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Development of Fast Identification Method using a DNA Microarray for *Candida* Species

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Abstract

The development of a fast identification method for *Candida* species has become increasingly important due to high mortality rates from opportunistic infection and the recognized need for optimal therapeutic treatment. Previous *Candida* identification methods have been somewhat limited due to the requirement for a large number of samples, the lack of a simultaneous identification technique, and the existence of a variety of target pathogens. Therefore, fast high-throughput methods need to be developed for an effective pathogen identification method. In this study, we developed fast identification methods for the 6 most common *Candida* species, using a DNA microarray. The *Candida*-specific segment of the ITS (Internal transcribed spacer) region was amplified for targeting and hybridized with the 6 common *Candida* species-specific probes onto aldehyde glass slides. Hybridization patterns in DNA microarrays were identical with the results of sequencing. This DNA microarray-based identification method constitutes a rapid, simultaneous, and high-throughput method for *Candida* species identification, and can be used as an alternative to currently-used methods.

Keywords: *Candida*, Species identification, Microarray, ITS, Opportunistic infection

Introduction

Many *Candida* species are commensal yeasts, but some *Candida* species can potentially cause opportunistic infections. *Candida albicans* is the most significant species in the *Candida*. However, in recent years, antifungal-resistant non-*Candida albicans* species infections have been observed to increase as the result of more widespread use of broad-spectrum antifungal agents[1]. Therefore, the identification of non-*Candida albicans* species, as well as *Candida albicans*, is a matter of critical importance. Many accurate identification methods have been applied to clinical pathogens thus far[2-6]. Conventional *Candida* species identification techniques are based on culture and biochemical experiments, but sometimes these methods have proven challenging, due to their low detection range and time-intensive nature[7]. Recently, PCR-based *Candida* identification techniques have been applied to the identification of *Candida* species[8,9]. However, these PCR-based methods have sometimes proven inconclusive as the result of non-specific PCR amplification. Additionally, PCR-based species identification is not appropriate for use with large numbers of clinical samples, and is too time-consuming to be used as an experimental process for the routine typing of *Candida* infections. Real-time PCR typing has been recognized as a fast method for the identification of *Candida* species, but is not capable of simultaneous typing of many pathogens[10]. Therefore, accurate, simultaneous, and high-throughput identification of *Candida* species is necessary for proper clinical treatment and efficient identification.

In this study, we developed a method for the identification of *Candida* species based on a DNA microarray. To discriminate between the 6 most common *Candida* species, we selected a *Candida*-specific ITS segment, which was the most frequently sequenced DNA region in fungal species. Amplified targets were fluorescently labeled for the detection of the probe signal, and then hybridized with specific oligonucleotide probes onto aldehyde glass slides. In conclusion, hybridization patterns in DNA microarrays were identical with the sequencing results. When applied to a DNA microarray, the entire experimental process was completed in 3.5 hours. This DNA microarray-based identification method allows for the fast, simultaneous and high-throughput identification of *Candida* species, and may be used as an alternative to the methods currently in use.

Results and Discussion

Candida-specific Probes for Species Identification

To discriminate between *Candida* species, we select-

Table 1. Sequence list of probes for *Candida* species identification.

Probe name	Probe sequence	Length (nucleotides)	Melting temperature	Target species
CP01	5′-(AMNIE)TTTTTTTTTAACATTGYTTGCGGCGGTAAC-3′	30	58˚C (mean value)	*Candida albicans*
CP02	5′-(AMNIE)TTTTTTTTTAGGTTTTACCAACTCGGTGTTGATCT-3′	35	58.3˚C	*Candida glabrate*
CP03	5′-(AMNIE)TTTTTTTTTAGTGGCCACCACAATTTATTTCATAAC-3′	36	56.2˚C	*Candida tropicalis*
CP04	5′-(AMNIE)TTTTTTTTTGCCGAGCGAACTAGACTTTTT-3′	30	55.4˚C	*Issatchenkia orientalis* (*Candida krusei*)
CP05	5′-(AMNIE)TTTTTTTTTGAAAGAAAGGCGGAGTATAAACTAAT-3′	35	53.2˚C	*Candida parapsilosis*
CP06	5′-(AMNIE)TTTTTTTTTAACATTGCTAAGGCGGTCTCT-3′	30	56.5˚C	*Candida dubliniensis*

[a]*Candida krusei*: asexual state of *Issatchenkia orientalis*

Table 2. Probe signal intensity of a DNA microarray for *Candida* species identification.

Target species	Signal intensities (background subtracted median value) ± Standard deviation					
	CP01	CP02	CP03	CP04	CP05	CP06
C. dubliniensis	136.2±4.3	174.5±4.4	195.5±4.9	116.2±2.7	128.3±3.9	**12,891.7±180.3**
C. tropicalis	32.7±7.7	81.2±6.0	**6599.8±376.6**	34.0±4.9	28.8±4.4	79.3±6.1
C. parapsilosis	39±17.7	68.7±12.0	95.0±8.1	19.7±2.8	**13,454.8±374.4**	98.0±3.7
C. glabrate	28.2±4.3	**13,671.5±788.4**	152.7±15.4	20.0±3.1	16.7±3.3	80.5±6.1
C. albicans	**8205±162.4**	113.2±5.3	153.8±17.3	24.3±5.1	26.3±4.9	240.8±9.5
Issatchenkia orientalis	20.8±3.6	84.2±7.0	120.8±17.2	**8,624.8±376.7**	42.5±8.1	76.8±3.1

Bold numbers represented positive signal intensity for each specific probe.

ed the ITS region because it harbors generally widely used DNA markers for the identification of fungal species[11,12]. Optimal positioning of probes was based on the alignment of the *Candida* ITS sequence and the specificity of the selected probes was confirmed by a BLAST search. Each specific probe was based on *Candida*-specific sequences and composed of 3 functional structures[13]. First, amine groups on the 5′ end of the probe were a functional group that bound to the microarray substrate. Second, oligonucleotide spacers, to relieve steric hindrance between the solid substrate and the target position of the probe, were composed of nine thymines. Third, the specific binding region, hybridized with the target sequence, was positioned. Each probe on a DNA microarray was identical with the sequence of each species. A *Candida albicans*-specific probe was positioned at nucleotides number 126-146 in the target products, and the melting temperature was 58˚C. When compared with the others, the probe sequence was unique and evidenced a similarity range of 38-76%. In a previous study, when discordance between a perfectly matched target and a non-perfectly matched target was more than two bases, hybridization patterns were clearly observed[13]. The hybridization kinetics were dependent on the nucleotide base contents of the matched position. When the probing position in the target DNA was mismatched by more than two bases[14], the degree of discrimination achievable between the positive and negative targets was sufficient for species identification. The specific probe sequence and properties are shown in detail in Table 1.

Hybridization Patterns using a DNA Microarray for *Candida* Species

The signal intensity of specific probes was used to determine the amount of binding on fluorescently labeled targets. When compared with non-targets in the experimental results, the perfectly matched target signal was sufficient for species identification. In the case of *Candida albicans*, the signal intensity was 8205 (arbitrary units). On the other hand, the non-specific targets evidenced signal intensities of 20.8-139.2. When *Candida dubliniensis* was compared with other species, the positive signal intensity of the perfectly matched signal was approximately 53 times higher than the highest negative signals. In the case of *Candida tropicalis*, the most negative signal intensity was 33 times lower than the positive signal intensity. The *Candida parapsilosis*-specific probe signal was measured at 13,454. When compared with *Candida glabrate* and *Candida dubliniensis*, the signal intensities were 805 and 104 times higher, respectively. The *Candida glabrate*-specific probe signal intensity was

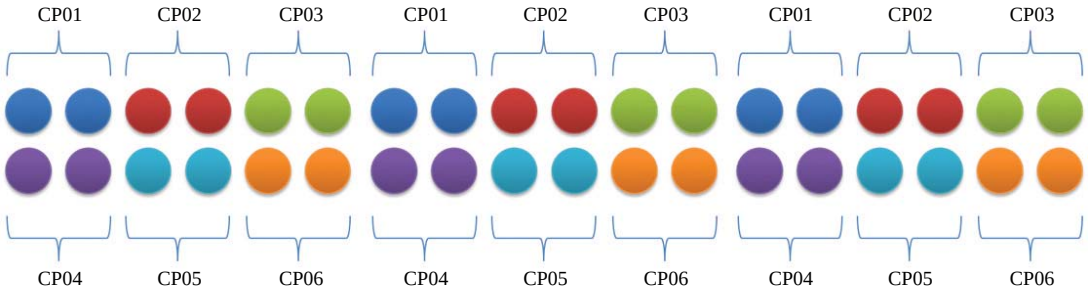

Figure 1. A DNA microarray layout for *Candida* species identification.

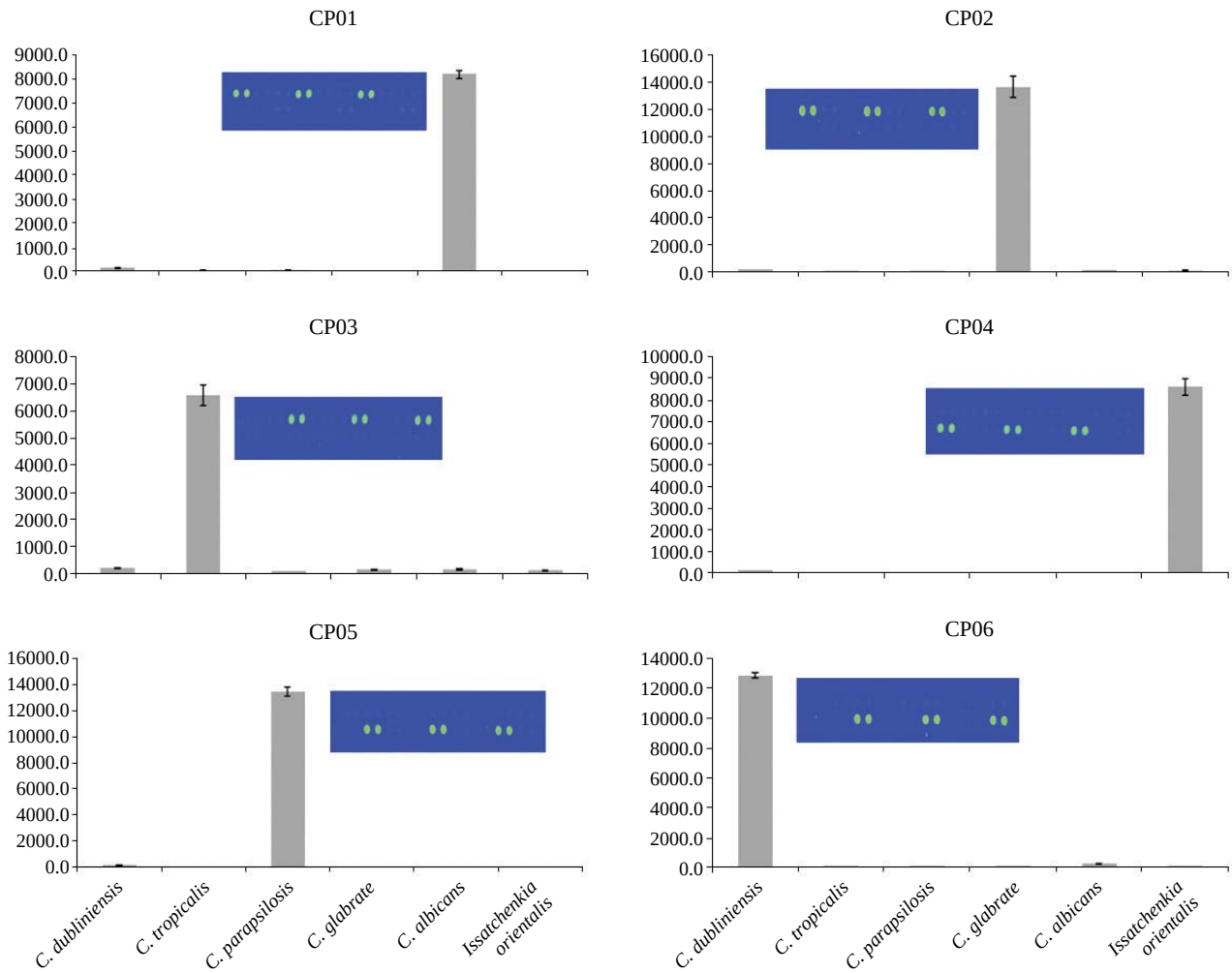

Figure 2. Hybridization patterns and signal intensity plots of *Candida* species identification using a DNA microarray. The Y axis represents the background subtracted from the median value, and the error bars show the standard deviation. The X-axis represents *Candida* species in this study.

13,671, and this signal intensity was 78 times higher than *Candida dubliniensis*. In the case of *Issatchenkia orientalis*, the positive signal intensity was 8,624, and this signal intensity was 74 times higher than *Candida dubliniensis*. The positive signals of each specific probe are shown in Table 2.

It was determined from the results that the hybridization patterns using microarrays were sufficient for

Candida species identification. All hybridization patterns were unambiguous and evidenced high specificity for each target (Figure 2). In a previous study, an important factor, which was a determining factor in the high specificity of the probe, was the position of the fluorescent label relative to the hybridization reaction in a short oligonucleotide microarray experiment[15]. However, the position of the fluorescent label effect was not shown in this study. This result was attributed to the fact that the target products were too short for the position of label effecting the specificity of probes. Also, because the target products were small, in general, all signal intensities of each probe were high as compared with the background signal.

Conclusions

In this study, we applied DNA microarray technology to *Candida* species identification. We selected a specific sequence for each *Candida* species and then a BLAST search was used to confirm specificity. The selected probe sequences utilized a synthesis of specific probe and printing arrays onto aldehyde-modified glass slides. In the series of hybridization experiments, *Candida* species identification using a DNA microarray was shown to be the ideal method for the fast and simultaneous detection of *Candida* species. When each specific probe was compared with non-specific targets, the discrimination of species could be clearly and easily determined by observation of the hybridization patterns. Therefore, the simplicity of a DNA microarray demonstrated its efficacy and practicality as an identification method.

Materials and Methods

Candida Species Samples and DNA Extraction

In this study, 6 common *Candida* species, *Candida albicans* (KCTC7270), *Candida dubliniensis* (KCTC-17427), *Candida tropicalis* (KCTC7212), *Candida parapsilosis* (KCTC7214), *Candida glabrata* (KCTC-7219) and *Issatchenkia orientalis* (KCTC17696), were applied in the development of a *Candida* DNA microarray. All *Candida* species in this study were acquired from the Biological Resource Center (BRC, Daejun, Korea). The genomic DNA of *Candida* species were extracted directly from resuspensions of cell stock using a PureCheck yeast kit (Genocheck, Korea) in accordance with the manufacturer's recommended protocols. Isolated genomic DNA samples were analyzed in terms of quality and quantity via spectrophotometry and gel electrophoresis.

PCR Amplification and Sequencing for *Candida* Species Identification

For *Candida* species identification, we selected the ITS region as a target product. The primers used for the amplification of the target region were based on the results of a previous study[16]. The PCR mixture for sequence analysis consisted of 1X PCR buffer with 1.5 mM magnesium chloride, 0.5 units of *Taq* polymerase (Promega, USA), 0.5 μM of *Candida* specific primer, 200 mM each dNTP, and 10 ng of each *Candida* genomic DNA. Amplification of the target region was conducted using a DNA-ENGENE apparatus (MJ Research, USA); 35 cycles of denaturation at 95˚C for 15 s, annealing at 55˚C for 30 s and 20 s of extension at 72˚C. The PCR products were visually assessed via electrophoresis with 2.0% agarose gels. The visualized products were purified from agarose gels using an Expin Combo GP kit (GeneAll, Korea). After the purification of PCR products as target material, the PCR products were cloned into pGEM-T Easy Vector (Promega, USA) and then transformed into HIT Competent Cells-DH5α (RBC, Taiwan). Clone sequencing was conducted via fluorescent dye-termination methods (Applied Biosystems, USA) and sequence analysis using a genetic analyzer (Applied Biosystems, USA).

Fabrication of a DNA Microarray for *Candida* Species Identification

A total of 6 specific probes were used in this study for the identification of *Candida* species. Each probe was composed of 3 functional structures: an amine group, an oligonucleotide spacer, and a specific binding region. The probe sequence in this study was utilized as described previously with minor modifications[16] (Table 1). The synthesized oligonucleotide probes were resuspended at 100 μM in nuclease-free water. Each specific probe prepared was then mixed with the same volume of Spot buffer (6X SSC, 3M betaine) and stored in 384-well microtiter plates until microarray printing. A *Candida* species identification microarray was printed with each specific probe onto silylated slides (Cell Associate, USA) using a robotic microarrayer (Cartesian Technology, USA). Each probe was printed six times (Figure 1). After the microarrays were printed, the microarrays were incubated to allow for reaction between the amine group of each probe and the aldehyde group onto microarray substrate in a humidity chamber at 25˚C and 60% humidity. Prior to hybridization, the printed microarrays were washed in 0.1% SDS solution to remove non-binding probes and immobilized by reduction using sodium borohydride

solution ($NaBH_4$ 1 g : PBS 300 mL : Ethanol 100 mL). After post-printing treatment, the solution remaining on the microarrays was washed out three times using nuclease-free water and then centrifuged for 5 min at 800 rpm to dry the microarrays.

Hybridization and Scanning of Microarray

The single-strand PCR products required hybridization with specific probes. This process can be done via an asymmetric PCR strategy[17]. In PCR amplification, fluorescently-labeled reverse primer was added to reaction at a concentration 10 times that of the forward primer, and then directly hybridized with the microarrays. All components of the PCR reaction were subjected to the same process for sequencing, with the exception of the concentration of the forward primer. Positive reactions were detected using a Genepix 4000B (Axon Instruments, USA) at a PMT (Photomultiplier Tube) gain of 550, with a laser power of 99%. The fluorescence intensity of each probe was calculated using Genepix 4.1 software (Axon Instruments, USA). Each probe's signals were normalized via the subtraction of the local background median value from the median value.

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