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Genetic Variations of *Candida glabrata* Clinical Isolates from Korea using Multi-locus Sequence Typing

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Abstract

Background: Although *Candida albicans* is considered to be the major fungal pathogen of candidemia, severe infections by non-*albicans Candida* (NAC) spp. have been on the increase in recent years. Among NAC spp., *C. glabrata* has emerged as the second most common pathogen. Unlike other *Candida spp*., it is often resistant to various azole antifungal agents, such as fluconazole. However, few studies have been conducted to investigate its structure, epidemiology, and basic biology. Recently, multi-locus sequence typing (MLST) has been developed as a highly useful and portable molecular biology technique.

Methods: In the present study, MLST was performed with a total of 102 *C. glabrata* clinical isolates that were isolated from various types of clinical specimens. The present study was performed with a total of 102 *C. glabrata* clinical isolates that were isolated from various types of clinical specimens. The fungal internal transcribed spacer (ITS) gene wad amplified and sequenced to identify and confirm *C. glabrata* clinical isolates. For MLST, six housekeeping genes including 1,3-beta-glucan synthase (FKS), 3-isopropylmalate dehydrogenase (LEU2),myristoyl-CoA, protein N-myristoyltransferase (NMT1), phosphoribosyl-anthranilate isomerase (TRP1), UTP-glucose-1-phosphate uridylyltransferase (UGP1), and orotidine-5'-phosphate decarboxylase (URA3) were amplified and sequenced. The results were analyzed by using the *C. glabrata* database.

Results: Of a total of 3,345 base-pair DNA sequences, 49 (1.5%) variable nucleotide sites were found and the results showed that a total of 12 different sequence types (STs) were identified from the 102 clinical isolates. As classified by STs, The ST138 was the most predominant sequence type (ST) in this study as a result of 52.9% (54/102), and the following most predominant ST was the ST63 as a result of 23.5% (24/102).

Conclusion: In conclusion, this data demonstrated that the ST138 was the most predominant ST in Korea. Further, we found eight undetermined STs (USTs) and then seven STs among these STs were given the number by PubMLST database. The data from this study might provide a fundamental database for further studies on *C. glabrata*, including its epidemiology, and evolution. Furthermore, the data might also contribute to the development of novel antifungal agents and diagnostic tests.

Keywords: *Candida glabrata*; Candidiasis; Multi-locus sequence typing; Sequence types; Genetic variations

Abbreviations:

MLST: Multi-locus sequence typing; STs: Sequence types; AIDS: Acquired immune deficiency syndrome; NAC: Non-albicans Candida; ITS: Internal transcribed spacer; KCMF: Korean Culture Collection of Medical Fungi; Blast: Basic local alignment search tool; NCBI: National Center for Biotechnology Information; MEGA: Molecular Evolutionary Genetics Analysis; UPGMA: Unweighted Pair Group Method using Arithmetic algorithm

Introduction

Candida species belong to the normal flora of the vaginal tract, the gastrointestinal tract, and the oral cavity in human [1,2]. However, rarely, serious infections, ranging from mucosal infections to systemic infection have been caused by *Candida spp.* [1,2]. Fungal infections caused by *Candida spp.* have increased significantly, especially in acquired immune deficiency syndrome (AIDS) and immunocompromised individuals, including intensive care and, elderly patients [2,3]. Also, candidemia is associated with a high mortality rate approximately 30 to 40% in hospitalized patients and is difficult to treat, thus increasing the cost of medical care [2,3].

C. glabrata has emerged as the second or third most common Candida pathogen after *C. albicans* in the United States, depending on the site [3-5]. Despite its increased prevalence, there have been relatively few studies on the population structure, epidemiology, and basic biology of *C. glabrata* compared to those conducted on other *Candida spp.* [2,4,6].

As mentioned above, *C. albicans* is considered to be the major fungal pathogen of candidemia in the past [6,7]. However, as the number of severe infections caused by non-albicans *Candida spp.* (NAC) have increased, studies have shifted from *C. albicans* to NAC such as *C. glabrata* in recent years [5,8]. Furthermore, since *C. glabrata* infections are often resistant to azole antifungal drugs, especially fluconazole, it is important to distinguish NAC from *C. albicans* to ensure the appropriate antifungal therapy and clinical management [2,5,9]. Thus, the discrimination of subtypes in these species are required for investigating their epidemiology and evolutionary biology [7,10,11].

In recent years, there has been substantial progress in the development of several molecular methods for typing subspecies and strains of fungi [12]. For instance, pulsed-field gel electrophoresis (PFGE) compares total DNA band patterns with or without restriction enzyme digestion, while multilocus variable-number tandem-repeat (VNTR) analysis examines length variations in six to nine PCR-amplified loci that contain polymorphic tandem repeats. Further, the random amplification of polymorphic DNA compares banding patterns following PCR with a nonspecific primer. Finally, multilocus enzyme electrophoresis, studies the different electrophoretic mobility of multiple core metabolic enzymes. These four approaches have some limitations, such as a lower reproducibility and portability [13,14], and the results obtained in different laboratories are difficult to compare [15,16].

Among these genotyping methods, multilocus sequence typing (MLST) is a useful tool to assign single nucleotide polymorphisms as allele numbers, which are stored in a database on line (PubMLST) and determine the differences from between closely related isolates by their geographical origins, sources, and other properties [7]. Also, it is possible that database are accessed by laboratories worldwide [11,15].

In the present study, MLST targets six independent housekeeping genes including 1,3-Beta-glucan synthase (FKS), 3-isopropylmalate dehydrogenase (LEU2), myristoyl-CoA, protein N-myristoyltransferase (NMT1), phosphoribosyl-anthranilate isomerase (TRP1), UTP-glucose-1-phosphate uridylyltransferase (UGP1), and orotidine-5'-phosphate decarboxylase (URA3) was performed with a total of 102 *C. glabrata* clinical isolates from various clinical specimens such as blood, urine, and other body fluids in Korea and results were analyzed by using the *C. glabrata* MLST database (http://pubmlst.org/cglabrata/). The aim of the study is to discriminate sequence types (STs) in the same *C. glabrata spp.* by using common MLST and investigate the most prevalent ST from the *C. glabrata* in Korea.

Methods

Clinical strains

A total of 102 *C. glabrata* clinical isolates were provided from Korean Culture Collection of Medical Fungi (KCMF) and those isolates were collected from tertiary hospitals in Korea. Clinical isolates were isolated from a wide variety of clinical samples, including blood, catheterized urine, bile and other body fluids (Table 1).

Table 1: Details of diverse yeast isolates used in this study.

Clinical specimens	No. of samples (%)
Blood	64 (63)
Urine	14 (14)
Bile	9 (9)
Others[a]	14 (14)
-b	1 (1)
Total	102 (100)

[a]Others : ascitic fluid, joint fluid, pleural fluid, tissue etc.

[b]No clinical information.

Genomic DNA extraction from fungal isolates

Genomic DNA (gDNA) of *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 [17]. The concentration and purity of the genomic DNA were checked by 260/280 optical density using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The extracted gDNA was stored at 4°C until use.

Polymerase chain reaction and sequence analysis of the fungal ITS region for precise identification of *C. glabrata* clinical isolates

The fungal internal transcribed spacer (ITS) region, the conserved region between the 18S and 28S ribosomal RNA (rRNA) was amplified and sequenced using each primer pairs (Table 2). Target amplification was carried out in 20 µL reaction mixture containing 10 µL Prime Taq Primix (Genet Bio Inc., Daejeon, Korea), 5 µL of distilled ultra-pure water, 1 µL of each primer (10 pmol/µL), and 3 µL of genomic DNA template. The

PCR condition was: an initial denaturation at 94°C for 1 min, 30 cycles including subsequent denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 45 sec followed by final extension at 72°C for 7 min and holding at 4°C. The amplified products were visualized by gel electrophoresis to confirm the presence of desired product.

Table 2: Sequences of primer pairs for fungal species identification.

C. glabrata sequence types	No. of isolates (%)
ST63	24 (23.5)
ST22	6 (5.9)
ST55	3 (2.9)
ST43	2 (1.2)
ST138	54 (52.9)
ST139	6 (59)
ST140	2 (1.2)
ST141	1 (1)
undetermined ST1	1 (1)
ST142	1 (1)
ST143	1 (1)
ST144	1 (1)
Total	102 (100)

The resulting amplicon was purified and sequenced by Macrogen Inc. (Daejeon, Korea). All sequences with low-quality bases in the chromatogram were re-sequenced for the high-quality results.

The obtained sequences were aligned with reference sequences in the Genebank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI), and percent homology scores were generated to precise identification of *C. glabrata* clinical isolates.

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

Table 3 shows primers for the amplification and sequence analysis of *C. glabrata* six housekeeping gene fragments including FKS, LEU2, NMT1, TRP1, UGP1, and URA3. For the PCR amplification, 20 µL of final mixture contained 10 µL of Prime Taq Premix, 5 µL of distilled ultra-pure water, 1 µL of each forward and reverse primer (10 pmol/µL), and 3 µL of genomic DNA template.

To amplify each six gene, the PCR reaction conditions were as follows: 7 min at 94°C, 30 cycles of 1 min at the relevant annealing temperature (Table 3), and 1 min at 74°C, followed by 10 min at 74°C.

Table 3: List of gene fragments and primer sequences for *C. glabrata* MLST analysis.

Locus	No. of polymorphic sites (%)	No. of alleles defined	Variable positions
FKS	5 (0.9)	5	403, 352, 154, 118, 43
			392, 384, 336,
LEU2	6 (1.2)	4	290, 111, 54
			575, 551, 512, 480,
			434, 396, 341, 328,
NMT1	12 (2.0)	8	305, 301, 243, 155
			387, 378, 357, 352,
			333, 309, 272, 268,
TRP1	13 (3.1)	8	255, 229, 176, 162, 158
UGP1	4 (0.7)	4	585, 435, 413, 195, 99
			581, 574, 556, 549,
URA3	9 (1.5)	7	440, 380, 257, 164, 44
Total	49 (1.5)		

The resulting sample was analyzed by gel electrophoresis. The PCR product of all loci were purified and sequenced using reverse sequence primer at Macrogen Inc.. The obtained sequences were analyzed by using the *C. glabrata* MLST database (http://pubmlst.org/cglabrata/). Each unique sequence at a locus defined an allele number, and unique combinations of alleles assigned as a ST.

Data Analysis

The alignment of combined six target gene sequences and loci (3,345bp) was performed using the Molecular Evolutionary Genetics Analysis (MEGA) v. 7.0 software [20]. For relatedness of the same species, the phylogenetic tree was drawn with the Unweighted Pair Group Method using Arithmetic algorithm (UPGMA) with randomized 1,000 bootstrapping. And then the eBURST package (http://eburst.mlst.net/) was used to determine that all related isolates were grouped into clonal complexes.

Results

Results of PCR and sequence analysis of the fungal ITS region for species identification

1.5% TBE agarose gel DNA electrophoresis data showed that the size of amplified fungal ITS region was 978 bp, and amplicons have shown one clear band (data not shown). As a analysis result of comparison by Genebank BLAST tool for verifying the amplified PCR products, all clinical isolates used in

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this study were identified as *C. glabrata* with high concordance rate (97.8% ± 2.9).

Results of PCR and sequence analysis, and obtaining allele number of six housekeeping genes for the MLST analysis

In order to perform the MLST analysis, six housekeeping genes of 102 C. *glabrata* clinical isolates were amplified by PCR. The size of amplified fragments of FKS, LEU2, NMT1, TRP1, UGP1, and URA3 were 589 bp, 512 bp, 607 bp, 419 bp, 616 bp, and 602 bp, respectively as the expected size and they represented clear band (data not shown).

Amplified six housekeeping gene fragments were sequenced and then sequenced data was trimmed as each length manually by using Chromas software. Trimmed sequence of six housekeeping gene fragments was analyzed at the *C. glabrata* PubMLST database then allele number of each gene was obtained. Obtained allele number of FKS gene were 3,5,7,8 and 10. Those of LEU2 gene were 5, 6, 16, and 17. Those of NMT1 gene were 3, 4, 6, 8, 11, 14, 19, and 22. Those of TRP1 gene were 2, 3, 5, 7, 10, 12, 19, and 50. Those of UGP1 gene were 1, 3, 5, and 51. Those of URA3 gene were 2, 4, 6, 8, 9, 17, and 20 (Table 4).

A total of 49 (1.5%) polymorphic sites were found among six housekeeping gene fragments. The number of polymorphic site in each gene fragment were as follows: FKS (5, 0.9%); LEU2 (4, 1.2%); NMT1 (8, 2.0%); TRP1 (8, 3.1%); UGP1 (4, 0.7%); and URA3 (7, 1.5%), as shown in Table 4. Additionally, insertions, deletions, or heterozygosity were only detected in the NMT1 fragments.

Table 4: The number of polymorphic site and different alleles, and sequence variable position of six housekeeping gene fragments in 102 *C. glabrata* clinical isolates.

Target region	Primer	Nucleotide sequences (5' to 3')	Amplicon size (bp)	Tm (°C)	Reference
ITS	pITS-F	GTCCTAACAAGGTTAACCTGCGG	970-980	62.4	Pryce et al. [18]
	pITS-R	TCCTCCGCTTATTGATATGC		55.3	

Sequence type and cluster of *C. glabrata* clinical isolates

The MLST scheme revealed a high diversity of *C. glabrata* isolates with a total of 12 STs, 8 of which were identified as undetermined STs (USTs) that were not discovered in the previous studies.

The data demonstrates that the ST138 among these USTs was the most predominant ST in this study as a total of 54 clinical isolates (52.9%) were contained in this ST, and the following most predominant ST was the ST63 as a total of 24 clinical isolates (23.5%) were contained in this ST. In addition, this study obtained the ST55, ST22, and ST43 were as a total of 3 (2.9%), 6 (5.9%), and 2 (1.2%) clinical isolates were contained in respective ST and the ST139 was identified in 6 isolates (5.9%). The ST140 was identified in 2 isolates (1.2%). The remaining 5 STs (UST1, ST141, 142, 143, 144) were classified only once each (1%) (Table 5).

Table 5: Unique sequence types determined with a combination of six loci.

Target gene	Gene product	GenBank accession no.	Primer	Nucleotide sequences (5'-3')	Sequenced fragment size (bp)	Annealing temp (°C)	Reference
FKS	1,3-Beta-glucan synthase	AF229171	FKS F	GTCAAATGCCACAACAACAACCT	589	55	
			FKS R	AGCACTTCAGCAGCGTCTTCAG			
LEU2	3-Isopropylmalate dehydrogenase	U90626	LEU 2F	TTTCTTGTATCCTCCCATTGTTCA	512	54	
			LEU 2R	ATAGGTAAAGGTGGGTTGTGTTGC			
NMT1	Myristoyl-CoA, protein N-myristoyltransferase	AF073886	NMT 1F	GCCGGTGTGGTGTTGCCTGCTC	607	59	
			NMT 1R	CGTTACTGCGGTGCTCGGTGTCG			
TRP1	Phosphoribosyl-anthranilate isomerase	U31471	TRP 1F	AATTGTTCCAGCGTTTTTGT	419	50	Dodgson [6]
			TRP 1R	GACCAGTCCAGCTCTTTCAC			

UGP1	UTP-glucose-1-phosphate uridylyltransferase	AB037186	UGP 1F	TTTCAACACCGACAAG GACACAGA	616	57	
			UGP 1R	TCGGACTTCACTAGCA GCAAATCA			
URA3	Orotidine-5'-phosphate decarboxylase	L13661	URA 3F	AGCGAATTGTTGAAGT TGGTTGA	602	53	
			URA 3R	AATTCGGTTGTAAGAT GATGTTGC			

Combined sequence (3,345 bp) of six housekeeping genes was used for the phylogenetic tree analysis. With the exception of 3 outliers, the isolates were divided into 2 major clusters: cluster 1 and cluster 2 (Figure 1). Cluster 1 consisted of the ST138 and cluster 2 consisted of the ST63 (Figures 2 and 3).

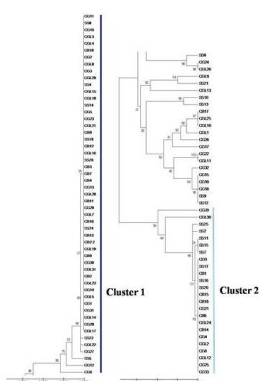

Figure 1: Evolutionary relationships of distinct sequence types-UPGMA dendrogram used to determine the pairwise differences of the concatenated sequence of C. glabrata clinical isolates.

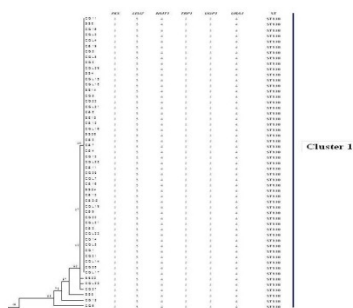

Figure 2: Phylogenetic tree generated from each isolate. The MLST allele profile, sequence type (ST), and eBURST group (Cluster 1) are listed.

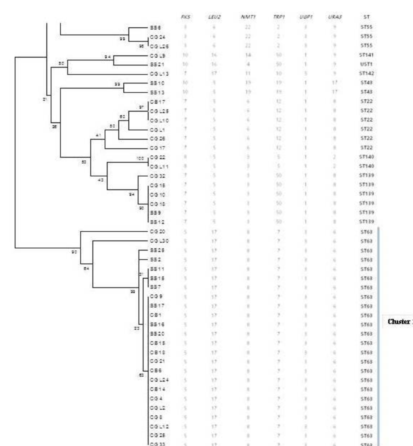

Figure 3: Phylogenetic tree generated from each isolate. The MLST allele profile, sequence type (ST), and eBURST group (Cluster 2) are listed.

Discussion

C. glabrata is a highly opportunistic pathogen of the urogenital tract and the bloodstream in humans [21]. It is especially prevalent in the elderly and within the human immunodeficiency virus positive population [22]. Although candidiasis is frequently treated with azole antifungal agents, treatment failure has become a serious concern with azole-resistant clinical isolates due to widespread and long-term use of these agents. Nevertheless, few studies have been conducted on the structure, epidemiology, and basic biology of *C. glabrata*.

Healthcare-associated infections may be endogenous in origin or nosocomially transmitted, and the only way to distinguish them is through strain typing. Recently, MLST directly investigated the DNA sequence variations in a set of housekeeping genes and characterized the strains by their unique allelic profiles. The principle of MLST is simple, involving PCR amplification followed by DNA sequence analysis. Nucleotide differences between strains can be verified at a variable number of genes depending on the desired degree of discrimination. MLST schemes now exist for a number of important bacterial pathogens including *Neisseria meningitidis*, *Streptococcus pneumoniae, Staphylococcus aureus*, *Streptococcus pyogenes*, and *Campylobacter jejuni*. The technique has also been used to assess genetic relatedness among strains of *Candida spp*. including *C. glabrata, C. albicans, C. tropicalis*, and *C. krusei*. However, MLST scheme for *C.*

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glabrata has only been used by Dodgson et al. [6] and Katiyar et al. [14].

Hence, in this study, the first MLST analysis with the yeast pathogen *C. glabrata* was performed and evaluated in Korea. 6 loci were selected for this study, as recommended by previous studies. While a ST3 was defined as prevalent ST in Dodgson et al. [6], the data in this study demonstrates that the ST138 was the most predominant ST. Additionally, the data defined a total of 12 STs among the 102 clinical isolates, and found 8 USTs as a result and these sequence was given the number except for one ST.

Conclusion

In conclusion, prevalent and novel *C. glabrata* STs were found in the present study. The data might provide a fundamental database for further studies on *C. glabrata*, including its epidemiology and evolution. Furthermore, these data might also contribute to the development of novel antifungal agents and diagnostic tests. It might even be possible to discover the virulence factors associated with disease, which population genetic studies currently struggle to monitor.

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