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Comparison of ITS and IGS1 regions for strain typing of clinical and non-clinical isolates of *Pichia anomala*

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Pichia anomala is an emerging nosocomial pathogen and there is a need for methods that distinguish between different *P. anomala* strains. In the typing of several clinical as well as non-clinical *P. anomala* strains, the sequence variation of the internal transcribed spacer (ITS) was found to be inadequate for typing purposes. The intergenic spacer 1 (IGS1) region of the rDNA of several *P. anomala* strains was therefore investigated in detail. The IGS1 region (which varied from 1213 to 1231 bp in length) was interspersed with repeats and had more variation than the ITS regions. Comparative analysis in cases where analysis by the ITS was ambiguous clearly revealed the IGS1 region to be a more discriminatory tool in the typing of *P. anomala* strains.

INTRODUCTION

Pichia anomala (formerly *Hansenula anomala*), a free-living ascosporogenous yeast, has become increasingly important as an opportunistic nosocomial fungal pathogen (Murphy *et al.*, 1986; Nohinek *et al.*, 1987; Klein *et al.*, 1988; Qadri *et al.*, 1988). Multiple outbreaks due to *P. anomala* have been reported in recent years (Thuler *et al.*, 1997; Chakrabarti *et al.*, 2001; Kalenic *et al.*, 2001). Due to the increasing incidence of *P. anomala* as a fungal pathogen, there is an increasing need to develop rapid means of identification of this organism at the species and subspecies level. In addition to early diagnosis, the process of strain typing is important epidemiologically for recognizing outbreaks of infections, detecting cross-transmission of nosocomial pathogens, determining the source of the infection and also recognizing particular virulent strains of organisms.

The molecular characterization of *P. anomala* has, like that of other yeasts, been undertaken by electrophoretic karyotyping, PCR-based analysis, sequencing of D1/D2 region of the large subunit of rDNA as well as by sequencing and RFLP of the internal transcribed spacer 1 (ITS1)–5·8S–ITS2 region (Naumov *et al.*, 2001; Caggia *et al.*, 2001; Kurtzman, 1984). However, while the ITS1/ITS2 and D1/D2 regions may still be best in terms of both rapidity and convenience for distinguishing organisms at the species level, recent evidence from other organisms (Sugita *et al.*, 2002) suggests that these regions still lack sufficient variation to distinguish between strains and, in some cases, between species.

It is therefore important to evaluate and incorporate either more regions or more hypervariable regions into typing strategies for other yeasts/fungi, including *P. anomala*. Among the more recent approaches that have been directed towards this end is multi-locus sequence typing (MLST), where multiple genetic loci are chosen from different chromosomal locations, amplified and sequenced to determine the extent of variation; it has the additional advantage that the different regions are unlikely to be co-inherited in a single genetic event (Maiden *et al.*, 1998; Urwin & Maiden, 2003). A second approach uses a single locus, the intergenic sequence (IGS), and has the advantage that the IGS locus is more variable than existing loci that have been exploited so far (Sugita *et al.*, 2002). In the latter case, the importance of IGS region sequences needs more rigorous evaluation in different organisms. However, owing to the much larger regions involved, IGS sequences have not been exploited in a routine manner. In fact, complete IGS regions are known for only a few organisms that include *Neurospora crassa* (Dutta & Verma, 1990), *Saccharomyces cerevisiae* (Molina *et al.*, 1993), *Filobasidiella* (*Cryptococcus*) *neoformans* (Fan *et al.*, 1995), *Neotyphodium lolii* (Ganley & Scott, 1998), *Collybia fusipes* and *Trichosporon* species (Sugita *et al.*, 2002).

In the present study, we describe the complete IGS1 sequences of several *P. anomala* strains for the first time and evaluate and compare the ITS and IGS1 regions of clinical and non-clinical strains of *P. anomala* for the development of a simple typing method.

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

The GenBank/EMBL/DDBJ accession numbers for the IGS1 and ITS sequences of *P. anomala* strains determined in this study are AY231599–AY231612, as detailed in Table 1.

METHODS

Strains and growth conditions. Three clinical isolates from blood of neonates [two isolated during an outbreak (Chakrabarti *et al.*, 2001) and one isolated in a post-outbreak period], three non-clinical isolates and one standard strain, NCYC 1509^T (Table 1), were included in the study. All yeast strains were grown on malt/yeast extract agar medium (0·3 % malt extract, 0·3 % yeast extract, 0·5 % peptone, 1 % glucose and 2 % agar) at 30 °C for 3 days.

Isolation of genomic DNA. Rapid preparation of genomic DNA from strains was performed by the glass bead lysis method (Kaiser *et al.*, 1994). A loopful of culture grown on malt/yeast extract agar was placed in 200 µl lysis buffer (2 % Triton X-100, 1 % SDS, 10 mM $NaCl_2$, 10 mM Tris/HCl, pH 8·0, 1 mM EDTA) and 200 µl phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and cells were lysed with 20 mg glass beads by vortexing for 4 min. The supernatant was extracted once more with phenol/chloroform/isoamyl alcohol. The DNA was precipitated with cold ethanol and washed with 70 % ethanol; the DNA pellet was finally resuspended in TE (10 mM Tris/HCl, pH 8·0, 1 mM EDTA).

PCR amplification. ITS regions were amplified using universal primers ITS1 and ITS4 (Table 2), sequences of which were respectively designed from conserved regions of the 18S and 26S rDNA (White *et al.*, 1990). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). PCRs were performed in final reaction mixtures (50 µl) containing 50 µg genomic DNA, 25 pmol of each primer (ITS1 and ITS4), 200 nM dNTPs (Promega), 25 mM $MgCl_2$, 2 U *Taq* polymerase (Promega) and 5 µl 10× reaction buffer (Promega). Amplification reactions were performed in a PTS 100 MiniCycler (MJ Research) with the following cycling parameters: initial denaturation for 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C with a final extension for 10 min at 72 °C and cooling to 4 °C.

IGS regions were amplified using primers IGS1F and IGSR (Table 2). The primers were designed on the basis of conserved regions of 28S and 18S rDNA. The PCR was performed with a similar reaction mixture to that described above and the following cycling parameters: initial denaturation for 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C with a final extension for 10 min at 72 °C and cooling to 4 °C. Using conserved regions of 28S and 5S rDNA, the IGS1 region was amplified with primers IGS1F and 5SR1 (Table 2).

DNA sequencing. Amplified products were purified after agarose gel electrophoresis using the Qiagen gel extraction kit. The DNA was quantified and subjected to sequencing using the ABI PRISM Big Dye Terminator cycle-sequencing protocol using an automated sequencer (ABI PRISM model 310; Applied Biosystems). Both strands of the PCR products were cycle sequenced with primer ITS1 and ITS4 separately. The sequences were confirmed using sequences of both strands and also confirmed sequences with primers ITS2 and ITS3 (Table 2).

IGS1 sequencing reactions were initially primed using IGS1F and 5SR1 and 15 internal primers were designed for primer walking in order to determine the complete sequence on both strands of the IGS1 PCR product; these primers are listed in Table 2.

Sequence analysis. Searches for sequence homologues in the non-redundant DNA database of NCBI were carried out using BLASTN

Table 2. Oligonucleotides used in this study

Primer	Sequence (5′–3′)
ITS region	
ITS1	TCCGTAGGTGAACCTGCGG
ITS2	GCTGCGTTCTTCATCGATGC
ITS3	GCATCGATGAAGAACGCAGC
ITS4	TCCTCCGCTTATTGATATGC
IGS region	
IGS1F	TTGTTGTTAACGATCTGCTGAGATT
IGS2F	TGGTCATGCGGGTAAATAGCC
IGS3F	GTGGAAATAGGGTGGTAA
IGS4F	CATGGGTGGTCATGAGGG
IGS5F	GGTGATGGTTATGGATGG
IGS6F	GTTTCCAAATAGGCCCGC
IGS7F	TGTAGATGGTGGTAGATAGT
IGS8F	GATCGGAGATGTTGTGGTGT
5SR1	CACCGTTTCCGTTCCGATC
5SR2	TTACCACCCTATTTCCAC
5SR3	GGCTATTTACCCGCATGACCA
5SR4	GATCGGGAAAATTTTTCACC
5SR5	CTATAAGGGATATCAGCTCAA
5SR6	ACCCCATATTTAAAAAAATCT
5SR7	TTTGACCATAACCATATTTT
IGSR	GTAGAGTAGCCTTGTTGTTACGATC

Table 1. *Pichia anomala* strains used in this study

Culture collection abbreviations: MTCC, Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India; MCCL, Mycology Culture Collection Laboratory, Postgraduate Institute of Medical Education and Research, Chandigarh, India.

Strain	Source/original source	Clinical/non-clinical	Accession no.	
			ITS	IGS1
MTCC 237^T	NCYC 1509^T, isolated from fermenting cassava tubers	Standard strain (non-clinical)	AY231606	AY231599
MTCC 462	Isolated from molasses	Non-clinical	AY231607	AY231600
MTCC 3033	Isolated from spoiled sweet potato	Non-clinical	AY231608	AY231601
MTCC 3815	Isolated from marcha, a traditional fermented food	Non-clinical	AY231612	AY231602
MCCL 13	Isolated from blood during outbreak, 1996–1997	Clinical	AY231609	AY231603
MCCL 1569	Isolated from blood during outbreak, 1996–1997	Clinical	AY231610	AY231604
MCCL 209	Isolated from blood during 2000 (post-outbreak)	Clinical	AY231611	AY231605

(Altschul *et al.*, 1997). Multiple sequence alignments were carried out using CLUSTAL W (Thompson *et al.*, 1994) and reformatted by CLOURE (Kohli & Bachhawat, 2003) to highlight differences between the sequences. All ITS and IGS1 region sequences were deposited in GenBank (Table 1).

RESULTS AND DISCUSSION

The amplified products of the ITS1–5·8S–ITS2 regions of all clinical and non-clinical isolates of *P. anomala* were approximately 600 bp long on agarose gel electrophoresis. These products were sequenced on both strands and the sequences were compared and analysed. For comparison, we used the sequence of the ITS1–5·8S–ITS2 region of MTCC 237^T (= NCYC 1509^T) as the standard. The sequence of this region from this strain was also identical to one of the *P. anomala* sequences deposited in GenBank (accession no. AF270936; strain FY 102).

Analysis of the sequences revealed identical sequences for all strains for the 5·8S rDNA and ITS2 region. However, two strains (MTCC 3033 and MTCC 3815) had an extra T at position 125 of ITS1 and MTCC 462 had seven single base variations in ITS1. All three clinical isolates had identical sequences that were surprisingly and unexpectedly also identical to the sequence of the non-clinical, geographically distinct standard strain (MTCC 237^T) in the ITS region, strongly suggesting that the ITS region sequence is not able to differentiate these strains.

Owing to the suspected inadequacy of the ITS regions in strain differentiation, the IGS regions were investigated. These regions have not been studied previously in *P. anomala*. The amplified IGS region was approximately 3 kb in size and was interrupted by a 5S rDNA, separating it into IGS1 and IGS2. The IGS1 region was amplified and had a sequence of about 1·2 kb in all strains, as seen on agarose gel electrophoresis. The entire IGS1 region was sequenced on both strands by primer walking. The sequencing reactions appeared to terminate abruptly on many occasions, leading to suboptimal sequence information being obtained with many of the primers. This was subsequently analysed and appears to be a result of excessive repeats in some regions. Because of this problem, a larger number of primers than usual were needed in order to ensure that the sequence was obtained on both strands (Table 2). The IGS1 sequences of *P. anomala* were comparatively long, and the presence of repeats made the sequencing of this region particularly difficult; for these reasons, we restricted the present analysis to seven strains. Despite the analysis being limited to only seven strains of *P. anomala*, the increased variability in both size and sequence nevertheless revealed several interesting aspects that have consequences both in typing methods in general as well as in the analysis of the present set of strains.

The length of the IGS1 region varied between 1213 and 1231 bp. Comparison of the nucleotide sequences of the entire IGS1 region for the seven strains revealed that (i) the IGS1 sequences of the three clinical strains (two epidemic and one post-epidemic) were completely identical to each other and showed approximately 96·6 % identity to the sequence of the type strain, MTCC 237^T, (ii) the three non-clinical isolates that were from different locations/sources showed 95·5–97·5 % identity to the sequence of MTCC 237^T and (iii) the IGS1 sequences of the different isolates were characterized by several imperfect repeats (Fig. 1).

Analysis of the repeat regions revealed that the most common tandem repeat was ATGGTT. At position 160, there were three tandem repeats in the standard strain, two in clinical isolates and one in non-clinical isolates. Further examination revealed that these sequences were within a larger repeat, although the repeat unit was not exact (Fig. 2). Similar observations were made for other repeats, GGGTGGTAATA (at position 780), GGAAAGATTAT (at position 817) and GTAGATAGTC (at position 865). The repeat regions of IGS1 of MTCC 237^T are represented schematically in Fig. 1.

One of the reasons for sequencing the entire IGS1 region was to identify possible restriction enzyme polymorphisms; one way of getting around the need to sequence the entire region is to exploit the sequence variation through restriction site polymorphisms. As mentioned earlier, owing to the presence of repeats, sequencing of these regions is quite problematic, with only short stretches of sequence obtained for some primers. We examined the IGS1 region and found several restriction enzymes that cleave within IGS1 and are invariant among all the isolates, as well as a few enzymes that can be used to differentiate between the strains. The enzyme *Bam*HI, which cuts twice, at positions 548 and 636 (with respect to MTCC 237^T), *Eco*RV, which cuts at 940, and *Hin*cII, which cuts at 940, are invariant among all the strains tested, while *Bal*I (or its isoschizomers *Msc*I and *Mlu*NI) can be used to differentiate MTCC 462, while *Tsp*RI can be exploited for checking variation between the three clinical isolates and MTCC 237^T, since the enzyme cuts once in the latter strain and in MTCC 3815 but twice in the remaining strains. Strain MTCC 3815 could likewise be differentiated from the other strains by *Bst*EII, *Bsp*MI and *Dra*I, which cut several times in MTCC 3815 but only once in the other strains.

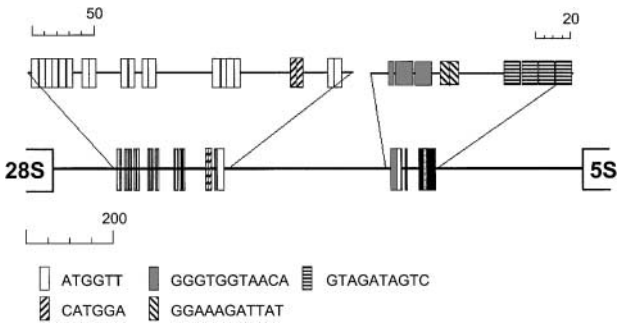

Fig. 1. Schematic representation of repeat regions of *P. anomala* MTCC 237^T (= NCYC 1509^T).

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83  GGGTAAAC--------GCCTATGGCACAACCATGGTTGTAGTT-TAACCATGGG--TGGTCATGA------------ 136
137 GGGTAAAT--------GGTTATGGTTGTGG-ATGGTGGTGGTGATGGTGGTGGATATGGTTATGGTTTAACCATGGAT 205
206 GGGTAACCAT------GGTTGTGGTTAAAATATGGTTATGGTCAAAAGTTGAAGCGTGGTCGCGC------------ 264
265 GGGTAACTT-------GGTGATGGTTATGG-ATGGCGATGGTTAAAATTTTGG-CATGGTCATAA------------ 320
321 GGGTAAGTT-------GGTGATGGTTACAC-ATTGACATGGACATGGTCATTA------------------------ 365
366 GGGTAATTTCACAAATGGTTATGGTTTGACCATGGTCAACCACCAAAATCAAACCGTGGTCATGC------------ 430
431 GGGTAAATAG------CCATATGGTTACAACAAAAAAGTGAAAAAATCTACGTTTA--------------------- 480
    ******              ***       *
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Fig. 2. Multiple alignments of larger repeats of *P. anomala* MTCC 237^T (= NCYC 1509^T). Numbers indicate the start and end of the larger repeat in the IGS region sequence of MTCC 237^T. Identical bases are represented by asterisks (*) and missing bases are shown by dashes (-). Tandem repeats are represented alternatively in bold and in bold underlined italic type.

The possibility of using the repeat variation (length variation) in a PCR-based strategy was also examined; however, since the variation in repeats was seen only in the case of MTCC 462, which had a single insertion of GATGGT in the repeat region, it did not appear to be a very convenient method of evaluating differences between the strains.

The availability of the complete IGS1 sequences of seven strains of *P. anomala* enabled us to carry out several comparisons and analyses that are not possible with incomplete sequences of these regions. Only for *Trichosporon* species has a large number of strains/species been completely characterized in their IGS1 regions, and this was made easier in *Trichosporon* by the shorter IGS regions of *Trichosporon* species (195–719 bp), compared with >1200 bp for the IGS1 region alone of *P. anomala* strains. In fact, to our knowledge, such a large number of complete IGS1 sequences (of size >1 kb) from a single species has not been reported so far from any organism. We have used the complete IGS1 sequences of these strains to throw light on how the region compares with or complements the ITS region in terms of both species and strain differentiation. Analysis of the IGS1 sequence clearly revealed more variation in these regions than is seen in the ITS (depicted schematically in Fig. 3). Thus, despite the difficulties encountered in obtaining full IGS1 sequences, it would be important to consider these sequences when analysing different strains. We discuss below how these IGS1 sequences have, even in the present study, thrown greater light on the analysis and conclusions based on ITS sequences (either lack of variation or excessive variation in the ITS region) and how they can help to resolve ambiguous situations in specific instances.

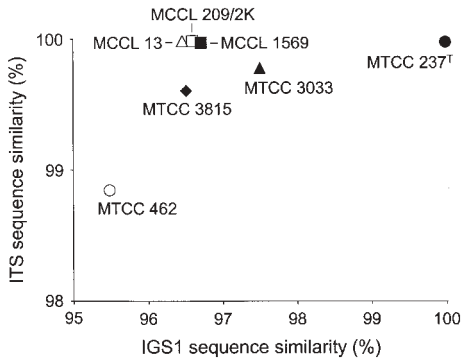

Fig. 3. Schematic/graphical representation showing sequence similarities between ITS and IGS1 regions, showing percentage similarity of clinical isolates and non-clinical isolates to MTCC 237^T (= NCYC 1509^T) in pairwise alignment.

In the first example from our study, the three clinical strains and the standard strain, MTCC 237^T (geographically distinct, non-clinical), were identical in the ITS regions. However, the IGS1 region clearly resolved that strain MTCC 237^T was, in fact, different from the clinical strains.

In the second example from our study (dealing with an opposite situation; greater than normal variation in the ITS), we examined the case of strain MTCC 462, which showed large variation in the ITS region (>1 %). The variation was large enough for MTCC 462 to be considered seriously as a member of a different species (as opposed to being merely a different strain). We decided to examine this with the IGS1 data and have used the conclusions reached in a study carried out on the complete IGS regions in *Trichosporon* species in our analysis (Sugita *et al.*, 2002). It was observed in this study that, for species differentiation, the variation in the IGS1 region was at least 5 % (only 95 % identity). If we base our analysis of this strain on these findings, the IGS1 region of MTCC 462 showed 96 % identity (or only 4 % variation). This 4 % variation was not large enough for the strain to be classified as a novel species. In other words, greater variation was observed in IGS1 compared with ITS, but it was not yet sufficient to be classified as a novel species. On the basis of the IGS1 region, therefore, MTCC 462 is likely to be *P. anomala* (and not a different species, as might be suggested from ITS region data). In phenotypic characters (biochemical and morphological study), strain MTCC 462 is in fact identical to the standard *P. anomala* strain MTCC 237^T (data not shown).

Despite the greater variation seen in the IGS1 regions, it is interesting to observe that all the clinical isolates were identical in the IGS1 region, even though one strain was isolated during the post-outbreak period (2 years after the outbreak). This strongly suggests that the same strain is persisting in the hospital environment even after the apparent control of the outbreak.

In conclusion, the present study emphasizes the need to go beyond the ITS region for strain differentiation in *P. anomala*. As a single locus, the IGS1 region has more discriminatory potential, due to the presence of multiple repeats and greater variation. These regions can potentially be used to differentiate *P. anomala* at the strain and even species level.

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REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

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

Chakrabarti, A., Singh, K., Narang, A. & 10 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.

Dutta, S. K. & Verma, M. (1990). Primary structure of the non-transcribed spacer region and flanking sequences of the ribosomal DNA of *Neurospora crassa* and comparison with other organisms. *Biochem Biophys Res Commun* **170**, 187–193.

Fan, M., Chen, L. C., Ragan, M. A., Gutell, R. R., Warner, J. R., Currie, B. P. & Casadevall, A. (1995). The 5S rRNA and the rRNA intergenic spacer of the two varieties of *Cryptococcus neoformans*. *J Med Vet Mycol* **33**, 215–221.

Ganley, A. R. D. & Scott, B. (1998). Extraordinary ribosomal spacer length heterogeneity in a neotyphodium endophyte hybrid: implications for concerted evolution. *Genetics* **150**, 1625–1637.

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). *Hansenula anomala* outbreak at a surgical intensive care unit: a search for risk factors. *Eur J Epidemiol* **17**, 491–496.

Klein, A. S., Tortora, G. T., Malowitz, R. & Greene, W. H. (1988). *Hansenula anomala*: a new fungal pathogen. Two case reports and a review of the literature. *Arch Intern Med* **148**, 1210–1213.

Kohli, D. K. & Bachhawat, A. K. (2003). CLOURE: Clustal Output Reformatter, a program for reformatting CLUSTAL X/CLUSTAL W outputs for use in SNP analysis and molecular systematics. *Nucleic Acids Res* **31**, 3501–3502.

Kurtzman, C. P. (1984). Synonomy of the yeast genera *Hansenula* and *Pichia* demonstrated through comparisons of deoxyribonucleic acid relatedness. *Antonie van Leeuwenhoek* **50**, 209–217.

Maiden, M. C., Bygraves, J. A., Feil, E. & 10 other authors (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* **95**, 3140–3145.

Molina, F. I., Jong, S. C. & Huffman, J. L. (1993). PCR amplification of the 3′ external transcribed and intergenic spacers of the ribosomal DNA repeat unit in three species of *Saccharomyces*. *FEMS Microbiol Lett* **108**, 259–263.

Murphy, N., Buchanan, C. R., Damjanovic, V., Whitaker, R., Hart, C. A. & Cooke, R. W. I. (1986). Infection and colonisation of neonates by *Hansenula anomala*. *Lancet* i, 291–293.

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

Nohinek, B., Zee-Cheng, C. S., Barnes, W. G., Dall, L. & Gibbs, H. R. (1987). Infective endocarditis of bicuspid aortic valve caused by *Hansenula anomala*. *Am J Med* **82**, 165–168.

Qadri, S. M. H., Al Dayal, F., Strampfer, M. J. & Cunha, B. A. (1988). Urinary tract infection caused by *Hansenula anomala*. *Mycopathologia* **104**, 99–101.

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

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.

Thuler, 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.

Urwin, R. & Maiden, M. C. J. (2003). Multi-locus sequence typing: a tool for global epidemiology. *Trends Microbiol* **11**, 479–487.

White, T., Burns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal rRNA genes for phylogenetics. In *PCR Protocols, a Guide to Methods and Applications*. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press.