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Original Article

Trf4 is a Useful Gene for Discrimination of *Candida tropicalis* from other Medically Important *Candida* Species

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Abstract

Comparative studies of random amplified polymorphic DNA (RAPD) band patterns of *Candida tropicalis* with those of clinically important *Candida* species have shown the presence of specific RAPD bands for *C. tropicalis*. A band specific to *C. tropicalis* strains (ca. 400 bp) was extracted and sequenced. It was found to belong to a fragment of the *Trf4* gene, which is essential for growth of these strains and has a characteristic sequence of *C. tropicalis*. A PCR primer was designed specifically for *C. tropicalis* which amplifies the 324 bp band. The PCR primer amplified DNA products for all *C. tropicalis* strains tested, but did not amplify any PCR bands from *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. kefer*, *C. krusei*, *C. parapsilosis*, or *C. zeylanoides*. Usefulness of the PCR primer in differentiating from clinical isolates of other fungal species is discussed.

Key words： *Candida tropicalis*, RAPD, specific PCR primer, *Trf4* gene

Introduction

Invasive fungal pathogens such as *Candida albicans*, especially in immunocompromised hosts, can cause life-threatening infections[1]. *Candida* species are the most common fungi that cause such fungal infections: infections caused by *C. albicans* are common in cases of hematogenous candidiasis, followed by *C. krusei*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*. In urinary infections, frequent isolations of *C. glabrata* and *C. tropicalis* have been confirmed, although most are caused by *C. albicans*[2]. Such a significant increase in the frequency of candidiasis attributable to non-*albicans Candida* also promoted the development of new molecular-based techniques aimed at the replacement of traditional methods used for identification, because identification of non-*albicans Candida* species is time-consuming[1]. Among such molecular methods, PCR-based techniques have been widely used for taxonomic and epidemiologic studies. Random amplified polymorphic DNA (RAPD) pattern blot analysis has been used broadly because it is less time consuming and easy[3]. We reported the usefulness of the RAPD band pattern for identification of *Cryptococcus neoformans*[4]. Therefore, RAPD pattern analyses of *C. tropicalis* strains were conducted in comparison with those of other *Candida* species in our laboratory. The DNA that was band-specific for *C. tropicalis* was extracted and sequenced. These band sequences suggest that the band belongs to the *Trf4* gene fragment of *C. tropicalis* and the *Trf4* gene sequence is specific for each *Candida* species. Based on the sequence information, a new PCR primer specifically for the identification of *C. tropicalis* was designed; in this paper, usefulness of the proposed PCR primer for such identification is discussed.

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Materials and Methods

Fungal species

This study used the following species and strains: *Candida tropicalis* IFM 46816^{T} (and 20 other reference and clinical isolates), *C. albicans* ATCC 90028 (including 5 other strains), *C. parapsilosis* ATCC 22019 (and 6 other strains), *C. dubliniensis* IFM 53846 (and 6 other strains), *C. krusei* IFM 51992 (and 1 other strain), *C. glabrata* ATCC 90030 (and 5 other strains), *C. utilis* ATCC 9550 (and 5 other strains), *C. guilliermondii* ATCC 9058 (and 4 other strains), *C. catenulate* IFM 5471, *C. rugosa* IFM 5474, *C. kefyr* IFM 5448, IFM 5450, IFM 40090, IFM 46921, *C. pelliculosa* ATCC 2149, *C. famata* IFM 40123 (and 3 other strains), *Cr. neoformans* ATCC 32045, *Saccharomyces cerevisiae* ATCC 9763, *Trichosporon asahii* IFM 53858, *Trichophyton mentagrophytes* IFM 5210, *Fusarium solani* IFM 5340. Their cultures were inoculated on potato dextrose agar (PDA; Difco Laboratories) slants, then incubated for 48−72 h at 37℃.

DNA extraction

Two loopfuls of the fungal yeast cells or fungal debris from PDA slants were resuspended in 200 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), 250 μl GPT reagent (6 M guanidine thiocyanate dissolved in 50 mM Tris pH 8.3) and 450 μl Tris-buffered phenol (pH 8.0) in an Eppendorf tube (1.5 ml). The DNA extraction was carried out according to the procedures described by Tamura *et al.*[3]

RAPD analysis and DNA sequencing of the band

For RAPD analysis, oligo 1 [5'-ATTGCGTCCA] primer was used. Amplification reactions were performed in a volume of 25 μl of distilled water containing 2.5 μl of primer (20 pM), 2.5 μl of genomic DNA (5 μg/ml) and PCR bead (Ready-to-Go PCR bead; Amersham Pharmacia Biotech). The PCR conditions were the following: 35 cycles of denaturation at 94℃ for 60 s, primer annealing at 50℃ for 60 s, and primer extension at 72℃ for 90 s. The amplified PCR product was extracted and sequenced using a previously described method[3]. A new PCR primer was designed based on the sequenced DNA information using software (Genetyx-Mac; Genetyx Cor.). PCR for identification of *C. tropicalis* was performed with a DNA thermal cycler (TaKaRa) using 35 cycles of denaturation at 94℃ for 60 s, primer annealing at 50℃ for 60 s, and primer extension at 72℃ for 90 s. The DDBJ accession number for the 411 bp band is EU037093.

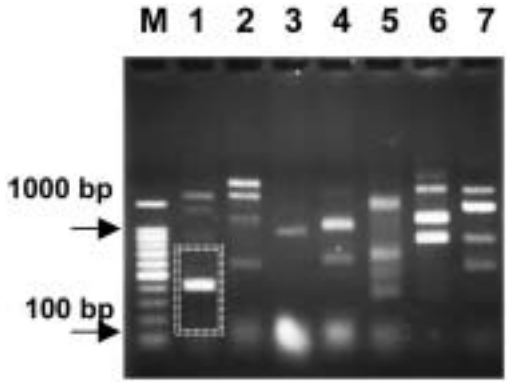

Fig. 1. RAPD band patterns of clinically important *Candida* species in comparison with those of *C. tropicalis*.

M: indicates DNA ladder marker (100 bp). Numbers on the left of the figure indicate molecular sizes (bp), lanes 1: *C. tropicalis* IFM 46816, 2: *C. krusei* IFM 51992, 3: *C. glabrata* ATCC 90030, 4: *C. guilliermondi* ATCC 9058, 5: *C. parapsilosis* ATCC 22019, 6: *C. dubliniensis* IFM 53846, and 7: *C. albicans* ATCC 90028. A 400 bp band surrounded by dotted square in lane 1 indicates a characteristic of *C. tropicalis* strain.

Results

DNA sequence analysis of RAPD band specific for *C. tropicalis* and design for species identification

Our preliminary studies of RAPD band pattern blot analysis against various *Candida* species using oligo 1 primer suggest that *C. tropicalis* has a characteristic band pattern differing from those of other *Candida* species. Therefore, the RAPD band patterns of *C. tropicalis* IFM 46816 were compared with those of the other clinically important *Candida* species (*C. krusei*, *C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, *C. dubliniensis*, *C. albicans*); one PCR band (about 400 bp) (Fig. 1), which was considered to be specific for the *C. tropicalis* strain, was extracted and sequenced (Fig. 1). The determined band size was 411 bp. Results of a BLAST search using the determined sequence suggest that the band belongs to a fragment of the *Trf4* gene[6, 7] of *C. tropicalis*. Comparison of the 411 bp fragment of this gene with those of *Trf4* gene regions of other medically important *Candida* species, *C. albicans* and *C. glabrata* is shown in Fig. 2. The sequence was found to be highly specific for the fungal species, and clearly differentiated from those of *C. albicans* and *C. glabrata*. Therefore, based on the sequence information, the following PCR primer pair CT-oligo 1, which was designed to amplify 324 bp, was prepared: CT- oligo 1, 5'-ATTGGCAACTGAGGTAGA and 5'-CCC-

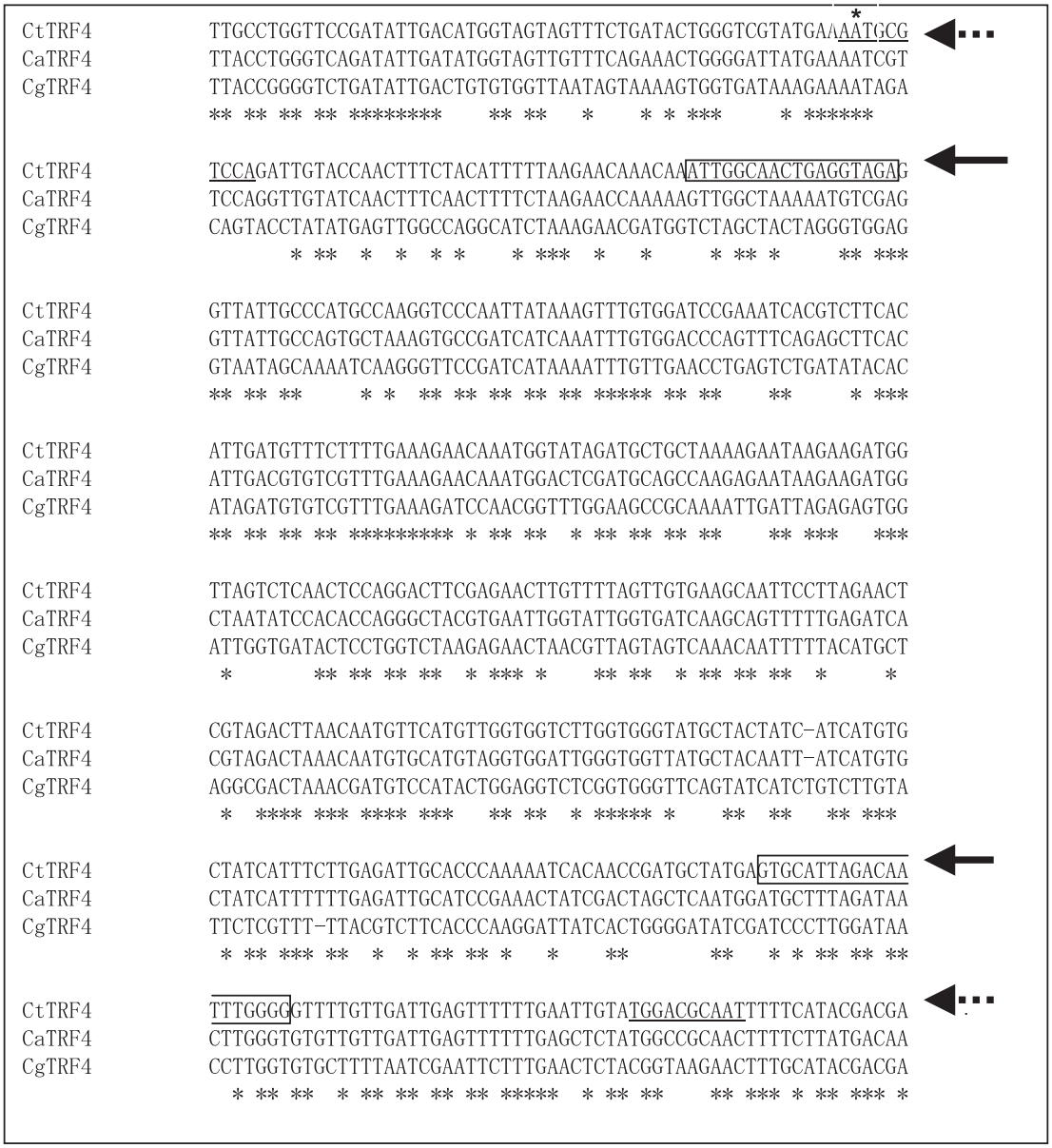

Fig. 2. Comparison of the 411 fragments of *Trf4* genes in *C. tropicalis*, *C. albicans* and *C. glabrata*, and designed PCR primer regions in *C. tropicalis*.

Underlines show PCR oligo 1 primer (5'-ATTGCGGTCCA shown in a dashed arrow) region in *Trf4* gene of *C. tropicalis*. One base (second base "A" from left of underline region) was different from the oligo 1 primer sequence because the base was "T" in the oligo 1 primer. Surrounded dotted square regions correspond to newly prepared primer region (CT-oligo 1 primers in a straight arrow are forward or reverse).

CAAATTGTCTAATGCAC (Fig. 2).

Specificity of the PCR primer for detection of *C. tropicalis*

Figure 3 shows that the CT-oligo 1 primer amplified the DNA band in five strains of *C. tropicalis* including the IFM 40816 type strain. However, no amplification of the DNA band was observed when the following six different *Candida* species were tested: *C. albicans*, *C. dubliniensis*, *C. krusei*, *C. parapsilosis*, *C. glabrata*, and *C. utilis*. When we used living fungal cells from the plate colony instead of the extracted DNA samples used in our previous experiment, all *C. tropicalis* strains amplified the DNA band, suggesting the usefulness of the PCR primer for direct identification of *C. tropicalis* from the colony without DNA extraction. Further con-

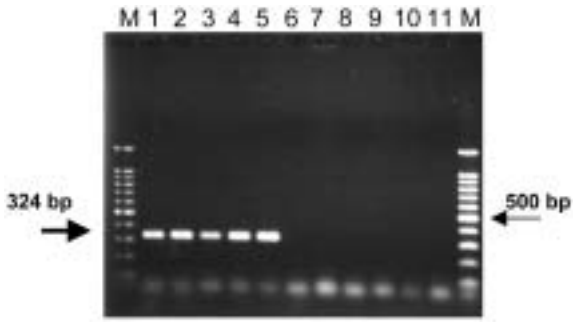

Fig. 3. Specific amplification of *C. tropicalis* strains by newly proposed PCR primer.
M: indicates DNA ladder marker (100 bp). Numbers on the left and right of the figure indicate molecular sizes (bp), lanes 1-5: *C. tropicalis* IFM 46816, IFM 40018, IFM 40085, IFM 40120, IFM 47099, 6: *C. albicans* ATCC 90028, 7: *C. parapsilosis* ATCC 22019, 8: *C. dubliniensis* IFM 53846, 9: *C.krusei* IFM 51992, 10: *C. glabrata* ATCC 90030, and 11: *C. utilis* ATCC 9550.

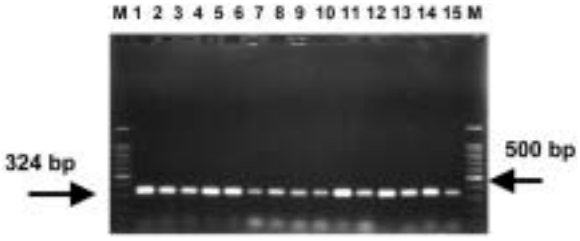

Fig. 4. Direct amplification of *C. tropicalis* IFM 46816 and 15 clinical isolates of *C. tropicalis* strains from Japan, USA and Brazil using colonies from which DNA has not been extracted.
M: indicates DNA ladder marker (100 bp). Numbers on the left and right of the figure indicate molecular sizes (bp); lanes 1: *C. tropicalis* IFM 46816, and lanes 2-15: 14 strains of *C. tropicalis* clinical isolates.

firmation studies using 15 *C. tropicalis* strains showed that all the tested strains amplified the DNA band directly from the colony without extraction of DNA (Fig. 4).

Specificity of this PCR primer was also confirmed using colonies of the following different species and strains of clinically important *Candida* species: *C. dubliniensis* IFM 53846 (and 6 other strains), *C. krusei* IFM 51992 (and 1 other strain), *C. glabrata* ATCC 90030 (and 5 other strains), *C. utilis* (ATCC 9550 (and 5 other strains), and *C. guilliermondii* ATCC 9058 (and 4 other strains), *C. catenulate* IFM 5471, *C. rugosa* IFM 5474, *C. kefyr* IFM 5448, IFM 5450, IFM 40090, IFM 46921, *C. pelliculosa* ATCC 2149, and *C. famata* IFM 40123 (and 3 other strains) (data not shown). The specificity of this PCR primer was also confirmed when we used pathogenic fungal colonies: *Cr. neoformans* ATCC 32045, *S. cerevisiae* ATCC 9763, *Tr. asahii* IFM 53858, *T. mentagrophytes* IFM 5210, and *F. solani* IFM 5340 (data not shown).

Discussion

Recent increases of infections attributable to non-*albicans Candida* have underscored the need for simple and accurate identification methods for such *Candida* species in clinical laboratories. Although several PCR primers have been developed for *C. albicans* strains, few useful PCR primers for *C. tropicalis* are available[3, 4]. During our molecular studies of pathogenic *Candida* species, analysis of *C. tropicalis* by RAPD method showed that fungus-produced specific bands that were not observed in any other *Candida* species were frequently isolated from clinical specimens. Based on sequence information of the determined bands, the band was a fragment of *Trf4* gene. *Trf4* genes have been reported to be essential for cell viability in eukaryotic microorganisms[6]. Aligned fragmented sequences of *Trf4* genes of *C. tropicalis*, *C. albicans* and *C. glabrata* are shown in Fig. 2. The sequence information suggests that sequences of *Trf4* genes are highly specific for each fungal species and such sequences are useful for discrimination of species level of fungi, especially in *Candida*. Based on this information, we prepared a specific PCR primer for identification of *C. tropicalis*, and the present studies confirmed the usefulness of the PCR primer pair for its specific identification. These data further suggest that *Trf4* genes are also useful for the preparation of specific PCR primers for other *Candida* species such as *C. glabrata* and *C. parapsilosis*. Since the *Trf4* gene is an essential gene for eukaryotes[7, 8], the presence of sequences common to all *Candida* species in distinguishing fungal species level is expected. However, we compared the *Trf4* genes among *C. albicans*, *C. glabrata* and *C. tropicalis*, and no common signature sequence useful as a PCR primer was observed. For the preparation of a useful common PCR primer pair to identify medically important *Candida* species at the genus level, the further experiments are required.

Results also confirmed that DNA extraction is unnecessary and that PCR identification using intact fungal cells in the PCR tube is also effective for species identification of *C. tropicalis*, which shortens the PCR procedure.

Our continuing studies suggest that *Trf4* genes give us characteristic signature sequence information for

species identification of highly pathogenic fungi such as *Coccidioides immitis* and *Coccidioides posadasii*. Based on such information, the development of a useful molecular identification system using the *Trf4* gene for the classification of pathogenic fungi belonging to biosafety class 3 is in progress in our laboratory.

Nucci and Colomb (2007) reported that *C. tropicalis* is the second most frequent agent of candidemia in tertiary hospitals in Brazil (20-24%) and cancer is the most frequent underlying disease[9]. Furthermore, it has been reported that the ratio of antimicrobial resistance of *C. tropicalis* clinical isolates to fluconazole is high (14.2%) in comparison with those of other *Candida* species[10]. Therefore a newly developed simple and accurate identification system is expected to fulfill the increased need for specific identification of *C. tropicalis* species in clinical laboratories.

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