Molecular screening for *Candida orthopsilosis* and *Candida metapsilosis* among Danish *Candida parapsilosis* group blood culture isolates: proposal of a new RFLP profile for differentiation

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Candida orthopsilosis and *Candida metapsilosis* are recently described species phenotypically indistinguishable from *Candida parapsilosis*. We evaluated phenotyping and molecular methods for the detection of these species among 79 unique blood culture isolates of the *C. parapsilosis* group obtained during the years 2004–2008. The isolates were screened by PCR amplification of the secondary alcohol dehydrogenase-encoding gene (*SADH*) followed by digestion with the restriction enzyme *Ban*I, using *C. parapsilosis* ATCC 22019, *C. orthopsilosis* ATCC 96139 and *C. metapsilosis* ATCC 96144 as controls. Isolates with RFLP patterns distinct from *C. parapsilosis* were characterized by sequence analysis of the ITS1–ITS2, 26S rRNA (D1/D2) and *SADH* regions. Restriction patterns for the 3 species with each of 610 restriction enzymes were predicted *in silico* using 12 available sequences. By PCR-RFLP of the *SADH* gene alone, four isolates (5.1 %) had a pattern identical to the *C. orthopsilosis* reference strain. Sequence analysis of *SADH* and ITS (internal transcribed spacer) regions identified two of these isolates as *C. metapsilosis*. These results were confirmed by creating a phylogenetic tree based on concatenated sequences of *SADH*, ITS and 26S rRNA gene sequence regions. Optimal differentiation between *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis* was predicted using digestion with *Nla*III, producing discriminatory band sizes of: 131 and 505 bp; 74, 288 and 348 bp; and 131, 217 and 288 bp, respectively. This was confirmed using the reference strains and 79 clinical isolates. In conclusion, reliable discrimination was obtained by PCR-RFLP profile analysis of the *SADH* gene after digestion with *Nla*III but not with *Ban*I. *C. metapsilosis* and *C. orthopsilosis* are involved in a small but significant number of invasive infections in Denmark.

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Abbreviations: AFLP, amplification fragment length polymorphism; AFST–EUCAST, Antifungal Susceptibility Testing Subcommittee–European Committee on Antimicrobial Susceptibility Testing; ATCC, American Type Culture Collection; ITS, internal transcribed spacer; RAPD, randomly amplified polymorphic DNA. The GenBank/EMBL/DDBJ accession numbers for the *SADH* sequences reported in this study are FJ746046–FJ746069.

INTRODUCTION

Candida parapsilosis is one of the five most common yeasts involved in invasive candidiasis and is particularly common in neonates, in catheter-associated candidaemia and in association with intravenous hyperalimentation (Krcmery & Barnes, 2002). In Denmark, it is involved in 4% of the fungaemia cases (Arendrup *et al.*, 2008). *C. parapsilosis* is normally susceptible to most antifungal compounds but elevated MICs for the echinocandin agents are consistently reported for this species due to an intrinsic mutation in the *FKS* hot spot (Garcia-Effron *et al.*, 2008). Susceptibility differences within the *C. parapsilosis* group could affect therapeutic choices (van Asbeck *et al.*, 2008).

C. parapsilosis is a complex of three genetic groups (I, II and III) according to the results from genotypic methods, including randomly amplified polymorphic DNA (RAPD) analysis (Lehmann *et al.*, 1992), karyotyping (Lott *et al.*, 1993), multilocus enzyme electrophoresis, ribosomal internal transcribed spacer (ITS) sequencing (Lin *et al.*, 1995), DNA reassociation analysis (Roy & Meyer, 1998), DNA topoisomerase II gene sequencing (Kato *et al.*, 2001), complex DNA probe pattern analysis by Southern blotting (Enger *et al.*, 2001) and mitochondrial DNA sequence differences (Nosek *et al.*, 2002). In 2005 Tavanti *et al.* (2005) reported a multilocus sequence typing scheme and suggested *C. parapsilosis* groups II and III should be reclassified as the species *Candida orthopsilosis* and *Candida metapsilosis*, respectively. *C. parapsilosis* was retained for group I. PCR amplification of the secondary alcohol dehydrogenase-encoding gene (*SADH*) followed by digestion of the PCR product with the restriction enzyme *BanI* was proposed for differentiation of these three new species. Subsequently, some retrospective epidemiological studies have been undertaken to screen for *C. orthopsilosis* and *C. metapsilosis* among previously identified *C. parapsilosis* isolates showing that a small proportion of such blood culture isolates are actually *C. orthopsilosis* or *C. metapsilosis*, with a significant geographical variation (Asadzadeh *et al.*, 2009; Gomez-Lopez *et al.*, 2008; Gonçalves *et al.*, 2010; Kocsuó *et al.*, 2007; Lockhart *et al.*, 2008; Pryce *et al.*, 2006; Silva *et al.*, 2009; Tavanti *et al.*, 2005, 2007; Tay *et al.*, 2009).

In the present study, *Candida* isolates identified as *C. parapsilosis* group by conventional phenotyping methods during an ongoing population-based surveillance of candidaemia (Arendrup *et al.*, 2008) were genotypically screened for *C. orthopsilosis* and *C. metapsilosis*. Sequence analysis of selected gene targets suggested that PCR-restriction fragment length polymorphism (RFLP) of the *SADH* gene by *BanI* is not sufficient for differentiating the three closely related species in the *C. parapsilosis* group, and therefore, an alternative RFLP profile algorithm is proposed for reliable identification.

METHODS

Isolates. A total of 79 clinical *C. parapsilosis* group isolates and 3 reference strains, *C. parapsilosis* ATCC 22019, *C. orthopsilosis* ATCC

96139 and *C. metapsilosis* ATCC 96144, were used in this study. Clinical strains were obtained from Danish patients with candidaemia during 2004–2008 (Arendrup *et al.*, 2008). Species identification was based on colony colour and morphology on CHROMagar, microscopic morphology on cornmeal agar and use of a commercial system (ATB ID32C). The isolates had been stored in glycerol/water at -80°C . All strains were subcultured on CHROMagar and incubated at 37°C for 48 h for reconfirmation of the identification as *C. parapsilosis sensu lato*. Susceptibility testing was performed according to the European Committee on Antimicrobial Susceptibility Testing Edef 7.1 guidelines (AFST-EUCAST, 2008).

PCR. Reference and clinical strains were subjected to amplification of the *SADH* gene. A small part (about 0.5–1 μl) of a single fresh colony was added directly, and without prior DNA extraction (Mirhendi *et al.*, 2007) to the PCR premix, which contained 0.5 μM of the forward (5'-GTT GAT GCT GTT GGA TTG T-3') and reverse (5'-CAA TGC CAA ATC TCC CAA-3') primers (Tavanti *et al.*, 2005), 400 μM deoxynucleoside triphosphate mix, 2.5 μl magnesium-free buffer, 1.5 mM MgCl_2 and 1.25 U *Taq* DNA polymerase (Sigma-Aldrich) in a final volume of 25 μl . A negative control (water) was included in all PCR experiments. Conditions for PCR amplification were: 94°C for 7 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 60 s and elongation at 72°C for 90 s; and a final extension step at 72°C for 5 min.

RFLP. The *SADH* PCR products were digested for 120 min with the restriction enzyme *BanI* or *NlaIII* (Fermentas), in a 15 μl reaction volume containing 0.5 μl (5 U) enzyme, 1.5 μl 10 $\times$ buffer (supplied with the enzyme), 5 μl PCR product and 8 μl molecular grade water.

Electrophoresis. A total of 5 μl amplified DNA and 8 μl digested DNA product were separated by electrophoresis on 1.5 and 2% agarose gels, respectively. The electrophoresis buffer was TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.3). A 100 bp DNA ladder was used as a molecular size marker. DNA bands were visualized by UV transillumination and photographed.

Sequencing. The following forward and reverse primer pairs were used for amplification and sequencing of the target DNA regions: 5'-TCC GTA GGT GAA CCT GCG G-3' and 5'-TCC TCC GCT TAT TGA TAT GC-3' for the ITS1–5.8S rRNA gene–ITS2 region (White *et al.*, 1990); 5'-GCA TAT CAA TAA GCG GAG GAA AAG-3 and 5'-GGT CCG TGT TTC AAG ACG-3' for the D1/D2 domain of the large subunit of rDNA gene (26S rDNA) (Fell, 1993); and 5'-GTT GAT GCT GTT GGA TTG T-3' and 5'-CAA TGC CAA ATC TCC CAA-3' for the *SADH* gene (Tavanti *et al.*, 2005). Sequencing was performed in all cases by the company MWG using a BigDye terminator cycle sequencing kit and an ABI-3730 genetic analyzer.

Sequence analysis. Forward and reverse sequences were aligned and compared to ensure agreement, and then subjected to nucleotide BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and DNASIS multiple alignment. Four separate phylogenetic analyses of sequence data were conducted: three using each individual *SADH*, ITS1–ITS2 and 26S rRNA gene sequence data for sequences determined in the present study and employing the same *Candida dubliniensis* data for the outgroup; and one using concatenated *SADH*+ITS1–ITS2+26S rRNA data representing the total nucleotide variation in all three regions of the tested strains, with *C. dubliniensis* as the outgroup. The analyses were run using the Bayesian inference method with the program Mr-Bayes v.3.1.2. The evolutionary distance was calculated by employing the general time reversible evolutionary model (nset=6), which utilizes a gamma-shaped variation in mutation rates between codons (rates=gamma). Posterior probabilities were calculated via 2 000 000 generations (ngen=2 000 000, burnin=20 000) using the Monte Carlo

Markov chain method and four simultaneous tree building chains (nchains=4), with every 100th tree saved (samplefreq=100).

Designing a new RFLP profile. The *SADH* sequences obtained in this study (GenBank accession nos FJ746046–FJ746069) and all available *SADH* sequences deposited in GenBank for isolates belonging to the *C. parapsilosis* group (<http://www.ncbi.nlm.nih.gov/>) were compared and analysed by DNASIS software. The cutting sites of enzymes listed in the DNASIS software were predicted and evaluated for potential to discriminate between *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis*.

RESULTS AND DISCUSSION

The colony colour of the 79 *C. parapsilosis sensu lato* isolates was white-cream to pale pink on CHROMagar Candida with slight, but not species-specific, differences among the isolates. ITS and the 26S rRNA gene sequence are well-known targets for most of the taxonomic studies in nearly all micro-organisms. They are the basis of routine and research identification of fungal pathogens and for recognizing new species (Iwen *et al.*, 2002). The *SADH* gene has been the basis of sequencing analysis or *BanI*-RFLP for identification of the *C. parapsilosis* group since the new species were described in 2005 by Tavanti and colleagues (Tavanti *et al.*, 2005). Thus these genes were chosen for comparative studies of the isolates. The *SADH* gene was successfully amplified in all the clinical isolates, as well as the reference strains, and a single sharp band with a size of approximately 720 bp was seen for all samples. Digestion of PCR products by the restriction enzyme *BanI* resulted in three different band patterns (Fig. 1). A double band pattern (approx. 520 and 200 bp in size) was observed for 75/79 clinical isolates. A single band representing the absence of a cutting site for *BanI* was seen for *C. orthopsilosis* ATCC 96139 (Fig. 1, lane 2) and four clinical isolates (Fig. 1, lanes 4, 5, 10 and 21). These results were reproduced three times for these strains in order to exclude any errors in the enzymic reaction. Finally, as expected, a three band pattern (approx. 390, 190 and 100 bp) was observed for *C. metapsilosis* ATCC 96144 (Fig. 1, lane 1) (a fourth 40 bp band is not visible by gel electrophoresis). Thus, according to the digestion patterns described previously (Tavanti *et al.*, 2005), 75 blood culture isolates were identified as *C. parapsilosis*, 4 as *C. orthopsilosis* (strains W36279, T27682, T19754 and F10552), and none as *C. metapsilosis* (Table 1).

For confirmation of the species identification obtained by *SADH*-RFLP, each of the three reference strains, the four blood culture isolates identified as *C. orthopsilosis* by RFLP and three randomly selected *C. parapsilosis* isolates, were sequenced for the three DNA markers: *SADH*, the entire ITS1–ITS2 region and 26S rRNA (D1/D2) gene sequence. Forward and reverse sequences determined were completely compatible. The sequences were subjected to a nucleotide BLAST search and sequence analysis of the *SADH* and ITS1–ITS2 regions, which resulted in identification of two isolates (strains T27682 and F10552) as *C. orthopsilosis* (Fig. 1, lanes 5 and 21) and two isolates (strains T19754 and W36279) as *C. metapsilosis* (Fig. 1, lanes 4 and 10) (Table 1). There was

no cutting site for the enzyme *BanI* at *in silico* digestion of the sequences of these isolates. For the American Type Culture Collection (ATCC) reference strains and three randomly selected *C. parapsilosis* isolates sequence analysis of the *SADH* gene and ITS region confirmed the species identification obtained by *SADH*-RFLP (Table 1). Sequence analysis of the 26S rRNA (D1/D2) region failed to differentiate *C. parapsilosis* from *C. metapsilosis* and *C. orthopsilosis*, even for the *C. orthopsilosis* and *C. metapsilosis* ATCC reference strains (Table 1).

To evaluate the nucleotide variation, the sequences obtained in this study were also subjected to multiple alignment and analysis of phylogenetic trees, separately for each region and also concatenated for all regions. A total of 592 nt for the *SADH* gene, 434 nt for the ITS1–ITS2 region, 591 nt for 26S rRNA (data not shown) and 1617 nt for the concatenated analysis were used. As shown in Fig. 2, two of four clinical isolates that initially were identified as *C. orthopsilosis* by *SADH* PCR-RFLP, grouped with *C. metapsilosis* ATCC 96144. The *SADH* sequence was identical when compared to the two Danish *C. metapsilosis* isolates and did not contain a *BanI* cutting site. The ATCC 96144 *C. metapsilosis* *SADH* sequences were 95 % similar to the two Danish isolates, and the reference strain contained a *BanI* cutting site.

With the objective of designing a better *SADH*-RFLP profile for species separation and identification of the *C. parapsilosis* group, all available sequences of the *SADH* gene, including the sequences obtained from the present study and sequences previously deposited in GenBank, were analysed and restriction patterns were predicted for

Fig. 1. Agarose gel electrophoresis of *SADH* PCR products digested by the restriction enzyme *BanI*. Lane 1, *C. metapsilosis* ATCC 96144; lane 2, *C. orthopsilosis* ATCC 96139; lane 3, *C. parapsilosis* ATCC 22019; lanes 4–30, blood culture isolates; lane M, 100 bp molecular size marker. Note the single band pattern in lanes 4, 5, 10 and 21, as well as for the reference strain *C. orthopsilosis* (lane 2). Note also the slightly smaller band size for samples 4 and 10 (which were eventually identified as *C. metapsilosis*).

Table 1. Molecular identification of 79 clinical *C. parapsilosis sensu lato* blood culture isolates and 3 reference strains using phenotyping and a complement of molecular methods

Strain	Species identification obtained by:				
	SADH PCR-RFLP with <i>Ban</i> I	SADH gene sequencing	ITS1–ITS2 sequencing	26S rRNA (D1/D2) gene sequencing	SADH PCR-RFLP with <i>Nla</i> III
ATCC 22019	<i>C. parapsilosis</i>	<i>C. parapsilosis</i>	<i>C. parapsilosis</i>	<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
ATCC 96144	<i>C. metapsilosis</i>	<i>C. metapsilosis</i>	<i>C. metapsilosis</i>	<i>C. orthopsilosis/parapsilosis/metapsilosis</i>	<i>C. metapsilosis</i>
ATCC 96139	<i>C. orthopsilosis</i>	<i>C. orthopsilosis</i>	<i>C. orthopsilosis</i>	<i>C. orthopsilosis/parapsilosis/metapsilosis</i>	<i>C. orthopsilosis</i>
W36279 & T19754	<i>C. orthopsilosis</i>	<i>C. metapsilosis</i>	<i>C. metapsilosis</i>	<i>C. orthopsilosis/parapsilosis/metapsilosis</i>	<i>C. metapsilosis</i>
T27682 & F10552	<i>C. orthopsilosis</i>	<i>C. orthopsilosis</i>	<i>C. orthopsilosis</i>	<i>C. orthopsilosis/parapsilosis/metapsilosis</i>	<i>C. orthopsilosis</i>
Remaining 75 blood isolates	<i>C. parapsilosis</i>	Not done*	Not done*	Not done*	<i>C. parapsilosis</i>

*Three of these blood isolates underwent sequencing of the *SADH* gene and of the 26S rRNA (D1/D2) gene region, and one blood isolate underwent ITS1–ITS2 sequencing, confirming the isolates to be *C. parapsilosis*.

610 (almost all available) restriction enzymes. The most applicable enzymes were *Mwo*I, *Hpy*8I, *Mfe*I, *Bst*ENII, *Afa*I, *Alw*I and *Nla*III, but only *Nla*III (*Fat*I) and *Bst*ENII were predicted to have cutting sites for all three species. Comparing the predicted fragment sizes of *Nla*III (*Fat*I) and *Bst*ENII, *Nla*III was superior, due to having the greatest difference in the main fragment sizes between the species. For *Nla*III the following fragment sizes were found: *C. parapsilosis* – 131 and 505 bp, *C. metapsilosis* – 74, 288 and 348 bp, and *C. orthopsilosis* – 131, 217 and 288 bp. In comparison the following fragment sizes were found for *Bst*ENII: *C. parapsilosis* – 141 and 576 bp, *C. metapsilosis* – 158 and 559 bp, and *C. orthopsilosis*, 141, 158 and

363 bp. The *SADH* PCR products of all blood culture isolates and reference strains were digested with *Nla*III (examples are shown in Fig. 3). The fragment patterns were as expected and in agreement with the *SADH* gene sequence data. No additional *C. orthopsilosis* or *C. metapsilosis* isolates were found among clinical isolates by this new technique. Finally, susceptibility to amphotericin B, caspofungin, fluconazole, itraconazole and voriconazole were compared. Given as MICs for the two *C. metapsilosis* isolates, MICs for the two *C. orthopsilosis* isolates and MIC₅₀ (range) for the 75 *C. parapsilosis* isolates, the values ($\mu\text{g ml}^{-1}$) were as follows: amphotericin B, 0.5 and 0.25, 0.5 and 0.5, and 0.5 (0.125–1), respectively; caspofungin,

Fig. 2. Genetic relationships of *C. parapsilosis sensu lato* isolated from Danish patients with candidaemia and the three reference strains, *C. parapsilosis* ATCC 22019, *C. orthopsilosis* ATCC 96139 and *C. metapsilosis* ATCC 96144. *C. dubliniensis* was included as the outgroup. The relationships were inferred based on concatenated *SADH*, ITS and 26S rRNA gene sequence data. Nodal support is given as a posterior probability value. Bar, distance.

Fig. 3. Agarose gel electrophoresis of *SADH* PCR products of *C. parapsilosis sensu lato* after digestion with *NlaIII*. Lanes 1, 8 and 9 are *C. parapsilosis* ATCC 22019 and two clinical isolates of *C. parapsilosis*, respectively; lanes 2, 5 and 6 are *C. orthopsilosis* ATCC 96139 and the clinical isolates T27682 and F10552, respectively; lanes 3, 4 and 7 are *C. metapsilosis* ATCC 96144 reference strain and clinical isolates W36279 and T19754, respectively; lane M, 100 bp molecular size marker.

0.5 and 1, 0.5 and 0.5, and 2 (0.5–4), respectively; fluconazole, 2 and 0.25, 0.5 and 1, and 1 (0.5–8), respectively; itraconazole, 0.125 and ≤ 0.032 , ≤ 0.032 and 0.125, 0.064 (≤ 0.032 –0.125), respectively; and voriconazole, 0.064 and ≤ 0.032 , ≤ 0.032 and 0.064, and ≤ 0.032 (≤ 0.032 –0.125), respectively. Thus, no significant differences were observed.

In this study we have reported the prevalence of the recently described species *C. orthopsilosis* and *C. metapsilosis* in a well-defined sample of blood culture isolates from Denmark and a new low cost PCR-RFLP method for the reliable separation of the *C. parapsilosis* group into the three species. Overall, 2.5 % of the isolates phenotypically identified as *C. parapsilosis* were *C. orthopsilosis* and 2.5 % were *C. metapsilosis*. Table 2 summarizes the frequencies of *C. orthopsilosis* and *C. metapsilosis* in other studies of *C. parapsilosis sensu lato*. In the majority of studies, including all those with more than 100 isolates, 88 % or more of the isolates were confirmed to be *C. parapsilosis* in agreement with the current findings. In Portugal, Spain and Denmark similar proportions of *C. orthopsilosis* and *C. metapsilosis* isolates were detected

Table 2. Summary of published studies on identification methods with distribution of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*

Method used for differentiation	Source of specimens	No. of isolates (%) by species			Country or region	Reference
		<i>C. parapsilosis</i>	<i>C. orthopsilosis</i>	<i>C. metapsilosis</i>		
MLST, RAPD-PCR, <i>SADH</i> RFLP	Various clinical sources	20 (74.1 %)	7 (25.9 %)	0	USA, Europe, Africa, Asia	Tavanti <i>et al.</i> (2005)
Direct PCR-sequencing of BacTec	Blood and other clinical sources	8 (88.9 %)	1 (11.1 %)	0	Australia	Pryce <i>et al.</i> (2006)
ITS sequencing, RAPD PCR, phenotyping data	Blood and other clinical sources	18 (90 %)	0	2 (10 %)	Hungary	Kocsubé <i>et al.</i> (2007)
<i>SADH</i> -RFLP, AFLP	Various clinical sources	277 (95.5 %)	13 (4.5 %)	NI	Italy	Tavanti <i>et al.</i> (2007)
<i>SADH</i> -RFLP, AFLP	Various clinical sources	375 (94.9 %)	NI	20 (5.1 %)	Italy	Hensgens <i>et al.</i> (2009)
ITS sequencing	Blood	67 (85.9 %)	5 (6.4 %)	6 (7.7 %)	Spain	Gomez-Lopez <i>et al.</i> (2008)
<i>SADH</i> -RFLP	Invasive clinical samples	1762 (92.1 %)	117 (6.1 %)	34 (1.8 %)	Global study (89 countries)	Lockhart <i>et al.</i> (2008)
ITS-sequencing, RAPD PCR	Blood	29 (70.7 %)	10 (24.4 %)	2 (4.9 %)	Malaysia	Tay <i>et al.</i> (2009)
PCR with specific primers, <i>SADH</i> -RFLP, D1/D2 and ITS sequencing	Blood (66) and other clinical sources (48)	109 (95.6 %)	5 (4.4 %)	0	Kuwait	Asadzadeh <i>et al.</i> (2009)
<i>SADH</i> -RFLP	Clinical and environmental sources	160 (94.67 %)	4 (2.37 %)	5 (2.96 %)	Portugal	Silva <i>et al.</i> (2009)
ITS sequencing	Blood	126 (88.1 %)	13 (9.1 %)	4 (2.8 %)	Brazil	Gonçalves <i>et al.</i> (2010)
<i>SADH</i> -RFLP, <i>SADH</i> , ITS and 26S sequencing	Blood	75 (95 %)	2 (2.5 %)	2 (2.5 %)	Denmark	Present study

NI, Not indicated.

(Gomez-Lopez *et al.*, 2008; Silva *et al.*, 2009), while *C. orthopsilosis* was more frequent than *C. metapsilosis* in a worldwide study with almost 2000 isolates (Lockhart *et al.*, 2008) and in two smaller studies from Malaysia and Kuwait (Tay *et al.*, 2009; Asadzadeh *et al.*, 2009). To what extent these observations reflect true differences compared to method-dependent effects remains to be elucidated. Some studies have used *SADH*-RFLP with *BanI*, which we found unreliable for identifying *C. metapsilosis*. Some studies have used PCR sequencing of the D1/D2 region of 26S rRNA gene; however, by sequence analysis (Table 1) including the use of phylogenetic trees, we showed that this region is not a suitable target for separation of the species within the *C. parapsilosis* group. The 26S rRNA gene sequence is generally a highly conserved region, particularly in yeasts (Iwen *et al.*, 2002). Thus it is foreseeable that 26S rRNA (D1/D2) gene sequence may not be an optimal marker for differentiating closely related species.

Since the description of *C. orthopsilosis* and *C. metapsilosis* in 2005 (Tavanti *et al.*, 2005), several methods have been proposed to identify and differentiate these species from *C. parapsilosis sensu stricto*. Tavanti *et al.* (2005) proposed PCR amplification of the *SADH* gene followed by restriction digestion with *BanI*, and used amplification fragment length polymorphism (AFLP) for identification and genotyping of about 400 isolates previously classified as *C. parapsilosis*. AFLP was shown to identify *C. orthopsilosis* (Tavanti *et al.*, 2007) and *C. metapsilosis* (Hensgens *et al.*, 2009) to the species level, and to be an efficient genotyping tool as well, delineating intraspecific genetic relatedness. Campa *et al.* (2008) developed an oligonucleotide microarray based on the arrayed-primer extension technique to simultaneously identify pathogenic fungi. They designed probes complementary to the ITS1 and ITS2 region for identification of 24 species, including *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*. Based on the major DNA fragments of the RAPD analysis profiles, Tay *et al.* (2009) identified the isolates as types P1 to P5. Sequence analysis of the ITS region of the isolates identified RAPD type P1 as *C. parapsilosis*, P2 and P3 as *C. orthopsilosis*, and P4 as *C. metapsilosis*. van Asbeck *et al.* (2008) reported the correlation of RFLP genotyping with ITS sequence, RAPD and multilocus sequencing for the *C. parapsilosis* group. According to Borman *et al.* (2009) pyrosequencing analysis of 20 nucleotides of the ITS2 region rapidly and robustly distinguished the three species. Finally, Asadzadeh *et al.* (2009) introduced primers derived from unique sequences within the ITS1–5.8S rDNA–ITS2 region for the purpose of differentiating the three species. All these methods have the advantages of a sequencing-based approach, but are time consuming and labour intensive, and some are expensive.

In our hands the *SADH* PCR-RFLP with the enzyme *BanI* successfully separated *C. parapsilosis sensu stricto* from the two new species, but the band patterns of *C. orthopsilosis* and *C. metapsilosis* were so similar that misclassification of *C. metapsilosis* as *C. orthopsilosis* must be expected in a routine setting. As PCR-RFLP is an attractive method due

to low cost, we carefully analysed the *SADH* region in the *C. parapsilosis* complex for differences that predicted individual RFLP patterns and confirmed that the use of *SADH*-PCR followed by digestion by *NlaIII* could be a more reliable method for differentiating the three species. Further studies using this method and a higher number of *C. metapsilosis* and *C. orthopsilosis* isolates are needed to confirm the robustness of the method.

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