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Rapid Discrimination between *Candida glabrata*, *Candida nivariensis*, and *Candida bracarensis* by Use of a Singleplex PCR[▽]

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We report here a PCR-based assay using a single primer pair targeting the *RPL31* gene that allows discrimination between *Candida glabrata*, *Candida bracarensis*, and *Candida nivariensis* according to the size of the generated amplicon.

Candida albicans remains the *Candida* species most commonly recovered from clinical specimens. However, the incidence of non-*C. albicans Candida* species has increased, notably *Candida glabrata*, which now ranks second among the etiologic agents of invasive candidiasis (7, 11, 13, 14, 16, 17).

Along with *C. glabrata* and belonging to the *Nakaseomyces* clade, two new species, *Candida nivariensis* and *Candida bracarensis*, have recently been reported to be emerging pathogens (1, 4, 6, 8, 12, 18). Differentiation of these species from *C. glabrata* is of major importance to understand their clinical and epidemiologic role in candidiasis.

Conventional routine identification using a combination of chromogenic media, rapid trehalose assimilation test, biochemical panels such as the ID32C (bioMérieux, Marcy l'Etoile, France) may fail to differentiate *C. nivariensis* and *C. bracarensis* from *C. glabrata*. Thus, to unambiguously identify these species, molecular methods, mainly sequencing of the internal transcribed spacer (ITS) or D1/D2 domain of the large subunit of the ribosomal DNA (rDNA) are required (1, 4, 5, 12). A multiplex PCR targeting the internal transcribed spacer 1 (ITS1) region and using specific forward primers for each of the three species has recently been proposed (15). A peptide nucleic acid fluorescence *in situ* hybridization method has been developed with specific probes for *C. nivariensis* and *C. bracarensis* (4). Overall, these methods, while reliable, are either time-consuming or have been evaluated on a rather low number of strains. In this work, we describe a PCR-based assay using a single primer pair to differentiate *C. glabrata*, *C. nivariensis*, and *C. bracarensis* with no need of sequencing.

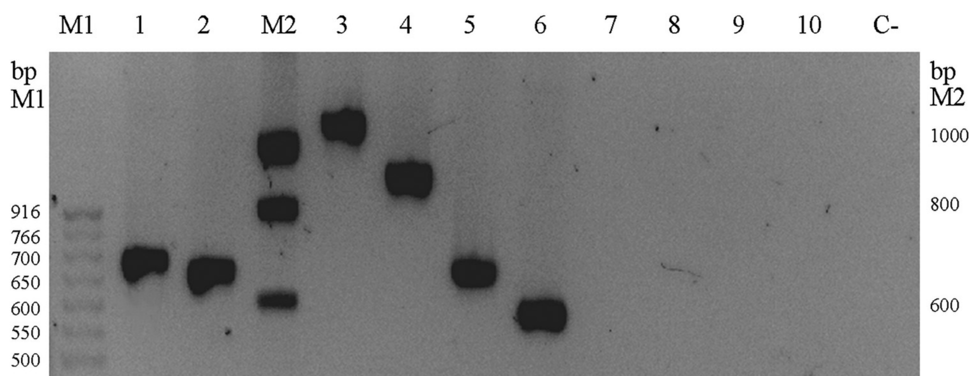

FIG. 1. Agarose gel electrophoresis of *RPL31* PCR products. Lanes: M1, 50-bp ladder (New England BioLabs, Evry, France); M2, Smart ladder (Eurogentec, Liege, Belgium); 1, *S. cerevisiae* FY73; 2, *C. nivariensis* CBS 9983; 3, *C. glabrata* CBS 138, 4, *C. bracarensis* CBS 10154; 5, *C. nivariensis* CBS 9983; 6, *N. delphensis* CBS 2170; 7, *C. albicans* ATCC 90028; 8, *C. parapsilosis* KB10016244; 9, *C. tropicalis* KB10016966; 10, *C. krusei* KB10016563; and C−, control without a DNA template.

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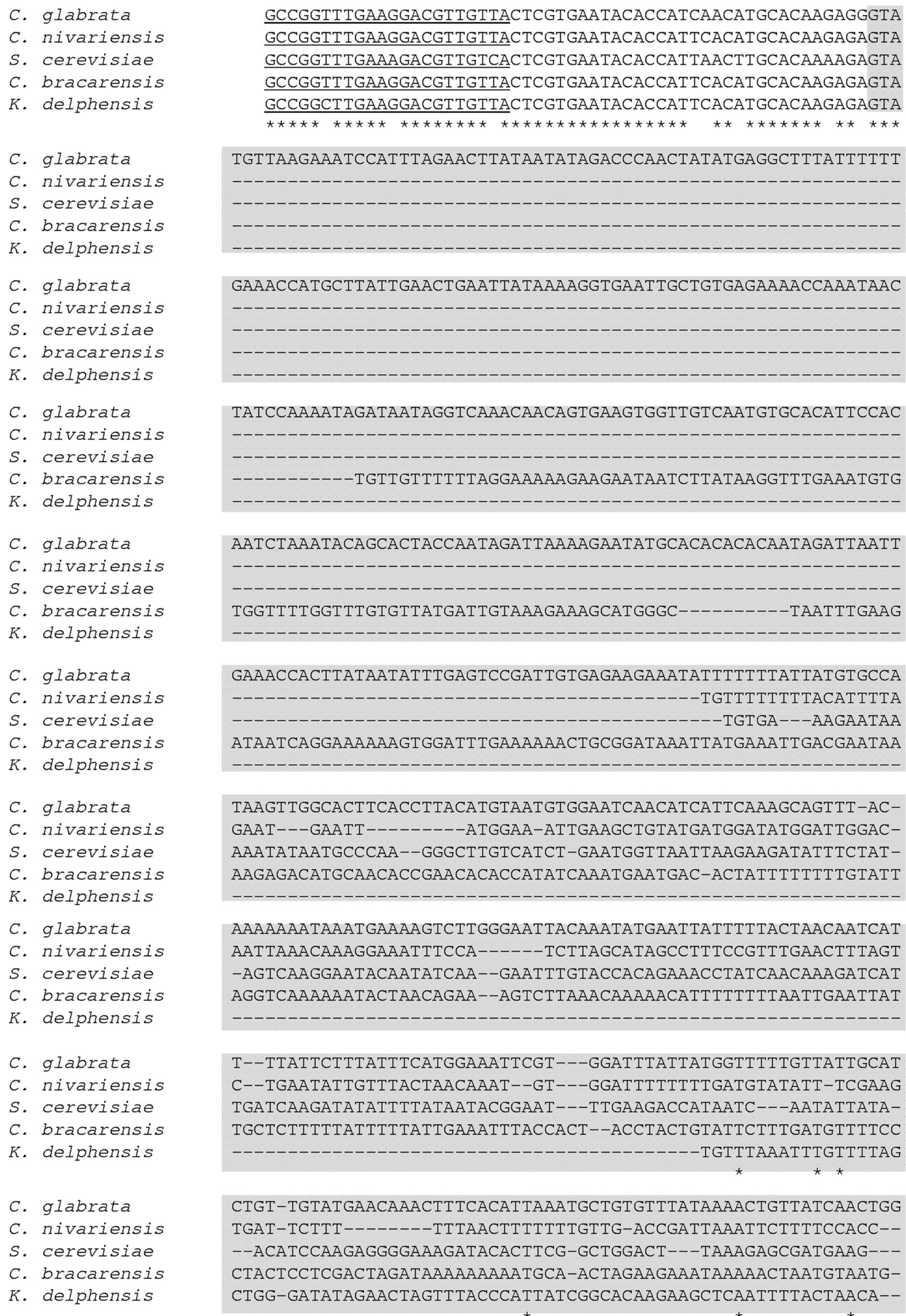

FIG. 2. Multiple-sequence alignment of *RPL31* orthologs in *C. glabrata* (CBS 138), *C. nivarensis* (CBS 9983), *C. bracarensis* (CBS 10154), *N. delphensis* (CBS 2170) and *S. cerevisiae* (FY73) (CLUSTALX 2.0.12). Primers RPL31cgF and RPL31cgR are underlined. The intronic portions of the genes are indicated by a gray background. Gaps introduced to maximize alignment are indicated by the dashes. Nucleotides that are identical in all five species are indicated by an asterisk below the sequences.

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C. glabrata       AAATCGTATTATGTGGCTGTAATGATTGCAGTGCTCAAAACTGTTATTTTGTTATTTAGA
C. nivariensis    ---CTGTATTGAGCAATTGGTATTATATCACAGG—AGAATTCTTATTAGTTTTGATGGA
S. cerevisiae     --ACAGAAAT-AGTTATCTTAAATAGTG-GAATCCTAGATTT-TAAGTCGAC------AA
C. bracarensis    -AATCGAATTGAATTGCCTTTATTAATACGAATCCTTAATGAATAATTAGGA------AG
K. delphensis     AACCAGTATT-TGCA-TTATTTGATGATAATTTCTTAATCTATGATTCAACAAACCCCC
                       * * *              *             *    * * *

C. glabrata       TTTCCGATACGGAATATAACACTGTTGTGTGGTCATTTTATTAGAGCATCCCCAGTTATG
C. nivariensis    TAGTAGATATTGAACTGCAAAAGGTG----GGAAATATTATAAGA---------------
S. cerevisiae     TTATCCATA------------TCCCA------AGTTTGACTAAA---------TATGA--
C. bracarensis    AAATTGAAAGAAGTGAGAGAACTGCA------AGATGGTATGGAAGGAATCCGTATGAC-
K. delphensis     CCAACCTCCCCGACTTGGAAGAATTAGAAGGTACGATTGACGAAGGAAGTATAGAAACTG

C. glabrata       CCACCGATACTGTTTTTGTAAATTTGAATTGGAAGGATTAGCCAGCTATTAATGGAACAA
C. nivariensis    --AATGATA----------GGGTGGGAAATGGAA---------------TGATGCAATAA
S. cerevisiae     --ATCAGAAC----AAATTGGGTCTCCAATATACAAAACCATTCATCAAGATT-ACACTA
C. bracarensis    TGATTAAGATGG--AAAAAGAGGTTGAAATA-ATCTAAGCATTTATAAGGGGGGAAACAA
K. delphensis     GGGACTACATTAGGAGCTATATTATAACCTAGAA-----AGCATGAAATATTTGGAAAGA
                          *                    *  *                       *  *

C. glabrata       GTTTGGATCATAGCAGA-TGTGATTCTACTATCTTAGATCACACTTGTTAAACA-TAAAC
C. nivariensis    GGGTAAA--------GA-AGTGAACATATCATCA----------------------AATT
S. cerevisiae     ATATTAAAATGAACAAT---CGTTACTAACAAAAAA-------------------TTTAC
C. bracarensis    AGAAAAATACTGACAGT-TCCGATATGAATAAAAAGAGGATTAAAAAAAATTGGTTTTGT
K. delphensis     GGGTGGGGAATTACGGAATCTGAGGAGAGAAATATTGATG----TCATAAATATCAAAAT
                                       *     *  *

C. glabrata       AAGAATATCTTTTATTAACAGTTGCATGGTGTTTCTTTCAAGAAGAGAGCCCCAAAGGCC
C. nivariensis    GAAATTAATTTTTCTT--TAGTTGCATGGTGTTTCTTTCAAGAAGAGAGCTCCAAGAGCT
S. cerevisiae     CATTTTATTTTTAAT----AGTTGCACGGTGTCTCCTTCAAGAAGAGAGCTCCAAGAGCT
C. bracarensis    TATGAAATATCTAATT---AGTTGCATGGTGTTTCTTTCAAGAAGAGAGCCCCAAGAGCT
K. delphensis     AAAAAAATATTCAATT---AGTTGCATGGTGTTTCTTTTAAAAAGAGAGCCCCAAGAGCT
                   *    *  *    *    ******* ***** ** ** ** ******** ****  **

C. glabrata       GTCAAGGAAATCAAGAAGTTCGCTAAGTTGCACATGGGTACTGAAGATGTCCGTTTGGCT
C. nivariensis    GTCAAGGAGATCAAGAAGTTCGCCAAGTTGCACATGGGTACTGAAGATGTCCGTTTGTCT
S. cerevisiae     GTCAAGGAAATTAAGAAGTTCGCCAAGTTACACATGGGTACTGATGATGTCCGTCTAGCT
C. bracarensis    GTCAAGGAGATCAAGAAGTTCGCCAAGTTGCACATGGGTACTGAAGATGTCCGTTTGTCT
K. delphensis     GTCAAGGAGATCAAGAAGTTCACCAAGTTGCACATGGGTACTGAGGATGTCCGTTTGTCT
                  ******** ** ********* * ***** ************** ********* *  **

C. glabrata       CCAGAATTGAACCAAGAAATCTGGAAGAGAGGTGTCAAGGGTGTTGCTTTCAGATTGAGA
C. nivariensis    CCAGAGTTGAACCAAGAAATCTGGAAGAGAGGTATCAAGGGTGTTCCATTCAGAATGAGA
S. cerevisiae     CCAGAATTGAACCAAGCTATCTGGAAGAGAGGTGTCAAGGGTGTTGAATACAGATTAAGA
C. bracarensis    CCAGAATTGAACCAAGAAATCTGGAAGAGAGGTATCAAGGGTGTTCCATTCAGAATGAGA
K. delphensis     CCAGAATTGAACCAAGAAATCTGGAAGAGAGGTATCAAGGGTGTTCCATTCAGAATGAGA
                  ***** **********  *************** ***********   * **** * ***

C. glabrata       TTGAGAATCTCCAGAAAGAGAAACGAAGAAGAAAACGCCAAGAACCCATTGTTC
C. nivariensis    TTGAGAATCTCCAGAAAGAGAAACGATGAAGAAGACGCCAAGAACCCATTGTTC
S. cerevisiae     TTGAGAATTTCCAGAAAGAGAAACGAAGAAGAAGACGCCAAGAACCCATTGTTC
C. bracarensis    TTGAGAATCTCCAGAAAGAGAAACGATGAAGAAGACGCCAAGAACCCATTGTTC
K. delphensis     TTGAGAATTTCCAGAAAGAGAAACGATGAAGAAGACGCCAAGAACCCATTGTTC
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FIG. 2—*Continued.*

We first performed an *in silico* search for highly conserved genes between *C. glabrata* and *Saccharomyces cerevisiae* that contain intron(s) on the Genolevures (http://www.genolevures.org/) and *Saccharomyces* Genome Database (http://downloads.yeastgenome.org/) (accessed 8 August 2010) websites. We postulated that conservation between *Nakaseomyces* species should be even higher than with *S. cerevisiae* and that because intron size is not being selected, it may differ between the considered species. We selected *RPL31*, a gene coding for a protein component of the large (60S) ribosomal subunit that fulfils our requirements. This gene exists in two copies in *S. cerevisiae* (*RPL31A* and *RPL31B*), but in a single copy in *C. glabrata*. A primer pair (RPL31cgF [cg stands for *C. glabrata*, and F stands for

forward] [5′-GCCGGTTTGAAGGACGTTGTTACT-3′] and RPL31cgR [R stands for reverse] [5′-GAACAATGGG TTCTTGGCGT-3′]) was designed to amplify a fragment of 1,061 bp in *C. glabrata* (CBS 138). This pair, under the conditions described here, specifically amplifies *RPL31B* in *S. cerevisiae* and not *RPL31A*.

Seventy-five strains, including *C. glabrata* CBS 138, *C. bracarensis* CBS 10154, *C. nivariensis* CBS 9983 type strains, and closely related *Nakaseomyces* (syn. *Kluyveromyces*) *delphensis* CBS 2170 type strain (10), *S. cerevisiae* FY73, and *C. albicans* ATCC 90028 reference strains, and 68 clinical isolates (34 *C. glabrata* isolates, 26 *C. nivariensis* isolates, 5 *C. bracarensis* strains and one isolate of each *C. parapsilosis*, *C. tropicalis*, and *C. krusei*) were used in this study. The *Candida* isolates were obtained from different patients from three different hospitals in France. All *C. glabrata*, *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *S. cerevisiae* strains were phenotypically identified with the commercial ID32C strip. The *C. bracarensis* and *C. nivariensis* strains were identified by sequencing the ITS and the D1/D2 domain of the large subunit regions of rDNA by the method of White et al. (19) (data not shown). A monoclonal subculture of each was stored at −80°C before testing.

DNA was extracted using a rapid method with Chelex resin and heat shock (9). The PCR was done with 0.25 μg of DNA in a 50-μl reaction mixture volume containing 1× PCR buffer, 0.5 μM each primer (EuroGentec, Belgium), 0.25 mM each deoxynucleoside triphosphate (equimolar concentrations of dATP, dCTP, dGTP, and dTTP) (New England BioLabs, Evry, France) and 0.5 U of Dream*Taq* polymerase (Fermentas, Saint Rémy lès Chevreuse, France). A touch-down amplification program was performed as follows: 3 min at 95°C; 3 cycles, with 1 cycle consisting of 30 s at 95°C, 30 s at 62°C, and 30 s at 72°C; 3 cycles, with 1 cycle consisting of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C; 3 cycles, with 1 cycle consisting of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; 28 cycles, with 1 cycle consisting of 30 s at 50°C and 30 s at 72°C; and a final extension step of 10 min at 72°C. The amplified products were separated by electrophoresis in an agarose gel (2% in Tris-borate-EDTA [TBE] buffer) stained with ethidium bromide (0.5 μg/ml) at 90 V for 2 h. Amplification products of *C. nivariensis*, *C. bracarensis*,. and *N. delphensis* type strains were directly sequenced and deposited under the accession numbers GenBank ID: JF690246, JF690247, and JN088223, respectively. Applied to our whole collection of strains, the method allowed DNA amplification for all *C. glabrata*, *C. nivariensis*, and *C. bracarensis* strains and the *S. cerevisiae* strain but not DNA amplification for *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* (Fig. 1).

DNAs from *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* strains were checked by successful amplification using the ITS1 and ITS4 primer pairs (data not shown). Amplification products had expected sizes of 1,061 bp for *C. glabrata* and 691 bp for *S. cerevisiae*. All *C. bracarensis* and *C. nivariensis* strains and the *N. delphensis* CBS 2170 strain had an amplification product estimated at 900 bp, 670 bp, and 550 bp, respectively. These sizes were confirmed by directly sequencing amplicons from type strains (902 bp, 665 bp, and 591 bp). Indeed, alignment of homolog sequences shows that the differences in size are due to the intron (Fig. 2).

The recently described *Candida* species *C. bracarensis* and *C. nivariensis* are closely related to *C. glabrata*. Based on phenotypic characteristics, these species were initially misidentified as *C. glabrata* (2, 3). Molecular methods to differentiate the three species have been reported (1, 4, 5, 12, 15), but none used a simple PCR with a single pair of primers.

We report here a PCR-based assay using a single primer pair that allows us to distinguish species: 1,061 bp for *C. glabrata*, 902 bp for *C. bracarensis*, 665 bp for *C. nivariensis*, and 591 bp for *N. delphensis.* Experimental simplicity (a single primer pair, analysis on agarose gel electrophoresis) and the rapidity and accuracy of this method make it a powerful tool to both investigate collections of strains that possibly contain *C. bracarensis* and/or *C. nivariensis* strains and to confirm the identification of strains isolated from invasive infection. This should improve our knowledge on the respective epidemiology and pathogenic importance of these three species.

Nucleotide sequence accession numbers. Amplification products of *C. nivariensis*, *C. bracarensis*, and *N. delphensis* type strains were sequenced and deposited in GenBank under accession numbers JF690246, JF690247, and JN088223, respectively.

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