LightCycler™ real-time polymerase chain reaction and melting temperature assay targeting the ribosomal DNA gene for the identification of clinically isolated fungal strains, with special reference to *Scedosporium* spp.

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INTRODUCTION

*Scedosporium* is a non-*Aspergillus* filamentous fungal pathogen reported to cause bloodstream infections, including meningitis and endocarditis.¹,² In a report of the epidemiology of invasive fungal diseases caused by non-*Aspergillus* filamentous fungi, *Scedosporium* spp. was the second most common after *Mucor* spp.³ The genus *Scedosporium* consists of two medically important

We developed a LightCycler™ real-time polymerase chain reaction (RTPCR) and melting temperature \((T_m)\) assay targeting the internal transcribed spacer 2 (ITS2) and D1/D2 regions of the fungal ribosomal DNA gene (rDNA), and applied it to differentiate and identify clinically isolated fungal strains, including 2 *Scedosporium* spp., 3 *Aspergillus* spp., and 8 yeast-like fungal species isolated in our laboratory between August 2008 and July 2016. PCR was carried out using the LightCycler™ 2.0 Instrument and \(T_m\) values of the two PCR products in each species were measured. The \(T_m\) values of the ITS2 and D1/D2 amplicons were (mean ± standard deviation [SD]) 89.6 ± 0.20 °C and 89.9 ± 0.07 °C for 3 strains of *S. prolificans*, and 91.0 °C and 91.7 °C for a single strain of *S. apiospermum*, effectively separating them. Furthermore, these \(T_m\) values were lower than those of *Aspergillus* spp., enabling differentiation between the two filamentous fungi. Of the 8 yeast-like fungi tested, 7 exhibited unique \(T_m\) profiles, whereas *Candida krusei* had a similar \(T_m\) profile to *S. apiospermum*. Taken together, the assay enabled the identification of 9 of 13 species studied. The \(T_m\) values of strains of *C. albicans* and *A. fumigatus* were determined with a sensitivity of \(1 \times 10^4\) (ITS2 and D1/D2), and \(1 \times 10^5\) (ITS2) and \(1 \times 10^2\) (D1/D2) colony forming units per mL, respectively, and the assay consistently yielded \(T_m\) values for the ITS2 and D1/D2 products with SDs of ± 0.04–0.21 °C and ± 0.02–0.05 °C, respectively. The LightCycler™ RTPCR-\(T_m\) assay provides fast and reliable information for the differentiation and identification of clinically relevant fungal pathogens, and is of value to initiate effective antifungal therapy promptly.

Keywords: LightCycler™ real-time polymerase chain reaction, melting temperature \((T_m)\) assay, ribosomal DNA gene, *Scedosporium* spp., filamentous and yeast-like fungi
species, i.e. *S. prolificans* and *S. apiospermum*, and in terms of antifungal susceptibility, the former species demonstrates high-level resistance to antifungal agents. Due to the high mortality and increased recognition of *Scedosporium* spp. infections, as well as the species-specific antifungal susceptibility patterns, fast and reliable tests for the identification of these pathogens are required.

The internal transcribed spacer (ITS) and D1/D2 regions of the fungal ribosomal DNA gene (rDNA) exhibit marked interspecies sequence diversity. Thus, polymerase chain reaction (PCR-) amplification and sequencing of these regions, and sequence comparison using available databases provide a useful tool to identify fungal pathogens. However, DNA sequencing is time-consuming and labor-intensive. On the other hand, as the PCR products of ITS regions amplified by pan-fungal primers exhibit a species-specific melting temperature ($T_m$), which is determined by the length of amplicons and the number of guanine and cytidine residues, this property has been applied to species differentiation. To date, two approaches for $T_m$ analysis have been proposed: one is to combine $T_m$ data of multiple amplicons and the other is to increase the resolution capability, enabling detection of minimal $T_m$ differences. LightCycler™ real-time (RT) PCR and $T_m$ assay following the completion of PCR were introduced in diagnostic laboratories, and confirmed to be useful for the fast and reliable differentiation of clinically isolated *Candida* spp. and *Aspergillus* spp.; however, there are few reports on *Scedosporium* spp. The advantages of this assay include molecular probes not being required to be synthesized or labeled, amplification and melting steps being incorporated in a single test tube, and the entire process being completed within one hour.

In this study, we applied the LightCycler™ RTPCR-$T_m$ assay targeting the ITS2 region enclosed by the 5.8S and 28S rDNA genes, in addition to the D1/D2 region within the 28S rDNA, to fungal strains isolated in our laboratory, and investigated whether this assay is capable of effectively differentiating *Scedosporium* spp. from other fungal species.

**MATERIALS AND METHODS**

**Fungal Strains**

The study included 2 *Scedosporium* spp. (*S. prolificans* [n = 3] and *S. apiospermum* [n = 1]), 3 *Aspergillus* spp. (*A. fumigatus* [n = 6], *A. niger* [n = 6], and *A. flavus* [n = 2]), and 29 strains of 8 yeast-like fungi (*Candida albicans* [n = 6], *C. glabrata* [n = 4], *C. guillermondii* [n = 2], *C. parapsilosis* [n = 6], *C. tropicalis* [n = 2], *C. krusei* [n = 4], *Clavispora lusitaniae* [n = 1], and *Cryptoccus neoformans* [n = 4]), all of which were isolated in our laboratory between August 2008 and July 2016. *Scedosporium* strains were cultured in a potato dextrose medium. DNA was extracted using CicaGenius™ DNA Extraction Reagent ST (Kanto Chemical Co. Inc, Tokyo, Japan), and the species was determined by PCR and direct sequencing targeting the D1/D2 region of the 28S rDNA gene. *Aspergillus* spp. were identified by morphological appearance. Yeast-like fungi were subjected to the formic acid/ethanol tube extraction method and processed for matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), and the species was determined by pattern matching using the BioTyper database and software (Bruker Daltonik GmbH, Lepzig, Germany). *C. albicans* ATCC24433 and *A. fumigatus* ATCC204305 were used as type strains.

**LightCycler™ RTPCR-$T_m$ Assay**

DNA from filamentous fungal strains (i.e. *Scedosporium* spp. and *Aspergillus* spp.) was prepared using the MORA-EXTRACT kit (Kyokuto Pharmaceutical Industrial Co., Ltd.). DNA from yeast-like fungi was extracted using the MagNA Pure Compact System (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions.

PCR was carried out using the LightCycler™ 2.0
LightCycler™ RTPCR- \( T_m \) assay for *Scedosporium* spp.

Instrument (Roche Diagnostics). The primers for the ITS2 region were ITS86 (5′-GTGAATCATCGAA TCTTTGAAC-3′) as the forward primer and ITS4 (5′-TCCTCCGCTTATTGATAGC-3′) as the reverse primer, amplifying 200- to 500-bp products, and those for the D1/D2 region were 5′-GCATATCAATAAG CGGAGGAAAAAG-3′ as the forward primer and 5′-GG TCCGTGTTCGAACGG-3′ as the reverse primer, generating approximately 600-bp products. The PCR mixtures in a volume of 20 \( \mu \)L comprised 5 \( \mu \)L (for ITS2) or 2 \( \mu \)L (for D1/D2) of template DNA, each primer at 0.5 \( \mu \)M, 3.5 mM MgCl, and 10 \( \mu \)L of 2 \( \times \) CYBR Green I master mix (Roche Diagnostics). The PCR parameters were: 50 cycles of 10 sec at 95 °C for denaturation, 10 sec at 55 °C for annealing, and 20 sec at 72 °C for extension; for the D1/D2 PCR, the extension time was adjusted to 30 sec due to the large products. After amplification, the temperature was raised from 70 to 95 °C at 0.1 °C per second to melt the double-stranded DNA products apart. The melting curve data were presented as the negative value of \( \frac{d}{dT} \) fluorescence using the LightCycler™ Melting Curves analysis software, providing the melting peak that estimates the \( T_m \) of a given sample.

To assess the sensitivity of the LightCycler™ RTPCR- \( T_m \) assay, we prepared a 10⁰ to 10⁻⁵ series of dilutions of the two type strains, and performed the assay targeting the ITS2 and D1/D2 amplicons. We next repeated the assay 10 times for the two type strains and the two clinically isolated *Scedosporium* species, and investigated whether the assay yielded consistent \( T_m \) values.

RESULTS

\( T_m \) Values Measured by the LightCycler™ RTPCR- \( T_m \) Assay of Fungal Strains

The \( T_m \) values of the ITS2 and D1/D2 products of a total of 47 fungal strains belonging to 13 species are presented in Figure 1. For 8 species, for which three or more strains were tested, the standard deviation (SD) was calculated, ranging from ± 0.03 to ± 0.21 °C for the ITS2 products and from ± 0.04 to ± 0.21 °C for the D1/D2 products.

On comparison of the \( T_m \) values of the ITS products of the two *Scedosporium* spp., the mean (± SD) value of 3 strains of *S. prolificans* (89.6 ± 0.20 °C) was lower than that of a single strain of *S. apiospermum* (91.0 °C). The values of *Aspergillus* spp. were higher than those of *Scedosporium* spp. and *A. fumigatus* had the highest \( T_m \) value (93.0 ± 0.19 °C); the values of the other two species were comparable. Among 5 filamentous fungal species, *S. prolificans* alone had a \( T_m \) value of below 90 °C. On the other hand, for yeast-like fungi, the \( T_m \) values of the ITS2 products were below 90 °C in 7 of the 8 species tested, and overall values of yeast-like fungi were lower than those of filamentous fungi. One exception was *C. krusei*, which had a \( T_m \) value over 90 °C, being comparable to that of *Scedosporium* spp.

Regarding the D1/D2 products, the \( T_m \) values of filamentous fungi were comparable to those of ITS2 products, i.e. *S. prorificans* had the lowest value (89.9 ± 0.07 °C), and those of the remaining 4 species were over 90 °C. In contrast, \( T_m \) values of the D1/D2 products of yeast-like fungal species were higher than those of the ITS2 products, except for *C. neoformans*, which had comparable \( T_m \) values for the two amplicons (86.1 ± 0.21 °C and 86.3 ± 0.12 °C).

**Discrimination of Fungal Species by Combining \( T_m \) Values of the ITS2 and D1/D2 Products**

The correlation between \( T_m \) values of the ITS2 and D1/D2 products of each fungal species is shown in Figure 2. Filamentous fungi had high \( T_m \) values for the two products and were plotted close to the equal line, whereas *S. prolificans* was well separated from the cluster of *Aspergillus* spp and *S. apiospermum* was plotted between the two. In contrast, yeast-like fungi were widely distributed within the area below 90 °C and over the equal line, except for *C. krusei*, which was plotted at a similar position to filamentous fungi.
Figure 1. $T_m$ values of the ITS2 (left) and D1/D2 (right) products of 13 fungal species. The values or mean values with or without SDs for the two amplicons are presented in parenthesis. 1, Scedosporium prolificans (89.6 ± 0.20 °C and 89.9 ± 0.07 °C); 2, Scedosporium apiospermum (91.0 °C and 91.7 °C); 3, Aspergillus fumigatus (93.0 ± 0.19 °C and 91.9 ± 0.10 °C); 4, Aspergillus niger (92.3 ± 0.12 °C and 92.3 ± 0.21 °C); 5, Aspergillus flavus (92.3 °C and 92.0 °C); 6, Candida albicans (86.3 ± 0.09 °C and 89.3 ± 0.11 °C); 7, Candida glabrata (85.2 ± 0.03 °C and 89.4 ± 0.10 °C); 8, Candida guilliermondii (84.4 °C and 87.4 °C); 9, Candida parapsilosis (84.7 ± 0.14 °C and 85.6 ± 0.06 °C); 10, Candida tropicalis (84.0 °C and 86.5 °C); 11, Candida krusei (90.6 ± 0.05 °C and 91.5 ± 0.04 °C); 12, Clavispora lusitaniae (87.2 °C and 90.2 °C); and 13, Cryptococcus neoformans (86.1 ± 0.21 °C and 86.3 ± 0.12 °C).

Figure 2. Correlation between the $T_m$ values of ITS2 and D1/D2 products of each fungal species. When two or more strains were tested, the mean values were presented. 1, Scedosporium prolificans; 2, Scedosporium apiospermum; 3, Aspergillus fumigatus; 4, Aspergillus niger; 5, Aspergillus flavus; 6, Candida albicans; 7, Candida glabrata; 8, Candida guilliermondii; 9, Candida parapsilosis; 10, Candida tropicalis; 11, Candida krusei; 12, Clavispora lusitaniae; 13, Cryptococcus neoformans; 14, Candida albicans ATCC24433; and 15, Aspergillus fumigatus ATCC204305. The last two type strains are indicated by open circles.
**Sensitivity and Consistency of the LightCycler™ RTPCR-\(T_m\) Assay**

We applied the LightCycler™ RTPCR-\(T_m\) assay to the dilution series of type strains. As shown in Table 1, the assay was able to measure the \(T_m\) values of both ITS2 and D1/D2 products at \(1 \times 10^4\) colony forming units (CFU)/mL for *C. albicans*, whereas for *A. fumigatus*, the \(T_m\) of the D1/D2 products was measured at \(1 \times 10^2\) CFU/mL. To evaluate the consistency of the assay, we next performed 10 consecutive runs for the two type strains and the two *Scedosporium* species, and calculated the run-to-run variation. As a result, the assay consistently yielded \(T_m\) values for the ITS2 and D1/D2 products with SDs of ± 0.04–0.21 °C and ± 0.02–0.05 °C, respectively. The melting profiles of 10 consecutive runs of the two type strains are shown in Figure 3, confirming consistent \(T_m\) values with little run-to-run variation.

**DISCUSSION**

We developed the LightCycler™ RTPCR-\(T_m\) assay targeting both ITS2 and D1/D2 amplicons, and investigated whether the combination of \(T_m\) values of the two PCR products effectively differentiates *Scedosporium* spp. from other fungal species. As the expected sizes of PCR products amplified by the D1/

![Figure 3. Screenshot of the LightCycler™ RTPCR-\(T_m\) assay, showing 10 consecutive runs for *C. albicans* ATCC24433 (A) and *A. fumigatus* ATCC204305 (B).](image)

**Table 1. Sensitivity and run-to-run variation of the LightCycler™ RTPCR-\(T_m\) assay for 2 type strains and 2 *Scedosporium* species**

| Fungal strains          | ITS2  | D1/D2  |       |       |       |       |
|-------------------------|-------|--------|-------|-------|-------|-------|
|                         | Sensitivity (CFU/mL) | \(T_m\) (°C)* | SD*   | Sensitivity (CFU/mL) | \(T_m\) (°C)* | SD*   |
| *Candida albicans*      | \(1 \times 10^4\)  | 86.4    | 0.15  | \(1 \times 10^4\)  | 89.3    | 0.02  |
| ATCC 24433              |       |        |       |       |       |       |
| *Aspergillus fumigatus* | \(1 \times 10^3\)  | 93.6    | 0.21  | \(1 \times 10^2\)  | 91.9    | 0.03  |
| ATCC 204305             |       |        |       |       |       |       |
| *Scedosporium prolificans* | Not tested | 89.6    | 0.06  | Not tested | 90.0    | 0.04  |
| *Scedosporium apiospermum* | Not tested | 90.5    | 0.04  | Not tested | 91.6    | 0.05  |

*Mean \(T_m\) values and SDs were calculated by 10 consecutive runs.*
D2 primer set (~600 bp) were larger than the preferable product size (< 500 bp) for LightCycler™ RT-PCR, whether the D1/D2 region is a suitable target was of concern. However, the assay readily measured the $T_m$ values of D1/D2 products with a high consistency and higher sensitivity than the ITS2 products (Table 1). To the best of our knowledge, this study is the first to combine $T_m$ values of ITS2 and D1/D2 in order to identify fungal species isolated from clinical materials.

We first found that *Scedosporium* spp. had lower $T_m$ values than *Aspergillus* spp., effectively differentiating the two filamentous fungal pathogens. We next found that the mean $T_m$ value of 3 strains of *S. prolificans* was lower than that of a single strain of *S. apiospermum*. As the SDs of both ITS2 and D1/D2 amplicons in the former species were small (i.e. $\pm 0.20$ °C and $\pm 0.07$ °C, respectively), and the assay yielded consistent $T_m$ values in multiple runs (Table 1), we concluded that the $T_m$ values of *S. prolificans* and *S. apiospermum* do not overlap. Therefore, the two species were reliably differentiated. Lastly, we found that, of the 8 yeast-like fungi examined, 7 species had unique $T_m$ profiles, as illustrated in the ITS2-D1/D2 scatterplot (Figure 2). Taken together, the LightCycler™ RT-PCR-$T_m$ assay successfully identified 9 of the 13 species examined.

The $T_m$ values of species other than *Scedosporium* spp. measured in this study were not necessarily identical to those in earlier studies, which may have been due to the instruments used in each laboratory or inter-laboratory differences in the composition of the PCR reaction mixture. However, as noted in this and previous studies, filamentous fungi had higher $T_m$ values for the ITS2 products than yeast-like fungi and *C. krusei* had markedly high $T_m$ values compared with other yeast-like fungal species. On the other hand, in a study detecting and identifying fungal pathogens directly from blood culture specimens, the authors instead targeted the $\beta$-tubulin gene to identify *S. prolificans*; they did not include *S. apiospermum* and the method required 2 rounds of PCR.

As the $T_m$ values of *S. apiospermum* and *C. krusei* were similar, the two species were not differentiated by the LightCycler™ RT-PCR-$T_m$ assay alone, and careful examination of morphological characteristics is therefore essential for their differentiation. Bloodstream infections of *Scedosporium* spp. in immunocompromised patients have been reported in Japan and antifungal susceptibility varies among species. As *S. prolificans* is resistant to many antifungal agents, rapid identification of this pathogen is of value to promptly initiate effective antifungal therapy. Limitations of this study include only a single strain of *S. apiospermum* being examined, materials being limited to strains isolated and previously characterized in our laboratory, and dermatophytes, for which 7 types of primers were proposed for identification, were not investigated. Our LightCycler™ RTPCR-$T_m$ strategy is unique in that both ITS2 and D1/D2 amplicons are targeted, providing fast and reliable information for the differentiation and identification of clinically relevant fungal pathogens. To confirm the clinical value of our assay, we are planning to apply it to filamentous and yeast-like forms obtained from positive blood culture bottles, and evaluate $T_m$ data of a large number of fungal strains under a variety of clinical settings.

**CONCLUSION**

We developed the LightCycler™ RT-PCR-$T_m$ assay targeting both ITS2 and D1/D2 amplicons. The combination of $T_m$ values of the two PCR products effectively differentiated and identified clinically isolated filamentous and yeast-like fungal pathogens, including *Scedosporium* spp.

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LightCycler™ RTPCR- \( T_m \) assay for \textit{Scedosporium} spp.

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リボゾーム DNA 遺伝子を標的とした LightCycler™リアルタイム PCR・融解温度分析法を用いた臨床分離真菌株の鑑別と同定 - Scedosporium 属に着目して-

今回は我々は、真菌リボゾーム DNA 遺伝子の internal transcribed spacer 2 (ITS2) と D1/D2 の 2 つの領域を標的とした LightCycler™ リアルタイム PCR・融解温度 (Tm) 分析法を開発し、本法を、Scedosporium 属を含む臨床分離真菌株の鑑別と同定に応用した。菌株は 2008 年 8 月から 2016 年 7 月までの間に当院で分離された糸状真菌 5 菌種と酵母様真菌 8 菌種を対象とした。LightCycler™ 2.0 を用いて ITS2 と D1/D2 の PCR 産物の Tm 値を求め、2 つの Tm 値の組み合わせから分離株を鑑別・同定した。S. prolificans 3 株の ITS2 と D1/D2 領域の PCR 産物の Tm 値（平均値±標準偏差 [SD]）は、それぞれ 89.6 ± 0.20 °C と 89.9 ± 0.07 °C、S. apiospermum 1 株の Tm 値は、それぞれ 91.0 °C と 91.7 °C で、2 菌種を鑑別することができた。Scedosporium 属の Tm 値は Aspergillus 属のそれよりも低く、これらの 2 つの糸状真菌の鑑別も可能であった。酵母様真菌では、8 菌種中 7 菌種で特異的な Tm 値プロフィルを示した。例外的に、Candida krusei は S. apiospermum に類似の Tm 値プロフィルを示した。従って、検討した 13 菌種中 9 菌種の鑑別・同定が可能であった。ATCC 株を用いた検出感度は、C. albicans では ITS2、D1/D2 とも 1 × 10^5 CFU/mL、A. fumigatus では ITS2 が 1 × 10^5 CFU/mL、D1/D2 が 1 × 10^2 CFU/mL であった。測定を 10 回繰り返し Tm 値の再現性を確認したところ、ITS2 と D1/D2 領域の SD は、それぞれ ± 0.04–0.21 °C と± 0.02–0.05 °C であった。今回開発した LightCycler™ リアルタイム PCR・Tm 分析法は、臨床分離真菌の鑑別・同定に関する迅速かつ信頼性の高い情報を提供し、有効な抗真菌治療を早期に開始するうえで価値が高い。

キーワード：LightCycler™リアルタイム PCR、融解温度 (Tm) 分析、リボゾーム DNA 遺伝子、Scedosporium 属、糸状真菌と酵母様真菌