

Hyphal wall protein 1 gene: A potential marker for the identification of different *Candida* species and phylogenetic analysis

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Abstract

Background and Purpose: Hyphal wall protein 1 (*HWPI*) is an important adhesin which usually is expressed on the germ tube and hyphal surface produced by different *Candida* species. The hyphal wall protein-coding gene (*HWPI*) was evaluated as a novel identification and phylogenetic marker in *Candida tropicalis*, *C. orthopsilosis*, *C. parapsilosis* and *C. glabrata*.

Materials and Methods: Initially, four specific primer pairs were designed, and the target was amplified and finally sequenced. A total of 77 *Candida* isolates from four different species were included in the study. Consensus sequences were used for the evaluation of phylogenetic tree using the CLC Genome Workbench, GENEIOUS, and MEGA softwares and the levels of nucleotide and amino acid polymorphism were assessed.

Results: According to the results, the specific amplified fragments of *HWPI* gene were useful for the differentiation of four species. Intra-species variation was observed only in *C. tropicalis* with two DNA types. The phylogenetic tree of *Candida* species based on the *HWPI* gene showed consistency in topology with those inferred from other gene sequences.

Conclusion: We found that *HWPI* gene was an excellent marker for the identification of non-*albicans* *Candida* species as well as the phylogenetic analysis of the most clinically significant *Candida* species.

Keywords: *Candida* species, *HWPI* gene, Identification, Phylogenetic analysis

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Introduction

A few *Candida* species are among the most pathogenic human fungi. Depending on the underlying host defect, *Candida* species cause a variety of infections, ranging from superficial mucocutaneous candidiasis to blood-stream infections [1-3]. The incidence of candidiasis has been notably increased over the recent decades due to the increasing in the number of immunocompromised patients. Among over 200 identified *Candida* species, *Candida albicans* remains the major fungal pathogen of human, followed by non-*Candida albicans* species, which has a remarkably growing prevalence [4-7].

This genus has been characterized with a set of virulence factors, such as reversible morphological transition between yeast, pseudohyphal, and hyphal, adhesion to biological substrates, transmigration by enzymatic and/or physical processes, and

modulation of host immune defense [8-10]. During the last decades, the molecular methods applied different genetic markers, such as ITS and D1/D2 regions in rDNA, for the identification of *Candida* species [11].

The hyphal wall protein (*HWPI*) is a main adhesin protein, commonly expressed on the germ tube and hyphal surface of *Candida* species as a substrate attach covalently to host cells transglutaminases and cross-links this genus to epithelial cells of mucosa [10, 12]. Moreover, in the *in vivo* model for biofilm formation, it is proposed that *HWPI* adhesin retains *Candida* in the biofilm [11].

The nucleotide sequences of *HWPI* gene were previously amplified only for *C. albicans*, *C. dubliniensis* and *C. africana* by Romeo and Criseo [12]. Nevertheless, there are no available data regarding the nucleotide or amino acid sequences of

this gene for other species. Furthermore, the efficacy of *HWP1* gene in the identification of non-*albicans* *Candida* species has not been reported. With this background in mind, we provided the sequence data, phylogenetic analysis, and polymerase chain reaction (PCR) discriminatory pattern for *Candida* isolates of four different species, including *C. parapsilosis*, *C. orthopsilosis*, *C. tropicalis*, and *C. glabrata*.

Materials and Methods

Fungal strains

For the purpose of the study, 70 clinical and 7 reference strains of *Candida* species isolates were used for the optimization of the PCR reaction. The reference strains, including *C. albicans* (CBS 2747), *C. dubliniensis* (CBS 8501), *C. tropicalis* (ATCC 750), *C. parapsilosis* (ATCC 90018), *C. orthopsilosis* (ATCC 96139), *C. africana* (IFRC 707), and *C. glabrata* (CBS 138) were obtained from the American Type Culture Collection, CBS-KNAW Fungal Biodiversity Centre, and Invasive Fungi Research Center.

We investigated 70 *Candida* isolates from a variety of specimens, including *C. albicans* (n=4), *C. africana* (n=4), *C. glabrata* (n=15), *C. parapsilosis* (n=7), *C. tropicalis* (n=15), *C. dubliniensis* (n=18), and *C. orthopsilosis* (n=7). These specimens were

obtained from the medical mycology laboratories in Tehran, Iran and Mazandaran University of Medical Sciences.

All samples were cultured on Sabouraud dextrose agar (S) and incubated at 35°C. Species identification of the clinical isolates was initially performed based on the conventional methods, including morphology on cornmeal agar and colony color on CHROMagar. The results were confirmed by performing the PCR-restriction fragment length polymorphism (RFLP) on the secondary alcohol dehydrogenase-encoding gene (SADH) and ITS1-5.8S-ITS2 rDNA region [13, 14].

Primer Design

Four primer pairs (Table 1) were specifically designed based on *HWP1* gene sequences of various *Candida* species. These *Candida* species including *C. parapsilosis*, *C. orthopsilosis*, *C. tropicalis*, and *C. dubliniensis* retrieved from the NCBI (<http://www.ncbi.nlm.nih.gov/pubmed/>). Primer designing was carried out using the AlleleID software, version 7.0 (Premier Biosoft International, Palo Alto, CA, USA).

The name, sequences, and length of these primers are displayed in Table 1. The PCR amplification of *HWP1* gene of other species, including *C. glabrata*, *C. africana*, and *C. albicans*,

Table 1. Primers used for the identification of *Candida* species

Species and primers used	Sequence	Fragment length	PCR program		Accession numbers
<i>C. glabrata</i> Forward Reverse	GCT ACC ACT TCA GAA TCA TCA TC GCA CCT TCA GTC GTA GAG ACC	250 bp	95°C	5'	KX758626-28
			94°C	30"	
			52	30"	
			72°C	1'	
			72°C	7'	
<i>C. parapsilosis</i> Forward Reverse	CGA GGT GAA TAT GAT GCT TGT A CCA ACA GAA TTG CTT AAT ACC ATA	840 bp	95°C	5'	KX758617/KX758618 KX758619/KX758620 KX758621/KX758622 KX758629
			95°C	30"	
			59.4	30"	
			72°C	55"	
			72°C	5'	
<i>C. orthopsilosis</i> Forward Reverse	ACC ACCACC TAG TTC TGA G TCA CIT GGA AGA TTG AGA ATA ACA	900 bp	95°C	5'	KX758615-16
			95°C	30"	
			61.9	30"	
			72°C	68"	
			72°C	5'	
<i>C. tropicalis</i> Forward Reverse	TAC TGT TAC TTC TTG CTC CAG GCT TGC CAT TGC TTA GTG	1236 bp	95°C	5'	KX898983-85
			94°C	60"	
			65.2°C	60"	
			72°C	110'	
			72°C	10'	
<i>C. dubliniensis</i> Forward Reverse	ACA GGA ATC TCC AAT AGT CAC AGA ACA GAC ACG GAT TCA G	1300 bp	95°C	5'	KX758623-25
			94°C	30"	
			60.1	30"	
			72°C	82"	
			72°C	7'	

was performed using the primers designed by Romeo and Criseo [12] as follows: forward, 5' -GCTACCACTTCAGAATCATCATC-3' and reverse, 5'- GCACCTTCAGTCGTAGAGACG-3').

DNA extraction and polymerase chain reaction amplification

DNA of the *Candida* strains was extracted from the fresh colonies using the previously described methods [15, 16]. The PCR reactions consisted of 12.5 µl Master Mix Red (Ampliqon, Copenhagen, Denmark), 1 µl template DNA, 1 µl of each forward and reverse primers, and enough water up to a final volume of 25 µl. The PCR programs are illustrated in Table 1.

Sequencing and Sequence Analysis

The PCR products of *HWP1* gene were purified using QIAquick Purification Kit (Qi-agen, Valencia, CA, USA) and subjected to ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). For all isolates, the forward and reverse primers were used for the sequencing of purified gene fragments by an automated DNA sequencer (ABI Prism™ 3730 Genetic Analyzer, Applied Biosystems). The sequences were assembled and edited with CLC Genome Workbench (version 7), Bioedit (version 7.2), and GENEIOUS softwares (<http://www.geneious.com>). The consensus sequences were annotated and deposited in the GenBank, and the accession numbers of the sequences were received.

Phylogenetic construction

The pairwise and multiple comparisons of sequences were performed to evaluate the levels of similarity and difference between nucleotide and amino acid sequences using GENEIOUS and MEGA softwares version 6. Phylogenetic evaluation used the neighbor joining method with 1,000 bootstrap simulations, conducted with the CLC Genome Workbench, GENEIOUS, and MEGA softwares. Bootstrap values greater than 70% were regarded significant. *Scheffersomyces stipitis* was used as the outgroup.

This research was approved by the Ethics Committee of the university with the ethical code of IR.MAZUMS.REC.94-1816.

Results

In this study, a newly simple PCR on *HWP1* gene was optimized and tested on 70 clinical and 7

reference strains of *Candida* species isolates. The *HWP1* gene was successfully amplified for all strains using new specific primers and generated PCR products ranging in size from 840 bp for *C. parapsilosis*, 900 bp for *C. orthopsilosis*, 1236 bp for *C. tropicalis* to 1300 bp for *C. dubliniensis*, providing specific patterns for the differentiation of these species (Figure 1, A-D).

Furthermore, *HWP1* gene [12] was partially amplified for *C. glabrata*, *C. albicans*, and *C. africana* using previously designed primers, yielding a single band with sizes of 250, 941, and 700 bp, respectively (Figure 1, E). Table 1 presents the exact size of partial amplified products of the *HWP1* gene for all species. The GenBank accession numbers of the 18 reference and clinical strains are displayed in Table 1.

Based on the sequence analysis performed through the Geneious software, the pairwise and total sequence homology of partial *HWP1* gene among species were 43% and 35.2%, respectively. The nucleotide and amino acid sequence alignment of the strains revealed that the similarity rate of *C. parapsilosis* with *C. orthopsilosis* was 60% and 23.07%, respectively. Among all isolates, *C. albicans* had the most similarity rate with *C. africana* and *C. dubliniensis* with 88.9% and 60.34% for nucleotide sequences and 9.52% and 38.36% for amino acid sequences, respectively.

Notably, the comparison of nucleotide and amino acid sequences displayed that the most pairwise homology belonged to *C. albicans* and *C. africana*, rendering rates of 90.1% and 89.6%, respectively. Furthermore, the least similarity of nucleotide and amino acid between species was observed in *C. orthopsilosis* and *C. dubliniensis* (50.8%) as well as *C. tropicalis* and *C. dubliniensis* (0%), respectively. Intra-species variation was observed only in *C. tropicalis* strains with two DNA types.

Phylogenetic analysis on hyphal wall protein 1 gene

The maximum likelihood phylogenetic tree constructed with *HWP1* gene sequences for the representative strains of each species (Figure 2) showed that the members of the genus *Candida* were divided into three clades. The *C. africana*, *C. dubliniensis*, and *C. albicans* were placed in clade I. The *C. glabrata* and *C. tropicalis* were located in clade II, and *C. parapsilosis* and *C. orthopsilosis* were in clade III. The phylogeny of *Candida* strains inferred from each *HWP1* gene was

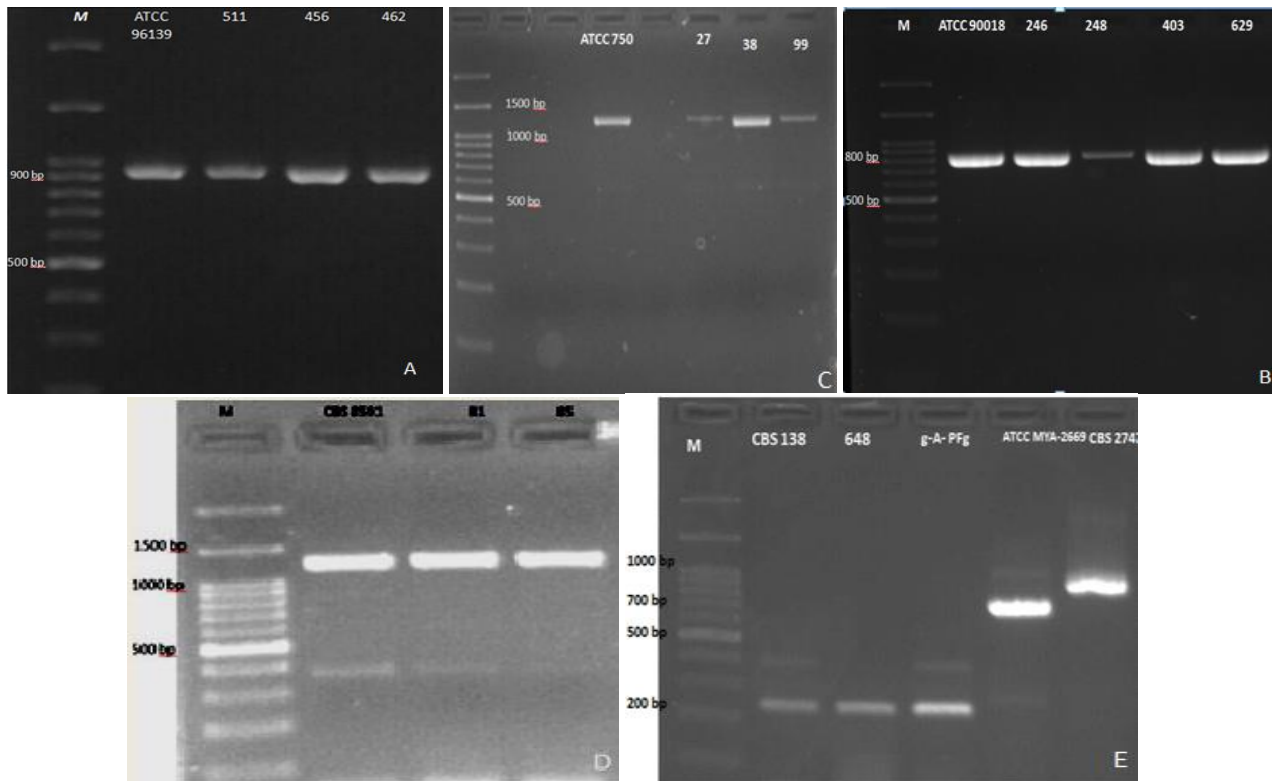


Figure 1. Agarose gel electrophoresis of *HWP1* gene PCR products

A: *C. orthopsilosis*, lanes 1, 2, 3, and 4 respectively denoting *ATCC 96139* and clinical isolates of 511, 456, and 462
 B: *C. parapsilosis*, lanes 1, 2, 3, 4, and 5 respectively signifying *ATCC 90018* and clinical isolates of 246, 248, 403, and 629
 C: *C. tropicalis*, lanes 3, 4, and 5 respectively displaying *ATCC 750* and clinical isolates of 27, 38, and 99
 D: *C. dubliniensis*, lanes 1, 2, and 3 respectively showing *ATCC 8501* and clinical isolates of 81 and 85
 E: Lane 1, *C. glabrata* CBS 138; lanes 2 and 3, clinical isolates: 648 and g-A-PFg, lane 4, *C. africana* ATCC MYA-2669; lane 5, *C. albicans*, CBS 2747

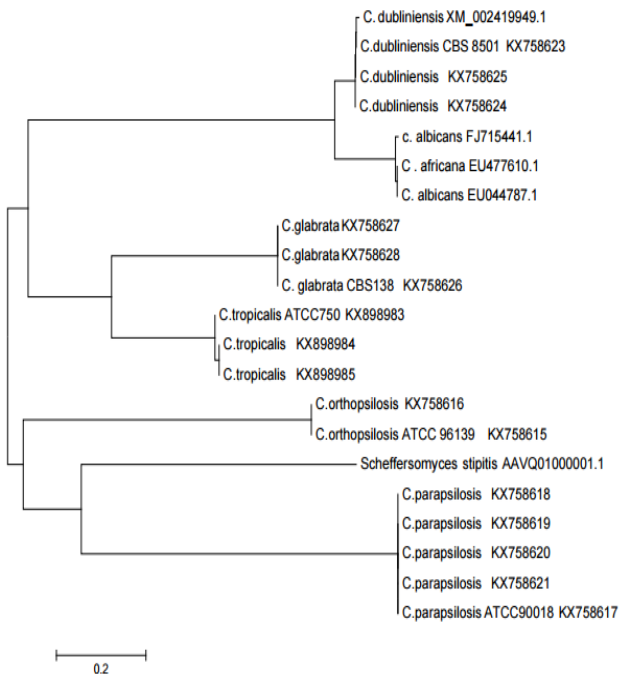


Figure 2. Maximum likelihood phylogenetic tree constructed with hyphal wall protein 1 gene sequences

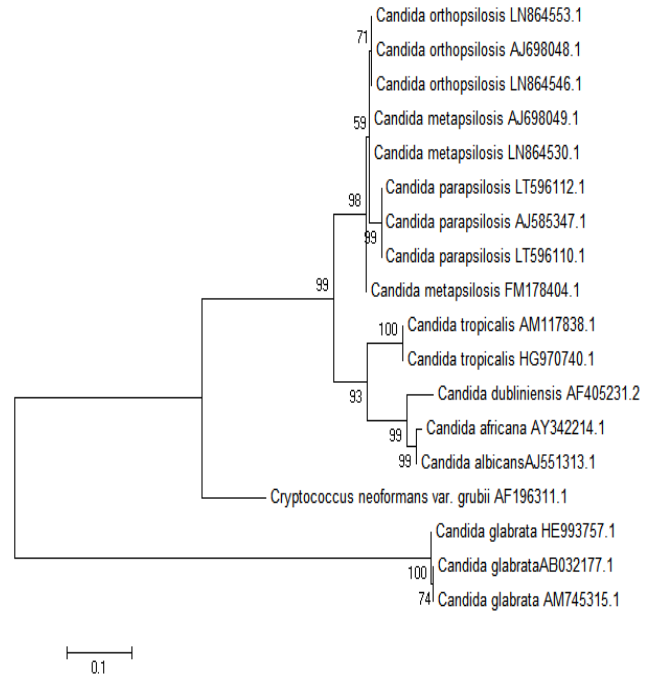


Figure 3. Maximum likelihood phylogenetic tree constructed with ITS1-5.8S-ITS2 rRNA gene sequences

compared with the phylogenetic tree obtained from the Maximum Likelihood analysis of the ITS1-5.8S-ITS2 rRNA gene (Figure 3).

Discussion

In spite of advances in the diagnosis and treatment of invasive candidiasis, this disease is still

the principal cause of death in the critically ill patients. Given the remarkable expansion of *Candida* species infections as well as the differences in pathogenicity and antifungal susceptibility pattern, the rapid and accurate identification at the species level seems to be crucial for clinical management [17].

During the last decades, the utility of the PCR-based techniques focusing on various genetic markers have gained popularity to identify the pathogenic fungi. This increased application is due to the fact that the PCR-based techniques have higher speed, sensitivity, specificity, and reproducibility, compared to the conventional methods [18]. Several authors have previously described the PCR-based procedures, which used specific primers to amplify the DNA fragment of different genes, such as pH-regulated *PHR1* and *PHR2* [19], mitochondrial cytochrome b [20], pH-regulated *KER1* [21], and *HWPI* [6] for the discrimination of *C. albicans*, *C. dubliniensis*, and *C. africana*.

In the current study, we focused on *HWPI* gene as a marker for the identification of the common species of non-*albicans* *Candida*, including *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. orthopsilosis*. However, the previous reports have demonstrated that this gene is highly expressed after transition from blastoconidia to germ tube and hyphae in *C. albicans*, *C. dubliniensis*, and *C. africana* species [22].

Currently, only few genetic markers have been reported for the specific identification of *C. parapsilosis* complex. Some of the markers introduced in different studies include the SADH [14], RPS0 [23], mitochondrial antioxidant manganese superoxide dismutase [24], vacuolar membrane ATPase, and intein genes [25]. Mirhendi et al. [14] developed a PCR-RFLP method for the species identification of *C. orthopsilosis*, *C. metapsilosis*, and *C. parapsilosis*. In their study, the PCR amplification of the secondary alcohol dehydrogenase-encoding gene (SADH) followed by digestion with a single restriction enzyme, NlaIII. They found that this genetic marker is suitable for separation of the species within the *C. parapsilosis* complex. In the present study, a simple and cost-effective PCR test was described to differentiate the two closely related species of *C. parapsilosis* complex (i.e., *C. parapsilosis* and *C. orthopsilosis*) using two specific primers. Our findings revealed that *C. orthopsilosis*, which is phenotypically indistinguishable from *C. parapsilosis*, can be easily identified with our strategy. The *C. parapsilosis* strains have less susceptibility to

triazole compounds in comparison to *C. orthopsilosis*; furthermore, they have a key role as the first-line therapy for candidiasis. Regarding this, the accurate and rapid differentiation between these species may have important therapeutic effects [26].

The majority of molecular procedures have focused on ITS1-5.8S-ITS2 of rRNA gene for the identification of *C. glabrata* species, using such methods as PCR-RFLP [27], multiplex nested PCR [28], and Real-Time PCR [29]. Therefore, this study was the first attempt for finding a unique discriminatory pattern for *C. glabrata* isolates based on *HWPI* gene. However, this species is a non-dimorphic unicellular budding yeast, which does not form germ tube and pseudohyphae; therefore, *HWPI* gene is not expressed in *C. glabrata* isolates [17].

In contrast to *C. glabrata* isolates, *C. tropicalis* undergoes the yeast-to-germ tube and hyphal switching and are shown to have *HWPI* expression [30-32]. There are a limited studies on the role of adhesion proteins, such as *HWPI*, in *C. tropicalis* [33, 34]. Our study provided the nucleotide and amino acid sequences of *HWPI* deposited in the Genbank as well as a specific PCR pattern for the rapid identification of this species.

Previously, Cornet et al. [34] and mirhendi et al. [27] introduced the PCR-RFLP, targeting the intergenic spacer and ITS1-5.8S-ITS2 of ribosomal DNA, respectively, as a reliable method to differentiate *C. tropicalis* from other common pathogenic species of *Candida*. The phylogenetic analysis provided statistically significant support for the clustering of *Candida* species based on *HWPI* gene. The topology was largely consistent with the phylogenetic tree obtained from the previously analyzed genes, such as COX3, SADH, *SYA1*, ALS, and ribosomal RNA [35-38].

Consistent with the last phylogenetic evidence [17, 39], we found that *C. dubliniensis* and *C. africana* fell into *C. albicans* complex as shown for ITS1-5.8S-ITS2 gene in Figure 3. The phylogenetic tree of *HWPI* gene indicated that the *C. dubliniensis* isolates formed a monophyletic group nested within the clade of *C. albicans*.

In addition, the phylogeny of *C. parapsilosis* complex based on the *HWPI* gene showed a completely similar topology with the phylogenetic tree based on the D1/D2 region of rDNA reported by Herkert et al. [40] and ITS (Figure 3), SADH, and 26S rRNA gene sequences presented by Mirhendi et al. [14]. However, in our study, *HWPI* gene was not amplified for *C. metapsilosis*. Our finding confirmed the previous analysis in which *C. parapsilosis* and *C. orthopsilosis* were placed in

"*psilosis*" clade [38, 40, 41].

In agreement with the phylogenetic tree based on D1/D2 region of rDNA [41], ALS [37], and cytochrome b genes [42], the current phylogeny inferred from *HWP1* displayed that *C. tropicalis* was phylogenetically distinct from other species of *Candida* and a sister taxon (75% bootstrap support) of the *C. glabrata* species. While in the phylogenetic tree of the DNA topoisomerase II and ITS1-5.8S-ITS2 genes (Figure 3) reconstructed by the neighbor-joining and Maximum Likelihood methods, respectively [43], *C. tropicalis* placed in a separate cluster next to *C. albicans*, *C. dubliniensis*, and *C. parapsilosis* isolates. Substantially, the differences between phylogenetic results seem to be related to the methods of analysis and genetic markers [42].

The analysis of *HWP1* gene revealed that except for *C. africana* and *C. albicans*, no other species showed more than 90% homology based on nucleotide sequences. Therefore, this gene presents an excellent genetic marker for the identification of different species of *Candida*. In the previous studies, the sequence homology rate for the different species of *Candida* was reported as 89.9%, 97.52%, and 97.9% for cytochrome b, V3 variable region of the LSU of rRNA gene, and *ACT1*, respectively [42].

The genetic diversity of *HWP1* gene has already been reported for *C. albicans* isolates originated from Yaoundé HIV-infected patients [44]. In a study, Kammalac Ngouana et al. described five genotypes (i.e., H1-H5) using the amplification of *HWP1* gene. In the current study, we obtained two single-nucleotide polymorphisms in one isolate of *C. tropicalis*.

The present study was the first report of *HWP1* gene analysis for the identification and phylogenetic evaluation in some *Candida* species, including *C. parapsilosis*, *C. orthopsilosis*, *C. tropicalis*, and *C. glabrata*. In this study, 77 *Candida* isolates causing different infections were identified at the species level based on the developed PCR pattern on *HWP1* gene. We concluded that the proposed simple PCR test was a rapid and cost-effective method. Therefore, this method could be used routinely in the research and clinical laboratories for the reliable identification of the most common species of *Candida*.

Conclusion

Finally, we found that *HWP1* gene was an excellent marker for the identification of non-*albicans Candida* species.

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Author's contribution

M.A. designed and supervised the research, S.H., N.R., N.A., I.H., and S.G. performed the tests. Furthermore, M.A. wrote the draft, and M.H., T.S., H.M., S.R.A., and R.M. edited the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest. The authors are responsible for the content and writing of the paper.

Financial disclosure

The authors declare no financial interests related to the materials of the study.

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