Genetics and Molecular Biology, 35, 1, 122-125 (2012)
Copyright © 2012, Sociedade Brasileira de Genética. Printed in Brazil
www.sbg.org.br

Research Article

Molecular identification of *Pichia guilliermondii*, *Debaryomyces hansenii* and *Candida palmioleophila*

Adolfo Jose Mota[1], Graziella Nuernberg Back-Brito[2] and Francisco G. Nobrega[2]

[1]*Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil.*
[2]*Departamento de Biociências e Diagnóstico Oral, Faculdade de Odontologia de São José dos Campos, Universidade Estadual Paulista "Júlio de Mesquita Filho", São José dos Campos, SP, Brazil.*

Abstract

Traditional phenotypic methods and commercial kits based on carbohydrate assimilation patterns are unable to consistently distinguish among isolates of *Pichia guilliermondii*, *Debaryomyces hansenii* and *Candida palmioleophila.* As result, these species are often misidentified. In this work, we established a reliable method for the identification/differentiation of these species. Our assay was validated by DNA sequencing of the polymorphic region used in a real-time PCR assay driven by species-specific probes targeted to the fungal ITS 1 region. This assay provides a new tool for pathogen identification and for epidemiological, drug resistance and virulence studies of these organisms.

Key words: Candida palmioleophila, *Debaryomyces hansenii*, differential identification, *Pichia guilliermondii*, real-time PCR.

Received: April 29, 2011; Accepted: September 29, 2011.

Introduction

The precise identification of some fungal species is often very difficult when using only biochemical or phenotypic methods (Dooley *et al.*, 1994; Fenn *et al.*, 1994). However, the advent of DNA-based methods largely overcame the limitations of traditional methods and studies using molecular approaches revealed a greater diversity in fungi (Odds *et al.*, 1998; Chen *et al.*, 2000).

Some closely related species are often misidentified because of the great similarity in their biochemical and morphological characteristics. Desnos-Ollivier *et al.* (2008) reported the misidentification of *Pichia guilliermondii* (teleomorph *Candida guilliermondii*), *Debaryomyces hansenii* (teleomorph *Candida famata*) and *Candida palmioleophila.* Their results showed that only 23 of 36 isolates identified as *P. guilliermondii* and three of 26 identified as *D. hansenii* were confirmed by sequencing the ITS1-5.8S-ITS2 and D1/D2 regions of the ribosomal cistron. Other species such as *Candida albicans* (Odds *et al.*, 1998; Jabra-Rizk *et al.*, 2000; Tietz *et al.*, 2001) and *Candida parapsilosis* (Lasker *et al.*, 2006) show the same problem.

In our laboratory, we have had problems differentiating (1) *P. guilliermondii*, *C. palmioleophila* and *D. hansenii*, (2) *Candida krusei* and *Candida inconspicua*, and (3) *Candida pelliculosa* and *Candida subpelliculosa* when using the commercial kit API® 20 C AUX (Biomérieux, France) (Table S1). The Vitek Yeast Biochemical Card and the ID 32C, two widely used methods for yeast identification, were tested by different groups (Dooley *et al.*, 1994; Lo *et al.*, 2001; Burton *et al.*, 2010) and their findings confirmed the problem of incorrect or inconsistent identification. Lo *et al.* (2001) suggested that the laboratory routine should include at least two methods for yeast identification.

Misidentification of the fungal species can compromise epidemiological or antibiotic susceptibility studies and over- or underestimate the species abundance. Precise identification is therefore necessary and molecular approaches can provide the tools for a fast method. In this study, we developed a real-time PCR method that can differentiate/identify *P. guilliermondii*, *D. hansenii* and *C. palmioleophila*, and sequencing the ITS 1 region of these species confirmed our results.

Materials and Methods

Total DNA was extracted as described by Philippsen *et al.* (1991). DNA was quantified with a Qubit® fluorometer and a Quant-iT PicoGreen® dsDNA BR assay kit (Invitrogen, Life Technologies, Eugene, OR, USA) according to the manufacturers recommendations.

A Go® Taq Flexi DNA Polymerase kit (Promega, Madison, WI, USA) was used to amplify the ITS 1 region

Send correspondence to Francisco G. Nobrega. Departamento de Biociências e Diagnóstico Oral, Faculdade de Odontologia de São José dos Campos, Universidade Estadual Paulista "Júlio de Mesquita Filho", Av. Francisco J. Longo 777, Jd. São Dimas, 12245-000 São José dos Campos, SP, Brazil. E-mail: francisco.nobrega@gmail.com.

with ITS1/ITS2 primers (White and Lee, 1990) and a TopoTA Cloning® kit (Invitrogen) was used for the constructions. The sequences were obtained using a Big Dye® Terminator v3.1 Cycle Sequencing kit followed by automatic sequencing in an ABI 3100 (Applied Biosystems, Life Technologies, Foster City, CA, USA).

The sequences obtained from the three American Tissue Culture Collection (ATCC) species, *D. hansenii* ATCC 36239, *C. famata* ATCC 62894 and *P. guilliermondii* ATCC 6260, were compared by the BLASTN program (Zhang *et al.*, 2000) against the GenBank non-redundant database (nr), EMBL, DDBJ and PDB nucleotide collections. The sequences obtained were aligned with ClustalW software (Larkin *et al.*, 2007) and then used to create a consensus sequence for each species and to choose target regions (data not shown). The primers and TaqMan® Minor Groove Binder (MGB) probes were designed using the software Primer Express v. 2.0 and default parameters (Applied Biosystems).

A real-time PCR was done using the TaqMan® Universal PCR Master Mix (Applied Biosystems) in the following singleplex reaction mixture: 10 ng of sample DNA, 1X TaqMan® Universal PCR Master Mix, 200 nM of each primer, 300 nM of TaqMan® MGB probe (Table 1) and water to a total volume of 25 μL. All reactions were done in duplicate. The cycling conditions were set in an ABI 7300 real-time PCR cycler fitted with SDS software v. 1.2.3 (Applied Biosystems) as follows: 10 min at 95 °C followed by 35 cycles of 95 °C for 30 s and 60 °C for 1 min.

The efficiency of multiplex reactions was tested by mixing all of the probes together in the same concentrations as shown above.

Results and Discussion

The sequences obtained from the ATCC strains (data not shown) were matched in a search against the (nr) nucleotide bank using the BLAST program (the alignment files are provided in Supplementary Material Figure S2) in order to choose the potential target region for genotyping by real-time PCR (Figure 1).

Comparison of the two methods of identification (API 20C AUX and ribotyping by sequencing) revealed the difficulty in differentiating *P. guilliermondii* and *D. hansenii*, in the correct identification of *C. parapsilosis* versus *D. hansenii* (Burton *et al.*, 2010 and Table S1) and the impossibility of differentiating *C. krusei* and *C. insconspicua*.

Initially, three polymorphic domains (ITS 1, ITS 2 and D1/D2) of the ribosomal cistron from these species were aligned using ClustalW (Larkin *et al.*, 2007). Of these three regions, only the ITS 1 region provided suitable discrimination (Figure 1). This region is ideal because it is flanked by two conserved domains: the end of 18S rRNA and the beginning of 5.8S rRNA. The amplicon is short (about 300 bp) and the sequence is variable among different species but conserved among strains of the same species.

Figure 2 shows the amplification plot of the real-time PCR genotyping done using species-specific probes. The region indicated as NTC (no template control) confirmed the specificity of each probe since only in the presence of the specific target was there amplification. To confirm this finding, we analyzed the real-time PCR products in a 2% agarose gel stained with ethidium bromide and detected the expected amplicons (data not shown).

The multiplex reaction worked as well as the singleplex test (Figure S1) and can be used for fungus identification, thereby reducing the costs of the assay.

The close relationship between *C. palmioleophila* and *D. hansenii* has previously been shown by a phylogenetic analysis using data from the D1/D2 and ITS regions (Desnos-Ollivier *et al.*, 2008). In our study, the strain *C. famata* ATCC 62894, used as a positive control, was identified as *C. palmioleophila* by sequence analysis of the D1/D2 region of rRNA (Table S1) and by the real-time PCR assay described here (Figure 2).

In conclusion, the three species examined here are difficult to identify using standard laboratory tests. Ribosomal RNA sequencing is the gold standard for identification but is generally expensive and time consuming. The real-time PCR assay described here is a very effective, rapid, low-cost alternative. The method can unambiguously identify isolates and confirm the identification of strains analyzed by traditional methods, with the advantage of measuring species abundance if necessary.

Table 1 - Sequences of primers and probes used in this work. In the probes, the reporter dye is indicated in bold and the "No fluorescent quencher (NFQ)" region is underlined. The probe sequence is inserted between these two regions.

Primers/probes	Sequence	Tm (ºC)
ITS1 Fw	TGAACCTGCGGAAGGATCAT	59
ITS2 Rev	TCCGTTGTTGAAAGTTTTGAAGATT	59
TaqMan® MGB probes		
D. hansenii	**VIC**TTGTTATTACAAGAACTTTTGC<u>MGBNFQ</u>	70
P. guilliermondii	**6FAM**TTGATACAGAACTCTTGCTTTG<u>MGBNFQ</u>	70
C. palmioleophila	**NED**CTTTATTAGAAACTATTGCTTTGGC<u>MGBNFQ</u>	70

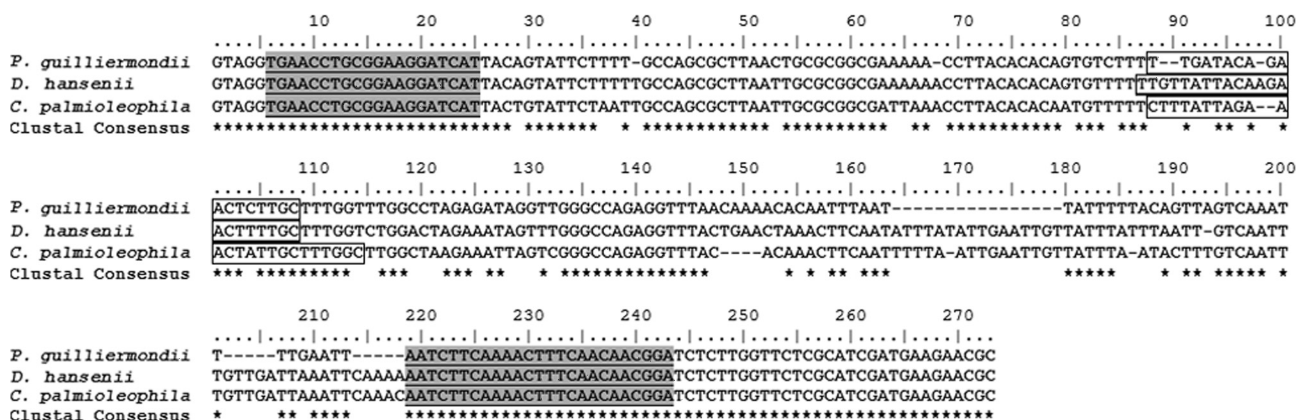

Figure 1 - Sequence alignment of the polymorphic ITS 1 region. The ITS 1 regions of the three species were aligned using ClustalW. The numbers above the sequences represent base positions, the gray boxes are the targets of forward and reverse primers and the clear boxes indicate the probe targets.

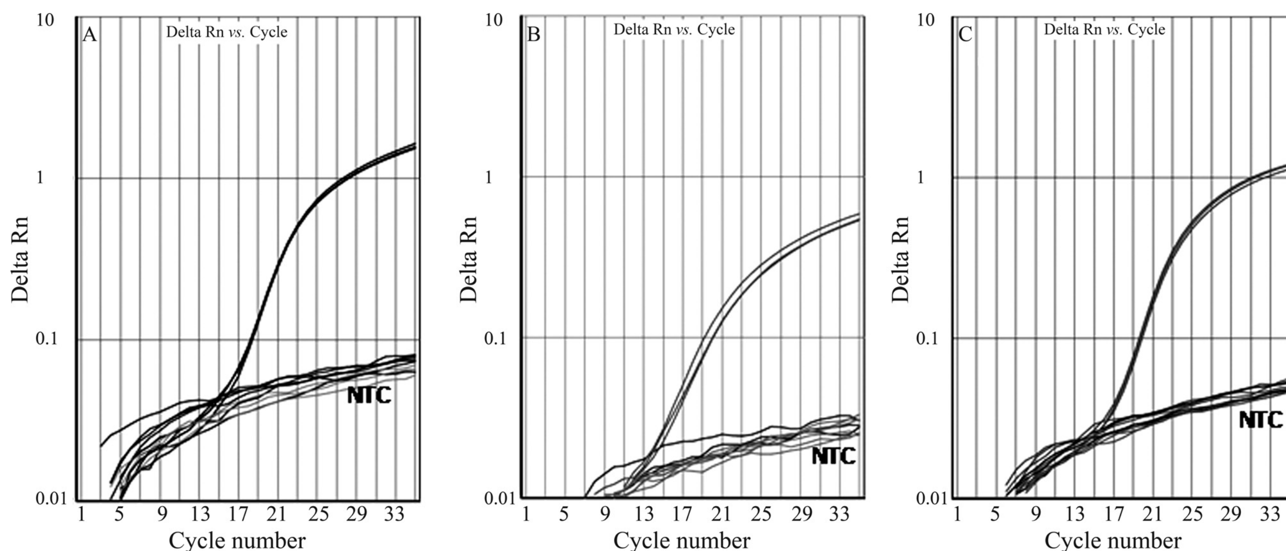

Figure 2 - Plots showing real-time PCR amplification of singleplex assays monitored with fluorescent TaqMan® MGB probes. Delta Rn versus Cycle amplification plots were obtained using probes for (A) *P. guilliermondii*, (B) *C. palmioleophila* and (C) *D. hansenii* templates*.* The NTC (no template control) curves show no detection in the absence of templates and in the presence of non-specific targets, *i.e.*, *C. palmioleophila* and *D. hansenii* in (A), *P. guilliermondii* and *D. hansenii* in (B) and *P. guilliermondii* and *C. palmioleophila* in (C).

Acknowledgments

This research was partly supported by a Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) grant to FGN (302992/2005-7). AJM and GNBB were supported by doctoral scholarships from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). We thank INCQS-FioCruz and Biomerriaux for supplying the ATCC species used in this work.

References

Burton MJ, Shah P and Swiatlo E (2010) Misidentification of *Candida parapsilosis* as *C. famata* in a clinical case of vertebral osteomyelitis. Am J Med Sci 341:71-73.

Chen YC, Eisner JD, Kattar MM, Rassoulian-Barrett SL, LaFe K, Yarfitz SL, Limaye AP and Cookson BT (2000) Identification of medically important yeast using PCR-based detection of DNA sequence polymorphism in the internal transcribed spacer 2 region of the rRNA genes. J Clin Microbiol 38:2302-2310.

Desnos-Ollivier M, Ragon M, Robert V, Raoux D, Gantier JC and Domer F (2008) *Debaryomyces hansenii* (*Candida famata*), a rare human fungal pathogen often misidentified as *Pichia guilliermondii* (*Candida guilliermondii*). J Clin Microbiol 46:3237-3242.

Dooley DP, Miriam BL and Jeffrey BS (1994) Misidentification of clinical yeast isolates by using the Vitek Yeast Biochemical Card. J Clin Microbiol 32:2889-2892.

Fenn JP, Segal H, Barland B, Denton D, Whisenant J, Chun H, Christofferson K, Hamilton L and Carroll K (1994) Comparison of updated Vitek Yeast biochemical card and API 20C Yeast identification systems. J Clin Microbiol 32:1184-1187.

Jabra-Rizk MA, Falkler Jr WA, Merz WG, Baqui AAMA, Kelley JI and Meiller TF (2000) Retrospective identification and characterization of *Candida dubliniensis* isolates among *Candida albicans* clinical laboratory isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected individuals. J Clin Microbiol 38:2423-2426.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, *et al.* (2007) Clustal W and Clustal X ver. 20. Bioinformatics 23:2947-2948.

Lasker BA, Butler G and Lott TJL (2006) Molecular genotyping of *Candida parapsilosis* group I clinical isolates by analysis of polymorphic microsatellite markers. J Clin Microbiol 44:750-759.

Lo HJ, Ho YA and Ho M (2001) Factors accounting for misidentification of *Candida* species. J Microbiol Infect 34:171-177.

Odds FC, Nuffel LV and Dams G (1998) Prevalence of *Candida dubliniensis* isolates in a yeast stock collection. J Clin Microbiol 36:2869-2873.

Philippsen P, Stotz A and Scherf C (1991) DNA of *Saccharomyces cerevisiae*. Methods Enzymol 194:169-171.

Tietz HJ, Hopp M, Schmalreck A, Sterry W and Czaika V (2001) *Candida africana* sp. nov. a new human pathogen or a variant of *Candida albicans*? Mycoses 44:437-445.

White TB and Lee S (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ and White TJ (eds) PCR Protocols. A Guide to Methods and Applications. Academic Press, San Diego, pp 315-322.

Zhang Z, Schwartz S, Wagner L and Miller W (2000) A greedy algorithm for aligning DNA sequences: J Comput Biol 7:203-214.

Supplementary Material

The following online material is available for this article:

Table S1 - Comparison of results obtained DNA sequencing.

Figure S1 - Real-time PCR amplification of multiplex assays.

Figure S2 - BLAST alignments for *C. palmioleophila*; *D. hansenii* and *P. guilliermondii*.

This material is available as part of the online article from http://www.scielo.br/gmb.

Associate Editor: Célia Maria de Almeida Soares

License information: This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.