Romanian Biotechnological Letters
Vol. 14, No. 1, 2009, pp. 4180-4186
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Romanian Society of Biological Sciences

**ORIGINAL PAPER**

# Molecular identification of some yeast strains involved in oral candidosis

Received for publication, August 08, 2008
Accepted, October 10, 2008

**ENACHE- SOARE SIMONA[1]*, PELINESCU DIANA[2], IONESCU ROBERTINA[1], AVRAM IONELA[1], STOICA ILEANA[2] AND VASSU-DIMOV TATIANA[2]**
[1] *MICROGEN- Center for Research in Microbiology, Genetics and Biotechnology, University of Bucharest, Faculty of Biology, 1-3 Aleea Portocalilor, sector 5, Bucharest 060101, Romania E-mail: simona_soare1979@yahoo.com (Simona Enache -Soare)*
[2] *University of Bucharest, Faculty of Biology, Department of Genetics, 1-3 Aleea Portocalilor, sector 5, Bucharest 060101, Romania E –mail: tatiana@botanic.unibuc.ro (Tatiana Vassu-Dimov), Tel/ fax: 0040.213118077*
** Author to whom correspondence should be addressed; E-mail: simona_soare1979@yahoo.com*

## Abstract

*The aim of this work was to identify four yeast strains using molecular techniques, i.e.* ***PCR, RFLP*** *and* ***RAPD****. Two of these molecular techniques (PCR and RFLP) are based on the size and structural variation of 5.8S rDNA. The amplicon was further individually digested with four restriction enzymes DdeI, Hae III, CfoI and MspI. The restriction pattern obtained for all four strains was identical with the restriction pattern of Candida albicans reference strain and different from all references strains (C. parapsilosis and C. guillermodii).*

*Regarding at RAPD analysis our data showed that the RAPD profiles of all isolated strains was similar with that of C. albicans strain and different from the other references strains. An exception is represented by CMGB 318 strains that exhibited a different RAPD profiles comparatively with C. albicans references strain. The different profile of CMGB 318 strain proves the existence of intraspecies DNA-length polymorphisms.*

*Our study demonstrated that the identification of Candida species by the amplification of 5.8S rDNA and RFLP analysis is a practical, short, and a reliable method when comparing to the conventional methods.*

Keywords*: Candida albicans,* RFLP, RAPD

## Introduction

In recent years, despite the advances in health care and therapeutic methods, the incidence of invasive systemic candidosis has markedly increased. This is thought to be the result of the increase of populations at risk, such as transplant recipients, cancer patients, HIV- infected patients, and those receiving immunosuppressive and broad-spectrum antibiotic therapy. During last decade yeast strains of *Candida* have produced an increasing number of human infections in either acute or chronic forms, frequently on mouth or vagina mucosa. Of particular importance proved to be the chronic hyperplasic forms of the oral candidosis as it has been associated with the development of squamous cell carcinoma. In the past, many studies of candidosis have not identified candidal isolates to species level [13]**.**

The majority of *Candida* infections are caused by *C. albicans* strains. Yet, non-albicans species of *Candida* such as *C.dubliniensis*, *C. glabrata*, *C. krusei* which are less susceptible to azoles derivates have been reported with increasing frequency, as well as an emergence of resistance to antifungal drugs. Early diagnosis of invasive fungal infections is

essential to reduce the mortality rates. In addition, in order to achieve an efficient antifungal therapy is essential to have an accurate taxonomic identification of the *Candida* strains [8].

Conventional methods for the identification of *Candida* species are based on assimilation profile, fermentation reactions and morphology [2]. Morphological features and reproductive structures useful for identifying isolated fungi may take days to weeks to develop in culture, and evaluation of these characteristics requires expertise in mycology [9]. More recently, molecular techniques provide substantial data in a global, polyphasic taxonomical approach on pathogenic fungi. Molecular methods with high discriminatory power are required for reliable identification of *Candida* at the species level, especially in epidemiological studies to asses the transmission routes as well as to determine appropriate antifungal drugs [8]. Our study was carried out on four yeast strains isolated from patient with oral candidosis and the ITS1-5.8S-ITS2 regions of fungal rRNA genes were amplified with universal primers (pITS1 and pITS4). Digestion of the PCR products with four restriction enzymes (*Dde* I*, Hae* III*, Cfo* I *and Msp* I) allowed discrimination of medically important *Candida* strains. We also report the use of RAPD assays as a molecular typing technique to differentiate distinct clinical isolates of *Candida*.

## Materials and Methods

**Strains -** This study was conducted on 4 clinical isolates of *Candida* from patients with oral candidosis and *Candida albicans* ATCC 10231, *Candida guillermondii* (CMGB 244 -Collection of Center for Research, Education and Consulting in Genetics, Microbiology and Biotechnology- MICROGEN) and *Candida parapsilosis* CBS 604 were used as reference strains. Yeasts were stored at -70°C on peptone glucose (YPG) medium (yeast extract 5g/l, peptone 10g/l, glucose 20g/l) supplemented with 20% glycerol.

**DNA isolation -** Yeast strains were grown on liquid YPG for 20 h at 37°C. Volumes of 1.5 ml cultures were sedimented at 6500 5 min. Cell pellet was resuspended and incubated at 30°C for one hour in 0.2 ml solution of sorbitol 1.2M, 50µl EDTA 0.5M and 125 µl lyticase (3mg/ml). Cells were again pelleted, resuspended in 250 µl Tris-EDTA (10X) and 25 µl SDS (10%) and incubated at 65°C for 30 minutes. 200 µl potassium-acetate (5M) were added followed by a new incubation at -20°C for one hour. DNA was pelleted by centrifugation at 10.000 rpm for 10 minutes and sediment was washed twice with 150 µl of 75% ethanol. DNA was resuspended in 80 µl free-nuclease-water and an aliquot was removed to determine nucleic acid purity and concentration by spectrophotometry. DNA purity can be estimated from the $A_{260}/A_{280}$ ratio. An $A_{260}/A_{280}$ ratio between 1.7 and 2.0 generally represents a high-quality DNA sample. DNA concentration can be estimated by measuring the absorbance at 260nm ($A_{260}$), and using the relationship that an $A_{260}$ of 1.0 = 50µg/ml pure DNA. DNA was also checked by agarose (0.8 %) gel electrophoresis.

**PCR Amplification -** PCR reactions were performed using a MJ Research thermal cycler in a total reaction volume of 50 µl, consisting of 1 ng isolated DNA, 0.2mM each dATP, dCTP, dGTP, dTTP, 0.2nm each primers (ITS1: 5'- TTC GTA GGT GAA CCT GCG G-3' and ITS4: 5' TCC TCC GCT TAT TGA TAT GC -3'- "Biosearch Technologies, Inc") and 10U of Taq DNA polymerase (Promega). After initial denaturation of DNA at 95°C for five minutes, each of the 35 cycles consisted of denaturation step at 95° C (1 min), an annealing step at 50° C (10 min) and an extension step at 72° C (2 min). Final extension step was performed at 72°C 10 min. Samples were stored at -20° C until used. PCR products were

analyzed in 2% agarose gel with 0.5X Tris-Borate-EDTA buffer, stained with ethidium bromide and visualized in UV light.

**Restriction Enzyme Analysis** – Ten microliters of each PCR product were separately digested with 10U of *Dde* I, *Hae* III*, Cfo* I and *Msp* I (Promega) at 37° C overnight. Restriction fragments were analyzed in 2.7% agarose gel electrophoresis in 0.5X Tris-Borate-EDTA buffer.

**RAPD profiles -** RAPD reactions were conducted in 30 µl volumes containing 0.2mM of each dATP, dGTP, dTTP, dCTP (Promega), 50 ng of genomic DNA, 0.2 nmoles of primer and 1U of Taq polymerase. The amplification parameters consisted of 35 cycles of denaturation at 95° C (45 secs), primer annealing at 36° C (2 mins) and extension at 72°C (2 mins). In the first cycle the denaturing step was set for 5 mins and the final extension step was set for 7 mins.

Samples of 10 µl of RAPD reactions were run in 6% polyacrylamide gel electrophoresis with 0.5X Tris-Borate-EDTA, stained with ethidium bromide and visualized in UV light. The molecular sizes of DNA fragments were determined in relation to molecular standards 50bp. All enzyme and leders were acquisition from Promega Company.

## Results and Discussion

Four clinical yeast strains were previously isolated from oral cavity and named CMGB 318, 319, 320 and, respectively, 321. Conventional tests showed that all 4 of them belong to *Candida albicans* (data not shown).

In the present study we performed several molecular analyses in order to obtain a more precise taxonomic identification of the four strains, as well as to estimate the genetic relatedness between them. Therefore, our experiments followed several main steps:

**A – Isolation and purification of genomic DNA**

Genomic DNA was isolated and purified using a classical technique with minor modifications [4]. DNA samples from our four strains (CMGB 318-321) were run in agarose gel electrophoresis and showed low RNA contamination .The DNA had not been sheared during extraction manipulations.

Spectrophotometrical analysis showed that the DNA samples had high purity and concentration (ranging between 2.75 and 7.65 µg/µl).

All these data showed that our genomic DNA samples were suitable for further molecular analyses.

**B – PCR amplification of [ITS1 – 5.8S – ITS2] region**

In order to amplify the ITS1 – 5.8S – ITS2 region we used the fungal specific primers pITS1 and pITS4 [13] flanking the ITS1 and, respectively, ITS2 (Fig.1).

As seen in fig.2 amplicons were obtained for all *Candida* strains. PCR products of approximately 500-550 bp for *C. albicans* ATCC 10231, C. *parapsilosis* CBS 604 and the four clinical specimens and, respectively, products of about 600 bp for *C. guillermondii* CMGB 224. The molecular weight for *C. albicans* and *C. parapsilosis* are similar with those indicate by Cirak [2]**.**

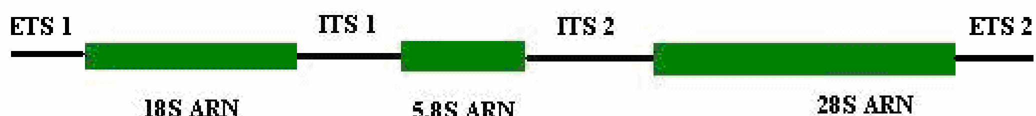


**Figure 1.** Nuclear rDNA region transcribed in fungi [3].
ITS = Internal Transcribed Spacer

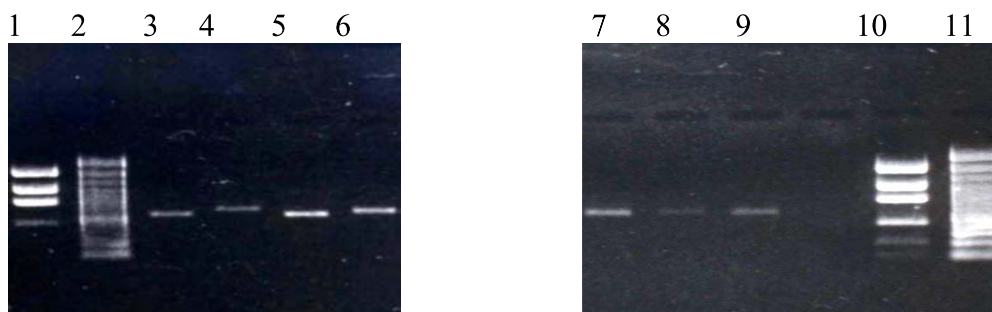


**Figure 2.** PCR amplification of rDNA with ITS1 and ITS4 primers.
Lane 1-11: 50pb DNA Step ladder, Bench Top 100pb DNA Ladder, *C. albicans* ATCC 10231, *C. guillermondii*, CMGB 318, CMGB 319, CMGB 320, CMGB 321, *C parapsilosis* CBS 604, 50pb DNA Step ladder, Bench Top 100pb DNA Ladder

Other previous studies in which the same rDNA region was amplified reported similar sizes for *C. albicans* (550 bp), and *C. parapsilosis* (550pb) [5-10]. Evenmore, homologous sequences in GeneBank have sizes between 525 – 560 bp [7]**.** Regardin to our strains we showed that for all isolates were obtained a PCR products with the same molecular weight like *C. albicans* ATCC 10231.

So we are concluded that our PCR products were fit for further molecular analyses.

### C – RFLP typing of [ITS1 – 5.8S – ITS2] region

Each amplicon was digested with four restriction enzymes - *DdeI, CfoI, HaeIII and MspI*, in separate reactions. The approximate length of the restriction fragments observed after digestion are summarized in table 1.

**Table 1.** Size of the fragments obtained from the restriction products enzymes

| Strain | *Dde*I (bp) | *Cfo*I (bp) | *Msp*I (bp) | *Hae* III (bp) |
|---|---|---|---|---|
| *C. albicans* ATCC 10231 | 100+450 | 290+260 | 300+250 | 100+450 |
| *C. parapsilosis* CBS 604 | - | 310+240 | - | 120 +430 |
| CMGB 318 | 100+450 | 290+260 | 300+250 | 100+450 |
| CMGB 319 | 100+450 | 290+260 | 300+250 | 100+450 |
| CMGB 320 | 100+450 | 290+260 | 300+250 | 100+450 |
| CMGB 321 | 100+450 | 290+260 | 300+250 | 100+450 |

When the rRNA gene region was digested with DdeI enzyme, each of the investigated strains (CMGB 318, 319, 320 and 321) exhibited similar restriction pattern with reference strains *C. albicans* ATCC 10231 and different pattern of *C. parapsilosis* CBS 604. Similar results were also obtained with *CfoI, MspI and Hae III* restriction enzymes.

Figure 3 shows a gel electrophoresis of PCR products obtained from *C. albicans* ATCC 10231, CMGB 318, CMGB 319, CMGB 320, CMGB 321, *C. parapsilosis CBS 604* strains

digested by restriction enzyme *DdeI* and *Hae III* and figure 4 shows a gel electrophoresis of the same PCR products obtained after digested with *Cfo* I and *Msp I* restriction enzyme. All of the studied isolates were found to yield the expected band patterns [5].

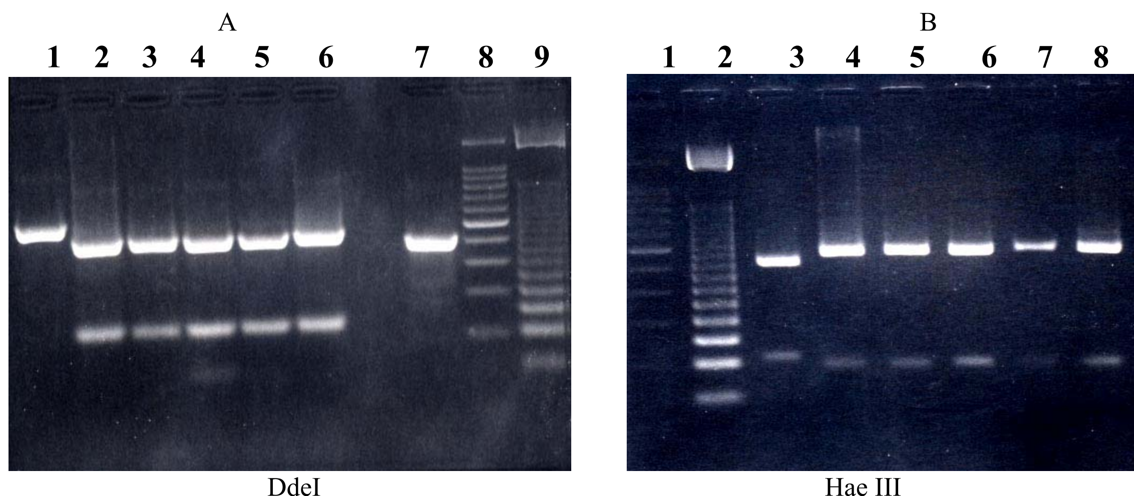


**Figure 3.** Restriction digestion of polymerase chain reaction products of clinical isolates with restriction enzyme *Dde*I (A) and *Msp*I (B)

A: Lane 1-9: *C. albicans* 10231 amplicon, *C. albicans* ATCC 10231, CMGB 318, CMGB 319, CMGB 320, CMGB 321, *C. parapsilosis CBS 604,* Bench Top 100pb DNA Ladder, 50pb DNA Step ladder

B: Lane 1-8: Bench Top 100pb DNA Ladder, 50pb DNA Step ladder, *C. parapsilosis* CBS 604, *C. albicans* ATCC10231, CMGB 318, CMGB 319, CMGB 320, CMGB 321

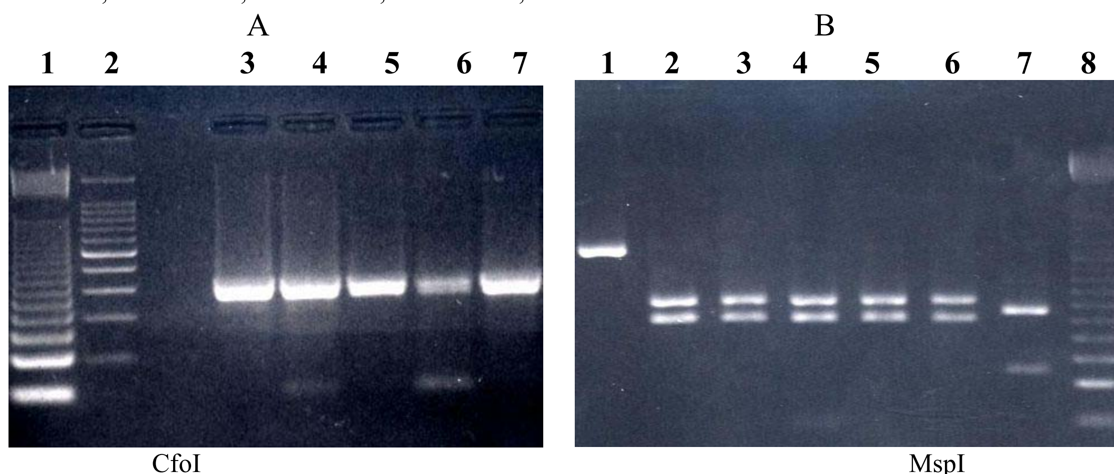


**Figure 4**. Restriction digestion of PCR products of clinical isolates with restriction enzyme CfoI (A) and MspI (B)

A) Lane 1-7: 50pb DNA Step ladder, Bench Top 100pb DNA Ladder, *C.albicans* ATCC 10231, CMGB 318, CMGB 319, CMGB 320, CMGB 321,

B) Lane 1-8: *C. albicans* 10231 amplicon, *C.albicans* ATCC 10231, CMGB 318, CMGB 319, CMGB 320, CMGB 321, *C. tropicalis* , 50pb DNA Step ladder

RFPL analysis of the PCR products of the isolates demonstrated that *Hae III* and *DdeI* were the most differentiating enzymes among the others. *DdeI* digestion was used to confirm the *Candida albicans* species. All of the *Candida albicans* species yielded 100 and approximately 450 bp products with *Dde I* restriction.

**D- RAPD analysis**

This technique produced a profile of bands that allowed the identification of intra- and inter-specific polymorphism among isolates [11]. The analysis was performed with two different primers **OPA 18** and **RP 4-2.** The effect of changing the primer on the DNA fragment profiles was profound, as both the number and the size of DNA fragments were observed to change with each primer [1-6]. Following the PCR reaction where the OPA 18 primer was used, there were obtained the identical amplification patterns for CMGB 318, 320 strains and 321 with the control strain *C. albicans* ATCC 10231 and different from the *C. parapsilosis* CBS 604. Regarding strain CMGB 318 the pattern obtained for this strain couldn't be found in the patterns of control strains. These results suggest the presence of intraspecific polymorphism among *Candida* species [12]**.** For the amplification reaction where we used both primers in the same reaction all strains presented the identical pattern with the one of control strain *C. albicans* ATCC 10231 (fig. 5).

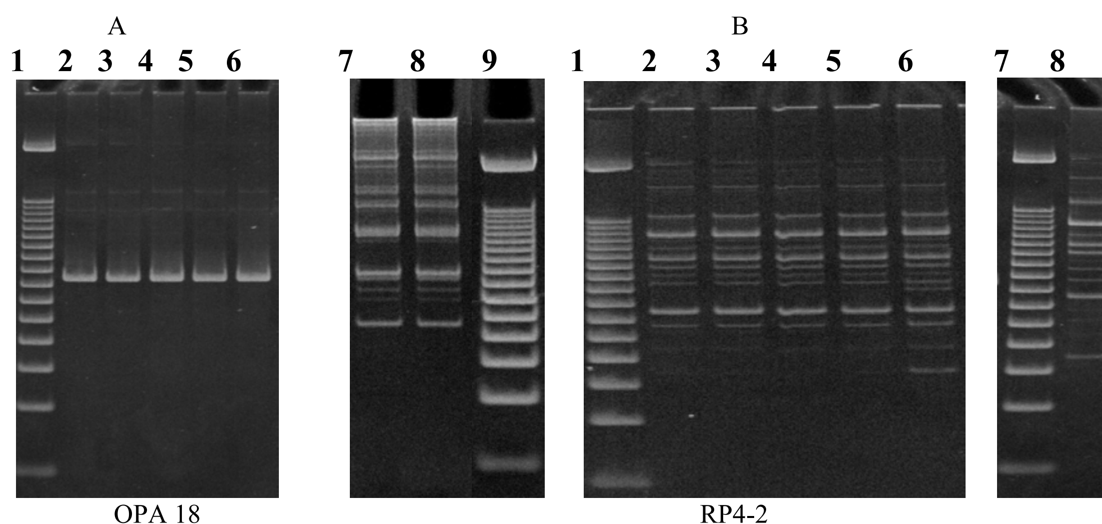


**Figure 5.** Amplified RAPD products from *Candida* species. Amplification with OPA 18 primer (A) and RP4-2 primer (B) are shown.

(A)Lanes 1-9: 50pb DNA Step ladder , *C. albicans* ATCC 1023, CMGB 318, CMGB 319, CMGB 320, CMGB 321, *C. parapsilosis* CBS 604 (8,9), 50pb DNA Step ladder

(B) Lane 1-8: *C. albicans* ATCC 1023, CMGB 319, CMGB 320, CMGB 321, CMGB 318, *C. parapsilosis* CBS 604, 50pb DNA Step ladder.

## Conclusion

In conclusion, the present study demonstrates that genotyping of *Candida* species could be accomplished on the basis of size and structural differences in the 5.8S rDNA regions using some molecular techniques like PCR, RFLP and RAPD. The profiles obtained by restriction of PCR products using different enzymes show that all strains included in this study exhibited a great similarity with the reference *C. albicans ATCC* 10231 strain**.** Moreover this method proved to be reproducible and very easy and, probably the major advantage, rapid to perform. The RAPD profiles using OPA 18 and RP4-2 primers were able to characterize intraspecific polymorphisms among the *Candida* species.

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