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One Step Multiplex PCR for Identification of *Candida haemulonii* complex and *Candia auris*

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Abstract

Purpose: Recently, *Candida haemulonii* complex (*Candida haemulonii*, *Candida duobushaemulonii* and *Candida haemulonii* var. *vulnera*) and two genetically close species (*Candida pseudohaemulonii* and *Candida auris*) have emerged as an opportunistic fungal pathogen associated with various infectious diseases of humans, and most of those isolates have displayed antifungal resistance. Because it is difficult to differentiate these microorganisms by a current technique, unfortunately, it is important to establish a method for identifying them accurately. The purpose of the present study was to design species-specific primers in order to identify and detect *C. auris*, *C. pseudohaemulonii*, and *C. haemulonii* complex, *i.e.*, *C. haemulonii*, *C. duobushaemulonii* and *C. haemulonii* var. *vulnera* using a multiplex PCR. **Methods:** Polymerase chain reaction (PCR) primers were designed based on partial sequences of the 26S rRNA, 18S rRNA, and *RPB*1 genes and ITS region of five *Candida* species. **Results:** The multiplex PCR method developed in this study was able to distinguish five *Candida* species clearly. **Conclusion:** Our developed one-step multiplex PCR method is accurate, specific, cost-effective, time-saving, and works without requiring DNA extraction.

Keywords

Genus *Candida*, *Candida haemulonii* complex, Multiplex PCR, *Candida auris*

1. Introduction

It is estimated that there are as many as 80,000 fungal species in the fungal kingdom, but less than 1% of these species are involved in infection. Even among these few species, the classification of fungi has long been confusing, but in recent years, with the introduction of DNA sequencing, the isolation of pathogenic fungi has made remarkable progress. The significance of isolation in the field of medical mycology is the contribution it makes to diagnosis and treatment by identifying the veritable causative organisms.

The genus *Candida* is the most important fungus in medical mycology [1], and the incidence of candidiasis has been on the rise in recent years [2]. Candidiasis is mainly caused by four *Candida* species, *i.e.*, *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* [3] [4]. *Candida albicans* is the most virulent and commonly isolated species from clinical samples, even though the reports of non-albicans species are increasing [5] [6]. These microorganisms frequently cause fungemia [7] and vulvovaginal infections [8]. Moreover, some species have been regarded as important nosocomial pathogens in newborns [9], elderly people [10], transplant recipients [11], and immunocompromised patients [12].

Some *Candida* species have been reclassified due to the description of new genetically related species. These microorganisms are currently divided into four complexes of cryptic species [13]. *C. albicans* complex comprises *C. albicans*, *C. dubliniensis* [14], and *C. africana* [15]. *C. glabrata* complex comprises *C. glabrata* [16], and *C. nivariensis* [17]. *C. parapsilosis* complex comprises *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis [18]*. A fourth complex of cryptic species called *C. haemulonii* complex includes *C. haemulonii*, *C. haemulonii var. vulnera*, and *C. duobushaemulonii* [19]. Other phylogenetically closely related species to *C. haemulonii* complex have been also registered, *i.e.*, *C. pseudohaemulonii*, *C. auris* and others [20] [21].

An accurate identification of cryptic species in the clinical setting is required in epidemiology and medicine. It is also important to better understand the evolution of antifungal resistance. The most notable example of the importance of identifying cryptic species might be the emergence and rapid diffusion of *C. auris*. This organism is considered a serious threat to public health worldwide due to frequent relapses and treatment failures [22] [23]. The emergence of new cryptic species of *Candida* poses a challenge for clinical laboratories because it is not always possible to have updated methodologies for their correct identification, particularly in low-income countries. Conventional methods based on carbohydrate assimilation or chromogenic media are designed to identify the most common yeast species but cannot detect all cryptic species. The introduction of matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) in the clinical laboratory has greatly improved the identification of fungi [24]. As a result of the implementation of this approach, it is possible to identify most cryptic species, however, this technology is still expensive, requires constant database updates, and its use is limited to high-income countries or third/fourth-level hos-

pitals.

C. haemulonii complex (*C. haemulonii*, *C. haemulonii var. vulnera*, and *C. duobushaemulonii*), *C. pseudohaemulonii* and *C. auris* are rarely detected in human specimens; however, it remains unclear whether those are the human resident microorganisms or not, and where the source of infection is. The purpose of the present study was to design species-specific primers in order to identify and detect five *Candida* species, *i.e.*, *C. haemulonii*, *C. haemulonii var. vulnera*, *C. duobushaemulonii*, *C. pseudohaemulonii* and *C. auris*, using a multiplex PCR.

2. Materials and Methods

2.1. Fungal Strains and Culture Conditions

Fungal strains were obtained from the Japan Collection of Microorganisms (JCM; Japan) and Medical Mycology Research Center, Chiba University (IFM; Japan). The following bacterial strains were used in the present study: *C. haemulonii* JCM 3762, *C. duobushaemulonii* IFM 64590, *C. pseudohaemulonii* JCM 12453, and *C. auris* JCM 15448. *C. haemulonii var. vulnera* NUM-CHV 1010 was isolated with a non-selective medium, *i.e.*, BHI-Y, from the human sample. These strains were maintained by cultivating them on Bact™ Brain Heart Infusion (BHI, Becton, Dickinson and Co., Sparks, MD, USA) and 1.5% agar (BHI agar), adjusted to pH 7.2. These microorganisms were cultured at 30˚C overnight under an aerobic condition.

2.2. Design of Species-Specific Primers for Five *Candida* Species

Design of species-specific primers for five *Candida* species, *i.e.*, *C. haemulonii*, *C. haemulonii var. vulnera*, *C. duobushaemulonii*, *C. pseudohaemulonii* and *C. auris* was performed as described previously [11]. Briefly, the 26S rRNA gene sequences of *C. haemulonii* (accession no. AY267823), *C. haemulonii var. vulnera* (JX459789), *C. duobushaemulonii* (JX459765), *C. pseudohaemulonii* (AB118792), and *C. auris* (AB375773) and the 18S rRNA gene sequences of *C. haemulonii* (AY500375), *C. haemulonii var. vulnera* (JX459688), *C. duobushaemulonii* (LC317494), *C. pseudohaemulonii* (MT974625), and *C. auris* (AB375772) and the internal transcribed spacer (ITS) region sequences of *C. haemulonii* (JX459660), *C. haemulonii var. vulnera* (JX459686), *C. duobushaemulonii* (JX459666), *C. pseudohaemulonii* (AB118792), and *C. auris* (EU884177) and the largest subunit of RNA polymerase II (*RPB*1) gene sequences of *C. haemulonii* (JX459692), *C. haemulonii var. vulnera* (JX459719), *C. duobushaemulonii* (JX459698), *C. pseudohaemulonii* (JX459704), and *C. auris* (JX459712) were obtained from the DNA Data Bank of Japan (DDBJ; https://www.ddbj.nig.ac.jp/services.html, Mishima, Japan), and a multiple sequence alignment analysis was performed with the CLUSTAL W program; *i.e.*, each gene sequence of five *Candida* species was aligned and analyzed, respectively. Homology among the primers selected for each *Candida* species and their

respective gene sequences was confirmed by a BLAST search.

2.3. Development of a Multiplex PCR Method Using Designed Primers

Bacterial cells were cultured in BHI supplemented with 0.5% yeast extract for 24 h, and 1 ml of the samples were then collected in microcentrifuge tubes and re-suspended at a density of 1.0 McFarland standard (approximately 10^7 colony-forming units (CFU)/ml) in 1 ml of sterile distilled water. A total of 5.6 µl of the suspension was then used as a PCR template. The detection limit of PCR was assessed by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The multiplex PCR mixture contained 0.2 µM of each primer, 10 µl of 2 × MightyAmp Buffer Ver.3 (Takara Bio Inc., Shiga, Japan), 0.4 µl of MightyAmp DNA Polymerase (Takara), and 5.6 µl of the template in a final volume of 20 µl. PCR reactions were performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, CA, USA). PCR conditions included an initial denaturation step at 98˚C for 2 min, followed by 30 cycles consisting of 98˚C for 10 s and 68˚C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis before being visualized by electrophoresis in 1 × Tris-borate-EDTA on a 2% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a molecular size marker. All experiments were performed in triplicate.

3. Results

3.1. Primer Design

Ten specific primers covering the upstream regions of the 26S rRNA gene, 18S rRNA gene, ITS region and *RPB*1 gene sequences of five *Candida* species were designed in the present study (**Figures 1-4**). The specific forward primers were designated as CAUF for *C. auris*, CPSF for *C. pseudohaemulonii*, CH + CHVF for *C. haemulonii var. vulnera and C. haemulonii*, CDUF for *C. duobushaemulonis,* and CHF for *C. haemulonii*, whereas the specific reverse primers were designated as CAUR for *C. auris*, CPSR for *C. pseudohaemulonii*, CH + CHVR for *C. haemulonii var. vulnera and C. haemulonii*, CDUR for *C. duobushaemulonis,* and CHR for *C. haemulonii*. The amplicon sizes of *C. duobushaemulonis*, *C. pseudohaemulonii*, *C. auris* and *C. haemulonii var. vulnera* were 106 bp, 260 bp, 346 bp, 420 bp, respectively. That of *C. haemulonii* was 248 bp and 420 bp.

3.2. Multiplex PCR

Our multiplex PCR method for identifying and detecting five *Candida* species, *i.e.*, *C. auris*, *C. pseudohaemulonii*, *C. haemulonii*, *C. duobushaemulonii* and *C. haemulonii* var. *vulnera* successfully amplified DNA fragments of the expected size for each species and produced no extra bands at all (**Figure 5**). Moreover, no amplicons were produced from any of representative *Candida* species other

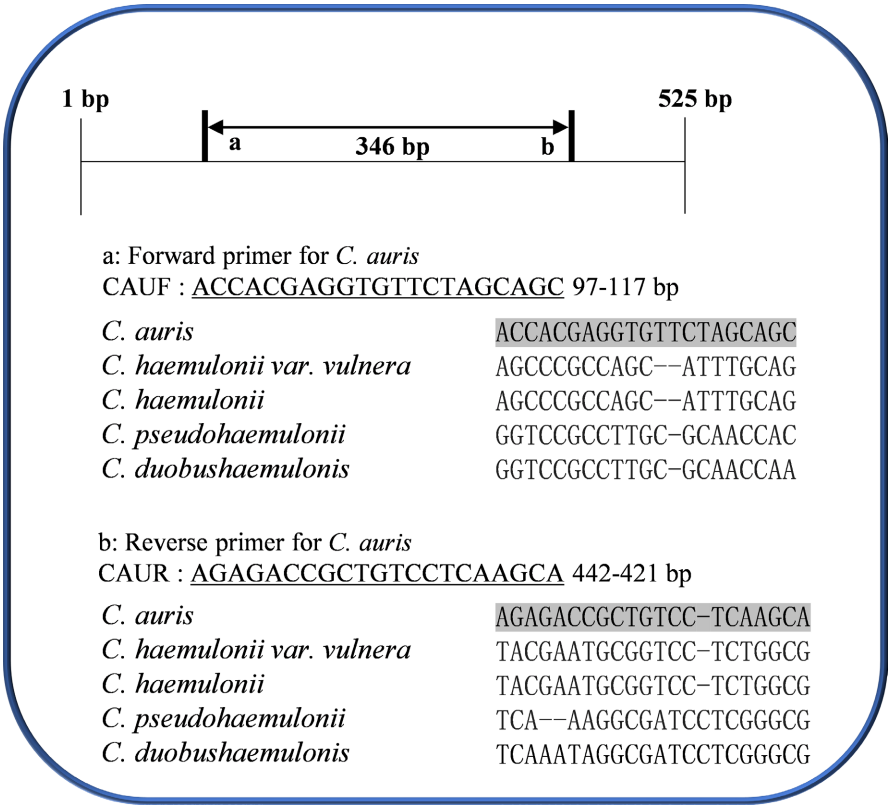

Figure 1. Locations and sequences of species-specific primers for the 26S rRNA gene of *Candida auris*. The nucleotide sequence of each primer has been underlined.

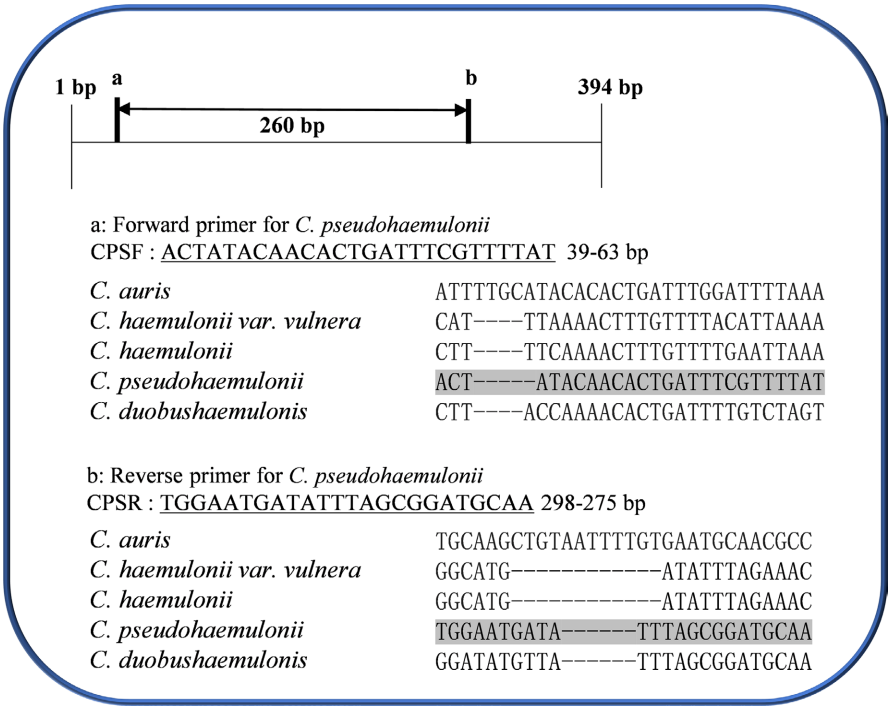

Figure 2. Locations and sequences of species-specific primers for the 18S rRNA gene of *Candida pseudohaemulonii*. The nucleotide sequence of each primer has been underlined.

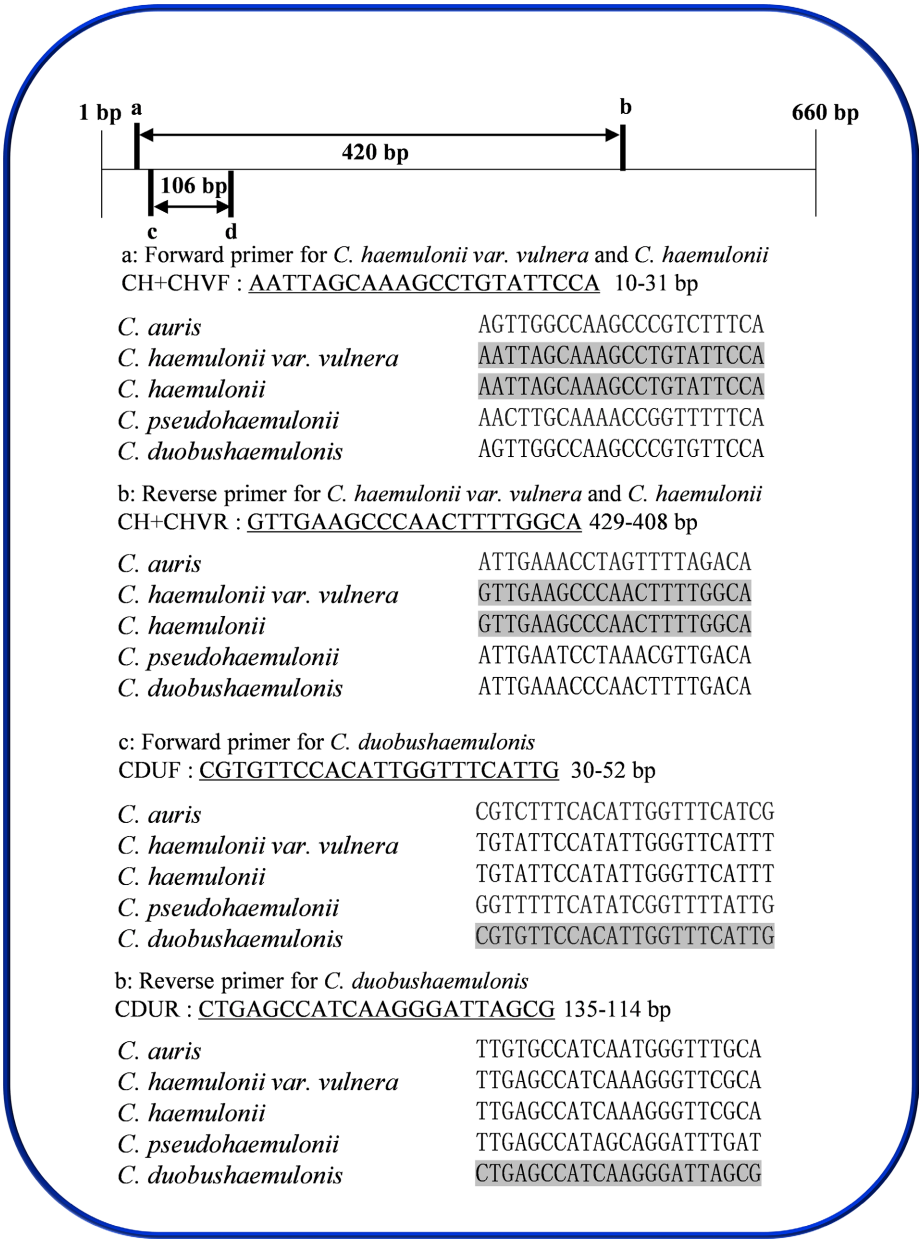

Figure 3. Locations and sequences of species-specific primers for the *RPB*1 gene of *C. haemulonii, C. haemulonii var. vulnera* and *C. duobushaemulonis*. The nucleotide sequence of each primer has been underlined.

than five fungal species. In addition, similar results were obtained using several different models of DNA thermal cyclers (data was not shown).

The detection limit was assessed in the presence of titrated bacterial cells, and the sensitivity of the PCR assay was between 5 × 1 and 5 × 10 CFU per PCR template (5.0 μl) for the *C. auris*-specific primer set with strain JCM 15448, the *C. pseudohaemulonii*-specific primer set with strain JCM 12453, the *C. duobushaemulonis*-specific primer set with strain IFM 64590, the *C. haemulonii*-specific primer set with strain JCM 3762 and the *C. haemulonii var. vulnera*-specific primer set with strain NUM-CHV 1010 (data was not shown).

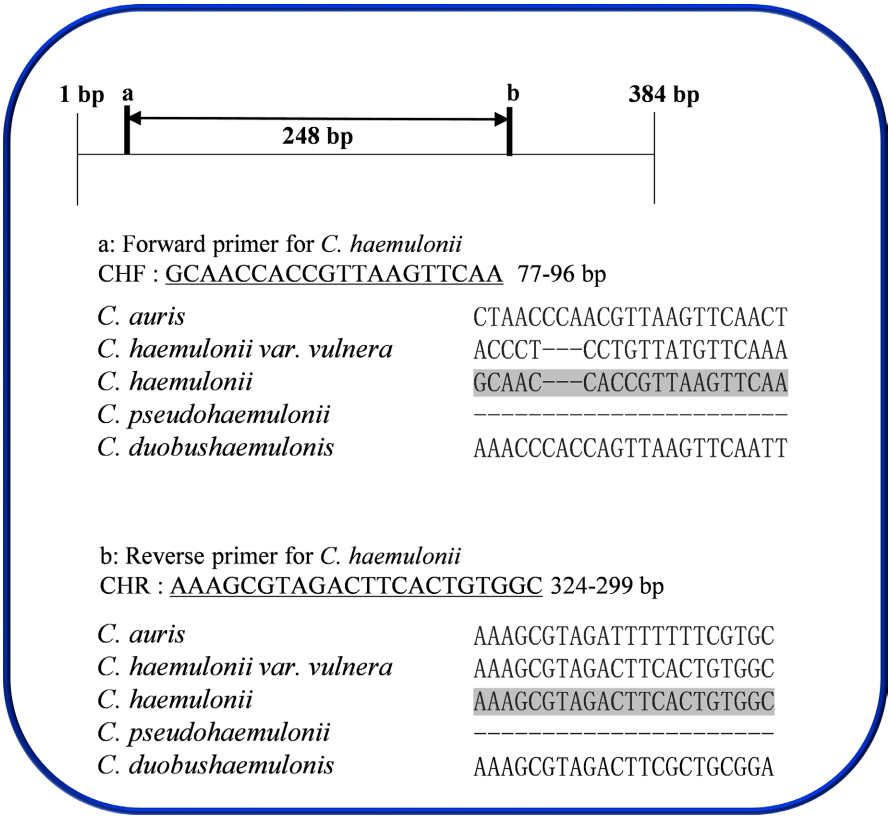

Figure 4. Locations and sequences of species-specific primers for the ITS region of *C. haemulonii*. The nucleotide sequence of each primer has been underlined.

Figure 5. PCR assay for identifying five *Candida* species. The primer mixture contained CAUF, CAUR, CPSF, CPSR, CH + CHVF, CH + CHVR, CDUF, CDUR, CHF and CHR. Lanes: 1, *C. duobushaemulonis* IFM 64590; 2, *C. pseudohaemulonii* JCM 12453; 3, *C. auris* JCM 15448; 4, *C. haemulonii var. vulnera* NUM-CHV 1010; 5, *C. haemulonii* JCM 3762; 6, *Candida lusitaniae* JCM 1814; 7, *Candida krusei* JCM1609; 8, *Candida glabrata* JCM3761; 9, *Candida tropicalis* JCM 1541; 10, *Candida parapsilosis* JCM 1612; 11, *Candida dubliniensis* IFM 54605; 12, *Candida guilliermondii* JCM 1539; 13, *Candida albicans* JCM 1537; 14, *Candida kruisii* JCM 1779; 15, *Candida orthopsilosis* JCM 1784; 16, *Candida kefyr* JCM 9556; 17, *Candida aaseri* JCM 1689; 18, *Candida inconspicua* JCM 9555; M, molecular size marker (100-bp DNA ladder).

4. Discussion

Some *Candida* species have been reclassified due to the description of new genetically related species. The independence of *C. dubliniensis* from *C. albicans* is all too well known; *C. dubliniensis* is primarily isolated from the oral cavity of

HIV patients [25]. This microorganism is genetically very close to *C. albicans*. Other fungal species such as *C. guilliermondii*, *C. parapsilosis*, *C. famata*, and *C. haemulonii* can be taxonomically subdivided into several fungal species, but the isolation frequency of atypical fungal species from clinical material is very low and their antifungal susceptibilities do not differ significantly. An accurate identification of cryptic species in the clinical setting is required in epidemiology and medicine. It is also important to better understand the evolution of antifungal resistance. The most notable example of the importance of identifying cryptic species might be the emergence and rapid diffusion of *C. auris*. This organism is considered a serious threat to public health worldwide due to frequent relapses and treatment failures [26] [27].

To develop a PCR-based technique more applicable for clinical use than conventional PCR, we established a multiplex PCR system for identifying and detecting simultaneously four medically important *Pseudomonas* species, using only one PCR tube per sample. A multiplex-PCR method is a rapid tool that allows for the simultaneous amplification of more than one sequence of target DNA in a single reaction, thereby saving time and reagents [15]. The most significant problem with regard to this method is the possibility of hybridization among the different sequences of primers. A multiplex PCR for the detection of five *Candida* species has not ever been developed. Therefore, a reliable identification method is needed to accurately assess the prevalence of five *Candida* species, *i.e.*, *C. auris*, *C. pseudohaemulonii*, *C. haemulonii*, *C. duobushaemulonii* and *C. haemulonii* var. *vulnera.*

It is estimated that there are about 80,000 fungal species in the fungal kingdom, but less than 1% of them are involved in infection. Even among these few species, there has long been confusion over their classification, but recent years have seen remarkable progress in the taxonomy of pathogenic fungi with the introduction of DNA sequencing. For example, *Malassezia furfur* has been implicated as an aggravating factor in atopic dermatitis, but recent studies have shown that this organism is not a major fungal species [28]. This is due to the fact that *M. furfur* was a complex of five fungal species. It was DNA sequencing that revealed this taxonomic heterogeneity. Similar examples apply to *C. albicans*/*C. dubliniensis* and T*richosporon cutaneum*/*T. asahii*. Almost all pathogenic fungi can be identified by sequencing the D1/D2 26S rDNA or ITS region.

Genes used for fungal taxonomic identification must be present in all fungi and show a moderate evolutionary rate. A widely used gene for both bacteria and fungi is rRNA. Fungal rRNA genes have four subunits: 18S (small subunit), 5.8S, 26S (large subunit), and 5S, as well as an ITS region between 26S and 18S and an IGS (intergenic spacer) region between 26S and 18S. The lengths of the four subunits are almost the same regardless of fungal species. On the other hand, the lengths of the ITS and IGS vary markedly among fungal species. For example, the total length of the ITS in *C. albicans* is about 300 bp, whereas in the same genus, *C. glabrata*, it is more than twice as long. In general, partial sequences of the 26S subunit (about 600 bp long in the Domain 1 and 2 regions)

and the ITS1/2 region are suitable for classification and identification. When attempting to identify between variants or at the strain level, analysis of the IGS region between 26S and 18S, or *RPB*1 gene is an excellent tool. In the present study, the 26S rRNA gene, 18S rRNA gene, *RPB*1 gene and ITS region sequences were used in order to design species-specific primers to selectively and simultaneously detect five *Candida* species.

In the present study, we designed species-specific primers with the already mentioned means, for the identification of *C. auris*, *C. pseudohaemulonii*, *C. haemulonii*, *C. duobushaemulonii* and *C. haemulonii* var. *vulnera* with a PCR method. These primers were able to distinguish each *Candida* species and did not display cross-reactivity with each other. Moreover, similar results were obtained using several different models of DNA thermal cyclers, indicating that the multiplex method developed in this study is highly reproducible. In addition, we developed a one-step multiplex PCR method with the ability to identify and differentiate five *Candida* species using only one PCR tubes per sample.

Our multiplex PCR method is easy because the use of MightyAmp DNA Polymerase Ver.3 (Takara) means that DNA extraction may be avoided, and the subspecies identification and detection using this method only takes approximately 2 hours. Thus, the method described herein will allow the prevalence of *C. haemulonii* complex (*Candida haemulonii*, *C. duobushaemulonii* and *C. haemulonii* var. *vulnera*) and two genetically close species (*C. pseudohaemulonii* and *C. auris*) and their involvement in the various infections, to be fully clarified in future studies.

5. Conclusion

Our developed multiplex PCR method enables the reliable identification of five clinically important *Candida* species. Its simplicity means that it can be employed readily in most laboratories, where it might contribute to a better understanding of the epidemiology and clinical significance of the most important *Candida* species, *i.e.*, *Candida haemulonii*, *C. duobushaemulonii*, *C. haemulonii* var. *vulnera*, *C. pseudohaemulonii* and *C. auris*.

Authors' Contributions

Fuchigami M, Tsuzukibashi O, Fukatsu A, Takahashi Y, Yamamoto H, Komine C, Hagiwara-Hamano M and Iizuka Y corrected the data. Fuchigami M, Tsuzukibashi O, Hayashi S, Umezawa K, Fukatsu A, Wakami M, Murakami H, Kobayashi T and Fukumoto M drafted and wrote the manuscript. The concept of this manuscript was devised by Fuchigami M. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

References

[1] Silby, M.W., Winstanley, C., Godfrey, S.A.C., Levy, S.B. and Jackson, R.W. (2011) Pseudomonas Genomes: Diverse and Adaptable. *FEMS Microbiology Reviews*, **35**, 652-680. https://doi.org/10.1111/j.1574-6976.2011.00269.x

[2] Argudín, M.A., Deplano, A., Meghraoui, A., Dodémont, M., Heinrichs, A., Denis, O., Nonhoff, C. and Roisin, S. (2017) Bacteria from Animals as a Pool of Antimicrobial Resistance Genes. *Antibiotics*, **6**, Article No. 12. https://doi.org/10.3390/antibiotics6020012

[3] O'Brien, H.E., Desveaux, D. and Guttman, D.S. (2011) Next-Generation Genomics of *Pseudomonas* syringae. *Current Opinion in Microbiology*, **14**, 24-30. https://doi.org/10.1016/j.mib.2010.12.007

[4] Wu, X., Monchy, S., Taghavi, S., Zhu, W., Ramos, J. and van der Lelie, D. (2011) Comparative Genomics and Functional Analysis of Niche-Specific Adaptation in *Pseudomonas putida*. *FEMS Microbiology Reviews*, **35**, 299-323. https://doi.org/10.1111/j.1574-6976.2010.00249.x

[5] Lalucat. J., Bennasar, A., Bosch, R., García-Valdés, E. and Palleroni, N.J. (2006) Biology of *Pseudomonas stutzeri*. *Microbiology and Molecular Biology Reviews*, **70**, 510-547. https://doi.org/10.1128/mmbr.00047-05

[6] Gudiol, C., Tubau, F., Calatayud, L., Garcia-Vidal, C., Cisnal, M., Sánchez-Ortega, I., Duarte, R., Calvo, M. and Carratalà, J. (2011) Bacteremia Due to Multidrug-Resistant Gram-Negative Bacilli in Cancer Patients: Risk Factors, Antibiotic Therapy and Outcomes. *Journal of Antimicrobial Chemotherapy*, **66**, 657-663. https://doi.org/10.1093/jac/dkq494

[7] Pfeifer, Y., Cullik, A. and Witte, W. (2010) Resistance to Cephalosporins and Carbapenems in Gram-Negative Bacterial Pathogens. *International Journal of Medical Microbiology*, **3**, 371-379. https://doi.org/10.1016/j.ijmm.2010.04.005

[8] Upadhyay, S., Sen, M.R. and Bhattacharjee, A. (2010) Presence of Different Beta-Lactamase Classes among Isolates of *Pseudomonas aeruginosa* Expressing AmpC Beta-Lactamase Enzyme. *The Journal Infection in Development Countries*, **4**, 239-242. https://doi.org/10.3855/jidc.497

[9] Lima, A.B.M, Leão-Vasconcelos, L.S.N.O., Costa, D.M., Vilefort, L.O.R., André, M.C.D.P.B., Barbosa, M.A. and Prado-Palos, M.A. (2015) Pseudomonas spp. Isolated from the Oral Cavity of Healthcare Workers from an Oncology Hospital in Midwestern Brazil. *Revista do Instituto de Medicina Tropical de São Paulo*, **57**, 513-514. http://dx.doi.org/10.1590/S0036-46652015000600009

[10] Chan, X.Y., Chua, K.O., How, K.Y., Yin, W.F. and Chan, K.G. (2014) Global Genome Comparative Analysis Reveals Insights of Resistome and Life-Style Adaptation of *Pseudomonas putida* Strain T2-2 in Oral Cavity. Hindawi Publishing Corporation. *The Scientific World Journal*, **2014**, Article ID: 930727. http://dx.doi.org/10.1155/2014/930727

[11] Fukatsu, A., Tsuzukibashi, O., Suzuki, H., Asaka, K., Ono, Y., Fuchigami, M., Kobayashi, T., Uchibori, S., Takahashi, Y., Komine, C., Konishi, Y., Ogura, Y., Omori, H., Wakami, M., Murakami, H. and Fukumoto, M. (2021) One-Step Multiplex PCR for Simultaneous Detection and Identification of Eight Medically Important Candida Species. *Open Journal of Stomatology*, **11**, 14-24. https://doi.org/10.4236/ojst.2021.111002

[12] Vos, D.D., Pirnay, A.L.J.J.P., Struelens, M., Vandenvelde, C., Duinslaeger, L., Vanderkelen, A. and Cornelis, P. (1997) Direct Detection and Identification of *Pseudomonas aeruginosa* in Clinical Samples Such as Skin Biopsy Specimens and Ex-

pectorations by Multiplex PCR Based on Two Outer Membrane Lipoprotein Genes, oprI and oprL. *Journal of Clinical Microbiology*, **35**, 1295-1299. https://doi.org/10.1128/jcm.35.6.1295-1299.1997

[13] Walker, T.S., Bais, H.P., Déziel, E., Schweizer, H.P., Rahme, L.G., Fall, R. and Vivanco, J.M. (2004). *Pseudomonas aeruginosa*-Plant Root Interactions: Pathogenicity, Biofilm Formation, and Root Exudation. *Plant Physiology*, **134**, 320-331. http://dx.doi.org/10.1104/pp.103.027888

[14] Noble, C.R. and Overman, S.B. (1994) *Pseudomonas stutzeri* Infection: A Review of Hospital Isolates and a Review of the Literature. *Diagnostic Microbiology and Infectious Disease*, **19**, 51-56. https://doi.org/10.1016/0732-8893(94)90051-5

[15] Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.H. and Vogt, P.H. (1997) Multiplex PCR: Critical Parameters and Step-by-Step Protocol. *BioTechniques*, **23**, 504-511. https://doi.org/10.2144/97233rr01

[16] Dogan, B. and Boor, K.J. (2003) Genetic Diversity and Spoilage Potential among Pseudomonas spp. Isolated from Fluid Milk Products and Dairy Processing Plants. *Applied Environmental Microbiology*, **69**, 130-138. https://doi.org/10.1128/AEM.69.1.130-138.2003

[17] Locatelli, L., Tarnawski, S., Hamelin, J., Rossi, P., Aragno, M. and Fromin, N. (2002) Specific PCR Amplification for the Genus Pseudomonas Targeting the 3' Half of the 16S rDNA and the Whole 16S-23S rDNA Spacer. *Systematic and Applied Microbiology*, **25**, 220-227. https://doi.org/10.1078/0723-2020-00110

[18] Tryfinopoulou, P., Tsakalidou, E. and Nychas, G.J.E. (2002) Characterization of Pseudomonas Spp. Associated with Spoilage of Gilt-Head Sea Bream Stored under Various Conditions. *Applied Environmental Microbiology*, **68**, 65-72. https://doi.org/10.1128/AEM.68.1.65-72.2002

[19] Wiedmann, M., Weilmeier, D., Dineen, S.S., Ralyea, R. and Boor, K.J. (2000) Molecular and Phenotypic Characterization of Pseudomonas spp. Isolated from Milk. *Applied Environmental Microbiology*, **66**, 2085-2095. https://doi.org/10.1128/AEM.66.5.2085-2095.2000

[20] Anzai, Y., Kim, H., Park, J.Y., Wakabayashi, H. and Oyaizu, H. (2000) Phylogenetic Affiliation of the Pseudomonads Based on 16S rRNA Sequence. *International Journal of Systematic Evolutionary Microbiology*, **50**, 1563-1589. https://doi.org/10.1099/00207713-50-4-1563

[21] Ercolini, D., Russo, F., Torrieri, E., Masi, P. and Villani, F. (2006) Changes in the Spoilage-Related Microbiota of Beef during Refrigerated Storage under Different Packaging Conditions. *Applied Environmental Microbiology*, **72**, 4663-4671. https://doi.org/10.1128/AEM.00468-06

[22] Bellingham, N.F., Morgan, J.A.W., Saunders, J.R. and Winstanley, C. (2001) Flagellin Gene Sequence Variation in the Genus Pseudomonas. *Systematic and Applied Microbiology*, **24**, 157-165. https://doi.org/10.1078/0723-2020-00031

[23] Hilario, E., Buckley, T.R. and Young, J.M. (2004) Improved Resolution of the Phylogenetic Relationships among Pseudomonas by the Combined Analysis of *atpD*, *carA*, *recA* and 16S rDNA. *Antonie van Leeuwenhoek*, **86**, 51-64. https://doi.org/10.1023/B:ANTO.0000024910.57117.16

[24] Yamamoto, S., Kasai, H., Arnold, D.L., Jackson, R.W., Viavian, A. and Harayama, S. (2000) Phylogeny of the Genus Pseudomonas: Intrageneric Structure Reconstructed from the Nucleotide Sequences of *gyrB* and *rpoD* Genes. *Microbiology*, **146**, 2385-2394. https://doi.org/10.1099/00221287-146-10-2385

[25] Sllivan, D.J., Westerneng, T.J., Hsynes, K.S., Bennett, D.E. and Coleman, D.C.

(1995) *Candida dubliniensis* Sp. Nov.: Phenotypic and Molecular Characterization of a Novel Species Associated with Oral Candidosis in HIV-Infected Individuals. *Microbiology*, **14**, 1507-1521. https://doi.org/10.1099/13500872-141-7-1507

[26] Snyder, G.M. and Wright, S.B. (2019) The Epidemiology and Prevention of *Candida auris*. *Current Infectious Disease Reports*, **21**, Article No. 19. https://doi.org/10.1007/s11908-019-0675-8

[27] Rhodes, J. and Fisher, M.C. (2019) Global Epidemiology of Emerging *Candida auris*. *Current Opinion in Microbiology*, **52**, 84-89. https://doi.org/10.1016/j.mib.2019.05.008

[28] Sugita, T., Suto, H., Unno, T., Tsuboi, R., Ogawa, H., Shinoda, T. and Nishikawa, A. (2001) Molecular Analysis of Malassezia Microbiota on the Skin of Atopic Dermatitis Patients and Healthy Subjects. *Journal of Clinical Microbiology*, **39**, 3486-3490. https://doi.org/10.1128/jcm.39.10.3486-3490.2001