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RESEARCH ARTICLE

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Rapid identification of the *Candida glabrata* species complex by high-resolution melting curve analysis

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Abstract

Background: *Candida glabrata* is a common pathogen that causes invasive candidiasis. Among non-*albicans Candida* infections, *C glabrata* infections are associated with the highest fatality rates. *Candida glabrata sensu stricto*, *Candida nivariensis*, and *Candida bracarensis* have been identified and together form the *C glabrata* species complex. It is difficult to detect the two rare species by traditional laboratory methods. This study established a method for the rapid identification of members of the *C glabrata* species complex based on high-resolution melting curve (HRM) analysis and evaluated its practical application.

Methods: The internal transcribed spacer (ITS) region was used as target gene region to design specific primers. HRM analysis was performed with three subspecies of the *C glabrata* species complex and negative controls to test its specificity and sensitivity. To evaluate its practical application, the HRM technique was tested with clinical isolates, and the results were compared with the DNA sequencing results.

Results: Differences were detected among the melting profiles of the members of the *C glabrata* species complex. The negative controls were not amplified, indicating the high specificity of the method. The minimum detection limits of *C glabrata sensu stricto*, *C nivariensis*, and *C bracarensis* were approximately 1×10^1 copies/μL or less. The results of the HRM analysis of the clinical isolates were consistent with the DNA sequencing results.

Conclusions: The HRM method is sensitive and can be used to rapidly identify the members of the *C glabrata* species complex. The method can allow early and targeted treatment of patients with invasive candidiasis.

KEYWORDS

Candida glabrata, high-resolution melting curve, identification, internal transcribed spacer, molecular typing

1 | INTRODUCTION

Invasive candidiasis is a common clinical fungal disease with a high mortality rate up to 40%.[1] Common risk factors include advanced age, tumor radiotherapy and chemotherapy, organ transplantation, interventional therapy, and HIV infection.[2,3] Although *Candida albicans* remains the predominant fungus of nosocomial infections, an increasing number of non-*C albicans* species, such as *Candida glabrata*, *Candida tropicalis*, and *Candida krusei*, have emerged as significant opportunistic pathogens in recent years.[4] Among these non-*C albicans* species, *C glabrata* is associated with the highest mortality rate[5] and can rapidly acquire increased resistance to fluconazole.[6,7] In the United States and Europe, *C glabrata* is the second most common pathogen causing candidemia.[8,9] In China, *C glabrata* has been listed as an important pathogenic agent of nosocomial infections, and it ranks fourth among invasive *Candida* spp.[10]

Since the development of gene-sequencing technology, two new species of *Candida* have been discovered, *Candida nivariensis*[11] and *Candida bracarensis*,[12] which together with *C glabrata sensu stricto* form the *C glabrata* species complex. Due to the many phenotypic characteristics that are shared among the three species, *C nivariensis* and *C bracarensis* can be unambiguously identified only by molecular identification methods.[13-15] Differences in virulence and antifungal susceptibility patterns exist among the members of the *C glabrata* species complex.[16,17] For example, *C nivariensis* has been reported to be less sensitive to fluconazole, itraconazole, and voriconazole than *C glabrata sensu stricto*.[18,19] Therefore, to develop effective antifungal therapies, a method for the fast and accurate identification of members of the *C glabrata* species complex is needed.

Molecular identification methods reported to date in the literature include sequencing, multiplex PCR,[13] restriction fragment length polymorphism (RFLP) analysis,[20] PNA-FISH analysis,[17,20] and pyrosequencing.[21] These methods have high requirements for operators and cannot be carried out in clinical laboratories on a large scale at present.[7] Therefore, a simple, rapid, and low-cost method that can meet clinical requirements is needed. High-resolution melting curve (HRM) analysis is a closed-tube operation that can be rapidly carried out following real-time quantitative PCR (qPCR). Different lengths, GC contents, and sequences of DNA lead to different melting profiles, which can allow detection of microdifferences among target sequences.[22,23] To date, HRM analysis has been successfully applied for taxon identification, for example, the identification of medically important *Candida* spp. (21 species),[24] members of Pasteurellaceae (6 species),[25] and members of *Trichinella* (8 species).[22] In this study, we report a qPCR-HRM method that uses a pair of primers to produce different melting profiles allowing discrimination among *C glabrata sensu stricto*, *C nivariensis*, and *C bracarensis*. Clinical isolates were subjected to HRM analysis and ITS1-ITS4 sequencing to evaluate the practical application of the HRM method.

2 | MATERIALS AND METHODS

2.1 | Strains and sources

Three strains, comprising one strain each of *C glabrata sensu stricto*, *C nivariensis*, and *C bracarensis*, were selected as reference strains, and forty-seven non-*glabrata* strains were used as negative controls (Table 1). All of the strains were provided by the Chinese Center for Disease Control and Prevention and had previously been identified by internal transcribed spacer (ITS) or large subunit of rDNA (LSU) sequencing.[26,27] Thirty clinical isolates of *Candida* spp. identified by MALDI-TOF MS were derived from outpatient and inpatient samples of Peking Union Medical College Hospital.

2.2 | Fungal culture

The reference strains, negative controls, and clinical isolates were cultured on potato dextrose agar (PDA) at 28°C for 48 hours.

2.3 | DNA extraction

The strains and isolates were individually collected in centrifuge tubes, each containing glass beads and 400 μL ultrapure water. The cell walls of the fungi were broken by bead beating, and then, the DNA was isolated by using the fungal DNA isolation protocol with QIAamp DNA Blood Mini Kit (Qiagen). The DNA products were stored at −20°C for further analysis.

2.4 | Design of primers and plasmids

The internal transcribed spacer (ITS), as a non-coding region that has been under low natural selection pressure during evolution, can tolerate many mutations, which results in sequence polymorphism. Accordingly, ITS is widely used for fungal identification.[13] Due to its rapid evolution, the region amplified by primers ITS1 and ITS2 exhibits interspecies specificity and intraspecies conservation.[28] Therefore, we compiled the sequences of the ITS1-ITS2 region of the *C glabrata* species complex published in GenBank, and multiple pairs of primers were designed with Lasergene Seqman software. The DNA of the reference strains was used as templates to perform gradient PCR, and a pair of appropriate primers that could amplify all three species was screened by agarose gel electrophoresis. The primers sequences are as follows:

forward primer: 5′-TCAACAATGGATCTCTTGGT-3′
reverse primer: 5′-CCAACAATTTCAAGCTAACT-3′

The alignment of the amplified fragments and primer positions is shown in Figure 1. Sequences of the amplified fragments were submitted to Sangon Biotech (Shanghai) Co., Ltd., where the plasmids were synthesized.

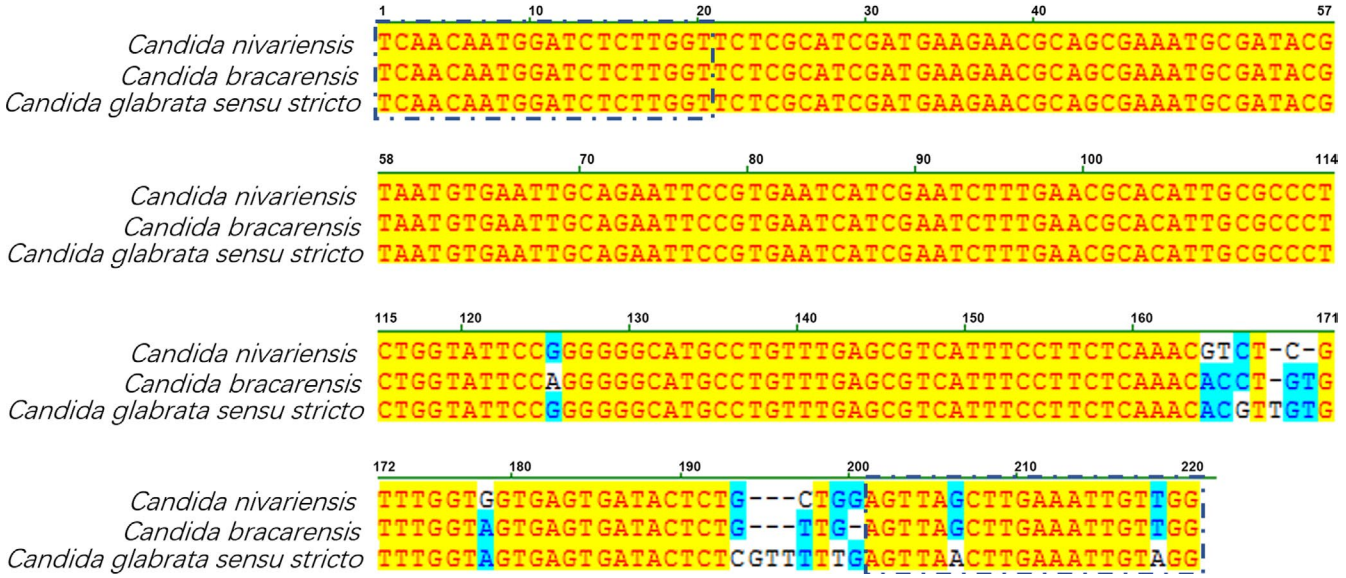

FIGURE 1 Aligned sequences of the amplified ITS region of the three reference strains. Conserved bases are represented in yellow, variable sites are indicated in blue, and gaps within the sequence are indicated by hyphens. The binding sites of the primers are indicated by rectangles

2.5 | Establishment of the HRM method and its specificity and sensitivity

qPCR was performed in a 30 μL reaction mixture containing 15 μL Taqman qPCR Mix (2×) (Takara), 0.9 μL of each forward primer and reverse primer, 0.3 μL Rox Reference Dye II (100×), 1.5 μL Evagreen (20× in water), 2 μL DNA template, and 9.4 μL ultrapure water. The PCR conditions were as follows: 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 58°C for 30 seconds. The heating and cooling speeds were 1.6°C/s, and HRM ramping was performed from 60-95°C. Fluorescence data were acquired at 0.025°C increments every second to generate specific melting curves.

The qPCR-HRM reaction was carried out with the DNA of the reference strains and the negative controls to examine the method's specificity.

The concentrations of the plasmids were measured by Qubit, and their unit was converted to copies per microliter (copies/μL) with the following formula: copies/μl = $(6.02 \times 10^{23}) \times (\text{ng}/\mu\text{L} \times 10^{-9})/(\text{DNA length} \times 660)$. Then, the plasmids were gradiently diluted with ultrapure water (1×10^{6}~1×10^{0} copies/μL), and qPCR-HRM analysis was performed again to examine the method's minimum detection limits.

All of the above procedures were performed three times.

2.6 | Detection of clinical isolates

To evaluate the practical application of this method, the DNA of clinical isolates was used as a template to carry out the qPCR-HRM method, and three reference strains were included to produce melting curve standards.

2.7 | Sequencing of clinical isolates

Sequence analysis of all clinical isolates was performed by using the primers ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ (forward) and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ (reverse).[29] Then, the PCR products were sent to Sangon Biotech (Shanghai) Co., Ltd., for sequencing. The sequencing results were analyzed by BLAST in the GenBank database to obtain the species name.

2.8 | Statistics

All data were entered into Excel 2007, and the means and SD of melting temperature (Tm) were calculated.

3 | RESULTS

3.1 | The specificity of the HRM method for the *C glabrata* species complex

HRM analysis was performed with the reference strains and the negative controls. The results showed that the negative controls were not amplified. The reference strains showed three specific melting profiles (Figure 2A). Taking the first derivative of the fluorescence signal, we obtained three reproducible melting peaks (Figure 2B). The melting curves of the three species were unimodal, and their corresponding melting temperature (Tm) values differed. Different ranges of Tm values were evaluated, and the mean Tm ± 3 SD range was selected to overcome intra-assay variability and attain maximum sensitivity and reproducibility. The mean Tm ± 3 SD ranges of *C glabrata sensu stricto*, *C nivariensis*,

TABLE 1 Overview of reference species and assignment of negative controls

Species	No. of examined strains	Strain code
Candida glabrata sensu stricto[a]	1	CDCF4750
Candida nivariensis[a]	1	CDCF4790
Candida bracarensis[a]	1	CDCF4794
Candida krusei[a]	3	IC00366, CDCF4400, CDCF5719
Candida tropicalis[a]	2	CDCF3540, CDCF4402
Candida albicans[a]	3	CDCF3156, CDCF4392, CDCF4404
Candida duobushaemulonii[a]	2	CDCF4458, CDCF4872
Candida haemulonis[a]	2	CDCF4462, CDCF2626
Candida haemulonis var. vulnera[a]	3	CDCF4476, CDCF4564, CDCF4584
Candida pseudohaemulonii[a]	1	CDCF4558
Candida auris[a]	2	CDCF4588, CDCF4590
Candida metapsilosis[a]	3	CDCF2596, CDCF4204, CDCF4732
Candida orthopsilosis[a]	3	CDCF4708, CDCF4710, CDCF4724
Candida parapsilosis[a]	3	CDCF4678, CDCF5060, CDCF5026
Candida guilliermondii[a]	1	CDCF0994
Candida oceani[a]	1	CDCF2574
Candida zeylanoides[a]	1	CDCF5314
Candida railenensis[a]	1	CDCF1621
Candida oleophila[a]	1	CDCF1655
Candida uthaithanina[a]	1	CDCF5438
Candida pseudointermedia[a]	1	CDCF5400
Aspergillus flavus[b]	1	CDCF1536
Aspergillus versicolor[b]	2	CDCF0515, CDCF1480
Aspergillus niger[b]	2	CDCF2256, CDCF2826
Aspergillus fumigatus[b]	1	CDCF5114
Fusarium solani[b]	2	CDCF2498, CDCF5088
Fusarium proliferatum[b]	2	CDCF1699, CDCF4634
Trichosporon asahii[b]	3	CDCF2012, CDCF5036, CDCF5044

[a]*Candida* spp. were identified by sequencing the ITS (internal transcribed spacer) region.

[b]*Aspergillus* spp., *Fusarium* spp., and *Trichosporon* spp. were identified by sequencing the LSU rDNA (large subunit of rDNA) region.

and *C bracarensis* were 84.371 ± 0.093°C, 84.681 ± 0.052°C, and 84.15 ± 0.048°C, respectively. The maximum Tm variation of the three reference strains was 0.07°C in triplicate assays. Since the mean Tm values for the three strains differed by at least 0.18°C, they were deemed suitable for discriminating among the reference strains. This approach followed that used in Manuel Miller's experiment.[25]

3.2 | The sensitivity of the HRM method for the *C glabrata* species complex

HRM analysis was performed on plasmids with gradient dilution. Appendix S1a, S2a, and S3a show that the amplification curves exhibited an "S" shape only within the concentration range of 1×10^6~1×10^1 copies/μL. We constructed standard curves (Appendix S1b, S2b, and S3b; data are listed in Table 2) and obtained corresponding regression equations. The maximum Ct value in the linear range corresponded to the concentration of 1×10^1 copies/μL. Therefore, the minimum detection limits of *C glabrata sensu stricto*, *C nivariensis*, and *C bracarensis* were approximately 1×10^1 copies/μL or less.

3.3 | Analysis of the clinical isolates

According to the amplification curves of the clinical isolates, only thirteen isolates were amplified, and their melting curves matched only one of the reference strains. Based on the species-specific melting curves of the reference strains (Figure 3), 7 isolates of *C glabrata sensu stricto*, 4 isolates of *C nivariensis*, and 2 isolates of *C bracarensis* were identified, and the results showed 100% agreement with the sequencing results (Table 3). The seventeen unamplified isolates were identified by sequencing as *C albicans*, *C tropicalis*, *C krusei*, and other species.

4 | DISCUSSION

Among infections due to non-*C albicans* taxa, *C glabrata* infections are associated with the highest mortality. Given that the members of the *C glabrata* species complex vary in their antifungal susceptibility patterns, methods to accurately identify the three species are necessary to allow early, targeted antifungal therapy. The conventional identification methods rely upon a combination of morphological and biochemical features and consistently fail to identify two less common species of this complex, tentatively identifying them as *C glabrata*.[20] Currently, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) is widely used in the clinical laboratory, but identification scores are consistently low and require confirmation by sequencing. The genotype differences among the members of the *C glabrata* species complex have prompted the application of various PCR-based techniques for species identification, such as pyrosequencing, multiplex PCR, and RFLP analysis. The conventional mycological procedures are time-consuming, and the molecular methods mentioned above are laborious and cannot be used at large scale in clinical laboratories.

The HRM method established in this study utilizes a saturation dsDNA-binding dye to monitor the shedding of dye during the heating process, which enables the differentiation of genetic variation

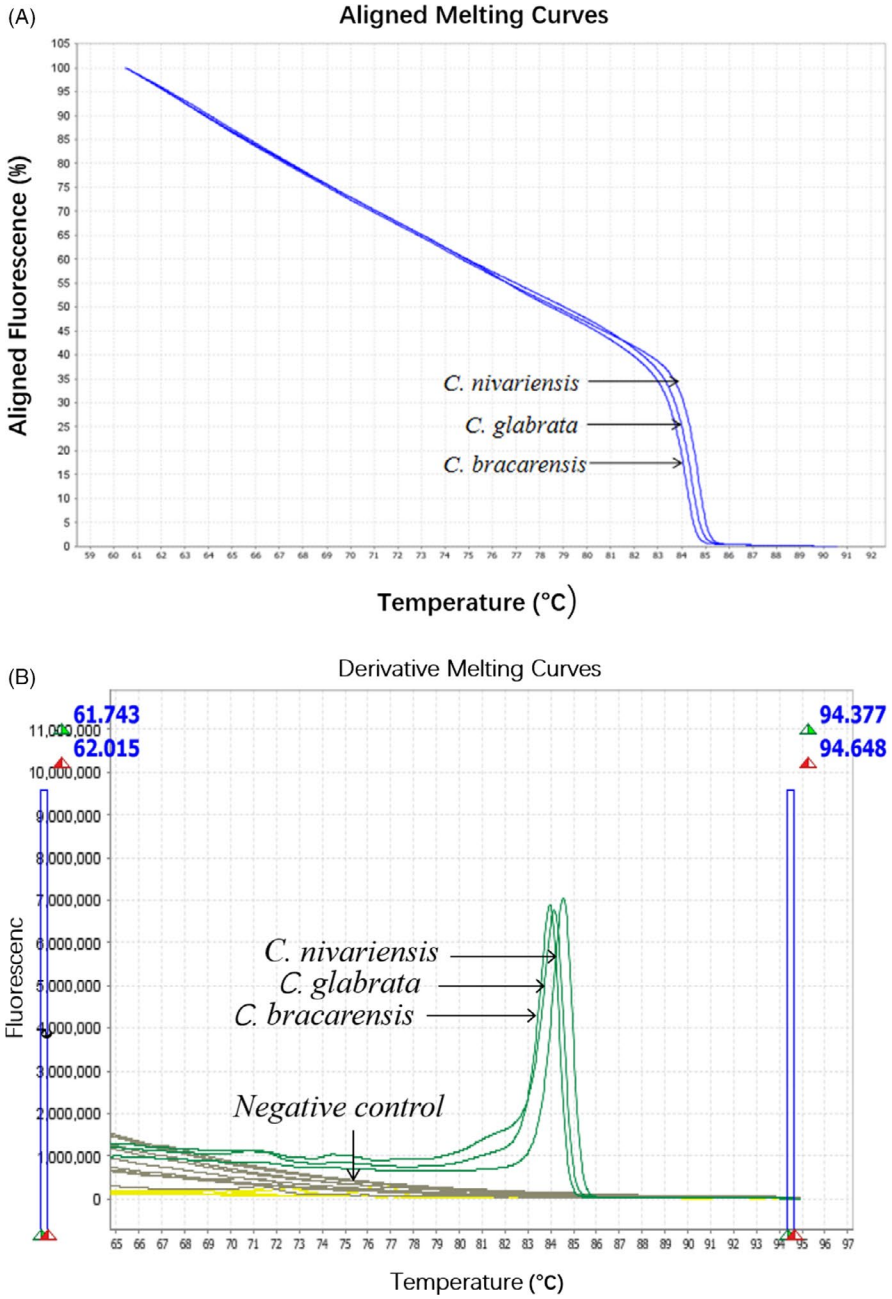

FIGURE 2 A, The melting curves of the *Candida glabrata species complex*. In the acceleration of the fluorescent signal decline period, three curves were mutually distinct, which was used for identification. B, Peak diagram of the *Candida glabrata species complex*. The melting curves of the three subspecies are unimodal, and the mean Tm values of *C glabrata sensu stricto*, *C nivariensis*, and *C bracarensis* are 84.371°C, 84.681°C, and 84.155°C, respectively. The negative controls were not amplified

TABLE 2 The Ct values and the logarithm starting quantity of the Candida glabrata species complex

	Log					
Ct values	**6**	**5**	**4**	**3**	**2**	**1**
C glabrata sensu stricto	18.359	23.468	26.854	31.74	35.308	37.55
C nivariensis	18.625	23.717	27.385	30.281	33.704	36.584
C bracarensis	18.733	22.613	25.976	29.706	34.774	38.114

(to the resolution of single nucleotide polymorphisms) in amplified DNA fragments without sequencing.[22,23] Unlike other PCR-based technologies, the HRM method does not require postprocessing, such as electrophoresis or enzymatic digestion, and can therefore avoid external nucleic acid contamination. Furthermore, the method can be used to quantify the concentrations of nucleic acids in the original specimen (even under very limited initial template concentrations), indicating its promise for clinical application.[23]

The key to success in HRM analysis is the design of specific primers that generate discernibly different melting profiles with appropriate amplification lengths. It has been reported that single nucleotide modifications in short amplicons can cause unique changes in the

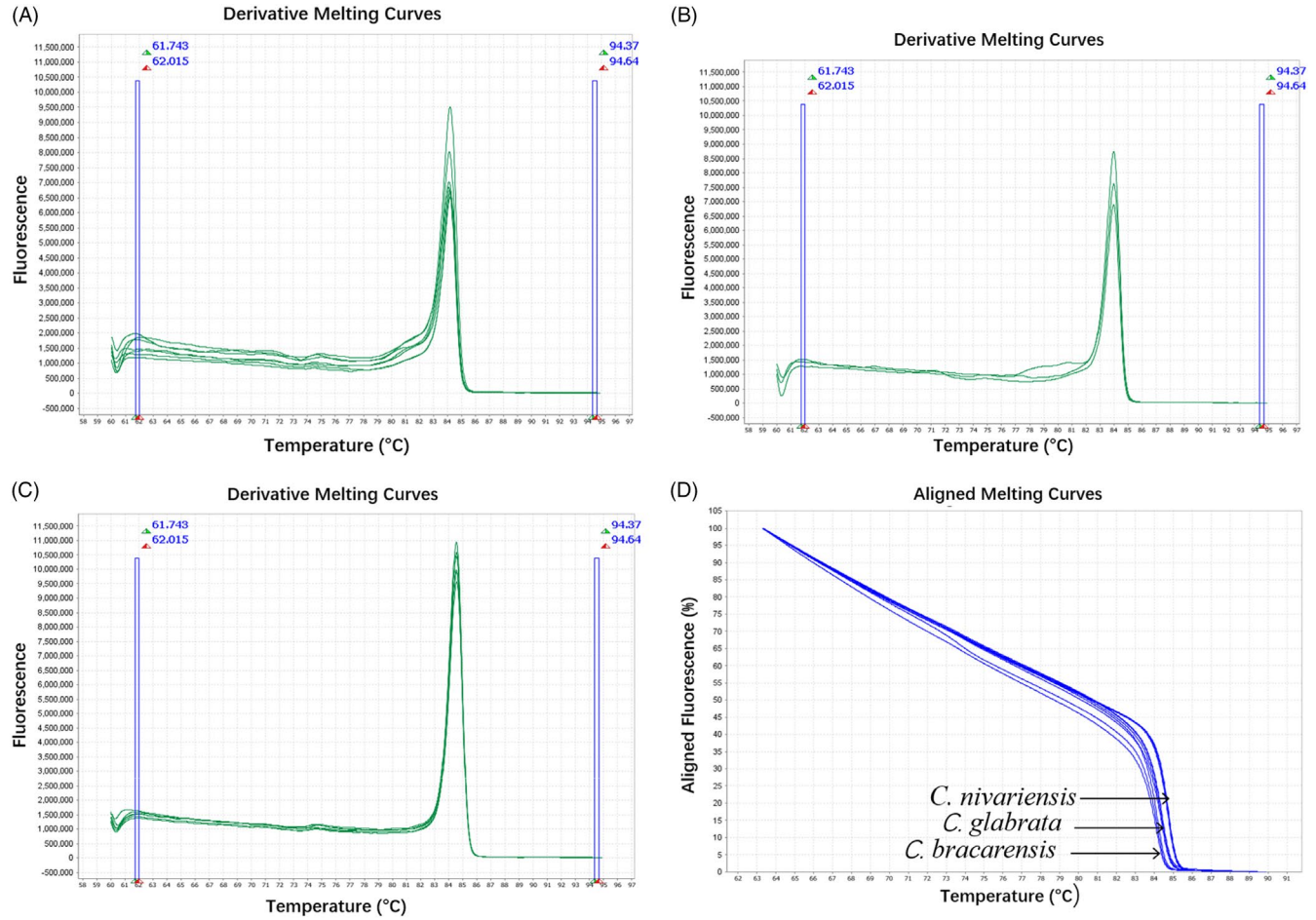

FIGURE 3 Only 13 clinical isolates were amplified. There were 7 isolates of *C glabrata sensu stricto* (A), 4 isolates of *C nivariensis* (B), and 2 isolates of *C bracarensis* (C) based on the species-specific melting curves of the reference strain. D shows the melting curves of all amplified isolates

TABLE 3 Comparison of HRM and sequencing of ITS

Species	HRM	Sequencing
Candida glabrata sensu stricto (n = 7)	*Candida glabrata sensu stricto* (n = 7)	*Candida glabrata sensu stricto* (n = 7)
Candida nivariensis (n = 4)	*Candida nivariensis* (n = 4)	*Candida nivariensis* (n = 4)
Candida bracarensis (n = 2)	*Candida bracarensis* (n = 2)	*Candida bracarensis* (n = 2)
Candida krusei (n = 4)	–	*Candida krusei*(n = 4)
Candida tropicalis(n = 3)	–	*Candida tropicalis*(n = 3)
Candida albicans(n = 5)	–	*Candida albicans*(n = 5)
Candida parapsilosis(n = 3)	–	*Candida parapsilosis*(n = 3)
Candida haemulonis(n = 2)	–	*Candida haemulonis*(n = 2)

Note: The overall agreement between HRM and sequencing of ITS was 100%.

melting curve but that as the amplicon is extended, the effectiveness of detecting single-base substitutions decreases.[25] Eva Nemcova and colleagues achieved rapid identification of medically important *Candida* isolates, including *C glabrata* (408 bp), by HRM[24] but have not been able to distinguish the members of the *C glabrata* species complex. To verify the results of the experiment, we performed qPCR-HRM again with primers UNF1 and UNF2[30] with isolates of the *C glabrata* species complex. The results showed that the ranges of the Tm ± 3 SD values for *C glabrata sensu stricto* and *C nivariensis* repeatedly overlapped. Therefore, we redesigned primers for the ITS1-ITS2 region, and the length of the amplicons was approximately 220 bp. Supplementing the results of the study by Eva Nemcova and colleagues, the results showed that the peaks of the three reference strains were distinguishable and unimodal. The Tm values of *C glabrata sensu stricto*, *C nivariensis*, and *C bracarensis* were 84.371°C, 84.681°C, and 84.155°C, respectively. The difference among taxa in Tm value

was >0.18°C. According to the amplified sequence alignment map of the *C glabrata* species complex (Figure 1), there are only a few base differences in the amplified fragments among the three species, which might explain why the peak diagrams of the three species were not clearly differentiated and why the Tm values were not significantly different. There was no amplification of the negative control fungi, indicating that the primers were species specific.

Evaluation of the newly established method using 30 unknown *Candida* spp. isolates revealed accurate identification of all specimens as confirmed by ITS and LSU sequencing. However, a limitation of the present study is the lack of verification with direct clinical specimens and mixed-infection specimens, and clinical application values remain to be studied.

Infections due to *C nivariensis* and *C bracarensis* are frequently reported.[14,17,31-33] We believe that the HRM technique can be used to rapidly and accurately identify three subspecies of the *C glabrata* species complex and allow rapid and targeted treatment of patients with invasive candidiasis.

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AUTHOR CONTRIBUTIONS

CSQ performed the molecular work, analyzed the data and sequences, and wrote the manuscript. XJ contributed to the molecular work and created image attachments. GJ designed the study, secured the biological material, provided financial support, consulted on data interpretation, and participated in revising the manuscript. ZF created sequence alignments, consulted on the experimental work, and participated in revising the manuscript. All authors contributed to the final version of the manuscript.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the research in this article is related to microorganisms.

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REFERENCES

1. Kullberg BJ, Arendrup MC. Invasive candidiasis. *N Engl J Med*. 2015;373:1445-1456.
2. Pappas PG, Kauffman CA, Andes D, et al. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2009;48:503-535.
3. Criseo G, Scordino F, Romeo O. Current methods for identifying clinically important cryptic Candida species. *J Microbiol Methods*. 2015;111:50-56.
4. Enoch DA, Yang H, Aliyu SH, Micallef C. The changing epidemiology of invasive fungal infections. *Methods Mol Biol*. 2017;1508:17-65.
5. Abi-Said D, Anaissie E, Uzun O, Raad I, Pinzcowski H, Vartivarian S. The epidemiology of hematogenous candidiasis caused by different Candida species. *Clin Infect Dis*. 1997;24:1122-1128.
6. Li L, Redding S, Dongari-Bagtzoglou A. Candida glabrata: an emerging oral opportunistic pathogen. *J Dent Res*. 2007;86:204-215.
7. Mandviwala T, Shinde R, Kalra A, Sobel JD, Akins RA. High-throughput identification and quantification of Candida species using high resolution derivative melt analysis of panfungal amplicons. *J Mol Diagn*. 2010;12:91-101.
8. Pfaller M, Neofytos D, Diekema D, et al. Epidemiology and outcomes of candidemia in 3648 patients: data from the Prospective Antifungal Therapy (PATH Alliance(R)) registry, 2004–2008. *Diagn Microbiol Infect Dis*. 2012;74:323-331.
9. Bolotin-Fukuhara M, Fairhead C. Candida glabrata: a deadly companion? *Yeast*. 2014;31:279-288.
10. Xiao M, Fan X, Chen S-A, et al. Antifungal susceptibilities of Candida glabrata species complex, Candida krusei, Candida parapsilosis species complex and Candida tropicalis causing invasive candidiasis in China: 3 year national surveillance. *J Antimicrob Chemother*. 2015;70:802-810.
11. Alcoba-Florez J, Mendez-Alvarez S, Cano J, Guarro J, Perez-Roth E, del Pilar Arevalo M. Phenotypic and molecular characterization of Candida nivariensis sp. nov., a possible new opportunistic fungus. *J Clin Microbiol*. 2005;43:4107-4111.
12. Correia A, Sampaio P, James S, Pais C. Candida bracarensis sp. nov., a novel anamorphic yeast species phenotypically similar to Candida glabrata. *Int J Syst Evol Microbiol*. 2006;56(1):313-317.
13. Romeo O, Scordino F, Pernice I, Lo Passo C, Criseo G. A multiplex PCR protocol for rapid identification of Candida glabrata and its phylogenetically related species Candida nivariensis and Candida bracarensis. *J Microbiol Methods*. 2009;79(1):117-120.
14. Hou X, Xiao M, Chen S-A, et al. Identification and antifungal susceptibility profiles of candida nivariensis and candida bracarensis in a multi-center Chinese collection of yeasts. *Front Microbiol*. 2017;8:5.
15. Figueiredo-Carvalho MH, Ramos LDS, Barbedo LS, et al. First description of Candida nivariensis in Brazil: antifungal susceptibility profile and potential virulence attributes. *Mem Inst Oswaldo Cruz*. 2016;111:51-58.
16. Treviño-Rangel RDJ, Espinosa-Pérez JF, Villanueva-Lozano H, et al. First report of Candida bracarensis in Mexico: hydrolytic enzymes and antifungal susceptibility pattern. *Folia Microbiol*. 2018;63(4):517-523.
17. Bishop JA, Chase N, Magill SS, Kurtzman CP, Fiandaca MJ, Merz WG. Candida bracarensis detected among isolates of Candida glabrata by peptide nucleic acid fluorescence in situ hybridization: susceptibility data and documentation of presumed infection. *J Clin Microbiol*. 2008;46:443-446.
18. Borman AM, Petch R, Linton CJ, Palmer MD, Bridge PD, Johnson EM. Candida nivariensis, an emerging pathogenic fungus with multidrug resistance to antifungal agents. *J Clin Microbiol*. 2008;46:933-938.
19. Fujita S-I, Senda Y, Okusi T, et al. Catheter-related fungemia due to fluconazole-resistant candida nivariensis. *J Clin Microbiol*. 2007;45(10):3459-3461.
20. Mirhendi H, Bruun B, Schønheyder HC, et al. Differentiation of Candida glabrata, C. nivariensis and C. bracarensis based on fragment length polymorphism of ITS1 and ITS2 and restriction fragment length polymorphism of ITS and D1/D2 regions in rDNA. *Eur J Clin Microbiol Infect Dis*. 2011;30:1409-1416.
21. Borman AM, Linton CJ, Oliver D, Palmer MD, Szekely A, Johnson EM. Rapid molecular identification of pathogenic yeasts by pyrosequencing analysis of 35 nucleotides of internal transcribed spacer 2. *J Clin Microbiol*. 2010;48:3648-3653.

22. Reslova N, Skorpikova L, Slany M, Pozio E, Kasny M. Fast and reliable differentiation of eight trichinella species using a high resolution melting assay. *Sci Rep*. 2017;7(1):16210.
23. Reed GH, Kent JO, Wittwer CT. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics*. 2007;8:597-608.
24. Nemcova E, Cernochova M, Ruzicka F, Malisova B, Freiberger T, Nemec P. Rapid identification of medically important Candida isolates using high resolution melting analysis. *PLoS ONE*. 2015;10:e0116940.
25. Miller M, Zorn J, Brielmeier M. High-resolution melting curve analysis for identification of pasteurellaceae species in experimental animal facilities. *PLoS ONE*. 2015;10:e0142560.
26. Stielow JB, Lévesque CA, Seifert KA, et al. One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Persoonia*. 2015;35:242-263.
27. <Assembling the fungal tree of life progress, classification, and evolution of subcellular traits.pdf>.
28. Irinyi L, Serena C, Garcia-Hermoso D, et al. International Society of Human and Animal Mycology (ISHAM)-ITS reference DNA barcoding database—the quality controlled standard tool for routine identification of human and animal pathogenic fungi. *Med Mycol*. 2015;53:313-337.
29. White TJ, Bruns T, Lee S, Taylor J. A guide to methods and applications. 1990. <Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics.pdf>.
30. Dendis M, Horváth R, Michálek J, et al. PCR-RFLP detection and species identification of fungal pathogens in patients with febrile neutropenia. *Clin Microbiol Infect*. 2003;9:1191-1202.
31. Tay ST, Lotfalikhani A, Sabet NS, et al. Occurrence and characterization of Candida nivariensis from a culture collection of Candida glabrata clinical isolates in Malaysia. *Mycopathologia*. 2014;178:307-314.
32. Morales-Lopez S, Dudiuk C, Vivot W, Szusz W, Córdoba SB, Garcia-Effron G. Phenotypic and molecular evaluation of echinocandin susceptibility of Candida glabrata, Candida bracarensis, and Candida nivariensis strains Isolated during 30 years in Argentina. *Antimicrob Agents Chemother*. 2017;61:e00170-17.
33. Cuenca-Estrella M, Gomez-Lopez A, Isla G, et al. Prevalence of Candida bracarensis and Candida nivariensis in a Spanish collection of yeasts: comparison of results from a reference centre and from a population-based surveillance study of candidemia. *Med Mycol*. 2011;49:525-529.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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