Species identification and strain differentiation of clinical *Candida* isolates using the DiversiLab system of automated repetitive sequence-based PCR

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The DiversiLab system, which uses repetitive sequence-based PCR (rep-PCR) to genotype micro-organisms, was evaluated as a molecular typing tool for members of the genus *Candida*. Initially, 41 clinical *Candida* spp. (7 *Candida krusei*, 10 *Candida parapsilosis*, 7 *Candida albicans*, 10 *Candida tropicalis* and 7 *Candida glabrata*), previously identified at the species level by morphological and biochemical analysis, were analysed with the DiversiLab system. Species identification was confirmed by DNA sequence analysis of the contiguous internal transcribed spacer (ITS) region (ITS1–5.8S–ITS2). On the basis of an 80 % similarity threshold, rep-PCR consistently clustered like species and this set of isolates, along with five ATCC reference strains, was used to create a DNA fingerprint library with the DiversiLab software. Subsequently, an additional set of 115 clinical *Candida* isolates, identified biochemically as *C. albicans* ($n=94$), *C. glabrata* ($n=8$), *C. parapsilosis* ($n=5$), *C. tropicalis* ($n=3$), *C. krusei* ($n=3$) and *Candida lusitanae* ($n=2$), isolated at a regional reference laboratory, were typed using DiversiLab. One hundred and six of these isolates clustered with members of the *Candida* library at >80 % similarity and thus could be assigned species identification, and initial calculations showed that identification via rep-PCR fingerprinting was 95 % concordant (101/106) with the biochemical/morphological identification. However, ITS region sequencing of the five discrepant samples, as well as the nine isolates that were <80 % similar to the database samples, showed that nine were misidentified with traditional biochemical/morphological methods. For the misidentified isolates, the sequence-based identification was in agreement with the DiversiLab clustering, yielding an actual correlation of >99 %. As traditional techniques can take several days to provide information about *Candida* at the genus/species level, genotyping with the DiversiLab system holds promise for more-rapid speciation of members of this genus. This system may also be useful for epidemiological studies such as source tracking that require *Candida* subspecies discrimination.

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INTRODUCTION

Candidiasis and candidaemia have emerged as common infections in debilitated hospitalized patients (Kauffman, 2006). Although *Candida albicans* remains the most

important species causing these infections, non-*albicans Candida* spp. such as *Candida glabrata* have emerged as common pathogens, often accounting for more than 50 % of candidiasis cases (Clark & Hajjeh, 2002; Eggimann *et al.*, 2003; Marchetti *et al.*, 2004; Tortorano *et al.*, 2004). Importantly, non-*albicans Candida* spp. frequently show decreased sensitivity to azoles compared with *C. albicans* (Imhof *et al.*, 2004; Li *et al.*, 2004; Pfaller *et al.*, 2002;

Abbreviations: ITS, internal transcribed spacer; rep-PCR, repetitive sequence-based PCR.

Vermitsky & Edlind, 2004) and are associated with high failure rates of azole-based treatment (Tortorano *et al.*, 2004; Viscoli *et al.*, 1999). Traditional automated and biochemical systems of identifying *Candida* organisms identify them only at the genus/species level (Warren & Hazen, 1999) and the procedures may take several days. Furthermore, some biochemical tests have been reported to misidentify clinical *Candida* isolates (Dooley *et al.*, 1994). Thus, there is a need for the development of rapid, reliable, diagnostic methods to support the selection of antifungal therapy. Accordingly, several studies reporting the development of rapid-detection methodologies based on real-time PCR (Chau *et al.*, 2004; Hsu *et al.*, 2003; Selvarangan *et al.*, 2003), multiplex PCR (Li *et al.*, 2003; Luo & Mitchell, 2002), fluorescent *in situ* hybridization with peptide nucleic acid probes (Rigby *et al.*, 2002) and flow cytometry (Page & Kurtzman, 2005) have been reported recently for identification of the clinically important *Candida* spp.

Similarly, there is an increasing realization that robust molecular subtyping methods for *Candida* are needed for epidemiological investigations. For example, although the source of candidiasis is endogenous in most patients, nosocomial transmission of *Candida* spp. has been recognized in intensive care units (Richards *et al.*, 1999). A variety of molecular methods for DNA-based subtyping of *Candida* spp. have been employed (Lockhart *et al.*, 2005), as no gold standard exists for the genus (Reiss *et al.*, 1998). Current molecular strain typing methods such as PFGE and RFLP are tedious (Fujita & Hashimoto, 2000), whilst others, such as randomly amplified polymorphic DNA, are prone to artefacts (Bart-Delabesse *et al.*, 2001). Additional molecular subtyping methods for *Candida* spp. include those based on polymorphism in microsatellite markers and loci (Botterel *et al.*, 2001; Dalle *et al.*, 2003; Foulet *et al.*, 2005; Lott *et al.*, 2003; Sampaio *et al.*, 2003; Stephan *et al.*, 2002). Multilocus sequence typing is a highly discriminatory and stable method of unambiguous characterization of *C. albicans* (Bougnoux *et al.*, 2002; Dodgson *et al.*, 2003; Robles *et al.*, 2004; Tavanti *et al.*, 2003). However, the costs associated with this method of sequencing can be prohibitive and, along with the intensive data analysis requirements, may keep such technologies out of reach for many clinical laboratories (Goulding *et al.*, 2000; Olive & Bean, 1999). Overall, few methods alone have the potential for providing both identification and strain-level differentiation in *Candida* in a time- and cost-effective manner.

Previous reports have described the application of repetitive extragenic palindromic sequence-based PCR (rep-PCR) for characterizing the genotypic relatedness among *Candida rugosa* isolates (Redkar *et al.*, 1996). The rep-PCR method uses primers that target non-coding repetitive sequences interspersed throughout the bacterial and fungal genome (Koeuth *et al.*, 1995; Stern *et al.*, 1984; Versalovic *et al.*, 1991) and is an established approach for classification of subspecies and strain delineation of bacteria (Versalovic & Lupski, 2002; Versalovic *et al.*,

1993). Recent studies have demonstrated the potential of a commercially available automated rep-PCR assay system, the DiversiLab system, for rapid identification and strain-level differentiation of *Aspergillus* (Healy *et al.*, 2004), *Fusarium* (Healy *et al.*, 2005b), dimorphic fungi (Pounder *et al.*, 2006), *Candida* (Chau *et al.*, 2004), *Zygomycetes* (Kontoyiannis *et al.*, 2005) and dermatophytes such as *Trichophyton* (Pounder *et al.*, 2005). This study explores the use of the DiversiLab system as a genotyping tool for discriminating clinical *Candida* isolates. Herein, we present a comparison of rep-PCR fingerprinting with sequence-based analysis of the internal transcribed spacer (ITS) region (ITS1–5.8S–ITS2) and assess the utility of the DiversiLab software to create a database of known clinical *Candida* isolates and assign a species designation to a set of 115 putative *Candida* isolates.

METHODS

Identification of *Candida* isolates by biochemical and culture characteristics. To construct the initial *Candida* library, 41 clinical isolates from 5 of the most common clinically encountered *Candida* species, *C. albicans* ($n=7$), *C. parapsilosis* ($n=10$), *C. glabrata* ($n=7$), *C. krusei* ($n=7$) and *C. tropicalis* ($n=10$), were characterized in the Clinical Microbiology Laboratory at the M. D. Anderson Cancer Center. Also included were five culture collection reference strains, *C. albicans* ATCC 10231, *C. glabrata* ATCC 15126, *C. krusei* ATCC 200339, *C. parapsilosis* ATCC 10232 and *C. tropicalis* ATCC 10610. The test sample isolates, identified initially as *C. albicans* ($n=94$), *C. glabrata* ($n=8$), *C. parapsilosis* ($n=5$), *C. tropicalis* ($n=3$), *C. krusei* ($n=3$) and *C. lusitanae* ($n=2$), were obtained from TriCore Reference Laboratories. The traditional *Candida* identification scheme was as follows. Briefly, after positive blood culture, samples were characterized by microscopy. Putative yeast cell-containing samples were tested for germ-tube formation, where a positive test resulted in a *C. albicans* identification (unless colony morphology was atypical). Germ-tube-negative cultures were resolved using the Vitek yeast biochemical card (YBC; bioMérieux) and the isolate was considered to be identified if the YBC confidence was $>85\%$ and colony morphology was typical. If a yeast showed atypical morphology or the YBC card confidence was low, an API 20C yeast identification system (bioMérieux) and morphological characteristics on cornmeal agar were used to obtain yeast identification. Overall, approximately 2 days and 60 min of technical hands-on time was necessary for completion of this identification procedure.

Culture and DNA extraction. *Candida* clinical isolates were cultured on trypticase soy agar II with 5% sheep blood for 24 h at 37 °C. DNA from each isolate was extracted using the UltraClean microbial DNA isolation kit (Mo Bio Laboratories) following the manufacturer's instructions. DNA was adjusted to approximately 25 ng μl^{-1} for each sample and the presence of high-molecular-mass DNA was confirmed by agarose gel electrophoresis.

rep-PCR DNA fingerprinting. All DNA samples were amplified using the DiversiLab Yeast kit for DNA fingerprinting (Bacterial Barcodes) following the manufacturer's instructions. Briefly, 50 ng genomic DNA, 2.5 U AmpliTaq polymerase, 2.5 μl 10 $\times$ PCR buffer (Applied Biosystems) and 2 μl primer mix were added to the rep-PCR master mix in a total volume of 25 μl per reaction. Thermal cycling parameters were as follows: initial denaturation of 94 °C for 2 min,

Fig. 1. rep-PCR-based dendrogram and virtual gel image fingerprints obtained from 41 clinical *Candida* isolates and 5 ATCC reference strains using the DiversiLab system with the yeast fingerprinting kit. Pearson's correlation coefficient was used to create a pairwise percentage similarity matrix and the tree was generated using UPGMA. An 80 % similarity threshold (vertical line) was chosen for *Candida* species identification. Bar, percentage similarity among strains.

Fig. 2. Phylogenetic tree based on the DNA sequence of the ITS1–5.8S–ITS2 region illustrating the relationships among the 41 clinical *Candida* isolates and 5 ATCC reference strains. Sequences were aligned using CLUSTALW and the tree was constructed from a matrix of pairwise genetic distances using the neighbour-joining algorithm (Saitou & Nei, 1987).

followed by 35 cycles of denaturation at 92 °C for 30 s, annealing at 50 °C for 30 s and extension at 70 °C for 90 s, with a final extension at 70 °C for 3 min. Detection and analysis of rep-PCR products were implemented using the DiversiLab system (Bacterial Barcodes) and analysis was performed with the DiversiLab software version 3.1. The resulting DNA fingerprint patterns were viewed as electropherograms and the results included a dendrogram with a similarity matrix and a virtual gel image of the fingerprint for each DNA sample.

DNA sequencing and analysis. Species identification of the *Candida* isolates was confirmed by sequencing the ITS1–5.8S–ITS2 region as described by White *et al.* (1990). Briefly, the ITS region was amplified using the ITS1 forward primer 5'-TCCGTAGGTGAAC-CTGCGG-3' and the ITS4 reverse primer 5'-TCCTCCGCTTATTG-ATATGC-3'. Amplicons were purified using the High Pure PCR

product purification kit (Roche Diagnostics). Bidirectional sequence data were generated using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). The products were purified using Performa DTR gel filtration cartridges (Edge BioSystems) or ethanol precipitation, and sequenced using an ABI 377 or ABI 3100 sequencer (Applied Biosystems). Contiguous sequences were assembled and edited using the SEQMAN software program, which is part of the Lasergene suite of sequence analysis software (DNASTAR). GenBank/EMBL/DBJ database searches were conducted for the full-length sequences using BLAST (Altschul *et al.*, 1990). Sequences were aligned using the CLUSTALW function of the MEGALIGN program (DNASTAR). To generate the phylogenetic tree, MEGALIGN uses the Kimura distance method to create a pairwise genetic distance matrix. The dendrogram was constructed using the neighbour-joining method (Saitou & Nei, 1987).

Fig. 3. rep-PCR-based dendrogram showing DiversiLab software analysis of 94 putative *C. albicans* isolates (circled) queried against the clinical *Candida* library. The dendrogram was collapsed at 95 % similarity due to space constraints, such that only one fingerprint profile of groups of query samples at >95 % similarity is shown. The number of samples at collapsed nodes is indicated in parentheses. For selected samples, the ITS sequencing identification is given in the rightmost column. Pearson's correlation coefficient was used to generate a pairwise percentage similarity matrix and the tree was created using UPGMA. An 80 % similarity threshold (vertical line) was chosen for *Candida* species identification. Bar, percentage similarity among strains.

RESULTS AND DISCUSSION

Discrimination of *Candida* isolates using rep-PCR-based DNA fingerprints and library construction

We obtained rep-PCR-based fingerprint patterns for all of the *Candida* isolates using the DiversiLab system and yeast DNA fingerprinting kit. For analysis, DiversiLab software used the Pearson correlation coefficient and UPGMA to compare automatically the rep-PCR profiles and create corresponding dendrograms (Healy *et al.*, 2005a). In addition, results included computer-generated virtual gel images and selected demographic fields to aid interpretation of the data. Guidelines have been suggested by the manufacturer and there are several publications for identification of an isolate to the species level (Healy *et al.*, 2004; Pounder *et al.*, 2005). Although there is no universal threshold for species identification via rep-PCR profiles, moulds and fungi can often be identified at the species level if the fingerprints are >80 % similar to reference library samples. The 41 clinical *Candida* isolates from the M. D. Anderson Cancer Center were used to generate an initial *Candida* library, and the analysis showed that the isolates clustered by species at the 80 % threshold (Fig. 1). Six of the seven *C. glabrata* isolates grouped tightly (approx. 95 % similarity) with the *C. glabrata* reference strain ATCC 15126. The remaining clinical *C. glabrata* isolate (CL09) was on average approximately 80 % similar to members of the main *C. glabrata* cluster. *C. glabrata* CL09 appeared to lack two major rep-PCR amplicons present in the other *C. glabrata* representatives isolates. The ten clinical *C. tropicalis* strains were part of a single clade at approximately 82 % similarity, which included reference strain ATCC 10610. All of the clinical *C. krusei* isolates clustered at >90 % similarity, but *C. krusei* ATCC 200339 was an outlier with a fingerprint that was only 68 % similar to its nearest neighbour in the dataset, *C. glabrata* CL10. The analysis split the *C. albicans* strains into two clusters, with *C. albicans* ATCC 10231 grouping with isolates CL02 and CL06, and the second cluster comprising the five remaining clinical isolates. Finally, the *C. parapsilosis* strains separated into three clades at the 80 % similarity cut-off; *C. parapsilosis* ATCC 10232 was 95.5 % similar to *C. parapsilosis* CL24.

Sequence analysis of the ITS1–5.8S–ITS2 region

The ITS regions are located between the 18S and 28S rRNA genes, separated by the 5.8S RNA gene. DNA sequence

variation in the ITS regions has led to their use in phylogenetic studies of many fungi (Guarro *et al.*, 1999; Turenne *et al.*, 1999). In our study, sequencing of the ITS1–5.8S–ITS2 region from each of the *Candida* isolates allowed confirmatory identification of the *Candida* strains via BLAST searches of the GenBank/EMBL/DDBJ databases. Fig. 2 illustrates a phylogenetic tree based on the aligned ITS1–5.8S–ITS2 region for the 41 *Candida* isolates and the 5 ATCC strains. In general, the sequence-based tree bore an overall topology similar to the rep-PCR dendrogram, with the isolates clustered in species-specific groups; however, rep-PCR subdivided the clinical *C. albicans* and *C. parapsilosis* strains into separate clades at the 80 % similarity threshold. The increased discriminatory ability of rep-PCR is not surprising considering the basis of the two methods. The sequence-based tree was derived solely from variations in the ITS and 5.8S rRNA regions, whereas the rep-PCR DNA profiles represent molecular patterns surveying the entire genome of a given isolate, providing an overview of genomic variation among strains. The rRNA genes can be considered to be molecular chronometers such that sequence variation can be used to infer phylogenetic relationships among organisms. The dendrogram that is created from rep-PCR fingerprints is based on a pattern-matching algorithm that does not necessarily reflect phylogenetic history. This most likely accounts for the fact that the sequence of the *C. krusei* reference strain ATCC 200339, although only about 72 % identical to its closest match, *C. krusei* CL17, grouped with the other *C. krusei* isolates in the sequence-based tree, but was an outlier with the rep-PCR analysis, as this strain failed to cluster with the clinical *C. krusei* isolates.

Identification of *Candida* isolates by comparison with the *Candida* library

One hundred and fifteen clinical *Candida* isolates from a large regional reference laboratory were typed with the DiversiLab system and queried against the *Candida* library as described above. The isolates were identified previously at the species level by conventional biochemical/morphological tests. Fig. 3 illustrates the results obtained with 94 isolates identified as *C. albicans*. Eighty-one isolates clustered at >80 % with *C. albicans* library entries. ITS sequencing of the remaining samples was conducted to determine whether the isolates might have been misidentified by the biochemical/morphological methods. One presumptive *C. albicans*, TCLC-089, grouped within the *C. parapsilosis* cluster and sequencing analysis via BLAST searches confirmed that the isolate was indeed *C.*

Fig. 4. rep-PCR-based dendrogram showing DiversiLab software analysis of eight *C. glabrata*, five *C. parapsilosis*, three *C. tropicalis*, three *C. krusei* and two *C. lusitaniae* isolates (circled) queried against the clinical *Candida* library. For the misclassified sample TCLC-114 (asterisk), ITS sequencing-based identification revealed that the isolate was *S. cerevisiae*. Pearson's correlation coefficient was used to create a pairwise percentage similarity matrix and the tree was created using UPGMA. An 80% similarity threshold (vertical line) was chosen for *Candida* species identification. Bar, percentage similarity among strains.

Table 1. Comparison of identification results for discrepant *Candida* samples

Isolate	Biochemical/morphological identification	rep-PCR-based identification	ITS sequence-based identification via BLAST*
TCLC-001	<i>C. albicans</i>	<i>C. tropicalis</i>	<i>C. tropicalis</i> (DQ680841) ($e=1e-137$)
TCLC-083	<i>C. albicans</i>	No identification	<i>C. albicans</i> (AF455524) ($e=0$)
TCLC-084	<i>C. albicans</i>	No identification	<i>C. albicans</i> (AF455531) ($e=0$)
TCLC-085	<i>C. albicans</i>	<i>C. glabrata</i>	<i>C. glabrata</i> (AY939793) ($e=0$)
TCLC-086	<i>C. albicans</i>	<i>C. glabrata</i>	<i>C. glabrata</i> (AY939793) ($e=0$)
TCLC-089	<i>C. albicans</i>	<i>C. parapsilosis</i>	<i>C. parapsilosis</i> (DQ683009) ($e=0$)
TCLC-090	<i>C. albicans</i>	No identification	<i>C. albicans</i> (AF455524) ($e=0$)
TCLC-091	<i>C. albicans</i>	No identification	<i>C. parapsilosis</i> (AY391843) ($e=0$)
TCLC-092	<i>C. albicans</i>	No identification†	<i>S. cerevisiae</i> (AB212260) ($e=0$)
TCLC-093	<i>C. albicans</i>	<i>C. krusei</i>	<i>C. albicans</i> (AJ551313) ($e=0$)
TCLC-094	<i>C. albicans</i>	No identification†	<i>C. dubliniensis</i> (AJ865083) ($e=0$)
TCLC-095	<i>C. albicans</i>	No identification†	<i>C. dubliniensis</i> (AJ865083) ($e=0$)
TCLC-096	<i>C. albicans</i>	No identification	<i>C. albicans</i> (AF455524) ($e=0$)
TCLC-114	<i>C. glabrata</i>	No identification	Uncultured saccharomycete (AY349457) ($e=0$)

*Highest scoring sequence, GenBank/EMBL/DDBJ accession no. and e value.

†Sample was positively identified by the commercially available DiversiLab yeast library.

parapsilosis. Similarly, isolate TCLC-001 grouped tightly with the *C. tropicalis* samples in the database and sequencing revealed that the rep-PCR analysis was accurate and that the isolate was indeed *C. tropicalis*. Also, two putative *C. albicans* isolates (TCLC-085 and TCLC-086) that clustered with the *C. glabrata* library entries were confirmed as *C. glabrata* by sequencing. One sample confirmed by sequencing as *C. albicans* (TCLC-093) was 81 % similar to *C. krusei* ATCC 200339; thus, by the 80 % similarity threshold rule-of-thumb, this sample was misclassified by the DiversiLab software.

The remaining eight isolates identified as *C. albicans* did not group with any library entries at the 80 % cut-off level. Four of these were confirmed as *C. albicans* by sequence analysis. Therefore, it appears that the *Candida* library was not of a sufficient scope to be useful for characterizing these particular strains. This is not surprising, as the library was constructed with isolates obtained from a limited patient population (cancer patients) at a single medical facility. The putative *C. albicans* TCLC-091 isolate was identified as *C. parapsilosis* by sequencing. Presumptive *C. albicans* TCLC-092 had a distinctive fingerprint and did not cluster closely with any library entries; sequencing revealed that it was actually *Saccharomyces cerevisiae*. Finally, the two isolates TCLC-094 and TCLC-095 with unusual rep-PCR profiles were identified as *Candida dubliniensis* by ITS sequencing and BLAST searches.

The fingerprints of *Candida* species identified biochemically as non-*albicans* and a comparison with the *Candida* library is illustrated in Fig. 4. All query isolates clustered with the appropriate library samples with the exception of one strain (TCLC-114). This isolate, identified as *C. glabrata* by biochemical methods, was 100 % similar by sequencing to uncultured *Saccharomyces* isolate A9

(GenBank accession no. AY349457). Table 1 summarizes the species-level identification results among all discrepant samples.

Conclusions

A simple, automated system of both species-level identification and strain-level differentiation of *Candida* organisms could be valuable both for accurate treatment decisions and for infection control interventions for these fungal infections. In this study, rep-PCR was able to discriminate between the medically important *Candida* spp., including *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. krusei* and *C. tropicalis*. The DiversiLab system was also successful at species-level identification of isolates by comparison with a reference library and in fact recognized several strains that were misidentified at the species level by the conventional biochemical/morphological techniques. The availability of large, well-characterized sample sets from dispersed geographical locations to build a comprehensive library of *Candida* spp. and other yeasts would enhance the ability to classify the samples that were not identified by rep-PCR in this study. In addition, this work suggests that rep-PCR is able to subtype *C. albicans* and *C. parapsilosis* species, indicating that this method may be particularly important if specific genotypes are shown to be associated with either increased virulence or unique antifungal-resistance profiles (Foongladda *et al.*, 2004). The DiversiLab system may also be an attractive tool for clinical laboratories where minimizing both the turnaround time and technical hands-on time is paramount. The most labour-intensive portion of the rep-PCR assay is the DNA extraction, and we have investigated replacing the manual DNA purification procedures with automated systems (data not shown). In particular, for non-*albicans*

Candida spp., which require multiple procedures to be performed to obtain species identification, the automation of rep-PCR would allow rapid genotyping, which could lead to the identification and subspecies differentiation of *Candida* organisms in hours instead of days. With regard to strain typing, further studies are needed to compare the various methods of molecular typing of sample sets that include demographic data to assess their usefulness for epidemiological studies.

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