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Pyrosequencing Analysis of 20 Nucleotides of Internal Transcribed Spacer 2 Discriminates *Candida parapsilosis*, *Candida metapsilosis*, and *Candida orthopsilosis*▽

Andrew M. Borman,[1]* Christopher J. Linton,[1] Debra Oliver,[1] Michael D. Palmer,[1] Adrien Szekely,[1] Frank C. Odds,[2] and Elizabeth M. Johnson[1]

Mycology Reference Laboratory, Health Protection Agency, Bristol, United Kingdom,[1] and Aberdeen Fungal Group, School of Medical Sciences, University of Aberdeen, Aberdeen, United Kingdom[2]

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Two new cryptic sister species, *Candida orthopsilosis* and *Candida metapsilosis*, were recently identified by consistent DNA sequence differences among several genes within the genetically heterogeneous *Candida parapsilosis* complex. Here, we present data demonstrating that Pyrosequencing analysis of 20 nucleotides of internal transcribed spacer region 2 rapidly and robustly distinguishes between these three closely related *Candida* species.

Invasive fungal infections, especially those caused by *Candida* spp., remain a significant cause of mortality in immunocompromised patients and those undergoing invasive procedures (reviewed in references 14 and 20). Although *Candida albicans* remains the most common *Candida* species encountered in human infections, *Candida parapsilosis* is frequently isolated from both mucosal and systemic infections worldwide (5, 13, 15). Previous studies have demonstrated that *C. parapsilosis* isolates were genetically heterogeneous, and could be separated into three groups (*C. parapsilosis* groups I to III) by a variety of approaches, including randomly amplified polymorphic DNA analyses (9), mitochondrial DNA (12), DNA topoisomerase II (8), or internal transcribed spacer (ITS) (10) gene sequencing and isoenzyme profiles (10). On the basis of sequence differences in multiple genes, two new sister species, *Candida metapsilosis* and *Candida orthopsilosis*, were proposed in 2005 to replace *C. parapsilosis* groups II and III (16).

Following their initial description, it was assumed that accurate discrimination between *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis* was important principally for epidemiological surveys, since few data existed concerning the antifungal susceptibility profiles of these new species. However, recent studies suggest significant differences between the antifungal susceptibility profiles of *C. orthopsilosis*/*C.metapsilosis* and *C. parapsilosis*, particularly with respect to the echinocandin antifungal agents (7, 11), indicating that correct identification may also eventually have therapeutic implications.

Here, we have investigated whether Pyrosequencing technology, which is a rapid and potentially robust method of yeast identification (2–4, 6, 18), could be used to discriminate between clinical isolates of *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*. Seventy clinical isolates presumed to be *C. parapsilosis* from a variety of biological specimens were included in the current study. These isolates had been referred to the United Kingdom Mycology Reference Laboratory (MRL) for identification and had been identified by us as *C. parapsilosis* by AUXACOLOR2 (Bio-Rad, Marnes-La-Coquette, France) testing. Six of these 70 isolates had also been previously confirmed as *C. parapsilosis* sensu stricto by rRNA gene sequencing (Table 1; data not shown). Reference isolates included the type strain of *C. parapsilosis*, the holotype strains of *C. metapsilosis* and *C. orthopsilosis*, and 10 isolates of *C. orthopsilosis* and 1 additional isolate of *C. metapsilosis* that had previously been characterized by multilocus sequence typing (MLST) (Table 1). Two clinical isolates of *Lodderomyces elongisporus*, which we had previously identified by rRNA gene sequencing, were also included since this yeast species had been proposed historically as a potential teleomorph for organisms that resemble *C. parapsilosis* physiologically (19). Twenty-one clinical isolates of *C. albicans* (including 8 which had undergone rRNA gene sequencing) were also included for comparison (Table 1).

Genomic DNA was prepared from all isolates using Whatman FTA filter paper technology exactly as described previously (1). PCR amplification of a fragment of the ITS2 region was performed using the primers supplied with the PyroMark fungus test (Biotage, Sweden). In all cases, PCR amplification (100-μl reaction volumes) was performed in the presence of 200 μM of each deoxynucleoside triphosphate (dNTP), 250 nM of the appropriate primers, 2 U of HotStar *Taq* polymerase (Qiagen, Valencia, CA), and a single FTA filter punch. Following enzyme activation at 94°C for 15 min, reactions were subjected to 40 thermal cycles with the following parameters: 94°C for 15 s, 55°C for 15 s, and 72°C for 90 s on a GeneAmp PCR Systems 9700 thermocycler (Applied Biosystems, Foster City, CA). Amplification success was evaluated by electrophoresis of a fraction of total amplification products in 1.2% (wt/vol) agarose gels run for 45 min at 120 V in Tris-borate buffer. ITS2 amplification products were subjected to Pyrosequencing analysis with the reagents supplied with the PYROGOLD SQA kit using a PyroMark ID Pyrosequencing instru-

* Corresponding author. Mailing address: Mycology Reference Laboratory, Health Protection Agency South-West Regional Laboratory, Myrtle Road, Kingsdown, Bristol BS2 8EL, United Kingdom. Phone: 0117 926 8683. Fax: 0117 922 6611. E-mail: Andy.Borman@uhBristol.nhs.uk.

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TABLE 1. Key isolates and reference strains used in this study

Species/isolate no.	Other strain identifier	Source	Molecular identification (reference)
Candida parapsilosis[a]			
NCPF 8766	CBS 604	Type, feces; Puerto Rico	MLST (16), D1-D2,[d] and this study
MRL1018881		Blood; Swansea, United Kingdom	D1-D2[d] and this study
MRL1317069		Mouth swab; Worthing, United Kindom	D1-D2[d] and this study
MRL1420493		CVP catheter tip; Leeds, United Kingdom	D1-D2[d] and this study
MRL1880293		Peritoneal dialysis fluid; Brighton, United Kingdom	D1-D2[d] and this study
MRL1867857		Blood; Isle of Wight, United Kingdom	D1-D2[d] and this study
MRL1993763		High vaginal swab; Stevenage, United Kingdom	D1-D2[d] and this study
Candida orthopsilosis			
ATCC 96139	NCPF 8767[e]	Holotype; central catheter tip, San Antonio, TX	MLST (16), D1-D2,[d] and this study
ATCC 96141	NCPF 8797[e]	Human blood; TX	MLST (16), D1-D2,[d] and this study
NCPF 8798	02-201	Blood; Italy	MLST (16), D1-D2,[d] and this study
NCPF 8799	02-212	Blood; Barcelona, Spain	MLST (16), D1-D2,[d] and this study
NCPF 3192	81/026	Unknown; United Kingdom	D1-D2[4] and this study
NCPF 8801	90/125	Unknown; San Francisco, CA	MLST (16), D1-D2,[d] and this study
NCPF 8802	92/181	Contaminated solution; Redwood City, CA	MLST (16), D1-D2,[d] and this study
NCPF 8803	J960679/2	Nail clipping; Belgium	D1-D2[d] and this study
NCPF 8804	J981226	Vaginal isolate; CA	D1-D2[d] and this study
NCPF 8795	J950813	Nail; Belgium	AFLP[f] (17)
MRL3144905		Unknown; Brighton, United Kingdom	This study
Candida metapsilosis			
ATCC 96144	NCPF 8768[e]	Holotype, hand; Tacoma, WA	MLST (16), D1-D2,[d] and this study
NCPF 8789	J960161	Nail; Belgium	D1-D2[d] and this study
MRL1136522		Blood culture; Kettering, United Kingdom	This study
Lodderomyces elongisporus[b]			
MRL1032435		Blood culture; Hemel Hempstead, United Kingdom	D1-D2[d] and this study
MRL1804516		Corneal scrape; London, United Kingdom	D1-D2[d] and this study
Candida albicans[c]			
MRL1154110		Unknown; Peterborough, United Kingdom	D1-D2[d] and this study
MRL1198490		Unknown; Guilford, United Kingdom	D1-D2[d] and this study
MRL1644128		Bile duct; Plymouth, United Kingdom	D1-D2[d] and this study
MRL1644115		Abdominal pus; Plymouth, United Kingdom	D1-D2[d] and this study
MRL1907106		High vaginal swab; Torbay, United Kingdom	D1-D2[d] and this study
MRL1912673		Blood; Nottingham, United Kingdom	D1-D2[d] and this study
MRL1898546		Genital swab; Dorchester, United Kingdom	D1-D2[d] and this study
MRL2082345		Throat swab; Plymouth, United Kingdom	D1-D2[d] and this study

[a] A further 62 clinical isolates of *C. parapsilosis* from the United Kingdom were included, isolated between 2005 and 2008 from blood culture ($n = 31$), CVP catheter tips ($n = 5$), high vaginal swabs ($n = 3$), sputum ($n = 3$), peritoneal dialysis fluid ($n = 2$), urine ($n = 2$), nails ($n = 2$), corneal scrapes ($n = 2$), ear swabs ($n = 2$), skin swabs ($n = 1$), fine-needle-aspirated knee fluid ($n = 1$), or nonspecified sites ($n = 8$). All were tentatively identified as *C. parapsilosis* by AUXACOLOR2 testing and confirmed as *C. parapsilosis* sensu stricto by Pyrosequencing analysis.

[b] The identity of the two clinical isolates of *L. elongisporus* was confirmed by sequencing of the D1-D2 portion of the large ribosomal subunit gene.

[c] A further 13 clinical isolates, all confirmed as *C. albicans* by germ-tube test and AUXACOLOR2, were included from unknown sites ($n = 11$), high vaginal swab ($n = 1$), and mouth ($n = 1$).

[d] The identity of these isolates was confirmed by PCR amplification and sequencing of the D1-D2 portion of the large ribosomal subunit gene, exactly as described previously (1, 2).

[e] These isolates are not commercially available through the National Collection of Pathogenic Fungi (NCPF).

[f] AFLP, amplified fragment length polymorphism.

ment (Biotage). Analysis of the resulting sequences was performed using the IdentiFire software (Biotage) with an extended sequence database generated at the MRL using reference and type species.

Table 2 shows the Pyrosequencing profiles generated for the various yeast species. Unique Pyrosequencing profiles were obtained for each of the species tested. Importantly, sequences from the isolates of *C. orthopsilosis*, *C. metapsilosis*, *L. elongisporus*, and *C. albicans* differed from the 33-nucleotide *C. parapsilosis* sequence at 1, 2, 11, and 15 positions, respectively. Indeed, isolates from the four species examined could be reliably distinguished using only the first 20 nucleotides of ITS2 sequence generated by Pyrosequencing analysis. It should be noted that the Pyrosequencing profiles reported here are unique to the yeast species examined. To date, none of the approximately 50 different species of yeast we have examined by Pyrosequencing analysis share identical profiles with *C. parapsilosis*, *C. metapsilosis*, or *C. orthopsilosis* (unpublished date), and BLAST searches using the sequences generated by Pyrosequencing analysis fail to produce any other significant, reliable matches in the public synchronized databases (data not shown). Additionally, no intraspecific sequence variations were detected in the relatively short region of ITS2 examined by Pyrosequencing analysis for all four species studied. This may seem to contrast with previous reports suggesting that both *C. orthopsilosis* and *C. metapsilosis* exhibit more genetic variability

TABLE 2. Pyrosequencing signature sequences for *C. parapsilosis* and related species[a]

Organism	Pyrosequencing profile	% Similarity	No. of strains sequenced	
			Reference	Clinical
C. parapsilosis	GTCGAA-TTT GGAAGAAGTT TTGG-AGTTT GTACC	33	1	68
C. orthopsilosis	GTCGAA-TTT GGAAGAA-TT TTGG-AGTTT GTACC	32	10	1
C. metapsilosis	GTCGAA-TTT GGAAGAAtgT TTGG-AGTTT GTACC	31	2	1
L. elongisporus	GTCGAAgTTT GaAAtAtaga TTGG-AGcTT tTAtt	23		2
C. albicans	GTCaAAgTTT GaAgatAtac gTGGtAGacg tTACC	20		21

[a] The first 33 nucleotides of the *C. parapsilosis* sequence generated by Pyrosequencing analysis are shown; dashes (denoting the positions of indels) have been introduced to improve alignments. Nucleotides in uppercase are conserved; those in lowercase are positions that differ from the *C. parapsilosis* sequence. The number of nucleotides shared with the *C. parapsilosis* sequence (similarity) and the number of clinical and reference isolates tested which gave identical Pyrosequencing signature profiles ("Strains sequenced") are shown. The signature sequences of *C. albicans* and *Lodderomyces elongisporus* (a teleomorphic yeast with physiological profiles similar to *C. parapsilosis*) are included for comparison.

than *C. parapsilosis* (7, 16, 17) and describing a subset of sequences purportedly from *C. albicans* in the public databases that lack the adenosine at nucleotide position 4 in the Pyrosequencing profile (data not shown). However, variability in highly conserved genes such as those encoding ribosomal DNA is more limited than that in many other genes, which is why such genes are used as the basis for discrimination of organisms at the species rather than the strain level. Future studies will be designed to test the possibility that some variability might be observed if a larger panel of isolates was studied or if a longer portion of ITS2 was analyzed by Pyrosequencing analysis.

Two of the 70 presumed isolates of *C. parapsilosis* included in the current study on the basis of physiological profiles were in fact identified as *C. metapsilosis* (1 isolate) and *C. orthopsilosis* (1 isolate) on the basis of Pyrosequencing analysis, as shown in Table 1 (MRL3144905 and MRL1136522) and Table 2 (prevalence of *C. metapsilosis* and *C. orthopsilosis* among "phenotypic *C. parapsilosis*" isolates of 1.4% for each species). The identities of these two isolates were formally confirmed by rRNA gene sequencing (data not shown). Previous studies have demonstrated important geographical variations in the prevalences of *C. metapsilosis* and *C. orthopsilosis* among "phenotypic *C. parapsilosis*" isolates (7, 11). However, it is not possible from the present data to draw firm conclusions regarding the relative prevalence of these species in the United Kingdom, since the 70 isolates of presumed *C. parapsilosis* studied here had been referred to our laboratory for identification, rather than collected through active surveillance. Nevertheless, the high-throughput nature of Pyrosequencing technology makes it ideally suited to evaluate the prevalence of cryptic minority species.

In conclusion, Pyrosequencing analysis of a small portion of ITS2 is sufficient to reliably discriminate between *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis*. The Pyrosequencing profiles produced for each of these species were reproducible and conserved across multiple isolates and are to date unique to each of these three species of pathogenic yeast. Given the relative rapidity and facility of Pyrosequencing analysis, we believe that this approach is ideally suited to the accurate identification of presumed isolates of *C. parapsilosis*, which in the light of recently reported antifungal susceptibility profiles (7, 11) may become increasingly important for informed therapeutic decisions.

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