Use of denaturing gradient gel electrophoresis for the identification of mixed oral yeasts in human saliva

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A PCR-denaturing gradient gel electrophoresis (DGGE) method was established for the simultaneous presumptive identification of multiple yeast species commonly present in the oral cavity. Published primer sets targeting different regions of the Saccharomyces cerevisiae 26–28S rRNA gene (denoted primer sets N and U) and the 18S rRNA gene (primer set E) were evaluated with ten Candida and four non-Candida yeast species, and twenty Candida albicans isolates. Optimized PCR-DGGE conditions using primer set N were applied to presumptively identify, by band matching, yeasts in the saliva of 25 individuals. Identities were confirmed by DNA sequencing and compared with those using CHROMagar Candida culture. All primer sets yielded detectable DGGE bands for all species tested. Primer set N yielded mainly single bands and could distinguish all species examined, including differentiating Candida dubliniensis from C. albicans. Primer set U was less discriminatory among species but yielded multiple bands that distinguished subspecies groups within C. albicans. Primer set E gave poor yeast discrimination. DGGE analysis identified yeasts in 17 of the 25 saliva samples. Six saliva samples contained two yeast species: three contained C. albicans and three C. dubliniensis. C. dubliniensis was present alone in one saliva sample (total prevalence 16 %). CHROMagar culture detected yeasts in 16 of the yeast-containing saliva samples and did not enable identification of 7 yeast species identified by DGGE. In conclusion, DGGE identification of oral yeast species with primer set N is a relatively fast and reliable method for the simultaneous presumptive identification of mixed yeasts in oral saliva samples.

INTRODUCTION

Yeasts, predominantly Candida spp., are part of the normal microbial flora of the human oral cavity. Candida albicans is the most common species, followed by Candida tropicalis, Candida glabrata, Candida parapsilosis, Candida guilliermondii, Candida krusei and Candida dubliniensis (Cannon et al., 1995; Martins et al., 2010). The oral carriage rate of Candida in healthy humans ranges from 40 to 60 % (Samaranayake, 2009). The non-Candida yeasts Pichia ohmeri, Geotrichum spp., Trichosporon spp., Rhodotorula spp., Cryptococcus spp. and Saccharomyces cerevisiae are found occasionally (Cannon et al., 1995; Gonçalves et al., 2006). In addition, multiple yeast species are frequently present (Soysa et al., 2006). Candida spp. are opportunistic pathogens causing a variety of oral pathologies (Williams & Lewis, 2011). C. albicans possesses adhesive, acidogenic and proteolytic properties that are characteristic of caries pathogens, and it is associated with caries and periodontal disease (Beighton et al., 1995; Klinke et al., 2011).

Accurate identification of Candida spp. is important for the treatment of infected patients, because significant attributes such as drug resistance and virulence differ among species (López-Martínez, 2010). Phenotypic tests are often ambiguous because of yeast strain variability and the high degree of phenotypic switching within a single species (Yang, 2003). Among the non-albicans Candida spp., C. dubliniensis is an increasingly important pathogen (Ells et al., 2011). It is closely related to C. albicans and exhibits similar phenotypic properties (Campanha et al., 2011).
As a consequence, C. dubliniensis is likely to be under-reported and misidentified using most of the available phenotypic identification methods, including culture on chromogenic media, which is used widely in clinical laboratories for presumptive yeast identification (Campanha et al., 2005; Madhavan et al., 2011).

Molecular techniques provide more accurate methods for identifying and fingerprinting micro-organisms (Borman et al., 2008; Kuba, 2008; Neppelenbroek et al., 2006; Sidrim et al., 2010; Trtkova & Raclavsky, 2006), and have been used widely to identify and type yeast. Techniques include PCR, real-time PCR, electrophoretic karyotyping, restriction fragment length polymorphism, fluorescence in situ hybridization, randomly amplified polymorphic DNA analysis, multilocus sequence typing and pyrosequencing (Dassanayake & Samaranyake, 2003; Moter & Göbel, 2000; Trtkova & Raclavsky, 2006). However, each technique has its own advantages and limitations, with many requiring a culture step to isolate the target species (Trtkova & Raclavsky, 2006). Hence, culture bias and the loss of minor species can occur. Real-time PCR methods have been developed to detect medically important Candida spp. in clinical samples (Fricker et al., 2010; Hsu et al., 2003; Khan et al., 2009; Pryce et al., 2003; White et al., 2004) using species-specific (Hsu et al., 2003) and pan-fungal (White et al., 2004) primers, and combinations of primers in multiplex real-time PCR methods (Carvalho et al., 2007). Although these novel techniques are sensitive, specific and rapid for Candida detection and estimation, they also have their own limitations. Generation of false-negative results can be an issue (White et al., 2004). The main limitation is their specificity for particular species or groups of species in a situation where multiple and/or unpredicted species may occur. The use of PCR together with denaturing gradient gel electrophoresis (DGGE) as described here enables detection of the presence of such species and frequently their presumptive identification, even if they are present as minor populations.

DGGE based on 16S and 26–28S rRNA gene sequences has been used to characterize complex microbial communities, including those in saliva, dental plaque and plaque microcosms (Ledder et al., 2006; Li et al., 2006; Rasiah et al., 2003; Zijnge et al., 2003). It has also been applied to monitor fungal communities in complex ecosystems such as soil, and in wine and food fermentations (Cocolin et al., 2000; Meroth et al., 2003; Muccilli et al., 2011; Oros-Sichler et al., 2006; Vilela et al., 2010). The use of this technique to identify yeasts in the oral cavity has potential both as a diagnostic tool and as a method to advance our understanding of these complex ecosystems.

This study had two aims: firstly, to develop a rapid and reliable DGGE method to detect and identify yeast species in human saliva samples where multiple species may be present by evaluating primer sets based on different regions of S. cerevisiae rRNA genes (Cocolin et al., 2000; Oros-Sichler et al., 2006; Van Elsas et al., 2000); and secondly, to compare the performance of DGGE with CHROMagar Candida culture medium in identifying yeasts from the saliva of different individuals.

**METHODS**

**Yeast strains and culture.** The yeast strains used as controls for the standardization of DGGE protocols, and to evaluate the level of discrimination between and within species, are listed in Table 1 (see also Fig. 2B). They were cultured in yeast peptone glucose (YPG) broth [10 g Bacto yeast extract (BD) 1^{-1}, 20 g Bacto peptone (Difco Laboratories) 1^{-1}], 20 g l−1 g−1 glucose (Merck) 1^{-1}] at 30 °C with shaking (200 r.p.m.) and maintained on YPG 1 % agar.

**Saliva collection.** Saliva was collected with informed consent from 25 healthy donors and with ethical approval from the Wellington Ethics Committee (WGT/04/02/003). Donors abstained from oral hygiene for 24 h prior to saliva collection. The chewing of chicle gum was used to stimulate salivary flow and increase plaque abrasion. Portions (1 ml) of each saliva sample were centrifuged at 11 760 g for 10 min and the pellets stored at −80 °C until analysed.

**Analysis of yeasts in saliva by culture.** Saliva (50 μl) was spread on CHROMagar Candida plates in triplicate and incubated at 35 °C for 48–72 h (Beighton et al., 1995). Colony counts (c.f.u. ml−1) were recorded. Presumptive identification by colony morphology and colour was recorded as: C. albicans, blue–light green/leaf green colour; C. tropicalis, dark blue–grey hue with purple halo in the agar; C. krusei, large rough colonies with pale pink colour; and C. dubliniensis, dark green (Coleman et al., 1997). C. albicans and C. dubliniensis appeared as different shades of green.

**Nucleic acid extraction.** Nucleic acid was extracted from 24 h YPG yeast cultures and from the 1 ml saliva pellets using a modification of a published bead-beater extraction procedure (Walter et al., 2000). From the yeast cultures, a 100 μl portion was transferred to a sterile bead-beater tube (BioSpec Products) containing 0.3 g sterile zirconium beads (0.1 mm diameter; BioSpec Products) and 900 μl sterile distilled water. Saliva pellets were resuspended in 1 ml sterile distilled water by vortexing prior to nucleic acid extraction. TN150 buffer [10 mM Tris/HCl (pH 8), 150 mM NaCl] (1 ml) was added to the samples in the bead-beater tubes, which were then vortexed and centrifuged at 11 760 g for 5 min at 4 °C. The supernatant was discarded and the pellets washed again with 1 ml TN150 buffer. Another 1 ml TN150 buffer was added to the washed pellets, which were processed in a mini bead beater (model 3110BX; BioSpec Products) at 480 r.p.m. for 3 min. The tubes were immediately cooled on ice and then centrifuged at 11 760 g for 5 min at 4 °C. The supernatant (300 μl) was placed in a sterile 2 ml microcentrifuge tube containing 400 μl Phase Lock Gel Heavy (Qiagen) and extracted twice with 200 μl UltraPure buffer-saturated phenol (pH 8; Bio-Rad) and 200 μl chloroform:isoamyl alcohol (24:1), followed by a final extraction with 400 μl chloroform:isoamyl alcohol (24:1). The upper phase was transferred to a sterile microcentrifuge tube, and 1 ml cold ethanol (100 %) and 50 μl 3 M Sodium acetate was added, and the nucleic acid precipitated at −20 °C for 18 h. The solution was centrifuged at 11 760 g for 20 min at −5 °C and the nucleic acid pellet air dried and then dissolved in 30 μl TE buffer [10 mM Tris/HCl (pH 8), 1 mM EDTA]. Each extraction batch included a negative control lacking the sample/organisms. The bead beating extraction procedure was compared with direct DNA extraction after enzymic lysis of the cell wall using two commercial kits: a High Pure PCR template preparation kit (Roche Diagnostics) and a Master Pure yeast DNA purification kit (Epicentre Biotechnologies).
**Table 1. Yeast species and sources**

The original sources of the yeast species were: a, American Type Culture Collection, Manassas, VA, USA; b, Department of Molecular & Cell Biology, Institute of Medical Sciences, University of Aberdeen, UK; c, A. Cassone, Instituto Superiore di Sanita, Rome, Italy; d, Centraalbureau voor Schimmelmicrocultures, Utrecht, The Netherlands; e, Department of Health, National Institute, Porirua, New Zealand; f, M. Niimi, Kagoshima University, Japan; g, D. C. Coleman, Dublin Dental University Hospital, Ireland; h, Institute of Medical Mycology, Teikyo University, Japan; i, K. Rogers, Mycology Laboratory, Food Sciences, Auckland, New Zealand; k, wild-type baker's yeast, DYC Foods, Auckland, New Zealand; l, M. Niimi, Kagoshima University, Japan; m, D. C. Coleman, Dublin Dental University Hospital, Ireland; n, Institute of Medical Mycology, Teikyo University, Japan; o, A. Fink, Massachusetts Institute of Technology, Cambridge, MA, USA; p, Wild-type Baker's yeast, DYC Foods, Auckland, New Zealand.

**Species** | **Strain ID** | **Source**
---|---|---
*C. albicans* | ATCC 10231 | a
 | SC5314 | b
 | AH72 | c
*C. glabrata* | CBS 138 | d
 | 850920 | e
*C. parapsilosis* | 90.493 | e
 | 59 (425) | f
*C. tropicalis* | 82.0738 | e
 | 61 (IFO 0618) | f
*C. kefyr* | B2455 | e
[C. pseudotropicalis]* | 78.1161 | e
 | 60 (7494) | f
*C. dubliniensis* | CD36 | g
 | CD41 | b
*C. guillermondii* | 89.14 | e
 | 57 (IFO 0838) | f
*C. kruzie* | 90.147 | e
 | 58 (IFO 0011) | e
*C. lusitaniae* | TIMM1668 | h
 | TIMM3482 | h
*C. rugosa* | 5.037 | i
 | 92.294 | i
*S. cerevisiae* | AH22 | j
 | DYC | k
*P. ohmeri* | 0412B1828 | i
 | 02/MR2067 | i
*G. capitatum* | 01/M11106 | i
 | 0412M8386 | i
*Trichosporon asahii* | 03/MR3060 | i
 | 05/DN1231 | i

*This C. kefyr strain was received as C. pseudotropicalis B2455 and is designated C. kefyr B2455.

DNA extracted using kits and total nucleic acid extracted using bead beating were quantified by measuring absorbance (at 260 and 280 nm) using a NanoDrop spectrophotometer (NanoDrop Technologies) and evaluated by electrophoresis. Nucleic acid extracted using the bead-beater method was tested for possible PCR inhibition, for example by RNA. No inhibition occurred. Hence, in further PCR-DGGE optimization steps, extracted nucleic acid was used. For electrophoresis, extracted nucleic acid (4 μl) was mixed with 1 μl 2 % gel loading dye [0.5 % (w/v) bromophenol blue sodium salt (Sigma), 2 % (w/v) 0.5 % xylene cyanol (Sigma), 70 % (v/v) glycerol] and separated by electrophoresis in 1 × TAE [40 mM Tris/ HCl (pH 8), 20 mM acetic acid, 1 mM EDTA] for 1 h at 120 V on a 1.5 % (w/v) agarose gel, stained with 1 × SYBR Safe gel stain (Invitrogen) and viewed by UV transillumination in a Syngene ChemiGenius bio-imaging system using GeneSnap version 6.00.19 software (Synoptics) to quantify the DNA.

**PCR conditions.** Three primer sets, designated N, U and E (Table 2) were evaluated. Primer set N comprised primer N1 (Kurtzman & Robnett, 1998) and LS2 (Coculin et al., 2000), and targeted the D1 region of the 26–28S rRNA gene, yielding amplicons of ~250 bp. Primer set U comprised primers U1 and U2, and also targeted the 26–28S rRNA gene, yielding amplicons of ~260 bp (Sandhu et al., 1995). Primer set E comprised primer NS1 (White et al., 1990), EF3 (Smit et al., 1999) and FR1 (Vainio & Hantula, 2000), which were used in a semi-nested PCR to amplify the V1–V9 region of the 18S rRNA gene, yielding a product of ~1650 bp (Oros-Sichler et al., 2006).

PCR was performed in 0.2 ml tubes using a Hybaid Px2 thermal cycler. For direct PCR, the 50 μl reaction mixture consisted of PCR buffer (5 μl) supplemented with 4 mM MgCl₂ (FastStart Taq DNA polymerase kit; Roche Applied Science), 0.2 mM each dATP, dCTP, dGTP and dTTP (0.4 μl; Invitrogen), 0.1 μl each primer (Invitrogen), 2.5 U FastStart Taq DNA polymerase (Roche Applied Science) and 2 μl template nucleic acid (~20 ng). The reactions were performed for 30 cycles for DNA from pure cultures and 35 cycles for DNA from saliva. Following an initial 4 min denaturation at 95 °C, the PCR cycle consisted of 95 °C for 30 s, 53 °C for 45 s and 72 °C for 30 s, with a final extension at 72 °C for 7 min. These optimized PCR conditions were then used for all the results for primer sets N and U described in this study.

Semi-nested PCR was performed by modification of a published procedure (Oros-Sichler et al., 2006). The first amplification step involved 25 PCR cycles with the NS1 and EF3 primers. The 25 μl reaction mixture consisted of 2 μl template nucleic acid (~20 ng), PCR buffer (5 μl) supplemented with 4 mM MgCl₂ (Roche Applied Science), 2 % (w/v) DMSO, 0.2 mM each dATP, dCTP, dGTP and dTTP (Invitrogen), 0.2 μM each primer (Invitrogen) and 2.5 U FastStart Taq DNA polymerase (Roche Applied Science). Reactions were performed with an initial denaturation at 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 57 °C for 45 s and 72 °C for 3 min at 72 °C, with a final extension at 72 °C for 10 min. Diluted amplicons comprising 2 μl (1:10) of the first step reaction were then used as template for the second amplification, which involved 20 PCR cycles with NS1 and the GC-clamped primer FR1. The cycle parameters were as for the first amplification except that the annealing temperature was 48 °C. All PCR experiments included a negative (no DNA) control. PCR products (4 μl) were visualized on a 1.5 % (w/v) agarose gel as described above.

**Optimization of PCR protocol**

**PCR additives.** The effects of adding betaine (0.8, 1.2 or 1.6 %, w/v), DMSO (1, 5 or 10 %, v/v) or formamide (1.25, 5 or 10 %, v/v) on PCR amplification were evaluated.

**Thermocycling parameters for primer sets N and U.** The annealing temperatures for *C. albicans* PCRs were optimized by testing temperatures between 50 and 60 °C. Different MgCl₂ concentrations (1–4 mM) were also tested. The PCR cycle number was varied: 30, 35, 40, 42, 45 and 50 cycles were examined.
Fig. 1. (A, B) DGGE profiles obtained from yeast species with primer set N (A) and set U (B). (C) DGGE profiles showing migration of bands amplified from different strains of C. dubliniensis and C. albicans using primer set U. (D) DGGE profiles obtained from yeast strains with primer set E. *Strain formerly identified as C. pseudotropicalis.

Fig. 2. Concentration of denaturant.
PCR inhibition by DNA samples. Equal volumes of extracted template nucleic acid from three replicate DNA extractions from saliva (one donor) were pooled and serially diluted tenfold in sterile TE buffer. A 10 μl portion of each dilution was mixed with 0.5 ng C. albicans ATCC 10231 DNA in 2 μl TE buffer, and PCR was performed using primer set N. The PCR products were stained and visualized by UV transillumination and were considered positive if the expected ~250 bp amplicons were detected.

DGGE conditions

The DCode universal mutation detection system (Bio-Rad) was used for DGGE analysis. For the N and U primer sets, electrophoresis was performed using a 1.0 mm polyacrylamide gel [ratio of 8 % (w/v) acrylamide to bis-acrylamide, 37.5 : 1] with a 35–50 % denaturing gradient [100 % denaturant was 7 M urea and 40 % (v/v) formamide], which increased in the direction of electrophoresis. The gels were prepared and run with 1 x TAE buffer at a constant voltage of 130 V at 60 °C for 4.5 h. The PCR products (10 μl) were mixed with 10 μl 2 % gel loading dye. After electrophoresis, the gels were stained at room temperature for 30 min in 1 x SYBR Safe gel stain and visualized by UV transillumination. A range of denaturant concentrations (0–80 %) and different durations of electrophoresis (3.5–5 h) were tested for primer sets N and U, resulting in 35–50 % denaturant being used as the standard condition except for primer set E. For primer set E, DGGE was performed using published conditions: a 7.5 % polyacrylamide gel, with an 18–38 % denaturing gradient and run with 1 x TAE at a constant voltage of 170 V for 18 h at 58 °C (Oros-Sichler et al., 2006).

DGGE analysis of saliva from 25 donors using primer set N. DGGE was performed as described with PCR products (35 μl) mixed with 15 μl 2 % gel loading dye and electrophoresis as described above.

Development of a yeast DGGE reference profile. To standardize among the DGGE gels and to facilitate identification of oral yeast species by band matching, a DGGE reference panel was developed. Yeast species that were known to be oral commensals and pathogens that generated rRNA gene amplicons with different mobilities on a denaturing gel were used as the reference panel. The species (strains) were: Candida rugosa (5.037), Geotrichum capitatum (01/M11106), C. tropicalis (82.0738), C. parapsilosis (90.493), C. guilliermondii (89.14), Candida kefyr (78.1161), C. glabrata (850920), C. dubliniensis (CD36), C. albicans (ATCC 10231), P. ohmeri (02/MR2067) and C. krusei (90.147). DNA from each species was amplified separately with primer set N and then mixed in equal volumes in order to ensure a reproducible reference ladder standard. A variable yield of the DNA of each species resulted if a mixture of DNA from the reference species was amplified, possibly due to template competition and PCR amplification bias. The reference mixture (10 μl) was combined with 15 μl gel loading dye and loaded to gels flanking the samples.

Sequence analysis of DGGE bands. The middle portion of the selected DGGE band was taken by inserting a sterile pipette tip into the polyacrylamide gel and removing a piece of the gel, which was transferred to a sterile tube containing 50 μl sterile DNase/RNase-free water and incubated overnight at 40 °C. The eluted DNA was amplified with the respective GC-clamped primer set (N or U) and the products visualized on a DGGE gel with the original sample as a reference to confirm that the reamplified product migrated to the original position. The eluted DNA from the original band was then reamplified using primers without the GC clamp. The resulting PCR products were purified using a GENECLEAN Turbo PCR purification kit (Q.BIOgene) according to the manufacturer’s protocol and quantified by measuring absorbance (at 260 and 280 nm) using a NanoDrop ND-1000 spectrophotometer and software (NanoDrop Technologies). PCR amplicons were sequenced in both directions using the PCR primers as sequencing primers (at the Allan Wilson Centre, Massey University, New Zealand).

![Fig. 2.](http://jmm.sgmjournals.org) Fig. 2. (A) DGGE profiles obtained from the indicated C. albicans strains with primer set N. Lane R shows the yeast reference profile: a, C. rugosa; b, G. capitatum; c, C. tropicalis; d, C. parapsilosis; e, C. guilliermondii; f, C. kefyr; g, C. glabrata; h, C. dubliniensis; i, C. albicans; j, P. ohmeri; k, C. krusei. (B) Cluster analysis of DGGE profiles of the 20 C. albicans strains with primer set U. V1–V6 represent different groups of strains identified at 60 % similarity.
Reproducibility, specificity and limit of detection (sensitivity) of DGGE for C. albicans. To evaluate the reproducibility of DGGE, two saliva samples from two different donors were split into three aliquots (1 ml each) and extracted in parallel, followed by DGGE with both N and U primer sets. The detection limit for yeast was evaluated by tenfold dilutions of an overnight culture of C. albicans (ATCC 10231) containing 10^8 cells ml^{-1} in 1 % peptone water and processed for DNA extraction, PCR and DGGE using both sets of primers. Twenty strains of C. albicans (listed in Fig. 2B) were used to determine the specificity of the DGGE system using the N and U primer sets. Each of these 20 C. albicans strains had been isolated from a different individual and had been fingerprinted by probing EcoRI digests of total DNA with the repetitive sequence Ca3 to determine the genetic relationships (Schmid et al., 1990), and were representative of all the major clades documented by Schmid et al. (1995). They comprised 14 commensal strains isolated from the oral cavity (9 isolates) or from anal specimens (5 isolates), 3 blood isolates and 3 isolates from oral lesions.

Statistical analysis. DGGE banding patterns were assessed by cluster analysis with a Dice similarity coefficient constructed using UPGMA within the BioNumerics program version 4 (Applied Maths).

RESULTS

Nucleic acid extraction and optimization of PCR procedures

Large and reproducible amounts of intact nucleic acid (up to 400 ng µl^{-1}) were obtained from pure yeast cultures and from saliva using the bead-beating method. The two commercial kits tested were quick and simple but the DNA yields were low (up to 25 ng µl^{-1} in 30 µl). PCR using either the N or U set of primers failed to amplify yeast DNA from saliva samples with 30 cycles. However, with 35 cycles, bands were visible. From single-species DNA, multiple bands appeared after 42 or more amplification cycles, probably due to non-specific amplification. The optimal concentration of MgCl₂ for PCR was 4 mM. The addition of PCR additives showed no detectable amplification change, or improvement, when N and U primer sets were used with 35 PCR cycles. Saliva-extracted nucleic acid was not inhibitory to C. albicans PCR amplification.

DGGE analysis of yeast species

The N, U and E sets of primers were evaluated based on their ability to distinguish 14 different yeast species by DGGE. A range of DGGE conditions was tested to determine the best separation and clarity of PCR products obtained from pure yeast cultures. Optimized DGGE conditions in 8 % (w/v) polyacrylamide for N and U primer sets were 35–50 % denaturant with a run time of 4.5 h. PCR fragments generated from all yeast species tested showed a distinct band in DGGE gels with all three primer sets (Fig. 1A, B, D). With primer set N, agarose gel electrophoresis showed no detectable differences in mobility among species and hence in amplicon size. With primer set U, there was some mobility variation, particularly evident with the non-Candida spp.

Primer set N yielded the best band separation by DGGE for individual species. Bands for the 14 yeast species migrated

Table 2. S. cerevisiae-derived rRNA gene primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon designation (size)</th>
<th>Primer</th>
<th>DNA sequence (5’→3’)</th>
<th>Position*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>26–28S rRNA</td>
<td>N (~250 bp)</td>
<td>NL1 (F)†</td>
<td>CCGCGCGCGCGCGCGCGCGG</td>
<td>-25–0</td>
<td>Kurtzman and Robnett (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LS2 (R)</td>
<td>ATTCACAAAGAAGGAGTC</td>
<td>203–222</td>
<td>Cocolin et al. (2000)</td>
</tr>
<tr>
<td>26–28S rRNA</td>
<td>U (~260 bp)</td>
<td>U1 (F)†</td>
<td>GCGCGCGCGCGCGCGCGCGG</td>
<td>403–422</td>
<td>Sandhu et al. (1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U2 (R)</td>
<td>GACTTCATGGAAGGTGT</td>
<td>645–662</td>
<td>Sandhu et al. (1995)</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>E (~1650 bp)</td>
<td>NS1 (F)</td>
<td>GTAGTCATATGCGTTGTC</td>
<td>17–36</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EF3 (R)</td>
<td>TCCTCTAAATGACCAAGTTT</td>
<td>1747–1727</td>
<td>Smit et al. (1999)</td>
</tr>
</tbody>
</table>

F, Forward primer; R, reverse primer.
*Relative to the start of the rRNA ORF of the gene.
†A GC clamp (bold, underlined) was added to primers NL1, U1 and FR1 for DGGE analysis.
‡Inosine nucleotides (I) were present at positions 2 and 17 of the primer.
at different positions on the gel (Fig. 1A). Duplicate strains of the same yeast species, except for the strains designated C. rugosa, yielded bands on the gel in the same position (data not shown). DGGE using primer set N differentiated C. albicans and C. dubliniensis (Fig. 1A, lanes 6 and 7). Apart from strain hp50an (Fig. 2A, lane 3), bands from the other 19 C. albicans strains migrated in the same position as the band from the reference C. albicans strain. The strain hp50an amplicon had 99% sequence identity with C. albicans strain TA62 (GenBank accession no. AF156537.1) and 98% sequence identity with C. dubliniensis (GenBank accession no. AB363780.1).

Primer set U did not provide adequate separation of amplicons from different yeast species (Fig. 1B). C. albicans (ATCC 10231), C. glabrata, S. cerevisiae, C. parapsilosis and C. dubliniensis yielded bands of different mobilities but they were very close to each other in a relatively narrow area of the upper gel. The two C. kefyr bands, including strain B2455 formerly identified as Candida pseudotropicalis, were at a similar position in the lower part of the gel. Each strain of C. albicans, Candida lusitaniae, C. parapsilosis, C. guilliermondii and C. rugosa shown in Table 1 migrated differently (data not shown). S. cerevisiae (DYC), C. krusei (90.147) and C. rugosa (5.037) yielded multiple bands, and bands from C. albicans ATCC 10231 migrated differently from those from C. albicans strains SC5314 and AH72 (Fig. 1C, lanes 3–5). C. albicans SC5314 and AH72 bands were separated from the C. dubliniensis bands, but those from C. albicans strain ATCC 10231 and C. dubliniensis (Fig. 1C, lanes 1, 2 and 4) co-migrated.

DGGE analysis of the 20 C. albicans strains yielded different multiple banding patterns in six groups, V1–V6 (Fig. 2B). The major cluster, V1, contained nine predominately commensal strains. The second major cluster, V4, contained four commensals. Overall, the commensal strains showed more variation than the strains isolated from clinical samples. C. albicans strain hp50an, which produced a band with primer set N that migrated differently from bands from other C. albicans strains, also yielded a different migration pattern with primer set U (designated group V5). Thus, DGGE using primer set U could differentiate between some C. albicans strains but was not suitable for use in identifying individual yeast species, especially in mixed yeast populations.

Semi-nested PCR with primer set E generated bands with poor separation between several species, and most bands were found in a narrow range on the gel (Fig. 1D). This was probably due to the high molecular mass of the PCR products (~1650 bp). Primer set E was not studied further.

Detection limit for C. albicans and the reproducibility of DGGE

C. albicans ATCC 10231 at a concentration of 10^3 cells ml⁻¹ produced visible bands on the DGGE gel, whilst no bands were detected with a concentration of 10^2 cells ml⁻¹, although products were detected on agarose gels following PCR. This gave a limit of detection under optimal conditions of 10^3 cells (as 1 ml of sample was processed to release nucleic acids), which was equivalent to DNA from 13 cells in the DGGE well, due to additional dilution steps. Identical triplicate DGGE patterns were generated from each of two saliva donors, indicating the reproducibility of the DGGE (data not shown).

Comparison of CHROMagar Candida and PCR-DGGE identification of yeasts in saliva

Using primer set N, DGGE was applied to examine the yeast populations in saliva samples from 25 donors (Fig. 3), and compared with isolation and presumptive identification by CHROMagar Candida culture (Table 3). Presumptive identification by DGGE was based on comparing the migration distances of bands in the DGGE gels with those of strains in the yeast reference panel (Fig. 3), and was confirmed by sequencing the individual bands (Table 3). Seventeen of the twenty-five saliva samples gave a positive result for yeast by PCR (Fig. 3). Six samples (35%) contained more than one yeast species (Fig. 3, lanes 3, 6, 13, 14, 16 and 17). Fourteen samples contained C. albicans, including saliva sample 12, which was negative by culture.

Culture analysis yielded positive results for 16 of the 25 saliva samples. Only two (numbers 6 and 16) showed evidence of multiple yeast strains. C. albicans was presumptively identified in 13 samples by its characteristic colony morphology of a light green to dark green colour (Table 3). Saliva sample 5 yielded pale pink colonies with a distinctively large rough shape and irregular edges, typical of C. krusei, which were identified as C. krusei by DGGE (Fig. 3, lane 5) and confirmed by sequencing.

DGGE identified C. dubliniensis in four samples (Fig. 3, lanes 3, 13, 15 and 17), three of which also contained C. albicans. However, no C. dubliniensis was detected in samples 3, 13, 15 or 17 by CHROMagar Candida; only C. albicans was detected in three of these samples (numbers 3, 13 and 17), indicating that the C. dubliniensis was misidentified on CHROMagar as C. albicans. In the fourth sample (number 15), an unidentified yeast (yeast C; see below) isolated on CHROMagar Candida was shown to be C. dubliniensis by DGGE and sequencing.

There were four unidentified yeast colonies isolated on CHROMagar Candida from saliva samples 2, 6, 15 and 16, and these were designated yeasts A, B, C and D (Table 3). These unknown colonies all had a typical yeast colony morphology: a round, smooth pasty appearance with regular edges. Yeast A was a cream colour, whilst yeast B changed colour from cream (24 h) to blue (72 h). Yeast C (subsequently found to be C. dubliniensis) was initially a cream colour and changed to dark green after 48 h. Yeast D remained white in colour (72 h). Yeast B (saliva sample 6) was presumptively identified as C. rugosa by DGGE (Fig. 3,
lane 6) but was in fact found to be *Candida pararugosa* by sequencing. The two additional non-*Candida* yeasts (yeasts A and D in saliva samples 2 and 16) that were unidentified on CHROMagar yielded unmatched DGGE bands and were identified by sequencing as *S. cerevisiae* and *Hanseniaspora uvarum*, respectively. A further unmatched DGGE band in saliva sample 14 (yeast E), undetected on CHROMagar, was identified by sequencing as *Pichia guilliermondii*.

**DISCUSSION**

DGGE using primer set N under the optimized conditions that were established in this study enabled the simultaneous presumptive identification of multiple oral yeast species. It yielded species-specific amplicons that were generally well separated and gave better species discrimination than those generated by primer sets U and E. It clearly separated *C. dubliniensis* from *C. albicans*, except for strain hp50an, which had atypical properties and has an uncertain taxonomic relationship to *C. albicans* (Schmid et al., 1990).

The DGGE analysis of 25 saliva samples showed that primer set N is suitable for identifying yeast communities in the oral cavity in which multiple species are present at significant frequency. This study is believed to be the first to describe DGGE yeast analysis of human samples. A report that stated that yeast DGGE had been applied to vaginal biofilms in fact described application of simple yeast-specific PCR and bacterial DGGE to these biofilms (Devillard et al., 2005). The present analysis was performed using saliva samples and is likely to be similarly applicable to dental plaque and other human-associated biofilms.

Primer set U was unsuitable for mixed yeast analysis because multiple banding patterns were observed in some species, resulting in ambiguous identification or misidentification of species. Several strains of *C. albicans* also co-migrated with *C. dubliniensis*. The different groups of *C. albicans* banding patterns observed with primer set U may be due to the highly variable nature of the 26–28S rRNA gene region and/or due to multiple copies of 26–28S rRNA.

**Table 3.** Presumptive yeast identification by CHROMagar *Candida* and DGGE, and confirmation of DGGE (primer set N) band species by sequence analysis for the 17 of the 25 saliva samples positive for yeast by PCR

<table>
<thead>
<tr>
<th>Saliva sample</th>
<th>CHROMagar analysis</th>
<th>Presumptive DGGE identification</th>
<th>Closest yeast by sequence analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
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<tr>
<td>2</td>
<td>Yeast A</td>
<td>Yeast A</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em> and <em>C. dubliniensis</em></td>
<td><em>C. albicans</em> and <em>C. dubliniensis</em></td>
</tr>
<tr>
<td>4</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
</tr>
<tr>
<td>5</td>
<td><em>C. krusei</em></td>
<td><em>C. krusei</em></td>
<td><em>C. krusei</em></td>
</tr>
<tr>
<td>6</td>
<td><em>C. albicans</em> and Yeast B</td>
<td><em>C. albicans</em> and <em>C. rugosa</em></td>
<td><em>C. albicans</em> and <em>C. pararugosa</em> (99%)</td>
</tr>
<tr>
<td>7</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
</tr>
<tr>
<td>8</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em> (99%)</td>
</tr>
<tr>
<td>9</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
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<tr>
<td>10</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
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<tr>
<td>11</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
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<tr>
<td>12</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em></td>
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<tr>
<td>13</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em> and <em>C. dubliniensis</em></td>
<td><em>C. albicans</em> and <em>C. dubliniensis</em></td>
</tr>
<tr>
<td>14</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em> and Yeast E</td>
<td><em>C. albicans</em> and <em>P. guilliermondii</em></td>
</tr>
<tr>
<td>15</td>
<td>Yeast C</td>
<td><em>C. dubliniensis</em></td>
<td><em>C. dubliniensis</em></td>
</tr>
<tr>
<td>16</td>
<td><em>C. albicans</em> and Yeast D</td>
<td><em>C. albicans</em> and Yeast D</td>
<td><em>C. albicans</em> and <em>H. uvarum</em></td>
</tr>
<tr>
<td>17</td>
<td><em>C. albicans</em></td>
<td><em>C. albicans</em> and <em>C. dubliniensis</em></td>
<td><em>C. albicans</em> and <em>C. dubliniensis</em></td>
</tr>
</tbody>
</table>

*Sequence identity of the DGGE band: all were 100% identical to the yeast sequence found in GenBank, except for those where 99% identity is shown.*
genes within a single strain possessing sequence diversity (Marshall et al., 2003). The advantage of this primer set is its potential to identify intra-species variation within C. albicans. With further evaluation, this might assist with the identification of particular strains associated with disease (Romeo & Cirseio, 2011).

Primer set E showed poor discrimination among yeast species. The 18S rRNA gene region amplified by primer set E targets a region of the gene that is highly conserved in the reference yeast species and yields amplicons longer than those optimal for DGGE analysis, probably accounting for its poor level of discrimination, which has been observed previously (Kowalchuk et al., 1997).

The comparison of DGGE with culture analysis applied to the total yeast community in saliva samples illustrated the strengths and weaknesses of each approach. Culturable yeast species can be characterized according to morphological and physiological characteristics, but this requires 2–3 days of culture, does not detect minor or uncultivable yeast species, and may also underestimate the number and diversity of the total oral yeast community present.

CHROMagar Candida could presumptively identify only three Candida species (C. albicans, C. krusei and C. tropicalis) by colony colour. Both C. albicans and C. dubliniensis strains give variable shades of green on CHROMagar plates, and it was not possible to differentiate accurately between these two species. Although CHROMagar Candida is sometimes used to differentiate these two species, the results are usually uncertain (Campanha et al., 2005; Ellepola et al., 2003). C. dubliniensis, C. parapsilosis and C. glabrata are frequently isolated from human immunodeficiency virus-infected patients and have different susceptibilities to azole antifungals; hence, accurate identification of these species is important. The development of this DGGE method using primer set N could be applied as a tool for their detection.

DGGE yeast analysis also has strengths and limitations. The technique requires specialist equipment and expertise. Its major strength is the detection of a wide range of yeast species in mixtures, identifiable by band matching, with further confirmation possible by sequencing. Due to potential PCR amplification bias, band quantification does not necessarily relate directly to microbial abundance. The likelihood of bias associated with differential and non-specific amplification during PCR (Polz & Cavanaugh, 1998) was reduced by optimizing the PCR and is of reduced importance where detection only of the presence of a species is sought. Using band matching, DGGE can only presumptively identify species. DNA from dead or uncultivable or low-prevalence cells is also likely to be amplified and visualized. Nucleic acid extraction by bead beating effectively takes 1 working day with a further day required for DGGE analysis, comparable with the time required for CHROMagar culture. Faster DNA isolation procedures would allow the analysis in 1 working day, although the extraction kits tested gave low yields. In practice, about 15 samples per gel, allowing for 3 sets of reference standards, can be analysed reliably. The detection limit was 10³ cells ml⁻¹ in the original saliva, a concentration found in some colonized individuals (R.D. Cannon, unpublished observations). Yeast was detected in 17 out of 25 saliva samples, one more than by CHROMagar culture.

**Yeast population dynamics of the oral cavity**

The prevalence of Candida spp. in the oral cavity has been documented as 40–60 % among healthy humans (Samaranayake, 2009). C. albicans can be detected in the mouths of between 20 and 60 % of healthy individuals (Samaranayake, 2009). Given that most studies of the incidence of yeasts and C. albicans carriage in the mouth are culture based and cross-sectional, not longitudinal, this is probably an underestimation.

In this study, DGGE identified yeasts in the saliva of 17 out of 25 donors (68 %). Six had multiple yeast species present. CHROMagar Candida culture found yeasts in only 16 of the 25 donors (64 %), and only 2 were identified as having multiple yeast species. Despite the small sample number, and the random convenience samples of apparently healthy adult donors, this overall yeast carriage rate in saliva, most commonly C. albicans, agrees with reported findings (Ellepola & Samaranayake, 2000; Samaranayake, 2009).

The second most common yeast identified by DGGE was C. dubliniensis (16 %), which was present at a much higher frequency than in previous reports from healthy populations (~3 %; Loreto et al., 2010). Our isolation of C. dubliniensis together with C. albicans is in agreement with previous reports (Willis et al., 2000). This apparently high prevalence of C. dublinensis may reflect misidentification in some earlier studies, as in the present study C. dublinensis could not be identified reliably by CHROMagar Candida. C. dubliniensis is often present at higher frequencies in human immunodeficiency virus-positive patients and patients with diabetes, and its pathogenic specificity is still uncertain (Moran et al., 2012). Hence, it is important to distinguish it from other Candida spp. (Loreto et al., 2010).

Several other species in addition to C. dubliniensis were identified by DGGE and sequencing, but were not identified using CHROMagar Candida: C. rugosa, C. pararugosa, S. cerevisiae, H. uvarum and P. guilliermondii. C. pararugosa (as identified by sequence analysis) was initially identified as C. rugosa by DGGE band matching. C. pararugosa is phylogenetically close to C. rugosa (Giammanco et al., 2004; Linton et al., 2007). On CHROMagar Candida plates, its colonies were very small and dark blue. Previous studies have reported that C. rugosa produces light blue/green-coloured colonies on CHROMagar Candida (Hospenthal et al., 2006) and C. pararugosa produces pale violet colonies (Giammanco et al., 2004).
C. pararugosa is rarely isolated from the human oral cavity – it was isolated from one healthy denture wearer (Giammanco et al., 2004). C. rugosa is isolated more frequently, especially in diabetes mellitus patients (Gonçalves et al., 2006). C. pararugosa has been isolated from raw milk and milk products (Cocolin et al., 2002; Seiler & Busse, 1990). Hence, this particular isolate of C. pararugosa could be food associated. H. uvarum, which was identified in saliva sample 16, is also rarely isolated from the oral cavity (Emmanouil-Nikoloussi et al., 1994), but is also associated with food (Hierro et al., 2006). P. guilliermondii, a teleomorph of C. guilliermondii, is routinely isolated from sputum (San Millán et al., 1997), and may also be derived from processed food (Nielsen et al., 2008) and wine grapes (Li et al., 2010).

In conclusion, DGGE analysis of yeast species associated with the oral cavity (saliva) using primer set N can be used for their presumptive identification in mixtures in a single analysis. It is a promising, effective and relatively fast method of obtaining species differentiation without the need to perform conventional culture and time-consuming phenotypic characterization, which can underestimate the diversity of yeast species present. It was applied successfully for the presumptive identification of mixed yeasts in human saliva samples and could probably be applied to the detection of yeasts in human-associated biofilms.

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