

## Probe-based qPCR Assay for Rapid Detection of Predominant *Candida glabrata* Sequence Type in Korea

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Recent years have seen an increase in the incidence of candidiasis caused by non-*albicans Candida* (NAC) species. In fact, *C. glabrata* is now second only to *C. albicans* as the most common cause of invasive candidiasis. Therefore, the rapid genotyping specifically for *C. glabrata* is required for early diagnosis and treatment of candidiasis. A number of genotyping assays have been developed to differentiate *C. glabrata* sequence types (STs), but they have several limitations. In the previous study, multi-locus sequence typing (MLST) has performed with a total of 101 *C. glabrata* clinical isolates to analyze the prevalent *C. glabrata* STs in Korea. A total of 11 different *C. glabrata* STs were identified and, among them, ST-138 was the most commonly classified. Thus, a novel probe-based quantitative PCR (qPCR) assay was developed and evaluated for rapid and accurate identification of the predominant *C. glabrata* ST-138 in Korea. Two primer pairs and hybridization probe sets were designed for the amplification of internal transcribed spacer 1 (ITS1) region and *TRP1* gene. Analytical sensitivity of the probe-based qPCR assay was 100 ng to 10 pg and 100 ng to 100 pg (per 1 µL), which target ITS1 region and *TRP1* gene, respectively. This assay did not react with any other *Candida* species and bacteria except *C. glabrata*. Of the 101 clinical isolates, 99 cases (98%) were concordant with MLST results. This novel probe-based qPCR assay proved to be rapid, sensitive, highly specific, reproducible, and cost-effective than other genotyping assay for *C. glabrata* ST-138 identification.

**Key Words:** *Candida glabrata*, Genotyping, Quantitative PCR (qPCR), Sequence types (STs), *TRP1*

### INTRODUCTION

Candidiasis is a widespread fungal infection caused by various *Candida* species, especially in acquired immune deficiency syndrome (AIDS) patients, immunocompromised individuals, patients with hematopoietic stem cells or organ transplantations, and those who present underlying valvular heart diseases or have received long-term antibiotic therapy (Bineshian et al., 2015; Rezazadeh et al., 2016; Ortiz et al.,

2018).

Although *C. albicans* is still the major cause of candidiasis, in recent years the number of infections caused by non-*albicans Candida* (NAC) species has increased (Ho and Haynes, 2015). Among them, *C. glabrata* has received considerable attention as one of the most common cause of invasive candidiasis (Shahrokhi et al., 2017). The mortality rate associated with candidemia by infection with *C. glabrata* is also becoming higher than with any other NAC species because of its resistance to azole antifungal agents (Gohar et

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al., 2017). Furthermore, with rapidly expanded use of echinocandin as the primary treatment for invasive candidiasis, echinocandin resistance in *C. glabrata* has posed a serious clinical challenge (Zhao et al., 2016). In spite of its growing prominence, however, little is known of the population structure, epidemiology, and basic biology of *C. glabrata* (Dodgson et al., 2003).

It is essential to develop DNA fingerprinting methods which can assess genetic distance between independent isolates in broad epidemiological studies (Abbes et al., 2010). A number of DNA fingerprinting methods have been developed to differentiate *C. glabrata* strains, such as random amplification of polymorphic DNA (RAPD) (Becker et al., 2000), pulsed-field gel electrophoresis (PFGE) (Lin et al., 2007), multi-locus enzyme electrophoresis (MLEE) (Pujol et al., 1997), multi-locus sequence typing (MLST) (Lott et al., 2010), and multi-locus variable-number tandem-repeat analysis (MLVA, also known as microsatellite analysis) (Abbes et al., 2012). Above all, the sequence-based method, MLST analyzes five to seven selected housekeeping genes for single nucleotide polymorphisms (SNPs) (Berila and Subik, 2010; Enache-Angoulvant et al., 2010). However, these approaches have several limitations. Although these genotyping methods can discriminate between closely related strains, they are costly, time-consuming, have lower reproducibility, and require highly trained clinicians for proper execution (Essendoubi et al., 2007). In order to overcome these limitations, a novel *C. glabrata* genotyping method is required.

In the previous study (Kang, 2017), MLST was performed with a total of 101 *C. glabrata* clinical isolates to analyze genetic polymorphisms and investigate the most prevalent sequence types (STs) in Korea. In a total of 3,345 base-pair DNA sequences, 49 variable nucleotide sites were found. A total of 11 different *C. glabrata* STs were identified, and among them, *C. glabrata* ST-138 was the most common (53%). Each ST has a number of nucleotide differences between alleles; therefore, these polymorphic sites can be utilized for PCR-based molecular assays (Pérez-Losada et al., 2013).

Quantitative PCR (qPCR) assays have advantages over other PCR-based molecular assays, such as higher sensitivity,

**Table 1.** Origin of *C. glabrata* clinical isolates used in this study

Origin of clinical isolates	Number of cases
Blood	64
Urine	14
Bile	8
Others <sup>1)</sup>	14
– <sup>2)</sup>	1
Total	101

<sup>1)</sup> Ascitic fluid, joint fluid, pleural fluid, tissue etc

<sup>2)</sup> No clinical information

less setup cost, shorter turnaround time, and reproducibility (Foongladda et al., 2014). In addition, amplification reactions and data analysis are processed in a closed-tube system, eliminating the post-amplification step and reducing chances for cross-contamination (Navarro et al., 2015).

In this study, a novel probe-based qPCR assay was developed for rapid and specific identification of *C. glabrata* ST-138 from other STs in Korea. Its performance was evaluated with a total of 101 *C. glabrata* clinical isolates provided from Korean Culture Collection of Medical Fungi (KCMF, Daejeon, Korea).

## MATERIALS AND METHODS

### Clinical strains

A total of 101 *C. glabrata* clinical isolates were provided from KCMF (Daejeon, Korea) and those isolates were collected from Asan Medical Center, Seoul, Yonsei University Wonju Severance Christian Hospital, Wonju, The Catholic University of Korea Seoul St. Mary's Hospital, Seoul, and Chungbuk National University Hospital, Cheongju, Korea. Clinical isolates were isolated from a wide variety of clinical samples, including bloodstream, catheterized urine, bile, and other body fluids (Table 1).

### Genomic DNA extraction

Genomic DNA (gDNA) from *C. glabrata* clinical isolates was extracted using a I-genomic BYF DNA Extraction Mini kit (iNtRON Inc., Seongnam, Korea) according to the manufacturer's instructions (Da Silva-Rocha et al., 2014). Briefly, cultured yeast samples were added 200 µL of MYP

**Table 2.** Uploaded MLST status of new *C. glabrata* sequence types

Assigned ST <sup>1)</sup>	<i>FKS</i>	<i>LEU2</i>	<i>NMT1</i>	<i>TRP1</i>	<i>UGP1</i>	<i>URA3</i>
ST-138	3	22	4	3	3	4
ST-139	7	22	3	50	1	8
ST-140	8	22	3	5	1	2
ST-141	10	21	14	50	1	9
ST-142	7	17	11	10	5	9
ST-143	5	17	8	7	3	50
ST-144	5	17	8	7	14	6

<sup>1)</sup> Sequence type.

\* All sequence types indicated in this table are novel combinations of existing database

buffer and 2 µL β-mercaptoethanol. After incubation at 37°C for 15 min, the samples were centrifuged and the supernatants were removed completely. Then the samples were lysed by 100 µL of MP buffer and 2 µL lyticase solution (4.2 unit/µL). After incubation at 37°C for 15 min, the samples were centrifuged and the supernatants were discarded, and added 200 µL of MG buffer, 20 µL of proteinase K solution (20 mg/mL) and 5 µL of RNase A solution (10 mg/mL). The samples were incubated at 65°C for 30 min for complete yeast lysis. The lysates were added 250 µL of MB buffer and 250 µL of 80% ethanol. Portions of the lysates were transferred to column in 2.0 mL collection tube and centrifuged. After washing stages, the gDNA was eluted by adding EB buffer. The concentration and purity of the gDNA was checked by 260/280 optical density using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) (Da Silva-Rocha et al., 2014).

#### MLST analysis for identifying sequence type of *C. glabrata* clinical isolates

MLST was performed using a procedure described previously (Kang, 2017). The six housekeeping gene fragments including *FKS*, *LEU2*, *NMT1*, *TRP1*, *UGP1*, and *URA3* were selected for MLST analysis. The reaction products were purified and sequenced at Macrogen Inc. (Daejeon, Korea). The obtained molecular sequences were analyzed by using the *C. glabrata* MLST databases (<http://pubmlst.org/cglabrata/>) (Dodgson et al., 2003). The allele profiles of

**Table 3.** MLST status of *C. glabrata* isolates

Sequence type (ST) by MLST	Number of isolates
ST-138	54
ST-63	24
ST-22	6
ST-139	6
ST-55	3
ST-43	2
ST-140	2
ST-141	1
ST-142	1
ST-143	1
ST-144	1
Total	101

the strains were defined according to the six MLST loci. Each unique allele profile was designated as a ST (Table 3).

#### Design of primer pairs and hybridization probes

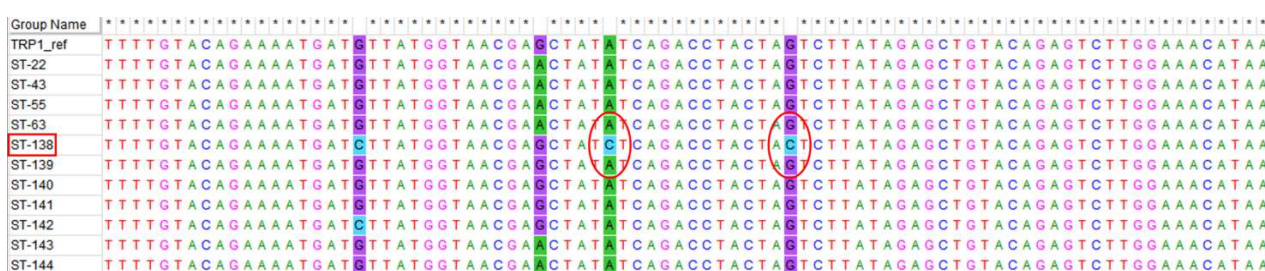
For identification of *C. glabrata* ST-138, the two primer pairs and hybridization probe sets were designed based on alignments of the ITS1 region, the conserved region between the 18S and 28S ribosomal RNA (rRNA) and the *TRP1* gene of *C. glabrata*, respectively, from the National Center for Biotechnology Information (NCBI) database. Optimal primer pairs and hybridization probes were designed by using Primer 3 (v. 0. 4. 0) online software (Rozen et al., 2012). The characteristics and the sequences of two primer pairs and probe sets are shown in Table 4. Two primer pairs (CgITS and CgTRP) recognized a 144 bp region of ITS1 and a 126 bp region of *TRP1* gene of *C. glabrata*. Two hybridization probes (Cf/Pan and Cf/138) were also designed by labelling different fluorophores (FAM and CY5) at 5' end, and non-fluorescent quencher (BHQ1 and BHQ3) at 3' end, respectively.

#### Probe-based qPCR assay

Two-tube TaqMan probe qPCR assay was carried out on the ABI 7500 Fast Real-Time PCR system (Life-Technologies, Waltham, MA, USA). Target amplification was performed in 20 µL reaction mixture containing 10 µL

**Table 4.** The primers and hybridization probes sequences for the qPCR assay

Name	Sequences (5' to 3')	Amplicon size (bp)	Target region
<b>Primers</b>			
CgITS-F	TCTGGGGAGGACCAGTGTAG	144	ITS
CgITS-R	ACACTCCCAGGTCTTTGTGCG		
CgTRP-F	AGCTATCTCAGACCTACTAC	126	TRP1
CgTRP-R	ATCCCGCAAACCTTTACCAG		
<b>Hybridization probes</b>			
Cf/Pan	CY5-GAATGCTATTTCTCCTGCCTGCGCT-BHQ3		ITS
Cf/138	FAM-ACCAACACACCAACAAGATGTCATTTG-BHQ1		TRP1



**Fig. 1.** *C. glabrata* ST-138 specific sequences of *TRP1* locus in Multi-align analysis.

THUNDERBIRD<sup>®</sup> Probe qPCR Mix (Toyobo, Osaka, Japan), 4.5 µL of ultrapure water, 1 µL of each primer (10 pmol/µL), 0.5 µL of each hybridization probe (10 pmol/µL), and 3 µL of gDNA template. The cycling conditions of qPCR were as follow: 2 min incubation at 95°C; 35 cycles of 10 sec at 95°C and 45 sec at 63°C. A positive result was indicated when the cycle threshold (CT) value was less than 30 after observing signal formation of a wavelength from each channel (FAM and CY5).

#### Determination of analytical sensitivity and specificity

The limit of detection (LOD) of the qPCR assay of the ITS1 region and *TRP1* gene was determined through the use of a 10-fold dilution [100 ng, 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg (per 1 µL)] along a standard curve of gDNA isolated from *C. glabrata* ST-138. Each dilutions were tested in triplicate, and the LOD of the qPCR assay was determined as the dilution at which all replicates were positive.

A total of five different strains were used to determine the specificity of the qPCR assay for detection of ITS1 region:

*C. albicans*, *C. glabrata*, *C. tropicalis*, *Staphylococcus aureus*, and *Escherichia coli*. A total of 11 *C. glabrata* STs were used to determine the specificity of the qPCR assay for detection of *TRP1* gene: ST-22, ST-43, ST-55, ST-63, ST-138, ST-139, ST-140, ST-141, ST-142, ST-143, and ST-144. Each gDNA samples were assayed by the same procedures for the qPCR assay application.

## RESULTS

#### Sequence type of *C. glabrata* clinical isolates

With a total of 11 *C. glabrata* STs, seven of which were identified as new STs that were not discovered in the previous study. These data of new *C. glabrata* STs were uploaded and assigned at *Candida glabrata* MLST Databases (<https://pubmlst.org/cglabrata/>) (Table 2).

The data demonstrates that the ST-138 was the most predominant ST in this study as a total of 54 clinical isolates were contained in this ST, and the following most predominant ST was the ST-63 as a total of 24 clinical isolates

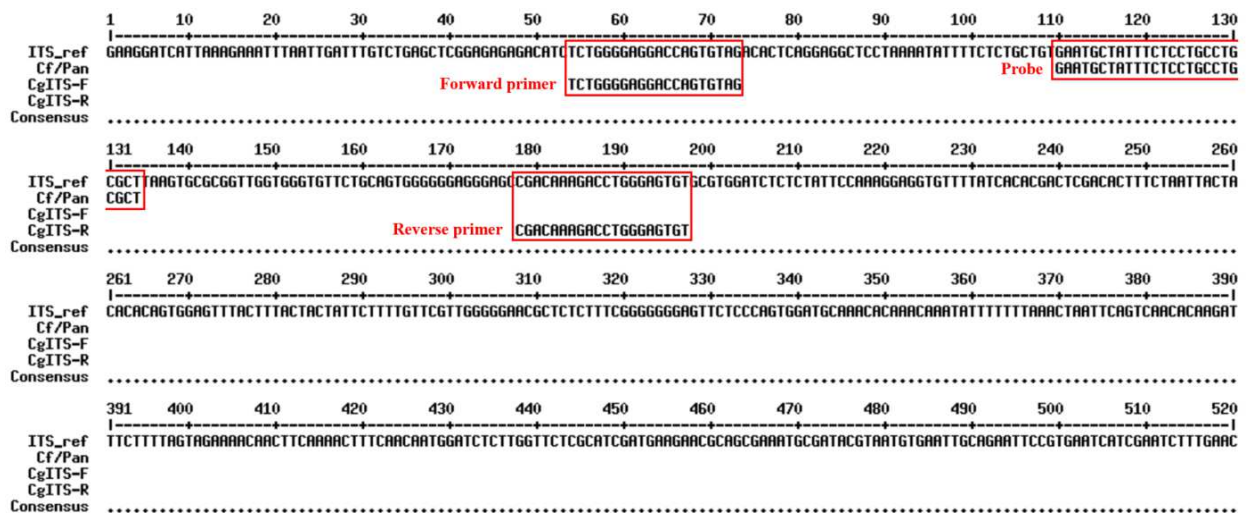


Fig. 2. Multi-alignment analysis of forward primers, reverse primers, and hybridization probes based on reference ITS1 gene sequences.

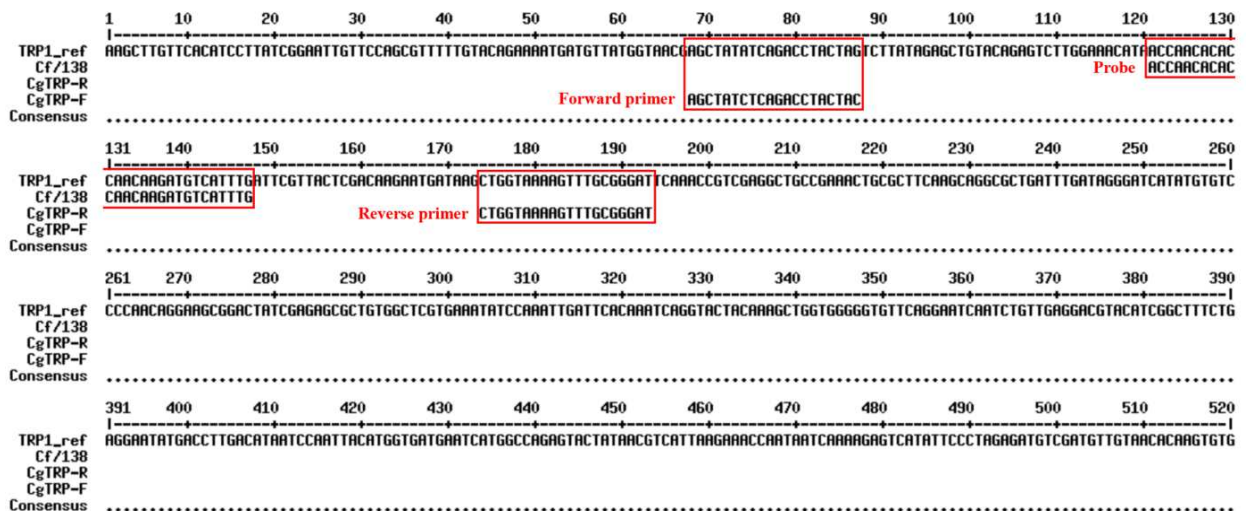


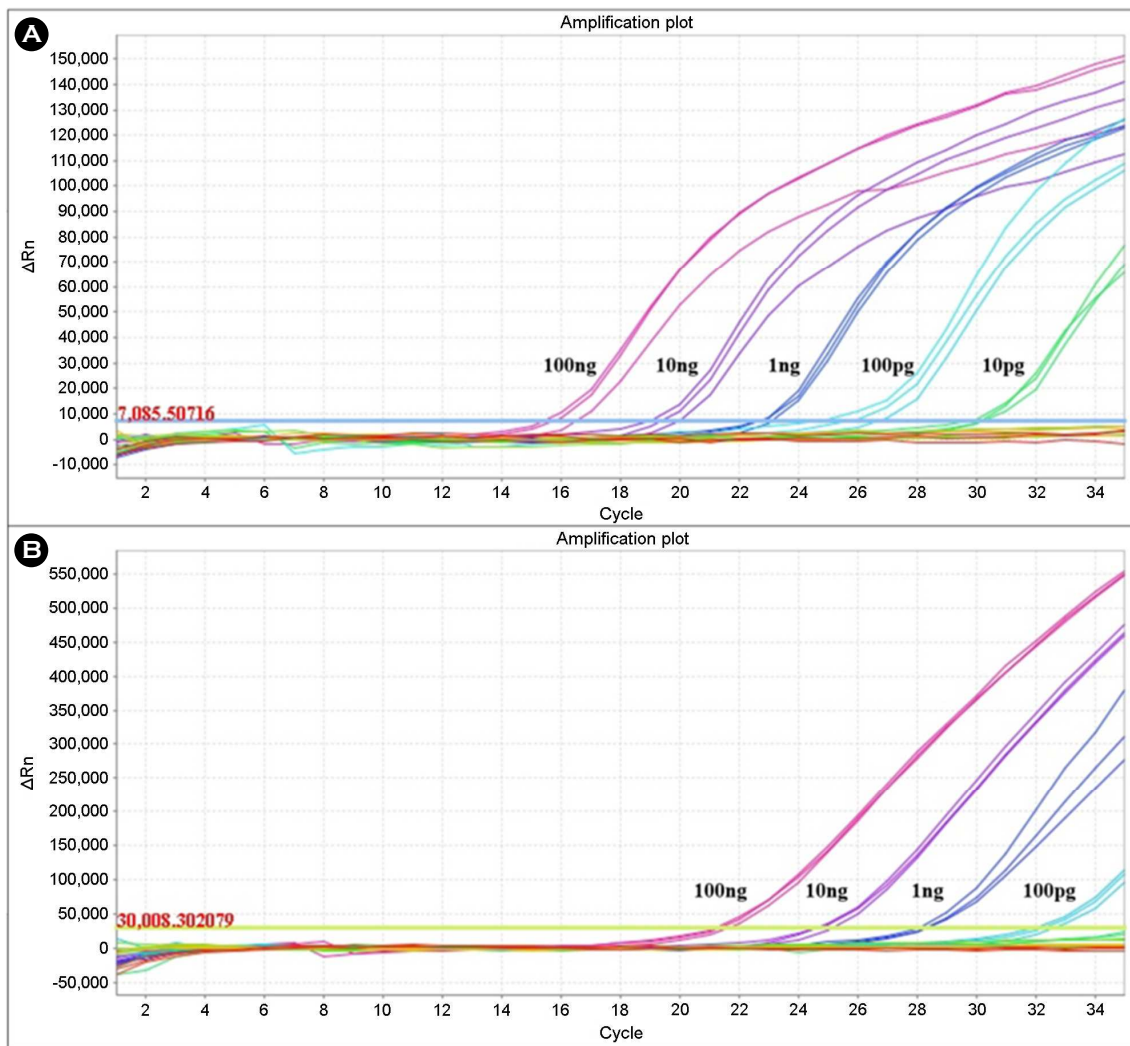
Fig. 3. Multi-alignment analysis of forward primers, reverse primers, and hybridization probes based on reference TRP1 gene sequences.

were contained in this ST. In addition, this study obtained the ST-22, ST-55, and ST-43 were as a total of 6, 3, and 2 clinical isolates were contained in respective ST and the ST-139 was identified in 6 isolates. The ST-140 was identified in 2 isolates and the remaining STs (141, 142, 143, and 144) were classified only once each (Table 3).

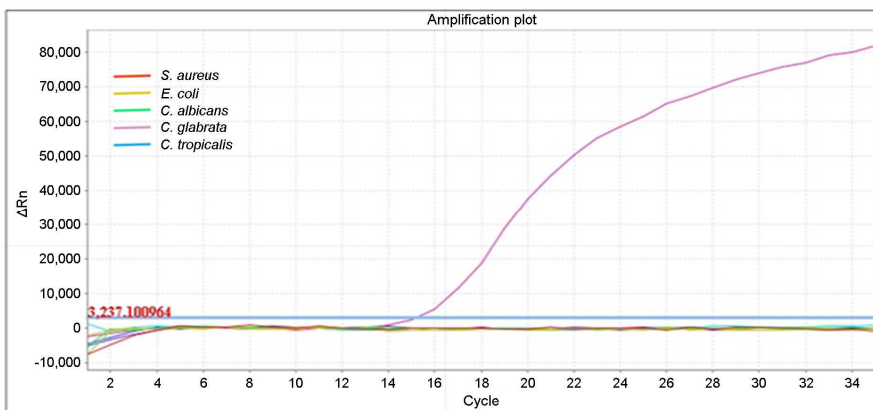
### Analytical sensitivity and specificity of probe-based qPCR assay

The LOD of the qPCR assay of the ITS1 region and TRP1

gene was determined through the use of a 10-fold dilution [100 ng, 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg (per 1 µL)] along a standard curve of gDNA isolated from *C. glabrata* ST-138. The LOD of the qPCR assay of the ITS1 region was 10 pg (per 1 µL) (Fig. 4A). The  $C_T$  values for each sample concentrate ranged from 15.898 to 30.043. The LOD of the qPCR assay of the TRP1 gene was 100 pg (per 1 µL) (Fig. 4B). The  $C_T$  values for each sample concentrate ranged from 21.358 to 32.382. The average  $C_T$  values for each sample concentration are shown in Table 5.

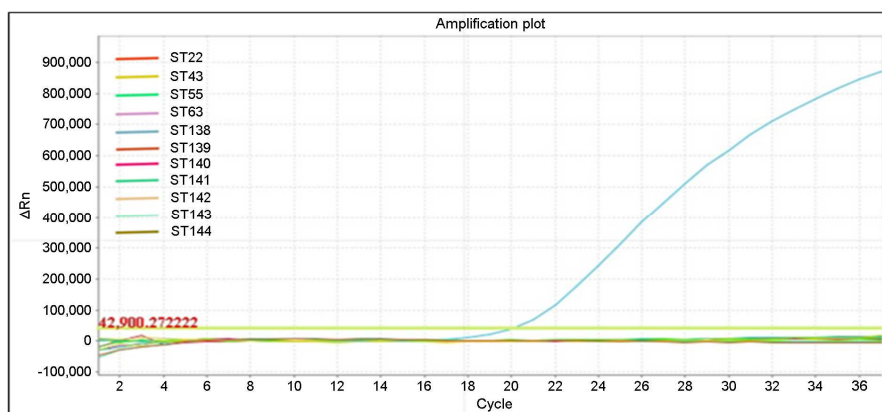


**Fig. 4. Limit of detection of real-time PCR assay.** The LOD of real-time PCR assay of the ITS1 region and *TRP1* gene was determined through the use of a 10-fold dilution [100 ng, 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg (per 1  $\mu$ L)] along a standard curve of gDNA isolated from *C. glabrata* ST-138. (A) the LOD of real-time PCR assay of the ITS1 region was 10 pg (per 1  $\mu$ L). (B) the LOD of real-time PCR assay of the *TRP1* gene was 100 pg (per 1  $\mu$ L).



**Fig 5. Specificity of real-time PCR assay for detection of ITS1 region.** A total of five different strains were used to determine the specificity of the real-time PCR assay for detection of ITS1 region: *C. albicans*, *C. glabrata*, *C. tropicalis*, *S. aureus*, and *E. coli*. The ITS1 region-specific primers detected *C. glabrata* accurately without cross-reaction.





**Fig 6. Specificity of real-time PCR assay for detection of *TRP1* gene.** A total of 11 *C. glabrata* STs were used to determine the specificity of real-time PCR assay for detection of *TRP1* gene: ST-22, ST-43, ST-55, ST-63, ST-138, ST-139, ST-140, ST-141, ST-142, ST-143, and ST-144. The *TRP1* gene-specific primers detected *C. glabrata* ST-138 accurately without cross-reaction.

**Table 5.** Analytical sensitivity of the qPCR assay

Concentration (ng/μL)	Ct Mean	
	ITS region	<i>TRP1</i> gene
100	15.898±0.51	21.358±0.26
10	19.441±0.42	24.865±0.26
1	22.825±0.21	28.244±0.19
0.1	25.739±1.10	32.382±1.10
0.01	30.043±0.32	n.d <sup>1)</sup>
0.001	n.d <sup>1)</sup>	n.d <sup>1)</sup>

<sup>1)</sup>Not detected

A total of five different strains were used to determine the specificity of the qPCR assay for detection of ITS1 region: *C. albicans*, *C. glabrata*, *C. tropicalis*, *S. aureus*, and *E. coli*. The ITS1 region-specific primers detected *C. glabrata* accurately without cross-reaction (Fig. 5). A total of 11 *C. glabrata* STs were used to determine the specificity of the qPCR assay for detection of *TRP1* gene: ST-22, ST-43, ST-55, ST-63, ST-138, ST-139, ST-140, ST-141, ST-142, ST-143, and ST-144. The *TRP1* gene-specific primers detected *C. glabrata* ST-138 accurately without cross-reaction (Fig. 6).

#### Performance of probe-based qPCR assay on *C. glabrata* clinical isolates

A total of 101 gDNA samples from *C. glabrata* clinical isolates were used for the qPCR assay. In a total of 54 *C. glabrata* ST-138 cases, all were positive in targeting the ITS1 region, and 52 cases (96.3%) were positive and two cases (3.7%) negative in targeting the *TRP1* gene. In a total of 47 cases of other STs (22, 43, 55, 63, 139, 140, 141, 142,

**Table 6.** Result of the qPCR assay

Sequence type (ST)	Total samples	Target region	Positive	Negative
ST-138	54	ITS	54 (100)	0 (0)
		<i>TRP1</i>	52 (96.3)	2 (3.7)
ST-63	24	ITS	24 (100)	0 (0)
		<i>TRP1</i>	0 (0)	24 (100)
Others <sup>1)</sup>	23	ITS	23 (100)	0 (0)
		<i>TRP1</i>	0 (0)	23 (100)

<sup>1)</sup>22, 43, 55, 139, 140, 141, 142, 143, and 144

143, and 144), all assays were positive in targeting the ITS1 region and were negative in targeting the *TRP1* gene (Table 6).

## DISCUSSION

Candidiasis is a fungal infection caused by *Candida* species that features various clinical expressions, including superficial, mucocutaneous, and invasive infection (Camacho-Cardoso et al., 2017). Although *Candida* species are commensal organisms that presented in healthy individuals and in the natural environment, some of these fungi can become opportunistic pathogens induced by alteration in the host environment, especially in immunocompromised patients (Sadeghi et al., 2018). *C. albicans* remains the major cause of candidiasis; however, infections by NAC species have also increased, recently. Among them, the incidence of *C. glabrata* infection ranks second in *Candida* species with a high mortality rate, and it is resistant to antifungal agents

(Becker et al., 2000). Notably, *C. glabrata* has a wide minimum inhibitory concentration (MIC) range ( $\leq 32 \mu\text{g/mL}$ ) for fluconazole susceptible-dose dependence (SDD) with no MIC cut-off for the susceptible range, according to the Clinical and Laboratory Standards Institute (CLSI). However, despite fluconazole's wide MIC range, *C. glabrata* has demonstrated reduced susceptibility and high-level resistance to fluconazole therapy (Whaley et al., 2018). The mortality rate of candidemia caused by *C. glabrata* is also increasing, becoming higher than other NAC species due to resistance to azole antifungal agents (Gohar et al., 2017). In spite of its growing prominence, little is known of the population structure, epidemiology, and basic biology of *C. glabrata* (Dodgson et al., 2003).

In order to investigate the epidemiology of these pathogens, it is crucial to perform genotype analysis of *C. glabrata* (Paluchowska et al., 2014). Several genotyping assays have been used to differentiate *C. glabrata*. PFGE compares total DNA banding patterns with or without restriction enzyme digestion. MLEE discriminates genetic variations of the DNA products for a number of loci. RAPD compares banding patterns following PCR with a non-specific primer. The sequence-based methods, MLST analyzes five to seven selected housekeeping genes for SNPs, whereas MLVA examines length variation markers in six to nine PCR-amplified sequences that contain polymorphic tandem repeats (Abbes et al., 2010; Berila and Subik, 2010; Enache-Angoulvant et al., 2010; Katiyar et al., 2016). But these methods have several limitations, in that they are high-priced, time-consuming, and low reproducibility.

In the previous study (Kang, 2017), MLST was performed to analyze prevalent *C. glabrata* STs in Korea. Of a total of 101 *C. glabrata* clinical isolates, 11 different STs were identified and, among them, ST-138 was predominant. Several studies have assessed the *C. glabrata* genotype in Korea by using MLST. In a study by Byun et al. (2018), MLST analysis showed *C. glabrata* ST-7 (100 isolates, 47.8%) was the most common type among 209 *C. glabrata* bloodstream isolates. A comparison between *C. glabrata* ST-7 and ST-138 allele profiles showed only the LEU2 locus was different in their overall allelic profiles. Due to its haploid nature, *C. glabrata* may undergo rapid genotypic change

during infection in humans, unlike the usually diploid *Candida* species. However, little is known about the relationship between the genotypic variation and antifungal therapy among isolates of *C. glabrata* (Shin et al., 2007). It is important to discriminate the most prevalent ST because the investigation of its virulence factor, resistance for antifungal agents or possible routes of transmission can assist in the prevention of pathogenic fungi (Paluchowska et al., 2014). For this reason, we developed a novel genotyping method for rapid and accurate identification of *C. glabrata* ST-138 using a probe-based qPCR assay. qPCR assays have advantages such as rapidity, high sensitivity, less setup cost and reproducibility (Takahashi et al., 2017). The rRNA gene operon, encoding the 18S, 5.8S, and 28S rRNA gene subunits, ITS1, ITS2, and ITS4 are commonly used for specific identification of *Candida* species in PCR-based assay (Silva et al., 2012). One of the housekeeping genes used for analysis *C. glabrata* ST in MLST, the *TRP1* gene has unique polymorphic sites that can identify ST-138 accurately. Of 101 samples, 99 (98%) were concordant compared with MLST results. The results of sequence analysis of two samples were uncertain which specifically targeted the *TRP1* region. Because the *TRP1* gene specific primer targets only two different polymorphic sites in *TRP1* gene, these two inconsistent samples require further sequence analysis for accurate identification.

In conclusion, the novel probe-based qPCR assay proved rapid, sensitive and highly specific identification of *C. glabrata* ST-138. This novel genotyping assay can be used for investigation of epidemiology, virulence factor or resistance to antifungal agents of predominant *C. glabrata* STs in Korea.

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#### CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.



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