

Differentiation of *Candida glabrata*, *C. nivariensis* and *C. bracarensis* based on fragment length polymorphism of ITS1 and ITS2 and restriction fragment length polymorphism of ITS and D1/D2 regions in rDNA

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Abstract Different molecular methods for the discrimination of *Candida glabrata*, *C. bracarensis* and *C. nivariensis* were evaluated and the prevalence of these species among Danish blood isolates investigated. Control strains were used to determine fragment length polymorphism in the ITS1, ITS2, ITS1-5.8S-ITS2 regions and in the D1/D2 domain of 26S rDNA using primers designed for this study. A total of 133 blood isolates previously identified as *C. glabrata* were examined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and the peptide nucleic acid-fluorescent in situ hybridization (PNA-FISH) method. The size of ITS1 allowed differentiation between *C. glabrata* (483), *C. nivariensis* (361) and *C. bracarensis* (385),

whereas the ITS2 region was of similar size in *C. nivariensis* (417) and *C. glabrata* (418). Sequence analysis of the ITS region suggested that many restriction enzymes were suitable for RFLP differentiation of the species. Enzymatic digestion of the D1/D2 domain with *TatI* produced unique band sizes for each of the three species. PCR-RFLP and PNA-FISH were in agreement for all of the isolates tested. None of the 133 Danish blood isolates were *C. nivariensis* or *C. bracarensis*. Fragment size polymorphism of ITS1 and RFLP of the D1/D2 domain or the ITS region are useful methods for the differentiation of the species within the *C. glabrata* group. *C. bracarensis* and *C. nivariensis* are rare among Danish *C. glabrata* blood isolates.

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Table 1 Sizes of the polymerase chain reaction (PCR) products of the entire ITS1-ITS2, ITS1 and ITS2 regions for the *Candida glabrata* group

Species	GenBank accession numbers of the analysed sequences	Fragment size for: (ITS1-ITS2) (ITS1) (ITS2)
<i>C. glabrata</i>	AY198398, U44808, EF489409, EF690393, EU445377, EU439448, Z48341, EF056321, EF056320, AF369518, AF369517, AF369512, AF369511, AF369507, FJ437059, AF369523, AF369522, AF369521, AF369520, AY601162, AB499020, GU199447*	(880) (483) (417)
<i>C. nivariensis</i>	EF056322, FN2984881, FM955316 AM745293, AM745291, AM745289, AM745287, AM745285, AM745283, AM745281, AM745279, AM745277, AM745275, AM745273, AM745271, AM745269, AY620959, AY620958, AY620957, FN298488, FM955316, GU199441*, GU199442*, GU199443*, GU199444*, GU199445*, GU199446*	(760) (361) (418)
<i>C. braccarensis</i>	AY589573, GU199438*, GU199439*, GU199440*	(805) (385) (440)

*Denotes sequences deposited in GenBank in the present study

Introduction

Although species identification of the most common *Candida* species is straightforward by conventional methods, a number of the rarer newly recognised species are more challenging to identify correctly. Recent advances in molecular techniques have allowed the discrimination between closely related species like *C. dubliniensis* and *C. albicans* [1], *C. orthopsilosis*, *C. metapsilosis* and *C. parapsilosis* [2, 3] and *C. nivariensis* [4], *C. braccarensis* [5] and *C. glabrata*. Discrimination between even closely related species may be of therapeutic importance. For example, *C. dubliniensis* more easily develops fluconazole resistance than *C. albicans* [1]. Similarly, intrinsic susceptibility patterns of *C. nivariensis* and *C. braccarensis* may differ from that of *C. glabrata* [6, 7] and in vitro susceptibility to azoles and flucytosine appears to be lower for *C. nivariensis* than for *C. glabrata* [8].

We have recently reported a reliable polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for the differentiation of *C. metapsilosis*, *C. orthopsilosis* and *C. parapsilosis*, and described the prevalence of these species among Danish blood isolates [3]. The objective of

this study was primarily to develop effective molecular methods for the identification of *C. glabrata*, *C. nivariensis* and *C. braccarensis*. The secondary objectives were to investigate the prevalence of *C. nivariensis* and *C. braccarensis* among Danish blood isolates identified as *C. glabrata* by conventional methods and to compare our genotypical methods with a commercially available peptide nucleic acid-fluorescent in situ hybridization (PNA-FISH) technology.

Materials and methods

Control strains and blood isolates

Ten control strains were included: two *C. glabrata* (ATCC [American Type Culture Collection] 90030 and CBS [Centraalbureau voor Schimmelcultures] 138), five *C. nivariensis* (CBS 10161, CBS 9983, CBS 9984, CBS 9985 and CPH-05-H41019) and three *C. braccarensis* (CBS 10154, NRRL Y-27794 and one strain from the company AdvanDx). One hundred and thirty-three Danish *C. glabrata* blood isolates were collected as part of an active population-based surveillance of candidaemia in Denmark

Fig. 1 Agarose gel electrophoresis of amplified entire ITS1-5.8SrDNA-ITS2 (left), ITS1 (middle) and ITS2 (right) regions. Lanes 1: *C. glabrata*, lanes 2: *C. nivariensis*, lanes 3: *C. braccarensis*, lanes NC: negative controls and lanes M: 100-bp ladder molecular size marker

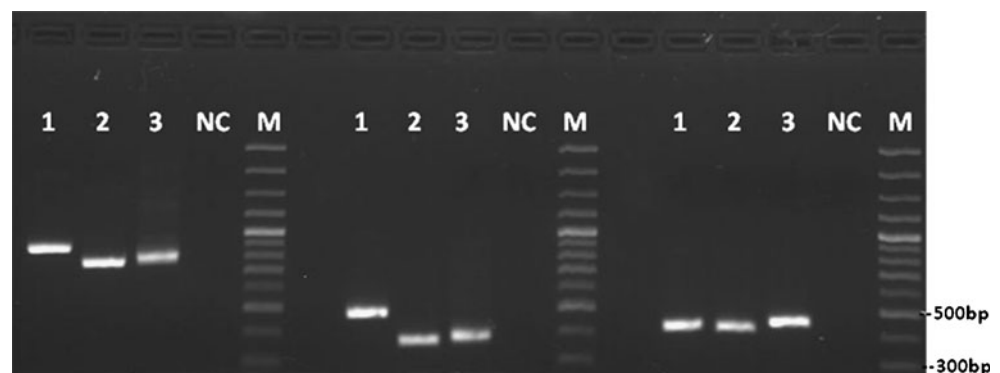


Table 2 Pair-wise sequence comparison of the entire ITS1-5.8-ITS2 region between the control strains of *C. glabrata* (*C. glab*), *C. nivariensis* (*C. niva*) and *C. braccarensis* (*C. brac*) used in this study. The numbers in the upper-right cells are the total number of nucleotide differences and the numbers in the lower-left cells are the percentage differences

No.	<i>Candida</i> sp. (reference number, sequence ID)	1 <i>C. glab</i> (GU199447) ATCC 90030 (AY939793)	2 <i>C. glab</i> (AY589573) NRRL Y- 27794 (GU199438)	3 <i>C. bracc</i> (AY589573) NRRL Y- 27794 (GU199439)	4 <i>C. bracc</i> (AY589573) NRRL Y- 27794 (GU199438)	5 <i>C. brac</i> AdvanDx (GU199439)	6 <i>C. brac</i> CBS 10154 (GU199440)	7 <i>C. niva</i> CBS 10161 (GU199441)	8 <i>C. niva</i> CBS 9983 (GU199443)	9 <i>C. niva</i> CPH-05- H41019 (GU199442)	10 <i>C. niva</i> CBS 9984 (GU199444)	11 <i>C. niva</i> CBS 9984 (GU199445)	12 <i>C. niva</i> (FM955316)
1	<i>C. glab</i> (GU199447)	–	346	347	346	346	346	335	337	333	337	337	352
2	<i>C. glab</i> ATCC 90030	–	342	343	342	342	342	338	339	334	339	339	345
3	<i>C. bracc</i> (AY589573)	35.1	–	12	0	0	0	240	240	233	240	240	246
4	<i>C. bracc</i> NRRL Y-27794	35.2	1.5	–	12	12	12	234	235	232	235	235	241
5	<i>C. bracc</i> (GU199438)	35.2	0	1.5	–	–	0	240	240	233	240	240	246
6	<i>C. bracc</i> CBS 10154	35.2	0	1.5	0	0	–	240	240	233	240	240	246
7	<i>C. niva</i> CBS 10161	34.9	35.2	26.8	27.4	27.4	27.4	–	3	8	3	3	10
8	<i>C. niva</i> CBS 9983	35.0	35.2	26.9	27.4	27.4	27.3	0.4	–	7	0	0	7
9	<i>C. niva</i> CPH-05-H41019	34.7	34.8	26.8	26.8	26.8	26.8	1.1	0.9	–	7	7	14
10	<i>C. niva</i> CBS 9985	35.0	35.2	26.9	27.4	27.4	27.3	0.4	0	0.9	–	0	7
11	<i>C. niva</i> CBS 9984	35.0	35.2	26.9	27.4	27.4	27.3	0.4	0	0.9	0	–	7
12	<i>C. niva</i> (FM955316)	36.4	35.7	27.9	27.9	27.9	27.9	1.3	0.9	1.8	0.9	0.9	–

and identified as previously described [9]. The isolates had been stored in glycerol/water at -80°C .

PCR

A small part ($0.5\text{--}1\text{ mm}^3$) of a single fresh colony was added without prior DNA extraction [10, 11] to the PCR premix containing $0.5\text{ }\mu\text{M}$ of each primer, $400\text{ }\mu\text{M}$ of deoxynucleoside triphosphate mix, $2.5\text{ }\mu\text{l}$ of magnesium-free buffer, 1.5 mM of MgCl_2 and 1.25 U of *Taq* DNA polymerase in a final volume of $25\text{ }\mu\text{l}$. A negative control (water) was included in all PCR experiments. The following primer pairs were used for PCR amplification: ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS2 (5'-GCT GCG TTC TTC ATC GAT GC-3') to amplify the internal transcribed spacer 1 (ITS1) region, ITS3 (5'-GCA TCG ATG AAG AAC GCA GC-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') for the ITS2 region and ITS1 and ITS4 for the entire ITS1-5.8SrDNA-ITS2 region [12–14]. DNA from all control strains and clinical isolates were also amplified using primers designed in the present study (26SglabF: 5'-GTA CCT TTG GTG CCC GAG TT-3' and 26SglabR: 5'-CCA TTA TGC CAG CAT CCT AGA-3') for partial amplification of the D1/D2 domain in the 26S ribosomal RNA gene (rDNA).

Sequencing

Control strains were sequenced using both forward (ITS1) and reverse (ITS4) primers [12–14]. Sequencing was performed by the company MWG (Eurofins MWG Operon, Ebersberg, Germany) with a BigDye Terminator Cycle Sequencing Kit on an ABI 3730 Genetic Analyzer.

Sequence analysis

Forward and reverse sequences were aligned to ensure validity. The sequences were subjected to nucleotide BLAST search

(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and DNASIS (Hitachi, Japan) multiple alignment. The sequences obtained in this project and all relevant sequences deposited in GenBank were also analysed for the assessment of the inter-/intra-species nucleotide and size variation of ITS regions. The levels of sequence difference (D) were calculated for pair-wise comparison using the formula $D=1 - (M/L)$ [15], in which M is the number of alignment positions at which the two sequences had a base in common and L is the total number of alignment positions over which the two sequences are compared. To select restriction enzymes suitable for RFLP analysis of the ITS1-5.8S-ITS2 and D1/D2 regions of the three species belonging to the *C. glabrata* clade, sequences were individually analysed against all 610 restriction enzymes present in DNASIS software and cutting sites and fragment sizes were predicted in silico. The enzymes with the highest discriminatory power were chosen for further studies.

RFLP

The PCR products of the D1/D2 region for all control strains and clinical isolates were digested by each of the restriction enzymes *TatI* and *AluI* (Fermentas, Lithuania); the enzymes *TasI*, *MnII*, *Hin6I*, *BstF5I*, *SatI* and *FatI* (Fermentas, Lithuania) were tested with selected control strains. The PCR products were digested for 120 min, in a $15\text{-}\mu\text{l}$ reaction volume containing $0.5\text{ }\mu\text{l}$ (5 units) of each enzyme, $1.5\text{ }\mu\text{l}$ of $10\times$ buffer (33 mM Tris-acetate [pH 7.9], 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA) supplied with the enzyme, $5\text{ }\mu\text{l}$ of PCR products and $8\text{ }\mu\text{l}$ of molecular grade water.

Electrophoresis

Five microlitres of amplified and $8\text{ }\mu\text{l}$ of digested DNA products were subjected to electrophoresis on a 1.5% and 2% agarose gel, respectively. The electrophoresis buffer was TBE

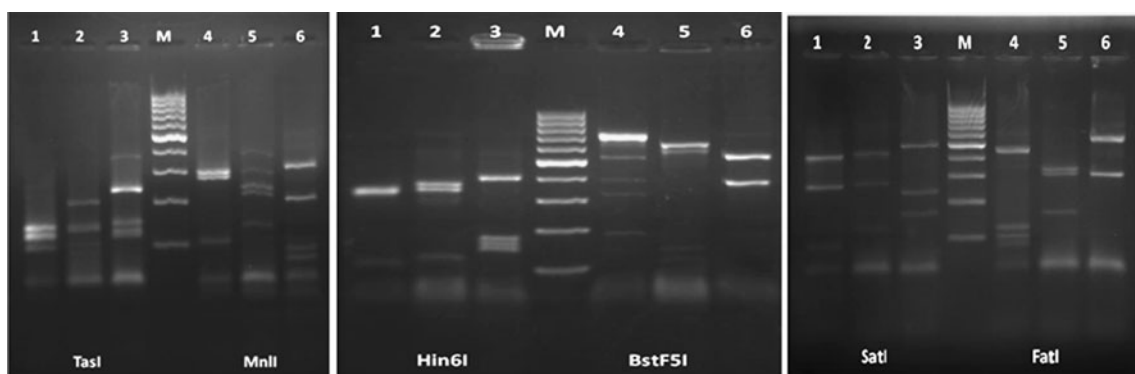


Fig. 2 Agarose gel electrophoresis of the amplified ITS1-5.8SrDNA-ITS2 region followed by digestion with some exemplified enzyme. Lanes 1 and 4 are *C. nivariensis*, 2 and 5 are *C. bracarensis*, and 3 and 6 are *C. glabrata*, respectively. Lanes M: 100-bp ladder molecular size marker

(90 mM Tris, 90 mM Boric acid and 2 mM EDTA, pH 8.3). A 100-bp DNA ladder (Fermentas, Lithuania) was used as a molecular size marker. DNA bands were visualised by UV trans-illumination and photographed.

PNA-FISH

Four types of probes (AdvanDx, Woburn, MA) were used in this study: all control strains and clinical isolates were initially screened using a *C. glabrata* clade-specific probe (staining all three species red) and a cocktail of three probes (*C. glabrata* Clade Probe Mix) staining *C. nivariensis* and *C. bracarensis* yellow and *C. glabrata* red. Isolates staining yellow using the probe cocktail were further tested using two species-specific probes for *C. bracarensis* and *C. nivariensis*, respectively, (green colour stain) for final species identification. The kits were used according to the manufacturer's protocol. Briefly, a drop of the fresh yeast suspension was placed onto a Teflon-coated microscope slide, dried at 70°C and fixed with methanol for 10 min. One drop of probe solution was added, a coverslip was applied and the slides were incubated at 55°C for 90 min using a slide heater supplied by the company. The slides were then submerged in a stringent wash solution at 55°C for 30 min in a water bath, mounted with mounting fluid and a new coverslip, and examined using a fluorescence microscope equipped with a filter (Filter cat. no. AC003, AdvanDX).

Results

ITS analysis

Sequence size comparisons of the entire ITS1-5.8-ITS2 region as well as for ITS1 and ITS2 separately are provided

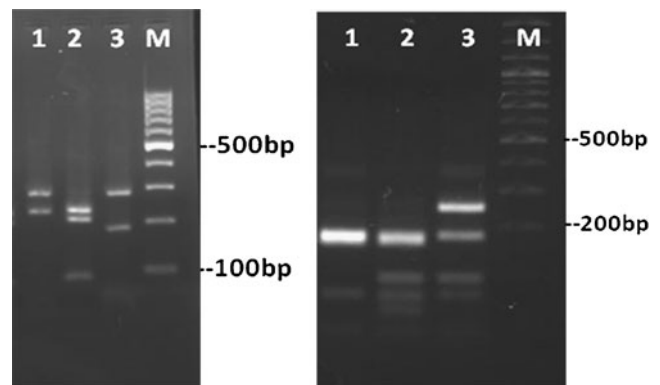


Fig. 3 Agarose gel electrophoresis of the D1/D2 domain of 26S rDNA from representative control strains, amplified by 26SglabF and 26SglabR followed by digestion with *TatI* (left) and *AluI* (right). In the left photo, lanes 1–3 are *C. bracarensis*, *C. nivariensis* and *C. glabrata*, respectively. In the right photo, lanes 1–3 are *C. glabrata*, *C. nivariensis* and *C. bracarensis*, respectively. Lanes M: 100-bp ladder molecular size marker

in Table 1 for a total of 53 strains (22 *C. glabrata*, 27 *C. nivariensis* and four *C. bracarensis*). The sizes of the entire ITS region and ITS1 were discriminative, while the ITS2 products of *C. nivariensis* and *C. glabrata* were similar (418 and 417 nucleotides [nt], respectively). The results obtained with PCR and gel electrophoresis for the three species confirmed the calculated band sizes (Fig. 1). A pairwise nucleotide sequence analysis of the entire ITS1-5.8-ITS2 region of the ten control isolates and two randomly chosen clinical isolates demonstrated a high inter-species difference (26.8% to 36.4% or 232 nt to 352 nt) in comparison with the low intra-species variation (0 to 1.8% or 0 to 14 nt). *C. bracarensis* and *C. nivariensis* were more similar to each other than to *C. glabrata* (Table 2).

Table 3 Enzymes suitable for differentiation of the *C. glabrata* group by restriction digestion of the D1/D2 domain of the large subunit of the rDNA region and their fragment sizes

Species	GenBank accession number	Size of PCR products	Cutting sites for:		Fragments after digestion with:
			<i>TatI</i>	<i>AluI</i>	<i>RsaI</i>
<i>C. glabrata</i>	Many	507	(49, 226)	(48, 177, 282)	(48, 177, 282)
			(164, 194, 360)	(30, 148, 163, 166)	(30, 148, 163, 166)
			(3, 35, 51, 228, 275)	(2, 16, 32, 47, 177, 233)	(2, 16, 32, 47, 177, 233)
<i>C. nivariensis</i>	AB500875, FM955317, AY627307, AY627305, AY627306, EF056323AB499984	508	(226, 426)	(83, 200, 225)	(83, 200, 225)
			(164, 194, 360, 412, 440)	(29, 30, 52, 68, 163, 166)	(29, 30, 52, 68, 163, 166)
			(3, 35, 228, 275, 428)	(2, 32, 47, 81, 153, 193)	(2, 32, 47, 81, 153, 193)
<i>C. bracarensis</i>	AY589572*	507	(226)	(225, 282)	(225, 282)
			(164, 194, 440)	(30, 68, 163, 246)	(30, 68, 163, 246)
			(3, 35, 228, 275)	(2, 32, 47, 193, 233)	(2, 32, 47, 193, 233)

*The only available sequence in GenBank

Table 4 Control strains and clinical isolates used in this study and their identification by different methods

Order	Species	Strain	Colony colour on CHROMagar Candida	PCR-RFLP of the D1/D2 region with <i>TatI</i>	PCR-RFLP of the D1/D2 region with <i>AluI</i>	ITS1-ITS2 sequencing	Fragment sizing of the ITS1 region	PNA-FISH
1	<i>C. glabrata</i>	ATCC 90030	Purplish-pink	<i>C. glabrata</i>	<i>C. glabrata</i>	Not done	<i>C. glabrata</i>	<i>C. glabrata</i>
2	<i>C. glabrata</i>	CBS 138	Purplish-pink	<i>C. glabrata</i>	<i>C. glabrata</i>	Not done	<i>C. glabrata</i>	<i>C. glabrata</i>
3	<i>C. nivariensis</i>	CBS 10161	White	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>
4	<i>C. nivariensis</i>	CBS 9983	White	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>
5	<i>C. nivariensis</i>	CBS 9984	White	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>
6	<i>C. nivariensis</i>	CBS 9985	White	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>
7	<i>C. nivariensis</i>	CPH-05-H41019	White	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>	<i>C. nivariensis</i>
8	<i>C. bracarensis</i>	CBS 10154	White	<i>C. bracarensis</i>	<i>C. bracarensis</i>	<i>C. bracarensis</i>	<i>C. bracarensis</i>	<i>C. bracarensis</i>
9	<i>C. bracarensis</i>	NRRL Y-27794	White	<i>C. bracarensis</i>	<i>C. bracarensis</i>	<i>C. bracarensis</i>	<i>C. bracarensis</i>	<i>C. bracarensis</i>
10	<i>C. bracarensis</i>	Strains from AdvanDx	White	<i>C. bracarensis</i>	<i>C. bracarensis</i>	<i>C. bracarensis</i>	<i>C. bracarensis</i>	<i>C. bracarensis</i>
11–143	<i>C. glabrata</i>	Blood isolates	Purplish-pink	<i>C. glabrata</i>	<i>C. glabrata</i>	Not done	Not done	<i>C. glabrata</i>

In order to select appropriate restriction enzymes for RFLP, restriction patterns were predicted for the 610 restriction enzymes included in the DNASIS software (version 2.09, 2007). A total of 87 restriction enzymes were identified as appropriate for differentiation (data not shown). Six enzymes (*TasI*, *MnII*, *Hin6I*, *BstF5I*, *SatI* and *FatI*) were randomly selected among these for laboratory evaluation (Fig. 2). The RFLP patterns on agarose gel for individual enzymes were exactly as expected from in silico analysis (data not shown).

Analysis of the D1/D2 domain of 26S rDNA

Computer analysis of the sequences of the D1/D2 region indicated that only three enzymes (*TatI*, *AluI* and *RsaI*) were suitable for digestion of the 507/8-bp PCR amplicon. Cutting sites and size of the individual fragments of the region for each species and these enzymes are shown in Table 3. *TatI* was predicted to have the highest discriminatory power, producing three bands for *C. glabrata* (48,

177, 282 bp), three bands for *C. nivariensis* (83, 200, 225 bp) and two bands for *C. bracarensis* (225, 282 bp). These results were confirmed by PCR and RFLP of selected isolates from the three species (Fig. 3).

PNA-FISH species identification

All control and clinical isolates were tested with the PNA-FISH method and the results were in agreement with the DNA-based identification. A summary of the species identification results is provided in Table 4.

Analysis of clinical blood isolates

The DNA of all clinical isolates were successfully amplified by the primers 26SglabF and 26SglabR and a 500-bp band was demonstrated for all isolates (data not shown). All 133 blood isolates were confirmed to be *C. glabrata sensu strictu* using PCR-RFLP, as well as PNA-FISH (Table 4).

Table 5 Summary of previous reports on *C. nivariensis* and/or *C. bracarensis* isolates

Country or region	No. of isolates (species)	Sort of specimens	Reference
Spain	3 (<i>C. nivariensis</i>)	Various clinical samples	[4]
Portugal	2 (<i>C. bracarensis</i>)	Vaginal exudates and blood culture	[5]
USA	3 (<i>C. nivariensis</i>)	Various clinical samples	[6]
Japan	1 (<i>C. nivariensis</i>)	Blood	[7]
Indonesia	1 (<i>C. nivariensis</i>)	Mucosa of HIV-infected patients	[21]
India	2 (<i>C. bracarensis</i>)	Various clinical samples	[22]
98 clinical centres in 28 countries	2 (<i>C. bracarensis</i>) and 1 (<i>C. nivariensis</i>)	Various clinical samples	[17]
UK	16 (<i>C. nivariensis</i>)	Various clinical samples	[8]

Discussion

In this study, we focussed on the molecular differentiation of *C. glabrata*, *C. bracarensis* and *C. nivariensis*. To some extent, *C. nivariensis* and *C. bracarensis* can be differentiated from *C. glabrata* morphologically. Thus, both species exhibit very similar carbohydrate source assimilation profiles, produce white colonies on CHROMagar® (Becton Dickinson) and do not show turbidity in YPD broth [4]. In contrast, *C. glabrata* has a typical pink colour on CHROMagar and shows turbidity in YPD broth. However, some strains of *C. glabrata*, 13/127 [16] and 11/1598 [17], produce white colonies on this medium; the three species may also be misidentified in the routine laboratories because all assimilate trehalose and none are able to form germ tubes, chlamydoconidia, pseudohyphae or ascospores. Furthermore, commercial identification systems such as VITEK 2 and API ID32C report all as *C. glabrata* [4, 5, 16, 17].

Sequencing the D1/D2 domain of the large subunit RNA gene and ITS1 and ITS2 regions of the ribosomal DNA have proven to be useful for the DNA-based identification of most fungi [18–20]. These targets have been the basis of the discovery of new pathogenic yeast species, including *C. dubliniensis* [1], *C. orthopsilosis* and *C. metapsilosis* [2]. Similarly, *C. nivariensis* was described as a novel species based on the sequences of the ITS and D1/D2 domain of rDNA [4] and *C. bracarensis* based on the sequencing of D1/D2 and PCR fingerprinting profile [5].

A limited number of methods have been used for the differentiation of *C. glabrata*, *C. nivariensis* and *C. bracarensis*, and the epidemiology of these species has not been well elucidated. So far, molecular identification methods for this purpose include sequencing [8, 21, 22], PNA-FISH [6, 17], PCR with specific primers [23, 24], ITS-PCR and microchip gel electrophoresis [7], and multiplex PCR [23, 24]. In the present study, we explored the possibility of differentiating *C. nivariensis* and *C. bracarensis* from each other and from *C. glabrata* using low-cost and simple DNA-based assays. We found the length of ITS1 to be a good marker for this purpose (Table 1 and Fig. 1). It may serve as a rapid screening of isolates presumptively identified as *C. glabrata*, since the method only requires a standard PCR and subsequent agarose gel electrophoresis. Secondly, we found several enzymes applicable for ITS-RFLP and a few for D1/D2. Since 26S rDNA is more conserved than the ITS regions in fungi [18], this region may be the preferable target for PCR-RFLP.

The 133 blood isolates were collected during a period of 7 years (2002–2008). They were all confirmed to be *C. glabrata* by genotypical methods and PNA-FISH, suggesting that *C. nivariensis* and *C. bracarensis* are rare causes of

candidaemia in Denmark. For comparison, 31 clinical isolates (25 *C. nivariensis* and six *C. bracarensis*) have, so far, been reported in the literature (Table 5).

In conclusion, fragment size polymorphism of ITS1 is a rapid and cost-effective method for differentiating the species in the *C. glabrata* clade, avoiding RFLP or sequencing after PCR. PCR-RFLP of the D1/D2 domain of 26S rDNA or ITS regions is a reliable and relatively inexpensive method also meeting this purpose. PNA-FISH using a commercial kit is a third effective method for the identification of *C. glabrata* and the two closely related species. Diagnostic efforts should be balanced against the fact that the prevalence of *C. bracarensis* and *C. nivariensis* in clinical samples, at the present time, appears to be low.

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