PCR-based identification and strain typing of *Pichia anomala* using the ribosomal intergenic spacer region IGS1

Sonia Bhardwaj,[1] Rajeshwari Sutar,[2] Anand K. Bachhawat,[2] Sunit Singhi[3] and Arunaloke Chakrabarti[1]

Correspondence
Arunaloke Chakrabarti
arunaloke@hotmail.com

[1]Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

[2]Institute of Microbial Technology, Sector 39-A, Chandigarh 160036, India

[3]Department of Paediatrics, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Frequent outbreaks of *Pichia anomala* fungaemia in paediatric patients have warranted the development of a rapid identification system for this organism. This study describes a specific PCR-based method targeting the rRNA gene intergenic spacer region 1 (IGS1) for rapid identification of *Pichia anomala* isolates and characterization at the strain level. These methods of species identification and strain typing were used on 106 isolates of *Pichia anomala* (77 from a previously described outbreak and 29 isolated post-outbreak from the same hospital). Using conventional morphological and biochemical methods, 11 strains isolated during the outbreak were misidentified as *P. anomala*. BLAST analysis of sequences of internal transcribed spacer (ITS) regions of rRNA genes confirmed that they were *Pichia guilliermondii* (eight isolates) and *Debaryomyces hansenii* (three isolates). Strain typing of *Pichia anomala* isolates confirmed the previous finding of a point-source outbreak. The results suggest that IGS sequences and their polymorphisms could be exploited for similar typing methods in other organisms.

INTRODUCTION

Increasing numbers of outbreaks of nosocomial fungal infections warrant early diagnosis, rapid and accurate identification of the causative fungus, and reproducible molecular typing methods to investigate the outbreaks. Conventional techniques are time-consuming and often pose difficulties in identification of the pathogen because of a lack of definitive morphological and biochemical characteristics. These problems have prompted the search for a rapid molecular method of identification. For identification of many yeasts and mycelial fungi, species-specific probes have been designed from 18S and 28S regions of the rRNA gene (Schmid *et al.*, 1990; Soll, 2000). Although reproducible and reliable for species identification, the 18S and 28S rRNA genes are less discriminatory for strain differentiation. Strain distinction resolves a number of epidemiological issues, such as recognizing outbreaks, detecting cross-transmission, determining source of infection and recognizing virulent strains of the organism.

Several studies have focused on the internal transcribed spacer regions (ITS1/ITS2) of rRNA genes to discriminate

strains of *Pneumocystis jiroveci* (*carinii*) and *Histoplasma capsulatum*, but the techniques necessitated sequencing of these regions (Jiang *et al.*, 1996; Latouche *et al.*, 1997; Tamura *et al.*, 2002). The ITS2 region has been used for specific diagnosis of *Candida* species (Lott *et al.*, 1998). In recent years, the intergenic spacer region (IGS) has also been explored for typing at the species and strain levels (Jackson *et al.*, 1999; Mochizuki *et al.*, 2001). However, this region has not been sufficiently studied or exploited (unlike the ITS regions) due to its large size and the difficulty in sequencing (due to repeat regions) (Sutar *et al.*, 2004).

Pichia anomala (anamorph: *Candida pelliculosa*), a free-living ascomycetous yeast, is gaining importance as an opportunistic pathogen. We previously reported an outbreak where 379 neonates and children (4.2 % of admissions) in our tertiary care hospital suffered from nosocomial fungaemia due to *Pichia anomala* (Chakrabarti *et al.*, 2001). Following this, we reported the complete sequence of the IGS1 region of seven *Pichia anomala* strains. We found that their IGS1 regions were more polymorphic than their ITS regions and were of help in resolving ambiguous cases of identification (Sutar *et al.*, 2004). The detection of hyper-variability in the IGS1 region of *Pichia anomala* prompted us to target it in the present study for development of a robust

Abbreviations: IGS, intergenic spacer region; ITS, internal transcribed spacer.

PCR-based method for species identification and to evaluate its potential for strain discrimination using the strains isolated by us during the outbreak caused by *Pichia anomala* (Chakrabarti *et al.*, 2001).

METHODS

Fungal strains and growth conditions. For initial standardization, eight *Pichia anomala* strains comprising three blood isolates (two randomly selected from the strains isolated during the outbreak at the Postgraduate Institute of Medical Education and Research, Chandigarh, India, in 1996–1998 and one from a child from the same hospital post-outbreak in 2001) and five unrelated environmental isolates were included (Table 1). To examine the specificity of the primers, seven clinically relevant yeasts (other than *Pichia anomala*) were included (Table 1). After standardization, 106 more isolates (77 from the outbreak and 29 post-outbreak; 103 of these isolates were from blood of paediatric patients with clinical sepsis, whilst the remaining three strains were from the hands of a resident doctor and a nurse and from a post-infusion drip set during the outbreak period) were included in the study for identification and strain typing. These isolates were conventionally identified as *Pichia anomala* based on morphology on cornmeal agar, ascospore formation, urease production, carbohydrate fermentation and assimilation, and nitrate utilization. All of the strains were stored at −80 °C and revived on malt yeast extract agar medium (0.3 % malt extract, 0.3 % yeast extract, 0.5 % peptone, 1 % glucose and 2 % agar; Himedia) incubated at 30 °C for 3 days.

Isolation of whole-cell DNA. Genomic DNA was isolated by the glass bead lysis method (Kaiser *et al.*, 1994). Briefly, a loopful of culture from malt yeast extract agar (Himedia) was transferred to 200 μl lysis buffer [2 % Triton X-100, 1 % SDS, 10 mM NaCl, 10 mM Tris/HCl (pH 8.0), 1 mM EDTA] in a 1.5 ml microcentrifuge tube and 200 μl phenol/chloroform/isoamyl alcohol (25 : 24 : 1) was added. Cells were lysed by vortexing for 4 min in the presence of 20 mg glass beads. The aqueous layer was treated once more with phenol/chloroform/isoamyl alcohol and the DNA was precipitated with chilled 2-propanol. The pellet was washed with 70 % ethanol and finally resuspended in 50 μl TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA].

Sequence analysis for primer designing. The sequences of IGS1 of the rRNA gene from the seven *Pichia anomala* isolates described previously (Sutar *et al.*, 2004) were aligned using CLUSTAL W (Thompson *et al.*, 1994) and differences were highlighted using CLOURE (Kohli & Bachhawat, 2003). Accordingly, five species-specific primers were designed from the conserved region and four strain-specific primers were designed from the variable region for strain typing (Table 2).

PCR amplification with species-specific primers. Different parts of the IGS1 DNA were amplified with different sets of primers (IGF1/IGR1, IGF1/IGR2, IGF2/IGR1, IGF1/5SR1 and IGF2/5SR1) in a 50 μl reaction mixture containing 50 ng genomic DNA, 25 pmol primer, 200 mM each dNTP, 2.5 mM $MgCl_2$ and 2 U *Taq* polymerase. Amplification was carried out in a PTS100 minicycler (MJ Research) with an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 10 min and a terminal hold at 4 °C. Amplified products were separated by electrophoresis in a 1 % agarose gel and stained with ethidium bromide (0.5 μg ml⁻¹).

PCR amplification with strain-specific primers. For strain variation, designed primers (Table 2) and the 28S region primer IGSF1 were used in combination (IGSF1/SR1, IGSF1/SR2 and IGSF1/SR3). PCR was carried out in a 50 μl reaction mixture containing

Table 1. Strains used in the study

In addition to the strains listed here, 106 isolates (77 isolated during the outbreak and 29 isolated in the post-outbreak period) were also included. They were initially identified as *Pichia anomala* by morphological and biochemical characteristics and were given MCCL numbers. Culture collection abbreviations: MCCL, Mycology Culture Collection Laboratory, Postgraduate Institute of Medical Education and Research, Chandigarh, India; ATCC, American Type Culture Collection, Manassas, VA, USA; MTCC, Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India; NCYC, National Collection of Yeast Culture, UK.

Strain	Source
Pichia anomala MTCC 237/NCYC 1509	Fermenting cassava tuber
Pichia anomala MTCC 462	Molasses
Pichia anomala MTCC 3033	Spoiled sweet potato
Pichia anomala MTCC 3815	Starter culture of marcha
Pichia anomala MTCC 4046	Rice beverage
Pichia anomala MCCL B13	Blood of a patient during the outbreak
Pichia anomala MCCL B1569	Blood of a patient during the outbreak
Pichia anomala MCCL B209/2K	Blood of a patient during the post-outbreak period
Candida albicans ATCC 90028	Not known
Candida guilliermondii ATCC 6260	Lung
Candida parapsilosis ATCC 90018	Not known
Candida krusei ATCC 6258	Lung
Cryptococcus neoformans ATCC 32045	Fermented fruit juice
Candida tropicalis ATCC 750	Lung
Candida glabrata ATCC 2001	Faeces

Table 2. Primers used in the study

Primer	Sequence (5′→3′)
IGF1	AGTATACTGGCTAACAGAAGTTGGCTA
IGF2	GGAATCGTGACCAAAAAATGGGAAAT
IGR1	ATAATTTGGCCCAAATCTATTCTCCCA
IGR2	CTAACGGGGGGAGGATCCAGTATAAAA
5SR1	CACCGTTTCCGTTCCGATC
IGSF1	GGGATAGATTTTTTTAAATATGGGGTA
SR1	GACTATCTACCACCATCTACAT
SR2	TTCCACCCGTTACCCCCCATCT
SR3	ACCACCATCTACATCCCTCCTT

50 ng genomic DNA, 25 pmol each primer, 200 mM each dNTP, 1.5 mM $MgCl_2$ and 2 U *Taq* polymerase using an Eppendorf gradient thermocycler with the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 73 °C for 1 min and amplification at 73 °C for 1 min, with a final extension at 72 °C for 5 min and cooling at 4 °C.

RESULTS AND DISCUSSION

Five sets of species-specific PCR primers were designed from the conserved regions of IGS1 of *Pichia anomala* following sequence alignment studies conducted on clinical and non-clinical strains (Sutar *et al.*, 2004). Different combinations of primers (IGF1/IGR1, IGF1/IGR2, IGF2/IGR1, IGF1/5SR1 and IGF2/5SR1) produced amplicons of 1.1, 0.6, 0.7, 1.25 and 0.75 kb, respectively. They were highly specific and showed no amplification of any of the clinically important and relevant yeasts tested other than *Pichia anomala* (Fig. 1a–d; results for IGF1/IGR2 not shown).

Consequently, from the results of suitability of the primers and standardization of the reaction conditions, the primer set IGF1/5SR1 was chosen for species identification of the 106 strains included in this study. Sixty-six of the 77 outbreak strains and all 29 post-outbreak strains were confirmed as *Pichia anomala* using this primer combination. The 11 discrepant outbreak strains (identified as *Pichia anomala* by morphological and biochemical characteristics) yielded an amplicon of 500 bp. Further analysis by sequencing of the ITS1 and ITS2 regions identified these strains as *Pichia guilliermondii* (eight isolates) and *Debaryomyces hansenii* (three isolates) based on a 100 % match with type strains of *P. guilliermondii* or *D. hansenii* ITS1/ITS2 sequences using BLAST analysis (data not shown).

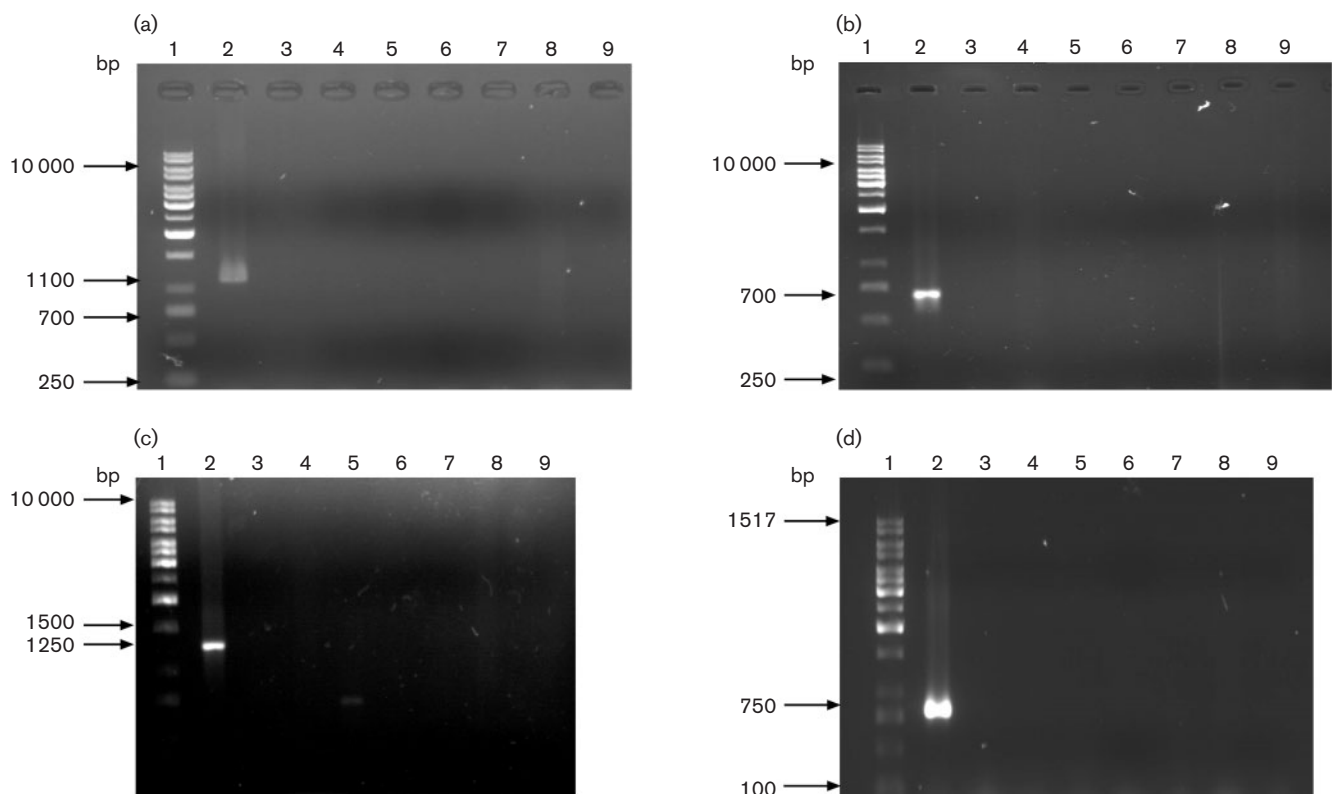*Pichia anomala* is an emerging agent of opportunistic mycoses in immunocompromised patients and premature

Fig. 1. Agarose gel electrophoresis of PCR-amplified products from different yeasts using the primer sets IGF1/IGR1 (a), IGF2/IGR1 (b), IGF1/5SR1 (c) and IGF2/5SR1 (d). Lanes: 1, DNA ladder; 2, *Pichia anomala*; 3, *Candida albicans*; 4, *Candida parapsilosis*; 5, *Candida guilliermondii*; 6, *Candida krusei*; 7, *Candida glabrata*; 8, *Candida tropicalis*; 9, *Cryptococcus neoformans*.

neonates with low birth weight, and on indwelling catheters and from a prolonged hospital stay. It has also been isolated from nosocomial fungaemia in paediatric patients and infections in surgical intensive care units. Many outbreaks by this organism have been reported (Thular *et al.*, 1997; Chakrabarti *et al.*, 2001; Kalenic *et al.*, 2001). Peripheral laboratories frequently fail to identify the organism because of the rarity of the infection and lack of expertise in identification, compounded by overlapping morphological and biochemical characteristics. To date, no acceptable scheme for typing of *Pichia anomala* is available.

In the present study, 11 isolates from the outbreak were wrongly identified as *Pichia anomala*. During the outbreak, any yeast producing ascospores (overlooking the detailed morphology of ascospores) was labelled as *Pichia anomala*. Several similarities of morphological and biochemical characteristics among *Pichia anomala*, *P. guilliermondii* and *D. hansenii* misled us in identification of those isolates. A detailed study of ascospore morphology and sugar assimilation is essential for accurate identification by conventional methods. Risk of misidentification among the above-mentioned species may pose a challenge to a routine laboratory during an outbreak that demands identification of a large number of isolates in a short time. The rapid method of identification by PCR reported here will overcome such difficulties and help in accurate identification of isolates.

For strain discrimination, three combinations of strain-specific primers, IGSF1/SR1, IGSF1/SR2 and IGSF1/SR3, were evaluated. IGSF1/SR2 displayed the best strain discrimination at an annealing temperature of 73 °C. It produced an amplicon of 730 bp in clinical isolates collected during and post-outbreak but no band in unrelated isolates (Fig. 2). This primer combination proved to be very efficient in differentiating outbreak (66 isolates) and post-outbreak (29 isolates) strains from unrelated isolates.
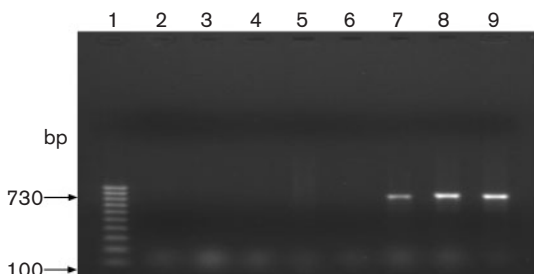

Fig. 2. Agarose gel electrophoresis of PCR-amplified products from different *Pichia anomala* strains using the strain-specific primer pair set IGSF1/SR2. Lanes: 1, 1 kb DNA ladder; 2–6, unrelated *Pichia anomala* isolates (MTCC 462, MTCC 237, MTCC 3033, MTCC 3815, MTCC 4046); 7–9, *Pichia anomala* strains isolated during the outbreak (MCCL 1454, MCCL 259, MCCL 742).

DNA-based typing methods have been used by several investigators, initially in yeasts and subsequently in filamentous fungi. The use of rRNA genes for strain discrimination and species identification is a popular approach in fungi. Sugita *et al.* (2002) developed specific oligonucleotide primers for *Trichosporon asahii* based on ITS regions. Analysis of *Candida* nucleotide sequences of the ITS2 region of 13 known species confirmed that no two species have identical size or sequence (Lott *et al.*, 1998). The IGS region has been found to be more polymorphic and has a higher discriminatory power than the ITS region (Sugita *et al.*, 2002; Sutar *et al.*, 2004). Polymorphisms of IGS in *Arthroderma benhamiae*, a teleomorph member of *Trichophyton menta-grophytes*, revealed five different types in eight strains (Jackson *et al.*, 1999). IGS-RFLP has also been investigated in *Trichophyton rubrum* where 14 different RFLP patterns were observed among 50 strains (Mochizuki *et al.*, 2001).

PCR-based methods and sequencing of the D1/D2 regions of the large subunit of the rRNA gene, as well as sequencing and RFLP of entire ITS regions, have been tried in *Pichia anomala* (Caggia *et al.*, 2001; Kurtzman, 2001; Lachance *et al.*, 2001; Naumov *et al.*, 2001; Las Heras-Vazquez *et al.*, 2003). Inter-repeat PCR has also been tried for differentiating clinical isolates of *Candida pelliculosa* (Barchiesi *et al.*, 2005). In the present study, *Pichia anomala* strains were analysed for the development of an epidemiologically relevant identification system based on hypervariability of IGS1 sequences. Our results describe a rapid, reliable and highly specific method for identification of *Pichia anomala* at the species level and for strain discrimination in the present study. The method allows one to exploit the IGS1 sequence polymorphism/hypervariability without sequencing, which is difficult and not practically feasible on a routine basis. Despite the presence of repeats, the presence of multiple bands following PCR (often seen with repeat sequences) was not observed.

The primer set for strain typing was found to be highly specific in amplifying the desired target of all strains (from both patient and environmental strains) isolated during the outbreak and post-outbreak periods. DNA was not amplified from any of the unrelated *Pichia anomala* strains. This confirmed our earlier observation of a single point source of the outbreak in 1996–1998 (Chakrabarti *et al.*, 2001). It was interesting to observe that the same strain persisted in our hospital despite a lull period from March 1998 to September 2000. The strain may have persisted as a colonizer among medical personnel in the hospital. With the possible deterioration of hospital practices at some point in time, the strain may have regained the opportunity to infect high-risk paediatric patients. However, the strain was restricted to the Advanced Pediatric Center, which is 300 yards away from the adult tertiary care centre.

Efforts to obtain strains from other *Pichia anomala* outbreaks around the world were unsuccessful so we could not extend this study to other clinical isolates. Although currently this method may appear limited in that

it produced results only with isolates from a particular outbreak, the observation that all of the primer combinations were effective for identification of *Pichia anomala* suggests that it will be possible to use a primer set and combine it in a multiplex system for species identification of the yeast. Similar methods using combinations of primers designed from IGS1 DNA may be used for strain typing of *Pichia anomala*. The present study also suggests that such IGS1-based PCR methods could also be developed for routine identification of other medically important yeasts.

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REFERENCES

Barchiesi, F., Tortorano, A. M., Di Francesco, L. F., Rigoni, A., Giacometti, A., Spreghini, E., Scalise, G. & Viviani, M. A. (2005). Genotypic variation and antifungal susceptibilities of *Candida pelliculosa* clinical isolates. *J Med Microbiol* **54**, 279–285.

Caggia, C., Restuccia, C., Pulvirenti, A. & Giudici, P. (2001). Identification of *Pichia anomala* isolated from yogurt by RFLP of the ITS region. *Int J Food Microbiol* **71**, 71–73.

Chakrabarti, A., Singh, K., Narang, A., Singhi, S., Batra, R., Rao, K. L. N., Ray, P., Gopalan, S., Das, S. & other authors (2001). Outbreak of *Pichia anomala* infection in the pediatric service of a tertiary-care center in northern India. *J Clin Microbiol* **39**, 1702–1706.

Jackson, C. J., Barton, R. C. & Evans, E. G. V. (1999). Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. *J Clin Microbiol* **37**, 931–936.

Jiang, B., Lu, J.-J., Li, B., Tang, X., Bartlett, M. S., Smith, J. W. & Lee, C.-H. (1996). Development of type-specific PCR for typing *Pneumocystis carinii* f. sp. *hominis* based on nucleotide sequence variations of internal transcribed spacer region of rRNA genes. *J Clin Microbiol* **34**, 3245–3248.

Kaiser, C., Michaelis, S. & Mitecheu, A. (1994). *Methods in Yeast Genetics: a Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Kalenic, S., Jandrlic, M., Vegar, V., Zuech, N., Sekulic, A. & Mlinaric-Missoni, E. (2001). *Hanselula anomala* outbreak at a surgical intensive care unit: a search for risk factors. *Eur J Epidemiol* **17**, 491–496.

Kohli, D. K. & Bachhawat, A. K. (2003). CLOURE: Clustal output Reformatter, a program for reformatting ClustalX/ClustalW outputs for SNP analysis and molecular systematics. *Nucleic Acids Res* **31**, 3501–3502.

Kurtzman, C. P. (2001). Four new *Candida* species from geographically diverse locations. *Antonie van Leeuwenhoek* **79**, 353–361.

Lachance, M.-A., Klemens, J. A., Bowles, J. M. & Janzen, D. H. (2001). The yeast community of sap fluxes of Costa Rican *Maclura* (*Chlorophora*) *tinctoria* and description of two new yeast species, *Candida galis* and *Candida ortonii*. *FEMS Yeast Res* **1**, 87–92.

Las Heras-Vazquez, F. J., Mingorance-Cazorla, L., Clemente-Jimenez, J. M. & Rodriguez-Vico, F. (2003). Identification of yeast species from orange fruit and juice by RFLP and sequence analysis of 5.8S rRNA gene and the two internal transcribed spacers. *FEMS Yeast Res* **3**, 3–9.

Latouche, S., Ortona, E., Mazars, E., Margutti, P., Tamburrini, E., Siracusano, A., Guyot, K., Nigou, M. & Roux, P. (1997). Biodiversity of *Pneumocystis carinii hominis*: typing with different DNA regions. *J Clin Microbiol* **35**, 383–387.

Lott, T. J., Burns, B. M., Zancope-Oliveira, R., Elie, C. M. & Reiss, E. (1998). Sequence analysis of the internal transcribed spacer 2 (ITS2) from yeast species within the genus *Candida*. *Curr Microbiol* **36**, 63–69.

Mochizuki, T., Kawasaki, M., Ishizaki, H., Kano, R., Hasegawa, A., Tosaki, H. & Fujihiro, M. (2001). Molecular epidemiology of *Arthroderma benhamiae*, an emerging pathogen of dermatophytoses in Japan, by polymorphisms of the non-transcribed spacer region of the ribosomal DNA. *J Dermatol Sci* **27**, 14–20.

Naumov, G. I., Naumova, E. S. & Schnürer, J. (2001). Genetic characterization of the non-conventional yeast *Hansenula anomala*. *Res Microbiol* **152**, 551–562.

Schmid, J., Voss, E. & Soll, D. R. (1990). Computer-assisted methods for assessing strain relatedness in *Candida albicans* by fingerprinting with the moderately repetitive sequence Ca3. *J Clin Microbiol* **28**, 1236–1243.

Soll, D. R. (2000). The ins and outs of DNA fingerprinting the infectious fungi. *Clin Microbiol Rev* **13**, 332–370.

Sugita, T., Nakajima, M., Ikeda, R., Matsushima, T. & Shinoda, T. (2002). Sequence analysis of the ribosomal DNA intergenic spacer 1 regions of *Trichophyton* species. *J Clin Microbiol* **40**, 1826–1830.

Sutar, R., David, J. K., Ganesan, K., Ghosh, A. K., Singhi, S., Chakrabarti, A. & Bachhawat, A. K. (2004). Comparison of ITS and IGS1 regions for strain typing of clinical and non-clinical isolates of *Pichia anomala*. *J Med Microbiol* **53**, 119–123.

Tamura, M., Kasuga, T., Watanabe, K., Katsu, M., Mikami, Y. & Nishimura, K. (2002). Phylogenetic characterization of *Histoplasma capsulatum* strains based on ITS region sequences, including two new strains from Thai and Chinese patients in Japan. *Nippon Ishinkin Gakkai Zasshi* **43**, 11–19.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Thular, L. C., Faivichenco, S., Velasco, E., Martins, C. A., Nascimento, C. R. & Castilho, I. A. (1997). Fungaemia caused by *Hansenula anomala* – an outbreak in a cancer hospital. *Mycoses* **40**, 193–196.