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RESEARCH ARTICLE

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Molecular identification of *Candida* isolates by Real-time PCR-high-resolution melting analysis and investigation of the genetic diversity of *Candida* species

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Abstract

Background: *Candida* species are considered as the cause of one of the most important opportunistic fungal diseases. Accurate identification of *Candida* species is important because of antifungal susceptibility patterns are different among these species, so proper identification helps in the selection of antifungal drugs for the prevention and treatment. Phenotypic methods for identification of *Candida* species, which are widely used in clinical microbiology laboratories, have some limitations. Real-time PCR followed by the high-resolution melting analysis (HRMA) is a novel approach for the rapid recognition of pathogenic fungi. Molecular phylogeny is essential for obtaining a better understanding of the evolution of the genus *Candida* and the identification of the relative degree of the *Candida* species. The purpose of this study was molecular identification of *Candida* isolates by Real-time PCR-high-resolution melting analysis and investigation of the genetic diversity of *Candida* species.

Methods: Two hundred and thirty-two *Candida* isolates including 111 *Candida* isolates obtained from 96 HIV/AIDS patients and 121 *Candida* isolates obtained from 98 non-HIV persons were identified by real-time PCR and high-resolution melting curve analysis. To evaluate genetic diversity and relationships among *Candida* species, PCR products of nine clinical *Candida* isolates, as a representative of each kind of species, were randomly selected for DNA sequence analysis.

Results: In HIV/AIDS patients, six species of *Candida* spp. were identified as follows: *C albicans* (n = 64; 57.7%), *C glabrata* (n = 31; 27.92%), *C parapsilosis* (n = 9; 8.1%), *C tropicalis* (n = 4; 3.6%), *C krusei* (n = 2; 1.8%), and *C kefyr* (n = 1; 0.90%). In non-HIV persons, we identified eight species of *Candida* including *C albicans* (n = 46; 38.33%) followed by *C glabrata* and *C krusei* (each one, n = 18; 15%), *C tropicalis* (n = 13; 10.83%), *C lusitaniae* (n = 12; 5.17%), *C parapsilosis* (n = 10; 4.31%), and *C kefyr* and *C guillermondii* (each one, n = 2; 1.66%). Also, the phylogenetic analysis showed the presence of two

Abbreviations: HIV/AIDS, Human immunodeficiency virus infection and acquired immune deficiency syndrome; HRM, High-resolution melting; HRMA, High-resolution melting analysis; OC, Oral candidiasis; PCR, Polymerase chain reaction; SDA, Sabouraud dextrose agar; SD, Standard deviation; Tm, Melting temperature.

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main clades and six separate subclades. Accordingly, about 88.9% of the isolates were located in clade I and 11.10% of the studied isolates were in clade II.

Conclusions: Real-time PCR followed by high-resolution melting analysis (HRMA) is known as a reliable, fast, and simple approach for detection and accurate identification of *Candida* species, especially in clinical samples.

KEYWORDS
Candida spp, high resolution melting, HIV/AIDS patients, non-HIV persons, phylogeny

1 | INTRODUCTION

The prevalence of fungal infections has been increased, especially in immunocompromised hosts such as consumers of corticosteroids and antibiotics, the patients with diabetes, malnutrition, severe malnutrition, alcoholics, HIV/ AIDS, cancer, and certain genetic disorders.[1] Candidiasis is considered as the most important opportunistic fungal diseases worldwide and remains a clinical problem, predominantly among the immunocompromised patients.[2,3]

Oral candidiasis (OC) is the commonest human fungal infection presented in the oral cavity among the HIV/ AIDS patients.[4] The different manifestations of OC in the immunocompromised patients especially among the HIV/ADIS patients involved oral thrush (pseudomembranous candidiasis), denture stomatitis, median rhomboid glossitis, hyperplastic candidiasis, erythematous candidiasis, linear gingival erythema, perleche or angular cheilitis, salivary gland swellings, sore formation in the oral cavity, and oral hairy leukoplakia.[5] Many people with OC can stay without any specific clinical symptoms for a long time. However, several symptoms in these people may include burning sensation and pain in the mouth, changes in taste sensation, and difficulty in swallowing liquids or solids, and or white creamy or creamy plaques in different parts of the oral cavity.[6,7]

C albicans is the major species in the development of oral candidiasis. Other *Candida* species including *C glabrata*, *C kefyr*, *C tropicalis*, *C parapsilosis*, *C krusei*, and *C guilliermondii* are found in different parts of the oral cavity.[6] The frequency of *Candida* species in the oral cavity was 40%-60% in healthy subjects,[8] while it was between 62% and 93% in the HIV/ AIDS patients.[9]

Accurate identification of *Candida* species is important, because antifungal susceptibility patterns are vary among these species, and also a proper identification helps in the selection of antifungal drugs for the prevention and treatment.[10] The methods used to identify *Candida* species are phenotypic and genotypic assays. Phenotypic assays for identification of *Candida* species involve carbohydrate assimilation and fermentation test, investigation of the morphological characteristics of yeasts on malt extract agar (MEA) and corn meal agar (CMA), production of germ tube and chlamydoconidia, used as a commercial chromogenic media such as CHROMAgar™ *Candida*, growth at different temperatures, cultured in Tween 80 agar, and serological methods.[11] These methods, which are widely used in clinical microbiology laboratories, have some limitations such as insensitive, expensive, time-consuming, and inability to identify important species, such as *C albicans* and *C dubliniensis*. Therefore, finding the reliable and fast approaches for their identification is needed, and proper identification helps in the selection of effective antifungal drugs.[12,13]

Molecular techniques are known as suitable approaches for identification of *Candida* species. These methods are expensive; however, their accuracy and speed are undeniable. Molecular techniques such as polymerase chain reaction (PCR), amplified fragment length polymorphism (AFLP), PCR-restriction length fragment polymorphisms (PCR-RFLP), random amplification of polymorphic DNA (RAPD), multiplex PCR, nested polymerase chain reaction (Nested PCR), DNA sequencing, matrix-associated laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), DNA fingerprinting, single-strand conformational polymorphism (SSCP) analysis, and real-time polymerase chain reaction (real-time PCR) are suitable for diagnosing and identifying *Candida* species.[10,14,15]

High-resolution melt analysis (HRMA) is a powerful molecular method to detect mutations, polymorphisms, epigenetic information, and also to identify species of different organisms by comparing relative positions and shapes of melting curves.[16] Real-time PCR followed by HRMA is a basic, fast, accurate, and closed-tube technique with the susceptibility for the single nucleotide.[17] Phylogenetic analysis is essential for obtaining a better understanding of the evolution of the genus *Candida* and detection of the relative degree of *Candida* species as well as infer evolutionary pathways of some of gene families.[18] Therefore, the aim of this study was molecular identification of *Candida* isolates by Real-time PCR high-resolution melting analysis and investigation of the genetic diversity of *Candida* species.

2 | MATERIAL AND METHODS

2.1 | Study design, population, and clinical specimens

The present cross-sectional study was performed in the Department of Medical Parasitology and Mycology, Kerman University of Medical Sciences, Kerman (southeast of Iran), from November 2018 to September 2019. Two hundred and thirty-two *Candida* isolates

including 111 *Candida* isolates from 96 HIV/AIDS patients and 121 *Candida* isolates from 98 non-HIV persons were used in this study. The oral specimens were taken from the HIV/AIDS patients who were referred to the Kerman Counseling Resource Center for behavioral disorders for taking periodic checkups and/or drugs or solving their health difficulties. As well, 121 *Candida* isolates from 98 non-HIV persons were collected from the different parts of the oral cavity of non-HIV subjects, who were referred to the Medical Mycology Laboratory of Afzalipoor Faculty of Medicine in Kerman. All the participants of this study completed the questionnaire and filled an informed consent form. Samples from different parts of the oral cavity such as the oral mucosa and tongue were collected with a sterile swab. The reason for the differences in the number of the individuals in these two groups was the lack of cooperation of some of the individuals in sampling. The obtained oral swabs were cultured on to chromo Agar *Candida* (HiMedia, Mumbai, India) and then incubated for 48 hours at 35°C to create specific colony colors.

2.2 | *Candida* reference strains

Eight species of *Candida* including *C albicans*, *C glabrata*, *C krusei*, *C tropicalis*, *C lusitaniae*, *C parapsilosis*, *C kefyr*, and *C guillermondii* were used in this study for optimizing the ITS2-melting curve analysis. These *Candida* species were characterized previously in the research mycological laboratory of our department using the standard methods such as the specific color the colony created on CHROMagar *Candida* media and PCR-RFLP with *Msp I* enzyme.[5,10] *Bln I* restriction enzyme was used for discrimination of between *C albicans* and *C dubliniensis*.

2.3 | DNA isolation

Eight *Candida* reference strains and clinical *Candida* isolates were grown on Sabouraud dextrose agar media for 18-24 hours at 37°C. The DNA of *Candida* isolates was extracted by the use of Favor Prep™ Blood/Cultured Cell Genomic DNA Extraction Mini Kit (Favorgen Biotech Co, Taiwan) in terms of the manufacturer's protocol.[19] DNA concentrations and A_{260}/A_{280} ratios of the extracted DNA were calculated by a ND-1000 spectrophotometer (NanoDrop, Fisher Thermo). An A_{260}/A_{280} ratio of 1.8 to 2.1 was selected for subsequent experiments, and all the extracted DNAs were stored at −20°C until use.

2.4 | Real-time PCR assay and HRMA analysis

Real-time PCR followed by HRMA was performed on eight *Candida* reference strains. The primer's sequences used for this study were *ITS86* as forward primer [5′- GTGCATCATCGAATCTTTGAAC-3′] and *ITS4* as a reversed primer [5′-TCCTCCGCTTATTAGGAC-3′], which were previously described by Gutzmer et al.[20] For gene amplification by HRM, volume of 20 μL comprising 4 μL 5× HOT FIREPol® EvaGreen® HRM Mix (Solis BioDyne Co), 4 μL of the extracted DNA from each *Candida* isolates, 1 μL of forward primer (10 pmol/L) (Macrogen Co), 1 μL of reverse primer (10 pmol/L) (Macrogen Co), and 10 μL of distilled water was prepared. HRM was done using Rotor-Gene 6000 (Corbett Research) using the following program: an initial denaturation step at 95°C for 12 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 2 seconds, and 72°C for 20 seconds. The amplification products for HRMA were cooled 1 minutes at 50°C and then heated from 80°C to 92°C monitoring fluorescence at the rate of 0.1°C/2 s. Melting data were normalized, and the temperature was shifted using Rotor-Gene 6000. Normalization regions for HRMA were set at 80.5°C to 90.9-91.3°C. The obtained results were analyzed using HRM Software Rotor-Gene series software ver. 2.3.1 (Corbett Research). All the experiments were done in duplicate for eight *Candida* reference strains and 232 clinical *Candida* isolates. All the reference *Candida* strains were used in each run. The mean Tm ± 4 SD and the dMelt curve shape of clinical *Candida* isolates were compared to the *Candida* reference strains panel.

2.5 | PCR Assay

To perform the accurate identification of the species using the results of HRM with the confidence level lower than 50% and for some species with closed melting Tm, for example, *C albicans* (87.385°C), *C lusitaniae* (87.84°C), and *C krusei* (87.04°C), PCR assay with *ITS 86* and *ITS4* primers, were used.

2.6 | Phylogenetic analysis

To evaluate genetic diversity and relationships among *Candida* species, PCR products of nine clinical *Candida* isolates, as a representative of any species, were randomly selected for DNA sequence analysis. The PCR products were submitted to Fazapajo Company. The obtained nucleotide sequences were aligned with the sequences registered in NCBI GenBank. All nucleotide sequences were trimmed by Sequence Scanner version 1.0 (Applied Biosystems). Afterward, the similarity percentage was determined by the online BLAST tool and Sequence Alignment software; BioEdit ver. 7.2.5. The phylogenetic analysis was performed following the investigation of the best nucleotide substitution's model using MEGA 7 software. The final phylogenetic tree was drawn in the same software with 1000 replicates using the Bootstrap topologies reliability test.

2.7 | Statistical analysis

After data collection, Graph Pad Prism version 8 (Graph Pad Software In, San Diego, USA) was used for descriptive statistical analysis.

3 | RESULTS

3.1 | Frequency of clinical *Candida* isolates according to CHROMagar *Candida* medium

The 232 *Candida* isolates were directly incubated on CHROMagar *Candida* medium. The number of *Candida* isolates observed based on colony color on CHROMagar *Candida* after 48 hours of incubation including 110 green colonies (47.4%), 46 pink colonies (19.32%), 38 purple colonies (16.37%), 17 dark blue and/or blue gray colonies (7.32%), 11 pinkish-purple colonies (4.74%), and 10 white colonies (4.31%), respectively.

3.2 | HRMA in *Candida* reference strains

Melting temperature (Tm) ± standard deviation (SD) of *Candida* reference strains are presented in Table 1. The results show reproducible melting peaks for each species. Most of the *Candida* reference strains presented single peak, whereas three *Candida* species including *C parapsilosis*, *C tropicalis*, and *C guilliermondii* melted in two domains (see Table 1). Figure 1 shows the melting curve analysis of *Candida* reference strains that was used in this study.

3.3 | Identification of clinical *Candida* isolates by high-resolution melting analysis

In patients with HIV/AIDS, six species of *Candida* spp. were identified as follows: *C albicans* (n = 64; 57.7%), *C glabrata* (n = 31; 27.92%), *C parapsilosis* (n = 9; 8.1%), *C tropicalis* (n = 4; 3.6%), *C krusei* (n = 2; 1.8%), and *C kefyr* (n = 1; 0.90%). In non-HIV persons, we identified eight species of *Candida* including *C albicans* (n = 46; 38.33%) followed by *C glabrata* and *C krusei* (each one, n = 18; 15%), *C tropicalis* (n = 13; 10.83%), *C lusitaniae* (n = 12; 5.17%), *C parapsilosis* (n = 10; 4.31%), and *C kefyr* and *C guillermondii* (each one, n = 2; 1.66%). The distribution of *Candida* clinical isolates in HIV/AIDS patients and non-HIV persons by HRMA in this study is presented in Table 2. The difference in the number of clinical *Candida* isolates in these two studied groups was related to the separation of several isolates from one individual in some cases.

3.4 | Phylogenetic analysis

Figure 2 presents the phylogenetic tree based on DNA sequenced isolate analysis. Phylogenetic analysis showed the presence of two main clades and six separate subclades. Accordingly, about 88.9% of the isolates were located in clade I and 11. 10% of the studied isolates were in clade II. Subclades were numbered from I to VI, with subclade I consisting of six subclades and isolates of H40, H60, H6, H65, H40, H599, and S7. Also, isolates H41, H60, and H63 isolated from the HIV/ AIDS patients were *C kefyr*, *C glabrata*, and *C tropicalis*, respectively. Isolates H6 and H65 obtained from the HIV/AIDS patients were *C albicans*. H59 and H40 isolates collected from the HIV/AIDS patients were *C parapsilosis*. Isolate S7 that was obtained from non-HIV individuals was *C guilliermondii*. The second clade consisting isolate S9 was isolated from non-HIV persons and was *C lusitaniae*. Origins, sources, and accession numbers of DNA sequenced *Candida* isolates registered in NCBI are presented in Table 3.

4 | DISCUSSION

The purpose of this study was molecular identification of clinical *Candida* isolates by real-time PCR-HRMA and investigation of the genetic diversity of these species. High-resolution melt analysis was able to identify among various clinical *Candida* species.[21] Real-time PCR-HRM could be considered as a quick, accurate, and inexpensive method for precise identification of yeast species such as *Candida* spp in clinical specimens.[13,22] Phenotypic assays, used for differentiation of *Candida* species, have also some limitations such as less accuracy, time-consuming, and inability to identify some species.[10] Superiorities of HRMA over conventional methods for *Candida* identification were demonstrated in Nemcova et al's study.[16] Although some methods such as API-ID32C, CANDIDA test 21, and pyrosequencing are not able to distinguish some *Candida* species, while these *Candida* species could be easily identified using HRMA.[16] In regard with that, due to its simplicity and rapidity, this method can be applied to predict the response of the treatment.[23]

In the present study, two hundred and thirty two *Candida* isolates including 111 *Candida* isolates from the HIV/AIDS patients and 121 *Candida* isolates obtained from non-HIV individuals were identified by real-time PCR and high-resolution melting curve analysis. Here, by considering the mean Tm ± 4 SD and the dMelt curve shape of eight *Candida* reference strains, six and eight different species in the HIV/AIDS patients and non-HIV persons were distinguished, respectively. Nemcova et al[16] differentiated 23 out of 27 *Candida* species based on real-time PCR amplification and HRMA of the ITS2 region with *UNF1* and *UNF2* primers. Mandviwala et al[24] used a relatively demanding

TABLE 1 Melting temperature (Tm) ± standard deviation (SD) of *Candida* reference strains

Candida reference strains	Melting peak 1	Melting peak 2
	Mean Tm (4 SD)°C	Mean Tm (4 SD)°C
C glabrata	85.915 (0.348712)	
C parapsilosis	83.985 (0.279544)	85.625 (0.315008)
C albicans	87.385 (0.440023)	
C guilliermondii	83.34 (0.085323)	84.725 (0.129692)
C lusitaniae	87.84 (0.106536)	
C kefyr	86.975 (0.188627)	
C krusei	87.04 (0.129538)	
C tropicalis	85.79 (0.141315)	87.059 (0.156141)

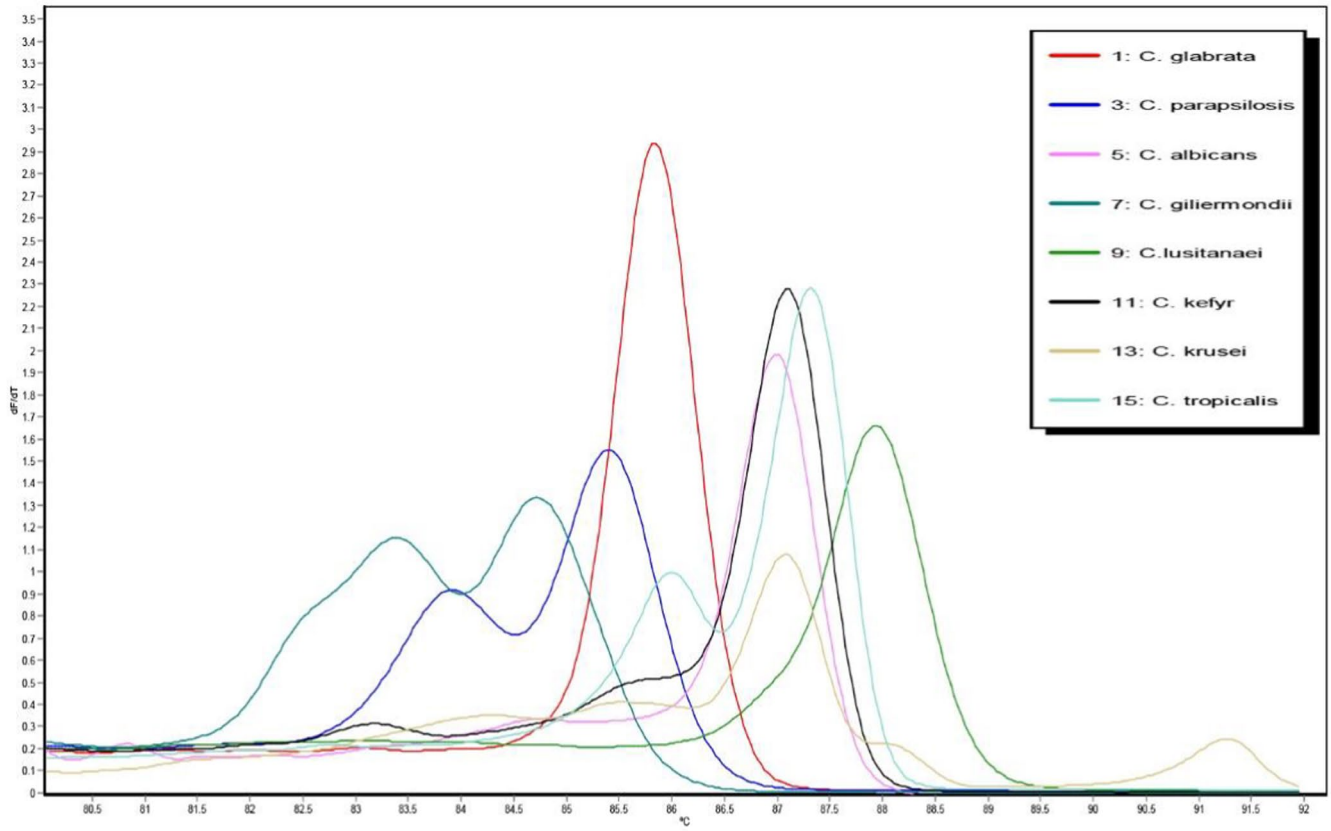

FIGURE 1 Melting temperature curve of amplicon of *Candida* reference strains used in this study

TABLE 2 Distribution of *Candida* clinical isolates in HIV/AIDS patients and non-HIV persons by HRMA in this study

Candida species	HIV/AIDS patients, No. (%)	Non-HIV persons No. (%)	Total No. (%)
C parapsilosis	9 (3.87)	10 (4.31)	19 (8.18)
C glabrata	31 (13.36)	18 (7.75)	49 (21.12)
C kefyr	1 (0.43)	2 (0.86)	3 (1.29)
C albicans	64 (27.58)	46 (19.82)	110 (47.41)
C tropicalis	4 (1.72)	13 (5.60)	17 (7.32)
C lusitaniae	0 (0)	12 (5.17)	12 (5.17)
C guilliermondii	0 (0)	2 (0.86)	2 (0.86)
C krusei	2 (0.86)	18 (7.75)	20 (7.75)
Total	111 (47.84)	121 (52.15)	232 (100)

analysis in MS Excel to identify 8 *Candida* species based on *ITS1* and *ITS2* sequences.

Arancia et al[21] distinguished five *Candida* species by HRMA analysis *MP65* gene. In Somogyvari et al's study,[25] 10 *Candida* spp were differentiated by analyzing the HRMA of the *ITS2* sequence. In a study concluded by Asadzadeh et al,[26] real-time PCR method based on melting point analysis with SYBR Green dye was introduced as a fast and reliable molecular method to perform accurate diagnosis and differentiation of *C albicans* and *C dubliniensis*. Alnuaimi et al[27] identified nine *Candida* spp using HRMA based on ITS1, 5.8S, and ITS2 sequences, in which they used eight *Candida* species as positive controls in each run. Decat et al[28] reported that the *ITS2* sequence using five positive controls is a useful approach for discrimination of 17 *Candida* species.

In the present study, prevalent species included *C albicans* followed by *C glabrata* in the HIV/AIDS patients and *C albicans* followed by *C glabrata* and *C krusei* in non-HIV persons. Similar to our results, *C albicans* was recognized as the most frequent species.[22,29,30] In contrast with the present study, *C tropicalis* was the second most common species. Similarly, the second most species was *C glabrata*.[13] In a study by Ziauddin Khan et al, the second most species were *C parapsilosis*, *C tropicalis*, and *C glabrata*.[30]

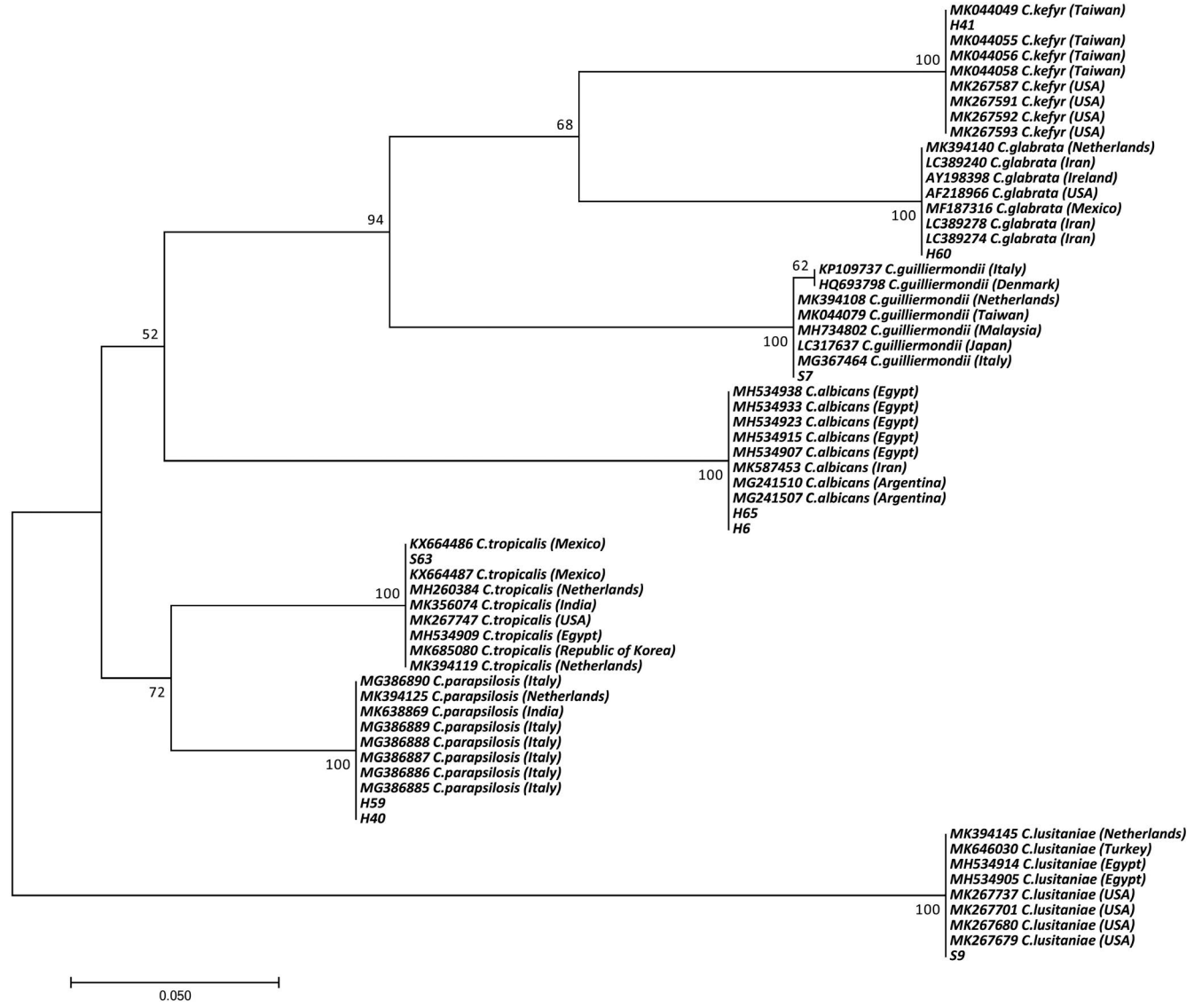

FIGURE 2 Phylogenetic tree based on sequencing results. The evolutionary history was conducted in MEGA7 by using the maximum likelihood method based on the Tamura 3-parameter model. All positions containing gaps and missing data were eliminated

According to the nucleotide changes in the ITS region and its importance in the molecular epidemiology of the disease, it seems that the phylogenetic study is essential. Also, these nucleotide variations would be more critical in the interpretation of PCR-based molecular experimental results. Therefore, we used HRM analysis of partial amplification ITS region as well as specific primers along with comparing the nucleotide sequence with other reported records of *Candida* species.

The results of the phylogenetic analysis show that there are two main clades and six separate subclades. Accordingly, about 88.9% of the isolates were located in clade I and 11.10% of the studied isolates were in clade II. Subclades were numbered from I to VI, with subclade I consisting of six subclades and H40, H60, H6, H65, H40, H599, and S7 isolates. The second clade consisting isolate S9 was isolated from healthy individuals, which was *C lusitaniae*.

Some limitations of our study were the presence of HRM results with the confidence level lower than 50% and the closed melting Tm for some of species, for example, *C albicans* (87.385°C), *C lusitaniae* (87.84°C), and *C krusei* (87.04°C). To overcome these limitations, PCR assay with *ITS 86* and *ITS4* primers was performed for performing an accurate identification of these species. In this study, 42 of 232 *Candida* isolates were identified by PCR technique. Therefore, the error rate of HRM in this study was 18.10%. Moreover, other limitations in this study were the lack of cooperation by some of the HIV/AIDS patients for sampling, and the low number of samples selected for phylogenetic analysis due to budget constraints and rising dollar price in Iran.

TABLE 3 Origins, sources, and accession numbers of DNA sequenced *Candida* isolates registered in NCBI

Species name	Origin	Source	GenBank sequence accession numbers
C parapsilosis (H59 isolate)	Kerman province, south east of Iran	HIV/AIDS patient	MT377825
C parapsilosis (H40 isolate)	Kerman province, south east of Iran	HIV/AIDS patient	MT377826
C albicans (H65 isolate)	Kerman province, south east of Iran	HIV/AIDS patient	MT377827
C albicans (H6 isolate)	Kerman province, south east of Iran	HIV/AIDS patient	MT377828
C glabrata (H60 isolate)	Kerman province, south east of Iran	HIV/AIDS patient	MT377829
C kefyr (H41 isolate)	Kerman province, south east of Iran	HIV/AIDS patient	MT377830
C tropicalis (S63 isolate)	Kerman province, south east of Iran	Non-HIV person	MT377831
C lusitaniae (S9 isolate)	Kerman province, south east of Iran	Non-HIV person	MT377832
C guilliermondii (S7 isolate)	Kerman province, south east of Iran	Non-HIV person	MT377824

5 | CONCLUSION

Real-time PCR followed by HRMA is a reliable, fast, and simple approach for performing an accurate identification of *Candida* species, especially in clinical samples. Accurate identification of *Candida* species is important due to antifungal susceptibility patterns that vary among these species, and a proper identification helps in the selection of antifungal drugs for prevention and treatment.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHORS' CONTRIBUTIONS

PGHA and SS developed the study concept and design. EEN and MAM collected the data. SS analyzed and interpreted the data. SS wrote the article. PGHA and SS revised and edited the article. All authors read and approved the final article.

ETHICAL APPROVAL

The study was evaluated and approved by the Ethics Committee of the Kerman Medical University and Kerman Research Council (IR.KMU.REC.1396.2500).

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