	471	*P.kudriavzevii* strain TB5Y	MT102788.1	539	99.79
C15_ITS-1	467	*P.kudriavzevii* strain CBS 5147	MH545928.1	2746	99.79
C18_ITS-1	467	*P.kudriavzevii* strain CBS 5147	MH545928.1	2746	100
C19_ITS-1	471	*P.kudriavzevii* strain CBS 5147	MH545928.1	2746	100
C21_ITS-1	468	*P.kudriavzevii* isolate C-335	MG015974.1	528	83.03

Discussion

Identification of *Candida* species that cause candidiasis is highly desirable in microbiological practice, as it may help in clarifying the prevalence and incidence of species that affect the susceptible population. Several studies have demonstrated the occurrence of candidiasis due to *Candida* species, indicating heterogeneity among isolates from different geographical regions. In the reported study, the prevalent species were *C. albicans*, C. *glabrata*, *C. guilliermondii*, *C. kefyr*, *C. dubliniensis* (Figures 1 and 3) and thereby suggesting *C. albicans* is the main species that affect most women in the Southern Highland Regions of Tanzania. This is in agreement with the studies reported (Spinillo 1999, Pennisi and Antonelli 2009). However, it has been reported (Achkar and Fries 2010) that an increase in infections that are caused by non-*albicans Candida species* has been registered, although *C. albicans* is still the most isolated species in vaginal candidiasis clinical cases. Furthermore, the geographical, cultural and ethnic differences may also influence the isolation rate of yeast from candidiasis samples (Wei *et al.,* 2010). In this study finding, it appears that most isolates in the *Candida albicans* group are more closely related among themselves than to the reference sequences from the database. This could again be attributable to geographical factors associated with genetic diversity resulting from mechanisms of adaptation (Douglas and Konopka 2019). For extensive comparison, molecular studies integrating sequenced ITS species are missing in Tanzania. More genetic variability and characterization are needed to reinforce the

importance of candidiasis epidemiological studies by molecular methods (Bonfim-Mendonca et al. 2013).

The isolated strains investigated in this study presented a wide diversity of species such as *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis and C. kruzei* (Figures 1, 2, 3, 4 and Tables 1, 2,). In addition, *C. albicans* was found to be more prevalent in the infection group and *C. dubliniensis* (n = 1) isolates were observed only in the complicated infection group. The microbiota species found in colonised women are the same as reported in vulvovaginal candidiasis (VVC) (Mahmoudi et al. 2012).

The phylogenetic pattern of *Candida albicans* in Figures 2, 3 and 4 shows that the isolates fall under two major clusters, which branch into four sub-clusters. Although the isolates conform to a single clade, the sprouting into such sub-clades is highly suggestive of intraspecific diversity. Candidiasis is among the most common clinical conditions associated with *Candida* infection. In Tanzania, candidiasis has been implicated in vaginitis in pregnant women in Mwanza (Mushi et al. 2019). These findings concur with those from other works, pointing to *Candida* species as the major contributor to vaginitis among women under different conditions, including pregnancy (Mushi et al. 2019).

Pichia kudriavzevii was among the isolates. While *Pichia* species are ubiquitous in the environment, little is discussed about their potential risk of infection, and their clinical implication is elusive. With current scanty data, *Pichia* is emerging as among the fungal pathogens of the human population and has been implicated in paediatric infections and inflammatory conditions, and women infections such as vaginitis and peritonitis (Zhu et al. 2021). The epidemiology of *Pichia* infection is not as widely established as that of *Candida spp*. This raises a surge in interest in understanding the prevalence of *Pichia* infection and its association with changes in the microbiome, underlying the pathogenesis of various diseases such as vaginitis. In Tanzania, the most reported local and systemic infections include *Candida* spp. in candidiasis, *Pneumocystis jirovecii* pneumonia and meningitis by *Cryptococcus neoformans* (Faini et al. 2015). Clinical isolates of *Pichia kudriavzevii* (*C. krusei*) in Dar es Salaam, Tanzania were reported about a decade ago using morphological and PCR amplification diagnostic methods from vaginal swabs of asymptomatic pregnant women (Namkinga et al. 2005). The latter reported a prevalence of 10% of 3000 samples, with *C. albicans* being the most prevalent. In a recent biochemical diagnostic study (Mushi et al. 2019), the prevalence of *Pichia kudriavzevii* was found to be 1.5% out of 300 clinical samples of symptomatic pregnant women. This is a strong line of evidence for the potential infection of pregnant women by *Pichia kudriavzevii* and this could be associated with vaginitis. In this study, our findings clearly demonstrate a significant number of samples with *Pichia kudriavzevii*. From Figure 3.7, samples in clusters H, J, L and M are all members of the genus *Pichia,* all representing *Pichia kudriavzevii.* Using molecular techniques and phylogeny as robust for the detection and characterization of fungal species, our findings cement the evidence of *Pichia* infection among pregnant women populations. We therefore infer a significant proportion of *Pichia kudriavzevii* in the vulvovaginal tissues of pregnant women, which could be attributable to the pathogenesis of vaginitis and conditions associated with candidiasis in Tanzania. These findings call for more studies to diagnose and establish the possible link between *Pichia* infection and vaginitis, as well as the potential risk to pregnant women.

Non-*Candida* species

Although *Candida* is the most predominant fungal genus in the vagina, evidence supports the presence and prevalence of other genera, accounting for both local, invasive and disseminated infections such as *Pichia kudriavzevii*, formerly known as *Candida krusei* (Douglas and Konopka, 2019 *Cyryptococcus* spp Ghajari *et al.,* 2018) as well as *Rhodotorula spp*. (Wirth and Goldani, 2012) In this work, sequence analysis revealed the presence of other fungal species

of the genus *Rhodotorula* and *Pichia*. Although *Rhodotorula* was previously considered noninfectious, current evidence shows that the fungus can opportunistically colonise and infect tissues of susceptible immune-compromised patients such as those with chronic diabetes mellitus and HIV/AIDS [36,38]. BLAST analysis revealed that one sequence (sample ID C16_ITS-1) out of 28 samples matched with several *Rhodotorula* strains. The topmost were *Rhodotorula* sp. strain SM03UFAM (Accession MN268779), *Rhodotorula* sp. strain DAMB1 (MK968443.1), *Rhodotorula* sp. strain KSB1 (MH782232.1) and *R. mucilaginosa* strain IMUFRJ 52392 (MK2631851), with E-value of 0.0 and percentage identity of 100%. This is the first incidence of *Rhodotorula* species from clinical samples in Tanzania. Evidence from a recent experimental study from Brazil demonstrates the infective potential of the *Rhodotorula mucilaginosa*, possessing a variety of virulence factors such as the production of extracellular polymeric substances (EPS) including eDNA, eRNA, proteins and polysaccharides, which are a major component of biofilm, critical for colonisation and invasion of the epithelial tissue, as shown in chronic renal patients (Jarros et al. 2020). The same study revealed that *R. Mucilaginosa* is resistant to antifungal azoles but susceptible to polyenes. Thus, these findings are an alarm for the possible involvement of *R. mucilaginous* in infection of the vulvovaginal tissue, possibly via the formation of biofilms and may pose an antifungal resistance problem. Extensive molecular studies are thereby crucial to unravel the epidemiology, virulence and potential antifungal drug resistance of *Rhodotorula* in Tanzania.

Conclusion

Molecular techniques confirmed species diversity and revealed intraspecific variations among genital *Candida* spp. in pregnant women. Findings underscore the need for molecular surveillance in Tanzania's clinical diagnostics and antifungal stewardship.

Conflicts of interest

The authors declare no conflicts of interest.

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