Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences

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The phylogenetic position of *Candida dubliniensis* has previously been established on the basis of the sequence of rRNA genes. In order to confirm the relationship between *C. dubliniensis* and other yeast species, particularly *Candida albicans*, using non-rRNA gene sequences the *ACT1* gene was chosen for analysis. Three overlapping fragments that together span the entire *C. dubliniensis ACT1* gene (*CdACT1*) were amplified from a recombinant phage isolated from a genomic DNA λ library using PCR. These were cloned and used to determine the contiguous sequence of the gene. Analysis of the sequence data revealed the presence of a 1131 bp ORF interrupted by a single 632 bp intron at the 5' extremity of the gene. Comparison of the *CdACT1* sequence with the *C. albicans* homologue (*CaACT1*) revealed that although the exons are 97.9% identical the introns are only 83.4% identical. Phylogenetic trees generated using *ACT1* exon and intron sequences from a range of yeast species unequivocally confirmed the phylogenetic position of *C. dubliniensis* as a unique taxon within the genus *Candida*. Analysis of the *ACT1*-associated intron sequences from 10 epidemiologically unrelated *C. dubliniensis* isolates from disparate geographical locations showed a very low level of intraspecies sequence variation. In order to develop an accurate and rapid method to identify *C. dubliniensis* from primary isolation plates the significant divergence between the *C. dubliniensis* and *C. albicans ACT1* intron sequences was exploited by designing *C. dubliniensis*-specific PCR primers. Using a rapid boiling method to produce template DNA directly from colonies from primary isolation plates in 10 min, these primers were used in a blind test with 122 isolates of *C. dubliniensis*, 53 isolates of *C. albicans*, 10 isolates of *C. stellatoidea* and representative isolates of other clinically relevant *Candida* and other yeast species. Only the *C. dubliniensis* isolates yielded the *C. dubliniensis*-specific 288 bp amplicon. Use of this technique on colonies suspected to be *C. dubliniensis* allows their correct identification as *C. dubliniensis* in as little as 4 h.

Keywords: *ACT1*, phylogenetics, *Candida dubliniensis*, PCR identification

INTRODUCTION

Candida dubliniensis is a yeast species first described in 1995 (Sullivan *et al.*, 1995). Although *C. dubliniensis* is

Abbreviations: HIV, human immunodeficiency virus.

The EMBL accession numbers for the nucleotide sequences reported in this paper are AJ236897 (*Candida dubliniensis* CD36), AJ237918 (*Candida tropicalis* NCPF 3111) and AJ237919 (*Candida stellatoidea* ATCC 11006).

phenotypically similar to *C. albicans*, the two species differ significantly at the genetic level. In particular, phylogenetic analysis of large- and small-subunit rRNA gene sequences provided the basis for the designation of *C. dubliniensis* as a separate species (Sullivan *et al.*, 1995, 1997; Gilfillan *et al.*, 1998). The first isolates identified as *C. dubliniensis* were recovered from the oral cavities of Irish human immunodeficiency virus (HIV)-infected individuals. However, over the last three

years there have been increasing numbers of reports of the recovery of *C. dubliniensis* isolates by laboratories throughout the world, including Europe, North and South America, and Australia (Coleman *et al.*, 1997b; Sullivan *et al.*, 1997; Sullivan & Coleman, 1998; Salkin *et al.*, 1998; Kirkpatrick *et al.*, 1998). Although the majority of these isolates have been primarily associated with oral candidosis in HIV-infected and AIDS patients (Coleman *et al.*, 1997a), *C. dubliniensis* isolates have also been recovered from the oral cavities, gastrointestinal tracts and vaginas of HIV-negative individuals (Moran *et al.*, 1997, 1998; Sullivan & Coleman, 1998; Odds *et al.*, 1998). There have also been reports of *C. dubliniensis* isolates associated with systemic disease (Pinjon *et al.*, 1998; Meis *et al.*, 1999). The majority of clinical isolates of *C. dubliniensis* have been shown to be susceptible to commonly used antifungal drugs, including fluconazole (Moran *et al.*, 1997, 1998; Kirkpatrick *et al.*, 1998; Pfaller *et al.*, 1999). However, fluconazole resistance has been detected in clinical isolates (Moran *et al.*, 1997, 1998; Kirkpatrick *et al.*, 1998; Pfaller *et al.*, 1999) and isolates of *C. dubliniensis* susceptible to fluconazole can be readily induced to produce fluconazole-resistant derivatives following exposure to the drug *in vitro* (Moran *et al.*, 1997, 1998).

The prevalence of *C. dubliniensis* in the oral cavities of HIV-infected individuals and AIDS patients and reports of its association with disease in other body sites warrant in-depth epidemiological analysis. However, these investigations have been hampered by the lack of a simple, reliable method capable of unequivocally differentiating between *C. dubliniensis* and *C. albicans* in the clinical laboratory. Indeed, since *C. dubliniensis* and *C. albicans* share the ability to produce germ tubes and chlamydo-spores, features previously used for the definitive identification of *C. albicans*, it is likely that many isolates of *C. dubliniensis* have been misidentified as *C. albicans*. Investigations of our own collection of stored oral *Candida* isolates, originally identified as *C. albicans*, have shown that 1.8% of isolates recovered from asymptomatic normal healthy individuals and 16.5% of isolates recovered from HIV-infected individuals were in fact *C. dubliniensis* (Coleman *et al.*, 1997a). In a similar study, Odds *et al.* (1998) have recently shown that approximately 2% of a stored archival culture collection of 2500 yeast isolates, originally identified as *C. albicans*, was *C. dubliniensis*. They found that the prevalence of *C. dubliniensis* was significantly higher among HIV-infected individuals than among HIV-negative individuals (Odds *et al.*, 1998). Although first described in 1995, the earliest known *C. dubliniensis* isolates were recovered in the 1950s, thus predating the HIV pandemic. One of these strains, NCPF 3108, was recovered in the UK in 1957 and was originally deposited in the British National Collection for Pathogenic Fungi as *C. stellatoidea* (Sullivan *et al.*, 1995), while another strain, CBS 2747, which was recovered in the Netherlands in 1952, was originally deposited in the Centraal Bureau fur Schimmelcultures as *C. albicans* (Meis *et al.*, 1999).

A variety of tests have been developed to discriminate between *C. dubliniensis* and *C. albicans* based upon phenotypic characteristics. These include carbohydrate assimilation profiles and colonial coloration on differential media such as CHROMagar Candida and methyl blue-Sabouraud agar (Sullivan *et al.*, 1995, 1997; Coleman *et al.*, 1997a; Schoofs *et al.*, 1997). However, some of these assays have been shown to be unreliable in some instances and should only be used for the presumptive identification of *C. dubliniensis* from clinical specimens (Schoofs *et al.*, 1997; Sullivan & Coleman, 1998; Kirkpatrick *et al.*, 1998). The accuracy of *C. dubliniensis* isolate identification based on carbohydrate assimilation profiles has been improved by the recent inclusion of the assimilation profiles of some *C. dubliniensis* strains in the databases of commercially available yeast identification systems, including the API ID 32C and 20C AUX systems. It has been reported recently that *C. dubliniensis* and *C. albicans* can be distinguished on the basis of differential growth at 45 °C, with isolates of the former species unable to grow at this temperature (Pinjon *et al.*, 1998). However, in a recent study a significant number of *C. albicans* isolates were found to be unable to grow at this temperature (Kirkpatrick *et al.*, 1998). Currently, the most reliable tests available to differentiate between these species are based on molecular techniques such as DNA fingerprinting with repetitive-sequence-containing probes, randomly amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis (Sullivan *et al.*, 1995), but these are not suitable for the analysis of large sample numbers in routine diagnostic laboratories. However, since the differences between *C. dubliniensis* and *C. albicans* are most pronounced at the genetic level such differences should provide the basis for a specific and rapid identification test. One molecular technique with the required degree of specificity and ease of use is the polymerase chain reaction (PCR). This technology is increasingly available in diagnostic laboratories and due to its speed, reproducibility and high sample volume throughput is ideally suited for application to large numbers of clinical isolates.

The phylogenetic position of *C. dubliniensis* in relation to other yeast species has been established on the basis of the comparison of small- and large-subunit rRNA gene sequences (Sullivan *et al.*, 1995, 1997; Gilfillan *et al.*, 1998). In the present study we sought to confirm these phylogenetic relationships using sequences of non-rRNA gene origin. It was also hoped that these sequence data would lead to the identification of *C. dubliniensis*-specific nucleotide sequences that could be exploited in the design of a rapid PCR-based identification test. To achieve these goals the *ACT1* gene of *C. dubliniensis* was chosen for analysis. *ACT1* encodes actin, a protein that is abundant in all eukaryotic cells, where it is the major component of cytoplasmic microfilaments. Due to structural constraints the amino acid sequence of actin proteins from different eukaryotic species is highly conserved (Korn *et al.*, 1978; Hightower *et al.*, 1986; Pollard *et al.*, 1990; Hennessey *et al.*, 1993; Welch *et al.*,

1994). Since *C. albicans* and *C. dubliniensis* are very closely related it was anticipated that the *ACT1* genes of these species would be very similar. Results presented in this study for *C. dubliniensis* and in a previous study for *C. albicans* (Losberger & Ernst, 1989) showed that both *ACT1* genes contain a single class IV intron and it was anticipated that these intron sequences would be subject to less evolutionary conservation than the actin-protein-coding exons. Therefore we decided to investigate whether the exons and introns of *C. albicans* and *C. dubliniensis* would be sufficiently divergent to allow an accurate determination of the phylogenetic relationship between the two species and to allow the design of *C. dubliniensis*-specific primers suitable for rapid and specific identification of this species in the clinical laboratory using a rapid template DNA preparation procedure.

METHODS

Candida strains and culture media. All *C. dubliniensis* strains were isolated by this laboratory or received from other laboratories and identified using the molecular and phenotypic methods described by Sullivan *et al.* (1995). All *Candida* strains and isolates were routinely grown on Potato Dextrose Agar (PDA, Oxoid) at pH 5.6 for 48 h at 37 °C. For liquid culture, *Candida* strains and isolates were grown at 37 °C in Yeast Peptone Dextrose Broth (YPD) in an orbital incubator (Gallenkamp) set at 150 r.p.m.

Bacterial strains and culture media. *Escherichia coli* DH5 α was used as the host strain for phagemid pBluescript II KS(+/–) (Stratagene) and was maintained on Luria–Bertani (LB) agar, supplemented with 100 μ g ampicillin ml^{–1} to maintain plasmids where appropriate. For liquid culture, strains harbouring plasmids were grown at 37 °C in LB broth containing 100 μ g ampicillin ml^{–1} in an orbital incubator set at

150 r.p.m. Transformation of *E. coli* DH5 α and identification of transformants containing recombinant plasmids were carried out by standard protocols (Sambrook *et al.*, 1989). *E. coli* LE 392 and its P2 phage lysogenic derivative (P2 392) were used for propagating the bacteriophage λ cloning vector EMBL3 and its recombinant derivatives. These strains were grown and maintained on LB agar supplemented with 10 mM MgSO₄ and 0.2% (w/v) maltose. Organisms for phage infection were grown in LB broth containing 10 mM MgSO₄ and 0.2% (w/v) maltose (Sambrook *et al.*, 1989).

Chemicals, enzymes, radioisotopes and oligonucleotides.

Analytical-grade or molecular-biology-grade chemicals were purchased from Sigma-Aldrich, BDH or Boehringer Mannheim. Enzymes were purchased from Promega or Boehringer Mannheim and used according to the manufacturer's instructions. [α -³²P]dATP (3000 Ci mmol^{–1}; 110 TBq mmol^{–1}) was purchased from Amersham. Custom-synthesized oligonucleotides were purchased from Genosys Biotechnologies (Europe).

DNA extraction procedures. Plasmid DNA for restriction endonuclease digestion and Southern hybridization was prepared by the alkaline lysis method described by Sambrook *et al.* (1989). Plasmid DNA for sequencing was prepared using a Quantum Prep Plasmid Miniprep kit (Bio-Rad). Total cellular DNA from *Candida* isolates was prepared as described by Gallagher *et al.* (1992). High-molecular-mass total cellular DNA from *C. dubliniensis* for the construction of a genomic library was isolated by the method described by Bennett *et al.* (1998). *Candida* template DNA for use in PCR experiments with the *C. dubliniensis*-specific oligonucleotide primer pair DUBF/DUBR (Table 1) was prepared as follows. A single colony from a culture grown for 48 h at 37 °C on PDA or CHROMagar *Candida* medium (CHROMagar *Candida*, Paris, France) was suspended in 50 μ l sterile distilled water. Cell suspensions were boiled for 10 min and the lysed cells subjected to a clearing spin for 5 min at 20000 g. Template DNA contained in 25 μ l supernatant was used for PCR amplification.

Table 1. PCR primers used in this study

Primer	Sequence	Nucleotide co-ordinates*	RE site†
<i>C. albicans</i>			
5'F	5'-CGGAATTCCTTAGAAACATTATCTCGAT-3'	–49 to –30	<i>Eco</i> RI
5'R	5'-GCTCTAGAGAGAAATATTATGTCGACAA-3'	126 to 145	<i>Xba</i> I
ACTF	5'-CGGAATTC AATGGACGGTGGTATGTT-3'	–1 to 17	<i>Eco</i> RI
ACTR	5'-CGGAATTC AATGGATGGACAGATTTCGTCG-3'	1746 to 1767	<i>Eco</i> RI
3'F	5'-CGGAATTC TAAGATTATTGCTCCACCAG-3'	1641 to 1660	<i>Eco</i> RI
3'R	5'-GCTCTAGACCAGATTTCAGAAATTTTCAC-3'	1792 to 1811	<i>Xba</i> I
INTF	5'-CGGAATTC AATGGACGGTGGTATGGT-3'	–1 to 17	<i>Eco</i> RI
INTR	5'-CGGAATTC GAGCGTCGTCACCGGC-3'	724 to 739	<i>Eco</i> RI
<i>C. dubliniensis</i>			
DUBF	5'-GTATTTGTCGTTCCCTTTTC-3'	251 to 270	
DUBR	5'-GTGTTGTGTGCACTAACGTC-3'	519 to 538	

* Primers were complementary to *ACT1* gene sequences as follows: *CaACT1*, accession no. X16377 (Losberger & Ernst, 1989); and *CdACT1*, accession no. AJ236897 (this study). Nucleotide co-ordinates shown are numbered in the 5' to 3' direction with the first base of the translation start codon being +1.

† Restriction endonuclease recognition sequence included within the primer sequence (underlined).

Fig. 1. Schematic diagram of the *C. dubliniensis* *ACT1* gene (*CdACT1*), which is represented by the large horizontal box. The black area corresponds to the position of the intron at the 5' end of the gene. The thin horizontal lines in the lower part of the figure represent sequences amplified from the *CdACT1*-encoding recombinant phage λ CDACT1 using the primer pairs 5'F/5'R, ACTF/ACTR and 3'F/3'R (Table 1). The names of the recombinant plasmids obtained when these amplimers were cloned in pBluescript are shown on the right. The location of sequences amplified with the *C. dubliniensis*-specific primer pair DUBF/DUBR is indicated by the shaded box. The nucleotide coordinates of the sequences contained in each amplimer relative to *CdACT1* sequences are shown at the top (numbering the *CdACT1* ATG start codon + 1).

Large-scale *E. coli* phage lysates were prepared according to the plate method of Sambrook *et al.* (1989) and recombinant phage DNA was purified from phage preparations using a Wizard Lambda Preps kit (Promega).

Construction of a *C. dubliniensis* CD36 genomic DNA library.

The DNA library was constructed by ligating *Sau*3A-generated partial digestion products of *C. dubliniensis* CD36 chromosomal DNA >10 kb in size with *Bam*HI-generated pre-prepared λ bacteriophage replacement vector EMBL3 arms (Promega) followed by packaging *in vitro* into pre-prepared phage heads and tails (Promega) according to the manufacturer's instructions. Previous studies have shown that DNA fragments ranging in size from 9 to 23 kb can be cloned into the EMBL3 vector (Frischauf *et al.*, 1983). The packaged recombinant phage particles were propagated on the *E. coli* lysogenic strain P2 392. A recombinant library containing 2.0×10^5 p.f.u. was obtained.

Recombinant phages were propagated on *E. coli* LE 392 to yield ~1000 p.f.u. per 90 mm Petri plate and were transferred from the plaques onto nitrocellulose membrane filters (Schleicher & Schuell) by overlaying the plaques with the filters, which were then screened by plaque hybridization (Sambrook *et al.*, 1989) using α - 32 P-labelled DNA probes.

DNA hybridization. Probe DNA used in screening the *C. dubliniensis* genomic DNA library and in Southern hybridization experiments was labelled with [α - 32 P]dATP (3000 Ci mmol $^{-1}$; 110 TBq mmol $^{-1}$) by random priming with a random hexanucleotide primer labelling kit (Prime-a-Gene, Promega). All hybridizations were performed under conditions of high stringency (65 °C), as described by Sambrook *et al.* (1989).

PCR isolation of *ACT1*-associated introns. Amplification of *ACT1*-associated intron sequences from *Candida* strains was performed in 100 μ l reaction mixtures containing 100 pmol each of the forward and reverse primers, INTF/INTR (Table 1), 250 μ M deoxynucleotide triphosphates, 2.0 mM MgSO $_4$, 20 mM Tris/HCl (pH 8.8 at 25 °C), 10 mM KCl, 10 mM (NH $_4$) $_2$ SO $_4$, 0.1% (v/v) Triton X-100, 1 U Vent $_R$ DNA polymerase (New England Biolabs) and 500 ng template DNA. PCR reactions were performed in a DNA Thermal Cycler

Ca	GTATG TTTAAATTTAGCTTCAATTCTAATTGATTGATTAATCAGTTGATTGGTTTCAATA
Cd	GTATG TTTATATTTAACTTAGATT-TAATTGATTGATTAATCAGTTGGATTTCATTT
Ca	TGACAAA-----TGGGTAG-----GGTGGGAAAACCTCAT-TTTCAATTGAGATCAAA
Cd	CGATAGAGTGTGTTGTTTGTAGCTGGGTGGGAAAAGAACCCATTTCATTGAGATCAAG
Ca	CTTTTTTGTGTCGACATAATATTTCTCGTTGGGATGTTACTGTGCACATTAATAATACA
Cd	-TTTTTGTGTCGACATAATATTTCTCGTTGGGATGTTACTGTGCACATTA-----ACA
Ca	CACACATCAGCTTATAATTTTGAAGTAATTTATCAGATATGTTGTGACGATCAATGGAA
Cd	CACA---AGCTTATAATTTTGAA-GTGGTACATCAGG--AGTT-TGACTACCATGGAT
Ca	ATGGCTAACTCAATGATCTGTTCTTCCCTTTTCAAAGTTCACGTTT---TTTGATT
Cd	GTGTCCAAATTTAGT GTATTTGTCGTTCCCTTTC ---AATTTCGTGTTAAGTTTAATT
Ca	GATTGATTGATTGATCTGTGCGGAGTGGTTTCAAAACCATTCGGTGAGTAATCCTATCAA
Cd	GATTGATTGATTGATCTGTGCGCAATGGTTTCAAA-CCATTCGGTGAGTAATATCATTTGA
Ca	TCAATGTACGACAAAAGGCTCAATATTCAAATTTGAATGTTTATGTTTTCCTACGTG
Cd	TCAA--TTAAAAACAAGGTTTAATACTTCAA-TGACAAATGTTTAATGTTTTCACAAAG
Ca	TACTTGTGCAAGGCAATTGATTCAACATTGCTTTGGTGTGTTGACGAGTTTCTAGTTTGG
Cd	CGTTTGTGCAAAATCAATTGATTGATTGCTTTGATGTT-GACGAGTTTCAATTTTCG
Ca	ACTTGTGTTGTTATCTGGACTATA-CAGATTTCCCGGCTCACTATGAATTTTTTTTCG
Cd	AGTTTCG--GTTATCTGACCTATAACAGATTTC--GGTTCATTGTAAT-----TTTTCG
Ca	ACGCTCAGTGCACACAACATAAAACACAAACACAAACAGCAAGAAAAA
Cd	ACG-TTAGTGCACACAAC -----ACACAA---CAAAACAGCAACAAAAA
Ca	GAACATTGAATTGAAACCAAGCCAACTGAAA-AATTCC-----TTATTTAAATGACTGT
Cd	-AATATTGTATTGAAACCAA--CAACTGCAACAGTCCCTTTTATTTTAAATGACTGT
Ca	CATACTAACCCATTTT-ATAG
Cd	CGTACTAACCCATTTTATAG

Fig. 2. Comparison of nucleotide sequences of the *C. albicans* and *C. dubliniensis* *ACT1*-associated introns. The *C. albicans* sequence was from strain ATCC 10123 (GenBank accession no. X16377; Losberger & Ernst, 1989) and the *C. dubliniensis* sequence from strain CD36 (EMBL accession no. AJ236897; this study). The 5' intron consensus sequence (GTATG), the 3' intron consensus sequence (YAG) and the branchpoint sequence (TACTAAC) are in bold type. Asterisks indicate identical nucleotides and dashes indicate deletions. Sequences of the *C. dubliniensis*-specific primers DUBF/DUBR (Table 1) are underlined.

(Perkin Elmer Cetus) with 30 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C, followed by 72 °C for 10 min. The amplimers were purified using the Wizard PCR Preps DNA Purification system (Promega) and cloned into pBluescript using standard procedures (Sambrook *et al.*, 1989).

DNA sequencing. This was performed by the dideoxy chain-terminating method of Sanger *et al.* (1977) using an automated Applied Biosystems 370A DNA sequencer and dye-labelled terminators (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit, Applied Biosystems). Searches of the GenBank database for nucleotide sequence similarities were performed using the BLAST family of computer programs (Altschul *et al.*, 1990). Nucleotide sequence alignments were carried out using the CLUSTAL W sequence analysis program (Thompson *et al.*, 1994).

PCR identification of *C. dubliniensis*. PCR identification of *C. dubliniensis* using the *C. dubliniensis*-specific primer pair DUBF/DUBR (Table 1) was carried out in a 50 μ l final volume containing 10 pmol each of the forward and reverse primers, 2.5 mM MgCl $_2$, 10 mM Tris/HCl (pH 9.0 at 25 °C), 10 mM KCl, 0.1% (v/v) Triton X-100, 2.5 U *Taq* DNA polymerase

(Promega) and 25 µl template-DNA-containing cell supernatant (prepared as described above). Each reaction mixture also contained 10 pmol each of the universal fungal primers RNAF/RNAR (Fell, 1993), which amplify approximately 610 bp from all fungal large-subunit rRNA genes and were used as an internal positive control. Cycling conditions consisted of 6 min at 95 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, followed by 72 °C for 10 min. Amplification products were separated by electrophoresis through 2.0% (w/v) agarose gels containing 0.5 µg ethidium bromide ml⁻¹ and were visualized on a UV transilluminator.

RESULTS

Isolation and sequence analysis of the *C. dubliniensis* ACT1 gene

A *C. dubliniensis* CD36 genomic library, constructed in bacteriophage λ EMBL3, was screened by plaque hybridization with a radioactively labelled probe consisting of the entire *C. albicans* ACT1 (*CaACT1*) gene cloned into

pBR322 (p1002, a gift from B. Magee, University of Minnesota). Approximately 10000 recombinant plaques were screened, from which 10 p1002-reactive plaques were detected. The plaque with the strongest signal was selected and subjected to single-plaque purification. DNA was extracted from the recombinant phage, termed λCDACT1, and restriction endonuclease digestion analysis and Southern hybridization analysis showed that λCDACT1 contained a DNA insert of approximately 15 kb. Attempts to subclone smaller hybridizing fragments from the cloned insert DNA of λCDACT1 into the vector phagemid pBluescript, to facilitate the sequencing of the *C. dubliniensis* ACT1 (*CdACT1*) gene, failed. A number of p1002-reactive recombinant plasmids were obtained. However, upon further investigation these were shown to contain both ACT1 and EMBL3 vector homologous sequences, and no recombinant plasmids harbouring only ACT1-homologous DNA were obtained. It was concluded that the ACT1-homologous insert DNA from λCDACT1 was

Table 2. Yeast species and strains used in the phylogenetic analysis of *C. dubliniensis*

Yeast strain*	ACT1 intron sequence†	Reference
<i>C. albicans</i>		
132A	This study	Gallagher <i>et al.</i> (1992)
179B	This study	Gallagher <i>et al.</i> (1992)
ATCC 10123	X16377	Losberger & Ernst (1989)
<i>C. dubliniensis</i>		
CD36 (Ireland)	AJ236897; this study	Sullivan <i>et al.</i> (1995)
CD91 (Ireland)	This study	This study
CD70 (UK)	This study	Sullivan <i>et al.</i> (1997)
NCPF 3108 (UK)	This study	Sullivan <i>et al.</i> (1995)
CD93 (Finland)	This study	This study
94191 (Spain)	This study	Pinjon <i>et al.</i> (1998)
P2 (Switzerland)	This study	Boerlin <i>et al.</i> (1995)
CD71 (Argentina)	This study	Sullivan <i>et al.</i> (1997)
CM2 (Australia)	This study	Sullivan <i>et al.</i> (1995)
CD92 (Canada)	This study	This study
<i>C. glabrata</i>		
ATCC 90876	AF069746	Unpublished data submitted to GenBank
<i>C. stellatoidea</i>		
ATCC 11006	AJ237919; this study	Kwon-Chung <i>et al.</i> (1989)
303530	This study	bioMérieux‡
303531	This study	bioMérieux‡
<i>C. tropicalis</i>		
NCPF 3111	AJ237918; this study	NCPF catalogue
<i>K. lactis</i>		
J7	M25826	Deshler <i>et al.</i> (1989)
<i>S. cerevisiae</i>		
A364A	L00026	Gallwitz & Sures (1980)

* Abbreviations: ATCC, American Type Culture Collection, (Manassas, VA, USA); NCPF, National Collection of Pathogenic Fungi, Bristol, UK. The country of origin of the *C. dubliniensis* isolates is shown in parentheses.

† Accession numbers are for the EMBL/GenBank nucleotide sequence databases.

‡ From the culture collection of bioMérieux, St Louis, MO, USA.

unstable when cloned in pBluescript. Therefore it was decided to amplify *CdACT1* from the recombinant phage by PCR using a mixture containing *Taq* polymerase and the proof-reading polymerase *Pwo* (Expand high-fidelity PCR system, Boehringer) and three primer sets homologous to regions of the *CaACT1* gene and flanking sequences, including 5'F/5'R, ACTF/ACTR and 3'F/3'R (Table 1, Fig. 1). The three amplimers, containing overlapping sequences, obtained following PCR with these primers were cloned into pBluescript to yield recombinant plasmids p5, pACT and p3, respectively (Fig. 1). The insert DNA cloned in p5 and p3 was sequenced fully in both directions using universal primers, while the insert DNA cloned in pACT was sequenced fully by primer walking. These three overlapping sequences yielded a contiguous sequence of 1827 bp revealing an ORF of 1131 bp interrupted by a single 632 bp intron at the 5' end (Fig. 1). The overall nucleotide sequence identity between this ORF (*CdACT1*) and the *CaACT1* gene was 90.6%. This divergence is mainly due to differences between the intron sequences, which are 83.4% identical (Fig. 2), while the spliced coding sequences, which are identical in length in both species, are 97.9% identical. The differences between the exon sequences correspond to a total of 24 base changes. However, only one of these base substitutions [A → G, at position 660, numbering the sequences in the 5'–3' direction from the first base (+1) of the translation start codon of *CdACT1*], results in a change in the predicted amino acid sequence, a conservative substitution from isoleucine to valine. At 632 bp the *CdACT1* intron is 25 bp shorter than the corresponding sequence in *CaACT1*; however, it is situated in exactly the same position at the 5' end of the gene and is recognizable by the presence of yeast intron consensus sequences (Fig. 2). These are the 5' consensus sequence GTATG, the 3' consensus sequence YAG, and the branchpoint sequence TACTAAC located near the 3' end of the intron which is essential for efficient splicing (Mount, 1982; Langford *et al.*, 1984; Leer *et al.*, 1984; Molenaar *et al.*, 1984; Teem *et al.*, 1984).

In order to determine the level of intraspecies intron sequence conservation in epidemiologically unrelated isolates from geographically divergent parts of the world the introns of nine additional *C. dubliniensis* isolates, two additional *C. albicans* isolates (132A and 179B) and three *C. stellatoidea* isolates (Table 2) were amplified using the primer set INTF/INTR, which were complementary to *CaACT1* sequences flanking the intron (Table 1). The intron sequences of the 10 *C. dubliniensis* isolates tested, including CD36 (Table 2), were found to be very highly conserved, with only one or two base changes found within each isolate. Similar intraspecies sequence conservation was observed with the *C. albicans* and *C. stellatoidea* strains studied.

Phylogenetic analysis based on *ACT1* sequences

The *ACT1* gene has been used extensively to infer interspecies relationships across broad evolutionary distances (Zakut *et al.*, 1982; Mertins & Gallwitz, 1987;

Table 3. Genetic distance matrix based on comparison of *ACT1* gene coding sequences

Values correspond to percentages of difference corrected for multiple base changes by the method of Jukes & Cantor (1969). The *ACT1* gene coding sequences used were as follows: *C. albicans* ATCC 10123 (X16377; Losberger & Ernst, 1989); *C. dubliniensis* CD36 (AJ236897; this study); *S. cerevisiae* A364A (L00026; Gallwitz & Sures, 1980); *C. glabrata* NCPF 90876 (AF069746; O. Kurzai and others, unpublished data) and *K. lactis* J7 (M25826; Deshler *et al.*, 1989). Neither the *C. tropicalis* nor the *C. stellatoidea* *ACT1* coding sequences are currently available in the databases, so they could not be compared with the sequences of the other yeast species used to construct the matrix.

	<i>C. al.</i>	<i>C. du.</i>	<i>S. ce.</i>	<i>C. gl.</i>	<i>K. la.</i>
<i>C. albicans</i>	–				
<i>C. dubliniensis</i>	2.1	–			
<i>S. cerevisiae</i>	12.9	12.6	–		
<i>C. glabrata</i>	13.2	12.3	9.8	–	
<i>K. lactis</i>	13.2	12.6	10.1	7.8	–

Wildeman *et al.*, 1988; Fletcher *et al.*, 1994; Cox *et al.*, 1995; Wery *et al.*, 1996). This part of the study was undertaken to confirm the phylogenetic position of *C. dubliniensis* in relation to other yeast species using *ACT1* sequences. This is the first time that the phylogeny of *C. dubliniensis* has been investigated using non-rRNA gene sequences. Since the *ACT1* gene of many yeast species contains highly conserved (i.e. exon) and less well-conserved (i.e. intron) sequences, these regions were compared separately. Firstly, the *ACT1* spliced coding sequences of *C. albicans*, *C. dubliniensis*, *C. glabrata*, *Kluyveromyces lactis* and *Saccharomyces cerevisiae*, obtained in this study or from the databases (Table 3), were compared using the CLUSTAL W sequence alignment software package. Secondly, the *ACT1*-associated intron sequences from selected strains of *C. albicans*, *C. dubliniensis*, *C. stellatoidea*, *C. tropicalis*, *C. glabrata* and *K. lactis* (Table 4) were obtained either from GenBank or following amplification using the INTF/INTR primer set (Table 1) and also compared using CLUSTAL W. An evolutionary distance matrix for each group of sequences was generated incorporating corrections for multiple base changes according to the method of Jukes & Cantor (1969) (Tables 3 and 4). These data indicated that the *C. dubliniensis* coding and intron sequences differ from the corresponding *C. albicans* sequences by 2.1% and 16.6%, respectively. Evolutionary trees constructed using the neighbour-joining method of Saitou & Nei (1987) based on these data are shown in Fig. 3. These trees and the bootstrap values determined for each node unequivocally confirmed the unique species designation of *C. dubliniensis* and its phylogenetic position in relation to the other yeast species, including *C. albicans*. In addition, these data also confirm that *C. albicans* and *C. stellatoidea* are

Table 4. Genetic distance matrix based on comparison of ACT1-associated intron sequences

Values correspond to percentages of difference corrected for multiple base changes by the method of Jukes & Cantor (1969). The intron sequences used were as follows: *C. albicans* ATCC 10123 (X16377; Losberger & Ernst, 1989); *C. stellatoidea* ATCC 11006 (AJ237919, this study); *C. dubliniensis* CD36 (AJ236897; this study); *C. tropicalis* NCPF 3111 (AJ237918, this study); *C. glabrata* NCPF 90876 (AF069746; unpublished data submitted to GenBank) and *K. lactis* J7 (M25826; Deshler *et al.*, 1989). The sequence of the *S. cerevisiae* ACT1-associated intron (L00026; Gallwitz & Sures, 1980) was not included in the construction of the matrix because it was only 308 bp in length, significantly shorter than the intron sequences of the other yeasts studied, and so valid genetic distance determinations with this sequence and the others used to construct the matrix could not be made.

	<i>C. al.</i>	<i>C. st.</i>	<i>C. du.</i>	<i>C. tr.</i>	<i>C. gl.</i>	<i>K. la.</i>
<i>C. albicans</i>	—					
<i>C. stellatoidea</i>	0.2	—				
<i>C. dubliniensis</i>	16.6	16.6	—			
<i>C. tropicalis</i>	43.4	43.5	47.1	—		
<i>C. glabrata</i>	54.8	55.0	57.1	54.0	—	
<i>K. lactis</i>	58.1	58.3	54.7	61.4	63.1	—

Fig. 3. Unrooted phylogenetic neighbour-joining trees generated from the alignment of the ACT1-exon (a) and -intron (b) sequences of *C. dubliniensis* and other yeast species. Numbers at the nodes were generated by bootstrap analysis (Felsenstein, 1985) and represent the percentage of times the arrangement occurred in 1000 randomly generated trees. The sequences used to construct the trees are indicated in the legends to Tables 3 and 4.

so closely related as to be considered a single species (Kamiyama *et al.*, 1989; Sullivan *et al.*, 1995).

PCR-based identification of *C. dubliniensis*

Because of the many phenotypic similarities shared by *C. albicans* and *C. dubliniensis* it is not easy to discriminate between isolates of these species in the clinical laboratory. However, examination of an alignment of the ACT1-associated intron sequences of these two species (Fig. 2) and the observation that they differ by 16.6% (Table 4) suggested that this significant sequence divergence could be exploited as a means to identify *C. dubliniensis* accurately and rapidly in combination with a rapid template DNA preparation

procedure. PCR primers specific for the *C. dubliniensis* intron (DUBF/DUBR; Table 1, Fig. 1) were synthesized and used to amplify a DNA fragment of 288 bp from *C. dubliniensis* template DNA obtained by boiling cells from a single 48 h colony suspended in 50 µl water for 10 min. PCR reactions also contained the fungal universal primers RNAF/RNAR (Fell, 1993), which amplify a product of approximately 610 bp from the fungal large-subunit rRNA gene and serve as an internal positive control. While all fungal species should produce a product of approximately 610 bp with the RNAF/RNAR primers, only *C. dubliniensis* isolates should yield the 288 bp amplicon with the DUBF/DUBR primer set. The *C. dubliniensis*-specific primer pair DUBF/DUBR was tested in a blind trial using template DNA

Table 5. Yeast species used in PCR identification experiments with the *C. dubliniensis*-specific primers DUBF/DUBR

Species	No. of isolates	Reference(s)
<i>C. albicans</i>	53	This study Pinjon <i>et al.</i> (1998); Jabra-Rizk <i>et al.</i> (1999)
<i>C. dubliniensis</i> *	122	This study; Sullivan <i>et al.</i> (1995); Sullivan <i>et al.</i> (1997); Coleman <i>et al.</i> (1997a); Moran <i>et al.</i> (1997, 1998); Pinjon <i>et al.</i> (1998); Jabra-Rizk <i>et al.</i> (1999); Pujol <i>et al.</i> (1997)
<i>C. glabrata</i>	1	Haynes & Westerneng (1996)
<i>C. kefyr</i>	1	NCPF 3234
<i>C. krusei</i>	1	Haynes & Westerneng (1996)
<i>C. norvegensis</i>	1	NCPF 3860
<i>C. parapsilosis</i>	4	This study
<i>C. sake</i>	1	NCPF 8360
<i>C. stellatoidea</i>	1	ATCC 11006
<i>C. tropicalis</i>	9	This study
<i>T. beigelii</i>	1	NCPF 3111
	1	NCPF 3857

* One hundred and fourteen of the *C. dubliniensis* isolates were recovered from oral specimens, five were recovered from faecal specimens and one each was recovered from a vaginal, sputum and a post-mortem lung specimen. The isolates were recovered from different countries as follows: Argentina, 1 isolate; Australia, 2; Belgium, 5; Canada, 6; France, 4; Germany, 4; Greece, 1; Ireland, 48; Scandinavia, 4; Spain, 5; Switzerland, 4; UK, 17; USA, 21.

Fig. 4. Agarose gel electrophoresis of PCR-amplified DNA products generated using the *C. dubliniensis*-specific primers DUBF/DUBR (Table 1, Fig. 2) and the universal fungal primers RNAF/RNAR (Fell, 1993) with template DNA from yeast strains. The profiles shown correspond to: the *C. dubliniensis* type strain CD36 (lane 1); *C. albicans* (lane 2); *C. glabrata* (lane 3); *C. kefyr* (lane 4); *C. krusei* (lane 5); *C. norvegensis* (lane 6); *C. sake* (lane 7); *C. stellatoidea* (lane 8); *C. tropicalis* (lane 9); *Trichosporon beigelii* (lane 10); *C. dubliniensis* American isolate (lane 11); *C. dubliniensis* Argentinian isolate (lane 12); *C. dubliniensis* Australian isolate (lane 13); *C. dubliniensis* Canadian isolate (lane 14); *C. dubliniensis* French isolate (lane 15); *C. dubliniensis* German isolate (lane 16); *C. dubliniensis* UK isolate (lane 17). A negative control in which no template DNA was used in the PCR reaction was also included (lane 18). The 288 bp *C. dubliniensis*-specific amplicon generated by the DUBF/DUBR primers is present in lane 1 and lanes 11–17. Lanes marked M contain 100 bp size reference markers.

from the yeast isolates listed in Table 5 as follows: *C. albicans* ($n=53$), *C. dubliniensis* ($n=122$), *C. glabrata* ($n=1$), *C. kefyr* ($n=1$), *C. krusei* ($n=1$), *C. norvegensis* ($n=1$), *C. parapsilosis* ($n=4$), *C. sake* ($n=1$), *C. stellatoidea* ($n=10$), *C. tropicalis* ($n=1$) and *Trichosporon beigelii* ($n=1$). All 196 yeast isolates yielded an amplicon of approximately 610 bp, but only the *C. dubliniensis* isolates yielded the 288 bp amplicon. Fig. 4

shows examples of the PCR amplicons obtained with representative strains belonging to a variety of different yeast species, including epidemiologically unrelated *C. dubliniensis* isolates from disparate geographical locations. Use of this PCR test in conjunction with the rapid template DNA preparation procedure used here means that a *C. dubliniensis* isolate can be identified unequivocally in less than 4 h.

DISCUSSION

Because *C. dubliniensis* was only described recently it is important to further investigate and confirm its taxonomic and phylogenetic relationship to other medically important yeast species, especially the closely related *C. albicans*. The close relationship between *C. dubliniensis* and *C. albicans* has resulted in difficulties in developing rapid and reliable identification techniques capable of definitively discriminating between the two species. As a result, in-depth epidemiological studies on the prevalence of this organism have been hampered. There is an urgent requirement for a test which can rapidly and definitively identify *C. dubliniensis* directly following primary culture from clinical specimens. Such a test must be simple to use, inexpensive, easily accessible to clinical diagnostic laboratories and suitable for application to large numbers of samples.

The objectives of this study were to investigate the phylogenetic relationship of *C. dubliniensis* to other yeast species using non-rRNA gene sequences, and to develop a rapid identification technique for this organism. To achieve these objectives the *ACT1* gene was chosen for investigation, firstly because it is ideal for inferring phylogenetic relationships due to its high degree of sequence conservation in all eukaryotes, and secondly because it is unusual among yeast genes in that it is interrupted by an intron in most yeasts studied. Cloning and gene sequence analysis revealed that the overall similarity between the *C. dubliniensis* and *C. albicans* *ACT1* genes is 90.6%. Comparison of the spliced coding sequences of the two species revealed that the exon sequences are 97.9% identical. This level of homology is comparable to the percentage sequence identity between the two species reported previously for the V3 variable region of the large-subunit rRNA gene (97.52–97.75%; Sullivan *et al.*, 1995, 1997) and the small-subunit rRNA gene (98.6%; Gilfillan *et al.*, 1998). The predicted *C. dubliniensis* *ACT1* protein sequence was identical to that of *C. albicans*, apart from a single conservative substitution. A phylogenetic tree generated from nucleotide comparisons of *ACT1* coding sequences from *C. dubliniensis* and a variety of yeast species showed that *C. dubliniensis* was grouped separately from *C. albicans* and the other yeast species in 100% of trees generated (Fig. 3a). These studies represent the first phylogenetic investigation of *C. dubliniensis* based on non-rRNA gene sequences, and they unequivocally confirm its unique position as a separate taxon within the genus *Candida* as determined previously by comparative rRNA gene sequence analysis (Sullivan *et al.*, 1995, 1997; Gilfillan *et al.*, 1998). In contrast to the highly conserved nature of the *C. dubliniensis* and *C. albicans* *ACT1* exon sequences there was considerable divergence (16.6%) between the *ACT1*-associated introns of the two species. When these and the *ACT1*-associated intron sequences from a number of other yeast species were used to generate a second phylogenetic tree (Fig. 3b) the unique position of *C. dubliniensis* as a separate taxon within the genus *Candida* was affirmed. These results also confirmed that *C.*

dubliniensis is most closely related to *C. albicans*. In addition, the *C. albicans* and *C. stellatoidea* *ACT1*-associated introns were found to differ by one basepair substitution, corresponding to a 0.2% sequence divergence. These findings provided further evidence that *C. albicans* and *C. stellatoidea* should be considered as the same species. This situation is analogous to that found between *S. cerevisiae* and *S. carlsbergensis*, where the *ACT1*-associated introns differ by one basepair deletion and one basepair substitution, and it is accepted that these two organisms are in fact the same species (Nellen *et al.*, 1981). The *C. tropicalis* intron sequence differs from that of *C. albicans* by 43.4%, confirming that it is more distantly related to *C. albicans* than *C. dubliniensis* (Table 4, Fig. 3b). All of these findings indicate that the *ACT1*-associated intron sequences are not subject to the same level of evolutionary constraint as the *ACT1* coding sequences.

The *ACT1* genes of fungal species, in general, are noteworthy because of the presence of introns (Gallwitz & Sures, 1980; Fidel *et al.*, 1988; Wildeman *et al.*, 1988; Deshler *et al.*, 1989; Losberger & Ernst, 1989; Fletcher *et al.*, 1994; Cox *et al.*, 1995; Matheucci *et al.*, 1995; Wery *et al.*, 1996). At present, most known introns can be assigned unambiguously to one of four classes, depending on the intron structure and location (Krainer & Maniatis, 1988). *ACT1*-associated introns belong to class IV, which are nuclear pre-mRNA introns. The *C. dubliniensis* *ACT1*-associated intron is located at the 5' end of the gene, where it interrupts the fourth codon. The *ACT1* genes of *C. albicans*, *C. glabrata*, *S. cerevisiae* and *K. lactis* all contain introns located at this codon (Losberger & Ernst, 1989; O. Kurzai and others, unpublished data submitted directly to GenBank – accession no. AF069746); Gallwitz & Sures, 1980; Deshler *et al.*, 1989). This position is conserved amongst fungi, as all fungal actin genes that contain an intron do so at the third, fourth or fifth codon. Three conserved sequence elements have also been identified in the nuclear pre-mRNA introns of yeasts, at the 5' and 3' splice sites and at a site within the intron near the 3' splice site, known as the branchpoint sequence. All three conserved elements have been shown to be important for the accurate and efficient splicing of introns in *S. cerevisiae* (Langford *et al.*, 1984; Leer *et al.*, 1984; Molenaar *et al.*, 1984; Teem *et al.*, 1984; Mount, 1982). The *C. dubliniensis*, *C. albicans*, *C. stellatoidea* and *C. tropicalis* *ACT1*-associated introns possess all three conserved elements, namely GTATG (5' consensus), TAG (3' consensus) and TACTAAC (branchpoint), (this study, Fig. 2; Losberger & Ernst, 1989). These sequences are also present in *C. glabrata* and *K. lactis* although the 3' consensus sequence is CAG (Deshler *et al.*, 1989; see GenBank accession no. AF069746 for the *C. glabrata* *ACT1*-intron sequence).

One striking feature of the *C. dubliniensis* introns was that they showed little intraspecies variation, even among isolates from geographically divergent locations. The small changes which were recorded consisted of single base changes, some of which were shared by more

than one strain, and deletions which occurred at the end of poly(T) and poly(A) stretches. Introns containing these deletions were sequenced on separate occasions using different preparations of template DNA to rule out the possibility of sequencing or amplification artefacts. We concluded that these deletions are genuine and are probably the result of slipped-strand mispairing during replication. Similar intraspecies sequence conservation was observed with the *ACT1*-associated introns from *C. albicans* and *C. stellatoidea*. Boucher *et al.* (1996) made similar findings with their analysis of the group I self-splicing intron present in the large-subunit rRNA gene, in which the intron is present in a similar location in *C. albicans*, *C. stellatoidea* and *C. dubliniensis*. Again there was no significant intraspecies variation in the intron sequence. Furthermore, the *C. albicans* self-splicing intron and that of *C. stellatoidea* showed a high degree of homology, differing only by three single basepair substitutions. They also found that the homology between the *C. albicans* and *C. dubliniensis* group I introns (*CaLSU* and *CdLSU*, respectively) was quite high except for two regions of divergence contained in two stem-loop regions, both of which are much longer in *C. dubliniensis* than in *C. albicans*. These two regions lie outside the catalytic core, and although they are predicted to have a more complex secondary structure than those of *C. albicans* they do not affect the self-splicing ability of the intron. Our analysis of the *C. dubliniensis* and *C. albicans* *ACT1*-associated introns showed that although identical conserved elements are present in both species, nucleotide differences accounting for a 16.6% sequence divergence were dispersed throughout the length of the intron (Fig. 2). With group I introns, conservation of the nucleotide sequence may be important as it dictates the secondary structure of the intron and therefore its self-splicing ability. However, with group IV introns, such as the *C. dubliniensis* and *C. albicans* *ACT1*-associated introns, the splicing event is mediated by the spliceosome and although maintenance of the three conserved elements is important for splicing there do not appear to be any other constraints upon conservation of the nucleotide sequence. This may explain why divergence between the *C. dubliniensis* and *C. albicans* *ACT1*-associated introns sequences is dispersed throughout the intron.

Genotypic tests such as DNA fingerprinting analysis, karyotype analysis and RFLP analysis have been used in the differentiation of *C. dubliniensis* and *C. albicans* isolates. However, these techniques cannot be easily applied to the analysis of large numbers of clinical isolates. In contrast, PCR, which may be applied to the detection of genetic differences, is rapid and relatively inexpensive. The low level of intraspecies sequence variation in the *C. dubliniensis* *ACT1*-associated intron, and the extent of divergence from the *C. albicans* *ACT1*-associated intron sequence, suggested that this region could provide the basis for the design of oligonucleotide primers capable of readily discriminating between isolates of both species using PCR. In order to facilitate the rapid processing of large numbers of samples and to

decrease the time required, template DNA was prepared by boiling a single fresh *Candida* colony suspended in 50 µl water. With template DNA preparation taking approximately 15 min, PCR amplification 2.5 h and electrophoresis of amplimers 1 h, *C. dubliniensis* colonies can be identified in as little as 4 h. Thus *C. dubliniensis* colonies can be identified directly from primary isolation plates without the requirement for additional subculture. This technique is particularly effective when used for the identification of presumptive *C. dubliniensis* isolates cultured from clinical specimens on CHROMagar *Candida* medium. The *C. dubliniensis*-specific primers were tested with a collection of 122 isolates of *C. dubliniensis* in a blind trial with isolates of 10 other yeast species, including 53 isolates of *C. albicans* and 10 isolates of *C. stellatoidea* (Table 5). The primers correctly identified only the *C. dubliniensis* isolates, with 100% accuracy. Recently, Mannarelli & Kurtzman (1998) also developed a PCR-based identification test for discriminating between *C. albicans* and *C. dubliniensis* isolates; however, these primers were only tested with seven *C. dubliniensis* isolates. Another study by Elie *et al.* (1998) reported the development of a *C. dubliniensis* probe, specific for the internal transcribed spacer region (ITS2) of the ribosomal gene cluster. However, this probe has been tested with a very small sample number ($n=5$) and the method itself involves a PCR-enzyme-linked immunoassay, which is relatively time consuming. In contrast, our method is a simple and rapid technique capable of identifying suspect colonies directly from a primary isolation plate. In addition it has been evaluated against a large number of isolates from diverse geographical locations. Our findings clearly demonstrate that PCR identification based upon the *ACT1*-associated intron sequence is a definitive and rapid technique for the identification of *C. dubliniensis*.

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