

Molecular Comparison of
Candida inconspicua* and *Candida krusei

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Abstract

PCR-based methods for the differentiation of *Candida inconspicua*, *C. krusei*, and *C. norvegensis* were developed on the basis of the differences in the internal transcribed spacer (ITS) 1 and 2 regions and 5.8S rRNA of the rDNA repeated locus. PCR-RFLP of rDNA using *Hha*I showed characteristic band patterns in these three species, allowing the identification of each species. Random amplified polymorphic DNA (RAPD) was also conducted for the purpose of species differentiation of three *Candida* species. RAPD patterns using primer R108 distinguished between the three species with potential species-specific band patterns in all the isolates. Variation of banding patterns between isolates of the same species were noted providing good preliminary evidence of a molecular typing system for each species with a single primer. The 5.8S, ITS 1 and 2 regions of ribosomal DNA in *C. krusei* and *C. inconspicua* were sequenced, and a phylogenetic tree was constructed. The data indicated that these two species are closely related to each other with only 2 bp differences in the highly conserved 5.8S rDNA sequence. The length variation of the ITS 1 and 2 regions between these species was found in the range of 89 to 163 bp. Species-specific oligonucleotides for the identification of *C. krusei* and *C. inconspicua* were designed based on the ITS 2 region sequence. Dot hybridisation for 1 h detected these species and proved that both probes could accurately distinguish the two species from each other and other species of fungi. These probes should provide the basis for a system to identify multiple species within a heterogeneous collection of yeasts from a variety of sources. In addition, direct PCR using the species-specific ITS primers was practicable and successful for presumptive identification of *C. krusei* and *C. inconspicua*.

Declaration

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Dedication

To my parents

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Manchester, 1997
Richard S. Nho

List of abbreviations

Units of measurement

day (s)	d
hour (s)	h
minute (s)	min
second (s)	s
meter	m
gram	g
liter	l
Molar	M
degrees Celsius	°C

Prefixes to units

milli-	m	10^{-3}
micro-	μ	10^{-6}
nano-	n	10^{-9}
pico-	p	10^{-12}

Abbreviations

bp	base pairs
ddNTP	dideoxyribonucleotide triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
<i>et al.</i>	et alia

e.g.	exempli gratia
>	greater than
pH	hydrogen ion concentration (minus log)
i. e.	id est
IGS	inter genic spacer
ITS	internal transcribed spacer
kb	kilobase pairs
<	less than
NTS	non transcribed spacer
PCR	polymerase chain reaction
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
SSC	sodium salt citrate
TBE	tris borate EDTA
TPE	tris phosphate EDTA
TE	10 mM Tris-Cl, 0.1mM EDTA
TPE	tris phosphate EDTA
U	unit
UV	ultra violet
v/v	volume to volume
w/v	weight to volume

Aims of this research

Several non *Candida albicans* species such as *C. inconspicua*, *C. krusei* and *C. norvegensis* have similar phenotypic and genotypic characteristics and are increasingly the cause of serious *Candida* infections. In particular, *C. krusei* is known to be naturally resistant to fluconazole, which is medically significant. Therefore several molecular techniques were conducted to achieve the following objectives.

- 1) To investigate the ITS 1, ITS 2 and 5.8S rDNA sequence length variation in clinically important *Candida* species as a preliminary step to assist in species identification.
- 2) To investigate whether restriction digestion of amplified PCR fragments from the rDNA could yield species identification among *C. inconspicua*, *C. krusei* and *C. norvegensis*.
- 3) To ascertain whether RAPD typing could provide species-specific banding patterns.
- 4) To ascertain the phylogenetic place of *C. inconspicua* and confirm the place of *C. krusei* using highly conserved 5.8S rDNA sequence.
- 5) To develop species-specific probes for *C. inconspicua* and *C. krusei* that should be able to be utilised on single colonies from agar plates.

Research strategies

Isolation of clinically important non *C. albicans* species from a variety of sources



PCR analysis using different primers

Determination of PCR products of *Candida* species



Restriction enzyme analysis of PCR products of rDNA

Differentiation and identification of
C. krusei, *C. inconspicua* and *C. norvegensis*



Sequencing of the 5.8S rDNA of *C. krusei* and *C. inconspicua*

Phylogenetic analysis



Sequencing of the ITS 1 and 2 regions of *C. krusei* and *C. inconspicua*

Design of the species-specific probes for
C. krusei and *C. inconspicua*



RAPD analysis

Molecular typing of isolates



PCR using a combination of universal and species-specific primers

Identification of target species,
C. krusei and *C. inconspicua*



Dot hybridisation using the species-specific probes

Identification of target species, *C. krusei* and
C. inconspicua as a confirmatory approach

1.

Chapter 1

Introduction

1.1 Candidiasis and medically important non *Candida albicans* species

Fungal infections can be broadly classified as either opportunistic mycoses or endemic mycoses (Pfaller 1994). Opportunistic mycoses occur primarily in immunocompromised patients, and in patients with malignancies, AIDS and those undergoing major surgery. In the case of endemic mycoses, susceptibility to the infection is acquired by living in a geographic area that is the natural habitat of the particular fungus.

Factors that contribute to fungal infections include exposure to broad spectrum antibacterial agents with adrenal corticosteroids, cytotoxic chemotherapy, antibiotic therapy, use of indwelling catheters, human immunodeficiency virus infection, organ transplantation and other immunosuppressive conditions (Scherer *et al.* 1987; Hazen 1995; Wardle *et al.* 1995; Dupont *et al.* 1994). The common agents of opportunistic mycoses include *Candida albicans*, *Aspergillus* species, *Cryptococcus neoformans* and zygomycetes.

Candida is the most widespread and prevalent mycotic disease causing organism of man. It may cause superficial or systemic infections. Superficial infections are the most common type of infection caused by *Candida* species. Systemic candidiasis is a serious problem, predominantly in chronically compromised hosts, and affects organs of vital importance. *C. albicans* was once regarded as the only medically important yeast species associated with human infections. However, other *Candida* species have now emerged as common pathogens. In particular, candidaemia can lead to focal complications such as arthritis, osteomyelitis, endocarditis, myocarditis, and meningitis (Hazen 1995). *C. albicans* is probably the most frequently isolated species from patients with oropharyngeal candidiasis, especially AIDS patients, and but also individuals undergoing immunosuppressive therapy for cancer or organ transplantation and those exposed to broad-spectrum antibacterial therapy (Pfaller *et al.*

1994). However, a large number of other species, especially *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei* and *C. inconspicua* are emerging as important opportunistic pathogens (Pfaller 1994; Just-Nübling *et al.* 1990).

1.2 Candidemia and definitions

Hematogenous candidiasis is used to identify all infections involving the bloodstream, explicitly or implicitly and refers to candidemia, or disseminated candidiasis (Bodey *et al.* 1993). Candidemia is defined as the presence of at least one positive blood culture containing pathogenic *Candida* species. However, *Candida* species isolated from blood are considered non-pathogenic if the following criteria are fulfilled: only one of at least two blood cultures was positive, the patient's symptoms resolved spontaneously without therapeutic intervention, and the impression by primary physicians and investigators based on clinical review, is that the organism is not pathogenic.

Candidemia can be a significant observation in the absence of clinical signs and symptoms. Therefore if *Candida* species are isolated from a blood culture collected from an asymptomatic patient without a catheter, additional blood culture must be examined (Bodey *et al.* 1993). If multiple blood cultures are also positive, a patient is considered to have significant candidemia even if asymptomatic.

1.3 Treatment of candidiasis

As *Candida* species are eukaryotic cells, like human cells, they share many metabolic, genetic and structural features. Antifungal agents are generally associated with substantially more toxicity than antibacterial agents. An antifungal agent must therefore inflict damage on one

eukaryotic type (the fungus) without adversely affecting the other eukaryotic type (the patients). *Candida* species, however, also contain constituents that are uniquely different from those of mammalian cells such as glucans, mannans, and glycoproteins (Bodey 1993). Therefore antifungal agents have been developed against these metabolic systems in order to minimise toxicity to human cells.

1.3.1 Topical and systemic antifungal agents

Topical therapy has been mainly used for the treatment of infection against the skin and nails. Mucosal candidiasis can be treated topically by single or repeated application of creams, lotions etc. to the infected sites. The treatment of deep seated fungal infections, most of which are diseases caused by opportunistic species of *Candida* or *Aspergillus* or by *Cryptococcus neoformans*. The antifungal drugs currently available belong to two major classes, polyenes (amphotericin B, nystatin) and azoles (miconazole, ketoconazole, itraconazole, clotrimazole, and fluconazole).

1.3.1.1 Polyenes

The polyenes are topically active agents and are not orally bioavailable. Amphotericin B is an antibiotic produced by *Streptomyces nodosus* and was first isolated and described in 1956. Since then it has been widely used for the treatment of superficial yeast infections and many deep-seated mycoses (Speller 1980). Amphotericin B is the preferred drug of choice for some disseminated fungal infections in immunocompromised hosts (Odds *et al.* 1996). Nystatin, originally called fungicidin, is a products of *S. noursei* and is too toxic to be given parenterally (Speller 1980). Polyene antifungal agents bind preferentially to ergosterol which is the sterol compound specific to most fungal cell membranes, leading to distortion and damage of normal membrane permeability with leakage of intracellular constituents and cell death (Odds 1996).

A secondary mode of action is known oxidative damage to cell components. However, nephrotoxicity is a common problem associated with amphotericin B. To overcome this problem, various lipid-based preparations have been introduced such as AmBisome which uses a true liposomal formulation of amphotericin B (White 1995). Recent studies reported that although fewer side effects were shown in patients with a lower dose of amphotericin B (total amphotericin B dose of ≤ 500 mg), no overall difference in mortality was evident compared with high-dose amphotericin B (total amphotericin B dose of >500 mg). The chemical structure of amphotericin B and nystatin is illustrated in Figure 1.1.

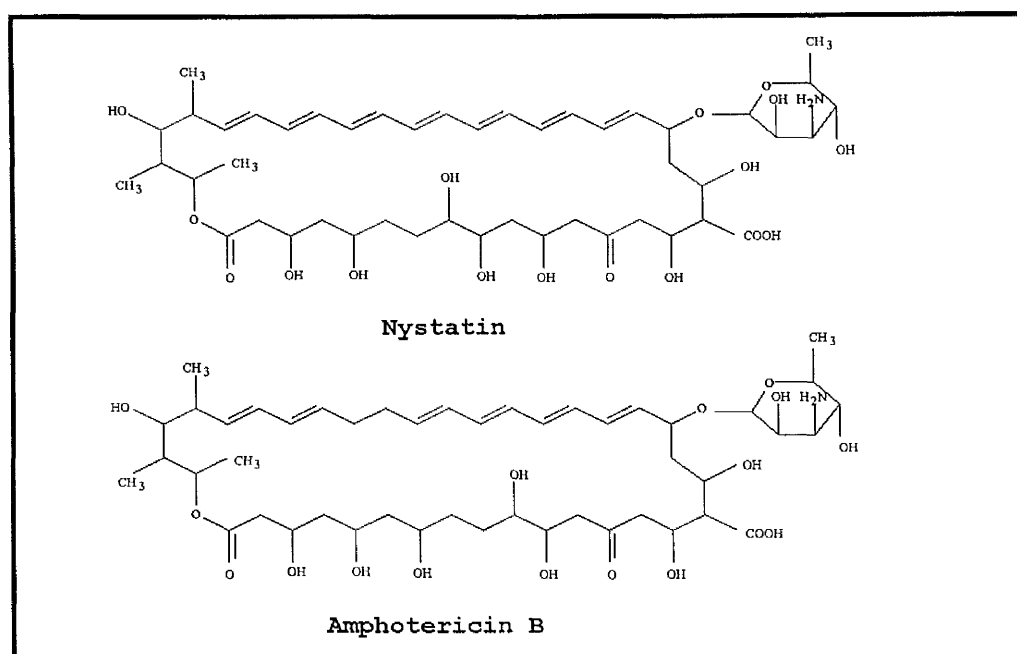


Figure 1.1 Chemical structure of polyenes.

1.3.1.2 Azole compounds

Several azole compounds are available for the treatment of *Candida* infections, including miconazole, ketoconazole, fluconazole, and itraconazole. Miconazole and ketoconazole are imidazole compounds, and have a five-membered ring structure containing two nitrogen atoms with a complex side chain attached to one of the nitrogen atoms (Figure 1.2).

Fluconazole and itraconazole are triazole compounds and contain an additional nitrogen in the ring. Triazole derivatives have a generally higher selectivity for the inhibition of fungal growth compared with imidazole derivatives. Two triazole derivatives, fluconazole and itraconazole, are now widely used for the management of deep-seated fungal infection. Recently, research has revealed that a new triazole agent called voriconazole (or UK 109,496) which is modified chemically from fluconazole, has a broader antifungal spectrum with an activity some 10 to 100 times more potent than fluconazole against some fungi (Barry *et al.* 1996).

a. Fluconazole

Fluconazole is one of the triazole antifungal agents and has been widely used for the treatment of oral / oesophageal *Candida* infection in AIDS as well as for primary and maintenance treatment of cryptococcosis. The fact that it is available orally as well as intravenously accounts for its widespread use (Just-Nübling *et al.* 1990; Dewsnup *et al.* 1994; Johnson *et al.* 1995). In addition, fluconazole is highly soluble in water and its very low protein binding characteristic allows rapid absorption into the blood stream and high levels of unbound fluconazole in most body fluids, including CSF. Furthermore, it has been known that this compound is not affected by food or gastric acidity. However, since it has been extensively used for the treatment of oropharyngeal and esophageal candidiasis in HIV infected individuals due to its safety and relatively few side effects, there has been increased concern about the emergence of fluconazole resistant *Candida* species (Rex *et al.* 1995a). Particularly, an increase in non *Candida albicans* species like *C. krusei*, *C. glabrata*, and *C. tropicalis* which are innately or relatively resistant to this agent have been reported over the past decade (Rex *et al.* 1995a). Research showed that there are three possible avenues by which a patient might acquire resistant organisms (Rex *et al.* 1995b):

- 1) a colonising or infecting organism is initially susceptible but mutates and becomes resistant,
- 2) the patient is colonised or infected with multiple strains or species and an inherently resistant strain or species is selected, or
- 3) the patient is initially colonised or infected with an inherently resistant species.

b. Miconazole

It was the first azole introduced for the treatment of systemic fungal infections. However, it has been known that miconazole has substantial toxicities, like local phlebitis, pruritus, rash, nausea, fever, chills, and hyponatremia. Subsequent clinical experience has been less promising and it is probably less effective than amphotericin B (Bodey 1993).

c. Ketoconazole

For ketoconazole therapy, only oral preparations are available to patients. Absorption in the stomach depends on acid because it must be converted to the hydrochloride salt. The administration of antacids greatly reduces its absorption. Although there is a major concern of potential hepatic toxicity (Odds 1996), ketoconazole is generally well tolerated, even with prolonged treatment. Resistance to ketoconazole in normally susceptible fungal species is also rare. It has been known that ketoconazole interferes with the synthesis of several mammalian hormones due to the inhibition of cholesterol synthesis which is the major precursor of these substances.

d. Itraconazole

Itraconazole is currently available in the form of oral capsules and an oral solution is now undergoing clinical trials (Odds *et al.* 1996). It has a broad range of activity in superficial and systemic mycoses including cryptococcosis, invasive aspergillosis, disseminated and oral /

oesophageal *Candida* infections in AIDS. But whether this activity is comparable or superior to other azoles remains unknown because of a lack of comparative trials (Bodey 1993). Because itraconazole is a lipophilic compound, it is only ionised at low pH (e.g. gastric juice). Studies showed that presence of antacids reduce the absorption of this drug. Itraconazole is an antifungal drug which is highly metabolised in the liver. Retrospective studies have shown that induction of resistance i.e. solely to itraconazole, has yet to be reported, and interestingly, *C. krusei* which is inherently resistant to fluconazole, is usually susceptible to itraconazole *in vitro* (Odds *et al.* 1996; Hazen 1995).

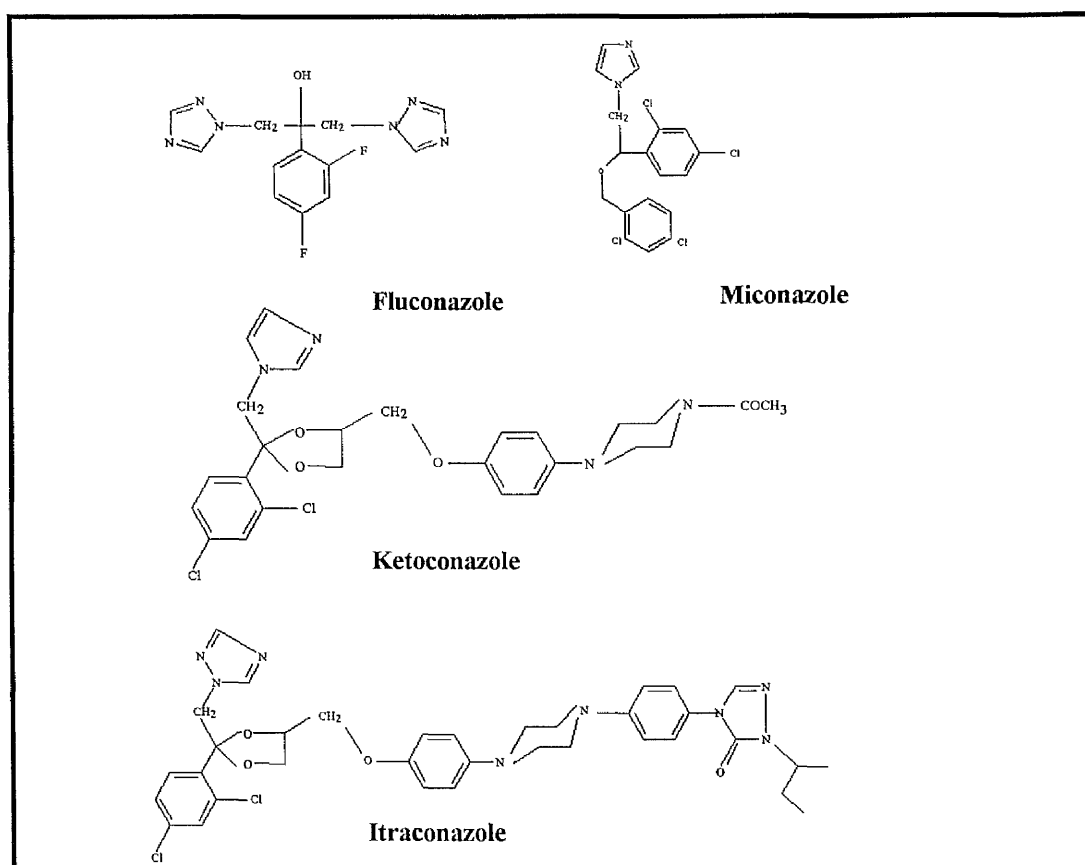


Figure 1.2 Chemical structure of azole antifungal agents.

1.4 Antifungal agents and their mechanisms of action

The fungal plasma membrane is the major interface between the cell and its environment. Sterols are key to the maintenance of the integrity of eukaryotic membranes. Cholesterol is the major sterol of the plasma membranes of most animals (Gooday, 1994). In contrast, for the majority of the fungi, ergosterol is the key membrane sterol. Ergosterol differs from cholesterol by being methylated at C-24 of the side chain. One consequence of having ergosterol as opposed to cholesterol in the membrane is increased fluidity. The presence of ergosterol provides the basis for selectivity of two types of antifungal agent, those interacting directly with the membrane and those interfering with its biosynthesis (Gooday, 1994). Nystatin and amphotericin B are characterised by a carbon ring containing a hydrophobic conjugated double-bond system and a hydrophilic region. A primary mode of action of these antibiotics is to become incorporated into the fungal plasma membrane in a group linked hydrophobically to ergosterol, thereby producing a pore and the loss of membrane integrity (Odds 1996; Deacon 1984).

Azole compounds, like fluconazole, miconazole, ketoconazole and itraconazole, inhibit preferentially 14 α -demethylase activity which is responsible for the conversion of lanosterol to ergosterol. To a lesser extent, these compounds interfere with the mammalian enzyme that converts lanosterol to cholesterol (Vanden Bossche 1991). The conversion of lanosterol to ergosterol is a complex process involving a host of other enzymes (Figure 1.3). The most studied enzyme in the pathway is the cytochrome P450-dependent 14 α -sterol demethylase, as this is the site of action of the azole antifungal agents (Parks *et al.* 1995). Treatment of fungi with sublethal concentrations of these agents leads to abnormal branching and deposition of chitin. Vanden Bossche (1991) has suggested that this inhibition of ergosterol biosynthesis results in an alteration of the fluidity of critical domains in the cell

membrane. This interference will lead to the production of a defective cell membrane with altered permeability, leading to growth inhibition and eventual cell death. Azole compounds may also exert a direct effect on the fatty acid content of the cell membrane, causing leakage of proteins and amino acids and therefore interference with the uptake of essential nutrients. Other effects of these compounds are to inhibit fungal adherence, and inhibit the formation of germ tubes and mycelia.

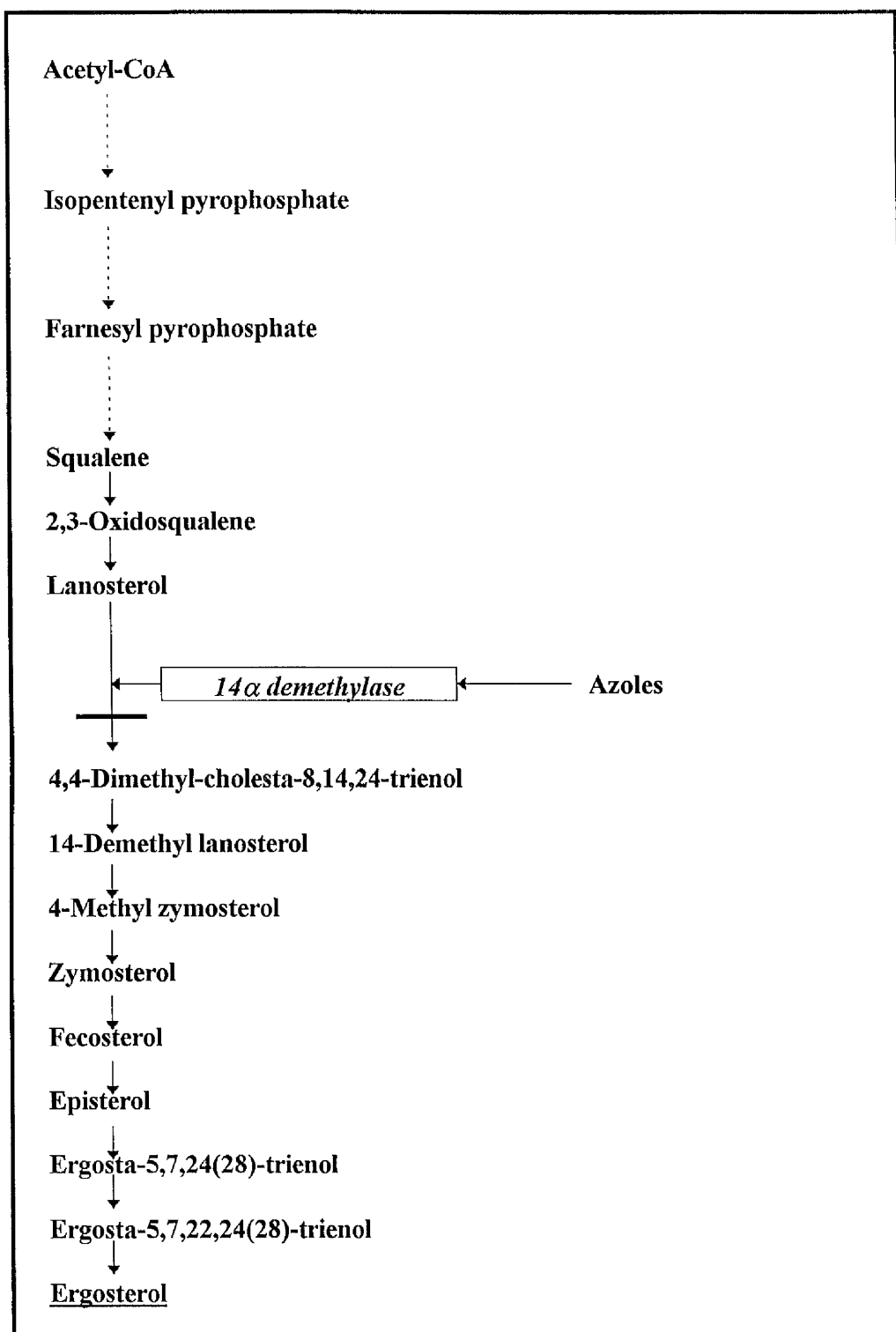


Figure 1.3 The simplified biosynthetic pathway for ergosterol and inhibition site of azoles.

Bar represents site of inhibition of the 14 α demethylase inhibitors.

1.5 Antifungal susceptibility test

Since new improved azoles are available for the treatment of *Candida* infections, the National Committee for Clinical Laboratory Standards (NCCLS) proposed a standardised procedure for *in vitro* antifungal susceptibility testing (Price *et al.* 1994). The role of this committee was to reduce the complexity of the standardisation problem by first looking at methodologies involving defined media (Ghannoum *et al.* 1996). A number of investigators have collaborated to examine the role of variables such as inoculum preparation, inoculum size, medium composition, incubation temperature, incubation time, and endpoint definition on inter-laboratory variability. The NCCLS have now established the M27 method which outlines their findings in detail (Table 1.1; Ghannoum *et al.* 1996).

However it is known that this method is cumbersome and time consuming. In addition the most critical step, endpoint determination, relies on the visual inspection of growth inhibition. Therefore, alternative methods have recently been evaluated (Espinel-Ingroff *et al.* 1995).

Table 1.1 Antifungal susceptibility test (M27 method).

Item	Implementation in the M27 method
Methodology	Broth macrodilution; final volume, 1ml
Medium	RPMI-1640 containing 0.165 M MOPS (pH 7.0)
Fungal inoculum	0.5×10^3 - 2.5×10^3 organisms/ml
Incubation temperature	35°C
Incubation time	48 h (<i>Candida</i> species) or 72 h (<i>C. neoformans</i>)
Endpoint	Amphotericin B, optically clear tube; azoles and flucytosine: 80% reduction in turbidity by comparison with growth control.

1.6 Laboratory diagnosis of *Candida* species

The anamorphic yeast genus *Candida* includes many pathogenic species of yeasts that cause a variety of clinical syndromes in humans. These range from superficial infection to invasive disease in immunocompromised patients. In particular, several *Candida* species are regarded as the most common hospital (Hayens *et al.* 1996; Jarvis 1995) and ubiquitous human pathogens, causing localised, invasive or disseminated disease in normal or immunocompromised hosts.

Although *C. albicans* is still the most frequently isolated species from patients with *Candida* infection (Hazen 1995), steady rise in the number of reports on the incidence of species of *Candida* other than *C. albicans* have been shown over the past decade in the western world. There are approximately 200 species in *Candida* genus, and at least 17 species have been shown to cause disease in humans (Rinaldi 1993). Those frequently isolated from HIV-positive patients with oropharyngeal candidiasis are typically *C.(T.) glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and more recently, *C. inconspicua* and *C. norvegensis* (Baily *et al.* 1994; Hazen 1995; Samaranayake *et al.* 1994; Hood *et al.* 1996).

C. inconspicua, first described in 1952, was not previously recognised as a pathogen and has not been discussed in any recent reviews of emerging pathogens (Hazen. 1995). Very little information on this species exists so far, although a recent study does suggest that this species was isolated from the upper and lower respiratory mucosa of AIDS patients as a result of prolonged drug pressure (Baily *et al.* 1996), giving clinical concern. It is known that *C. inconspicua* has similar phenotypic characteristics to *C. krusei*. As a result, identification and differentiation between the two using conventional methods are not straightforward (Mata

Essayag *et al.* 1996). *C. krusei* is now an important agent of opportunistic disease, inciting serious infections in the neutropenic patient population (Rinaldi 1993). In particular, *C. krusei* is known to be innately resistant to fluconazole and is causing great concern (Rex *et al.* 1995b). Research suggested that the increased isolation rates of *C. krusei* may be due to the use of fluconazole both for the prophylaxis and for treatment (Anaissie *et al.* 1994). Other *Candida* species, such as *C. lusitanae*, *C. tropicalis*, *C. guilliermondii*, and *C. glabrata*, have also been shown to develop resistance during antifungal therapy (Merz *et al.* 1986; Wade 1993; Law *et al.* 1996).

Several approaches have been used as diagnostic tools for the identification of *Candida* species but they do have limitations. For instance, phenotypic parameters such as substrate assimilation, cell wall composition, and production of extracellular proteolytic enzymes can vary greatly within some species like *C. albicans*. This would make the concept of species unclear to some extent (Williams *et al.* 1995; Martinez *et al.* 1990).

Therefore, a rapid and accurate identification of disease causing species of *Candida* is crucial for clinical treatment and epidemiological studies. So far several methodologies have been established to identify these species, and it could be classified into two categories as follows.

1.6.1 Conventional approaches

Methods in the laboratory commonly used for *Candida* species identification involve examination of their colonial morphology on specialised media such as corn meal agar. Germ tube test and assessment of various biochemical reactions are also used.

1.6.1.1 Direct examination of specimens using microscopy

Yeasts are traditionally identified by morphology, although metabolic characteristics require from a few days to many weeks to identify the. However, since fungal cells are usually much larger than bacteria and therefore more readily seen in microscopic preparations, the detection of fungal species is often easier.

The morphological features of some common *Candida* species are often instantly recognisable (Walsh *et al.* 1993). *C. albicans* has pseudohyphae with clusters of round blastoconidia at both junctions and septa. True hyphae may also be present. In the case of *C. tropicalis*, blastoconidia are distributed singly or in small clusters along the entire length of the pseudohyphae. In the case of *C. parapsilosis*, an overall pattern of curved hyphae with “sage-brush” pattern is a typical feature. For *C. krusei*, the overall pattern of pseudohyphae and elongated blastoconidia convey a “crossed sticks” pattern. Finally, *C. glabrata* has a small oval to round single-budding unencapsulated blastoconidia without formation of pseudohyphae or hyphae.

In case of invasive fungal elements, specific stains are usually required. Gram stain is used for yeast cells but less effective for staining hyphae. One of the most widely used staining procedures is periodic acid-schiff reagent (PAS) in which fungal cell wall polysaccharides become pink to deep red after staining. Another test based on microscopic characteristics is the germ tube test. Approximately 75% of the yeasts recovered from clinical specimens are *C. albicans* (Finegold *et al.* 1986), and the vast majority of isolates of *C. albicans* produce germ tubes. Therefore, this is now recognised as an excellent presumptive test for this species. The test is carried out by suspending a small portion of a colony in rabbit plasma, bovine serum albumin, fetal calf serum, or cell culture medium and incubating at 37°C

for 1-2 h. Germ tubes are recognised as direct parallel cell wall extensions from the yeast. The germ tube of *C. albicans* is distinguishable from the blastoconidial germination of organisms such as *C. tropicalis*. Other non *C. albicans* species are characterised by a constriction between the growing hyphal cell wall structure and yeast form if any hyphal formation is observed at all (Walsh *et al.* 1993). However, overinoculation of the test broth may lead to suppression of germination and a false-negative result in detection of *C. albicans*. In addition, parallel controls of known *C. albicans*, *C. tropicalis*, and *C. glabrata* should be used during the test to guide identification of key structures (Walsh *et al.* 1993).

1.6.1.2 Phenotypic characteristics of *C. inconspicua* and *C. krusei*

The identification of *C. inconspicua* and *C. krusei* is normally based on a set of phenotypic characteristics. The typical phenotypic characteristics for *C. krusei* are as follows (Mata Essayag *et al.* 1996): The presence of pseudohyphae, and elongated blastoconidia; the absence of chlamydoconidia and germ tubes; surface growth that has the ability to form a climbing film on the slides of glass containers; glucose fermentation; assimilation of glucose, glycerol, DL-lactic acid, succinate, ethanol, and N-acetylglucosamine; failure to assimilate galactose, L-sorbose, sucrose, D-ribose, L-rhamnose, maltose, cellobiose, trehalose, melibiose, raffinose, melezitose, insulin, starch, D-xylose, L-arabinose, D-arabinose, erythritol, ribitol, D-mannitol, salicin, inositol, lactose, galactitol, and glucosamine; failure to grow when potassium nitrate is the nitrogen source; and growth in a vitamin-free medium. In addition, *C. krusei* is thought to be the only species which can grow on Sabouraud dextrose agar as spreading colonies with a matt or a rough whitish yellow surface, in contrast to the convex colonies of other *Candida* species (Samaranayake *et al.* 1994).

C. inconspicua is distinguished from *C. krusei* by its failure to produce hyphae on corn meal agar, the presence of a primitive pseudomycelium, its inability to ferment glucose and assimilate N-acetylglucosamine, its ability to assimilate glucosamine, and its failure to grow in a vitamin-free medium.

Recent studies showed that conventional approaches, such as the API 32C, fermentation and assimilation test, were not found to be sufficiently discriminatory against some *Candida* species, such as *C. inconspicua*, *C. krusei* and *C. norvegensis* (Nho *et al.* 1997). In particular, it was found that *C. krusei* and *C. inconspicua* have very similar biochemical characteristics. One of the major differences detectable by biochemical testing is that *C. krusei* can ferment D-glucose whereas *C. inconspicua* cannot. However, some *C. inconspicua* isolates were found to ferment D-glucose like *C. krusei*. Furthermore, the API 32C test was not found to be reliable for the differentiation of these species, suggesting that further tests are required.

1.6.1.3 Special media for the recognition of yeast species

Most pathogenic fungi including *Candida* species generally grow well on common mycological and bacteriological media. Sabouraud glucose agar, sheep blood agar, and horse blood agar have all been used as a growth medium for *Candida* species (Walsh *et al.* 1993). Various preparations of Sabouraud dextrose agar, often supplemented with antibiotics to suppress bacterial growth, can also be used (Bodey 1993). In order to differentiate between several species in culture and for the direct and rapid identification of yeasts resistant to antifungal agents, several media have been developed. For example, CHROM agar (CHROMagar, Paris, France) for *C. albicans*, *C. tropicalis* and *C. krusei* (Odds *et al.* 1996; Patterson *et al.* 1996) and Albicans ID (BioMérieux, Marcy l'Etoile, France) for *C. albicans*.

identification which are based on colour change and appearance have been introduced and compare well with standard methods (Baumgartner *et al.* 1996). *C. albicans* may also be identified by terminal chlamydospore formation on corn meal agar with tween-80. The microscopic features of other *Candida* species can be distinguished on this medium by the arrangement of blastoconidia and the morphological features of pseudohyphae. Other yeasts, such as *Cr. neoformans* and *C. glabrata*, do not produce pseudohyphae in this medium. In general, these special media can be used to identify unknown species without difficulties. However, other tests should be performed to obtain conclusive results.

1.6.1.4 Biochemical tests

Biochemical tests have always complemented morphology when identifying *Candida* species. In general, there are two types of biochemical tests, assimilation and fermentation. Assimilation is the ability of an organism (measured by growth) to use a compound as the sole energy source in the presence of oxygen. Fermentation is the ability of an organism to use a compound as the sole source of energy in the absence of oxygen (measured by gas production and colour change). The API-20C carbohydrate assimilation test (Analytab products, Plainview, NY) is perhaps the most widely used rapid yeast identification system (Finegold *et al.* 1986). Recently, the API 32C system was introduced and has a better track record in identifying clinically important yeast species (Bruun *et al.* 1995). Other yeast identification systems including the Uni-Yeast-test System (Remel Laboratories, Lenexa, KS) and the automated systems, such as the MicroScan (Baxter Healthcare Corp., West Sacramento, CA) have been used. The data bases of these systems for commonly isolated yeasts appear to be adequate, but there are also problems with less commonly encountered yeasts.

1.6.2 Detection of *Candida* species using molecular approaches and their medical significance

In general, conventional approaches need a minimum of 24 to 48 h to identify a species of *Candida* from a clinical specimen. Frequent difficulties are encountered when trying to identify isolates definitively (Sullivan *et al.* 1996). Furthermore, for the identification of some medically important species, several problems exist with current laboratory methodology, particularly for *C. krusei* (Carlotti *et al.* 1996). *C. krusei* is widespread in nature, and represents one of the four most common yeast pathogens in highly compromised patient populations. HIV infection and the increasingly widespread use of the new triazole compound, fluconazole have contributed to a significant increase in *C. krusei* infection. It can cause life-threatening systemic infections and clinical studies showed that mortality rate varies from between 45 and 100% (Carlotti *et al.* 1994). Moreover, because most strains are naturally resistant to fluconazole and less susceptible to amphotericin B (Fisher *et al.* 1989), reliable, quick and accurate detection methods are crucial in the identification of this species to optimise therapy. In addition, *C. krusei* could be mistaken for *C. inconspicua* under conventional methodology (Mata Essayag *et al.* 1996), suggesting that more reliable methods towards species-specific detection of these species are needed.

1.6.2.1 Polymerase chain reaction based-methods (PCR)

Until the 1970s, several methods based on immunoblotting techniques were extensively employed to identify *Candida* species antigens, such as immunodominant cytoplasmic antigens. However these approaches were labour intensive and not able to distinguish between patients with candidiasis from those who were not actively infected with *Candida* species (Matthews *et al.* 1992). The polymerase reaction (PCR) was first introduced in 1985 by Kary Mullis and is now widely used in the laboratory. It is a *in vitro* technique which allows the amplification of a specific DNA region that lies between two regions of known

DNA sequence. A PCR reaction consists of four basic components (Newton *et al.* 1994): template DNA, *Taq* DNA polymerase, primers and nucleotides. Oligonucleotide primers, also known as amplimers, which are complementary to the ends of defined sequences of DNA template are used for the PCR amplification. *Taq* DNA polymerase is required for the extension of sequence from the primers. In the presence of deoxynucleoside triphosphates (dNTPs) in buffer containing an optimum concentration of $MgCl_2$, target DNA can be amplified (Figure 1.4). Strand synthesis can be repeated by heat denaturation of the double-stranded DNA, annealing of primers by cooling the mixture and primer extension by DNA polymerase at a temperature suitable for the enzyme reaction in a thermocycler (temperature cycling devices). Each repetition of strand synthesis comprises a cycle of amplification and each new DNA strand synthesised becomes a template for any further cycle of amplification. This way the amplified target DNA sequence is selectively amplified cycle after cycle (Figure 1.5).

Although the annealing and extension temperature are variable for each PCR, a typical temperature cycling profile is as follows. Initial denaturation of template DNA requires 95-100°C to completely denature complex genomic DNA. During the PCR, 92-95°C is normally sufficient for denaturation. The annealing temperature is an important parameter in optimising the specificity of a reaction and is commonly calculated by following formula, $T_m = [(number\ of\ A+T) \times 2^\circ C + (number\ of\ G+C) \times 4^\circ C]$. In general, many laboratories use annealing temperatures of 3-5°C below the calculated T_m as a starting point for PCR optimisation. Primer extension is usually performed at 72°C, which is the optimum temperature for *Taq* DNA polymerases. The availability of a purified heat-stable DNA polymerase from the thermophilic bacterium *Thermus aquaticus* (termed *Taq* polymerase) greatly facilitated the enzymatic catalysis of DNA amplification, owing to its

ability to withstand repeated cycles of high temperatures necessary for DNA denaturation. Up to 2 min are usually sufficient for the extension steps. The number of thermal cycles is usually between 25 and 35 because with increasing cycle numbers, it is common to observe an increase in the amount of unwanted artifacts.

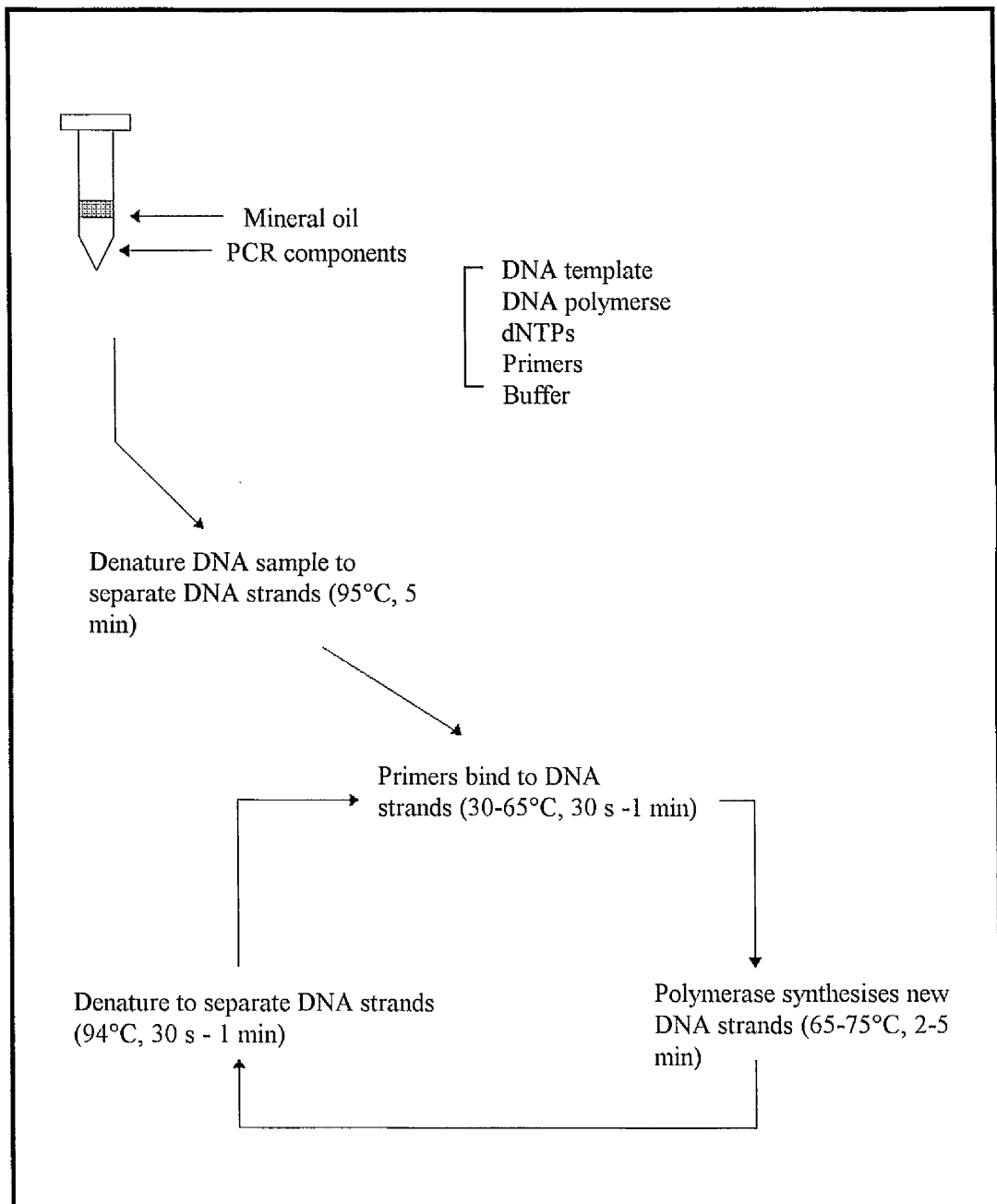


Figure 1.4 The PCR cycle.

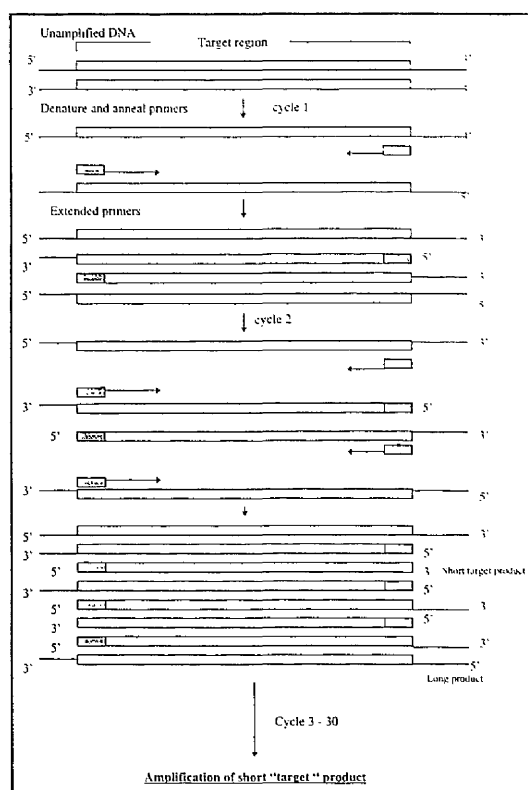


Figure 1.5 The polymerase chain reaction (PCR).

Recently, rapid PCR-based identification methods for several fungal groups (Makimura *et al.* 1994) as well as for *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei* have been introduced (Niesters *et al.* 1993; Holmes *et al.* 1994). Therefore, detecting circulating *Candida* genome from patients is possible by the *in vitro* DNA amplification technique.

Several PCR-based attempts have recently been made to identify *Candida* species using species-specific primers derived from conserved ribosomal DNA regions (rDNA) (Haynes *et al.* 1996). Also the genes coding for ribosomal RNA (rRNA) in the fungal genome have received considerable attention for phylogenetic analysis and molecular investigation (White *et al.* 1990). Because each *Candida* species has different fragment sizes in the internal transcribed spacer (ITS; Lott *et al.* 1993) 1 and 2 regions except the highly

conserved 5.8, 18 and 28S rDNA areas, PCR products of ITS regions using universal primers could be used for the identification of some medically important yeasts.

Another similar method called direct PCR has also been developed. This is identical to conventional PCR except that genomic DNA is not required. Maiwald *et al.* (1994) used restriction enzyme analysis of direct PCR products with six different restriction enzymes, *AluI*, *BanI*, *BbsI*, *DraII*, *Eco147I* and *NheI* for the identification of medically relevant yeasts, and proved a speedy identification method.

1.6.2.2 Restriction enzyme analysis and hybridisation techniques

Since PCR reactions usually generate enough DNA to be directly visible on a gel, amplified products can be digested with restriction enzymes and species-specific band patterns are visible on the gel. Several restriction enzymes can be employed for the analysis of digested band patterns. The standard molecular approach for the identification of *Candida* species involves Southern hybridisation analysis, and several probes specific for several *Candida* species have now been developed (Wickes *et al.* 1992; Sandhu *et al.* 1995; Carlotti *et al.* 1996). The amplified or digested DNA is transferred to a nylon membrane and hybridised with a labelled probe to identify targeted species *in vitro*. Another hybridisation method using oligonucleotides derived from ribosomal DNA internal spacers for the sensitive and rapid identification of *Candida* species is dot hybridisation technique (Botelho *et al.* 1994; Holmes *et al.* 1992). Once *Candida* genomic DNA is prepared, it is spotted on the nylon membranes and hybridised with species-specific probes. The membranes are then washed at stringent conditions followed by exposure to X-ray film. The colony hybridisation technique is also used for the direct identification of target colonies using species-specific probes (Min *et al.* 1995).

1.7 Universal ITS primers and ribosomal RNA processing

1.7.1 Ribosomal RNA in eukaryotes

In prokaryotes and eukaryotes, the regions of DNA that contain the genes for rRNA are called ribosomal DNA. There are four ribosomal DNAs in eukaryotic cells, 5S, 18S, 28S, and 5.8S. The ribosomal RNA genes are repeated several hundred times and clustered together in the nucleolus of the cell, and are separated by regions called non transcribed spacers (NTS). This distinguishes them from transcribed spacers which are regions of the gene that are transcribed as part of the rRNA precursor and then removed in the process to produce mature rRNA. RNA polymerase I transcribes the ribosomal RNA (rRNA) genes (except 5S RNA) for the processing of rRNA, while polymerase II transcribes genes coding for proteins and for small nuclear RNAs. Polymerase III is involved in the transcription of tRNA and 5S RNA (Kingsman *et al.* 1988).

1.7.2 Eukaryotic ribosomal DNA repeat unit and pre-rRNA

Nucleoli are factories for making ribosomes in eukaryotic cells. These distinctive nuclear substructures form in somatic cells at the one or more chromosomal sites where multiple copies of rRNA genes are present in tandem array (Watson *et al.* 1993). Nucleoli have distinctive protein compositions and possess all the enzymes required for synthesising and processing precursor ribosomal RNA into the 28S, 18S, and 5.8S molecules found in mature ribosomes. 5S rRNA molecules are synthesised elsewhere in the nucleus and are somehow specifically transported to the nucleolus for assembly into nascent ribosomes, as are almost all the 70 to 80 different ribosomal proteins (Rothwell 1993).

The genes for 18S, 5.8S and 28S rRNA are usually found adjacent to one another in the order 18S, 5.8S, 28S with each set of three genes repeated many times to form tandem arrays called rDNA repeat unit. (Figure 1.6). Three of these, 18S, 28S and 5.8 S are produced by post-transcriptional cleavage of a precursor RNA (pre-rRNA). There are typically 100 to 1000 copies of the rDNA repeat unit, the number varying between different eukaryotes. For instance, yeast has 140 rRNA gene sets and human cells have 1250 (Watson *et al.* 1993). Within each nucleolus, the rRNAs are synthesised and associate with ribosomal proteins to produce the ribosomal subunits which are then transported to the cytoplasm where they function in protein synthesis.

1.7.3 Transcription of rDNA repeat units by RNA polymerase

Each rRNA repeat unit (18S, 5.8S, 28S) is transcribed by RNA polymerase I to produce a large precursor rRNA (pre-rRNA) in the nucleolus. The external transcribed spacers -ETS- are transcribed sequences that are located immediately upstream of the 5' end of the 18S sequence and downstream of the 3' end of the 28S sequence. The regions coding for 18S, 5.8S and 28S are separated by the internal transcribed spacer (ITS) which is removed from the primary transcript by endonuclease during processing and is located on either side of the 5.8S sequences. Together, the ETS and NTS regions comprise the intergenic spacer (IGS) region (Mitchell *et al.* 1995; Figure 1.6). The non transcribed spacer sequence between each rRNA repeat unit is not transcribed. The RNA polymerase I is unique in that it only transcribes the rRNA repeat units, and the promoter for RNA polymerase I is upstream of the transcription initiation site in the NTS. The 5S rRNA is produced by transcription for the 5S rRNA genes by the RNA polymerase III. The ribosomal proteins are produced by transcription of the ribosomal protein genes by RNA polymerase II and subsequent translation of their mRNAs.

1.7.4 Processing of pre-rRNA into mature rRNA

The processing is carried out on nucleoprotein (pre-ribosomal) particles rather than on the naked RNA and includes modification as well as nucleolytic cleavage (Veldman *et al.* 1981). Important components of this processing include the small nucleolar ribonucleoprotein particles (snoRNPs), which consist of small nucleolar RNAs (snoRNAs) associated with proteins (Maxwell *et al.* 1995). A multifunctional ribonucleoprotein nuclease (MRP) which is responsible for a non essential cleavage in the formation of the 5' end of 5.8S rRNA in yeasts has been identified and studied (Schmitt *et al.* 1993). To produce the mature 18S, 5.8S, 28S rRNAs in eukaryotic cells, the 45S pre-RNA is processed at specific sites by special ribonucleases to remove the ITS and ETS sequences (Watson *et al.* 1993). The first cleavage removes the 5' ETS sequence and produces a precursor molecule containing all three rRNA sequences. Processing of this transcript then takes place as follows. 20S precursor to 18S rRNA, and the 32S precursor to 28S and 5.8S rRNA are produced by a second cleavage, and 18S rRNA is generated from the 20S precursor by the removal of the ITS sequence. In the processing of the 32S precursor, the molecule folds so that the 5.8S sequence hydrogen-bonds to 28S sequences, allowing the removal of the ETS from the 3' end. The 5S rRNA of the larger subunit is a transcript of DNA found outside the nucleolar organiser. The 5.8S, 28S and 5S subunits become part of large ribosomal subunit, 60 S, while 18S joins with the 40S subunit. Once assembled, the two ribosomal subunits migrate out of the nucleolus through the nucleus, and into the cytoplasm, where they associate with mRNAs and tRNAs and begin the process of protein synthesis (Rothwell 1993).

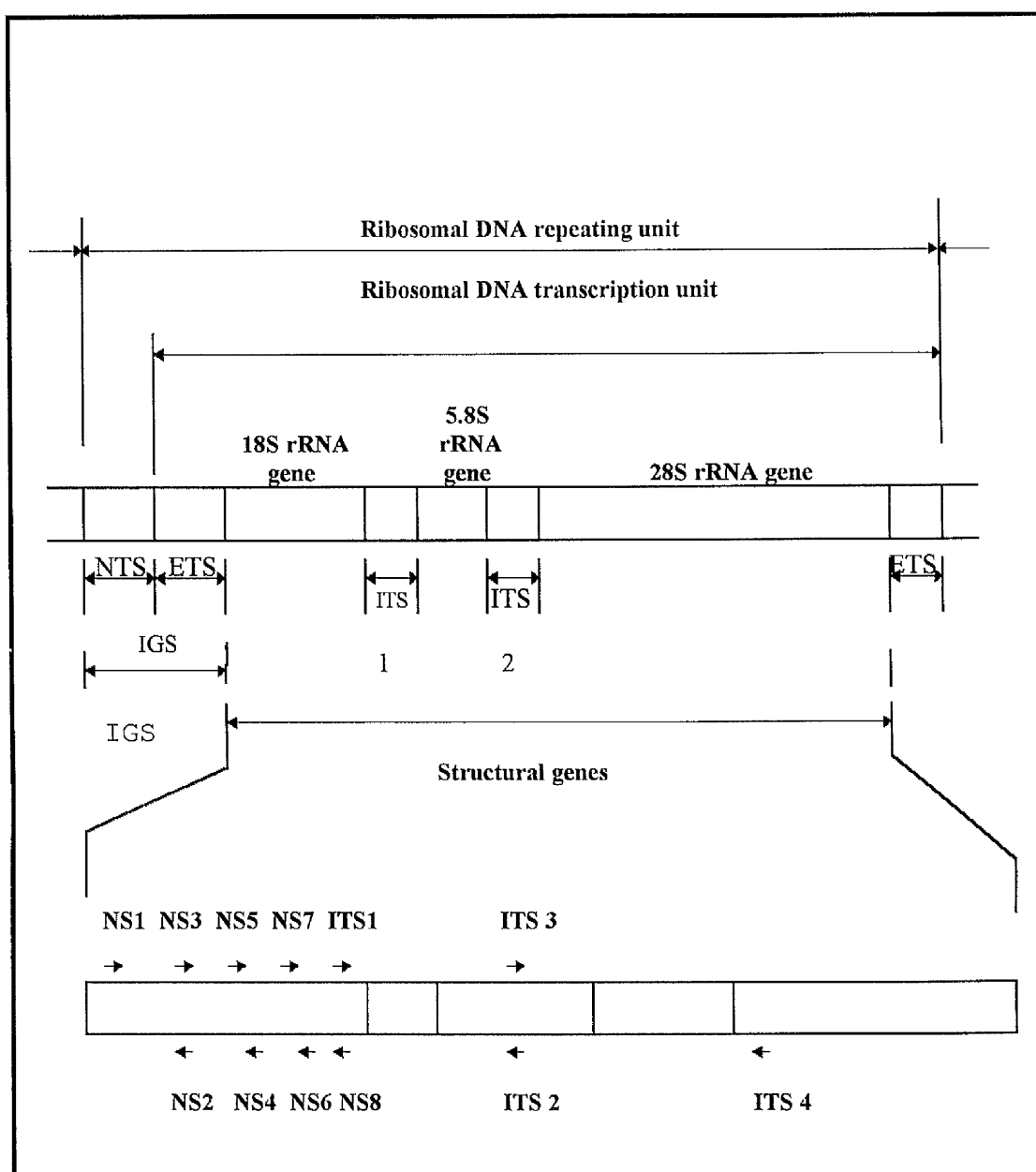


Figure 1.6 Diagram of a eukaryotic ribosomal DNA repeat unit and ribosomal RNA gene cluster showing positions of universal PCR primers.

NTS = non transcribed spacer; ETS = external transcribed spacer; ITS = internal transcribed spacer
IGS = intergenic spacer

1.8 Molecular Typing Methods

1.8.1 Definition and Applications

Molecular typing methods are aimed at identifying and discriminating between isolates at the sub-species level by a comparative fingerprinting approach. This establishes the relatedness of isolates, and its importance in terms of epidemiological analysis has now become evident over the past decade. The primary reason for this is to ascertain whether outbreaks of infection resulted from exposure to a common etiologic agent.

Many traditional methods, such as phage typing, antibiograms and plasmid profiles for bacteria, and serotyping, biotyping and resistotyping for yeasts have been used in the clinical laboratory (Hunter 1991), but these methods are not applicable to more advanced typing methods (Swaminathan *et al.* 1993). Utilisation of other protein-based methods like immunoblot fingerprinting is not frequently used because of unreliable discrimination and difficulties in standardisation. In order to fulfil the requirements of appropriate discrimination for novel methods of typing, particularly for those species that lack discriminating schemes, nucleic acid-based typing methods have been widely employed.

1.8.2 Nucleic acid-based methods

The nucleic acid-based typing methods have been developed and applied to address various epidemiological problems. These approaches include restriction endonuclease analysis (REA) of plasmid or genomic DNA, probe-based fingerprinting, and polymerase chain reaction (PCR) based fingerprinting and it may be categorised as follows. Examples of different molecular typing methods for fungi are described in Table 1.2.

Table 1.2 Examples of DNA typing methods for fungal species.

Fungal species and molecular techniques used	Paper
1) <i>Candida</i> species	
<i>C. krusei</i> , RFLP : <i>Hin</i> fI + Southern hybridisation CkF 1,2 DNA probe	Carlotti <i>et al.</i> (1994)
<i>C. albicans</i> , RFLP : <i>Eco</i> RI	Stevens <i>et al.</i> (1990)
Several <i>Candida</i> species, RFLP : <i>Eco</i> RI, <i>Hind</i> III	Scherer <i>et al.</i> (1987)
<i>C. albicans</i> , RFLP : Ca3	Schmid <i>et al.</i> (1990)
<i>C. albicans</i> , karyotyping, hybridisation with 27A probe, RAPD M13 primer	Berenguer <i>et al.</i> (1996)
<i>C. rugosa</i> , RAPD : two primers, Ca-21 and Ca-22	Redkar <i>et al.</i> (1996)
Several <i>Candida</i> species, RAPD : four primers	Thanos <i>et al.</i> (1996)
2) <i>A. fumigatus</i> ^a	
RFLP: <i>Eco</i> RI +rRNA intergenic spacer probe	Spreadbury <i>et al.</i> (1990)
RFLP : <i>Sal</i> I, <i>Xho</i> I	Denning <i>et al.</i> (1990)
RFLP : <i>Xba</i> I	Burnie <i>et al.</i> (1992)
RAPD : 44 primers tested	Aufauvre-Brown <i>et al.</i> (1992)
RAPD : 2 primers	Loudon <i>et al.</i> (1993)
RFLP : <i>Eco</i> RI + MRS ^b probe	Girardin <i>et al.</i> (1994)
RFLP : <i>Eco</i> RI + <i>Fusarium oxysporum</i> telomeric probe, RAPD four primers	Tang <i>et al.</i> (1994)
RAPD : One primer, immunoblot, SDS-PAGE	Loudon <i>et al.</i> (1994)
RFLP : <i>Eco</i> RI + MRS probe	Girardin <i>et al.</i> (1993)
RFLP : <i>Eco</i> RI +MRS probe	Girardin <i>et al.</i> (1994)
3) <i>A. niger</i> ^c	
RFLP : RFLP : <i>Hha</i> III and <i>Bgl</i> II	Varga <i>et al.</i> (1994)

^{a,c}: Information from Birch *et al* paper (1995); ^b: moderately repetitive sequence.

1.8.3 DNA - sequencing

For the differentiation of yeast isolates, the direct comparison of genomic DNA sequences is the best means of determining whether two strains are similar or different. The combination of PCR to amplify genomic DNA for sequence similarities and differences has recently been developed. However, DNA sequencing remains an expensive and complex method for typing (Swaminathan *et al.* 1993). Practical use of this method could be replaced in the future. The combination of PCR to amplify genomic DNA fragments (Mullis *et al.* 1987) and an automated DNA-sequencing procedure involving fluorescent dye labelled terminators (Applied Biosystems, Foster City, Calif.) to direct sequence the PCR amplified DNA fragments allows a 300 to 500 bp DNA fragment to be sequenced in 24 h (Swaminathan *et al.* 1993).

1.8.4 Chromosomal DNA restriction analysis

This method involves comparison of the number and the size of fragments produced by digestion of total chromosomal DNA with a restriction enzyme (RE). Each restriction enzyme is highly specific for its recognition site so complete digestion of a given DNA sample with a specific RE provides a reproducible array of fragments if digestion is complete. These fragments, usually ranging in size from 1,000 to 20,000 bp, can be separated by size by agarose gel electrophoresis and visualised by staining with ethidium bromide.

Variations in the array of fragments generated by a specific restriction enzyme are called restriction fragment length polymorphisms (RFLPs, Swaminathan *et al.* 1993; Krawczak *et al.* 1994), and have been used successfully for typing. RFLPs is often a result of sequence rearrangements, insertion or deletion of DNA, or base substitution within the RE cleavage sites (Swaminathan *et al.* 1993). The first two types of changes can be observed

with all REs, whereas the third type can be observed only with REs for which the recognition site encompasses the substitution site. The frequent cutting enzymes generate many DNA fragments which can be analysed through the gel electrophoresis. This has been successfully applied to many microorganisms including *Candida* species (Scherer *et al.* 1987; Stevens *et al.* 1990).

The selection of an RE for use in RFLP analysis is based on two important criteria. First, the restriction fragments must be suitable for analysis in terms of size and frequency. Best results are obtained with restriction fragments of 1,000 to 15,000 bp. Second, the fragments in this size range should not be too numerous to avoid overlapping bands that may obscure differences.

Isolation and purification of DNA are critical steps in RFLP analysis. The DNA should be sufficiently intact and free of impurities that may inhibit REs and lead to partial or no digestion. In general, genomic DNA is isolated from yeasts by lyticase, novozyme, zymolase or similar cell wall-digesting enzymes pre-treatment followed by lysis with a non-ionic detergent (e.g. Triton X-100) or an ionic detergent (e.g. SDS) and DNA purification. However, frequent-cutting restriction endonucleases are often not very useful for typing due to the large number of fragments generated and the relatively poor resolution achievable by a conventional agarose electrophoresis (Pitt 1992).

1.8.5 Pulsed-field gel electrophoresis (PFGE)

In the case of large fragments of DNA e.g. > 50 Kb to chromosome size, pulsed field gel electrophoresis (PFGE) in agarose gels has been frequently used as a method to separate DNA. The conformation of the DNA is altered by a voltage gradient, and when an electric

field is applied to the gel, DNA molecules elongate in the direction of the field and migrate into the gel. Then the first electric field is removed and a second field, at an angle to the first, is introduced (Cantor *et al.* 1988). The time required for this reorientation is proportional to the molecular weight. Therefore, molecules of increasing size must spend a larger portion of each switching cycle reorienting before they can migrate through the gel. The most popular technique is contour-clamped homogeneous electric field (CHEF) gel electrophoresis. Its main application is karyotyping analysis, and isolates of *Candida* have been typed by this method. Southern hybridisation with appropriate DNA probes (Magee *et al.* 1987; Mason *et al.* 1987) in conjunction with karyotyping method is also widely used. However, the disadvantages of karyotyping methods are that rather expensive equipment is required and run times take several days limiting the number of strains that can be tested. In addition, because intact DNA is required for PFGE analysis, conventional DNA isolation methods that cause shearing of DNA are not suitable (Swaminathan *et al.* 1993).

1.8.6 PCR-based typing

Recently, PCR-based methods have been used for fingerprinting micro-organisms by using paired primers derived from previously known sequence information (Williams *et al.* 1995). This method has been widely used in many laboratories for the molecular analysis.

1.8.6.1 PCR-RFLP

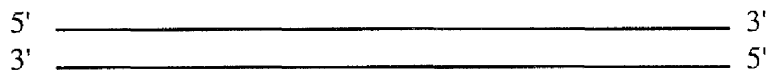
PCR-RFLP involves amplification of a known sequence, cutting with restriction enzymes, and comparing restriction fragments of the amplified DNA from different species (Maiwald *et al.* 1994; Williams *et al.* 1995; Swaminathan *et al.* 1993). This approach offers significant advantages over conventional DNA restriction analyses. Genetic differences are determined by

PCR amplification of a target locus (usually 1 to 2 kb), followed by cutting of the amplicon with different restriction enzymes. It allows differentiation of strains without performing Southern blotting or probing (Swaminathan *et al.* 1993). Another significant advantage of PCR-RFLP over genomic DNA restriction is that problems of poor restriction of genomic DNA as a result of DNA base modification (methylation) are less likely. The target loci for this approach are ribosomal operons and virulence genes.

1.8.6.2 Random amplification of polymorphic DNA (RAPD or AP-PCR)

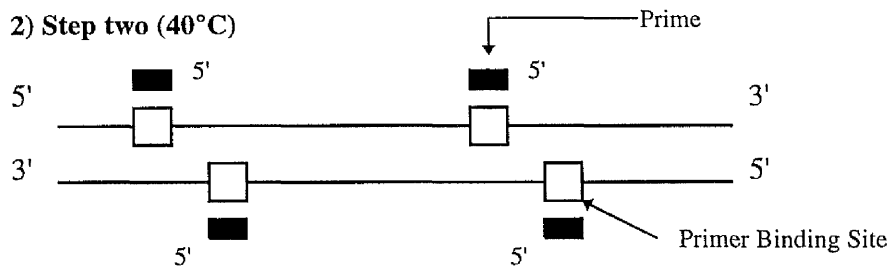
This is a recent molecular typing technique (Welsh *et al.* 1990) using short oligonucleotides (between 5 and 20 bases) to randomly amplify different portions of genomic DNA using the polymerase chain reaction (Figure 1.7). One of the merits of this approach is that an arbitrary primer can be used successfully without any sequence information (Williams *et al.* 1990). Low annealing temperatures (between 36 and 45 °C) are used to permit primer binding to template DNA with up to one or two mismatches. Amplification products are formed if the correctly oriented primer/template interactions are within 100 to a few thousand base-pairs of each other. After PCR cycles, characteristic band patterns are produced dependent on the primers used and targeted genome amplified. This method has been extensively used as a molecular typing technique in many laboratories because it is rapid and allows large numbers of isolates to be screened. A sample containing bacterial or fungal DNA which is either a crude extract or culture supernatant is sufficient for this experiment. RAPD can be used not only to distinguish strains within a species but also to build a genetic map of the degree of relatedness (Welsh *et al.* 1990). However there has been an increasing report of lack of reproducibility (Schierwater *et al.* 1993), suggesting standardisation of this method is a key in obtaining reproducible results.

1) Step one (94°C)



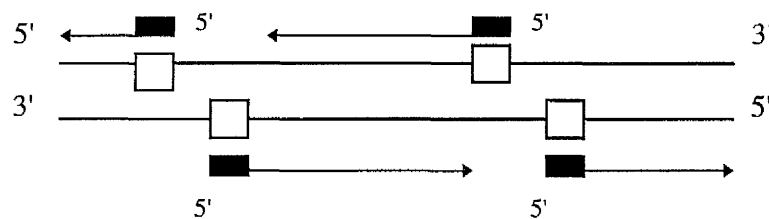
Double stranded genomic DNA is denatured at high temperature.

2) Step two (40°C)



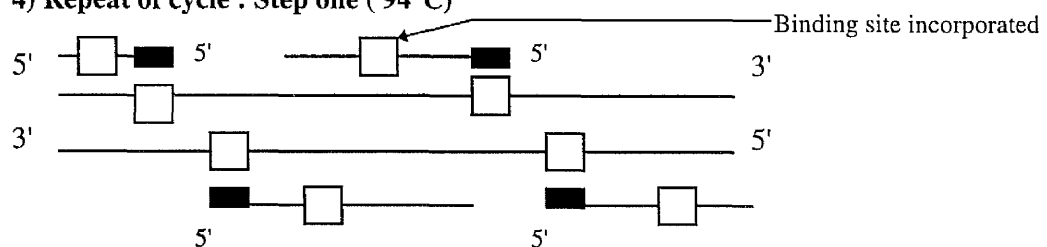
Annealing of randomly chosen primer to partially complementary genomic DNA. Low annealing temperature permits mismatches.

3) Step three (72°C)



Synthesis of DNA from primer by thermostable DNA polymerase (*Taq* enzyme)
Completion of one cycle of amplification.

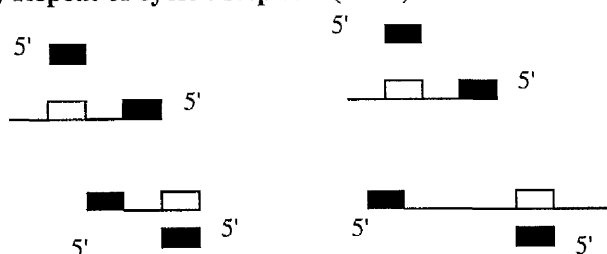
4) Repeat of cycle : Step one (94°C)



Denaturation of newly synthesised fragments. Depending on the location of the primer binding sites, these fragments may contain other primer binding sites.

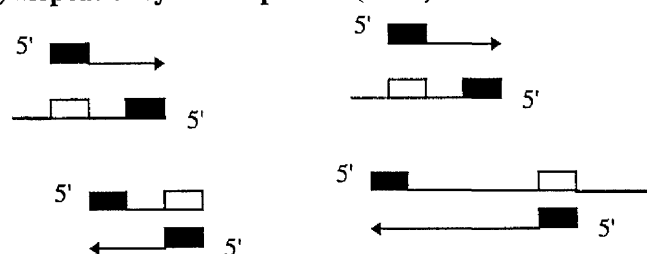
Figure 1.7 Random amplified polymorphic DNA (RAPD).

5) Repeat of cycle : step two (40°C)



Primers are in excess and at the permissive temperature anneal to their complementary sequences, either to template DNA again (not shown) or to the newly synthesised fragments.

6) Repeat of cycle : step three (72°C)



Synthesis of DNA from primer as before.

The fragment pattern is dependent on the original genomic DNA and primer chosen.

Figure 1.7 (continued).

1.8.7 Gene probe typing

The chromosomal DNA restriction fragments could be hybridised with probes, and only the digested DNA which are hybridised with probes are visible, simplifying the fingerprint by decreasing the number of fragments to be compared. Several attempts have been made to type *Candida* species using nucleic acid probes to improve the discrimination of Southern blots of restriction fragment digest electrophoresis (Hunter *et al.* 1991). Several probes such as random or specific chromosomal sequences, toxin and

antibiotic resistance genes, and ribosomal RNA cistrons (ribotyping) have been used for epidemiological study (Einstein 1990). Previously, Schmid *et al.* (1990) used a Ca3 probe to type *C. albicans*, and this probe was found to be highly discriminatory. Magee *et al.* (1987) also used labelled ribosomal DNA to probe an *EcoRI* digest of *C. albicans* genomic DNA and were able to find six different types in 12 strains. Recently, a modification of the enhanced chemiluminescence labelling system was used to produce sensitive rRNA probes. The entire plasmid or the ribosomal operon insert can be labelled with biotin or digoxigenin by using commercially available kits. Although ribotyping has been useful as a typing method, a disadvantage of ribotyping is that labour intensive steps are required (DNA preparations, restriction, electrophoresis, Southern blotting; Swaminathan *et al.* 1993). Furthermore, the appropriate RE(s) must be determined for each bacterial/fungal species.

1.9 Molecular taxonomy of the fungi

One of the important subjects in the study of molecular evolution is how to construct a phylogenetic tree from molecular data (Tateno *et al.* 1982). This subject has been called molecular taxonomy and various methods have been proposed for constructing phylogenetic trees.

1.9.1 Nucleic acid analysis

Increasingly the speciation of fungi with many morphological and phenotypic characteristics in common is likely depend on genetic characterisation. Recently, with the development of fast analytical techniques in molecular biology, the reliance on traditional microbiological tests for gathering phenotypic data has decreased. Of the various chemical components used for taxonomy, only chromosomal DNA and RNA are unaffected by growth conditions (Priest *et al.* 1993). The amounts of these molecules will fluctuate with growth rate, but the composition is invariant. Therefore, nucleic acids offer the only standard molecules by which the widest range of micro-organisms can be compared and classified (Priest *et al.* 1993). Nucleic acid analyses in the context of microbial systematics are described as follows.

1.9.1.1 Taxonomic application of G+C base composition

If two organisms possess DNA with widely different base composition, they will have few DNA sequences in common and are likely to be distantly related. This concept is also useful at the species level. It has been suggested that members of a species should differ by no more than 5% G+C; deviation beyond this limit being indicative of excessive genetic dissimilarity (Priest *et al.* 1993). However, the taxonomic uses of G+C values are mainly exclusionary because fungus species range in G+C content from approximately 30 to 70 mol%, and overlap between unrelated species is inevitable (Kurtzman 1985). For instance, *Debaryomyces*

hansenii and *D. marasmi* have G+C contents of 39.0 and 39.1 mol%, respectively, yet they show only 8.4% base sequence complementarity (Priest *et al.* 1978). In particular, the overlap among the higher fungi is much greater so the use of G+C values has limited taxonomic resolution (Kurtzman *et al.* 1985).

1.9.1.2 DNA reassociation

DNA reassociation technique has been extensively used as a means for determining relatedness within the genus *Candida* (Bak *et al.* 1969). Assessment of base sequence similarity between DNA from two organisms may be readily achieved by DNA reassociation experiments, in which DNA is rendered into single strands by thermal or alkali denaturation and subsequently allowed to anneal in the presence of a second denatured DNA molecule. If the nucleotide sequences of the two DNA samples are largely homologous, hybrid duplexes will be formed by base pairing. If there are few sequences in common, there will be negligible hybrid formation. Thus, this technique provides a quantitative estimate of DNA sequence identity between two organisms (Priest *et al.* 1993). Speciation in a number of ascosporogenous yeast genera has been classified as a result of DNA relatedness studies (Kurtzman 1985). Previously, the 75% relatedness detected between *Pichia lindneri* and *Hansenula minuta* prompted Kurtzman (1984) to propose that the two genera, which are separated on ability to assimilate nitrate, be combined. DNA reassociation studies also showed *C. krusei* to be the imperfect state of *I. orientalis* (Kurtzman *et al.* 1980). The DNA comparisons showed the extent of base sequence complementarity between *Aspergillus flavus* and *A. oryzae* to be 100% (Kurtzman 1985). In addition, studies demonstrated that *C. paratropicalis* possesses >95% DNA-DNA homology with *C. tropicalis* (Wickes *et al.* 1992). Therefore, DNA relatedness studies have had an great impact on our assessment of the criteria used to define species and genera among the fungi.

Although this technique has proven an invaluable tool to fungal taxonomists, there are still limitations in its application to systematic studies. It is known that this method is heavily influenced by external conditions as follows (Johnson 1991):

- 1) DNA reassociation is influenced by the size of the DNA fragments: the larger the fragment the greater the rate of association.
- 2) the rate and extent of renaturation increases as the ionic strength of the incubation buffer is increased.
- 3) purity of the DNA preparation is important (Kurtzman 1985).
- 4) DNA concentration and time of incubation are critical features in reassociation assays.

Furthermore, for the following reasons this technique has made little contribution to the study of genetic variation within species (Selander *et al.* 1986):

- 1) because of the relatively large experimental error associated with DNA hybridisation, the technique lacks the precision required for the analysis of genetic relationships of closely related strains.
- 2) reciprocal experiments often yield nonisomorphic values.
- 3) variation among strains in type and amount of extra chromosomal DNA is potentially a source of error in estimating genealogical (phylogenetic) relationships (Priest *et al.* 1993).
- 4) DNA-DNA homology generally not detect the presence of subgroups (Wickes *et al.* 1992). While this is not important for simple variant, the presence of a distinct subgroup or cluster should be determined in order to account for any potentially important distinguishing characteristics, especially if there are of diagnostic value or play a role in pathogenicity (Wickes *et al.* 1992).
- 5) If there are numerous inversions present within the genome, this technique could give an erroneously low homology value (Wickes *et al.* 1992).

1.9.1.3 DNA restriction patterns

Chromosomal DNA of isolates is digested with restriction enzyme and separated by electrophoresis in an agarose gel and the DNA is then denatured and transferred to a nylon membrane. The immobilised DNA on the filter is hybridised to a labelled DNA probe and the banding patterns visualised. Although this method has been used for distinguishing taxa, the choice of probes is important for reliable results. In addition, it is essential that the DNA is cut into completion otherwise multiple banding patterns from composite fragments will be revealed and will confuse the RFLP pattern (Priest *et al.* 1993).

PCR-RFLP technique has also been used for the phylogenetic relationships among yeasts. After PCR, the product is digested with the appropriate restriction enzyme and electrophoretic separation demonstrates the presence or absence of the restriction site. PCR-RFLP method offers advantages over conventional DNA restriction analyses. For instance, no Southern blotting or probing is necessary. Previously, this technique was used for the recognition of several medically important *Candida* species using rDNA sequences with a variety of restriction enzymes (Magee *et al.* 1987). In addition, estimates of evolutionary relationships based on restriction fragment length polymorphism patterns have been reported for species assigned to the genus *Cryptococcus* (Vilgalys *et al.* 1990). However, such estimates would be expected to be less accurate than estimates derived from sequence comparisons as evolutionary distances increase and the extent of pattern similarities become less certain (Kurtzman 1992).

1.9.1.4 Sequence analysis of rRNA

Analysis of RNA for fungal taxonomic purposes focuses on the three rRNAs; the 5.8S, 18S and 28S genes. In eukaryotes, two internal transcribed spacers (ITS 1 and ITS 2) separate

these three genes, and an external transcribed spacer (ETS) is located upstream of the 18S gene (Figure 1.8; Hills *et al.* 1991; Mitchell *et al.* 1995).

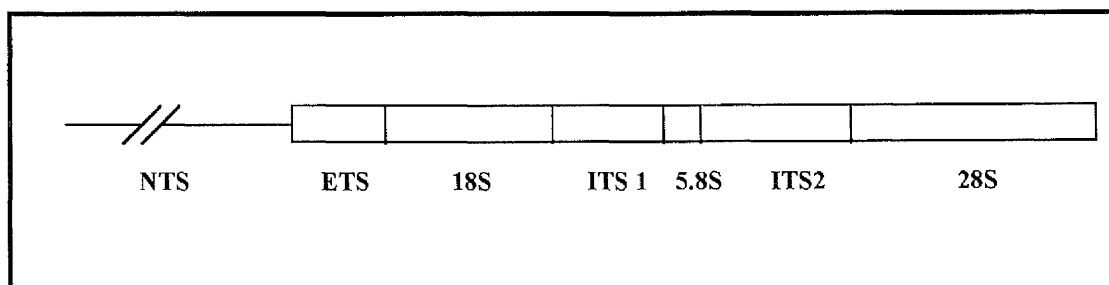


Figure 1.8 The rDNA array of a eukaryote.

Ribosomal RNAs are involved in the structure of the ribosome and the reading of messenger RNA and are present in all living organisms. The DNA coding for ribosomal RNA appears to be among the most highly conserved sequences known, and it offers a means for assessing affinities above the species level (Kurtzman 1985).

Recently, methods for the rapid sequencing of the ribosomal DNA of organisms using universal primers has been developed (White *et al.* 1990). Because rRNA genes are found in all species, they can be sequenced, aligned, and analysed to study phylogenetic relationships at the deepest part of the tree of life (Hills *et al.* 1991). Therefore, these molecules are valuable as indicators of taxonomic relatedness. For instance, the nucleotide sequences of the genes encoding the 18S rRNA has been used for the study of phylogenetic relationships between *Aspergillus flavus*, *A. nidulans*, *A. terreus* and *A. niger*. (Verweij *et al.* 1995). Extent of divergence in partial nucleotide sequences from large and small subunit ribosomal RNAs was used to estimate genetic relationships among ascomycetous yeasts and yeastlike fungi (Kurtzman 1993). The 5.8S rRNA gene was also used for the phylogenetic analysis of *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. krusei* (Lott *et al.* 1993). In addition, the ITS

regions have also been used for differentiating and delineating genealogically closely related species of the genera *Zygosaccharomyces* and *Torulaspora* (James *et al.* 1996). Because the small subunit and large subunit rRNA genes have different rates of evolution, some regions can be no useful for comparisons involving taxa. For instance, studies showed that the small subunit nuclear rRNA gene is among the slowest evolving sequences found throughout living organisms. In contrast, the large subunit nuclear rRNA gene shows more variation in rates of evolution of its different domains than does the small subunit (Hills *et al.* 1991). Furthermore, rRNA comparisons are probably too broad in resolution to detect closely related species. For instance, previous studies showed that 25S rRNA comparisons can not resolve the phylogeny of closely related species because of the highly conserved nature of the sequences (Bicknell *et al.* 1970).

1.9.1.5 Amino acid sequencing

The longer the time after the divergence of two organisms, the more changes (mutations) occur in the DNA sequence encoding the homologous protein in the two organisms (Tortora *et al.* 1992). Therefore, the comparison of amino acid sequences from proteins of two different organisms can help determine the evolutionary relatedness of the species. The determination of the sequence of amino acids in certain important enzymes, such as cytochrome c, glutamine synthetase and superoxide dismutase has been useful in the study of phylogenetic relationships among many different organisms (Baumann *et al.* 1980). However, amino acid sequence analysis are limited to those groups of organisms that share a common protein. Also it can not be assumed that all organisms that share a protein are genetically related (Tortora *et al.* 1992).

1.9.2 Protein analysis

Like the case of nucleic acid analyses, proteins can also be used for both classification and identification. Measurement of relationships between organisms using proteins focuses on either comparisons of individual molecules or gross evaluation of total cellular protein.

1.9.2.1 Multilocus enzyme electrophoresis (MEE)

Rather than an analysis of the total proteins in a cell, the electrophoretic properties of several enzymes that are common to a group can be readily detected in native (non-denaturing) gels through a specific reaction that results in colour formation. Therefore, strains can be classified into groups on the basis of the presence or absence of particular enzymes and by comparison of their electrophoretic mobilities. The rationale of the method lies in the fact that minor changes in gene structure between organisms can result in. The advantage of this technique is that variation in mobility can be directly related to allelic variation of specific genes encoding those proteins (Selander *et al.* 1986).

Multilocus enzyme electrophoresis has been used in large-scale studies to estimate genetic diversity between organisms (Selander *et al.* 1986). It has been demonstrated that estimates of genetic distance based on MEE correlate strongly with those derived from reassociation experiments. However, it would be preferable to compare the gene sequences directly, because comparison of the enzyme mobilities are made visually so there is a possibility of inaccuracies in their estimation. Care has to be taken with the choice of protein analysed as the post-translational modification of some enzymes such as xanthine dehydrogenase, esterase can be a potential source of error in the application of the multilocus enzyme technique to population genetics and systematics (Johnson *et al.* 1981).

1.9.2.2 Comparative electrophoresis of cellular proteins

It is thought that identical organisms have identical protein profiles, that the profiles of closely related organisms show only minor differences, and that the profiles of distantly related organisms show major differences (Tortora *et al.* 1992). Therefore, this method may provide a useful tool for identification as well as classification. Electrophoresis of the total cellular proteins in polyacrylamide gels (PAGE) provides a partial separation in which individual bands mostly represent several proteins. Sodium dodecyl sulphate (SDS)-PAGE has also been used. Protein electrophoresis has several advantages over DNA:DNA pairing: the technique is more rapid than DNA reassociation, there are no lengthy preparation procedures and the data can be entered directly from the densitometer into a computer. However, inter-laboratory comparisons may not always be entirely reliable (Priest *et al.* 1993). Another limitation of the PAGE analysis is that different proteins may not be resolved from one another because they migrate to the same position (Tortora *et al.* 1992). In addition, standardisation of growth conditions may be difficult or impossible. Therefore, comparative electrophoresis of proteins should be used in conjunction with other molecular methods (Priest *et al.* 1993).

1.10 Current taxonomy of *Candida*

Among the yeasts, assignment to genera and especially to species is based not only on morphology but also on the ability to ferment or assimilate various carbon compounds and on the ability to utilise nitrate as the sole source of nitrogen (Barnett *et al.* 1990). In other taxa, such as the rusts, smuts, and certain imperfect fungi, speciation has been based in part on the type of host plant parasitised.

Classification of yeasts relies upon testing a selected panel of biochemical reactions, with a determination made regarding the identity of the isolate supported, in part, by morphological characteristics because the single major factor unifying the species of the genus is the absence of a detectable sexual stage (Wickes *et al.* 1992; Sullivan *et al.* 1996). Although it was known that the assimilation and fermentation of many compounds were controlled by one or only a few genes, conventional classification approaches could result in an erroneous definition of species (Kurtzman 1993). Furthermore, certain physiological test results may be borderline, so that the same isolate may show a positive response on one occasion and a negative response on another (Hunter *et al.* 1989).

Wickes *et al.* (1991) reported that certain *C. stellatoidea* strains spontaneously gave rise to sucrose-positive colonies. In addition, a normal laboratory strain of *C. albicans* spontaneously produces mutants which acquire the ability to assimilate certain carbon sources that are not utilised by the parental strain (Rustchenko *et al.* 1994). Karyotype and fingerprinting data show that there are two kinds of *C. stellatoidea*, termed types I and type II (Kwon-Chung *et al.* 1988), and it has been suggested that type I *C. stellatoidea* should be considered as a subspecies of *C. albicans* (Odds 1988). Thus reliance on assimilation and fermentation of sugars is potentially problematic for species assignment.

Sexual compatibility and mating is another approach to taxonomy. Owing to tradition as well as to technical difficulties, relatively few taxa have been separated from one another because of demonstrated infertility. Therefore this approach has hardly been applied in *Candida*. However, the problems stemming from unknown sexual stages, lack of complementary mating types, and loss of fertility largely can be circumvented in *Candida* because genetic divergence also may be measured by the extent of nucleic acid complementary between taxa.

The species, the primary unit of taxonomy, represents, when defined in terms of genetics, a product of nature rather than an construct of the taxonomist. Species of the genus *Candida* are anamorphic (asexual /imperfect) yeasts classified in the Form-Division Fungi Imperfecti, Form-Class Blastomycetes (Rinaldi 1993). However, it is difficult to find a singular established candidal nomenclature satisfying the opinion of all mycological authorities. Table 1.3 shows current system of classification of *Candida* species.

Table 1.3 One classification system of *Candida* species (Rinaldi 1993).

A) Superkingdom : Eukaryota

B) Kingdom : Fungi (Mycota)

C) Form-Division : Fungi Imperfecti

D) Form-Class : Blastomycetes

E) Form-Order : Cryptococcales

F) Form- Family : Cryptococcaceae

G) Form-Genus : *Candida* Berkhout

**H) Species : *Candida albicans*, *C. guilliermondii*, *C. kefyr*, *C. krusei*,
C. parapsilosis, *C. tropicalis*, *C. glabrata*, *C. norvegensis*,
C. inconspicua, *C. utilis*, *C. lipolytica*, *C. zeylanoides*.**

Some *Candida* species have been found capable of mating with resultant formation of a teleomorphic (sexual/perfect) form. However, these organisms are still classified within the genus. Anamorphic and teleomorphic connections are listed in Table 1.4.

Table 1.4 Anamorph and teleomorph connections.

Anamorph name	Teleomorph name
<i>Candida ciferri</i>	<i>Stephanoascus ciferrii</i>
<i>Candida guilliermondii</i>	<i>Yamadazyma guilliermondii</i>
<i>Candida krusei</i>	<i>Issatchenkia orientalis</i>
<i>Candida lipolytica</i>	<i>Yarrowia lipolytica</i>
<i>Candida lusitanae</i>	<i>Clavispora lusitanae</i>
<i>Candida norvegensis</i>	<i>Pichia norvegensis</i>
<i>Candida pulcherrima</i>	<i>Metschnikowia pulcherrima</i>
<i>Candida utilis</i>	<i>Pichia jadinii</i>

Although the relationships between these *Candida* species and their respective teleomorphic forms have been determined by classical chemotaxonomic techniques, the synonymy of *C. krusei* and *I. orientalis* have been confirmed by molecular techniques (Wickes *et al.* 1992). *Candida* species are in a sense, dimorphic in that they form yeast cells with typical budding (blastoconidia), but also do form true hyphae and pseudohyphae.

The genus *Candida* has been described as a taxonomic pit (Odds 1987), a reflection of the diverse nature of the organisms placed there. The genus *Candida* has been frequently reclassified with respect to the composition of its members. Therefore species have been added, subtracted, and renamed. Much of this activity has involved members that are closely related, but are variable in too many characteristics to classify them as biochemically homogeneous (Wickes *et al.* 1992). Compounding the difficulty of proper classification is the potential for variation, of particular significance for the medically important members of this genus. A partial solution to this problem has been the employment of DNA-DNA homology

for the determination of relatedness by assaying an unknown isolate against a typical (usually the type culture) control. This DNA reassociation technique have been used as a means for determining relatedness among phenotypically similar *Candida* species of medical interest (Bak *et al.* 1969). Their results showed sufficiently high base sequence complementarity to regard *C. albicans*, *C. stellatoidea*, and *C. clausenii* as conspecific.

The taxonomic status of the genera *Candida* and *Torulopsis* has been a source of controversy for many years (Odds *et al.* 1997). The two genera have been distinguished by their ability (*Candida*) or lack of ability (*Torulopsis*) to form pseudohyphae. However this characteristic is not a sufficient criterion for the two genera to be separate, and pseudohyphae formation is not a reliable characteristic for identification of yeasts at the genus level (Odds *et al.* 1997). A constructive contribution to this debate would be to perform an analysis of type strains of the *Candida* and *Torulopsis* genera, and possibly the *Cryptococcus* genus, by molecular techniques, of which the most appropriate method would be comparative nucleotide sequence analysis of ribosomal genes (Sullivan *et al.* 1996).

Recently, molecular phylogenetic analyses based on the rRNA genes have been widely used (see 1.9.1.4 and Chapter 5). Most fungal phylogenetic studies have used sequences from the ribosomal gene cluster, and most of these studies have been performed on the small ribosomal gene sequences.

A recent search of the nucleotide sequence databases revealed that small ribosomal subunit genes have been reported only for a limited number of *Candida* species and other ascomycetous yeasts and not always for type strains (Sullivan *et al.* 1996). Therefore, in order to better understand the species concepts of the genus *Candida*, additional comparative

studies are needed, and future works should be performed with type strains from a wide range of *Candida* species (Sullivan *et al.* 1996).

2.

Chapter 2

Materials and methods

2.1 Chemicals

Chemicals and reagents were purchased from the following companies. Other chemicals used for specific experimental work are mentioned in the text.

- | | |
|---|-----------------------------|
| 1) Agarose | |
| - Molecular Biology Grade | Appligene |
| - NuSieve agarose | FMC |
| - SeaPlaque agarose | FMC |
| 2) Antibiotics | |
| - Ampicillin | Sigma Chemicals Co. |
| 3) Ethidium bromide | Sigma chemicals Co. |
| 4) Trizma [®] base | Sigma chemical Co. |
| 5) Yeast extract | Oxoid Ltd. |
| 6) Bromophenol blue | Fisons Scientific Apparatus |
| 7) Xylene cyanol | Fisons Scientific Apparatus |
| 8) Tween [®] 20 | Sigma Chemicals Co. |
| 9) Sodium citrate | BDH - Merick |
| 10) Phenol: equilibrated to pH 8.0
against Tris buffer | Fisons Scientific Apparatus |
| 11) Chloroform | BDH - Merick |
| 12) Isoamylalcohol | BDH - Merick |
| 13) Lauryl sulfate (SDS) | BDH - Merick |
| 14) Sodium hydroxide | BDH - Merick |
| 15) Tryptone | Oxoid Ltd. |
| 16) Ammonium acetate | BDH - Merick |
| 17) Ammonium persulphate | BDH - Merick |

2.2 Enzymes and markers

The restriction endonucleases and other enzymes used for this study were obtained from the companies listed below.

1) Sequenase [®] 2.0	United States Biochemicals Corporation
2) <i>Taq</i> DNA polymerase	Advanced Biotechnologies Boehringer Mannheim
3) Terminal transferase	Boehringer Mannheim
4) <i>Hha</i> I	New England Biolabs
5) <i>Eco</i> RI	Boehringer Mannheim
6) <i>Msp</i> I	Boehringer Mannheim
7) <i>Scr</i> F I	New England Biolabs
8) <i>Rsa</i> I	Boehringer Mannheim
9) <i>Dde</i> I	New England Biolabs
10) 100 bp DNA ladder	Advanced Biotechnologies
11) λ DNA <i>Pst</i> I digest marker	Sigma Chemicals Co.
12) RNase A	Sigma Chemicals Co.
13) Lyticase	Sigma Chemicals Co.
14) Lysing enzyme	Sigma Chemicals Co.

2.3 Buffers and other materials

1) 40% acrylamide	Ready made solution for sequencing work from Anachem, 380 g of acrylamide, 20 g of N,N'methylenebisacrylamide, made with water up to 600 ml.
2) 0.5 M Tris-Cl	Tris base, adjusted to pH 8.0 with concentrated HCl.

- 3) TE 10 mM Tris-Cl, 1 mM EDTA, pH 8.0.
- 4) 10 X TBE 108 g Tris base, 55.0 g boric acid, 40 ml 0.5 M Na₂ EDTA.2H₂O (pH 8.3).
- 5) 10 X TPE 108 g Tris base, 15.5 ml 85 % (v/v) phosphoric acid, 40 ml 0.5 M EDTA (pH 8.0).
- 6) Gel loading dye 30% (v/v) glycerol, 1% (w/v) bromophenol blue
1% (w/v) xylene cyanol FF,
5 mM EDTA, pH 8.0.

7) Deoxyribonucleoside triphosphates (dNTPs)

Purchased as a stock solution from Boehringer Mannheim, and 30 µl of 2 mM solutions (pH 7.0) were prepared. The 2 mM solution was then used to make the 200 µM dNTP mixtures for PCR and RAPD and stored at -20°C.

8) Easi gel

Purchased from Scotlab, UK

Acrylamide/Bis-acrylamide stock solution

6 % (w/v) acrylamide /0.3 % (w/v) bisacrylamide, 7 M urea, 1 X TBE (ratio 19:1).

- 9) 20 X SSC 3 M NaCl, 30 mM Na citrate, adjusted to pH 7.0
with NaOH.

- 10) Denaturation buffer 0.5 N NaOH, 1.5 M NaCl.

- 11) Neutralisation solution 0.5 M Tris-Cl, 3 M NaCl, pH 7.5.

- 12) 10 M Ammonium acetate Dissolve 770 g of ammonium acetate in 1 liter
of H₂O. Sterilise by filtration.

- 13) 10% Ammonium persulphate Dissolve 1 g of ammonium persulphate in 10 ml of
of H₂O.

- 14) Restriction enzyme buffers

Ready made buffers for restriction digests were provided by the various companies:

- Buffer H : 50 mM Tris-Cl, 10 mM MgCl₂, 100 mM NaCl, 1 mM Dithioerythritol
(DTE, pH 7.5) from Boehringer Mannheim.

- NE buffer 3 : 100 mM NaCl, 50 mM Tris-Cl, 10 mM MgCl₂, 1 mM DTT (pH 7.9) from New England BioLabs.
- NE buffer 4 : 50 mM Potassium acetate, 20 mM Tris acetate, 10 mM Magnesium acetate, 1 mM DTT (pH 7.9) from New England BioLabs.

2.4 Media for bacterial cultures

- 1) LB-Broth 1 % (w/v) Bacto-tryptone
 1 % (w/v) NaCl, 0.5 % (w/v) Bacto-yeast extract
- 2) YEPD Broth 1% yeast extract, 2% Bacto-peptone, and 2% glucose
- 3) L-B Medium As for L-Broth but with 1.5 % agar
- 4) Sabouraud dextrose agar Medium for yeast culture

2.5 Preparation of DNA

2.5.1 Isolates used in this study

A total of 79 isolates (Table 2.1) were obtained from a variety of sources for the experimental work. These included 68 *Candida* isolates representing organisms frequently isolated from patients with candidiasis. Thirty three isolates of *Candida* species were donated by bioMérieux, France, and ten isolates were from American Type Culture Collection (ATCC). Four isolates of *C. inconspicua*, which were clinical isolates from AIDS patients in the Monsall Unit, North Manchester General Hospital, Manchester and four *C. glabrata* strains from NCPF 3309 and Hope Hospital, Salford, Manchester were provided. In addition, 4 procaryotic genomic DNA samples were obtained from the Department of Biochemistry and Pharmacy at the University of Manchester and ATCC. Each isolates source and strain numbers are listed in Table 2.1. All *Candida* isolates were speciated using the API 32C identification strip (bioMérieux, Marcy l'Etoile, France). For the bioMérieux culture collection, the code for each isolate was provided, and the test was not repeated.

Table 2.1 Isolates tested in this study

Isolates / stains	Origin of culture
1. <i>C. inconspicua</i> (21 strains)	
FA / 390 ^a	Manchester
FA / 446 ^a	Hope Hospital, Manchester
FA / 985 ^a	Manchester
SM / 038 ^a	MRI
16783 ^e	ATCC
18949	bioMérieux
3187	bioMérieux
8507247	bioMérieux
8509110	bioMérieux
8509109	bioMérieux
8503199	bioMérieux
8509111	bioMérieux
8510102	bioMérieux
9312113	bioMérieux
8502122	bioMérieux
8502119	bioMérieux
8503197	bioMérieux
0870/IL/0012 (large colony)	Hope Hospital, Manchester
0870/IL/0012 (small colony)	Hope Hospital, Manchester
FA/2924	Hope Hospital, Manchester
087011/0012	Hope Hospital, Manchester
<hr/>	
2. <i>C. krusei</i> (15 strains)	
6258 ^e	ATCC
18888	bioMérieux
62404	ATCC
20298	ATCC
8908008	bioMérieux
8808110	bioMérieux
9105030	bioMérieux
8908006	bioMérieux
8510089	bioMérieux
9112022	bioMérieux
9112020	bioMérieux
9112021	bioMérieux
8908007	bioMérieux
9003063	bioMérieux
Unusual Hope strain	Hope Hospital, Manchester
<hr/>	
3. <i>C. norvegensis</i> (14 strains)	
20686	ATCC
22977 ^e	ATCC
8808100	bioMérieux
8904072	bioMérieux
8503050	bioMérieux
9502007	bioMérieux
8503051	bioMérieux

8903079	bioMérieux
9502006	bioMérieux
8504237	bioMérieux
8503198	bioMérieux
8807095	bioMérieux
FA/1589	Hope Hospital, Manchester
FA/2425	Hope Hospital, Manchester
<hr/>	
4. <i>C. rugosa</i> (3 strains)	
10571 ^a	ATCC
20306	ATCC
34637 ^b	ATCC
<hr/>	
5. <i>C. glabrata</i> (6 strains)	
FA/3309 (small colony) ^c	NCPF
FA/3309 (large colony) ^c	NCPF
FA/2867	Hope Hospital, Manchester
FA/2823	Hope Hospital, Manchester
FA/2983	Hope Hospital, Manchester
FA/2105	Hope Hospital, Manchester
<hr/>	
6. Other isolates (20 isolates)	
<i>C. tropicalis</i> , FA/3504	Hope Hospital, Manchester
<i>C. kefyr</i> , San Antonio	California, USA
<i>C. kefyr</i> , FA/1727	Hope Hospital, Manchester
<i>C. parapsilosis</i> , FA/3511	Hope Hospital, Manchester
<i>C. albicans</i> , FA/1470	Hope Hospital, Manchester
<i>C. albicans</i> , Y109	Hope Hospital, Manchester
<i>C. albicans</i> Hope strain	Hope Hospital, Manchester
<i>C. dubliniensis</i> , FA/448	Hope Hospital, Manchester
<i>C. cylindracea</i>	ATCC
<i>Rhodotorula rubra</i> , FA/3081	Hope Hospital, Manchester
<i>Aspergillus nidulans</i> , G15	University of Glasgow
<i>Aspergillus fumigatus</i> , AF210	Hope Hospital, Manchester
<i>Trichosporon beigelii</i> , FA/2441	Hope Hospital, Manchester
<i>Saccharomyces cerevisiae</i> , FA/3388	Hope Hospital, Manchester
<i>Cryptococcus neoformans</i> , FA/3239	Hope Hospital, Manchester
<i>Treponema denticola</i> , 35405	ATCC
<i>Escherichia coli</i> , PA 360	University of Manchester
<i>Staphylococcus epidermidis</i> F337 ^d	University of Manchester
<i>Staphylococcus anginosus</i> MS12a ^d	University of Manchester
Human placental DNA	Sigma Chemical Co.

^a : Clinical isolates from the patients with AIDS.

^b : Strain which was identified as *C. inconspicua* formerly but re-designated as *C. rugosa* by the author of this study.

^c : Isolates showing phenotypic differences.

^d : Obtained from the Department of Biochemistry, University of Manchester.

^e : Type strains

The aesculin test was included in the ID 32C strip and was read visually either as positive or negative. Rice Agar Tween (RAT) medium (bioMérieux) was also used to determine whether the isolates had the ability to produce pseudohyphae.

2.5.2 *Candida* strains and culture storage

All *Candida* isolates which were obtained from Hope Hospital, Manchester and Boehringer Mannheim including ATCC were inoculated on Sabouraud glucose agar for 48 h at 37°C. One single colony was then inoculated into Sabouraud broth (pH 7.0) for 16 h at 37°C. *Candida* isolates (850 µl) were then added to 150 µl of 100 % glycerol in small tubes and stored at -80°C for further experiments.

2.5.3 Preparation of *Candida* genomic DNA

A single colony from all the *Candida* isolates was incubated in 5 ml Sabouraud broth in universal bottles for 16 h at 37°C in a shaker set at 210 r.p.m. They were then centrifuged at low speed (6,500 rpm) for 5 min. The cells were then washed with 1 ml 1 M sorbitol and centrifuged again for 1 min at low speed. The cells were suspended in 1 ml of PSB buffer (1.2 M sorbitol, 10 mM Tris-Cl, 50 mM EDTA, pH 7.5) with 15 µl of lyticase (40 mg/ml). After incubation for 75 min at 37°C, the cells were lysed by the addition of 10 µl proteinase K (25 mg/ml, Boehringer Mannheim) and 1% (w/v) lauryl sulfate in 500 µl of buffer (0.15 M NaCl, 0.1 M EDTA). After incubation for 45 min at 55°C, the debris was removed by centrifugation and the supernatants were extracted twice with phenol/chloroform/isoamyl-alcohol (25:24:1, v/v/v) and once with chloroform. The supernatant was then transferred to a new microcentrifuge tube and the DNA was precipitated with 1 volume of isopropanol at -20°C for 1 h. The genomic DNA was

washed with cold 70% ethanol and centrifuged at high speed (25,000 g) before being dissolved in 50 µl TE buffer (pH 8.0). Genomic DNA was then stored at 4°C for further experiments.

2.5.4 DNA preparation from *Cryptococcus neoformans*

DNA preparation from *C. neoformans* has previously been described (Varma *et al.* 1991). Briefly, a single colony of *C. neoformans* was inoculated into 5 ml of YEPD broth and incubated at 30°C overnight. The culture was then spun down at 4,000 g for 5 min, and resuspended in 5 ml SCS buffer (20 mM sodium citrate (pH 5.8), 1 M sorbitol). The cells were then pelleted by centrifugation at 4,000 g for 5 min. The pellet was resuspended in 5 ml of protoplasting solution (10 mg/ml lysing enzyme dissolved in SCS) and incubated in a 37°C water bath. The pellet was then spun down and resuspended in 0.5 ml lysing solution (0.45M EDTA, 10 mM Tris hydrochloride, 1% sarkosyl, and 2 mg of proteinase K, pH 8.0). After a 30 min incubation at 37°C, the tube was transferred to a 65°C water bath and incubated for a further 15 min again before being cooled down to room temperature. The mixture was then centrifuged at 20,000 g for 15 min and the supernatant transferred to a fresh tube. An equal volume of chilled isopropanol was then added. After the tube was placed at - 20°C for 10 min, the pellet was collected by centrifugation, dried and then resuspended in 50 µl of 0.3 M sodium acetate. The DNA suspension was then transferred to a sterile tube and 2 µl of RNase (10 mg/ml) was added and incubated at 37°C for 30 min, followed by incubation with 2 µl of proteinase K (20 mg/ml) at 37°C for 15 min. The DNA was extracted twice with 0.5 volume of phenol and then once with 0.5 volume of chloroform. The DNA was precipitated with 2 volumes of 95% ethanol and washed with 70% ethanol. Finally, the DNA was resuspended in 50 µl of sterile distilled water.

2.5.5 Quantitation of DNA

After preparation of the genomic DNA, each sample was then diluted 1:200 in sterile distilled water. The concentration of genomic DNA was then measured in a quartz cuvette at 260 and 280 nm in a Cecil Digital Ultraviolet Spectrophotometer (Series 2, CE 292). The DNA concentration was then calculated by the value of A₂₆₀ on the spectrophotometer. A value of 1.0 is equivalent to a concentration of 50 µg/ml of double stranded DNA. The purity was also measured by calculating 260/280 ratio. Values between 1.8 and 1.9 indicate good DNA preparations and would be suitable for PCR and dot blotting experiments. DNA used in PCR was then prepared by adding an appropriate amount of distilled water to give a concentration of about 20 ng/µl.

2.5.6 Preparation of oligonucleotide primers and probes

Synthetic oligodeoxyribonucleotides (ITS 1: 5'- TCG GTA GGT GAA CCT GCGG -3', ITS 2: 5'- GCT GCG TTC TTC ATC GAT GC - 3', ITS 3 : 5'- GCA TCG ATG AAG AAC GCA GC - 3' and ITS 4 : 5'- TCC TCC GCT TAT TGA TAT GC - 3') were prepared by beta-cyanoethyl phosphoramidite chemistry with a 392 automated DNA/RNA synthesiser (Applied Biosystems, Calif, USA) in the Cell - Matrix Unit at Manchester University on the basis of data previously reported (White *et al.* 1990). The primer concentration was determined by measuring the A₂₆₀. It was assumed that a value of 1.0 was equivalent to a concentration of 20 µg/ml. Each primer was then diluted to 5 µM as a stock solution and stored at -20 °C.

Arbitrarily primed polymerase chain reaction (AP-PCR, RAPD) as described by Welsh *et al.* (1990) was carried out for fingerprinting *Candida* species. The synthetic oligo

deoxyribonucleotides primer (Aufauvre-Brown *et al.* 1992), R108 (5'- GTA TTG CCC T - 3') was also prepared by the same method described above. Each stock solution of primers was diluted to 5 μ M and stored at -20°C .

A species-specific probe for *C. inconspicua*, CIP (5'- GGA GCC ACC CAA AGG TGA CAA -3') which corresponds to the nucleotides of the ITS 2 region (26 bp to 46 bp) was prepared on the basis of sequencing results from the PCR products of *C. inconspicua* (bioMérieux, 18949) and amplified using ITS 1 and 4. Another species specific primer, CKP (5'- GCT CCG ACG CTC TTT ACA CGT-3') which flanks the ITS 2 region (from 42 bp to 62 bp) of *C. krusei* (ATCC 6258) was also prepared based on the data derived from sequencing results. The molarity of the synthetic oligonucleotide primers was calculated and diluted to 5 μ M for the PCR reaction and 100 μ M for dot blotting. The diluted primers were stored at -20°C.

2.6 Gel electrophoresis

2.6.1 High resolution gel electrophoresis

PCR amplicons were run at 5 V cm⁻¹ for 2.5 h on 3% (3 : 1) NuSieve agarose gels which have low viscosity in 1X TPE buffer. The gel was then stained with ethidium bromide solution (1 μ g/ml in 1 X TPE buffer) for 20 min and destained with distilled water for 15 min. The gel was photographed on a UV transilluminator using Polaroid 667 & 665 film.

2.6.2 Preparation of polyacrylamide gels

For DNA sequencing, ready made 40% (w/v) acrylamide/bis-acrylamide (37.5 :1 ratio) or Easi-gel was used for the preparation of 6% polyacrylamide gels. The V3-11 sequencing gel

unit (Anachem) was used as the electrophoresis apparatus. Fresh 10% ammonium persulfate (300 μ l) and 20 μ l of TEMED (N,N,N',N',tetramethylethylenediamine) were added to 40 ml of acrylamide solution for gel polymerisation. Two to three ml of dimethyldichlorosilane solution (BDH laboratories, England) was applied to one side of the glass sequencing plates in the hood and assembled to ensure that both plates were easily separated after running the gel. The polyacrylamide solution was then poured into the space between the two plates, whilst carefully avoiding the formation of air bubbles. The flat side of a shark's tooth comb was then inserted into to form the wells in which the samples were loaded.

For the longer sequences, the wedge gels which are thicker at the bottom than at the top were also prepared to produce a more even banding pattern. After polymerisation of the gel, the comb was removed and the gel mould was attached into the lower sequencing gel unit. One litre of 1 X TBE buffer was poured onto both the upper and lower tank before running the gel. The gel was pre-run at 55°C for 30 min before loading the samples.

2.7 Polymerase Chain Reaction (PCR)

2.7.1 PCR optimisation

The yields and specificity of PCR products have been influenced by many reaction conditions, therefore PCR was carried out under several different conditions described below in order to generate optimum results.

2.7.1.1 Preparation of MgCl₂

The magnesium (Mg²⁺) ion concentration in PCR reaction mixtures is the most important factor in determining specificity of PCR. Magnesium ions not only stimulate the *Taq*

polymerase activity, but increase the melting temperature (T_m) of the double stranded DNA and primer/template interaction (Newton *et al.* 1994). Increasing the concentration of magnesium not only has the net effect of decreasing the stringency of primer binding, but stabilises double stranded DNA and prevents complete denaturation of the product at each cycle, which would otherwise reduce yield. In contrast, low magnesium ion concentrations may also result in poor reaction efficiency (Persing 1993). The $MgCl_2$ titration was prepared in the range of 1 mM to 3.5 mM, and the reaction was carried out in order to find the optimum concentration of $MgCl_2$. The total volume of reaction mixtures was 50 μ l containing different Mg^{2+} ion concentration in 0.5 ml PCR tubes.

Two buffer systems, Buffer IV (Advanced Biotechnologies) and BM Buffer (Boehringer Mannheim) were tested under the same conditions in order to find the optimum conditions required. A 15 mM stock solution of $MgCl_2$ (Advanced Biotechnologies) was prepared and stored at -20°C.

2.7.1.2 Prevention and control of contamination

In order to minimise the contamination during PCR experiments, each reaction was carried out in separated rooms. Ultra-pure water for PCR was provided by the Department of Biochemistry, University of Manchester and stored at -20°C. In order to minimise the risk of contamination during experiments, products from completed reactions were handled with a separate set of micropipettors at different locations in the laboratory. Micropipettors for handling genomic DNA were also used only in a specified area. Pre-sterilised disposable microliter pipette tips with filters (Rainin, MA, USA) were used for PCR. Negative controls containing the same amount of water, dNTPs, $MgCl_2$ buffer but not genomic DNA were also

included in experiments as an assay for overt contamination. Positive controls with type strains (*C. inconspicua* ATCC 16783, *C. krusei* ATCC 6258, *C. norvegensis* ATCC 22977 and *C. rugosa* ATCC 10571) and several fungal and procaryotic DNA (*E. coli* PA 360, *S. epidermidis* F337 and *S. anginosus* Ms12a) were also included for the efficiency and specificity of PCR.

2.7.1.3 Calculation of melting temperature (T_m)

The G+C content of an oligonucleotide primer is important in determining duplex stability. G and C bases have 3 hydrogen bonds whereas A and T have 2 hydrogen bonds. The T_m is roughly calculated for oligonucleotides which are 20 bases long or less by the following equation (Meinkoth *et al.* 1984) : $T_m = 2 * (\text{number of A+T residues}) + 4 * (\text{number of G+C residues})$. T_m is the temperature at which half of the DNA strands have become separated in solution. Obviously, the T_m would also therefore depend on the oligonucleotide concentration, the sequence composition, and the composition of the solvent. The applicable annealing temperature could be 3 to 5°C lower than the T_m calculated from the above formula as a starting point for optimisation (Zyskind *et al.* 1992). Because this formula is inaccurate with primers longer than 20 nucleotides (Newton *et al.* 1994), T_m values for nucleotides between 20 and 35 can also be calculated by the following equation : $T_p = 22 + 1.46 \times (\ln)$ where T_p = optimised annealing temperature ± 2 -5; \ln = effective length of primer = $2 \times (\text{G+C}) + \text{AT}$ (Wu *et al.* 1994). For annealing temperature optimisation, different temperatures were tested under otherwise identical conditions.

2.7.1.4 Determination of primer concentration and buffer systems

In order to know whether different concentrations of primers can affect PCR patterns, concentrations in the range of 0.1 μM to 0.5 μM were prepared and tested. A slightly lower primer concentration (0.25 μM) was however used in our experiments to prevent primer dimer formation.

For the optimum PCR condition, different buffers, BM buffer (10 mM Tris-HCl, 25 mM KCl, 5 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO_4 , pH 8.85, Boehringer Mannheim) and buffer IV (20 mM $(\text{NH}_4)_2\text{SO}_4$, 75 mM Tris-HCl, 0.01 % (w/v) Tween pH 9.0, Advanced Biotechnologies) and 2 different *Taq* DNA polymerase enzymes (BM *Taq* polymerase, Boehringer Mannheim and AB *Taq* DNA polymerase, Advanced Biotechnologies) were tested. Buffer IV showed good results with AB thermostable enzyme (Advanced Biotechnologies). Increasing cycle numbers can lead to unwanted artifacts and reduced enzyme activity, therefore a total of 30 cycles was performed in a Crocodile II Thermocycler (Appligene).

2.7.1.5 PCR conditions used after optimisation

For the experiments described in this study, the following conditions were used; 0.1 μg of genomic DNA, 0.25 μM of each oligonucleotide, 200 μM of dNTPs, 1.5 mM MgCl_2 , 5 μl of buffer IV and 2.5 U of *Taq* polymerase (Advanced Biotechnologies). The master mix which contained dNTPs, MgCl_2 , primers, water and buffer was prepared to ensure that the correct amount of material was allocated into the reaction tubes after the genomic DNA was added. 50 μl of mineral oil was added to prevent evaporation. The PCR reaction tubes were then placed in a thermocycler for 5 min at 95°C without *Taq* polymerase. 0.5 μl (2.5 U) of *Taq* polymerase was then added and the reaction performed as follows : 94°C for 1 min, 56°C for

1 min, 72°C for 1 min for 30 cycles followed by 94°C for 1 min, 56°C for 1 min and 72°C for 5 min for 1 cycle. Samples were stored at 4°C after each run.

2.7.2 Direct PCR from colonies

An alternative approach was direct PCR which did not require preparation of genomic DNA (Niester *et al.* 1993). A single colony from an agar plate was picked and mixed with 1 ml of sterile distilled water and then vortexed vigorously for 20 s. 1 µl of mixture was then used as template and added to tubes containing the same amount of dNTPs, MgCl₂, primers, reaction buffer, and *Taq* polymerase as conventional PCR and performed using the same cycling conditions. The PCR products were visualised on an agarose gel and compared with the PCR patterns carried out with extracted genomic DNA.

2.7.3 PCR conditions for the detection of *C. inconspicua* and *C. krusei*

Rapid identification of *Candida* species, particularly *C. inconspicua* and *C. krusei*, which is often difficult due to their similar phenotypic (Mata Essayag *et al.* 1996) and genotypic characteristics, PCR-based methods using species specific primers and ITS 1 were therefore developed. All conditions used in this section were the same as described in PCR optimisation section except for annealing temperature. Several different annealing temperatures in the range of 58 to 65°C were carried out to find the optimum temperature which generated single amplified products of *C. inconspicua* and *C. krusei* using ITS1, CKP and CIP primers. In the end, 63 to 65°C appeared to be the optimum annealing temperature in these experiments.

2.7.4 Random amplified polymorphic DNA (RAPD)

2.7.4.1 RAPD conditions

RAPDs (Welsh *et al.* 1990) were carried out for the differentiation of yeast species using the R108 primer. For RAPD analysis, reaction mixtures containing 0.1 µg of genomic DNA, 0.5 µM single oligonucleotide, 200 µM dNTPs, 2.5 mM MgCl₂, and buffer IV were used. DNA was denatured at 95°C for 5 min without *Taq* DNA polymerase, and then after addition of 0.5 µl (2.5 U) *Taq* DNA polymerase as follows : 94°C for 1 min, 36°C for 1 min and 72°C for 1 min for enzymatic extension for 30 cycles followed by 94°C for 1 min, 36°C for 1 min and 72°C for 5 min for 5 cycles.

2.7.4.2 Analysis of RAPD profiles

RAPD profile comparison is best carried out in a two dimensional form as a similarity matrix. The matrix can be ordered to group together strains and displayed in a simplified format as a dendrogram. The algorithm used groups the profiles on the basis of their similarity unweighted pair group with mathematical averaging method (UPGMA). The Bioimage Whole Band Analysis (WBA) computer software package (Millipore, UK) on a SunSparc Station 2 was used for RAPD analysis. After scanning the gel picture, each band on the gel was identified and marked by the programme and visualised as a lane map.

For better RAPD analysis, the SPSS programme (Bandi *et al.* 1995) was also used to generate reliable dendrograms. Each band with the same molecular weight on the gel was compared visually, and marked 0 or 1 as a binary matrix on the basis of absence or presence of bands. The similarity was based on the following equation ; $(2 * a) / (2 * a + b + c)$; a = bands present on both strains at a given molecular weight; b, c = bands present on one strain at

given molecular weight, were then measured by Average Linkage Method (between groups). The numeric string generated in this manner was used to determine relatedness values among strains. The patterns of bands of 34 *Candida* isolates (10 *C. inconspicua*, 10 *C. krusei*, and 10 *C. norvegensis*) from bioMérieux including 4 reference strains (*C. inconspicua* SM/038 from patients with AIDS, *C. krusei* from ATCC 6258, *C. norvegensis* from ATCC 20686 and *C. glabrata* from NCPF 3309) along with size marker (λ *Pst*I digest) were then printed out by the computer.

2.7.5 Restriction enzyme digestion of PCR product

The polymerase chain reaction products were digested with several restriction enzymes as mentioned in the general information section under conditions recommended by the suppliers.

2.7.5.1 Preparation of amplified DNA in the reaction vial

After each PCR, 50 μ l of PCR product was transferred to a sterile centrifuge reaction vial (L.I.P., West Yorkshire, England) without disturbing the oil. One tenth volume of 3 M sodium acetate (pH 5.0) and 3 volumes of chilled absolute ethanol were then added. The DNA was then precipitated at -20°C for 1 h and centrifuged at high speed (12,000 g) at 4°C. The pellet was washed with chilled 70% ethanol and dried at room temperature. Finally the pellet was dissolved in 50 μ l of TE (pH 8.0).

2.7.5.2 Enzyme digestion of PCR products

The concentration of DNA was measured by agarose gel. A small aliquot of the purified DNA was run alongside DNA size markers (low ladder 100 bp, Advanced

Biotechnologies, UK) of known concentration. The intensities of DNA were compared visually allowing the concentration of amplified DNA to be measured. Approximately 0.2 µg of DNA was then mixed with one tenth volume of 10 - 20 U 10 X restriction enzyme and NE Buffer 3 or Buffer 4. The total volume was 20 µl, and reaction tubes were incubated for 2 to 3 h at 37°C. The resulting band patterns were visualised and photographed on a NuSieve agarose (3:1) gel.

2.7.5.3 Digestion of plasmid DNA with *EcoRI*

It was possible that false white colonies appeared when 3' T-overhangs were lost and the vector religated. Plasmid DNA from white colonies was therefore digested with *EcoRI* in order to confirm genuine clones for sequencing. Approximately 1 µg of plasmid DNA was digested with 2 Units of *EcoRI* in a reaction containing one tenth of *Sure/Cut* Buffer H (50 mM Tris-HCl, 10 mM MgCl₂, 100 mM NaCl, 1 mM Dithioerythritol, Boehringer Mannheim) to ensure correct clones were selected. Digested plasmid DNA was run on a 1.5% agarose gel and correct clones were selected and stored at -20°C.

2.8 Sequencing

2.8.1 PCR cloning using TA vectors (Harwood *et al.* 1994)

This protocol is based on the inherent terminal transferase activity of *Taq* polymerase, which tends to add a template independent single deoxyadenosine (A) residue to the 3' ends of the PCR product. The kit therefore uses a cloning vector which has a single thymidine (T) base overhang thereby boosting chances of a complementary successful ligation of a PCR products (Marchuk *et al.* 1991). This allows direct sticky end ligation of PCR products containing *Taq* polymerase catalysed an extension, without further enzymatic processing (Figure 2.1).

2.8.2 TA Cloning vector preparation and transformation test

The pCRTM TA cloning vector (Figure 2.2) was purchased from Invitrogen, De Schelp, Netherlands and prepared according to the manufacturer's instruction. Briefly, the lyophilised cloning vector was prepared by adding TE buffer (pH 8.0) to a final concentration of 25 ng/ μ l and stored at -20°C.

In order to test the stability of the vector before sequencing work, a self ligation test was performed as follows; the ligation buffer, resuspended vector and T4 DNA ligase (Invitrogen) were incubated at 12°C overnight. After transformation into *E. coli* INV α F', the cells were streaked on to LB agar containing ampicillin (50 μ g/ml) and 25 μ l of blueo-gal (40 mg/ml of blueo-gal in dimethyl formamide, Life Technologies, NY,USA) and then incubated at 37°C for 40 h. A T:T mismatch self ligation would show up as blue colonies. As the vector loses stability, more white colonies would be obtained because of the loss of 3' T overhangs leading to blunt end self ligation of the vector. The ligation reaction was then carried out in the presence of 10 X ligation buffer, pCRTM vector, 12.5 ng of diluted PCR product and T4 DNA ligase overnight at 12°C.

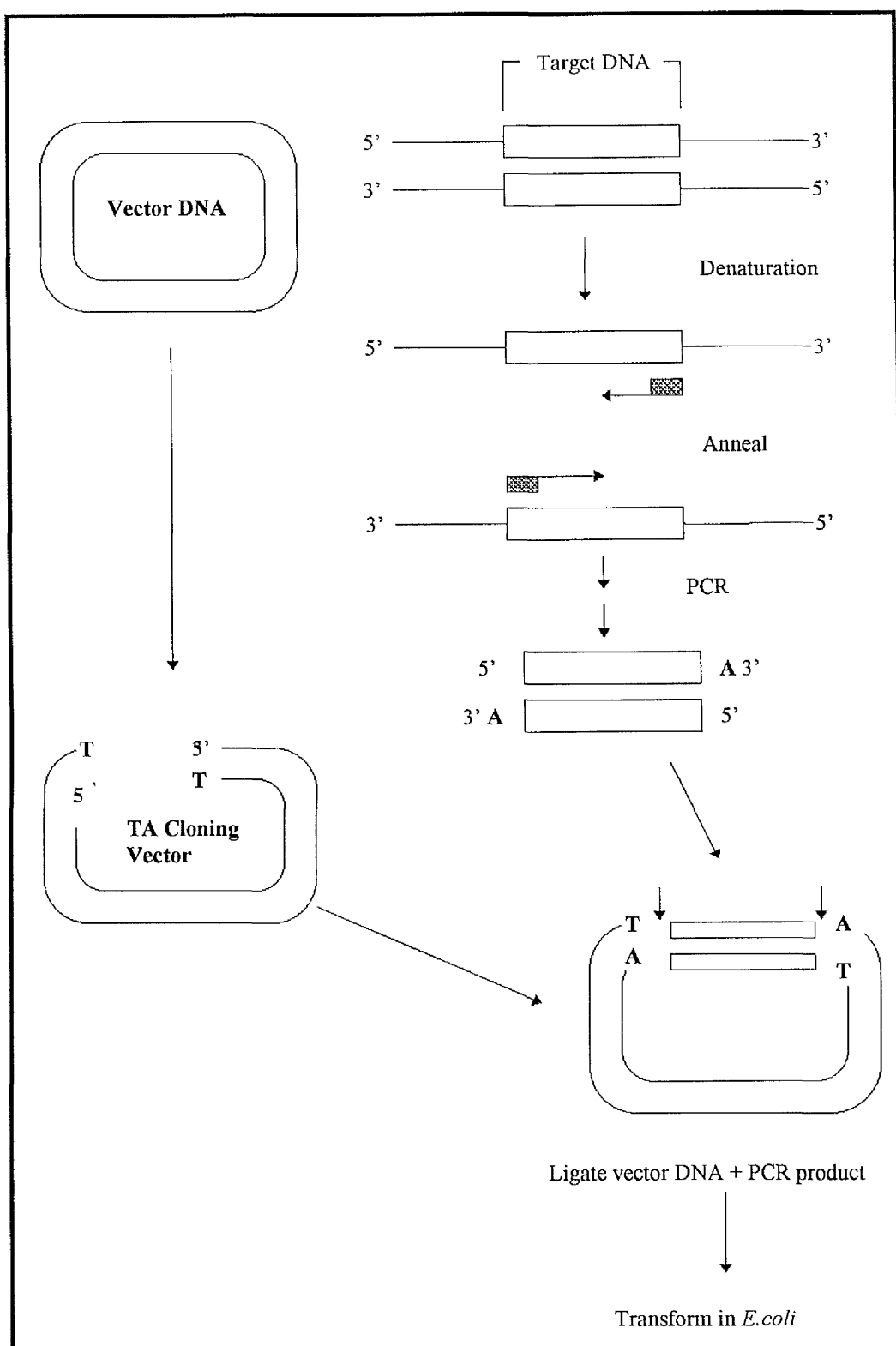


Figure 2.1 Cloning of PCR products into the TA cloning vector for sequencing.

2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added and incubated at 37°C for 1 h in a gyrator shaker-incubator at 225 rpm. 25 µl of blue-gal (40 mg/ml) was spread on LB agar plates containing ampicillin (50 µg/ml) using a glass spreader. 25 and 100 µl from each transformation vial was spread on the plates and incubated at 37°C overnight.

2.8.3 Recovery of amplified DNA from agarose gels

PCR using ITS primers (ITS1, 2, 3 and 4) was performed for sequencing under the same conditions as mentioned in the PCR optimisation section except that the dNTPs concentration was 20 µM. The PCR products were made visible on the gel by using long wave length UV (365 nm) as this causes less damage to the DNA (Sambrook *et al.* 1989). The DNA band was carefully cut out using a sterile razor blade on clean saran wrap. It was then cut up and placed into a sterile tube then vortexed vigorously for 30 s after which it was placed at -80°C for 10 minutes. The aqueous phase was then transferred to new tubes and re-extracted with phenol and chloroform twice. 3 volumes of chilled isopropanol and one tenth volume of 3 M sodium acetate (pH 5.2) were added and re-centrifuged at high speed (12,000 g) for 1 minute. Finally the DNA pellet was washed with 70% (V/V) ethanol, dried at room temperature and re-suspended in TE (pH 8.0). The concentration of recovered DNA was measured by a UV spectrophotometer and stored at -20°C.

2.8.4 Selection of white colonies on LB plates and plasmid preparation

After incubation, several white colonies on the LB plates were selected, and inoculated into an universal bottle containing 5 ml of LB broth with ampicillin (50 µg/ml) for 17 h at 37°C in a rotary shaker-incubator.

The plasmid preparation kit (Hybaid, Middlesex, UK) was used to isolate plasmid containing PCR products for sequencing. Briefly, after spinning 1.5 ml of an overnight bacterial culture for 30 s, the cells were resuspended in pre-lysis buffer (100 µg/ml RNase A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0). Alkaline lysis solution (200 mM NaOH, 1% lauryl sulfate) was then added and mixed until the solution was clear and viscous. Neutralising solution (3 M KAc pH 5.5) was then added before vortexing. A white precipitate consisting of cell membranes, proteins, and chromosomal DNA was formed which was pelleted by centrifugation for 2 min. The supernatant was transferred to a microcentrifuge tube kit supplied with a spin filter (Hybaid). 250 µl of binding buffer was then added to same tube and mixed. The liquid was collected by centrifugation for 1 min, then the same procedure was followed again. Finally, the spin filter was transferred to a new tube and mixed with 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and plasmid DNA was collected by centrifugation. After the concentration of plasmid DNA was measured, the DNA was stored at -20°C.

2.8.5 Sequencing reactions

Double stranded DNA was sequenced by the dideoxy chain termination method (Sanger *et al.* 1977). Because 2'3' dideoxynucleoside triphosphates have no hydroxyl residue at the 3' position of deoxyribose, no phosphodiester bond with dNTPs could be formed, therefore further extension of the growing chain is impossible. The sequenase (ver. 2.0, USB, USA) which has no 3' - 5' exonuclease activity was purchased and sequencing was carried out.

Approximately, 3 µg of plasmid DNA was mixed with 3 pmol of primer and made up a total volume of 11 µl with water. Several different universal primers : M13 forward primer (16

mer) 5'- TCG TGA CTG GGA AAA C- 3'; M13 reverse primer (17 mer) 5'- CAG GAA ACA GCT ATG AC -3'; M13 (-20) primer (16 mer) 5'- CTG GCC GTC GTT TTA C- 3' were used as sequencing primers; ITS primers, 1,3 and 4, were also used.

The DNA was denatured by the addition of 1 M NaOH. After incubation for 10 min at 37°C, 1 M HCl and plasmid reaction buffer (100 mM MgCl₂, 20 mM NaCl, 400 mM Tris-HCl, pH 7.5) were added to the reaction tubes and incubated for a further 10 min at 37°C. The 1 : 5 diluted 7-deaza-dGTP labelling mix (7.5 µM 7-deaza-dGTP, 7.5 µM dCTP, 7.5 µM dTTP) was then prepared, and sequenase enzyme was diluted with enzyme dilution buffer (5 mM DTT, 0.5 mg/ml BSA, 10 mM Tris-Cl, pH 7.5). The above template-primer mix, 0.1 M DTT, diluted labelling mix, and 0.5 µl [α -35S] dATP were then mixed together and 2 µl of diluted sequenase enzyme was added. After 5 min incubation at room temperature, 5.5 µl of this reaction mixture was transferred to the tube labelled ddT, ddA, ddG and ddC, and incubation was continued at 37°C for 5 min. Finally, the reaction was stopped by addition of 4 µl of stop solution (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol FF) to each reaction tube. When the polyacrylamide gel was ready to load, the samples were heated to 80°C for 10 min in order to detach the chain terminated molecules from the template DNA, and 2.5 µl of each sample was immediately loaded into the wells. The reaction samples were loaded again after 1 or 2 h in order to read sequence further away from the primer. The top and bottom strands of DNA from different clones were read using both forward and reverse primers to ensure that correct sequence was generated. After running the samples on a polyacrylamide gel, the gel was fixed in a tank containing 10% acetic acid and 10% methanol for 30 min to eliminate the urea followed by drying in a gel dryer (BioRad, UK) for 1 h at 80°C.

Automated DNA sequencing was also performed using *Taq* DyeDeoxy terminator chemistry (Applied Biosystems) in a 373A DNA sequencer (Applied Biosystems) according to the manufacturer's instructions. Because pure template DNA is important to generate good results, amplified DNA was purified again using phenol/chloroform methods, and the A_{260/280} ratio was checked to confirm purity of the template DNA. Four different fluorescent dyes were used for labelling, one attached to each of the four 3' dideoxynucleotide terminators. The ITS1 primer was used, and cycle sequencing with *Taq* polymerase was carried out according to the supplier's instruction. To avoid secondary structure problems, dITP was used in place of dGTP. After completion of the reactions, samples were loaded onto a polyacrylamide gel in the DNA sequencer for electrophoresis. Fluorescence was excited by laser and the signals were analysed by computer, and chromatograms were printed out.

2.8.6 Autoradiography and phosphoimaging

After electrophoresis the banding pattern was visualised by autoradiography. The dried gel was exposed onto X-ray film (X-ograph imaging system, England or BioMax MS, Kodak) at room temperature for 24 h. The X-ray film was developed in developer (Kodak) for 5 min followed by washing. The film was then fixed in a tank containing fixer (Kodak) for 3 min. The film was then washed again and air dried.

For faster autoradiography results, the sequence from PCR products were read through a Fujix BAStation ver 1.3 (Fuji photo film co., Ltd) A bioimage analyser on Solair workstation and a Fuji imaging plate (20 x 40Cm type Bas-III) on which 1 h exposure is equivalent to 24

h of standard X-ray film was used for reading sequences. The sequence on the film was printed out through LBP-8IV automatic image refinement printer (Canon) and carefully read twice.

2.8.7 Computer analysis of sequencing data

The nucleotide sequence database query/retrieval (Rodriguez-Tomé *et al.* 1996) from European Bioinformatics Institute (EBI, Hinxton Hall, UK), which is an EMBL outstation was used to compare sequence homology. A molecular probe data base (MPDB, Campi *et al.* 1996) using Network Information Retrieval tools on Netscape was used as a reference for synthetic oligonucleotides including their identification and application. For the analysis of restriction enzyme sites on the PCR products, DIGEST program (ver. 1.0) was used.

For the DNA parsimony, bootstrap analysis and preparation of phylogenetic tree, the Tree View program was used. Clustal W program on Windows 95 was used for the analysis of multiple and pairwise sequence alignment of the 5.8S rDNA region and ITS 1 and 2 areas.

2.9 Transfer of DNA solution onto membranes

Primers (CKP,CIP) were labelled using a non-radioactive labelling method (see next section). The DIG oligonucleotide 3'-end labelling kit (Boehringer Mannheim) was purchased for the detection of *C. krusei* and *C. inconspicua*. Only DIG-ddUDP molecule without dNTP is added to the 3' end of oligonucleotides by terminal transferase.

2.9.1 Preparation of labelled oligonucleotides

Candida species-specific probes were prepared based on the sequencing data. Each specific probe was diluted to 20 μ M as a stock solution which gave approximately 20 pmol/ μ l in the labelling reaction. 4 μ l of tailing buffer (1M potassium cacodylate, 0.125 M Tris-HCl, 1.25 mg/ml bovine serum albumin, pH 6.6 (25°C)), 4 μ l of CoCl₂ solution (25 mM CoCl₂), 1 μ l of 1 mM DIG-ddUTP solution, 50 U of terminal transferase (Boehringer Mannheim) and 100 pmol of oligonucleotide probe were mixed in a final volume of 20 μ l of sterile distilled water. After incubation for 15 min at 37°C, 2 μ l of the mixture in which 1 μ l glycogen solution was mixed with 200 μ l 0.2 mM EDTA (pH 8.0) was added to stop the reaction. Labelled oligonucleotides were precipitated with 2.5 μ l 4 M LiCl and 75 μ l chilled absolute ethanol. After incubation for 2 h at -20°C, the pellets were washed in 70% chilled ethanol and air dried. Each pellet was resuspended in 20 μ l sterile distilled water.

2.9.2 Dilution of genomic DNA

Genomic DNA extracted from isolates was diluted with dilution buffer (50 μ g/ml herring sperm DNA; 10 mM Tris/HCl, pH 8.0; 1 mM EDTA, pH 8.0) to 500 ng/ μ l, and DNA was denatured by heating at 95°C for 10 min. 1 μ l of each DNA sample was then spotted on the

nylon membrane. The denatured genomic DNA was then fixed using a UV transilluminator for 1.5 min each side.

2.9.3 Detection of minimum concentration of genomic DNA using labelled primers

For the determination of minimum concentration of *Candida* genomic DNA which can be detectable using 3' labelling primers, a series of *C. krusei* genomic DNA dilutions were prepared up to 43 pg/μl (10^{-4}), and the sensitivity of CKP probe was compared to previously designed CK probe which was derived from the ITS 2 region of *C. kruei*. DNA was denatured by heating for 10 min at 95°C and snap cooled. 1 μl of each diluted DNA was spotted on a positively charged nylon membrane (Boehringer Mannheim) and UV cross linking was then carried out for 3 min at on UV transilluminator. Approximately 20 ml of hybridisation solution (5 X SSC, 1% (w/v) blocking reagent, 0.1% (w /v) N-lauroylsarkosine, and 0.02% (w/v) lauryl sulfate) per 100 cm² were added to a nylon membrane in a sealed bag, and incubated for 2 h at 68°C. The labelled probe (10 pmol/ml) in 2.5 ml hybridisation solution per 100 cm² filter was then added to the bag and incubation was carried out at 52°C for 1 h. The filter was then washed twice for 15 min in 2 X SSC, 0.1% (w/v) lauryl sulfate at 52°C followed by washing twice for 15 min each in 0.1 X SSC, 0.1% (w/v) lauryl sulfate again at 52°C.

2.9.4 Prehybridisation, hybridisation and washing conditions

The optimum hybridisation conditions involved prehybridising the filters for at least 2 h followed by hybridisation with 5 ml of hybridisation solution per 100 Cm² membrane area. The optimal temperature of hybridisation was determined both empirically and from the Boehringer Mannheim formula ($Tm = 16.6 (\log Na^+) + 0.41 (\%G+C) + 81.5^{\circ}C - (600/N)$;

(%G+C) is the percentage guanine and cytosine residues in the hybridisation probe; N is the length of the probe. Therefore, different hybridisation temperatures, 48, 50 and 52°C were tried in this study. The hybridisation temperature 52°C produced good results. Washing conditions were determined using the following equation as a reference, $WT = 33^{\circ}\text{C} + 0.41 (\% \text{ G+C})$ (Saluz *et al.* 1990) and an empirical approach. After hybridisation, the membranes were washed twice at 52°C in 2 X SSC for 15 min followed by washing in 0.1 X SSC twice at 52°C for 15 min.

2.9.5 Detection of DIG-labelled nucleic acid

The DIG luminescent detection kit (Boehringer Mannheim) was used for the detection of *C. krusei* and *C. inconspicua* genomic DNA according to the manufacturer's manual. Briefly, there are 3 steps to the process. Firstly, membranes are treated with blocking reagent (1 to 3% Skimmed milk); secondly, membranes are incubated with diluted anti-digoxigenin Fab fragments, conjugated to alkaline phosphatase, and finally, in the presence of Lumigen PPD, membranes were exposed to X-ray film. After hybridisation and post hybridisation washes, the membranes were equilibrated in buffer 1 (100 mM maleic acid, 150 mM NaCl; pH 7.5). The membranes were then blocked by incubation in buffer 2 (1 % (w/v) skimmed milk in Buffer 1) for 1 h. The membranes were then incubated in buffer 2 containing 1:10⁴ (75 mU/ml) diluted anti-DIG alkaline phosphatase for 30 min. Membranes were then washed twice in buffer 1 containing 0.3% (w/v) Tween 20 for 20 min per wash. After equilibration of the membranes in buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5) for 2 min, membranes were immersed in 10 ml of 1:100 diluted Lumigen PPD followed by incubation for 5 min at 37°C. Finally the membranes were sealed in a plastic bag with care to avoid air bubbles and

incubated for 15 min at 37°C. They were then exposed to X-ray film at room temperature for between 30 min and several hours.

3.

Chapter 3

API 32C codings and MICs test

3.1 Introduction

3.1.1 API systems

The API systems have been adopted by large numbers of laboratories since their introduction in the late of 1970s. The API 20C for yeast identification, API 20E for identification of gram-negative bacilli, API 20S for identification of streptococci, and API 20A for identification of anaerobes have been used (Finegold *et al.* 1986).

The methods for the most part are rapid, and results are available within 24h. The major advantage is that the systems provide an identification based on a data base of thousands of yeast biotypes which considers a number of variations and substrate utilisation patterns. In the case of API 20E, reactions are read and results are converted to a seven-digit biotype profile number and the yeast identification is made from a profile register (Analytab Products). The identification steps using API 20E which is one of the API systems have been outlined in Figure 3.1.

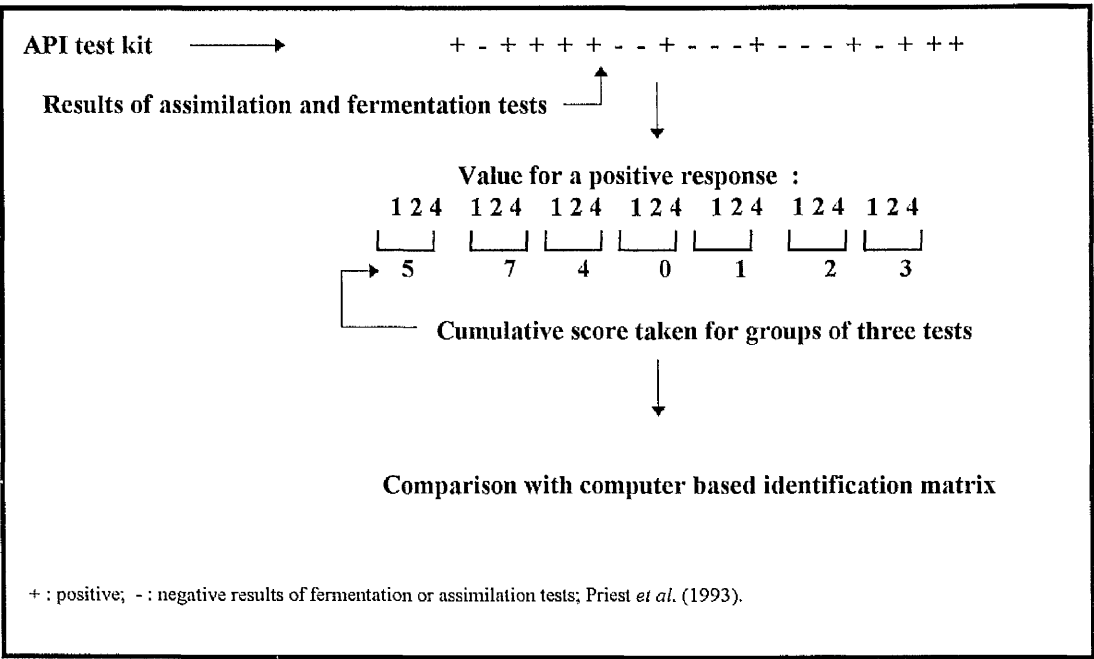


Figure 3.1 Use of API system.

Recently, the API 32C system (bioMérieux) was introduced and has been widely used in the clinical laboratory for the identification of clinically important yeast species. The API 32C system has been found to provide accurate information for the identification of common yeast species and has the advantage of having a more extensive database (Bruun *et al.* 1995; Fricker-Hidalgo *et al.* 1996). It consists of a single use disposable plastic strip with 32 wells to perform 29 assimilation tests (carbohydrates, organic acids, and amino acids), 1 assimilation test with a negative control, 1 susceptibility test (cycloheximide), and 1 colorimetric test (aesculin) (Gutierrez, *et al.* 1994). Although the API ID 32C system has been proven to be accurate for most common clinically important yeast, it has been found that there are limitations for the identification of some *Candida* species (Thanos *et al.* 1996).

3.1.2 Minimum inhibitory concentration (MIC)

The lowest concentration of the agent that inhibits growth of the organism, as detected by lack of visual turbidity (matching the negative growth control) is designated the minimum inhibitory concentration (MIC; Finegold *et al.* 1986). If the concentration of antimicrobial agent represented by the MIC can be readily achieved in the patient's serum by normal routes of delivery, the organism is said to be susceptible to that agent. If the MIC is above the achievable level or within a range toxic to the host, the microorganism is said to be resistant to the antimicrobial agent (Finegold *et al.* 1986). In this study, the MIC of amphotericin B was taken as the lowest drug concentration to inhibit $\geq 80\%$ of growth, and MICs of ≥ 0.25 $\mu\text{g/ml}$ were regarded as intermediate or resistant. In the case of fluconazole, MICs of ≥ 25 $\mu\text{g/ml}$ were regarded as resistant, and those of 6.25 to 12.5 $\mu\text{g/ml}$ were regarded as of intermediate (Baily *et al.* 1994).

Therefore this study investigated the reliability of API 32C, in order to address whether conventional tests are able to provide enough information for the differentiation of *C. inconspicua*, *C. krusei* and *C. norvegensis*.

3.2 Results

All API 32C codes and susceptibility test results were provided from the Microbiology Department in Hope Hospital. A total of thirty isolates of three *Candida* species from bioMérieux and three typing strains were investigated to check their identification, and antifungal susceptibility. API codings alone against *C. krusei* strains were not consistently identical, and five different codings, i.e. 0300000001, 0300010001, 0300010401, 0200000001, 0200010001, were recorded (Table 3.1). This result suggests that identification of *C. krusei* isolates based on the API 32C codings are not reliable. Furthermore, all *C. inconspicua* and all *C. norvegensis* isolates had an identical code, 0200010005, indicating further tests are required for the differentiation of these species.

However, it was found that these isolates could be differentiated from each other on the basis of the aesculin (AESC) test and rice agar tween medium (RAT) test. For instance, apart from one *C. norvegensis* isolate, only *C. krusei* isolates (all 11 isolates) were able to produce pseudohyphae. Although all *C. inconspicua* isolates were negative on rice agar tween medium (RAT) test while all *C. krusei* isolates showed a positive result, this was not found to be the case previously (Mata Essayag *et al.* 1996). Therefore it has been suggested that AESC in conjunction with the RAT test may provide information for the differentiation of these 3 *Candida* species.

C. krusei type strain, ATCC 6258 had 50 µg/ml of MIC, showing that this strain was clearly resistance to fluconazole. All other *C. krusei* isolates from bioMérieux were also resistant to fluconazole (MIC, 25-100µg/ml) as expected, and appeared to have the higher MICs than that of other two *Candida* species, indicating that *C. krusei* is innatively resistant to fluconazole. This data suggested that three *Candida* species, *C. inconspicua*, *C. krusei*, and *C. norvegensis* have different intrinsic *in vitro* susceptibilities to fluconazole. In the case of the MIC of type strain (ATCC 6258) to amphotericin B, 0.12 µg/ml was recorded, and was found as more susceptible to this antifungal drug than fluconazole. In addition, they all had relatively high MICs to amphotericin B with one isolate, 8808110 (MIC, 0.5 µg/ml) in the intermediate/resistant range (Rex *et al.* 1995a). This is in accordance with animal model data showing incomplete killing *in vivo* of *C. krusei* by amphotericin B (Fisher *et al.* 1989).

In the case of *C. inconspicua* and *C. norvegensis*, isolates were in the middle range of fluconazole susceptibility with many intermediate MICs (eg., 6.25-12.5 µg/ml), and one resistant isolate in each group (*C. inconspicua*: 8510102; *C. norvegensis*: 8503198) was found. In contrast, all the strains of these two species were amphotericin B susceptible. *C. norvegensis* ATCC 20686 also showed 12.5 µg/ml, 0.062 µg/ml of MICs to fluconazole and amphotericin B, respectively.

Table 3.1 *Candida* isolates tested in this study

Species	API 32C codes	AESC ^a	RAT ^b	Amp ^c .	Flu ^d .
All					
<i>C. inconspicua</i> (11 isolates)	0200010005	-	-	0.03 - 0.125	3.125 - 50 ^e
<i>C. krusei</i>					
5 isolates	0300010001	-	+		
2 isolates	0300000001	-	+		
1 isolates	0300010401	-	+	0.125 -	25 -
1 isolates	0200000001	-	+	0.5	100
1 isolate	0200010001	-	+		
(Type strain ATCC 6258)	0300010401	-	+	0.12	50
<i>C. norvegensis</i>					
10 isolates	0200010005	+	-	0.03 -	6.25 -
1 isolate	0200010005	+	+	0.062	12.5
a: Aesculin test; b: Rice agar tween medium for the determination of pseudohyphae production. c: MICs range of amphotericin B (µg/ml); d: MICs range of fluconazole (µg/ml); e, f: Only one isolate showed 50 and 25 µg/ml of MIC, respectively in <i>C. inconspicua</i> and <i>C. norvegensis</i> isolates.					

3.3 Discussion

Conventional identification of *Candida* species currently relies on many phenotypic characteristics including the production of hyphae or pseudohyphae on suitable media, fermentation and assimilation of sugars and others. Although the API ID 32C system (bioMérieux) has been proven to be accurate for most common clinically important yeasts (Bruun *et al.* 1995), it still has some limitations for the identification of several *Candida* species. For instance, a false reading based on visual interpretation is always possible and experience is required to obtain reliable results. Furthermore, this product does not

differentiate some medically important yeasts (Thanos *et al.* 1996). In most species of *Candida* there is considerable variation in API 32C codes, but the identification of *C. inconspicua*, *C. krusei* and *C. norvegensis* critically depends on one or two characters such as the aesculin test and /or production of pseudohyphae. Isolates of all three species among this collection, *C. krusei*, *C. norvegensis* and *C. inconspicua* can generate API 32C codings that alone do not give reliable identifications and therefore require additional tests (Nho *et al.* 1997). For instance, all the *C. inconspicua* and *C. norvegensis* isolates in this study have the same code (0200010005), differing only in their reaction to aesculin.

It was found that all *C. inconspicua* and *C. norvegensis* isolates other than one isolate fail to produce pseudohyphae. However, all *C. krusei* species produced pseudohyphae, so that conventional identification of these three species based on phenotypic characteristics is usually possible, although some isolates of *C. krusei* are still problematic (Lischewski *et al.* 1995; Mata Essayag *et al.* 1996).

Most isolates of *C. krusei* showed different API 32C codings, and it was found that there were 5 different codings, i.e. 5 isolates for 0300010001, 2 isolates for 0300000001, 2 isolates for 0300010401, 1 isolate for 0200000001 and 1 isolate for 0200010001, respectively. The identification probability of target species based on the information from the Microbiology Department of the Hope Hospital, Manchester, showed that API coding, 0200000001 in one of the *C. krusei* strains represents a 68.2% probability of *C. inconspicua* but only 5.1% probability of *C. krusei*. Besides, another API coding, 0200010001, in the *C. krusei* species, represents a 62.1% of possibility of being a *C. inconspicua* species, but only a 22.7% possibility of being *C. krusei*. The results showed

that the identification and differentiation of *C. krusei* needs further identification procedures.

Recently the API Candida system (bioMérieux) has been introduced to identify the most clinically important yeasts and yeast like organisms and its reliability has been evaluated (Fricker-Hidalgo *et al.* 1996). The new API *Candida* system gave good results in the identification of the commonly isolated yeasts without the need for extra tests, but there are still limitations in the use of this product for one of the medically important yeast species. For example, *C. krusei* could not be completely identified by this new approach (Fricker-Hidalgo *et al.* 1996). Therefore, it is suggested that a new approach based on the PCR applications like PCR-RFLP followed by a dot hybridisation test is necessary to confirm this species.

When it comes to the *in vitro* antifungal susceptibility tests of these 33 *Candida* species, many of the isolates in this study were shown to be relatively insensitive or resistant to fluconazole with MICs ranging from 3.13 to 100 µg/ml. Particularly, the *C. krusei* isolates were in general more resistant to fluconazole and amphotericin B than isolates from the other 2 species, supporting the concept that *C. krusei* is resistant to fluconazole both in the laboratory and when the drug is used therapeutically (Samaranayake *et al.* 1994).

C. krusei type strain, ATCC 6258 also had higher MIC (50 µg/ml) to fluconazole than that of other two *Candida* species. Our data suggested that *C. krusei* isolates are thought to be innately resistant to fluconazole. In addition, the high MICs to amphotericin B are

consistent with *in vivo* data showing that amphotericin B is also relatively ineffective against this species (Fisher *et al.* 1989). However, amphotericin B resistance is otherwise uncommon among clinical isolates of *Candida*.

In contrast, *C. norvegensis* ATCC 20686 strain appeared to be susceptible to both fluconazole and amphotericin B. Apart from one strain, all other *C. norvegensis* strains were found to be also susceptible to these antifungal agents, suggesting fluconazole and amphotericin B can be used for the treatment of patients with candidiasis caused by *C. norvegensis*.

Because there is no established definition of resistance to be applied to antifungal agents, the use of this term can be problematic (Rex *et al.* 1995). In some cases, the term resistance has been used when a patient fails to respond clinically to antifungal therapy. In others, it also has been used to describe a strain for which the MIC of an antifungal drug is greater than the MIC of the drug for other strains tested in the same laboratory (Odds 1993). Problems also arise when it comes to the establishment of correlation between fluconazole susceptibility and clinical outcome, because host factors such as undrained abscesses, removal of vascular catheters and penetration of the drug to the specific site of infection are as likely to influence clinical outcome as intrinsic drug susceptibility (Ghannoum *et al.* 1996). Although low MICs do not guarantee successful outcomes and high MICs do not guarantee failure, and prediction of resistance, and clinical outcome at least in the area of antibacterial agents, is far more reliable clinically than the prediction of susceptibility and clinical response (Ghannoum *et al.* 1996). *In vitro* susceptibility testing can predict outcome in certain well defined clinical situations such as fluconazole-treated AIDS patients. For instance,

based on the survey of the correlation of outcome with *in vitro* susceptibility testing in AIDS patients with oropharyngeal candidiasis (OPC), as individual patients are carefully followed, a rising MIC can be clearly correlated with a lessened clinical response to a given dose of fluconazole. Law *et al.* (1994) also support this point that the antifungal susceptibility test is helpful in clinical treatment because evidence showed that this test is correlated with the development of resistance to fluconazole in OPC and oesophageal candidosis in late stage AIDS.

C. inconspicua is generally believed to be resistant to fluconazole, but this species may be susceptible to itraconazole (Baily *et al.* 1996). However, the data available is from isolates obtained from patients with advanced AIDS receiving large quantities of fluconazole. MICs could be higher in this group of isolates than others, as the comparison with the isolates in this study suggest. More extensive studies are required to establish the susceptibility of *C. inconspicua* to antifungal agents. There may be differences in susceptibility compared with *C. krusei* which is of interest given the close phylogenetic relationship of these species (see Chapter 5).

Several studies have shown that fluconazole resistant *Candida* species are often susceptible to itraconazole. Although some *Candida* isolates are cross resistant to all azoles, itraconazole may be substituted as an alternative therapy for some infections. Such information can provide a guide line for the use of antifungal agents against these species for clinical treatment.

In conclusion, the conventional approach to identification might be able to identify most medically important species of *Candida*, but for certain species, molecular based techniques are possibly superior for reliable identification. Therefore, this study focused on the development of molecular techniques to fulfil these criteria.

4.

Chapter 4

Identification of *Candida* species using PCR products

4.1 Introduction

Each *Candida* species has a characteristic (species-specific) fragment size on the internal transcribed spacer (ITS) 1 and 2 regions except the highly conserved 5.8S rDNA area which appears to have an almost identical size in all species (Figure 4.1; Lott *et al.* 1993). Therefore, the size of amplified products by PCR using universal primers (ITS 1,2,3 and 4) which are complementary to the highly conserved nuclear rDNA (18, 5.8 and 28S) regions could be used for the identification of some medically important yeasts. Besides, for the detection of isolates into species level, species-specific probes could be designed for the ITS regions (Botelho *et al.* 1994). Studies showed that PCR using primers complementary to large ribosomal subunit sequences can be applied successfully to the rapid *in vitro* identification of several *Candida* species (Sullivan *et al.* 1996; Haynes *et al.* 1996).

Previously, universal primers, ITS 1 and 4 were tested for the identification of *Cryptococcus neoformans* in conjunction with unique oligonucleotide primers derived from the ITS regions of this species (Mitchell *et al.* 1994).

The aim of this study was to investigate the length variation of the ITS 1 and 2 regions including 5.8S rDNA as a preliminary step to assist in identification of several clinically significant *Candida* species. The location of the PCR primers (ITS 1,2,3, and 4) and the size differences of ITS regions in *Candida* species are shown in Figure 4.1.

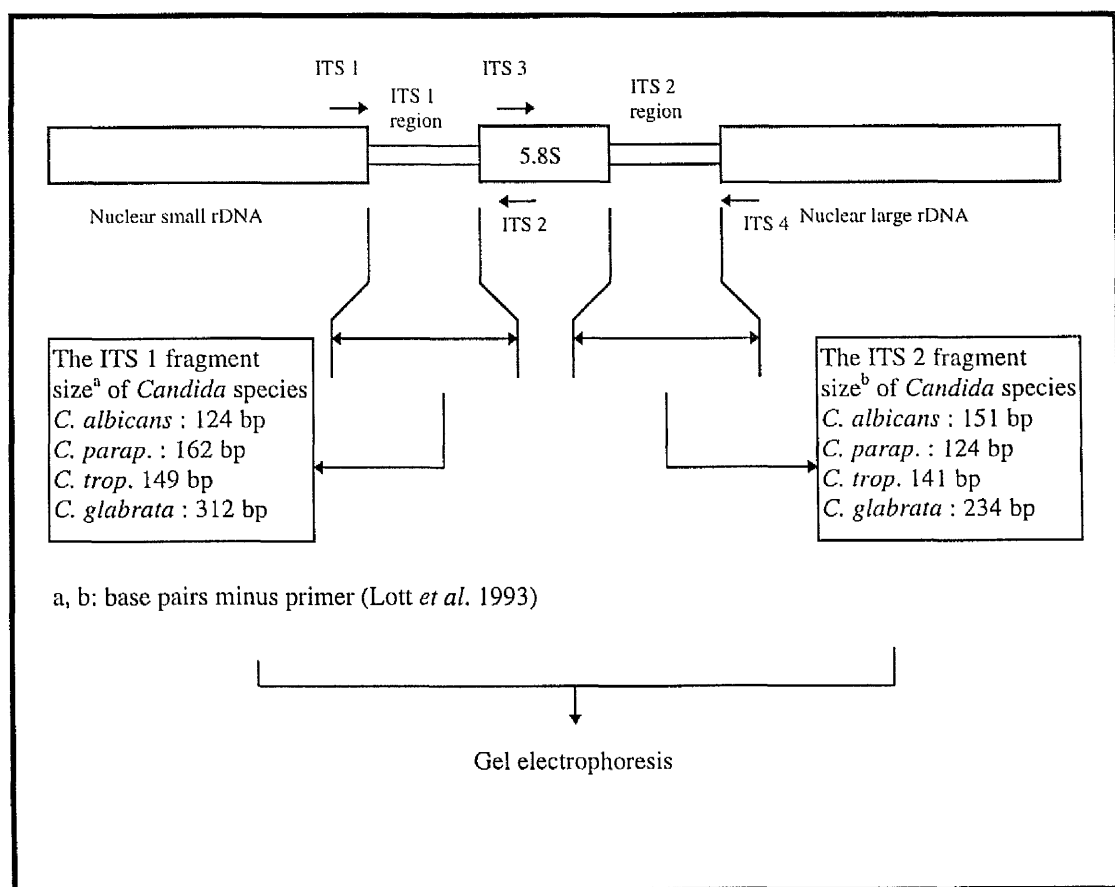


Figure 4.1 Location of the ITS primers on nuclear ribosomal DNA and the size differences of the ITS regions in *Candida* species.

4.2 Results

4.2.1 PCR optimisation and controls

Because no single protocol can be applicable to all situations (Innis *et al.* 1988), PCR optimisation was carried out before main experiments were conducted. Different concentrations of PCR primers in the final tubes in the range of 0.1 to 0.5 μ M were prepared to find the optimum concentration of primers. Results showed that amplified products of *C. inconspicua* and *C. krusei* were produced under the condition of 0.25 and 0.5 μ M primer concentrations (Figure 4.2.A). However, PCR products were not

generated when 0.1 μM of primers were used, suggesting that it is not the optimum concentration of primers. Therefore 0.25 μM primer concentration was used in this study.

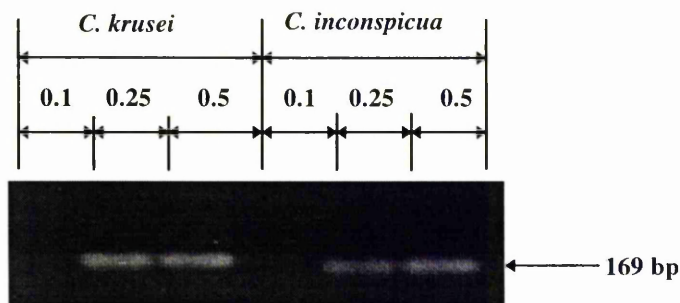
The effect of MgCl_2 concentrations on the specificity of PCR was then tested. The magnesium concentrations in the final reaction tubes of *C. inconspicua* were set to 1 mM to 3.5 mM (Figure 4.2.B). The optimum concentration of magnesium ion appeared to be between 1 and 2 mM, and 1.5 mM was therefore chosen as the optimum condition for MgCl_2 concentration for this study. The excessive amounts of non-target fragments were clearly visible when using 3.5 mM MgCl_2 . It is known that excess amounts of Mg^{2+} result in the accumulation of non-specific products (Newton *et al.* 1994).

PCR products were generated under conditions of 20 and 200 μM dNTPs, and 200 μM dNTPs were used for the study of sizes of PCR products. 20 μM dNTPs were used for sequencing because low dNTP concentrations reduce the likelihood of extending mis-incorporated nucleotides (Innis *et al.* 1988). *Taq* DNA polymerases from different suppliers can also affect the yield of PCR products. For instance, under the conditions in which *Taq* polymerase and buffer supplied from identical supplier were used, a single PCR band was visible (Figure 4.2.C). However, non-specific products were generated when *Taq* polymerase and buffer from different suppliers were used (Figure 4.2.D). It is known that different suppliers may use different formulations, assay conditions and unit definitions. Therefore in this study, all PCR experiments were carried out using materials provided from identical supplier.

For the determination of annealing temperature, several different formula, as described in materials and methods (see chapter 2), were used. For the application of the PCR identification technique using ITS and species-specific primers, a stringent annealing temperature was used. In general, it is known that annealing temperatures in the range of 55 to 72°C yield the best results (Michael *et al.* 1990).

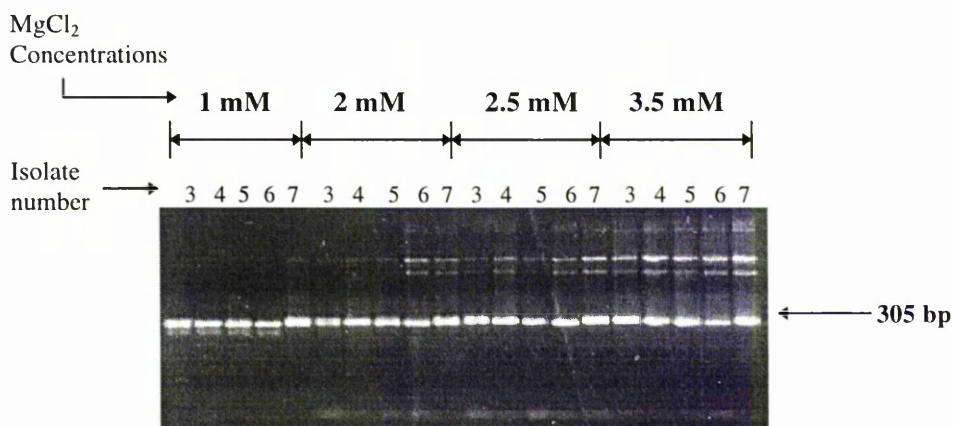
Because too many cycles can increase the complexity of non-specific background products, and too few cycles give low product yield, 30 cycles were used in this study. All other reaction components were provided from supplier and experiments were carried out according to the instructions.

For setting up PCR, control experiments were performed to avoid contamination and for the efficiency and specificity of PCR. Negative controls in which PCR was performed in the absence of DNA were included to check the PCR reagents for contamination with DNA (Figure 4.2 C, D and 4.3 B, C). Positive controls with type strains (*C. inconspicua* ATCC 16783, *C. krusei* ATCC 6258 and *C. norvegensis* ATCC 22977) and several fungal and prokaryotic isolates were also included (Figure 4.2 and 4.3). Results showed that the ITS PCR sizes of *Candida* isolates were found to have similar sizes on the gel compared to type strains. Prokaryotes were tested with the ITS 1 and 4 primers, and *T. denticola* generated faint PCR band. PCR products from other prokaryotic isolates were difficult to be seen on the gel (Figure 4.5).



A. Determination of primer concentration for PCR.

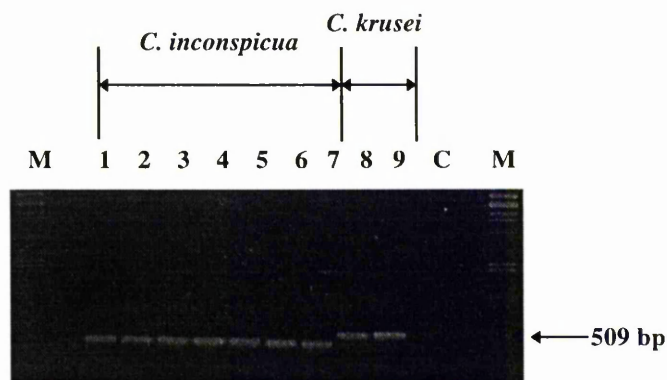
Different concentrations (μM) of ITS 1 and 2 primers were prepared and PCR was carried out.



B. Titration of MgCl₂ concentration in PCR.

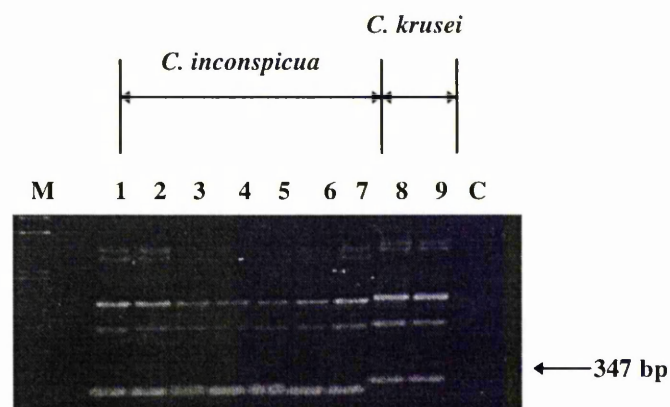
C. inconspicua genomic DNA was amplified with different MgCl₂ concentrations using ITS 3 and 4 primers. Isolates: 3, FA/985; 4, SM/038; 5, bioMérieux 3187; 6, ATCC 16783; 7, bioMérieux 18949.

Figure 4.2 PCR optimisation.



C. PCR using PCR buffer and thermostable DNA polymerase from identical supplier.

PCR products were generated with AB (Advanced Biotechnologies) PCR buffer IV and AB thermostable DNA polymerase using ITS 1 and 4 primers. Lane order is corresponding to Figure 4.3.A. M: Size marker λ *Pst*I digest DNA; C : control lane (PCR in the absence of genomic DNA).



D. PCR using AB PCR buffer and BM *Taq* polymerase from different suppliers.

PCR products were generated with AB buffer IV and BM (Boehringer Mannheim) *Taq* polymerase using ITS 3 and 4. Lane order is corresponding to Figure 4.3.A. Size marker : λ *Pst*I digest DNA. C : control lane (PCR in the absence of genomic DNA).

Figure 4.2 (continued).

4.2.2 PCR sizes using ITS 1 and 4 primers

4.2.2.1 *Candida* species

All the genomic DNA were amplified using universal primers (ITS 1,2,3 and 4) and the sizes are summarised in Table 4.1. *C. inconspicua* isolates from Hope Hospital and ATCC, including 3 bioMérieux isolates, showed amplified products at 450 bp on the gel (Figure 4.3.A). The other 10 isolates from bioMérieux also produced same the sized amplicons, with no size variations found (Figure 4.6.A). In the case of *C. krusei*, the product corresponding to about 510 bp was generated in all strains from bioMérieux, ATCC and type strain (Figure 4.3.A and Figure 4.6.B). The PCR products of *C. krusei* and *C. inconspicua* were confirmed by sequencing (see Chapter 5). The same pattern seen in *C. inconspicua* and *C. krusei* cases was also seen in *C. norvegensis* isolates with all amplified products placed at around 490 bp (Figure 4.3.A and C, 4.6.C).

In contrast, *C. rugosa* isolates showed size variations between strains, and all 3 strains from ATCC including type strain, ATCC 10571 had different sizes of amplicons in the range of 350 bp to 550 bp (Figure 4.3.A,C). Interestingly, the *C. glabrata* isolates had products at 850 bp, showing the biggest PCR product in our isolate collection (Figure 4.3.A). Other four *C. glabrata* isolates were also found to have an identical product size. In addition, the amplified products at 850 bp of two NCPF 3309 *C. glabrata* isolates (large and small colonies) were found (Figure 4.3.C).

4.2.2.2 Other species

Other fungal isolates were also tested under the same conditions using ITS 1 and 4 primers as a positive control. The products amplified were of different sizes within the

range of 520 to 850 bp (Figure 4.5). Many species like *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *Trichosporon beigeli* (*T. cutaneum*), *Cryptococcus neoformans* and *C. dubliniensis* gave fragments of similar sizes in the range of 520 to 550 bp, whilst others including *Saccharomyces cerevisiae* had a product at 840 bp. The sizes of amplified products are also listed in Table 4.1. Finally, in the case of prokaryotic isolates, i.e. *Escherichia coli* and *Staphylococcus epidermidis*, PCR products were not clearly visible. *Treponema denticola* produced a faint band at about 550 bp.

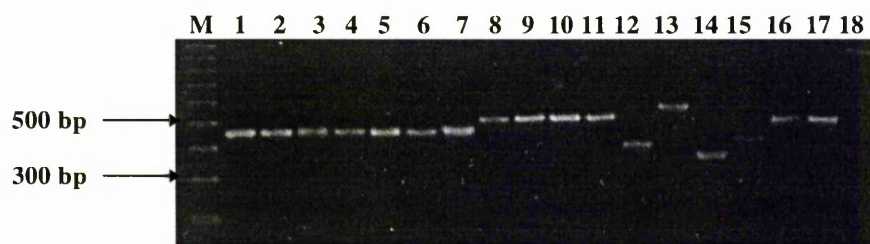
4.2.3 PCR sizes using primers ITS 1 and 2

ITS 1 and 2 primers were also used to produce PCR profiles of *C. inconspicua* and *C. krusei* isolates from bioMérieux including isolates from patients with AIDS. All *C. inconspicua* isolates including type strain, ATCC 16783 had similar PCR products at 170 bp whereas two *C. krusei* isolates including type strain ATCC 6258 had similar amplicons at 180 bp (Figure 4.3.B). The actual ITS fragment sizes were confirmed by sequencing (169 and 182 bp, respectively). The data suggested that *C. krusei* has a bigger ITS 1 region than that of *C. inconspicua* (see Chapter 5).

4.2.4 PCR sizes using primers ITS 3 and 4

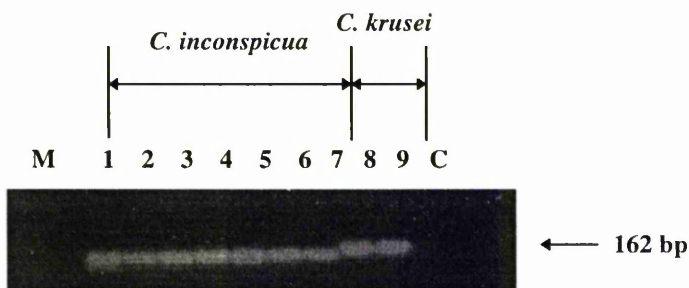
After running the gel using the ITS 3 and 4 primers, each *Candida* species showed characteristic band patterns. The size of product from all *C. inconspicua* isolates was 300 bp whilst other *Candida* isolates product sizes were: *C. krusei*, 350 bp, *C. norvegensis*, 310 bp and *C. glabrata*, 400 bp. *C. rugosa* had single PCR products in the range of 240 and 350 bp. In the case of *C. glabrata*, the amplicon size was about 400 bp, which implies that *C. glabrata* has the biggest ITS 2 fragment among medically important

Candida species. Some isolates of *C. inconspicua* were found to have doublet bands on high resolution agarose gel (Figure 4.4). Sequencing showed that there are 4 bp insertions of the larger band amplified using ITS 3 and 4 primers, and a polymorphic site on the ITS 2 region was found.



A. PCR products of *Candida* species using ITS 1 and 4 primers.

Lanes: M, size marker 100 bp ladder; 1, *C. inconspicua* FA / 390; 2, *C. inconspicua* FA/446; 3, *C. inconspicua* FA / 985; 4, *C. inconspicua* SM / 038; 5, *C. inconspicua* bioMérieux 3187; 6, *C. inconspicua* type strain ATCC 16783; 7, *C. inconspicua*, bioMérieux 18949; 8, *C. krusei* type strain ATCC 6258; 9, *C. krusei* bioMérieux 18888; 10, *C. krusei* ATCC 62404; 11, *C. krusei* ATCC 20298; 12, *C. rugosa* type strain ATCC 10571; 13, *C. cylindracea* ATCC 14830; 14, *C. rugosa* ATCC 20306; 15, *C. rugosa* ATCC 34637; 16, *C. norvegensis* ATCC 20686; 17, *C. norvegensis* type strain ATCC 22977; 18, *C. glabrata* NCPF 3309. Amplified products were run on the 3% NuSieve agarose gel for 2.5h in 1XTPE buffer.



B. PCR products of *Candida* species using ITS 1 and 2 primers.

Lanes: M: Size marker λ *Pst*I digest DNA; 1, *C. inconspicua* FA / 390; 2, *C. inconspicua* FA/446; 3, *C. inconspicua* FA / 985; 4, *C. inconspicua* SM / 038; 5, *C. inconspicua* bioMérieux 3187; 6, *C. inconspicua* type strain ATCC 16783; 7, *C. inconspicua* bioMérieux 18949; 8, *C. krusei* type strain ATCC 6258; 9, *C. krusei* bioMérieux 18888; C: control lane (PCR in the absence of genomic DNA).

Figure 4.3 Amplified products of *Candida* isolates using universal primers analysed by agarose gel electrophoresis.

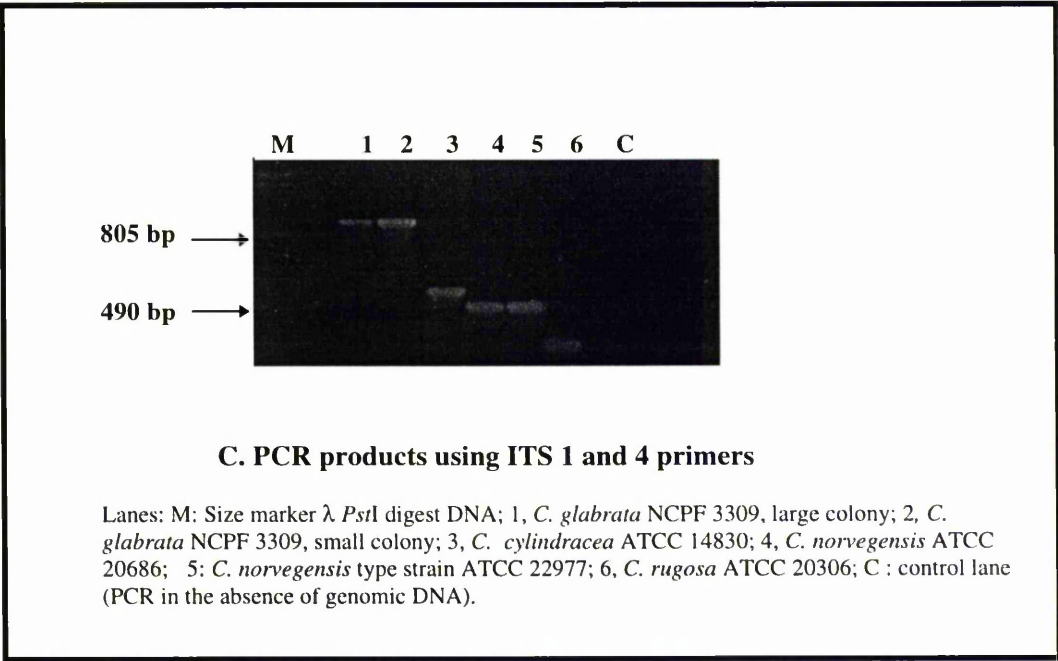


Figure 4.3 (Continued).

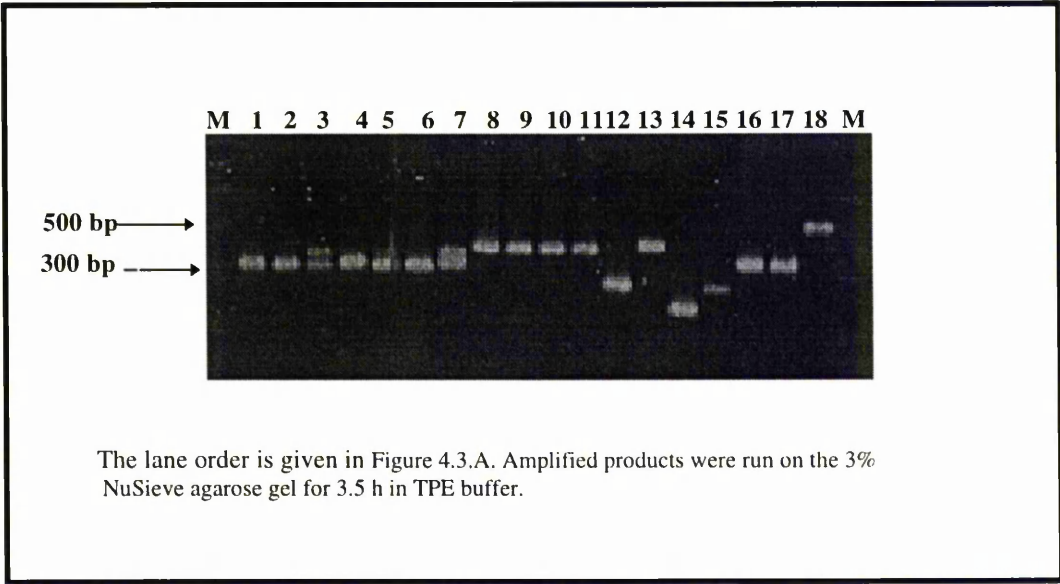


Figure 4.4 The amplified products of *Candida* species using ITS 3 and 4 primers analysed by agarose gel electrophoresis.

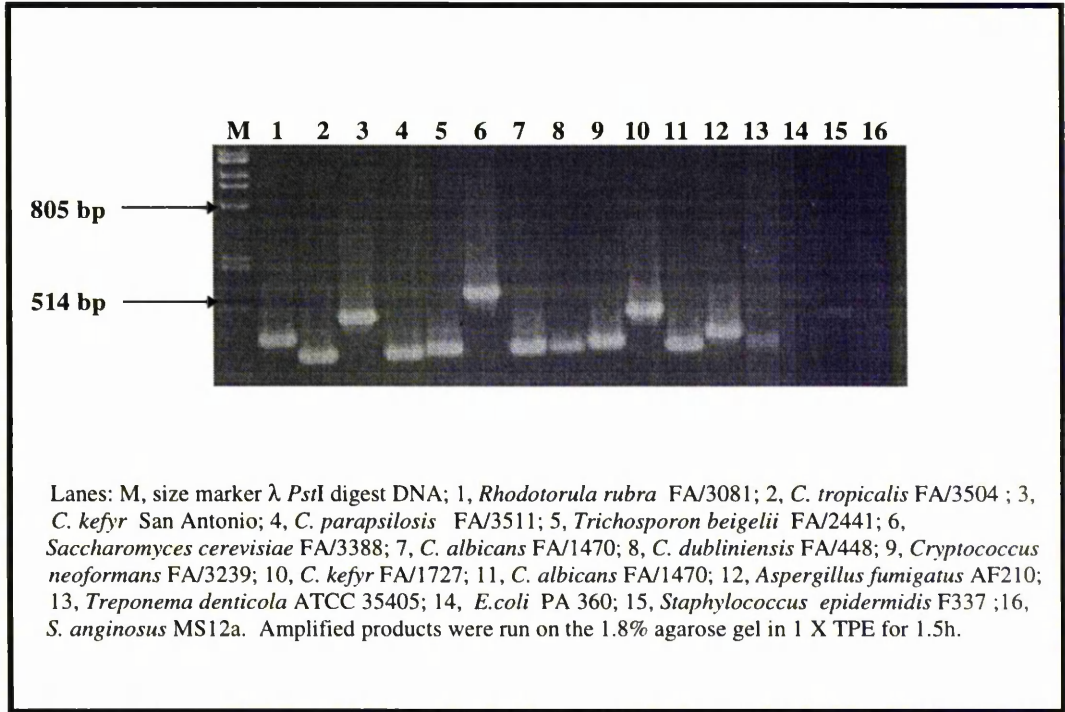


Figure 4.5 PCR products of other fungal species and prokaryotes using ITS 1 and 4 primers.

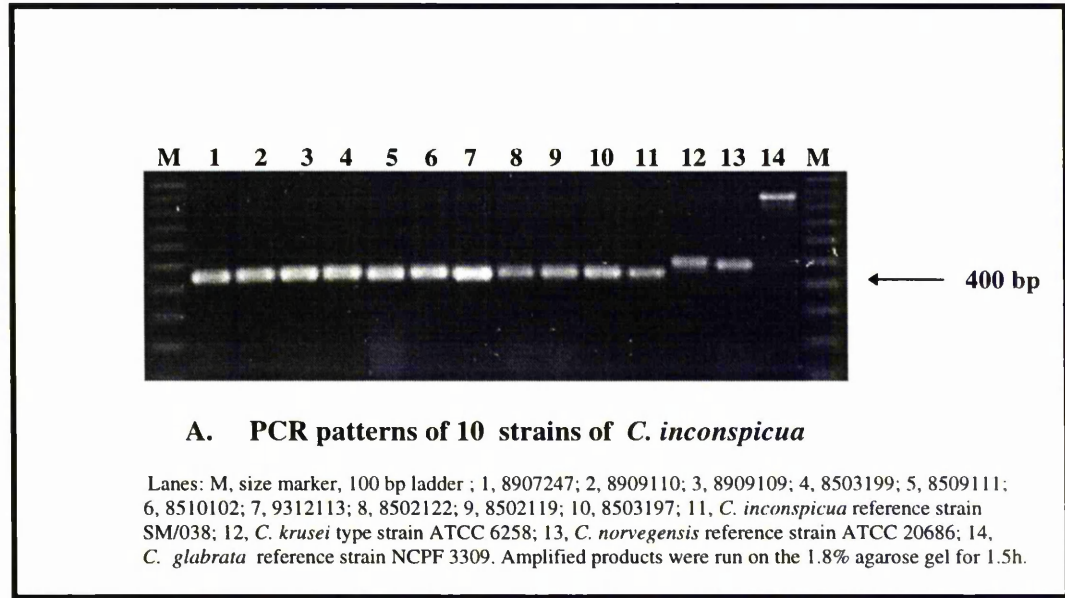
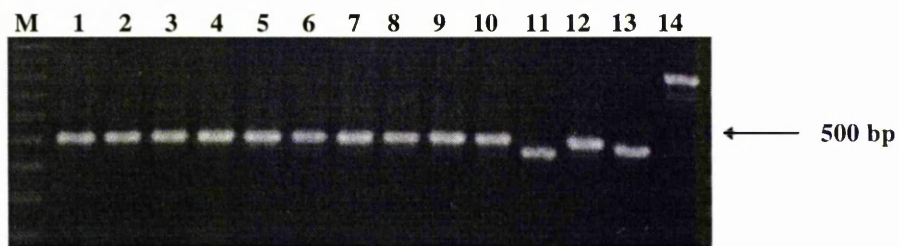
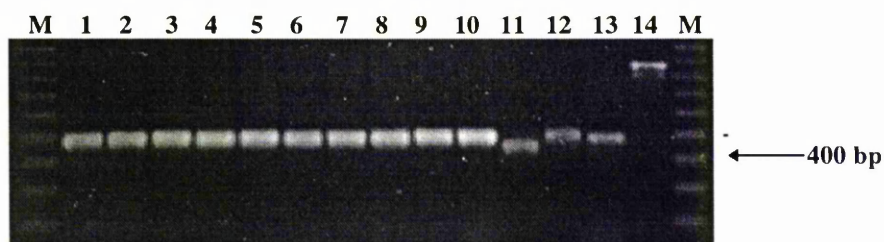


Figure 4.6 PCR patterns using ITS 1 and 4 primers of *Candida* species from bioMérieux.



B. PCR patterns of 10 strains of *C. krusei*.

Lanes: M, size marker, 100 bp ladder; 1, 8808100; 2, 8904072; 3, 8503050; 4, 9502007; 5, 8503051; 6, 8903079; 7, 9502006; 8, 8504237; 9, 8503198; 10, 8807095; 11, *C. inconspicua*, reference strain SM/038; 12, *C. krusei* type strain ATCC 6258; 13, *C. norvegensis* reference ATCC 20686; 14, *C. glabrata*, reference strain NCPF 3309. Amplified products were run on the 1.8% agarose gel for 1.5h.



C. PCR patterns of 10 strains of *C. norvegensis*.

Lanes: M, size marker, 100 bp ladder; 1, 8908008; 2, 8808110; 3, 9105030; 4, 8908006; 5, 8510089; 6, 9112022; 7, 9112020; 8, 9112021; 9, 8908007; 10, 9003063; 11, *C. inconspicua*, reference strain SM/038; 12, *C. krusei*, type strain ATCC 6258; 13, *C. norvegensis*, reference strain ATCC 20686; 14, *C. glabrata*, reference strain NCPF 3309. Amplified products were run on the 1.8% agarose gel for 1.5h.

Figure 4.6 (continued).

Table 4.1 Sizes of amplified products of isolates using ITS 1, 4 and 3, 4 primers.

Isolates (71)	ITS PCR sizes ^a (bp)	
	primer 1 and 4 ^b	primer 3 and 4 ^c
1) <i>Candida</i> species		
<i>C. inconspicua</i> all strains ^d (20)	450 (455) ^h	300 (305) ^h
<i>C. krusei</i> all strains (15)	510 (509) ^h	350 (347) ^h
<i>C. norvegensis</i> all strains (14)	490	310
<i>C. glabrata</i> all strains ^e (5)	850	400
<i>C. rugosa</i> ATCC 10571	370	280
ATCC 20306	350	240
ATCC 34637 ^f	400	270
<i>C. tropicalis</i> FA 3504	520	N/D ^g
<i>C. kefyr</i> San Antonio FA/1727	720	N/D
<i>C. parapsilosis</i> FA/3511	520	N/D
<i>C. albicans</i> Hope strain	540	N/D
FA/1470	540	N/D
<i>C. dubliniensis</i> FA/448	540	N/D
<i>C. cylindracea</i> ATCC 14830	550	350
2) Other species		
<i>Rhodotorula rubra</i>	590	N/D
<i>Cryptococcus neoformans</i>	550	N/D
<i>Trichosporon beigeli</i>	540	N/D
<i>Saccharomyces cerevisiae</i>	840	N/D
<i>Aspergillus fumigatus</i>	590	N/D

^a : PCR sizes were measured by either sequencing results or amplified products on the gel.

^b : Size including ITS 1 and 4 universal primers ; ^c : Size including ITS 3 and 4 universal primers.

^{d,e} : The amplified products from all isolates were identical except one unusual isolate in *C. inconspicua* and *C. glabrata* species, respectively. ^f : Unusual isolate which was originally identified as *C. inconspicua* but has been redesignated by author as *C. rugosa* based on the size of amplified product in this study, ^g : Not done.

^h : Actual sizes of PCR products confirmed by sequencing.

4.3 Discussion

PCR can be inhibited or enhanced by different substances, and many factors may lead to inhibition of PCR. Different types of biological specimens are used for PCR, including different types of biological fluids, bacterial and fungal samples, forensic and archaeological material and plant tissues. Many of these crude preparations contain inhibitory substances which have not been identified (Newton *et al.* 1994). In addition, there are many potential sources of pre-PCR contamination; therefore, PCR controls should be performed.

In this study, several factors which influence the fidelity of PCR were evaluated. Results showed that primer concentration, MgCl₂ concentration, and the supplier of the *Taq* polymerase and buffers all affect PCR results. Therefore, PCR optimisation was carried out before PCR-based techniques were performed. In order to avoid contamination of reagents with target DNA, PCRs in the absence of genomic DNA were carried out as a negative control. Positive controls with well-characterised isolates were also included for the specificity of ITS PCR. In addition, MgCl₂ and primer concentrations are important factors with regard to the specificity and yield in a PCR so that different concentrations of these materials were tested. In each definitive experiment, PCR with the type strains with ITS primers was also performed, and results showed that similar sized PCR products between type strains and isolates were found. Although very unlikely it is possible that there are very small size difference between isolates of the same species, which is difficult to detect following electrophoresis in high agarose NuSieve gels.

The ITS PCR results using ITS primers (ITS 1, 2, 3 and 4) showed that this technique is not a promising approach to identifying some medically important species, because the product sizes of different species were not characteristic. The sizes of products using ITS 1 and 4 primers on the gel were 450 bp for *C. inconspicua*, 510 bp for *C. krusei* and 490 bp for *C. norvegensis*, and 300, 350 and 310 bp, respectively when ITS 3 and 4 primers were used. Furthermore, several other *Candida* species had very similar amplified products at 540 bp. Therefore it is proposed that additional tests are required to identify target species.

In this study, the type strains were included in the analysis of the ITS and 5.8S rRNA sequence length. All isolates of *C. inconspicua*, *C. krusei* and *C. norvegensis* were found to have similar bands on the gel compared to type strains. Our data showed that PCR sizes using ITS primers might provide preliminary information for the identification of these species. However, three *C. rugosa* isolates were not identical to each other, indicating that further investigation of these species is required. In the case of *C. glabrata* isolates, the biggest PCR products at about 850 bp were produced so that simple PCR using universal ITS 1 and 4 primers might identify this species easily (Figure 4.3.A,C). Further studies are required to establish whether this approach can be employed as a method for identification of *C. glabrata*. Our work examined a limited set of organisms, and a larger study is required to address the issue of variability of amplified products using ITS primers. We therefore believe that this technique should be used with other diagnostic methods to assist the identification of fungal species.

Size variations of the ITS regions in the same *Candida* species had already been studied, and Lin *et al.* (1995) showed nucleotide insertions in the ITS 1 and 2 regions in three genetically distinct *C. parapsilosis* isolates. Likewise, in the study of the multiple sequence alignment of conserved 5.8S rDNA, only 2 bp differences were found in all fungal species (*C. inconspicua* and *C. krusei*, 157, *Ph. carinii*, 158, *C. glabrata*, 159, respectively) in this study, therefore it has been suggested that the size differences mainly come from the ITS regions rather than the conserved 5.8S rRNA. This concept was supported after sequencing on the ITS regions and the result of multiple alignments analysis. Fujita *et al.* (1995) mentioned that fungal DNA amplified with universal primers showed size variations in the amplified product.

Previously, Gonzales *et al.* (1985) revealed that a smaller amount of length variations occurs within individuals among the multiple copies of the genes. Each copy of an rRNA array is usually very similar to other copies within individuals and species. However there is a possibility that differences among species accumulate rapidly in parts of the array. Our data showed that not only are these size variations in the *Candida* genus but that RFLP in same *Candida* species are also possible.

Apart from *C. rugosa*, the profiles of amplified products of *Candida* species using ITS 1 and 4 primers have provided limited information in identifying some *Candida* species into species level. *C. rugosa* isolates showed size variations on both PCR products using ITS 1 and 4, and 3 and 4 primers. Therefore, results suggested that *C. rugosa* isolates in this study have variable size differences on both the ITS 1 and 2 regions, so reliance based on

the simple PCR sizes was not helpful in identifying this species and this species may not be homogeneous.

In conclusion, the amplified sizes using ITS primers of *Candida* species are generally consistent, with the exception of *C. rugosa* species. However, as the sizes for the differentiation of different species were similar, and amplified band patterns were not species-specific, additional molecular-based approaches should be employed to identify unknown species. Further studies are required to clarify *C. rugosa* species as it may be many subspecies together or even different species under a different name.

5.

Chapter 5

Sequence analysis of the 5.8S and ITS regions

5.1 Fungal taxonomy using genes coding for ribosomal RNA

Classical fungal taxonomy relies heavily on using the size and shape of fruiting structures, spore morphologies and release mechanisms, coloration and habitat to define taxa (Mitchell *et al.* 1995). However, there are very few homologous morphological characters that can be compared among all living organisms (Hills 1991). Furthermore, because phenotypic characteristics can vary considerably within some species, irregularities in the taxonomy of *Candida* are encountered frequently (Sullivan *et al.* 1996). In addition, recent studies have shown that there are isolates of *Candida* whose characteristics do not correspond precisely with classical species descriptions, causing confusion (McCullough *et al.* 1994; Sullivan *et al.* 1995). For instance, one of the species in *Candida* genus, *C. krusei* shows significantly different features compared with other medically important *Candida* species, suggesting the possibility of re-classification (Kogan *et al.* 1988; Samaranayake *et al.* 1994). *C. krusei* cell-wall mannan is different from that of other *Candida* species (Kogan *et al.* 1988). In addition, recent studies showed *C. krusei* appeared to have different average chromosomal numbers: eight, compared with 16 for *C. albicans* (Doi *et al.* 1992), giving credence to the belief that this organism can be re-classified. To resolve these ambiguities, a molecular phylogenetic approach is called for.

For the phylogenetic analysis of organisms, sequences with useful information come from genes that have the same function in all taxa, evolve at approximately the same rate, and are present in all living organisms. Genes that fulfil these criteria are the ribosomal RNA (rRNA) genes from the nuclear and mitochondrial genomes, cytochrome oxidase genes and certain ribosomal protein elongation factors (Mitchell *et al.* 1995), and most fungal phylogenetic studies have used sequences from the ribosomal gene cluster.

Several reasons can be given why rRNA sequences have been used as a indicator of phylogeny (Priest *et al.* 1993). Firstly, the rRNA molecules are essential in protein synthesis and are, therefore, found in all organisms except viruses. Secondly, because rRNA molecules are highly conserved and have changed very little during evolution, even the rRNA sequences from distantly related organisms can be compared, and therefore relatedness can be assessed. Ribosomal RNA is regarded as unique amongst macromolecules in this respect. Thirdly, some segments of rRNA evolve more rapidly than others and sequence variation occurs between closely related organisms, allowing comparisons to be made at the species level. Finally, phylogenetic lines of descent may be inferred from RNA sequences.

5.1.1 Ribosomal DNA as a taxonomic tool

Molecular taxonomy based upon the nucleotide sequence of various regions has been widely used to address species relatedness. There are several regions which can be useful for the phylogenetic analysis of *Candida* species in ribosomal DNA. The most studied rRNA is the small subunit nuclear gene. This gene has been studied most extensively because it is among the slowest evolving sequences found throughout living organisms (Hills *et al.* 1991). The large subunit nuclear rRNA gene is larger and shows more variation in rates of evolution of its different domains than the small subunit. Recently, the V3 variable region of the large ribosomal subunit genes from atypical oral *Candida* isolates was compared, and a novel species within the genus *Candida*, *C. dubliniensis* was proposed (Sullivan *et al.* 1995).

Previously, the 5.8S, one of the subunits of 60S demonstrates its utility as a taxonomic tool and its adaptability for *in vitro* amplification and automated sequencing (Lott *et al.* 1993). In addition, the 5.8S binds to the 28S by hydrogen bonding, to form part of the large ribosomal subunit (60S). Hence, this region is highly conserved throughout organisms (Mitchell *et al.* 1995). Although some success has been obtained in using 5.8S to examine relationships within major phyla of eukaryotes, this region is relatively short, so the number of phylogenetically informative sites can be limited (Hills *et al.* 1991).

Not only can the 5.8S region be used for the phylogenetic analysis of species, but also non conserved regions like ITS 1 and 2 can provide excellent information for the development of species-specific probes (Lott *et al.* 1993; Srikantha *et al.* 1994) in fungi. Therefore, phylogenetic analysis based on the sequence data of 5.8S was undertaken in this study with the subsequent development of species-specific probes in the ITS regions for the detection of *C. krusei* and *C. inconspicua* as a confirmatory identification step (see Chapter 9).

5.1.2 Phylogenetic analysis based on the sequences

Construction of a phylogenetic tree showing the evolutionary relationships of all organisms is the main aim of taxonomy (Priest *et al.* 1993). Since nucleotide changes happen more or less randomly in time, the rate of change of these nucleotide sequences can be used as a molecular clock. Recently, sequence data of small and large subunit rRNAs have had a great impact on yeast systematics.

The extent and nature of the differences among nucleotides sequences provides an incisive insight into the phylogenetic relationships of all organisms; the more

homogeneous the sequences, the more closely related the organisms and conversely, the more diverse the sequences the more distant the organisms. Closely related organisms are descended from more recent common ancestors than are distantly related ones. Therefore the aim of the phylogeneticist is to determine the pattern of this branching and represent it as a tree from ancestral forms through to the organisms as we see them today.

5.1.3 Multiple alignment

Multiple alignments have been used for the detection of homology between new sequences and existing families of sequences. Multiple alignment analysis is also used for finding diagnostic patterns to characterise protein families and for predicting the secondary and tertiary structures of new sequences. For the construction of phylogenetic trees, multiple alignment must be carried out. In this study, Clustal W was used for the analysis of the phylogenetic place of *C. inconspicua* and *C. krusei* among fungal species. Currently the most widely used approach is to exploit the fact that homologous sequences obtained from multiple alignment are evolutionarily related.

The basic multiple alignment algorithm is as follows (Thompson *et al.* 1994). Firstly, all pairs of sequences are aligned separately in order to calculate a distance matrix giving the divergence of each pair of sequences. Secondly, a guide tree is calculated for the distance matrix. Thirdly, the sequences are progressively aligned according to the branching order in the guide tree. For the construction of more reliable alignment analysis, several factors such as gap opening and extension penalties are used (Thompson *et al.* 1994). For instance, increasing the gap opening penalty will make gaps less frequent and increasing the gap extension penalty will make gaps shorter. Phylogenetic trees that use the

Neighbour-Joining method (Saitou *et al.* 1987) are based on a matrix of distance between all species. The procedure for the tree construction is illustrated in Figure 5.1.

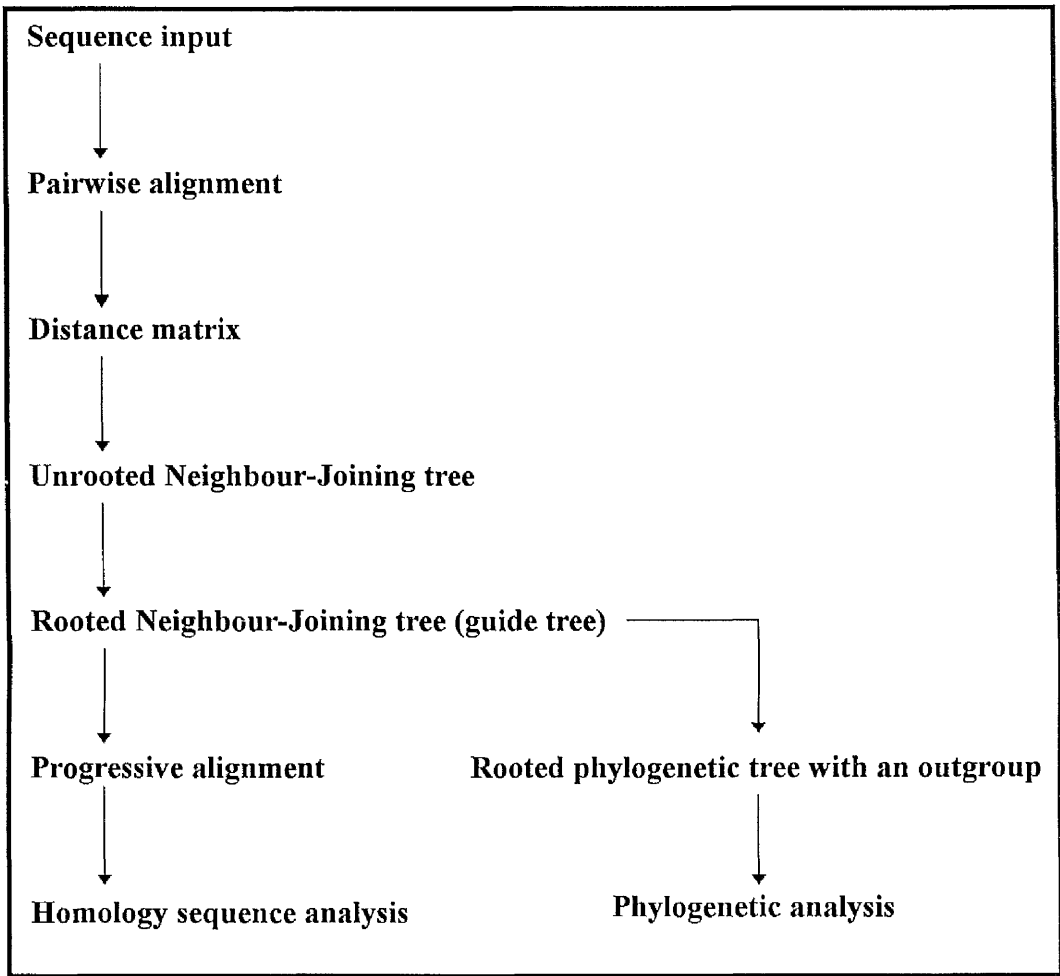


Figure 5.1 Sequence alignment and phylogenetic analysis procedure.

5.1.4 Tree construction

A phylogenetic tree is a graph composed of nodes and branches, and only one branch connects any two adjacent nodes. The nodes of the tree represent the taxonomic units which may be species, individuals or genes (Priest *et al.* 1993). The tree can be constructed in several ways, 1) unscaled tree or scaled tree (Figure 5.2). In an unscaled tree, the external nodes are aligned and branches are presented. In contrast, a scaled tree has the lengths of branches which are proportional to the number of molecular changes.

In addition, the tree is also constructed as rooted and unrooted (Figure 5.3). A rooted tree has a unique path leading to the branches showing the direction of each evolutionary path. An unrooted tree just shows phylogenetic relationships but does not show an evolutionary path.

To generate a rooted tree, an outgroup which is distantly related to the organisms under study is used. In order to construct a valid tree, the distances are calculated based on algorithms such as Jukes-Canor (Jukes *et al.* 1969), Kimura (Kimura, 1980), Tajima and Nei and Jin-Nei gamma methods. For the better parsimony analysis of *Candida* species using phylogenetic tree in our study, Kimura two parameter method with the assumption in which transition substitution (purine-purine, or pyrimidine-pyrimidine) often occur much more frequently than transversion (purine-pyrimidine) substitutions was used (Kimura 1980), and the evolutionary distance per site is represented as K: $K = -\frac{1}{2} \ln \{(1-2P-Q) \sqrt{1-2Q}\}$, P= position scored as transitions type; Q= position scored as transversions type

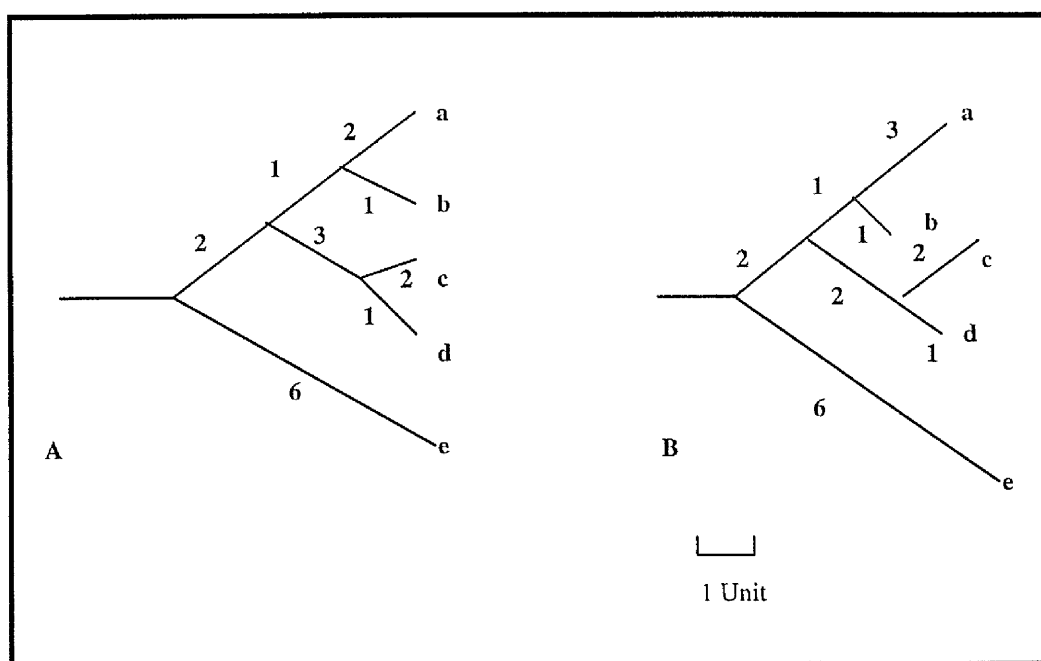


Figure 5.2 Two alternative representations of a phylogenetic tree.

A: Unscaled tree; B: Scaled tree: Lengths of branches are proportional to the numbers of molecular changes.

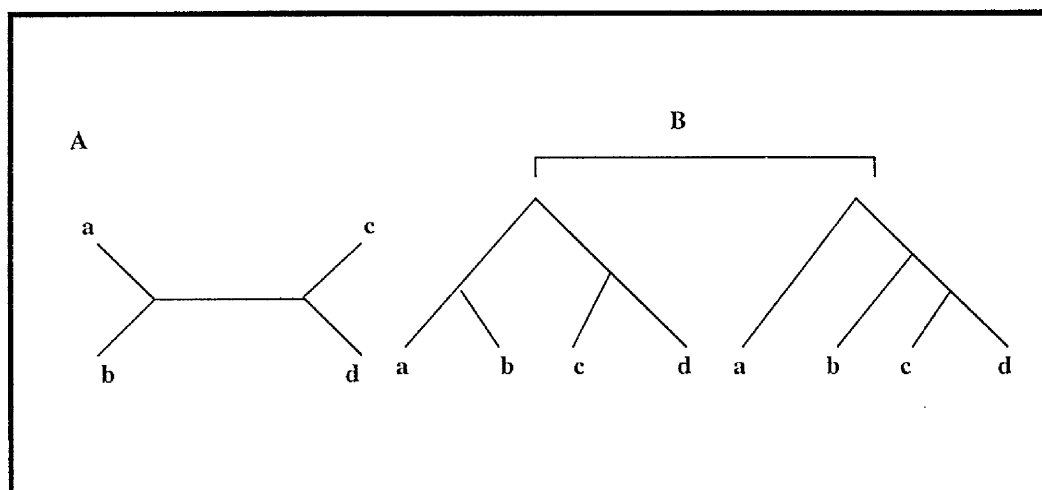


Figure 5.3 The rooted and unrooted trees.

A: unrooted tree; B: rooted trees

5.2 Results

5.2.1 Sequencing strategy

To obtain the correct sequences of the *C. krusei* and *C. inconspicua* PCR products, PCR was performed twice, and transformants from the first and second amplification products containing the ITS and 5.8S regions were then sequenced. The clone preparation for this study is outlined in Figure 5.4.

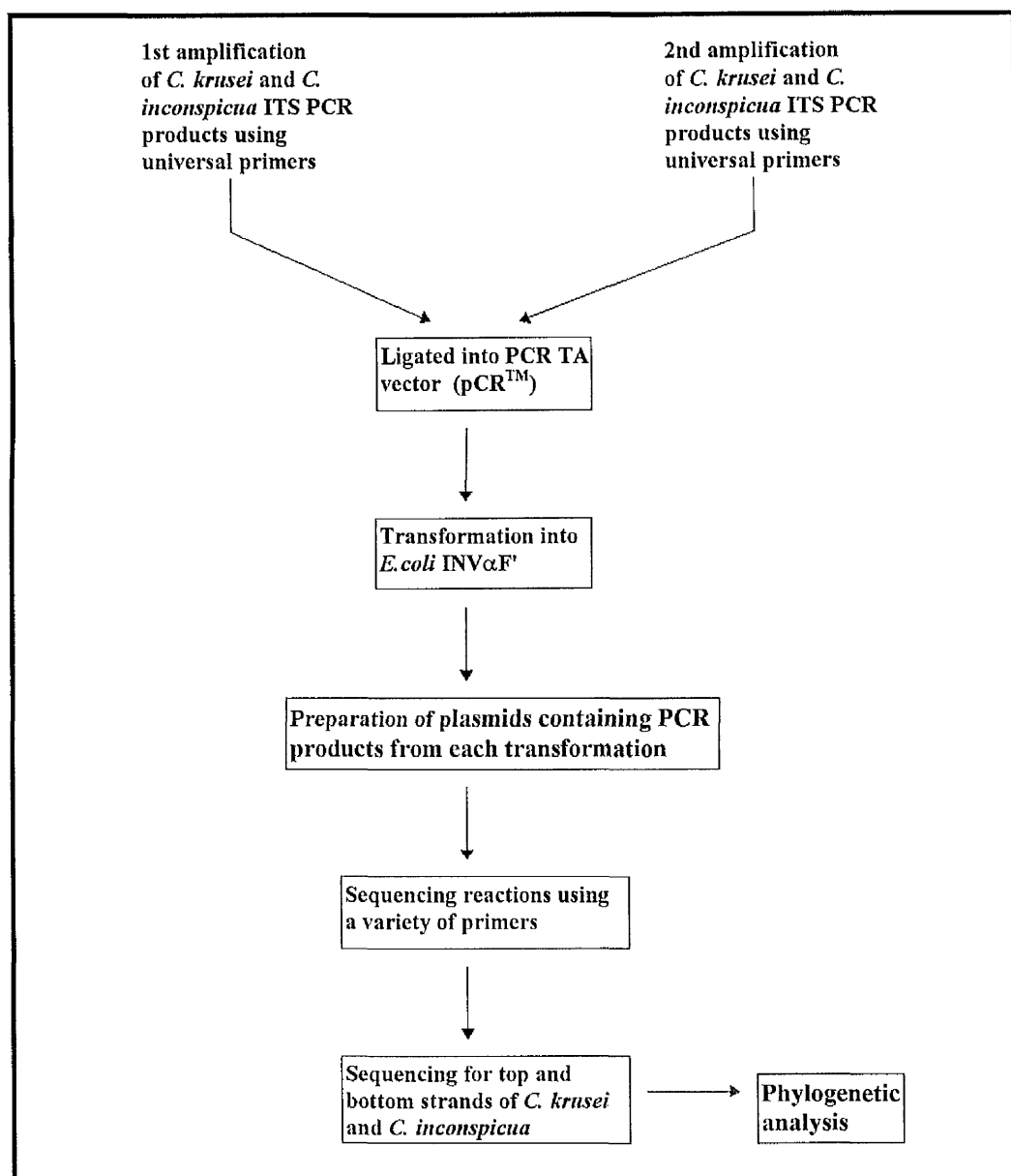


Figure 5.4 Cloning of *C. krusei* and *C. inconspicua* ITS 1 to 4 regions.

A variety of primers were used to ensure that the correct sequence was obtained. In addition, top and bottom strands of selected clones were also sequenced. A total of seven primers, i.e. four ITS primers (1, 2, 3, and 4) and three universal primers (M13 reverse, M13 forward (-40) and M13 (-20)), were used to generate sequence on both strands (Figure 5.5.A). Several clones of *C. inconspicua* and *C. krusei* used for the sequencing are listed in Figure 5.5.B. Figure 5.6 shows the sequencing strategy for *C. krusei* and *C. inconspicua*.

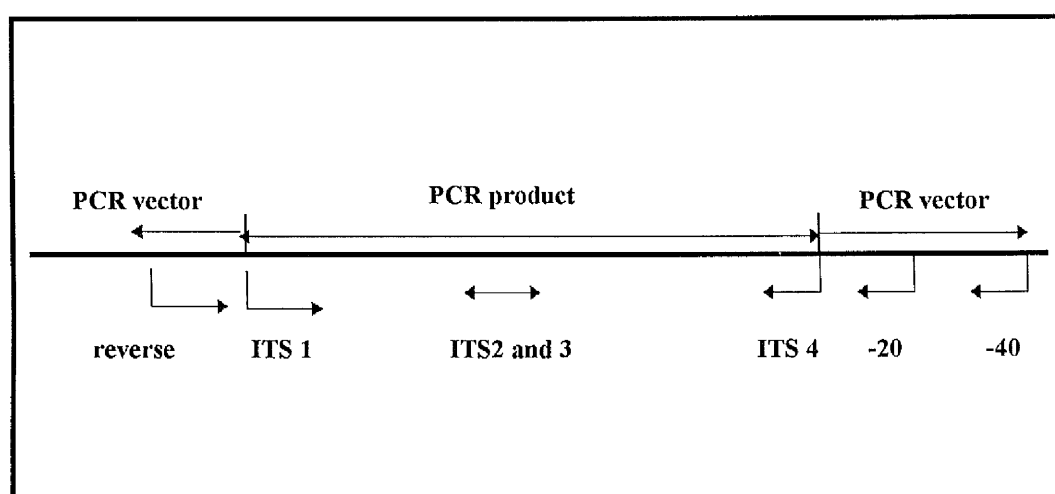


Figure 5.5.A Primer positions for PCR product sequencing.

C. inconspicua

clone 3 : reverse, ITS 1,2,3 and 4, forward (-40)

clone 4 : reverse, forward, ITS 3

clone 2 : reverse, forward (-20)

clone 6 : forward

clone 8 : forward

clone 3 (2)^a : ITS 1

C. krusei

clone 3 : ITS 2, 3 and 4

clone 2 (2)^b: ITS 1, 2, 3, 4, forward (-40), (-20)

clone 5 (2)^c: ITS 1, 2, 3, 4, reverse, forward (-40),(-20)

clone 5 : ITS 1, 2, 3 and 4, reverse, forward (-40)

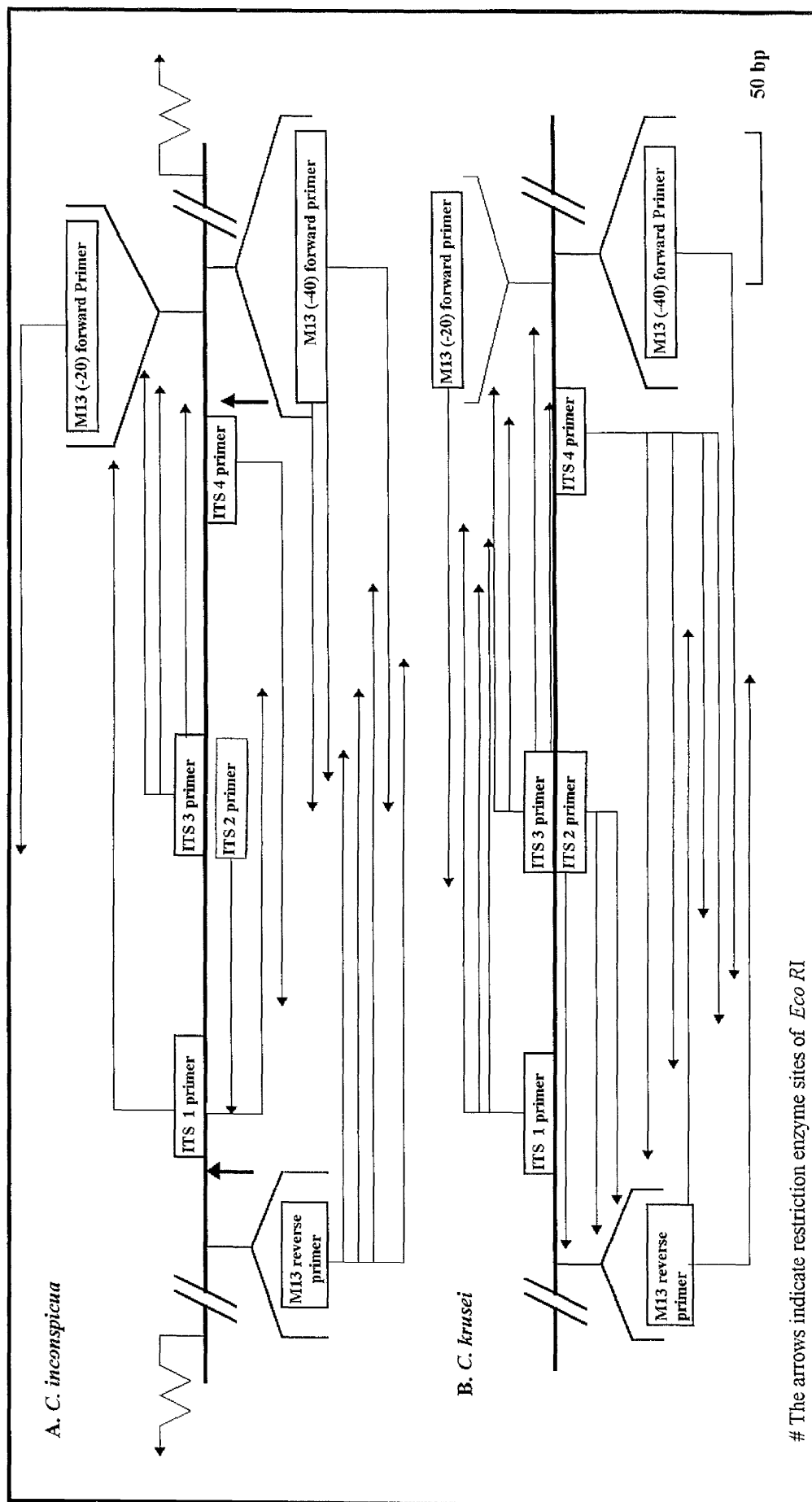
clone 1 : ITS 3 , reverse

clone 4 : ITS 4

Figure 5.5.B Clones of *C. inconspicua* and *C. krusei* using different primers for sequencing.

a,b,c : 2nd amplified PCR products of rDNA of *C. inconspicua* and *C. krusei*.

Figure 5.6 Sequencing analysis of *C. inconspicua* and *C. krusei*.



5.2.2 Comparative sequence analysis and phylogeny of the 5.8S rDNA and ITS regions of *Candida* species and fungal species.

5.2.2.1 Analysis of 5.8S rDNA

After comparative analysis of multiple sequence data from the 5.8S region of *C. inconspicua* and *C. krusei*, great sequence homology was found. The only sequence differences between these species was a TC inversion at 118 and 119 bp (Figure 5.7). The total length of the 5.8S sequence was 157 bp for both species, similar to other *Candida* which are 157, 158 and 159 bp. As there are 2 bp differences between these two species, 99% homology is recorded (Table 5.2), suggesting that these species are closely related to each other. The alignment of all 5.8S rDNA sequences submitted on the EMBL database (release on 23 Jul. 1996) of *Candida* and other fungal species is shown in Figure 5.8. One *C. albicans* strain (L47111) showed 100% sequence identity with *C. stellatoidea* (L47114). In the case of *C. tropicalis* species, there was 1 bp difference between the two *C. tropicalis* strains (L11349 and L47112). The two *C. parapsilosis* strains also showed one base pair difference. Parsimony analysis of *C. parapsilosis* and *C. tropicalis* sequence data indicate their close genetic relatedness.

ITS regions of each species appear to show variable size differences whereas all *Candida* species had similar sizes of 5.8S rDNA, at around 158 bp. The 5.8S sequence of *C. inconspicua* was then compared with the corresponding region of other *Candida* species (Table 5.3), and the lowest identity, 83%, between *Ph. carinii* and *C. inconspicua*, was recorded. Other species showed 85 to 92% homology compared with *C. inconspicua* over the same region.

The phylogenetic analysis, based on the sequence data of this region in *Candida* species, was performed using Clustal W and Treeview programmes and statistical bootstrap analysis was carried out (Figure 5.10). The genetic distance matrix based on a comparison of 5.8S sequences in fungal species are listed in Table 5.1. For the preparation of tree construction, *Ph. carinii* was used as an out group. The rooted tree using neighbour-joining method (Saitou *et al.* 1987) was then compared with the result of the parsimony analysis derived from the alignment of a 500 bp section of the V3 region (Sullivan *et al.*, 1995) from selected *Candida* species. Moreover, several analysis of the data with different conditions of gap and opening penalties, the tree topology was not changed. The unscaled rooted tree and scaled radial tree of this region were also constructed to give a better visual comparison of distance between species (Figure 5.11 and 5.12).

Over 99% of the positive arrangements between *C. krusei* and *C. inconspicua* on the 5.8S region were suggested by bootstrap analysis. This is strong evidence of their taxonomic closeness since a value over 70% would represent a significant degree of probability (Lott *et al.* 1993). Interestingly, these two species appeared to be distantly related to other *Candida* species.

5.2.2.2 Analysis of ITS 1 region

The entire sizes of the ITS 1 region of *Candida* species had variable lengths (White *et al.* 1990) in the range of 89 to 229 bp, suggesting this region is not conserved between species. In the case of *C. inconspicua*, the size of the ITS 1 fragment was 89 bp, and much smaller than most other *Candida* species. In contrast of those species so far sequenced, *C. kefyr* had the biggest fragment in this region, 229 bp. *C. krusei* had a size of 102 bp, and only differences, 13 bp compared with *C. inconspicua* was recorded. In particular, it was interesting that

sequencing data derived from this ITS 1 region of *C. krusei* and *C. inconspicua* showed that these species are also closely related to each other with a homology of 81% (Table 5.2).

Although previously published data from the corresponding region of another *C. krusei* isolate (L11350) had no *RsaI* recognition site on this region, five isolates from bioMérieux and ATCC showed the *RsaI* restriction site on the PCR products (Figure 5.14, 6.2 and 6.3.A). Sequencing also showed the *RsaI* site on the ITS 1 region of *C. krusei* ATCC 6258 isolate. In contrast, in the case of *C. inconspicua*, sequencing showed that there was no *RsaI* restriction sites in this region, and no polymorphisms were found (total 7 isolates; Figure 5.13 and 6.2).

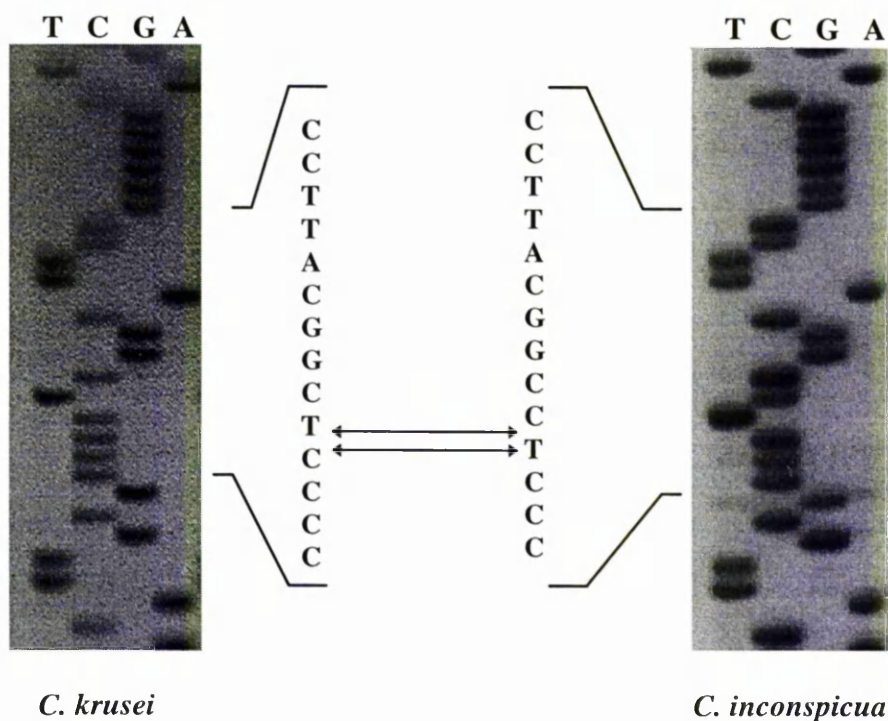
Although the ITS regions were not found to have great similarity between species, these regions were studied to analyse the possible ribosomal processing site. The sequence CTGATTTGC(G)AATT was found in the 5' start region of the ITS 1 region in *C. tropicalis*, *C. stellatoidea* and *C. albicans* (data not shown). In addition, ATAGTCA(C) was recorded in the 3' end region of ITS 1 for these species. Except for *C. kefyr*, conserved sequences were found in all *Candida* species in the 5' start region and 3' end region. For instance, TCATTA was found at the 3' end of 18S in *C. krusei* and *C. inconspicua* (Figure 5.15.A), suggesting a possible ribosomal processing site. Previously, TCAATA was proposed by De Jong *et al.* (1977) as a processing site.

The sequence similarity of other *Candida* species with *C. inconspicua* was studied. *C. kefyr* had the lowest homology, 49% (Table 5.3). Other *Candida* species were between 53 and 57% similar to *C. inconspicua* in this region.

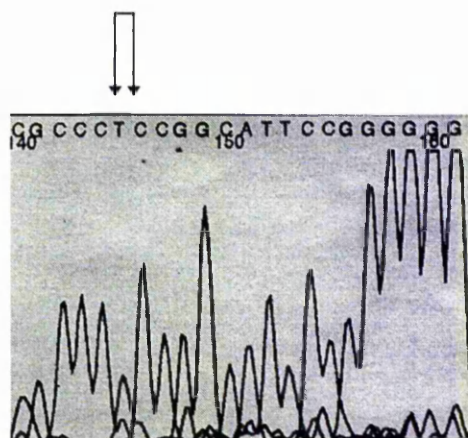
5.2.2.3 Analysis of ITS 2 region

Great sequence similarity was found in the 5' start sequence of all *Candida* species except *C. krusei* and *C. inconspicua*. The ITS 2 fragment of *C. inconspicua* was found to be 122 bp whereas it was 163 bp in *C. krusei*. The ITS 2 sequence length for *C. kefyr* length was 247 bp and therefore the largest ITS 2 fragment noted among the *Candida* isolates for which information is available. The sequence identity in this region between *C. inconspicua* and *C. krusei* was 62% (Table 5.2), which indicated that the ITS 2 region for these species has relatively low homology compared with the ITS 1 region (Figure 5.9). This information is potentially useful because species-specific probes could be designed for this region for use in specific identification between *C. krusei* and *C. inconspicua*. Sequence similarity between *C. inconspicua* and other *Candida* species was between 54 and 66% (Table 5.3). As was the case for ITS 1, *C. guilliermondii* and *C. kefyr* showed the lowest homology, 54 and 57%, respectively against *C. inconspicua*. *C. stellatoidea* was found to be very closely related to *C. albicans* as found for 5.8S region, with almost identical sequences. Likewise, higher homology between *C. parapsilosis* and *C. tropicalis* was recorded in this region compared to that of the ITS 1 region (data not shown).

Except for *C. krusei* and *C. inconspicua*, the conserved region of the ITS 2 area was CTCT (C)CTCAAACC at the 5' start followed by a great similarity of sequence up to base 37, and for the 3' end of ITS 2 region, AC(G)T(C)T was found. The whole sequence alignment of *C. krusei* and *C. inconspicua* is presented in Figure 5.13 and 5.14.



A. Nucleotide differences on the 5. 8S.
Arrows indicate nucleotide substitutions between two species



B. Automated sequencing (Chromatogram) on substitution sites of the 5. 8S of *C. inconspicua*.
Arrows indicate the site of nucleotide substitutions compared with the corresponding sites of *C. krusei*.

Figure 5.7 Nucleotide differences between *C. inconspicua* and *C. krusei* on the 5.8S rDNA.

	***** ** *** ***** ** ***** ** ** ** *
<i>C. albi.</i> (1)	AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGC-GA
<i>C. albi.</i> (2)	AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCCGA
<i>C. stella.</i>	AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGC-GA
<i>C. para.</i> (1)	AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGC-GA
<i>C. para.</i> (2)	AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCCGA
<i>C. trop.</i> (1)	AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCCGA
<i>C. trop.</i> (2)	NAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGC-GA
<i>S. cerevi.</i>	AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGC-GA
<i>C. guiller.</i>	AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGC-GA
<i>C. kefyfyr</i>	AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGC-GA
<i>C. glab.</i>	AAACTTTCAACAATGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCCGA
<i>C. krusei</i>	AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAAGCGCAGCGAAATGC-GA
<i>C. incons.</i>	AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAAGCGCAGCGAAATGC-GA
<i>Pn. carinii</i>	AAACTTTCAACAATGGATCTCTTGGTTCCCGCGTCGATGAAGAACGTGGCAAATGC-GA
<i>N. crassa</i>	AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC-GA
	** * * * ***** * ***** * * * * ***** *
<i>C. albi.</i> (1)	TACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTG-AACGCACATTGCGCC
<i>C. albi.</i> (2)	TACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTG-AACGCACATTGCGCC
<i>C. stella.</i>	TACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTG-AACGCACATTGCGCC
<i>C. para.</i> (1)	TAAGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTG-AACGCACATTGCGCC
<i>C. para.</i> (2)	TAAGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTG-AACGCACATTGCGCC
<i>C. trop.</i> (1)	TACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTG-AACGCACATTGCGCC
<i>C. trop.</i> (2)	TACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTG-AACGCACATTGCCCC
<i>S. cerevi.</i>	TACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTG-AACGCACATTGCGCC
<i>C. guiller.</i>	TAAGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTG-NACGCACATTGCGCC
<i>C. kefyfyr</i>	TATGTATTGTGAATTGCAGATNT-CGTGAATCATCAAATCTTTGGAACGCACATTTGCGCC
<i>C. glab.</i>	TACGTAATGTGAATTGCAGATATTCGTGAATCATCGAATCTTTG-AACGCACATTGCGCC
<i>C. krusei</i>	TACCTAGTGTGAATTGCAGCCAT-CGTGAATCATCGAGTTCTTTG-AACGCACATTGCGCC
<i>C. incons.</i>	TACCTAGTGTGAATTGCAGCCAT-CGTGAATCATCGAGTTCTTTG-AACGCACATTGCGCC
<i>Pn. carinii</i>	TAAGTAGTGTGAATTGCAGATTCAGTGACTCATCGAATTTTTG-AACGCATATTGCGCT
<i>N. crassa</i>	TAGGTAATGTGAATTGCAGATTCAGTGAATCATCGAATCTTTG-AACGCACATTGCGCT
	* *
<i>C. albi.</i> (1)	CTCTGGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTT 158 bp.
<i>C. albi.</i> (2)	CTCTGGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTT 159
<i>C. stella.</i>	CTCTGGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTT 158
<i>C. para.</i> (1)	CTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTT 158
<i>C. para.</i> (2)	CTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTT 159
<i>C. trop.</i> (1)	CTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTT 159
<i>C. trop.</i> (2)	CTTTGGTATTCCAAAGGGNATGCCTGTTTGAGCGTCATTT 158
<i>S. cerevi.</i>	CCTTGGTATTCCAGGGGGCATGCCTGTTTGAGCGTCATTT 158
<i>C. guiller.</i>	CNCTGGTATTCCAGAGGGGATGCCTGTTTGAGCGTCANNC 158
<i>C. kefyfyr</i>	CTCTGGTATTCCAGGGGGCATGCCTGTTTGAGCGTCATTT 158
<i>C. glab.</i>	CTCTGGTATTCCGGGGGGCATGCCTGTTTGAGCGTCATTT 159
<i>C. krusei</i>	CCTCGGCATTCCGGGGGGCATGCCTGTTTGAGCGTCGTTT 157
<i>C. incons.</i>	CTCCGGCATTCCGGGGGGCATGCCTGTTTGAGCGTCGTTT 157
<i>Pn. carinii</i>	CCTCAGTATTCTGTGGAGCATGCCTGTTTGAGCGTCATTT 158
<i>N. crassa</i>	CGCCAGTATTCTGGCGAGCATGCCTGTTTCGAGCGTCATTT 158

Figure 5.8 Multiple sequence alignment of 5.8S rDNA of fungal species.

Asterisks indicate the matching sequences. The EMBL accession numbers are as follows:

C. albi(1), (2): *C. albicans* (L47111),(L07796); *C. para.*(1),(2): *C. parapsilosis*, (L47109),(L11352); *C. incons.*:*C. inconspicua*, bioMérieux (18949); *C. krusei*: ATCC (6258) *S. cerevi.*:*S.cerevisiae* (K01048); *C. guiller.*:*C. guillermundii* (L47110); *C. trop.*(1)(2): *C. tropicalis*, (L11349),(L47112); *N. crassa* (M10692); *Pn. carinii* (M86760);*C. stella.*: *C. stellatoidea* (L47114).*C. kefyfyr*: (L47107);*C. glab.* : *C. glabrata* (L11351).

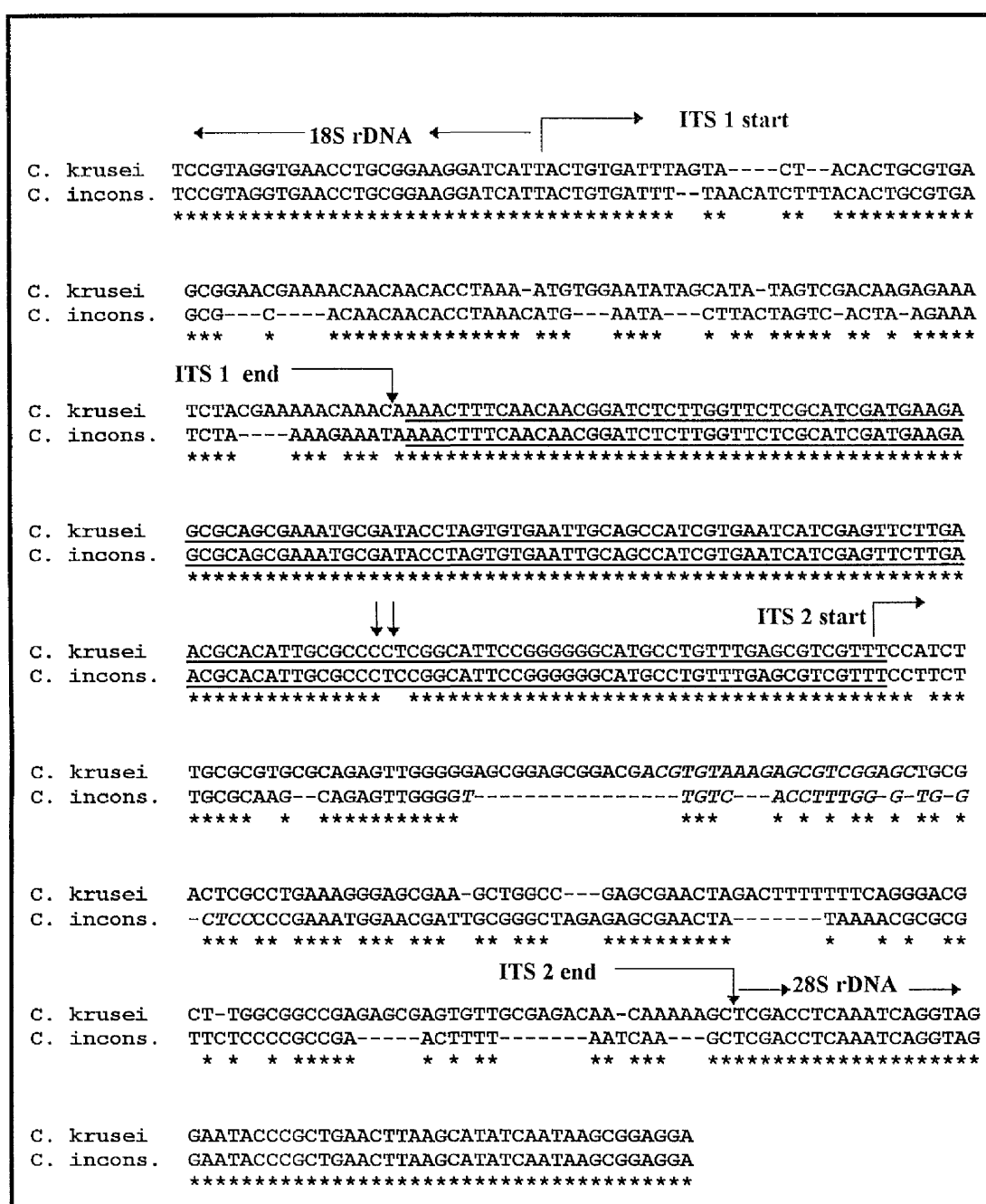


Figure 5.9 Sequence alignments of *C. krusei* and *C. inconspicua* in 5.8S rDNA including the ITS regions.

Asterisks indicate the matching sequences, and underlines represents 5.8S rDNA area. The positions where species-specific probes, CKP, CIP were designed are presented in italics. Arrows indicate nucleotide differences on 5.8S rDNA.

Table 5.1 Genetic distance matrix of fungal 5.8S rDNA.

	<i>C. albi.</i>	<i>C.stella.</i>	<i>C. para.</i>	<i>C. tropi</i>	<i>C. gla.</i>	<i>C. kru.</i>	<i>C. incons.</i>	<i>Pn.cari.</i>	<i>N. cra.</i>	<i>S.cere.</i>	<i>C. guiller.</i>	<i>C. kefy</i>
<i>C. albi.</i>	-											
<i>C. stella.</i>	0.0	-										
<i>C. para.</i>	3.3	3.3	-									
<i>C. tropi.</i>	2.6	2.6	0.6	-								
<i>C. gla.</i>	3.9	3.9	6.0	5.3	-							
<i>C. krusei</i>	9.7	9.7	12.0	11.2	10.4	-						
<i>C. incons.</i>	8.2	8.2	12.0	11.2	8.9	1.3	-					
<i>Pn. carinii</i>	19.3	19.3	17.6	18.4	14.3	18.3	20.0	-				
<i>N. crassa</i>	10.3	10.3	11.8	11.8	8.1	15.7	14.8	12.6	-			
<i>S. cerevi.</i>	5.3	5.3	4.6	3.9	2.6	8.9	10.4	14.3	8.8	-		
<i>C. guiller.</i>	4.7	4.7	4.0	4.7	6.2	14.7	13.8	20.0	11.4	5.4	-	
<i>C. kefy</i>	6.1	6.1	6.0	6.1	5.3	12.8	11.2	19.4	11.8	5.3	6.9	-

Values correspond to percentages of difference by the method of Kimura.

The species' EMBL accession numbers are as follows.

C. albi: *C. albicans* (L47111); *C. para.*: *C. prapsilosus*, (L47109); *C. incons.*: *C. inconspicua* (bioMérieux (18949); *C. krusei*: ATCC(6258) *S.cerevi.*: *S.cereviaiae* (K01048); *C. guiller.*: *C. guillermondii* (L47110); *C. tropi.*: *C. tropicalis*, (L11349); *N. crassa* (M10692); *Pn. carinii* (M86760); *C. stella.*: *C. stellatoidea* (L47114), *C. gla.*: *C. glabrata* (L11351)

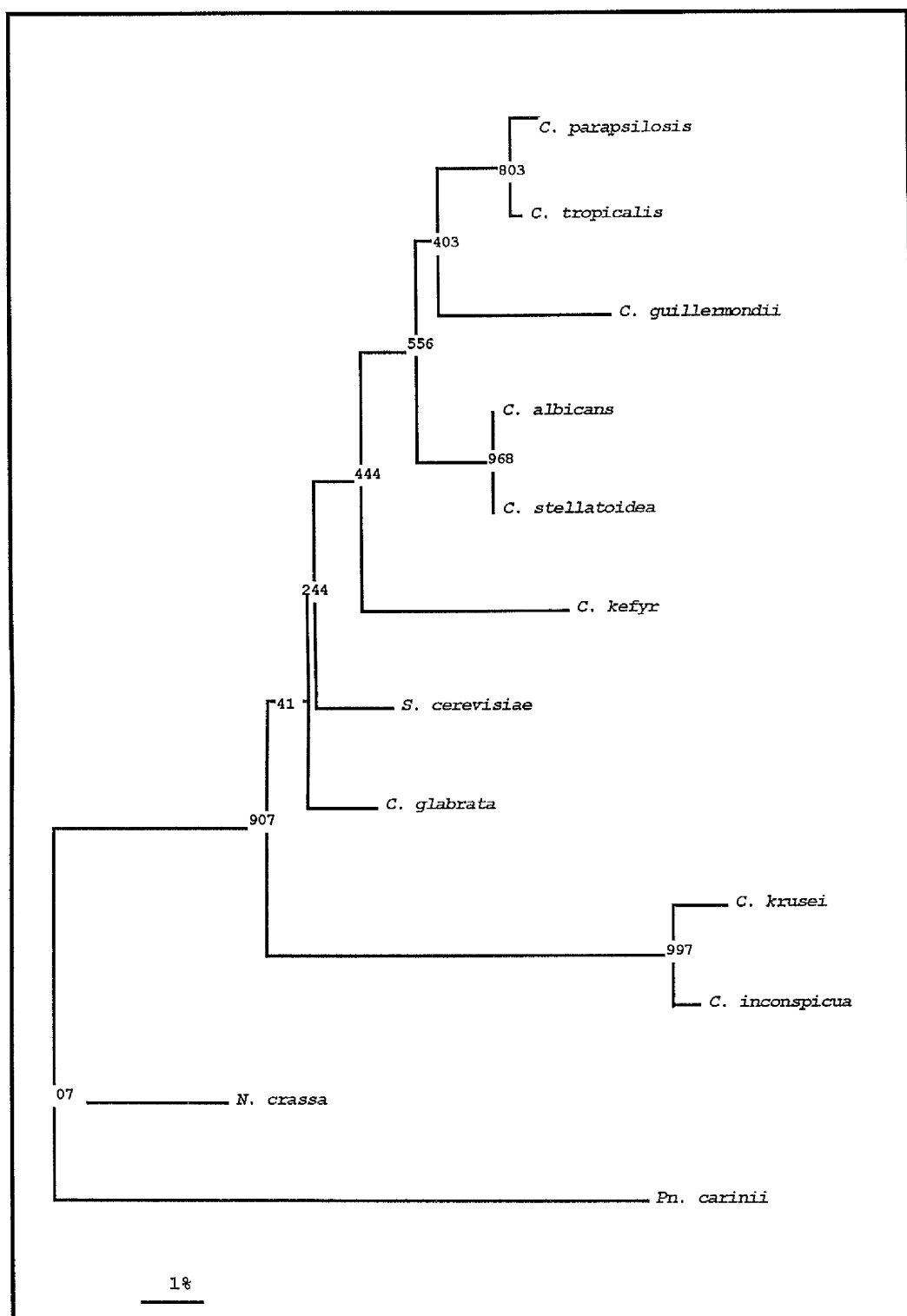


Figure 5.10 Phylogenetic tree (phylogram) of 5.8S rDNA.

The phylogenetic tree (scaled and rooted) was constructed based on the comparative analysis of 5.8S rDNA sequences. The numbers given at each branch point were derived from boot strap analysis, representing the number of times of positive selection out of 1000 total. The EMBL accession numbers of isolates are corresponding to the numbers listed in Table 5.1.

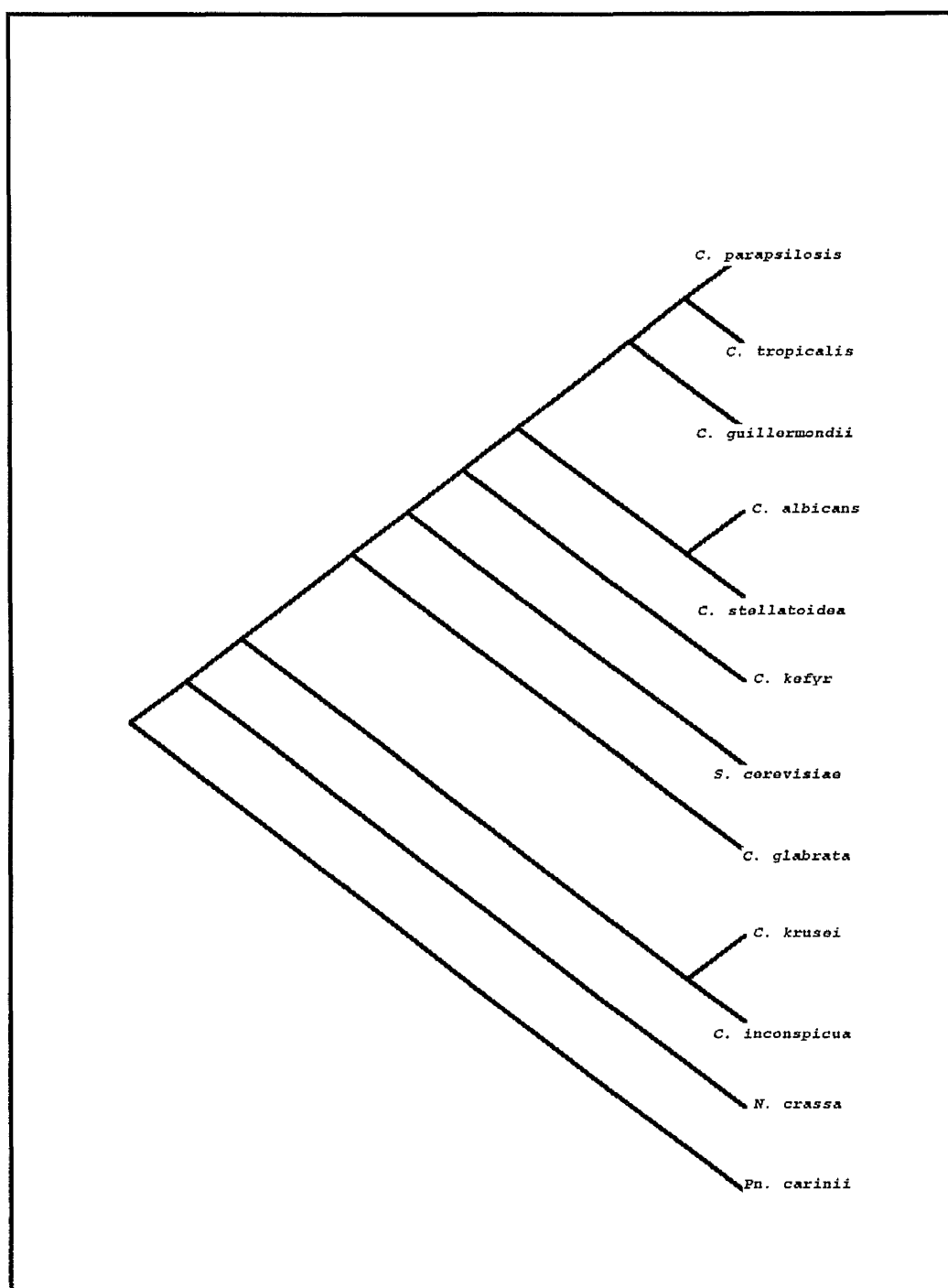


Figure 5.11 Slanted cladogram of 5.8S rDNA.

The phylogenetic tree (unscaled and rooted) was constructed based on the comparative analysis of 5.8S rDNA sequence. The EMBL accession numbers of isolates are corresponding to the numbers listed in Table 5.1.

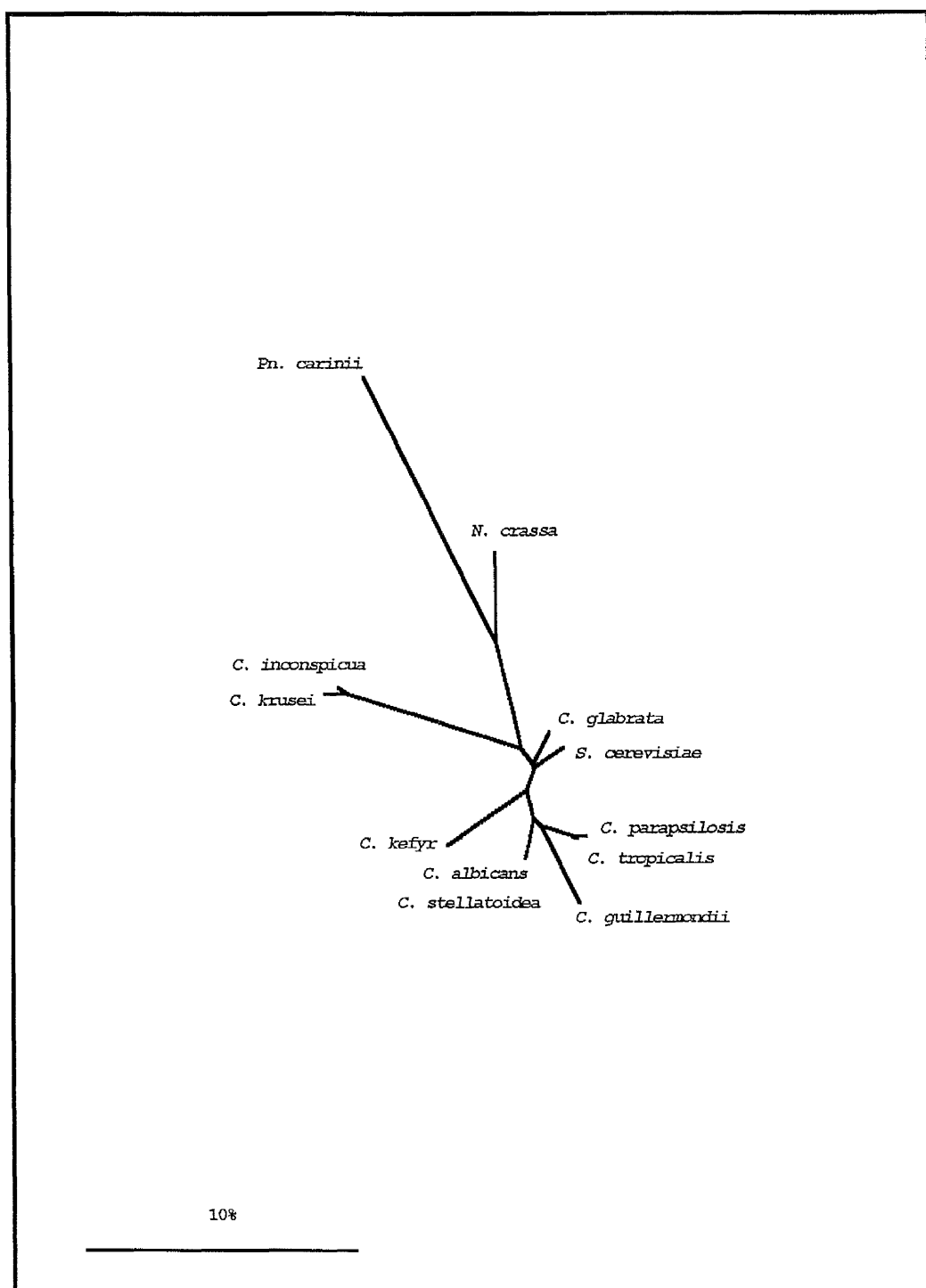


Figure 5.12 Phylogenetic radial tree of 5.8S rDNA.

The phylogenetic tree (scaled and unrooted) was constructed based on the comparative analysis of 5.8S rDNA sequences. The EMBL accession numbers of isolates are corresponding to the numbers listed in Table 5.1.

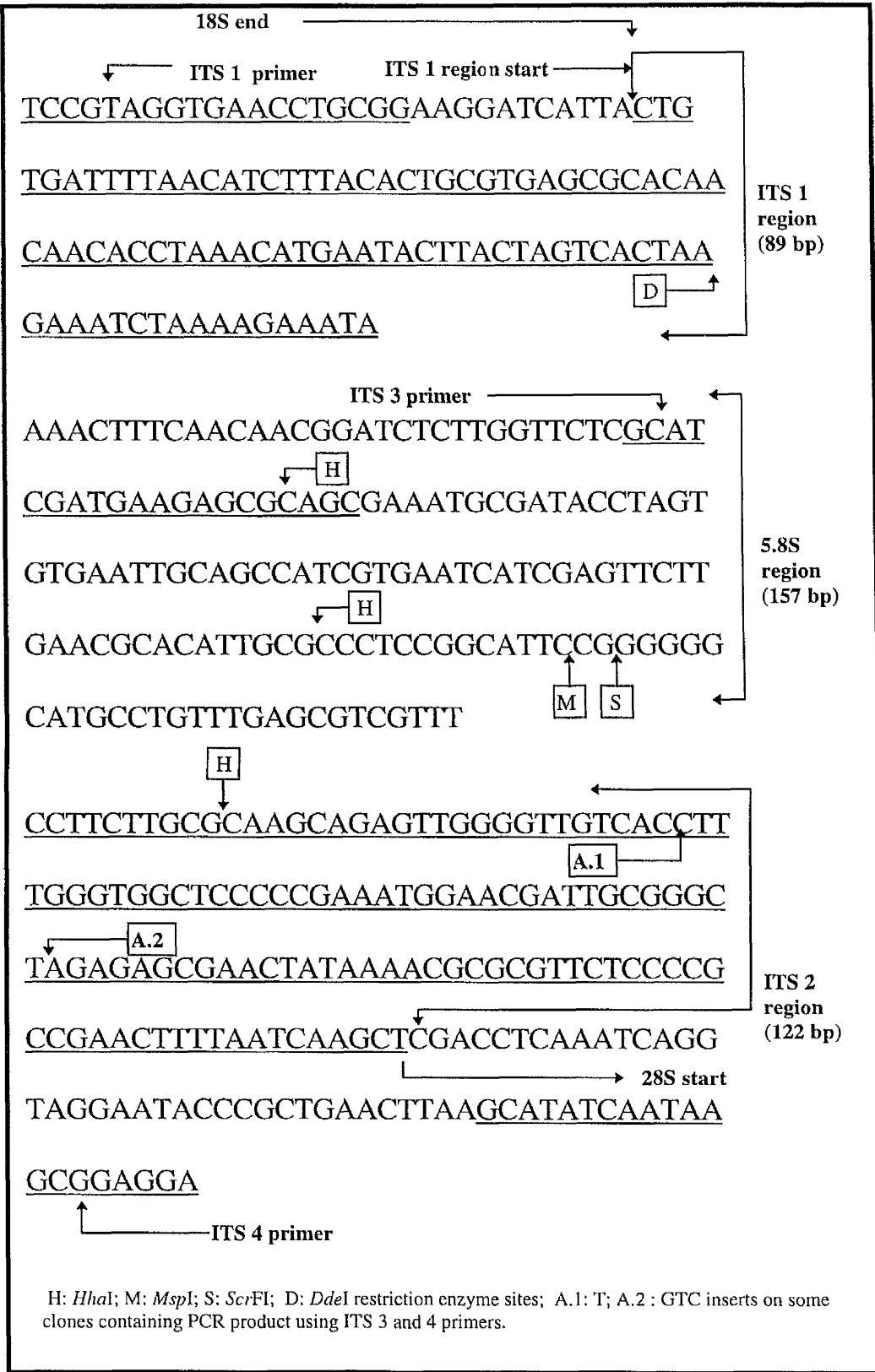


Figure 5.13 Nucleotide sequence of the *C. inconspicua* PCR product using ITS 1 and 4 primers.

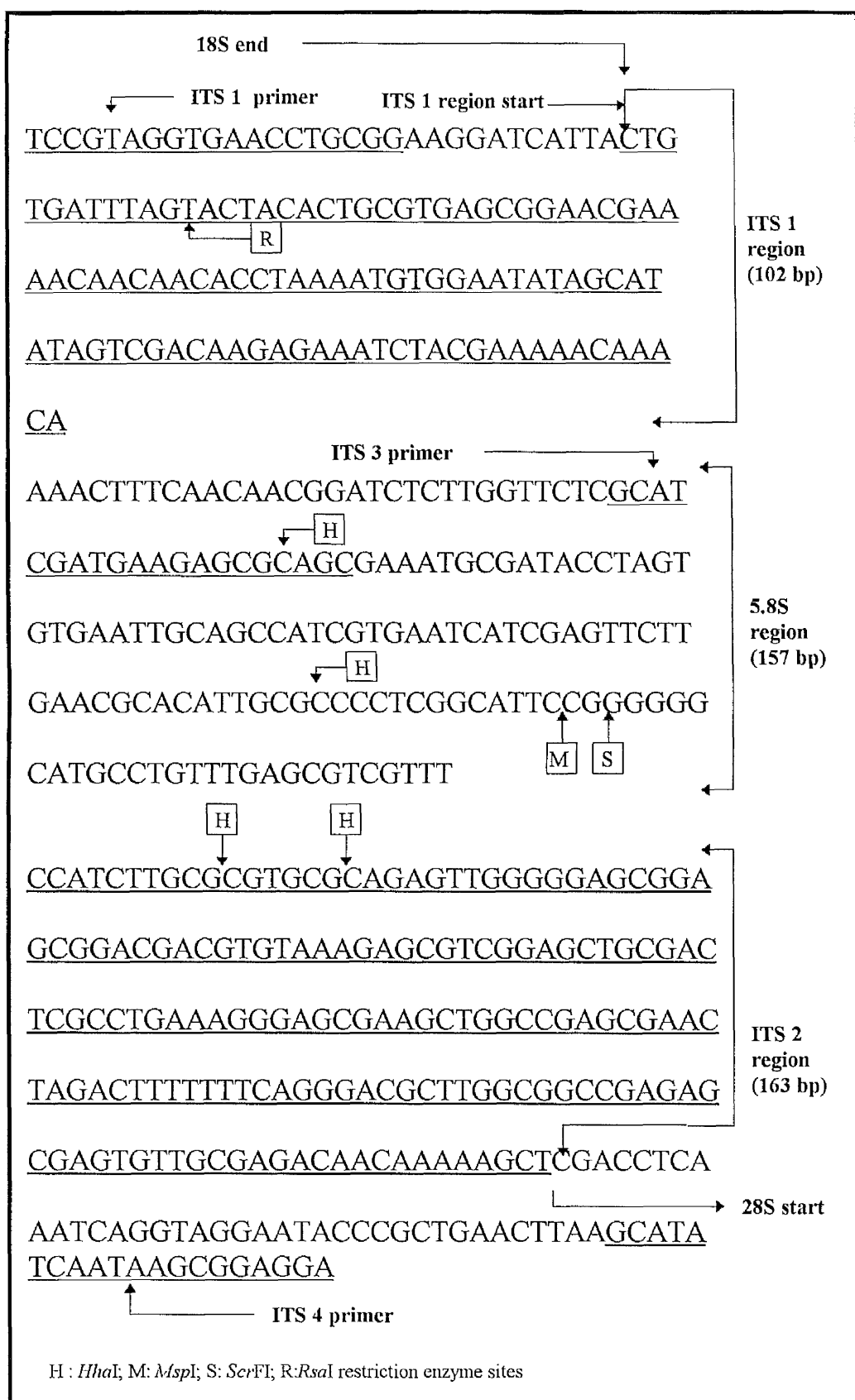


Figure 5.14 Nucleotide sequence of the *C. krusei* PCR product using ITS 1 and 4 primers.

Table 5.2 Comparison of sequence similarity of 5.8 S and ITS 1 and 2 regions between *C. inconspicua* and *C. krusei*

	Percent similarity (%) ^a		
	<i>C. inconspicua</i>		
	ITS 1	5.8S rDNA	ITS 2
<i>C. krusei</i>	81	99	62

^a Based on the equation calculated as follows : (2 x number of matches) / (total number of bases in both sequences)

Table 5.3 Comparison of sequence similarity of 5.8S, ITS 1 and 2 regions between *C. inconspicua* and other fungal species

	Percent similarity (%)		
	<i>C. inconspicua</i>		
	ITS 1	5.8S	ITS 2
<i>C. tropicalis</i> (L47112)	61	88	62
<i>C. parapsilosis</i> (L47109)	54	89	61
<i>C. albicans</i> (L47111)	60	92	66
<i>C. kefyr</i>	49	89	57
<i>C. guilliermondii</i>	53	85	54
<i>C. stellatoidea</i>	57	92	65
<i>C. glabrata</i>	N/D	91	N/D ^a
<i>S. cerevisiae</i>	N/D	90	N/D
<i>Pn. carinii</i>	N/D	83	N/D
<i>N. crassa</i>	N/D	87	N/D

Each *Candida* species has corresponding EMBL accession numbers as described in Table 5.1 ^a: Not defined.

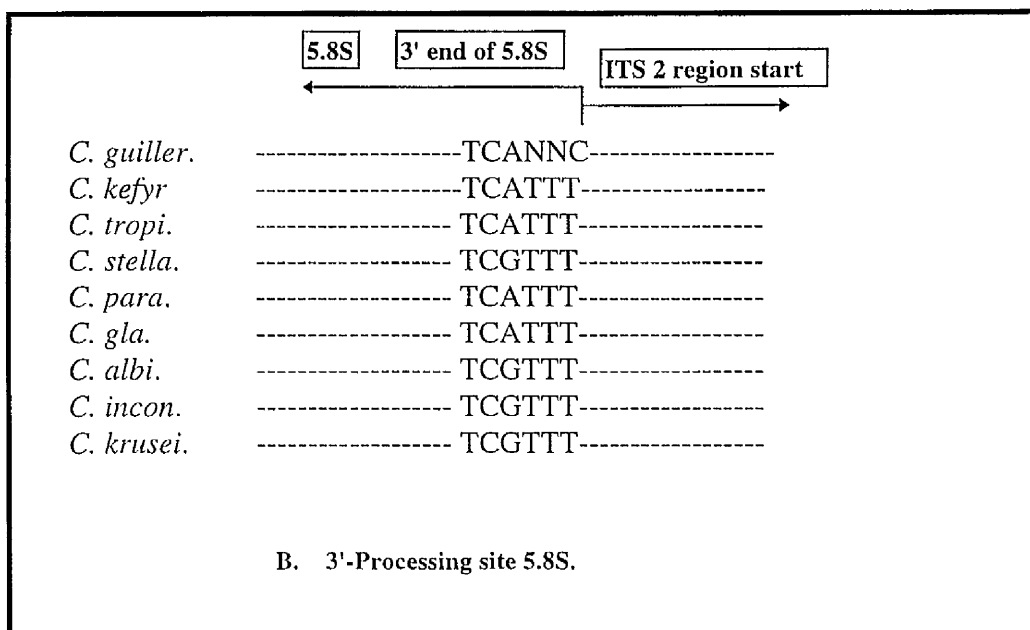
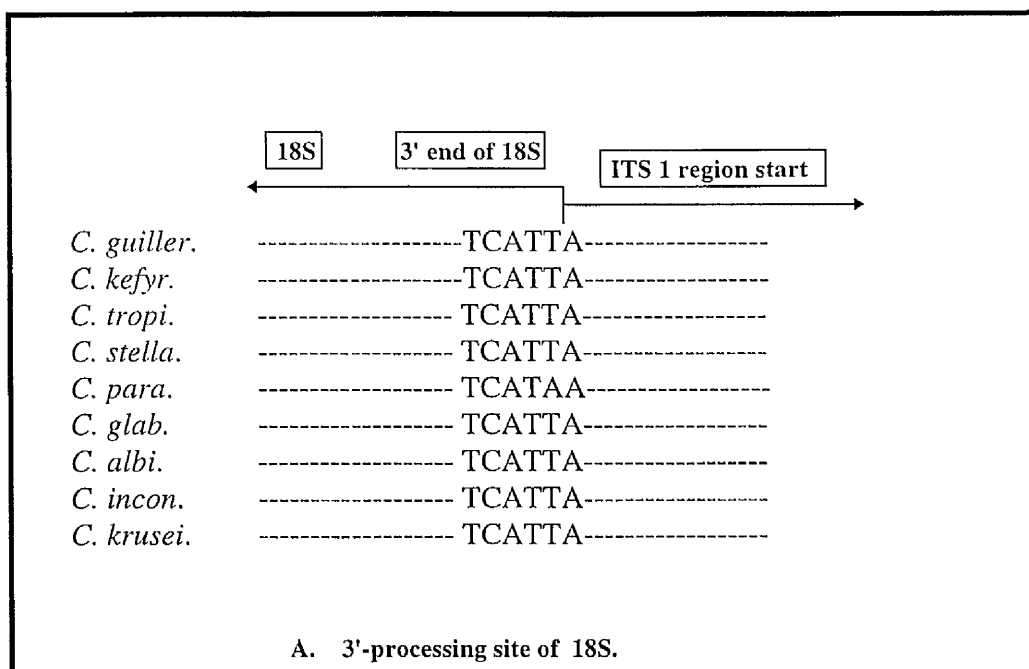


Figure 5.15 Processing sites of 18S and 5.8S of ribosomal DNA in *Candida* species.

EMBL accession numbers are as follows.

C. albi.: L47111; *C. guiller.*: L47110; *C. kefy.*: L47107; *C. tropi.*: L47112; *C. stella.*: L47114; *C. para.*: L47109; *C. gla.*: L47108.

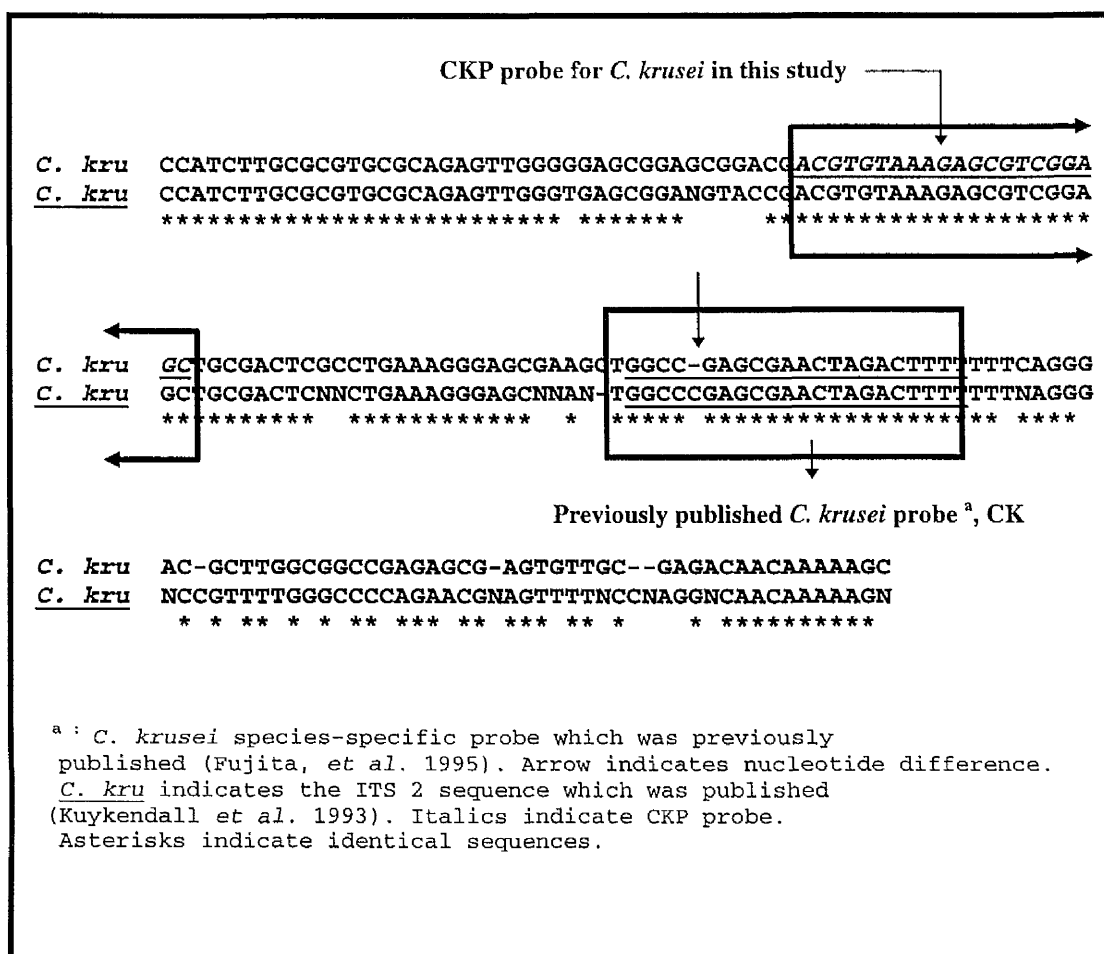


Figure 5.16 Comparison of sequences where *C. krusei* species-specific probes were designed.

5.3. Discussion

The sequence data show that *C. krusei* and *C. inconspicua* have a very similar sequence, suggesting that they are genetically closely related. Both species have an identical size of 5.8S, 157 bp with only 2 bp differences, showing 99% identity. In addition this suggestion was further reinforced by the 99.7% positive arrangement of the bootstrap analysis (Figure 5.10). Several pieces of data suggest that *C. krusei* is completely different from other *Candida* species. This study, in conjunction with other physical and molecular evidence, addresses the phylogeny of these species.

Several recent studies indicate that *C. krusei* might be re-classified taxonomically. This statement is based on evidence not only from metabolic and phenotypic features i.e. colonial morphology, cell wall structure, ultrastructure (Kogan *et al.* 1988), but also from genetic evidence such as sequence homology of ribosomal DNA, coenzyme Q numbers, and average chromosome numbers (Doi *et al.* 1992). For instance, *C. krusei* has an elongated and long grain shape in contrast to other *Candida* species which are ovoid in shape. *C. krusei* also has a modified α -D-mannan structure (Kogan *et al.* 1988). Furthermore, studies have shown that while the average chromosome number is eight, *C. albicans* has 16 (Doi *et al.* 1992). Mata Essayag *et al.* (1996) also showed that chromosome numbers vary from three to six in this species although chromosomal numbers were not used as they were not available. Although *Candida* is a genus of asexual yeasts, *C. krusei* is very closely related to certain sexual species (*Issatchenkia orientalis*; Samaranayake *et al.* 1994).

Previously, *C. krusei* has been classified as being distant from other *Candida* species on the basis of the V3 region of the large ribosomal RNA (Sullivan *et al.* 1995). Likewise, the 5.8S rRNA phylogeny for *C. krusei* supports the concept that *C. krusei* is distantly related to other species and that reassignment could be considered.

The question of the taxonomic closeness between *C. inconspicua* and *C. krusei* at a molecular level was addressed in this study. No published research has been carried out to date to determine the taxonomy of *C. inconspicua*. Both the phenotypic and genotypic characteristics of this species were studied. *C. inconspicua* has similar phenotypic characteristics to *C. krusei*. Physical characteristics such as the inability to develop pseudohyphae on rice agar tween (RAT) medium and the inability to assimilate N-acetyl glucosamine exemplify the conventional approach for the identification of *C. inconspicua*. However, current methodology based on fermentation and assimilation tests in the diagnostic microbiology laboratory does not always provide enough information for the identification and differentiation of *C. krusei* from *C. inconspicua* (Baily *et al.* 1996; Mata Esayag *et al.* 1996).

The analysis of sequence from the 5.8S, ITS 1 and 2 regions showed that *C. parapsilosis* and *C. tropicalis* are closely related to each other with a similar tree topology observed to that previously published by Sullivan *et al.* (1995). As expected, *C. stellatoidea* is closely related to *C. albicans*. Results derived from the large ribosomal V3 DNA region, (Sullivan *et al.* 1995) as well as the small ribosomal subunit (Hendriks *et al.* 1991) confirm these relationships.

Previously, Lott *et al.* (1993) used the 5.8S rRNA gene for the phylogenetic analysis of several *Candida* species including *C. krusei*. However, digestion using several restriction enzymes of the amplicon of all the studied *C. krusei* isolates did not correspond with previously published sequence data. To clarify this discrepancy, sequencing was performed and errors in the previously published sequence were found. Therefore this new sequence data was used to construct a new phylogenetic tree showing the placement of *C. krusei* and *C. inconspicua*. The 99% similarity recorded between *C. inconspicua* and *C. krusei* was the highest level of identity found among *Candida* species. Similarity in the range of 83 and 92% was recorded in the 5.8S region of other *Candida* species compared with that of *C. inconspicua*. Therefore it is proposed that *C. krusei* and *C. inconspicua* are themselves closely related but distantly related to other *Candida* species.

Although internal spacer regions and the intergenic spacer (IGS) are not highly conserved and not processed to mature ribosomal RNA, they are useful because they evolve more rapidly, providing information about the phylogeny of closely related taxa (i.e. those which have diverged within the last 50 million years; Hills *et al.* 1991; Appel *et al.* 1995). Therefore, studies have been undertaken to examine the taxonomy of organisms based on the ITS and IGS regions (Baura *et al.* 1992; Kim *et al.* 1992). Previously, the homology at the 5' end of the ITS2 region within the *C. albicans*/*C. parapsilosis*/*C. tropicalis* group was observed, giving evidence for their relatedness (Lott *et al.* 1993).

Given that these two species are closely related, the genetic relatedness between *C. krusei* and *C. inconspicua* on the basis of the ITS sequence was studied. In this study, no attempt to construct a dendrogram based on the ITS sequences was made because deletions and substitutions necessitate assumptions in the sequence alignment which are

essentially arbitrary. However, evidence from these regions of other *Candida* species suggest that *C. krusei* and *C. inconspicua* are in fact closely related to one another and both species show a very different sequence structure compared with other *Candida* species. The fragment size of the ITS 1 region in *C. inconspicua* (89 bp) is similar to that of *C. krusei* (102 bp), and alignment revealed that the similarity was 81% (Table 5.2). 62% similarity in the ITS 2 region was recorded between *C. inconspicua* and *C. krusei*.

The ITS 2 region has been described as a pseudo-intron which is cut off during processing, without subsequent ligation of the small and large subunit (Lott *et al.* 1993), and was used for the sequence analysis of evolutionary nucleotide replacement (Lott *et al.* 1996). It was found that *C. albicans*, *C. stellatoidea*, *C. tropicalis*, and *C. parapsilosis*, had an almost identical sequence structure (data not shown), suggesting the possible evolutionary conservation of structural and cleavage mechanisms in this region. *C. guilliermondii* and *C. kefyr* were also homologous over the 5' start region of ITS 2, but had different structures beyond that.

Given the similarity between the ITS 2 regions (62%) was lower than ITS 1 (81%) the development of species-specific probes was based on the ITS 2 region. Previously, Botelho *et al.* (1994) used the ITS 2 region for the preparation of two *C. albicans* probes (ANAB2 and ANAB 3). They mentioned that the ITS 2 region is larger in size with a lesser degree of sequence conservation than the ITS 1 region, and therefore should provide sites suitable for the preparation of probes (see Chapter 9).

Veldman *et al.* (1981) studied the secondary structure of the 5.8S, 18S and 28S rRNAs and their processing to make mature ribosomal RNA. A homologous sequence, TCATTA

was found at the 3' end of the 18S rRNA, just before the ITS 1 start site which they proposed to be a nuclease(s) recognition site for processing precursor rRNA. De Jonge *et al.* (1977) also proposed TCATTA and TCAATA to be the 3' processing sequences of the 17S and 18S rRNAs, respectively. TCA(G) TTT is found at the 3' end of the 5.8S rRNA in *Candida* species including *C. krusei* and *C. inconspicua* (Figure 5.15.A), and TCATTT has been suggested by Rubin (1973) to be the 5.8S rRNA 3' processing site. A sequence at the 5' end of the 28S rRNA, NNNNAAAG(C)TT-TGACCT is found in all *Candida* species, which is very similar to TCTTAAAGTT-TGACCT, published by Veldman *et al.* (1981) as the 5' processing site.

Although the 5.8S rRNA gene is relatively short compared with other regions such as the 28S and 18S rRNAs, the tree topology based on the 5.8S sequence is similar to the tree based on the V3 region of the large ribosomal gene (Sullivan *et al.* 1995). For instance, *C. stellatoidea* and *C. albicans* are clustered together in both trees, and high percentage bootstrap numbers were recorded between *C. tropicalis* and *C. parapsilosis*. Lott *et al.* (1993) also support the results that *C. parapsilosis* and *C. tropicalis* are closely related to each other based on the 5.8S rRNA sequences. However, *C. krusei* is clustered together with *C. albicans*, *C. parapsilosis* and *C. tropicalis*, showing different topology. Because the 5.8S rRNA sequence of *C. krusei* was not correctly generated, taxonomic relationships between *C. krusei* and other *Candida* species were not reliable.

However, as the number of informative sites in the 5.8S region is limited, several bootstrapping numbers are relatively low. Therefore, additional sequencing data are required for the construction of robust results in order to provide a better phylogeny of

Candida species. Our research objective was to suggest the relationship between *C. krusei* and *C. inconspicua* using the 5.8S gene, with other phenotypic and genetic characteristics to investigate whether this region can provide evidence of a close relatedness between these species. Other molecular information supporting their phylogenetic place in yeasts is necessary to establish the usefulness of ribosomal DNA analysis for the molecular taxonomy in *Candida* species.

In conclusion, *C. krusei* and *C. inconspicua* are closely related to each other based on the data derived from the 5.8S rRNA as well as the ITS regions. They are distantly related to other *Candida* species. These results support the growing concept that *C. krusei* might be re-classified to another genus other than *Candida*.

6.

Chapter 6

Restriction enzyme band patterns of *Candida* species

6.1 Restriction enzyme analysis and agarose gel electrophoresis

Restriction endonucleases are enzymes that recognise specific nucleotide sequences in double-stranded DNA. Generally, different enzymes will recognise different sequences that are four to six nucleotides long, and most restriction enzymes will reproducibly cleave DNA at a precise point within a recognition sequence. The population of DNA fragments produced by restriction enzymes will move through an agarose gel under the influence of an electric field, where negatively charged DNA molecules will be drawn to the anodes and rate of movement is based on size, with the largest molecules having the lowest mobilities. High percentage gels tend to compress the bands formed by higher molecular weight DNA fragments and improve the resolution of smaller fragments. In contrast, lower percentage gels allow better separation of the larger fragments, whilst tending to compress the smaller fragments.

6.1.1 Restriction fragment length polymorphism (RFLP) analysis

The ribosomal DNA regions have widely been used for the RFLP analysis of several fungal isolates. Because the rDNA is constant across isolates of the same species and rDNA occurs as multiple copies, it lends itself to analysis based on restriction fragment length polymorphisms. In particular, sensitivity is increased if rDNA region is amplified by PCR. Therefore, studies showed that RFLP patterns are sufficiently different to allow recognition of individual species, as well as individual strains of a species (Kurtzman 1992). Restriction fragment length polymorphisms are also frequently used in diagnostic laboratories.

For RFLP analysis, total genomic DNA or PCR product has been widely used (Newton *et al.* 1994). A PCR reaction will usually generate enough DNA to be directly visible on a gel after staining. As long as some of the surrounding DNA sequence is known, it can then serve to design PCR primers. PCR can be carried out using primers, and the amplified products are digested with the appropriate restriction enzyme and electrophoretic separation demonstrates the presence or absence of the restriction site. The cause of the length difference is sequence variation within the recognition sequence itself. Therefore, the polymorphism is a restriction site polymorphism (RSP; Krawczak *et al.* 1994). This method, PCR-RFLP has the following advantages.

- 1) being rapid.
- 2) requiring little starting sample DNA compared to conventional RFLP analysis.
- 3) the DNA can be extracted from many types of tissue.
- 4) the products can be detected without the use of radioisotopes.

The PCR-RFLP method offers significant advantages over conventional DNA restriction analyses (Swaminathan *et al.* 1993). Genetic differences are determined by PCR amplification of a target locus, followed by cutting of the amplicon with a RE with 4-base recognition sequence. PCR can be employed to detect a wide range of organisms (Makimura *et al.* 1994), whether they are present in foodstuffs, the environment or biological or histological materials. *Candida* species can also be detected using PCR-RFLP technique (Maiwald *et al.* 1994). Progress has also been reported in the diagnosis of systemic candidiasis (Hopfer *et al.* 1993). In particular, further characterisation of broad groups of fungal pathogens has also been achieved by PCR-RFLP analysis (Maiwald *et al.* 1994; Appel *et al.* 1995).

6.1.2 Direct PCR for the identification of fungal isolates

PCR-based methods have many advantages over standard methods. Firstly, they are rapid and convenient to use. Secondly, just a small quantity of a clinical specimen is required for the detection of target species. Thirdly, they are very sensitive. Although certain requirements such as specialised reagents and materials are necessary prior to use, in general, they do not require very expensive equipment.

Rapid PCR-based identification methods such as direct PCR have been used for the identification of some medically significant fungal species (Maiwald *et al.* 1994). Ordinarily a DNA or RNA extraction is undertaken prior to PCR for identification. However, direct PCR can be carried out directly from a single colony on agar plate without genomic DNA preparation with the potential advantage of rapidity, especially for identification.

In Chapter 4, the work described the utility of universal primers corresponding to the 18S and 28S ribosomal RNA region (White *et al.* 1990) for the differentiation of some *Candida* species. *C. glabrata* showed the largest PCR products to be about 850 bp while PCR products of most other species were in the range of 520 and 720 bp. This result indicated that additional procedures were necessary to differentiate most *Candida* species. For this reason, enzymatic profiles following endonuclease restriction digestion of the PCR products using direct PCR were assessed against three *Candida* species. It was found that *Hha*I digestion against PCR products using ITS 1 and 4 generated many characteristic digested bands patterns in *C. inconspicua*, *C. krusei* and *C. norvegensis*.

The aim of this study is to investigate whether digested band patterns of PCR products from the rDNA can yield species identification of three *Candida* species, *C. inconspicua*, *C. krusei* and *C. norvegensis* and distinguish them from other *Candida* species. In addition, for the RFLP analysis on the ITS regions and confirmation of sequencing results, several restriction enzymes were used. In addition, this chapter also addresses the question of whether the same data can be reliably generated by direct PCR thus avoiding the time consuming steps of DNA extraction. To test the procedure realistically it was used for some phenotypically 'atypical' isolates.

6.2 Results

Different restriction enzymes were used to digest amplified products derived from PCR products using ITS primers. Five restriction enzymes, *MspI*, *RsaI*, *ScrFI*, *HhaI* and *DdeI* were used in this study in order to find restriction enzyme which can generate characteristic band patterns of three *Candida* species.

6.2.1 *MspI*

In *C. inconspicua*, *MspI* generated two conspicuous digested bands of the expected sizes at 218 and 237 bp from the PCR product using ITS 1 and 4 primers (Figure 6.1). In the case of *C. krusei*, a single band was visible, and following sequencing confirmed that this consists of 2, similarly sized digested fragments, 249 and 260 bp. All the sizes of digested fragments from both species corresponded to subsequent sequencing data. *C. norvegensis* isolates produced two discernible double bands at 190 and 210 bp, and two digested bands, about 350 and 500 bp, were generated from PCR product of *C. glabrata*. In the case of *C. rugosa*, each isolate produced different sized fragments. For instance, ATCC

10571 had two bands at 100 and 250 bp while ATCC 14840 yielded fragments of 80 and 330 bp. ATCC 20306 had a single band at 350 bp, and ATCC 34637 also yielded a single band of around 400 bp, suggesting that there are no restriction sites.

6.2.2 *RsaI*

There were no restriction sites on PCR products of *C. inconspicua*. All isolate produced a single band at about 450 bp which is identical to the amplified product using ITS 1 and 4 primers (Figure 6.2). This result, confirmed the sequencing work that there are no *RsaI* restriction sites on *C. inconspicua* PCR products amplified using ITS 1 and 4 primers (Figure 6.7). Likewise, except 1 isolate from bioMérieux, 18888, all isolates of *C. krusei* also showed a single band at 470 bp which is a similar size to the PCR product of *C. inconspicua*.

Sequencing based on the type strain *C. krusei* ATCC 6258 indicated that isolate 18888 does not have restriction sites in the ITS 1 region as the PCR product was not digested with this enzyme. Ten other isolates of *C. krusei* from bioMérieux were also investigated with *RsaI*, showing that some isolates had similar digestion profiles to the 18888 isolate (Figure 6.3.A). The restriction site of four isolates in Figure 6.3.A is illustrated in Figure 6.3.B. In the case of *C. norvegensis*, the 2 isolates produced double bands which are at 90 and 400 bp. One *C. glabrata* isolate had a single band at 850 bp, and it suggested that there are no restriction sites (Figure 6.2). As in the case of *MspI*, all three isolates of *C. rugosa*, showed size variations.

6.2.3 *ScrF I*

All seven *C. inconspicua* isolates produced the expected doublet at 207 and 248 bp (Figure 6.4.A). All four *C. krusei* isolates also had a doublet at 248 and 261 bp. *C. norvegensis* produced digestion patterns very much like a *C. inconspicua* isolate but with a slightly larger band (about 270 bp). *C. glabrata* showed 3 bands which are placed at about 200, 300, 350 bp. The digestion band patterns of the *C. rugosa* isolates were not identical to each other as with previous enzymes, and all 4 isolates showed different sizes in the range of 350 to 450 bp.

In the case of digestion band patterns with this enzyme from amplified product using ITS 3 and 4, digestion band patterns of isolates did not show species-specific characteristics either (Figure 6.4.B). For example, most of the isolates produced an identical fragment at 100 bp, except 3 isolates of *C. rugosa*, and other bands were placed in the range of about 210 to 310 bp. *C. norvegensis* had very similar digestion banding patterns to the *C. inconspicua* case. Therefore this enzyme is not useful for distinguishing *C. inconspicua* and *C. norvegensis*.

6.2.4 *DdeI*

Seven *C. inconspicua* isolates amplified with ITS 1 and 4 primers were digested with *DdeI*, and all isolates produced two or three digested fragments (Figure 6.5). Four isolates, FA/390, FA 985, SM/038, 16783 showed an extra band at 250 bp, showing restriction fragment length polymorphisms whilst other isolates had just two bands corresponding to 109 and 346 bp, respectively. In the case of *C. krusei*, the sequencing result indicated that no restriction sites for *DdeI* were present. After digestion of *C.*

inconspicua PCR products using ITS 3 and 4 primers with this enzyme, polymorphic sites were found on the ITS 2 region (Figure 5.13).

6.2.5 *HhaI*

Unlike other RSP profiles, digestion of PCR product with *HhaI* produced characteristic banding patterns (Figure 6.6). Especially, for the differentiation of *C. inconspicua*, *C. krusei* and *C. norvegensis* which are difficult to differentiate using conventional approaches, the *HhaI* yielded highly discriminatory patterns allowing the identification of each species.

There were four fragments corresponding to 52, 69, 85 and 102 bp in *C. inconspicua* (Figure 6.8). In particular, *C. krusei* showed very characteristic digestion patterns, with the largest band at 178 and 204 bp and bands at 52 and 69 bp (Figure 6.9). Several fragments were produced in *C. norvegensis* strains and the banding patterns were similar to the *C. inconspicua* case (Figure 6.10). The sizes of the *HhaI* digested fragments as determined by image analysis are: 60 bp, 70 bp(x2), 90 bp(x2) and 110 bp.

The data suggests that *HhaI* digestion of PCR products can be used as a method for the identification of these three *Candida* species without other laboratory approaches. Additional isolates were then tested to know whether *HhaI* digestion can provide identical band patterns among our culture collection. All different *Candida* isolates of the same species (total 43 isolates, i.e. 30 isolates of *C. krusei*, *C. inconspicua* and *C. norvegensis* from bioMérieux, and 7, 4, and 2 isolates of *C. inconspicua*, *C. krusei* and *C. norvegensis*, respectively from a variety of sources) also showed identical digestion

profiles. In particular, type strains of three *Candida* species were included and results confirmed the usefulness of this approach. Figure 6.7 presents restriction enzyme sites and PCR-RFLP analysis of *C. inconspicua* and *C. krusei* isolates using the five different restriction enzymes examined.

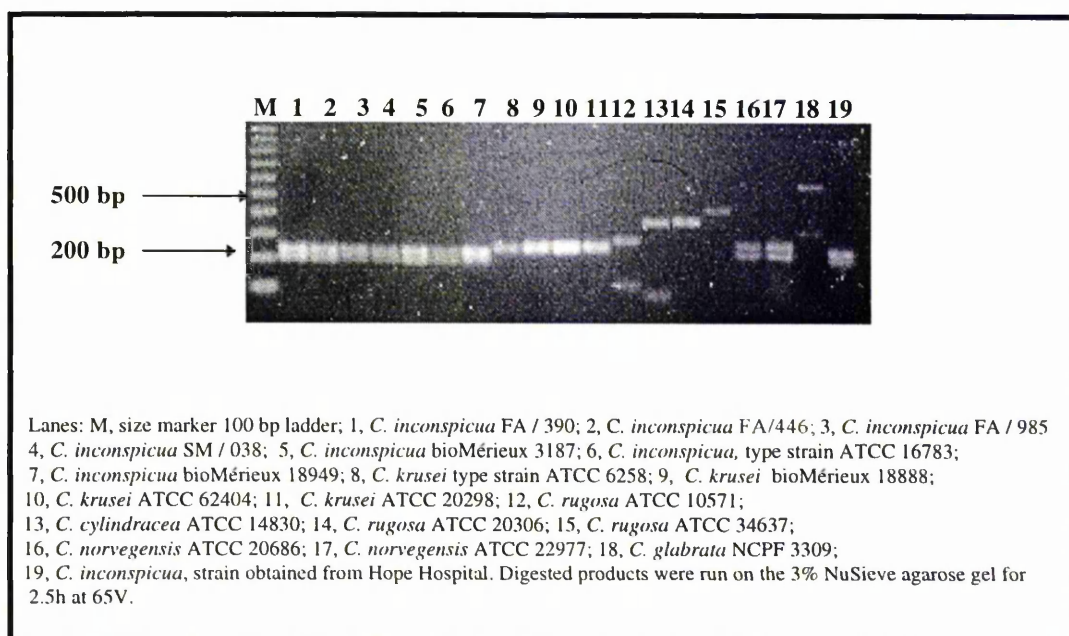


Figure 6.1 Digested band patterns of amplified products with *MspI*.

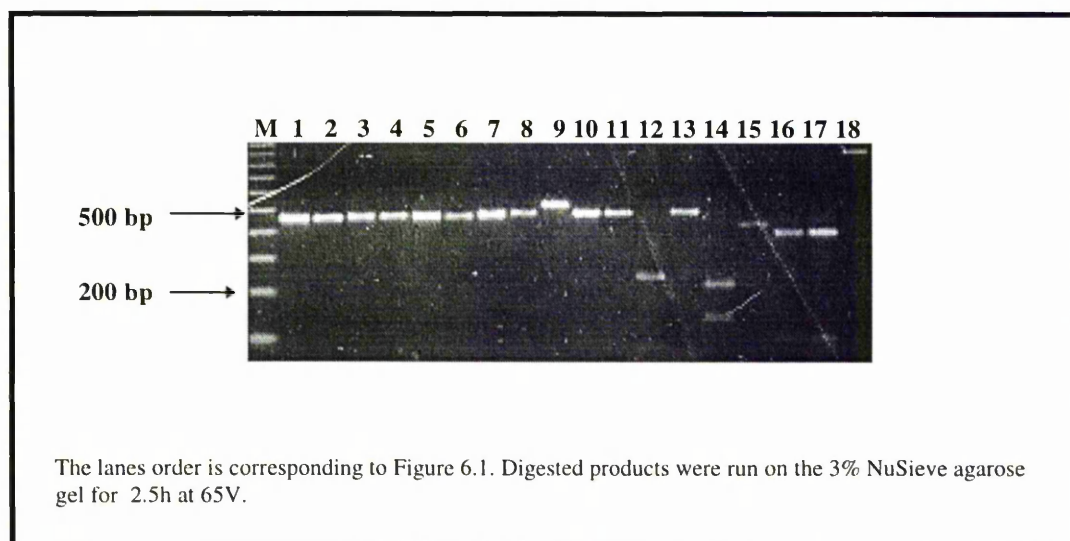
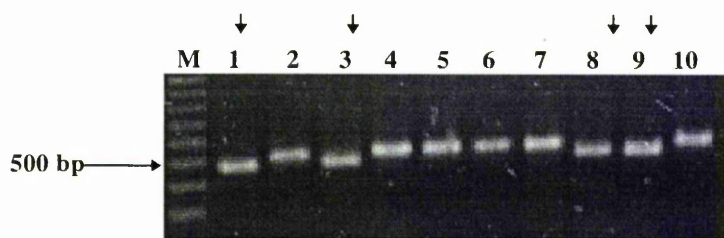
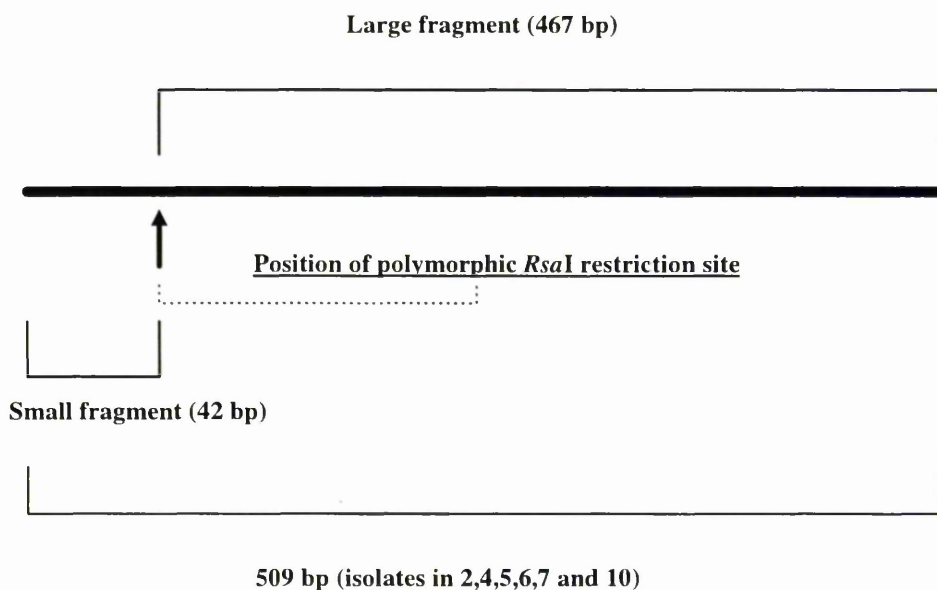


Figure 6.2 Digested band patterns of amplified products with *RsaI*.



Lanes: M, size marker, 100 bp ladder; 1, 8808100; 2, 8904072; 3, 8503050; 4, 9502007; 5, 8503051; 6, 8903079; 7, 9502006; 8, 8504237; 9, 8503198; 10, 8807095.
Arrows indicate isolates with a *RsaI* site. PCR products were run on 1.8% agarose gel for 2 h.

A. *RsaI* digested bands patterns of 10 *C. krusei* isolates from bioMérieux



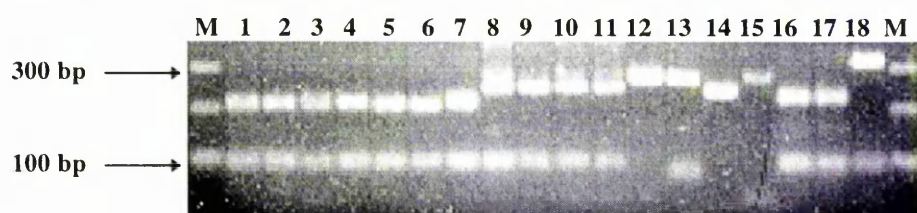
B. Schematic representation of *RsaI* digested sites of *C. krusei* isolates.

Figure 6.3 PCR-RFLP analysis of *C. krusei* isolates using *RsaI*



A. Digested band patterns with *ScrFI* from PCR products using ITS 1 and 4.

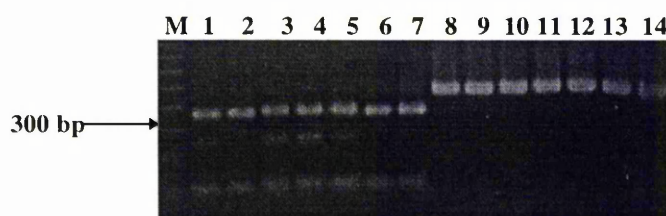
Lanes: M, size marker 100 bp ladder; 1, *C. inconspicua* FA / 390; 2, *C. inconspicua* FA/446; 3, *C. inconspicua* FA / 985; 4, *C. inconspicua* SM / 038; 5, *C. inconspicua* bioMérieux 3187; 6, *C. inconspicua* ATCC 16783; 7, *C. inconspicua* bioMérieux 18949; 8, *C. krusei* type strain ATCC 6258; 9, *C. krusei* bioMérieux 18888; 10, *C. krusei* ATCC 62404; 11, *C. krusei* ATCC 20298; 12, *C. rugosa* type strain ATCC 10571; 13, *C. cylindracea* ATCC 14830; 14, *C. rugosa* ATCC 20306; 15, *C. rugosa* ATCC 34637; 16, *C. norvegensis* ATCC 20686; 17, *C. norvegensis* ATCC 22977; 18, *C. glabrata* NCPF 3309; 19, *C. inconspicua*, strain obtained from Hope Hospital. Digested products were run on the 3% NuSieve agarose gel for 2.5h at 65V.



B. Digested band patterns with *ScrFI* from PCR products using ITS 3 and 4.

Lane order is corresponding to Figure 6.4.A.

Figure 6.4 Digested bands profiles with *ScrFI* of amplified products.



Lanes: M, size marker, 100 bp ladder; 1 to 7, PCR products of *C. inconspicua* digested with *DdeI*, FA/390, FA/44,FA 985,SM/038, 16783, 18949,3187, respectively; 8 to 9, PCR products of *C. inconspicua* using ITS 1 and 4, lane order is corresponding to lane 1 to 7. Digested products were run on 1.8% agarose gel for 2 h.

Figure 6.5 PCR-RFLP analysis of *C. inconspicua* strains using *DdeI*.

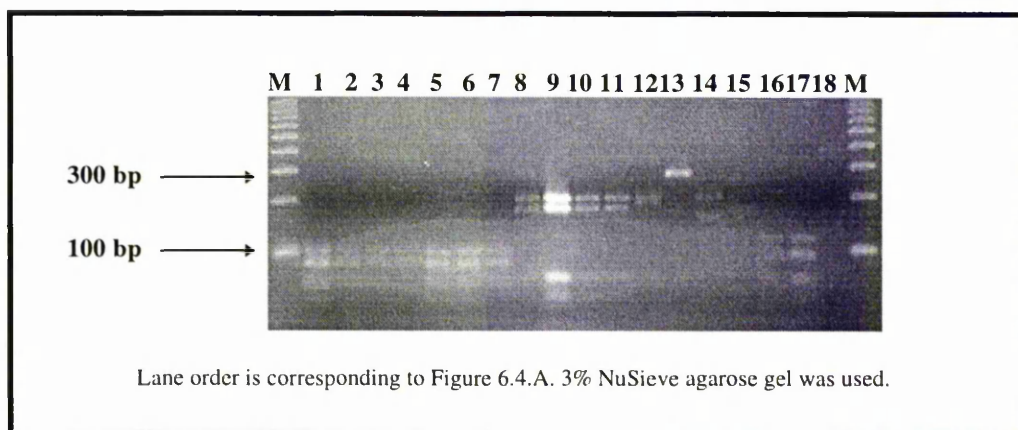


Figure 6.6 Digested band patterns with *HhaI* from PCR products using ITS 1 and 4 primers

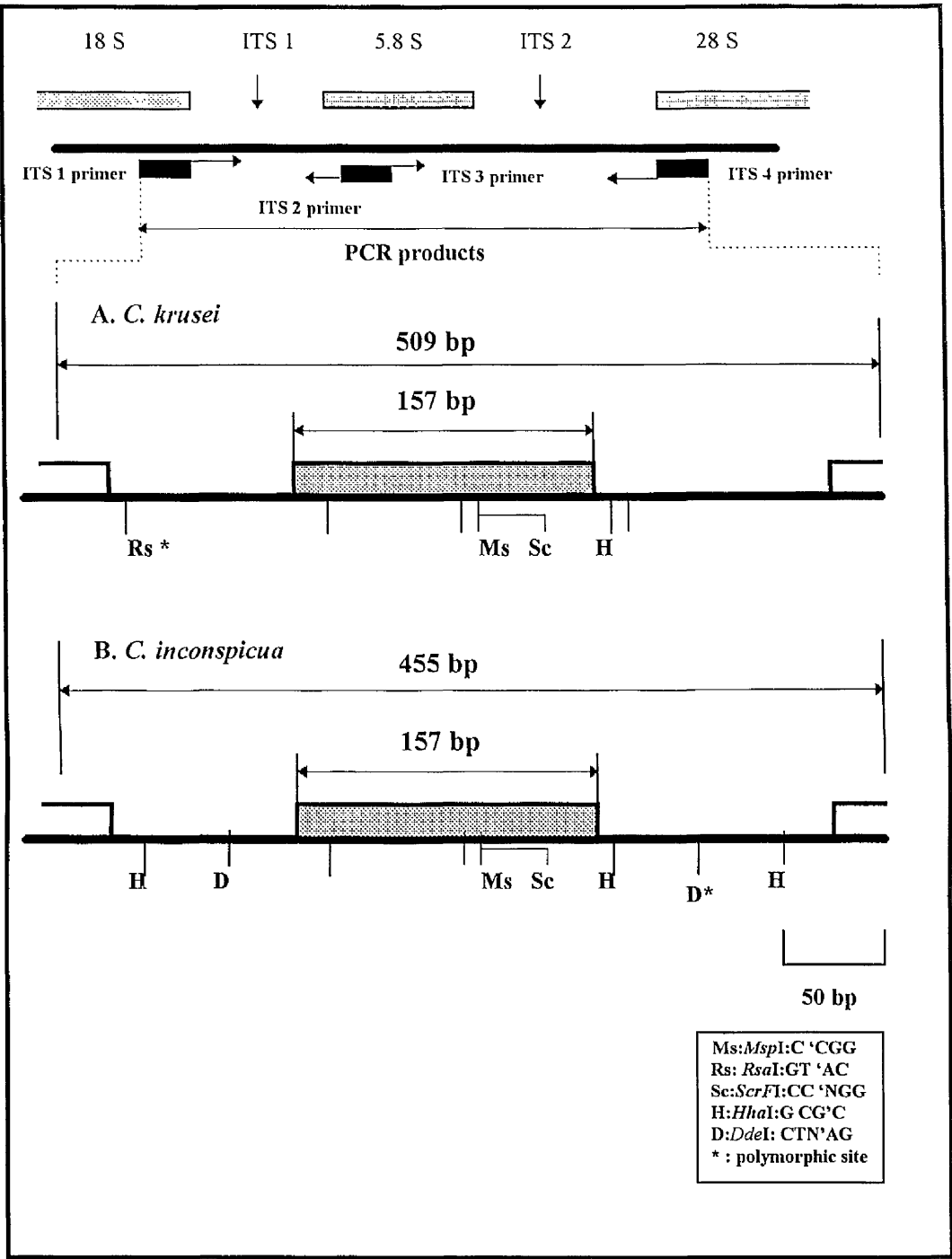


Figure 6.7 The restriction enzyme sites on PCR products of *C. krusei* and *C. inconspicua* as determined by sequencing and confirmed by PCR-RFLP.

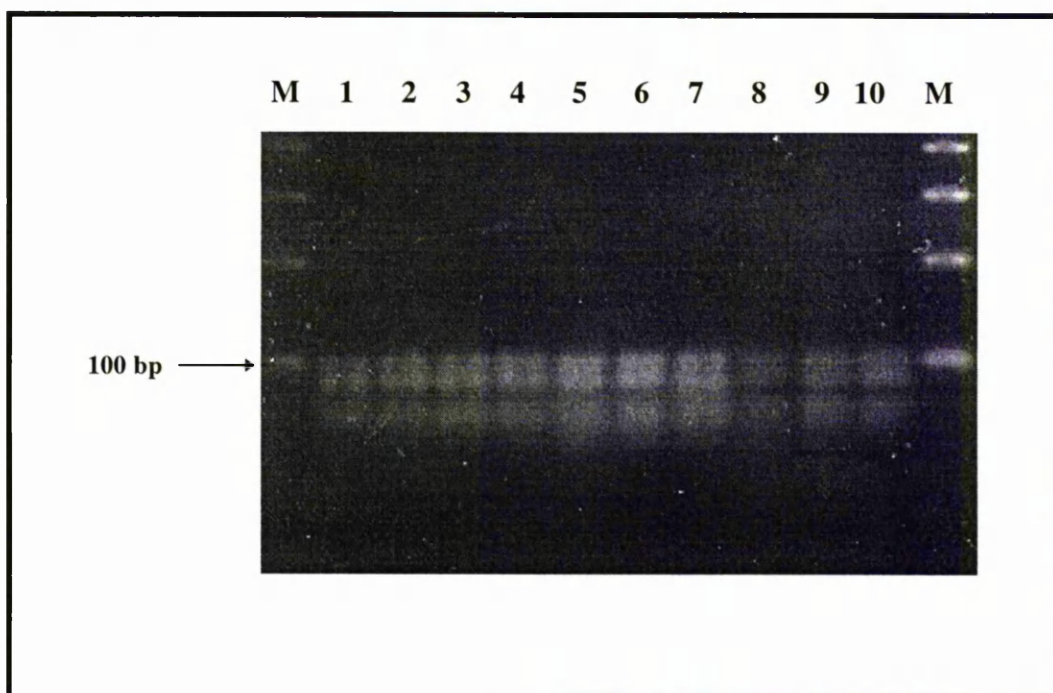


Figure 6.8. PCR products of *C. inconspicua* strains digested with *HhaI*.

The lane order of the *C. inconspicua* strains is as follows. M, size marker 100 bp-ladder; 1, 85 07 247; 2, 85 09 110; 3, 85 09 109; 4, 85 03 199; 5, 85 09 111; 6, 85 10 102; 7, 93 12 113; 8, 85 02 122; 9, 85 02 119; 10, 85 03 197. The sizes of the *HhaI* digested fragments as determined by sequencing are: 52 bp, 63bp, 69bp, 82bp, 87bp, and 102 bp. Digested fragments were run on the 3% NuSieve agarose gel.

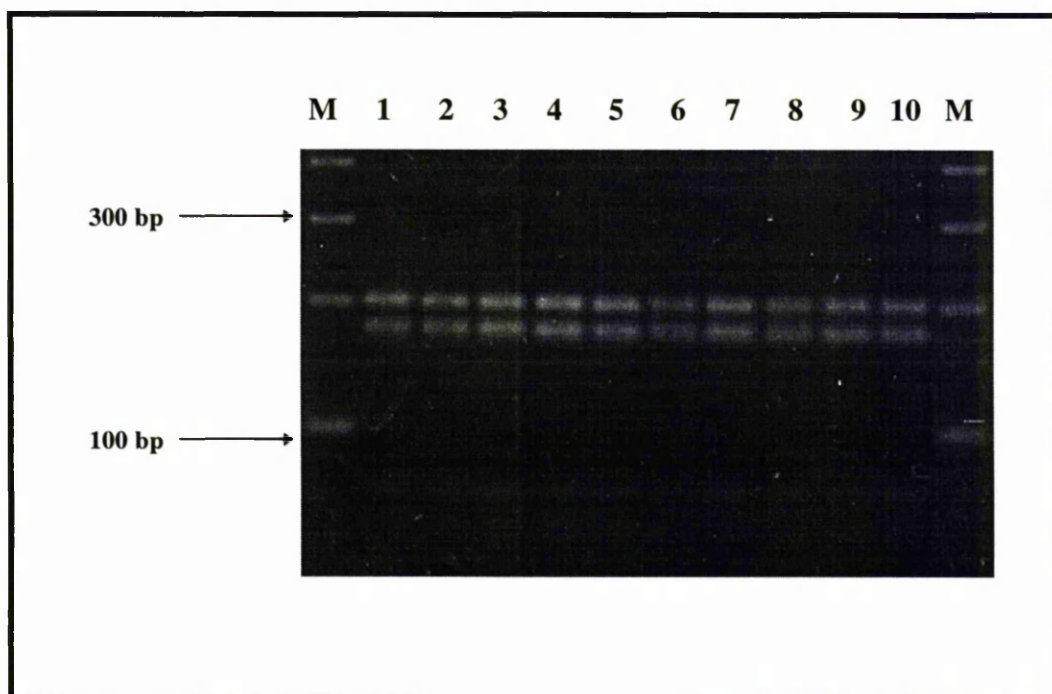


Figure 6.9 PCR products of *C. krusei* strains digested with *HhaI*.

The lane order of the *C. krusei* strains is as follows. M, size marker 100 bp-ladder; 1, 89 08 008; 2, 88 08 110; 3, 91 05 030; 4, 89 08 006; 5, 85 10 089; 6, 91 12 022; 7, 91 12 020; 8, 91 12 021; 9, 89 08 007; 10, 90 03 063. The sizes of the *HhaI* digested fragments as determined by sequencing are: 6 bp, 52 bp, 69 bp, 178 bp and 204 bp.

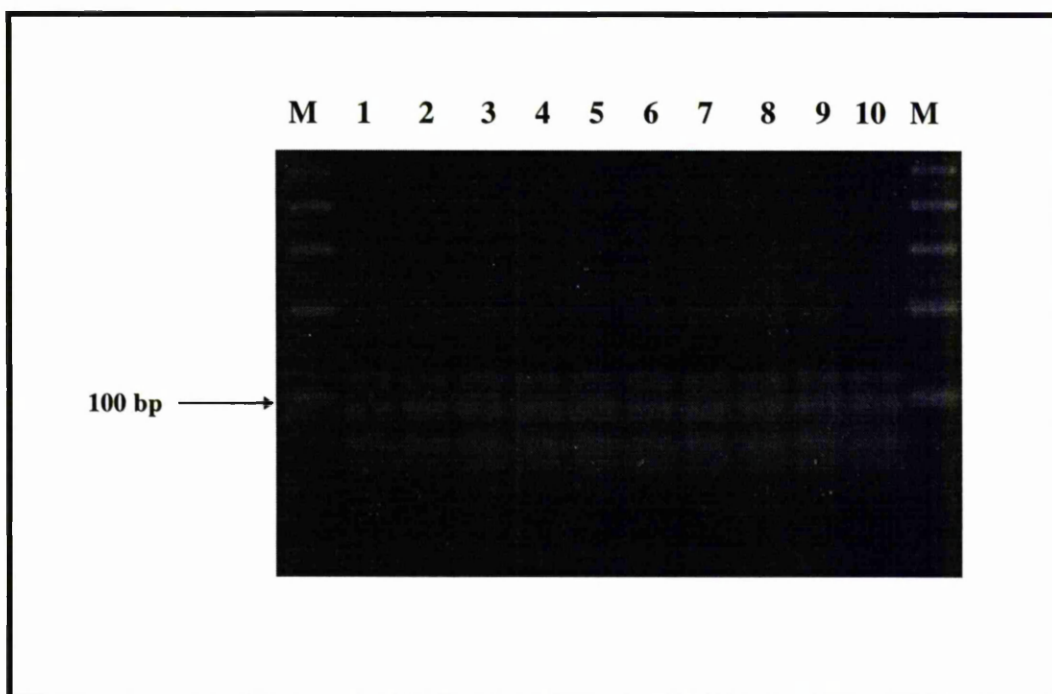


Figure 6.10 PCR products of *C. norvegensis* strains digested with *HhaI*.

The lane order of the *C. norvegensis* strains is as follows. M, molecular size marker, 100 bp-ladder; 1, 88 08 100; 2, 89 04 072; 3, 85 03 050; 4, 95 02 007; 5, 85 03 051; 6, 89 03 079; 7, 95 02 006; 8, 85 04 237; 9, 85 03 198; 10, 88 07 095. The sizes of the *HhaI* digested fragments as determined by image analysis are: 60 bp, 70, bp(x2), 90 bp(x2) and 110 bp.

6.2.6 Direct PCR profiles of fungal species

To determine whether the direct PCR could be employed for the presumptive identifications based on the PCR sizes using ITS 1 and 4 primers, several isolates were amplified under conditions identical to PCR carried out with purified genomic DNA. For optimisation, the same PCR conditions as previously described in materials and methods (Chapter 2) were used. With all primers, different sets of amplified products ranging from 500 to 580 bp were generated. Thus direct PCR can speed up the presumptive identifications of some unknown isolates. For instance, conventional PCR takes 2 day to obtain results (i.e. inoculation and incubation steps for overnight and DNA extraction and drying steps for at least 5 to 6 h). Because direct PCR use colony on the agar plate directly without these steps, it can speed up the procedure for the identification of target isolates.

Figure 6.11 shows direct PCR profiles of 11 isolates, and *S. cerevisiae* and *C. glabrata* had products at over 800 bp. Other species, like *T. beigelli*, *C. albicans*, *C. dubliniensis*, *Cr. neoformans* and *C. tropicalis*, had very similar PCR products about 540 bp on the gel. The results of PCR with genomic DNA also showed identical PCR profiles compared with that performed by the direct PCR method.

It was found that direct PCR and enzyme digestion profiles against three species (*C. krusei*, *C. norvegensis* and *C. inconspicua*) also had identical results compared with results obtained with purified DNA. This information suggests that direct PCR can be employed as a method for the presumptive identification of *Candida* species in the clinical laboratory.

6.2.7 Investigation of unusual *Candida* species

To assess the ability of direct PCR as a tool of identification of some species in clinical laboratory without laborious and time consuming methods, unusual yeast species which showed either abnormal range of MICs to fluconazole or unusual API codes were obtained from a variety of sources and tested using either direct PCR or digestion with restriction enzyme *Hha*I. Table 6.1 shows their phenotypic characteristics.

Figure 6.12 shows the direct PCR products using ITS 1 and 4 primers of unusual isolates. In lane 1, the PCR pattern showed the isolate which was thought to be *C. inconspicua* species (FA/2924) does not follow the PCR amplified pattern of typical *C. inconspicua*. The PCR size of this isolate was 500 bp and size difference was recorded compared with other *C. inconspicua* isolates (Figure 4.3.A). Furthermore, this species showed very high MIC, 50 µg/ml against fluconazole (Table 6.1), suggesting this isolate does not follow typical phenotypic and genotypic characteristics of *C. inconspicua*.

In lanes 2 and 3, the isolates having different colonial morphology from patients who had been given antifungal therapy showed that they have identical PCR sizes as the control *C. inconspicua*. Another *C. inconspicua* isolate in lane 7 which was also isolated from the patient on antifungal therapy showed the same PCR size as expected.

Two *C. glabrata* isolates in lanes 4 and 5 which had different colonial sizes were included, and they yielded a PCR product of 850 bp, the same size as reference isolate. The PCR product of another unusual *C. glabrata* isolate (FA/2867) which showed

significantly low MIC, 0.19 µg/ml against fluconazole yielded a very different and distinctive PCR product at 550 bp.

Another unusual isolate which had been presumptively identified as *C. krusei* in Hope Hospital was also tested using direct PCR followed by digestion with *HhaI* (Figure 6.13), and all amplified products using 4 universal primers, ITS 1 and 4, 3 and 4 combinations were identical to *C. krusei* type strain (ATCC 6258).

Finally, in the case of unusual *C. norvegensis* isolate, (FA/1589) and *C. albicans* FA/2425 isolate, direct PCR followed by digestion with *HhaI* were employed (Figure 6.14). FA/1589 isolate had the largest band at about 120 bp with several bands under 100 bp as is the case for reference isolates ATCC 20686. In the case of FA/2425 isolate, digested band pattern does not follow typical *C. norvegensis* profiles, and this species was identified as *C. albicans* in Hope Hospital.

Table 6.1 Unusual *Candida* species

Number	Hospital	Isolates (?)	Site	Flu ^a	Amp ^b	Itra ^c	API 20C
FA/2425	Hope	<i>C. albicans</i>	Tracheal aspirate	0.39	N/D ^d	N/D	N/D
FA/2867	Hope	<i>C. glabrata</i>	Urine	0.19	0.03	N/D	U ^g
FA/1589	NMG ^e	<i>C. norvegensis</i>	Throat	25	N/D	0.0625	000100002
FA/2924	NMG	<i>C. inconspicua</i>	Throat	50	0.125	1	N/D
870/IL/0012	RMC ^f	<i>C. inconspicua</i>	N/D	N/D	N/D	N/D	N/D

a: Fluconazole (µg/ml)

b: Amphotericin B (µg/ml)

c: Itraconazole (µg/ml)

d: Not defined

e: North Manchester General Hospital

f: Royal Manchester Children's Hospital

g: Unidentified

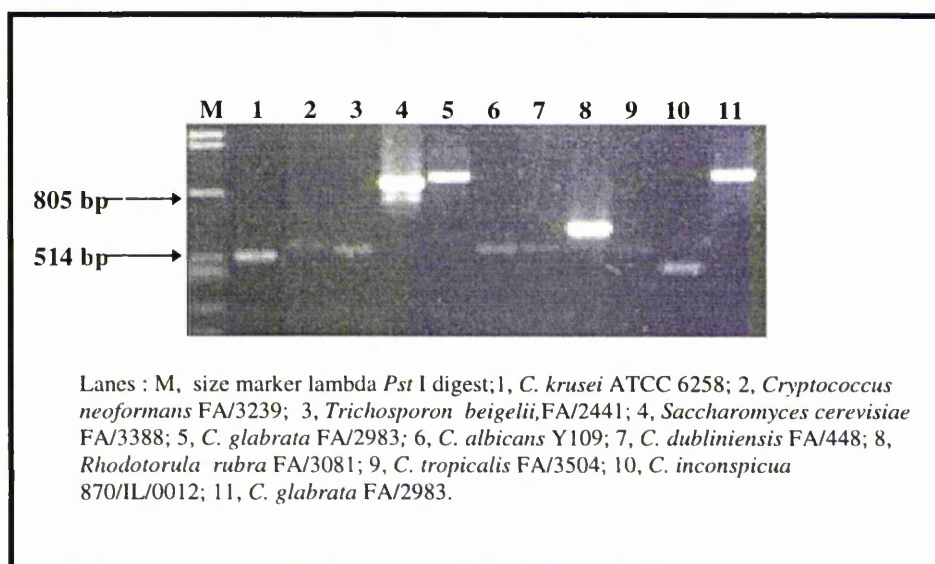


Figure 6.11 Direct PCR patterns of fungal species using ITS 1 and 4 primers

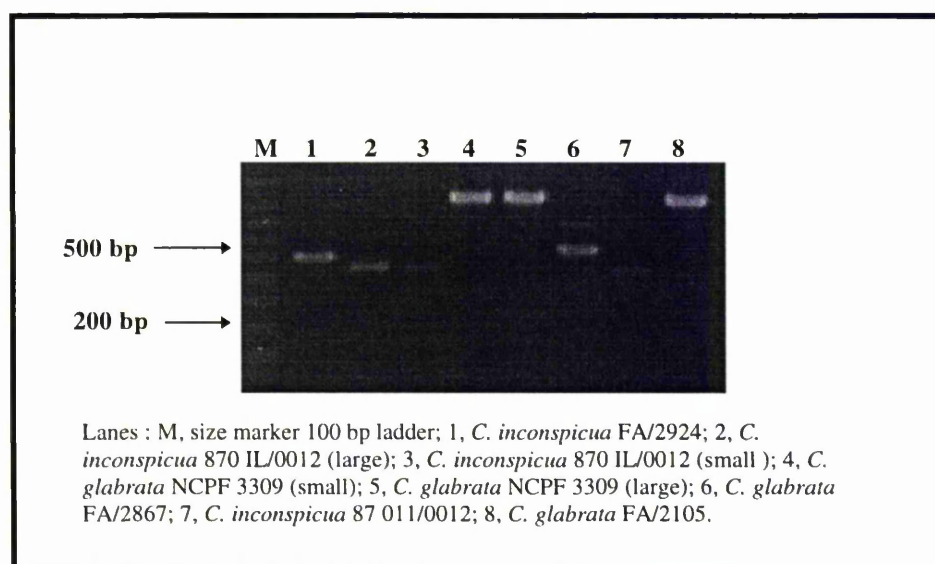
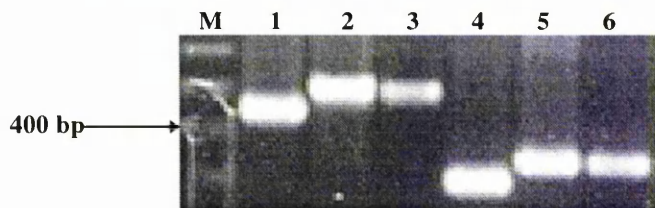
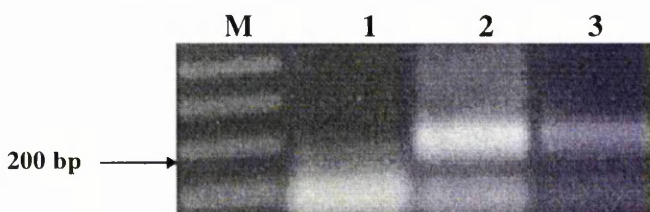


Figure 6.12 Direct PCR patterns of unusual isolates using ITS 1 and 4 primers.



A. Direct PCR of *C. krusei* strains using different ITS primers.

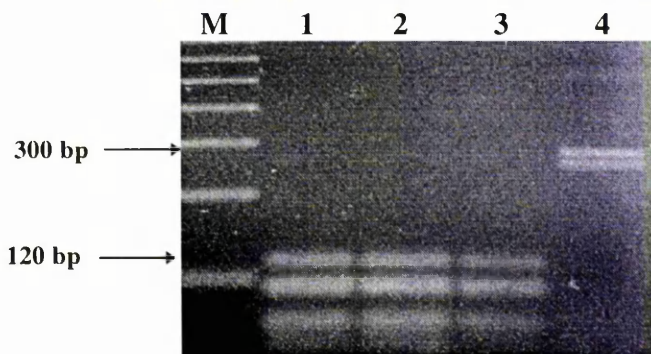
Lanes: M, size marker 100 bp ladder; 1 to 3, amplified products using ITS 1 and 4 primers; 1, reference strain, *C. inconspicua* SM/038; 2, type strain *C. krusei* ATCC 6258; 3, unusual *C. krusei* Hope strain; 4 to 6, amplified products using ITS 3 and 4 primers; 4, reference *C. inconspicua* SM/038; 5, type strain *C. krusei* ATCC 6258; 6, unusual *C. krusei* Hope strain.



B. *HhaI* digested band patterns of *C. krusei* strains from PCR products using ITS 1 and 4.

Lanes: M, 100 bp size marker; 1, reference *C. inconspicua* strain SM/038; 2, type strain *C. krusei* ATCC 6258; 3, unusual Hope *C. krusei* strain.

Figure 6.13 PCR and *HhaI* digested band patterns of unusual *C. krusei* isolates.



Digested band patterns of PCR products with *HhaI*.

Lanes: M, 100 bp size marker; 1 to 2, same *C. norvegensis* isolate, 1589; 3, *C. norvegensis* ATCC 20686; 4, *C. albicans* FA/2425.

Figure 6.14 Digested bands patterns of unusual *C. norvegensis* strains with *HhaI* from PCR products using ITS 1 and 4 primers.

6.3 Discussion

Because the nuclear ribosomal DNA repeat includes both highly conserved and more variable spacer regions like ITS and IGS (Appel *et al.* 1995), PCR-RFLP methods based on these regions have been conducted for the identification of medically important isolates. The non-concordance areas on the ITS regions not only provide sites for the development of species-specific probes, but also can be used for RFLP analysis. Several studies have been published using a combination of PCR with several primers corresponding to the regions of ribosomal DNA, followed by restriction digestion as a approach for species-specific identification.

PCR-restriction enzyme pattern analysis was previously used for the identification of medically important yeasts. For instance, Niesters *et al.* (1993) amplified the small subunit rDNA and using a combination of five enzymes could distinguish four *Candida* species. Maiwald *et al.* (1994) also used a similar procedure to identify presumptively eight *Candida* species using PCR products of the small subunit rDNA and digestion with six restriction enzymes. Williams *et al.* (1995) used PCR to amplify the internally transcribed spacer region of the ribosomal DNA repeat and digested the amplified fragments with three restriction enzymes. They concluded that 8 *Candida* species could be identified on the basis of size and sequence variation. Although three *Candida* species, *C. inconspicua*, *C. krusei* and *C. norvegensis*, which are closely related to each other, become clinically important in terms of treatment of patients using antifungal therapy, no attempt has been made so far to identify and differentiate these species using PCR and restriction enzyme analysis.

Therefore, PCR-RFLP was employed to examine whether species-specific band patterns were generated from these 3 *Candida* species and we have been able to differentiate them using a single restriction enzyme, *Hha*I. Results showed that the *Hha*I digestion profiles of the three species are readily distinguishable on high resolution agarose gels (Figure 6.8 to 6.10).

The use of PCR-RFLP has an advantage over other genetically based tests such as RFLPs on genomic DNA and electrophoretic karyotyping (Mata Essayag *et al.* 1996) in being simple and quick especially if the PCR is done directly from a colony. In particular, because this approach does not require hybridisation steps or the use of radioactivity, it can be completed within one working day. After digestion with different restriction enzymes of the amplified products using ITS 1 and 4 primers, all digestion band patterns were identical in the same *Candida* species (17 isolates of *C. inconspicua* 14 isolates of *C. krusei* and 12 isolates of *C. norvegensis*) except for *C. rugosa*. This results supports the trustworthiness of this approach as a method towards rapid identification and differentiation of several *Candida* species. In particular, it was found that *C. krusei* had very characteristic digestion band patterns so differentiation of this species from other closely related species is not difficult if this approach is employed. Previously, the differentiation of this species from other clinically important *Candida* species was not so straightforward (Mata Essayag *et al.* 1996).

PCR-RFLP can also be used for the analysis of polymorphisms as a result of nucleotide substitutions of the rDNA in *Candida* species. For instance, we found that 5 isolates of *C. krusei* from bioMérieux culture collections have different band patterns after digestion

with *RsaI*. After sequencing rDNA (5.8S, ITS 1 and 2 region) of one *C. krusei* isolate (ATCC 6258), a *RsaI* recognition site in the ITS 1 region was found. This is consistent with variations in sequence, in the same species, in the ITS regions but not highly conserved regions such as the 5.8S rDNA. This concept was described in *C. parapsilosis* strains in Lin *et al.* (1995). Likewise, digestion of the PCR products in *C. inconspicua* isolates with *DdeI* showed that the ITS regions are not highly conserved and nucleotide differences can accumulate in this region among species.

The direct PCR method has significant advantages over the conventional PCR method with purified genomic DNA (Maiwald *et al.* 1994). It is quicker. A colony on the Sabouraud agar plate is simply mixed with sterile distilled water and vortex mixed providing sufficient amount of DNA for PCR. Because PCR needs very little amount of genomic DNA, laborious methods like a purification of genomic DNA using phenol/chloroform, drying steps can be omitted.

Direct PCR was performed followed by restriction enzyme analysis, and it was found that the PCR products and digestion band patterns were identical compared to those performed with digestion of purified DNA, which indicates this method can be applicable to the clinical laboratory. In total 25 isolates, including some unusual isolates, were tested by the method of direct PCR in this study. All previously tested isolates were identified correctly based on the sizes of amplified products or digestion band patterns so this approach is thought to be worthy as a rapid identification method. Within 5 h (approximately, 3.5 h for PCR cycles and 1.5 h for gel running and staining),

identification of several *Candida* species is possible and could provide enough information for species identification.

Based on the usefulness of this approach, direct PCR against some unusual *Candida* species was then performed to examine whether this method can assist in species identification of these isolates. The results showed that one of the isolates, FA/2425, had very different band profiles compared with reference ATCC 20686. This species was identified as *C. albicans* in the Hope Hospital. In the case of another unusual isolate, FA/2867, biochemical tests were carried out for the identification of this species, and this species was identified as *C. glabrata*. However, the MIC of this isolate against fluconazole was significantly lower than other *C. glabrata* isolates. Furthermore, the size of amplified product using ITS 1 and 4 primers was 550 bp, which shows a 300 bp difference of the PCR product compared with other isolates (Figure 6.11, 6.12). Further investigation is required to address the identification of this species. In contrast, another unusual isolate, *C. krusei* isolated from Hope Hospital had similar digestion band patterns compared with type strain ATCC 6258 (Figure 6.13).

Although additional molecular approaches such as sequencing, karyotyping, etc. are required for the conclusive identification of these unusual isolates, this approach is worthy as a presumptive method for the identification of certain *Candida* species, particularly, when the PCR products or digestion band patterns are identical with type strains, for preliminary information on unusual isolates.

However, direct PCR is also limited as a method for the identification of unknown isolates. Because genomic DNA is not prepared, the intensities of bands after PCR are frequently not consistent. After digestion of the PCR products of some isolates with restriction enzymes, it might be not easy to interpret the data of some digested bands. Species identification by this method is therefore presumptive. Digestion of PCR products with two or more restriction enzymes is likely to provide more reliable results. Thus, phenotypic analyses such as API 32C testing followed by genotypic analysis as described here is to provide absolutely reliable results as the likely possible species identifications are narrowed substantially by the initial phenotypic testing. However, a large database is necessary to have confidence in this approach.

In conclusion, a simple genetic test, such as PCR-RFLP, can be used to provide confirmation of phenotypic identifications. It is possible to differentiate three *Candida* species, *C. inconspicua*, *C. krusei* and *C. norvegensis* using PCR-RFLP without laborious approaches. It is proposed that PCR-RFLP followed by dot hybridisation is a good approach for the identification and differentiation of unknown isolates at the species level. In addition, PCR using a combination of ITS and/or species-specific primers and direct PCR have shown reliability in terms of speed and convenience as diagnostic tools for identifying several *Candida* species. It is believed that direct PCR can provide one of the most efficient approaches to presumptively identify several *Candida* species. For other species, direct PCR followed by digestion with restriction enzymes could provide preliminary information for the identification of these species.

7.

Chapter 7

RAPD analysis of three *Candida* species

7.1 Molecular typing of three *Candida* species

Molecular typing methods have been used in many laboratories as the method of choice for the study of epidemiology, colonisation and the transmission of pathogenic isolates and are regarded as one of the most productive applications of molecular biology to medical microbiology (Matthews *et al.* 1992). The potential of DNA typing methods to address other questions, such as recurrent disease and epidemiology in selected disease categories or environments, is found to be great (Stevens *et al.* 1990).

As typing methods are considered, many outbreaks of infection result from exposure to a common source of the etiologic agent (Swaminathan *et al.* 1993), so reliable typing methods are vital in order to answer important questions. *Candida* species cause a variety of clinical syndromes in human, so it is essential to identify accurately the etiologic species of clinical isolates of the *Candida* species, not only for prognostic and etiological reasons, but also for epidemiological study (Thanos *et al.* 1996).

When it comes to the prevention and control of nosocomial candidiasis, one important consideration is whether the patients acquire the infection from an exogenous or endogenous source because endogenous forms of candidal infection require different strategies for prevention than exogenous infection caused by transmission of an organism from patient to patient (Pfaller 1994). Recently, molecular typing revealed that most hospitalised patients infected with *C. albicans* are colonised or infected with their own distinct strain (Pfaller 1995).

Several different molecular typing techniques have been tried to yield high discrimination without time consuming methods. Furthermore, with increasing numbers of mycoses being caused by species other than *Candida albicans* (Wade 1993), rapid and efficient typing methods are desirable for epidemiological reasons. Despite the large diversity of typing methods, no single procedure has become the accepted standard for *Candida* species (Hunter 1991). When it comes to the use of the typing approach in the laboratory, several criteria should be fulfilled prior to use (Birch *et al.* 1995). The technique should be able to produce an identifiable type for all isolates of a species. It should also provide reproducible results and isolates must be typed correctly independent of growth conditions and preferably after many generations. The technique should have the required level of discrimination as well. Several studies have showed appropriate primer choice to be a critical factor in influencing the discriminatory power of the RAPD method (Baleiras Couto *et al.* 1995).

Therefore, this study aimed to determine whether the molecular typing approach could be useful to type *C. inconspicua*, *C. krusei* and *C. norvegensis* for epidemiological purposes and furthermore whether RAPD might be a useful typing procedure. *C. krusei* has been typed by molecular methods previously (Carlotti *et al.* 1994) but no systems are published for *C. inconspicua* and *C. norvegensis* and no typing method has been introduced.

Although *Candida* isolates in this study did not directly represent the collection of species obtained from patients with a variety of *Candida* infections in different geographical areas, the power of RAPD as a preliminary molecular tool for showing the relative similarity of isolates was measured.

7.1.1 Random amplified polymorphic DNA (RAPD)

Recently, RAPD or arbitrary primed PCR have been widely used to address species identification and genetic relatedness using a single oligonucleotide primer. RAPD is a relatively straightforward procedure and offers several advantages such as speed and simplicity over other more cumbersome typing methods. RAPD allows the detection of polymorphisms without prior knowledge of nucleotide sequence. Several different lengths of single oligonucleotide primers in the range of 10 to 21 bases can bind to the target genome under a low annealing temperature in the PCR tubes. This low stringency of the early cycles ensures the generation of products by allowing priming with mismatches between primers and template. RAPD is ideally suited to fingerprinting applications because it is fast, requires little material and is technically easy to use. RAPD cycle is shown in Figure 1.7. However a lack of reproducibility, particularly between laboratories, is a limitation of RAPD. Even within a single laboratory, changes such as magnesium concentration, buffer, the manufacturer of *Taq* polymerase can alter results so controls and standardisation are essential to interpretation.

Previously, DNA polymorphisms in different species and strains of the genus *Candida* were assessed by amplifying genomic DNA with a single arbitrary AP3 primer (Thanos *et al.* 1996). Successes have also been achieved using RAPD for the characterisation of the fungal pathogen *Aspergillus fumigatus*, the fungus responsible for invasive aspergillosis, an often fatal pneumonia in immunocompromised patients (Birch *et al.* 1995; Anderson *et al.* 1996; Loudon *et al.* 1993). It has been thought that PCR fingerprinting could be used as an indicator of genetic distances because it randomly samples sequence polymorphisms distributed throughout the genome (Welsh *et al.* 1992).

The potential use of RAPDs in taxonomy and population genetics has been widely documented. It is known that this approach is easily performed and provides an efficient way to analyse a large number of isolates.

7.1.2 Similarity coefficients

For the construction of dendrogram, each band with the same molecular weight is visualised and marked as absence or presence as a binary matrix. For measuring similarity values based on the band profiles, several coefficients (Priest *et al.* 1993) have been used for the construction of a dendrogram and formula are listed in Table 7.1. Comparison of RAPD profiles comparison is best carried out in a two dimensional form as a similarity matrix. The matrix can be ordered to group together strains and displayed in a simplified format as a dendrogram.

For better RAPD analysis, the SPSS programme (Bandi *et al.* 1995) was used to generate reliable dendrograms. Each band with the same molecular weight on the gel was compared visually, and marked 0 or 1 as a binary matrix on the basis of absence or presence of bands. The numeric string generated in this manner was used to determine relatedness values among strains.

Table 7.1 Similarity coefficients for microbiology study

Coefficient	Abbreviation	Formula
Simple matching	S_{SM}	$(a+d)/(a+b+c+d)$
Jaccard	S_J	$a/(a+b+c)$
Dice	S_D	$2a/(2a+b+c)$

a and d : number of positive and negative matches, respectively

b and c : number of non-matching characters between pairs of operational taxonomic units.

7.2 Results

7.2.1 RAPD profiles of fungal isolates

Candida isolates from a variety of sources were tested for the RAPD profiles with the R108 primer (Figure 7.1). Results showed that each isolate of each species gave a similar pattern with several bands in common. *A. fumigatus* was found to have a different RAPD profile and could be differentiated visually compared to other *Candida* isolates. Strain variations of *Candida* species were also found.

7.2.2 The reproducibility test of *Candida* isolates

One *C. inconspicua* and one *C. krusei* type strain (ATCC 16783, ATCC 6258, respectively) were included to test the reproducibility of RAPD (Figure 7.1). Although banding patterns were reproducible, intensity difference of bands was found. The reproducibility of 10 *C. inconspicua* isolates from bioMérieux was then tested with the R108 primer, and overall similar banding patterns were recorded (Figure 7.2). However, minor difference in intensities of several bands was also recorded, indicating that RAPDs should be performed with master mixes containing all reagents to ensure that the same amount of materials could be allocated to test tubes in order to obtain high reproducibility.

7.2.3 RAPD profiles of 30 isolates from bioMérieux

Thirty isolates of *C. inconspicua*, *C. krusei* and *C. norvegensis* were then employed to demonstrate the discriminatory ability of the PCR typing method and compared to a type strain in each species. All the isolates of each species were shown to have similar or identical profiles by RAPD with a single primer, R108 (Figure 7.3, 4 and 5) compared

with isolates of the other two species. Some isolates of the same species showed slightly different profiles, indicating that there are genetic variations between isolates of the same species. Three *Candida* isolates from bioMérieux were then compared with reference isolates, and similar banding patterns were found. However, all *C. krusei* isolates were found to have slightly different patterns compared to other ATCC strains. The banding pattern of each of the three species is distinguishable by visual comparison. For instance, in the case of *C. inconspicua*, bands of approximately 370, 1300, 2000 and 2500 bp were common to all the strains, (Figure 7.3). All *C. krusei* isolates have a strong band at 900 bp, and there was a consistent band of variable intensity at 500 bp (Figure 7.4). For *C. norvegensis*, bands at 250, 800, 1700, 1900 and 2900 bp were present in all the strains. (Figure 7.5). The *C. glabrata* reference isolate showed very different and distinctive bands and was easily differentiated from other *Candida* species by visual comparison, suggesting that RAPD analysis could be used for the analysis of differentiation of some isolates at species level. The prominent bands in each lane from all three species were recorded as a lane map by bioimage software (Figure 7.6) as described in the materials and methods (Chapter 2). Finally, the RAPD primer, R108, used in this analysis gave a high level of discrimination and as a consequence, revealed variation between isolates of the same species, demonstrating a high degree of discriminatory power.

7.2.4 Preparation of dendrograms using 3 different similarity coefficients

Using the data of the binary matrix based on the presence or absence of bands on the gels, dendrograms were created on the basis of the three similarity coefficients, Jaccard, Simple matching, and Dice (Priest *et al.* 1993) for quantifying the similarity in these

Candida species (Figure 7.7 and 7.8). However, all three dendrograms were not identical, suggesting that dendrograms could be variable if different coefficients were employed. For instance, dendrograms based on the Simple matching and Jaccard similarity coefficients showed that the percentage of similarity amongst several isolates was not identical to each other, suggesting that the RAPD approach can not be directly applied to phylogenetic analysis. In particular, a dendrogram using the Simple matching coefficient was found to have a less reliable result compared to other dendrograms generated by two other coefficients (Figure 7.7). However, a similar topology based on the Dice and Jaccard coefficients was recorded. In this study, analysis was carried out based on the Dice coefficient because it showed better relationships between three *Candida* species (Figure 7.8).

With respect to *C. inconspicua*, isolates in lane 2, 3 and 8, 9 in Figure 7.3 (corresponding to lanes 2, 3 and 8, 9, respectively in Figure 7.6) showed high percentage of similarity (100%) to each other. *C. krusei* isolates in lanes 3 and 5 in Figure 7.4 (corresponding to lanes 13 and 15 in Figure 7.6) and lanes 8 and 10 (corresponding to lanes 18 and 20 in Figure 7.6) showed a high percentage of similarity to each other. In the case of *C. norvegensis*, a total of four isolates, in lanes 5,6,7 and 9 in Figure 7.5 (corresponding to in lanes: 25, 26, 27 and 29, respectively in Figure 7.6) were identical to each other using the scored bands. Visual inspection of the gels shows minor qualitative differences in the same isolates.

Based on the phenogram constructed using scores of bands on the gels, *C. krusei* isolates were more similar to *C. inconspicua* isolates than to *C. norvegensis* (Figure 7.8). All

isolates in *C. krusei*, *C. inconspicua* and *C. norvegensis* showed their interspecific band patterns. *C. krusei* was also found to be closely related to *C. inconspicua* based on the highly conserved 5.8S rDNA and ITS sequences (see Chapter 5).

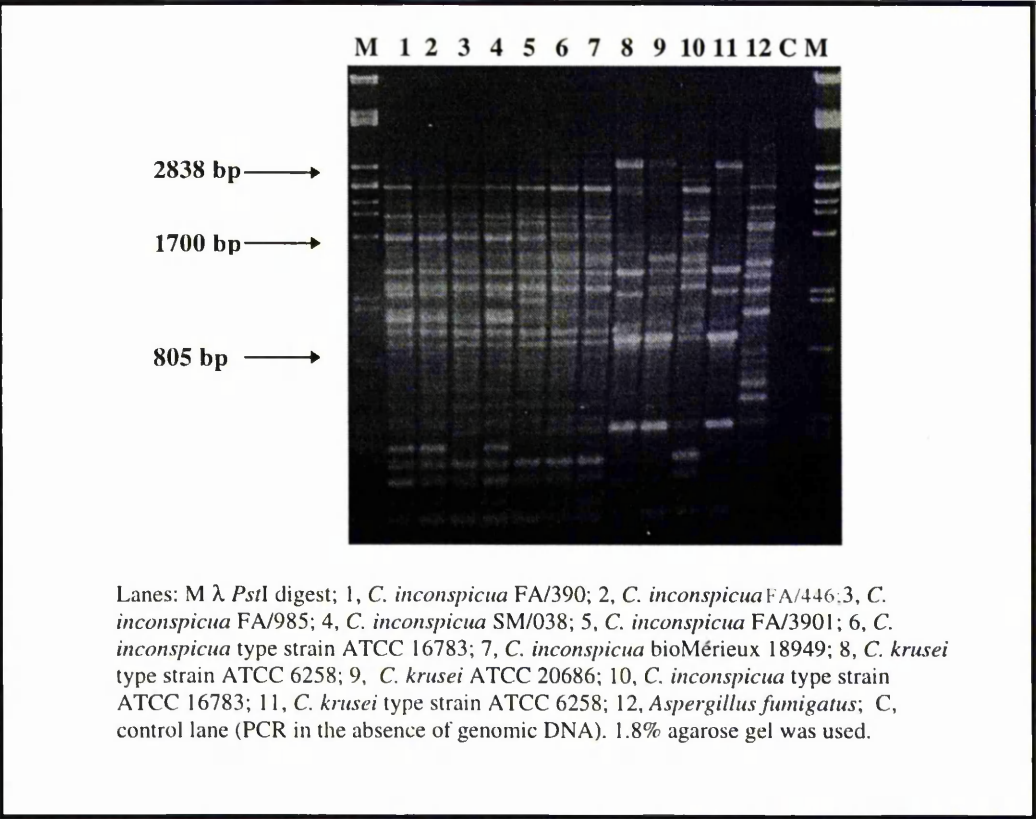


Figure 7.1 RAPD profiles of fungal isolates with R108 primer.

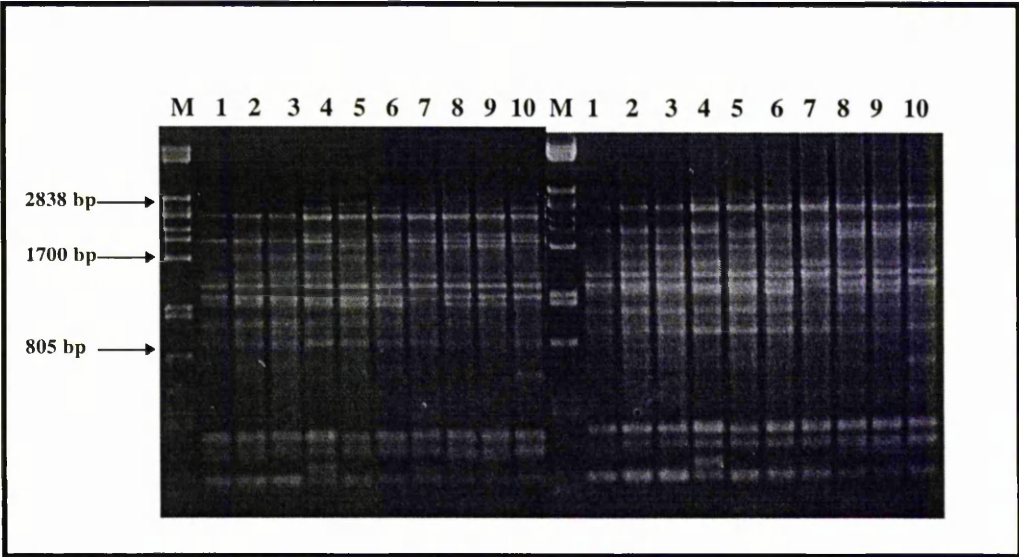


Figure 7.2 Reproducibility of *C. inconspicua* isolates with R108 primer.
The lane order of the *C. inconspicua* isolates is given in Figure 7.6.

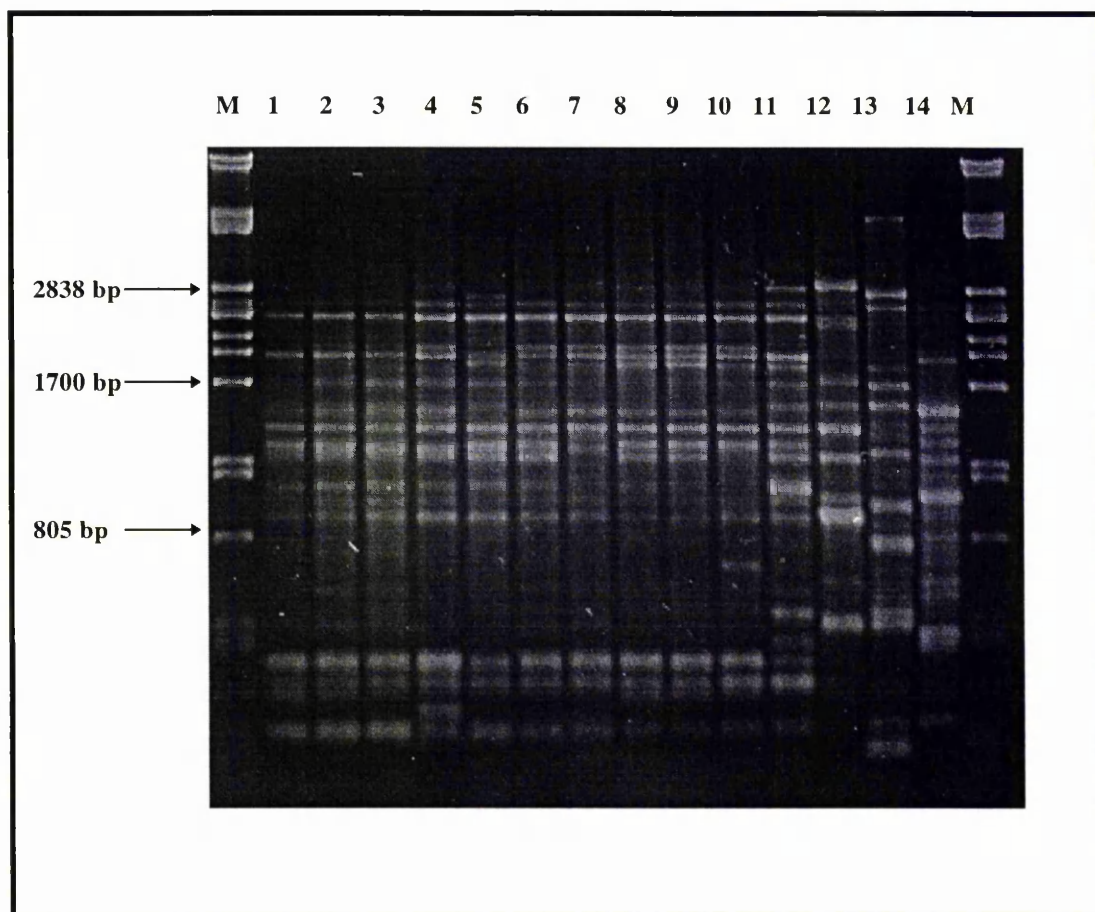


Figure 7.3 RAPD profiles of *C. inconspicua* isolates with R108 primer.

The lane order of the *C. inconspicua* strains is given in Figure 7.6. The molecular size markers (M) are λ digested with *Pst*I; lane 11 : *C. inconspicua* SM/038; lane 12 : *C. krusei* ATCC 6258; lane 13 : *C. norvegensis* ATCC 20686; lane 14 : *C. glabrata* NCPF 3309. 3% NuSieve agarose gel was used.

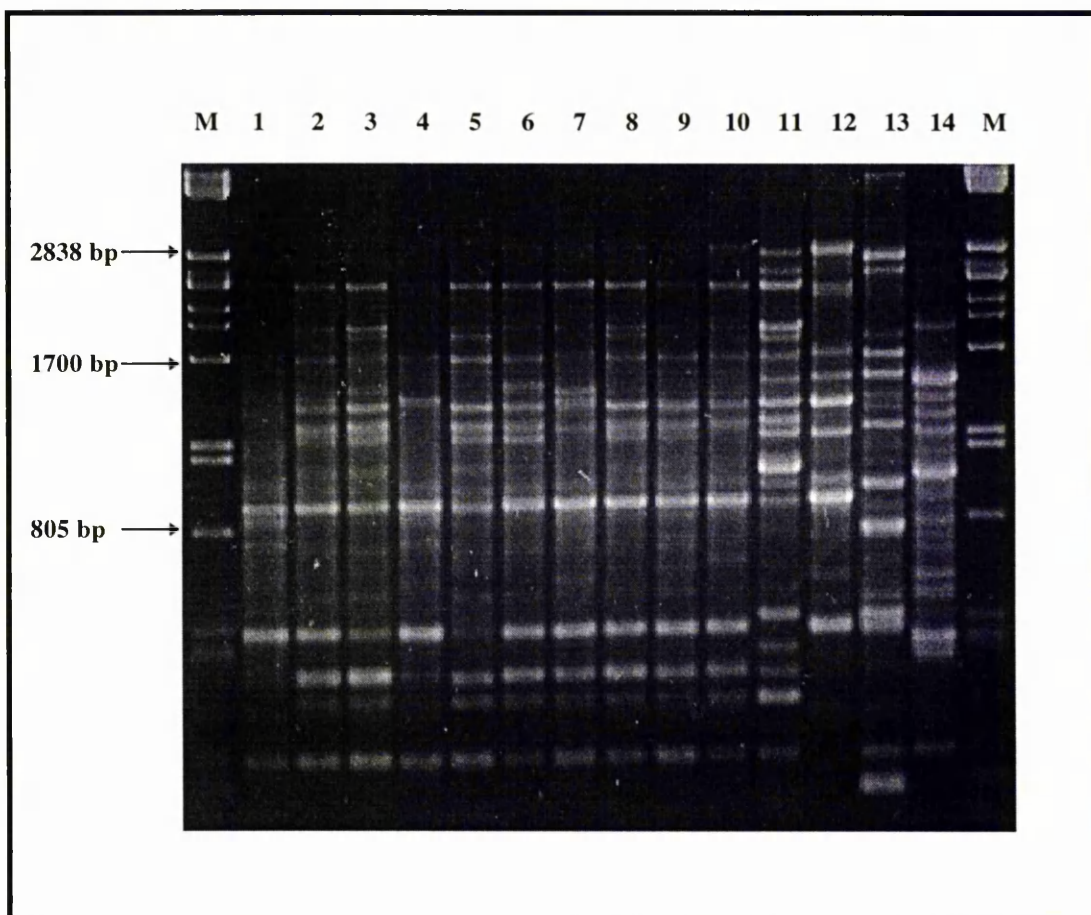


Figure 7.4 RAPD profiles of *C. krusei* isolates with R108 primer.

The lane order of the *C. inconspicua* strains is given in Figure 7.6. The molecular size markers (M) are λ digested with *Pst*I; lane 11 : *C. inconspicua* SM/038; lane 12 : *C. krusei* ATCC 6258; lane 13 : *C. norvegensis* ATCC 20686; lane 14 : *C. glabrata* NCPF 3309. 3% NuSieve agarose gel was used.

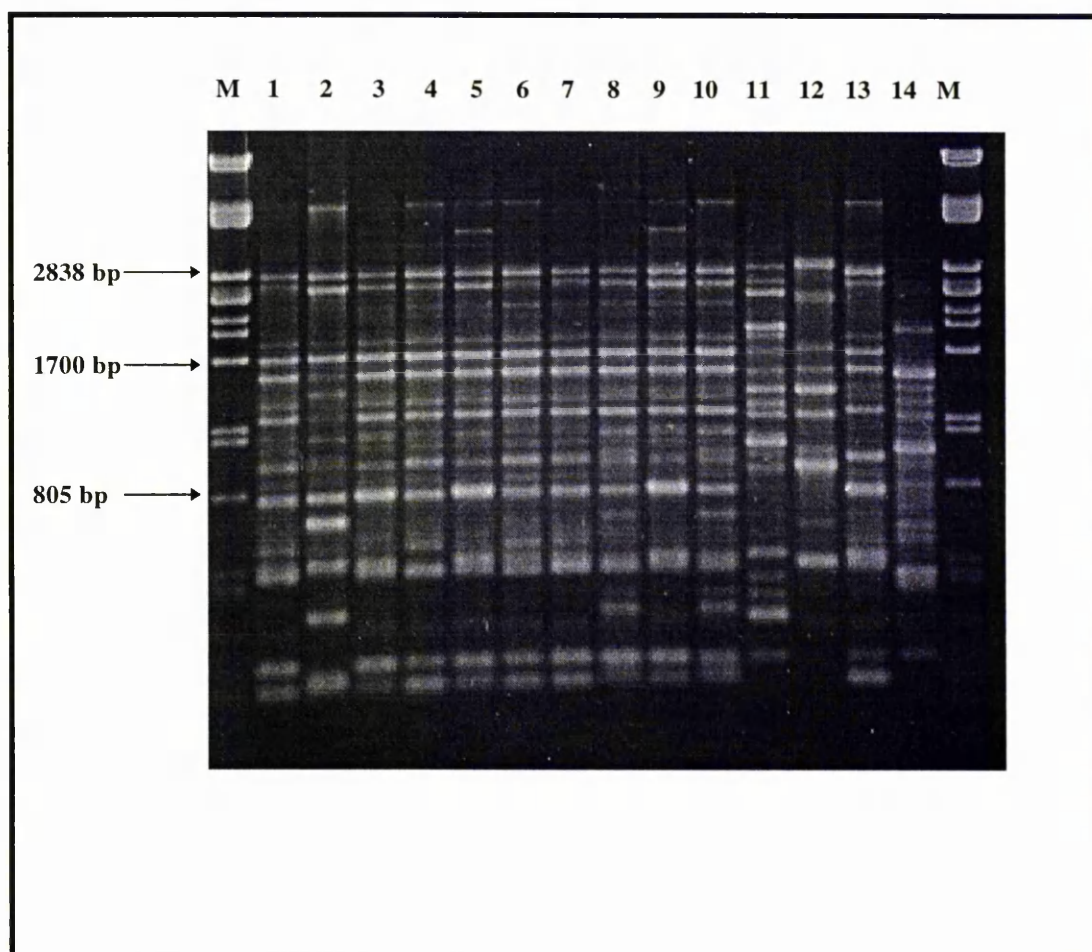


Figure 7.5 RAPD profiles of *C. norvegensis* isolates with R108 primer.

The lane order of the *C. inconspicua* strains is given in Figure 7.6. The molecular size markers (M) are λ digested with *Pst*I; lane 11 : *C. inconspicua* SM/038; lane 12 : *C. krusei* ATCC 6258; lane 13 : *C. norvegensis* ATCC 20686; lane 14 : *C. glabrata* NCPF 3309. 3% NuSieve agarose gel was used.

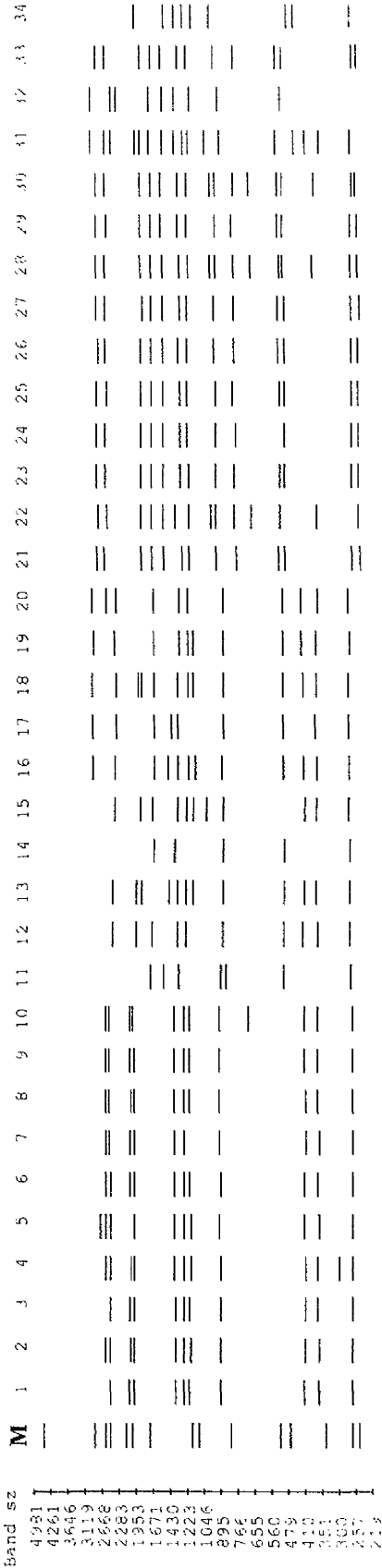


Figure 7.6 Lane map of 30 *Candida* isolates using R 108 primer

Lanes: M, λ *Pst*I digest DNA, 1 to 10, *C. inconspicua*, 8907247, 8909110, 8909109, 8503199, 8509111, 8510102, 9312113, 8502122, 8502119, 8503197, respectively; 11 to 20, *C. krusei*, 8908008, 8808110, 9105030, 8908006, 8510089, 9112022, 9112020, 9112021, 8908007, 9003063, respectively; 21 to 30, *C. norvegensis*, 8808100, 8904072, 8503050, 9502007, 8503051, 8903079, 9502006, 8504237, 8503198, 8807095, respectively; 31, *C. inconspicua*, reference strain SM/038; 32, *C. krusei*, type strain ATCC 6258; 33, *C. norvegensis* reference strain ATCC 20686; 34, *C. glabrata*, reference strain NCPF 3309.

Dendrogram using Average Linkage (Between Groups)

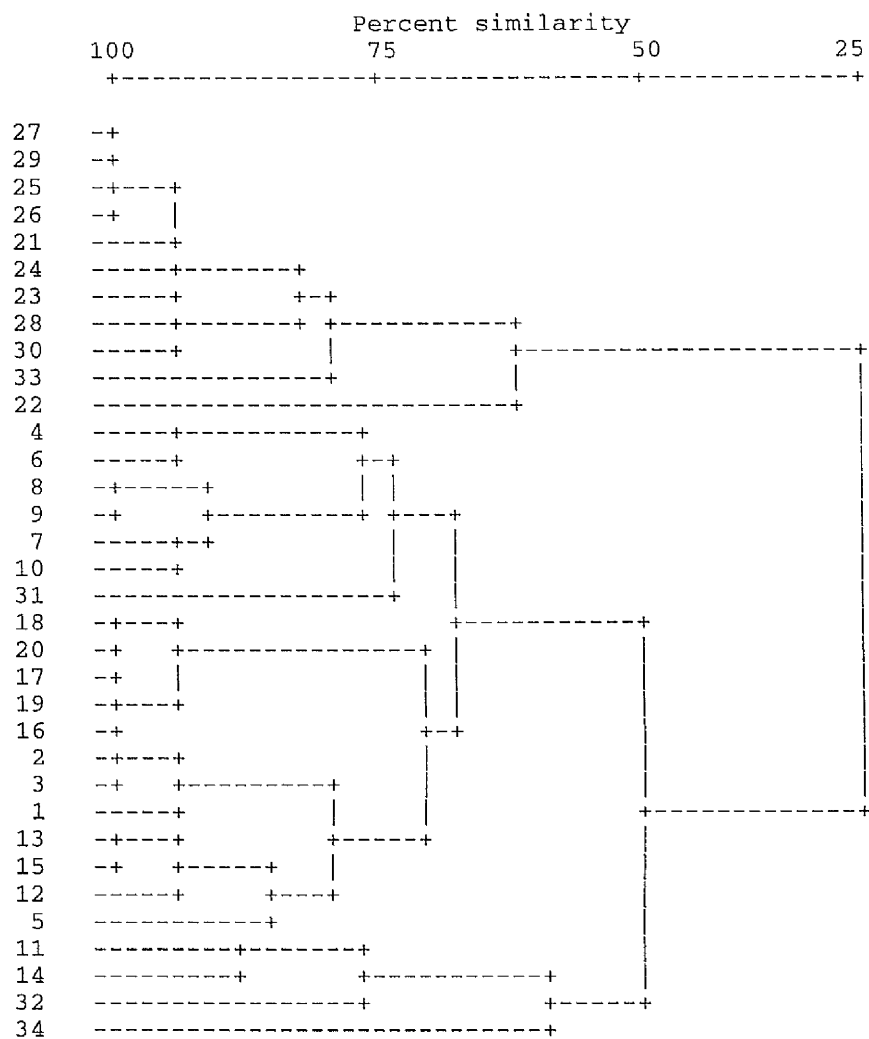


Figure 7.7 Phylogenetic analysis of 30 *Candida* isolates using the Simple matching coefficient.

Isolate numbers are corresponding to the lane numbers in Figure 7.6.

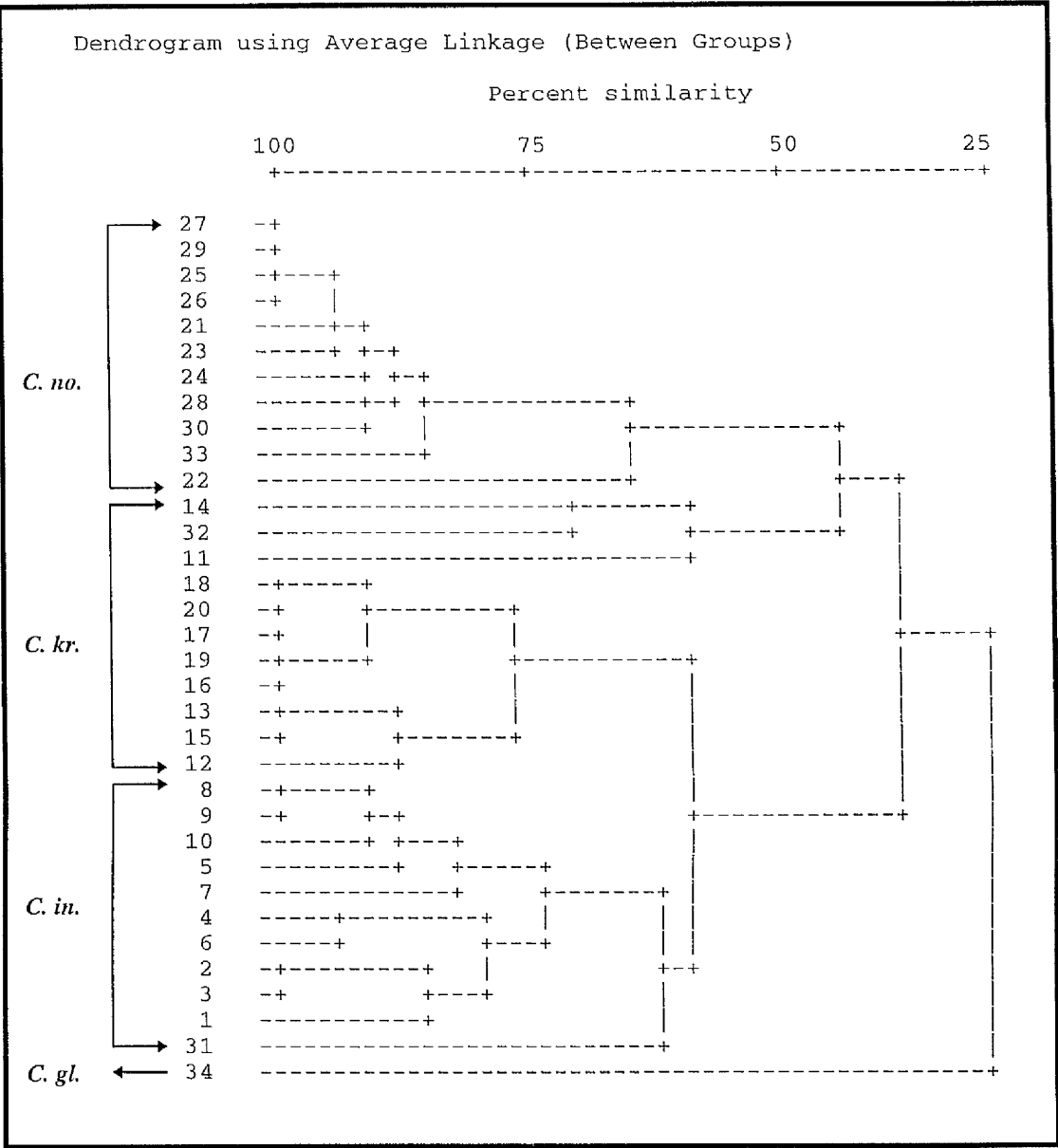


Figure 7.8 Phylogenetic analysis of 30 *Candida* isolates using the Dice coefficient.

Isolate numbers are corresponding to the lane numbers in Figure 7.6.

7.3 Discussion

The use of classical taxonomy based on qualitative data such as colonial morphology, growth characteristics, etc., often cannot provide reliable information because of the difficulties in interpreting data. Several techniques such as immunoblotting have been used but these are laborious and time consuming. Other methods like RFLP analysis on the basis of ribosomal DNA (Appel *et al.* 1995) or the mitochondrial genome have limitations in so far as only limited variations between species and subtype are encountered (Niesters *et al.* 1993).

RAPD is a technique ideally suited to fingerprinting applications because it is fast, requires little material and is technically easy (Newton *et al.* 1994). An accurate identification can be performed rapidly, and RAPD analysis is simpler to perform and interpret than most other genotypic methods used for fingerprinting of fungi (Lehman *et al.* 1992). A major advantage of RAPD analysis is the possibility of it providing a single procedure for the routine identification of both the species and the type of an organism obtained from clinical materials. Since RAPD fingerprinting has been widely used as one of the promising methods for molecular typing, major concerns about reproducibility have been reported, so that strictly controlled conditions are important to generate trustworthy results. PCR and RAPD were carried out using consistent conditions to generate reliable results. For instance, it has been reported that different *Taq* polymerases can generate different profiles from the same isolates (Schierwater *et al.* 1993). Therefore, an identical *Taq* polymerase (Advanced Biotechnologies, UK) was used in all experiments in this study. In addition, other factors like $MgCl_2$ concentration and types of buffer should also be considered when RAPD is employed; hence these experiments were carried out with identical $MgCl_2$ concentrations and buffers. A master mix containing dNTP, $MgCl_2$, primers except genomic DNA was prepared each time for

RAPD, to ensure the delivery of equal amounts of material to each test tube. In particular, cross-contamination with previously amplified DNA products can cause problems, and therefore, all experiments were performed in separate rooms using presterilised disposable microliter pipette tips with three different set of micropipettors. Ultra pure water was also prepared only for RAPD. In addition, the bench for PCR and RAPD was cleaned with disinfectant each time prior to use.

The type strains of *C. krusei* and *C. inconspicua* were included in the reproducibility test, and overall reproducible patterns were found with the R108 primer. RAPD against 10 *C. inconspicua* isolates was also performed twice to assess the reproducibility. It was decided that reproducibility was generally good if all reactions were carried out under strictly controlled conditions. However, there were intensity differences of several bands and there was the loss of higher molecular weight bands in some isolates, which might cause confusion, and make the interpretation of data for the preparation of dendrograms not reliable. Studies have also showed that DNA template quality greatly affects RAPD reproducibility (Micheli *et al.* 1994). In this study, identical procedures were used for the preparation of *Candida* genomic DNA. In addition, because there is a possibility that genomic DNA might be degraded, and the integrity of the DNA was checked on gels before use.

RAPD typing with R108 was also found to successfully demonstrate a great degree of discriminatory power against *Aspergillus fumigatus* (Lin *et al.* 1995; Birch *et al.* 1995). Our results also showed that RAPD with R108 primer generated a good degree of discriminatory power against these *Candida* species. The RAPD profiles with R108 showed clearly that there were interspecies differences between the three *Candida* species.

Ideally additional primers should be used for phylogenetic analysis. A search for additional discriminatory primers was not made in this study as the primary purpose was to confirm the broad relationships between the species and to show variation between the studied isolates. This was particularly important as one of the key objectives of the work was to identify species-specific probes which could work on many genetically different isolates of the same species.

Restriction fragment length polymorphism analysis has limitations as a method for epidemiological study because the probability of losing and regaining a bands are unknown and length polymorphisms are usually indistinguishable from nucleotide substitution polymorphisms (Thanos *et al.* 1996). RAPD studies of *C. inconspicua* (Baily *et al.* in press) and *C. krusei* have been reported (Thanos *et al.* 1996). In contrast, RAPD typing of *C. norvegensis* has not previously been reported.

Several problems are still open to address for the use of RAPD as a method for typing of *Candida* species. For instance, the quality of bands on the gel is not identical in each lane so that there is always difficulty in scanning the bands. When it comes to the construction of dendrograms based on similarity coefficients, different coefficients such as Simple matching, Jaccard, and Dice, the overall pattern of the trees is slightly different, making it uncertain which is the 'correct one'. Although we can make an elucidated assumption about which coefficient to use, our data suggested that RAPD data in conjunction with other comparative data can provide more reliable results. Therefore the inconsistency observed in the different topologies derived from the three different similarity coefficients implies that this method has

limitations when applied to *Candida* taxonomy. It was found that the differences in the trees values are due to the nature of the coefficients used. In particular, the presence of low similarity values between *C. inconspicua* and *C. krusei* isolates was recorded when the Simple matching coefficient which measures positive matches (a) and negative matches (d) as a proportion of the total number of characters (a+b+c+d) was used. Because negative matches (i.e. 'd') ($S_{SM} = (a+d)/(a+b+c+d)$; a, the number of positive matches; b and c, the number of non-matching characters between pairs of OTUs; d, the number of negative matches) are included as a measure of similarity, the S_{SM} coefficient generates different tree topology compared to the case of trees generated with the Dice and Jaccard coefficients. In contrast, negative matches are not used in the S_D and S_J coefficients so that the number of positive matches between isolates is stressed to construct dendrograms. In this study, the number of positive matches between isolates was highlighted for the analysis of percent similarity so that the S_{SM} coefficient generated different similarity values between isolates.

The Dice and Jaccard coefficients (i.e. $S_D = 2a/(2a+b+c)$; $S_J = a/(a+b+c)$) generated similar topologies, and close relatedness between *C. inconspicua* and *C. krusei* isolates was recorded, supporting the concept that *C. krusei* and *C. inconspicua* are closely related to each other. Therefore, in this study the S_D was used for the RAPD analysis of *Candida* isolates. The S_D coefficient has been used for the DNA fingerprinting of the eight taxa of *Trichinella* (Bandi 1995).

Because of the variable intensities of bands at the same molecular weight, subjective interpretation by individuals means that there is a possibility that the actual percent similarities will be different. In addition, transferring RAPD from one laboratory to another

laboratory can also be problematic because different band profiles could be generated so that interpretation of similarity results based on the RAPD patterns will not be consistent. We therefore believe that RAPDs alone cannot be applied in phylogenetic studies without a lot more comparative data.

Although RAPD showed good discriminatory ability in this study, this analysis was based on data with a single primer, and therefore percentage differences between isolates and species are preliminary. However, the general trend is visible and probably indicative of the relative relatedness of each species group. Therefore, this method may be employed as a preliminary molecular tool for phylogeny of these *Candida* species.

In conclusion, despite the real and possible concerns about RAPD, the results indicate that PCR fingerprinting using R108 can differentiate these three *Candida* species with possibly sufficient discriminatory power to distinguish between isolates. This approach is faster and simpler than most other genotype-based methods, and above all, the same methodology could potentially be used in a much larger study for the differentiation of species and the taxonomic study of a large numbers of isolates from geographically and epidemiologically diverse sources.

8.

Chapter 8

Identification of *Candida* species using CIP and CKP primers

8.1 Identification of *Candida* species using species-specific primers

Although PCR using ITS primers followed by restriction enzyme digestion were reliable, they required two steps, i.e. PCR and endonuclease digestion. Therefore, a single step procedure would be preferable. One such approach would be the use of species-specific probes for *in situ* hybridisation (Montone *et al.* 1994). Several other attempts have been made to identify clinically significant *Candida* species based on the use of a combination of species-specific oligonucleotide and universal primers. Previously, unique primers based on the ITS sequences of *C. neoformans* were also designed, and proved that an appropriate combination of species-specific (Mitchell *et al.* 1994) and universal primers (White *et al.* 1990) could be used to identify target organisms. Haynes *et al.* (1996) also used PCR techniques using species-specific primers with the universal reverse primer to identify *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. krusei*.

The aim of this study was to know whether newly designed species-specific probes, CKP and CIP could be used as PCR primers for rapid identification of target species. Figure 8.1 shows the location of the ITS 1 and species-specific CIP, CKP primers for the identification of *C. krusei* and *C. inconspicua* using PCR.

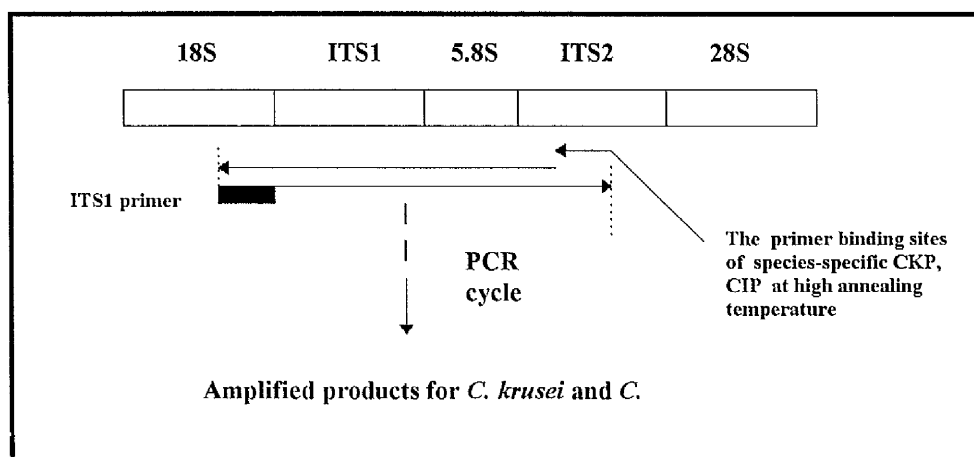


Figure 8.1 Identification of *C. krusei* and *C. inconspicua* using CKP or CIP-ITS 1 primer pairs.

8.2 Results

Based on the comparative analysis of sequencing results of the ITS 2 region as described previously, the species-specific probes, CIP and CKP for the detection of *C. inconspicua* and *C. krusei* were designed (Figure 5.9). CKP and CIP primer sequences are described in Figure 8.2. Each probe was 21 base long and designed from the ITS 2 regions. The G+C content and melting temperature of each probe were calculated. T_m was calculated by the following formula: $4 (G+C) + 2 (A+T)$, and 66°C was found to be the T_m of both primers, and the annealing temperature was decided empirically to obtain reliable results. Then each probe was used as a primer for the PCR amplification of the relevant *Candida* species (Figure 8.3.A). For the rapid detection of *C. krusei* and *C. inconspicua* species, the usefulness of these potentially unique oligonucleotide primers was examined.

Candida and other species genomic DNA in our culture collection were tested against CKP or CIP and ITS 1 primer pairs under different annealing temperatures as follows; 56, 60, 63 and 65°C, to enable identification of target *Candida* species. The annealing temperatures chosen were decided empirically.

At the 56°C annealing temperature in PCR cycles with CIP or CKP - ITS1 pair, many non specific amplified products appeared in both *C. inconspicua*, *C. krusei* isolates and other *Candida* species (data not shown). At the increased annealing temperature of 60°C, several non specific bands were still generated on both *C. inconspicua* and *C. krusei* isolates. Figure 8.3.B shows several non specific PCR products with CIP-ITS1 pair as PCR was carried out at 60°C annealing temperature. As the annealing temperature was

increased, non specific bands other than genuine PCR products of *C. krusei* and *C. inconspicua* gradually disappeared.

At an annealing temperature of 63°C, the 305 bp of *C. inconspicua* PCR product only was generated (Figure 8.3.C). Species-specific amplified products were also generated at 65°C (data not shown), suggesting that temperature in the range of 63 to 65 °C is good for the identification of these species.

In the case of *C. krusei*, six isolates of *C. krusei* from ATCC and bioMérieux were tested with four isolates of *C. inconspicua*, three isolates of *C. norvegensis*, two isolates of *C. glabrata*, and all *C. krusei* isolates were also successfully amplified at 63°C using the CKP and ITS 1 primer combination (Figure 8.4.A). CKP primer with ITS 1 was also tested against other fungal species at 63°C (Figure 8.4.B) and only *C. krusei* genomic DNA was amplified. A similar result was also recorded when the 65°C annealing temperature was used (data not shown). Based on these results, PCR-based approaches using species-specific primers could possibly be used as a rapid method for the identification of these *Candida* species.

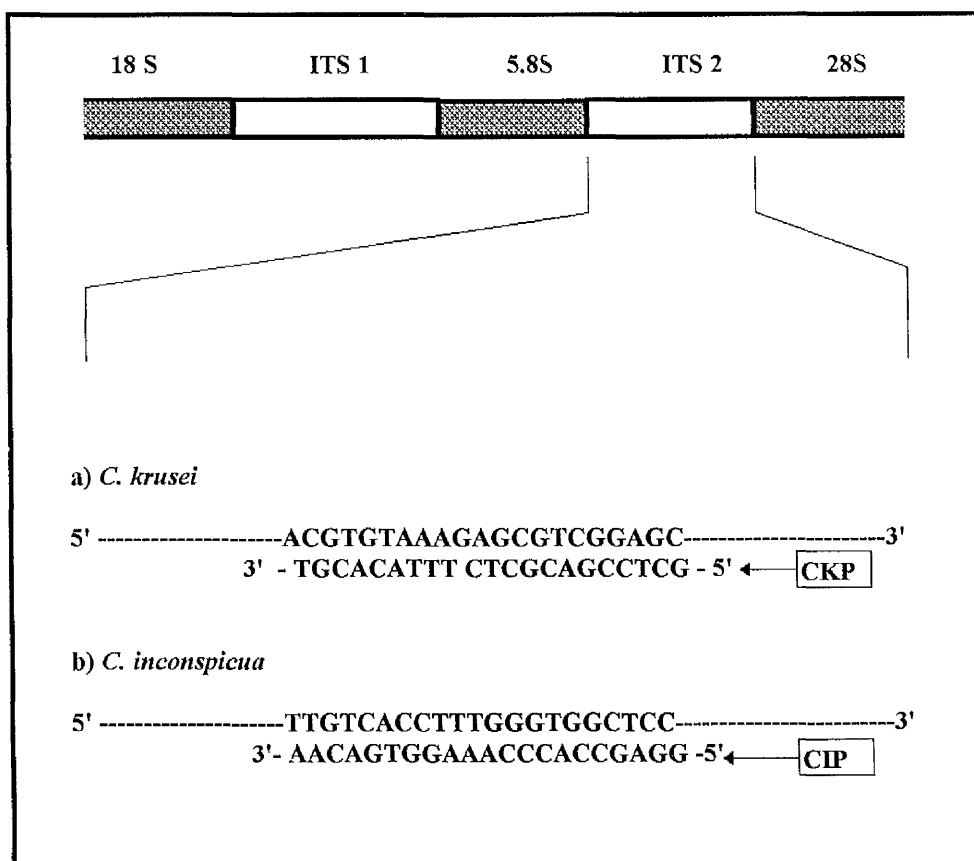
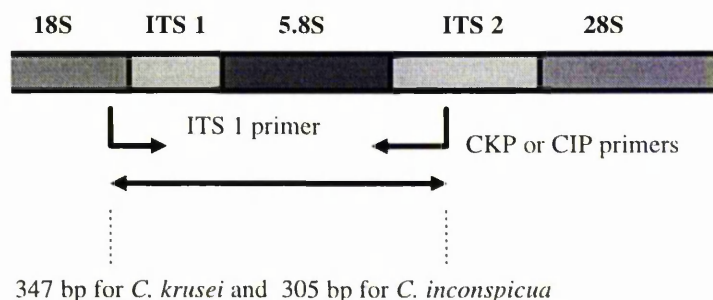
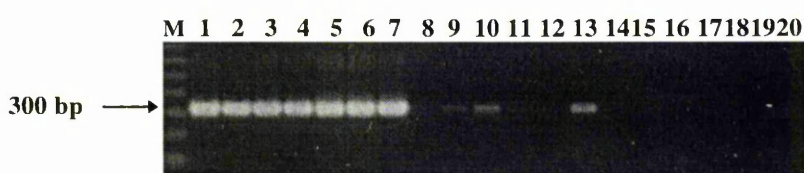


Figure 8.2 Preparation of species-specific primers, CKP and CIP on the ITS 2 region of *C. inconspicua* and *C. krusei*.

CKP and CIP were also used as species-specific probes (see Chapter 9). Detailed probe sites on the ITS 2 region are shown in Figure 5.9.

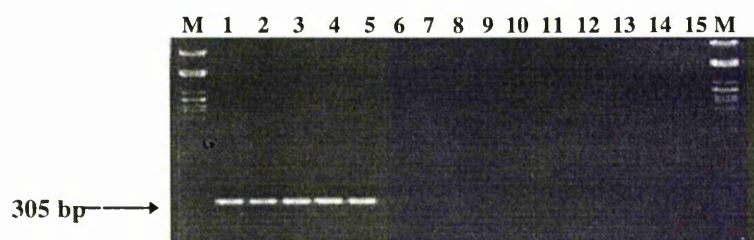


A. Schematic representation of primers binding sites on ribosomal RNA locus and PCR product sizes.



B. PCR using ITS 1 and CIP combination at 60°C annealing temperature.

Lanes: M. 100 bp ladder; 1 to 7, *C. inconspicua* FA/390, FA/446, FA/985, SM/038, bioMérieux ATCC 3187, type strain ATCC 16783, bioMérieux 18949, respectively; 8, *C. krusei* type strain ATCC 6258; 9, *C. krusei* bioMérieux 18888; 10 to 13, *C. krusei*, 8908008, 8808110, 9105030, 8908006; 14 to 16, *C. norvegensis* bioMérieux 8908100, 8904072, 8503050, respectively; 17 to 18, *C. glabrata* FA/3309 large, small, respectively; 20, *C. rugosa* ATCC 10571. PCR products were run on the 1.8% agarose gel for 1.5h.



C. PCR using ITS 1 and CIP combination at 63°C annealing temperature.

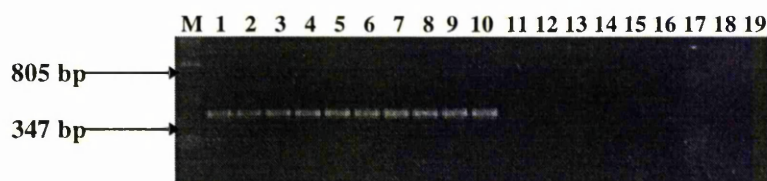
Lanes: M, 100 bp ladder; 1 to 5, *C. inconspicua* FA/390, FA/446, FA/985, SM/038, bioMérieux 3187, respectively; 6, *C. krusei* type strain ATCC 6258; 7, *C. krusei* bioMérieux 18888; 8, *C. krusei*, 8908008; 9 to 12, *C. norvegensis* bioMérieux 8908100, 8904072, 8503050, respectively; 13, *C. krusei* ATCC 20298; 14, *C. rugosa* ATCC 34637; 15, *C. glabrata* NCPF 3309.

Figure 8.3 PCR-based method for the identification of *C. inconspicua* using ITS 1 and CIP primers at different annealing temperature.



A. PCR amplified products using ITS 1 and CKP combination at 63°C annealing temperature.

Lanes: M, λ PstI digest; 1 to 4, *C. inconspicua* FA/390, FA/446, FA/985, SM/038, respectively); 5, *C. krusei* type strain ATCC 6258; 6, *C. krusei* bioMérieux 18888; 7 to 9, *C. krusei* bioMérieux 8908008, 8808110, 9105030, respectively; 10, *C. krusei* ATCC 20298; 11, *C. rugosa* ATCC 34637; 12, *C. glabrata* FA/3309 (large); 13 to 15, *C. norvegensis* bioMérieux 8908100, 8904072, 8503050, respectively; 16 to 17, *C. glabrata* FA/3309 (small), FA/2983, respectively; C: control lane (PCR in the absence of genomic DNA). Amplified products were run on 1.8% agarose gel for 1.5 h.



B. PCR amplified products of *C. krusei* from bioMérieux at 63°C annealing temperature.

Lanes : M, λ PstI digest 1 to 10, *C. krusei* strains from bioMérieux; 1, 8908006; 2, 8808110; 3, 9105030; 4, 8908006; 5, 8510089; 6, 9112022; 7, 9112020; 8, 9112021; 9, 8908007; 10, 9003063, respectively; 11, *C. glabrata* FA/2867; 12, *Rhodotorula rubra* FA/3081; 13, *C. tropicalis* FA/3504; 14, *C. kefyr* FA/1727; 15, *C. parapsilosis* FA/3511; 16, *Trichosporon beigeli* FA/2441; 17, *Saccharomyces cerevisiae* FA/3388; 18, *Cryptococcus neoformans* FA/3239; 19, *C. dubliniensis* FA/448. 1.8% agarose gel was used.

Figure 8.4 PCR-based method for the detection of *C. krusei* using ITS and CKP primers.

8.3 Discussion

The sequence data of 5.8S ribosomal DNA including ITS regions were used to design species-specific probes for *C. krusei* and *C. inconspicua*, exploiting the small sequence differences identified. These probes were called CKP or CIP primers for *C. krusei* and *C. inconspicua*, respectively.

Because CKP and CIP were developed as probes having a corresponding top strand sequence in the ITS 2 region, these probes could be used under identical PCR conditions as PCR primers for the identification of these species in conjunction with the ITS 1 primer derived from the end of 18S. Interestingly, the yield of amplified products using this primer combination was influenced by the annealing temperature, and it was found that annealing temperature at 63-65°C was optimal for the detection of target isolates.

Previously, Haynes *et al.* (1996) used a similar approach to identify several *Candida* species using primers derived from large subunit rDNA and proved their efficiency as a rapid PCR-based technique. Initially they used 64°C as an annealing temperature, and proved that the non target DNA band disappeared when the temperature was increased to 66°C. It was also found that the annealing temperature was an important factor in generating species-specific amplified bands in this study when universal and species-specific primers were used. Therefore it may be wise to test additional primers with different annealing temperatures to establish the most useful identification results of these species. In addition, a variety of isolates from geographically diverse sources should be tested to ensure that this approach is one hundred percent reliable.

In conclusion, it was suggested that the ITS1 and CIP, or CKP pair could be able to detect *C. krusei* and *C. inconspicua*. These primer combinations are particularly useful in the identification of target isolates among multiple isolate collections. Just PCR with an appropriate primer pair followed by gel electrophoresis can provide information for species identification. A large number of additional species should be tested if this approach alone (e.g. without conventional phenotypic tests) were to be used for identification of these species in the future.

9.

Chapter 9

Identification of *C. krusei* and *C. inconspicua* using dot hybridisation

9.1 Microbial identification using sequence-based technology

With the development of fast and reliable analytical techniques in molecular biology, the reliance on traditional microbiological tests for gathering phenotypic data has decreased. Genotypes are more specific and are more easily quantified and standardised among different organisms than the phenotypic markers used traditionally (Fredricks *et al.* 1996). There are various approaches based on nucleic acid analysis for the identification and detection of microorganisms in clinical samples. The flow diagram for the preparation of probes is illustrated in Figure 9.1.

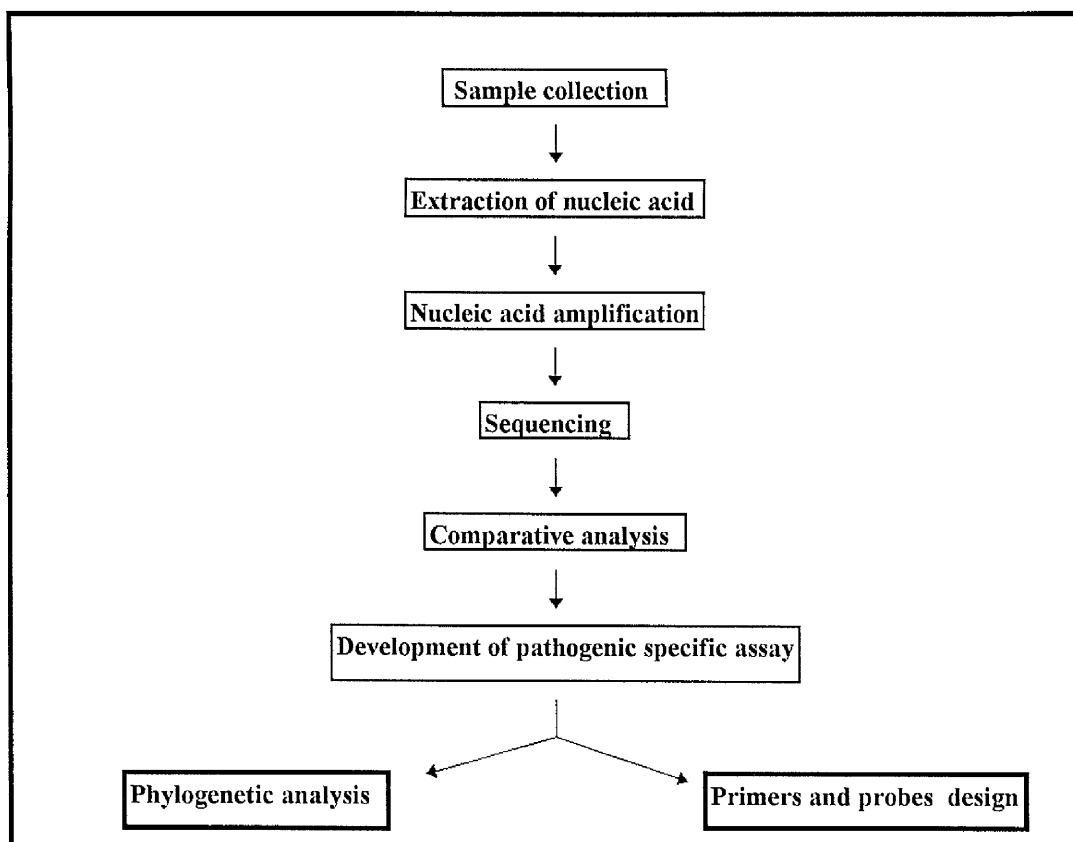


Figure 9.1 Nucleic acid-based approaches for the preparation of species-specific probes.

9.1.1 Dot hybridisation

Detection and /or confirmation of the identity of a PCR product can be performed by dot blot hybridisation with radioactive or nonradioactive specific probes. This technique is rapid method for the qualitative screening of DNA. The target DNA sample may be purified DNA, cell lysate or PCR-amplified DNA. There are many different protocols for dot blotting, and there are several steps to consider (Darling *et al.* 1994).

1) Preparation of the sample and species-specific probes.

The DNA sample must be denatured in order to hybridise with a probe. The DNA is usually denatured before it is applied to the membrane. The DNA is denatured at 95-100°C for 15 min.

2) Preparation of the membrane.

The nucleic acid binding capacity of a nylon membrane is in the order of 500 $\mu\text{g}/\text{cm}^2$, whilst that of a nitrocellulose filter is in the order of 100 $\mu\text{g}/\text{cm}^2$. A positively charged nylon membrane is therefore recommended for dot blotting.

3) Application of the sample and blotting.

Dispense sample DNA onto the membrane and apply a gentle vacuum.

4) Processing the membrane.

The DNA is then fixed to the membrane by UV crosslinking or baking. Prehybridisation and hybridisation steps are followed.

5) Detection.

With the digoxigenin system, detection of DIG-labelled nucleotides can be accomplished with a chemiluminiscent or colorimetric reaction.

9.1.2 Probes design

Several species-specific probes derived from ribosomal RNA genes have been designed (Fujita *et al.* 1995; Carlotti *et al.* 1996; Botelho *et al.* 1994) for the detection of *Candida* species so far, and none of these probes has been found to be satisfactory or reliable for the detection of *C. krusei*. As species-specific probes were designed in this study, several factors were considered in order to obtain reliable results. The correct sequence of probes, which match perfectly to the corresponding target DNA, is essential otherwise a prolonged hybridisation time is required, delaying the identification of target isolates. The rRNA gene was chosen in this study as a target region for the development of species-specific probes because it exists in multiple copies so that this approach was thought to be more reliable and sensitive than others.

With the concept of rDNA as a target site for molecular identification, several approaches have been employed to detect both *C. albicans* and non *C. albicans* species based on the species-specific ITS regions in ribosomal DNA. The ribosomal RNA regions consist of a highly conserved area (18S, 5.8S and 28S) including relatively variable sequence regions (ITS 1, 2 and IGS), tandemly repeated many times so that molecular approaches based on the rDNA ITS regions are suitable for the selection of clinically important *Candida* species-specific DNA probes (Botelho *et al.* 1994).

It has been found that the sites of the ITS regions where the species-specific probes are designed are an important factor in obtaining reliable results. Because high homology sequences in *Candida* species were found in some parts of the ITS regions, in particular, at the 5' start of ITS 1 and 2, and the 3' end of ITS 1 and 2, due to evolutionary

conservation and the mechanisms of processing, the sites aimed at species-specific probes should not be located in these conserved regions. Since *C. krusei* and *C. inconspicua* have very similar phenotypic and genotypic characteristics, this study focused on the discovery of sensitive approaches using species-specific probes which would enable the identification of these species more reliably.

9.2 Results

CIP and CKP were designed as described in the previous Chapters and used as probes for dot hybridisation. CKP was then compared with another probe derived from the ITS 2 region which has already been published for the detection of *C. krusei*. (Fujita *et al.* 1995; see Chapter 5). The species-specific probes were then analysed to examine whether they are capable of identifying targeted species without cross-hybridisation.

9.2.1 Sensitivity of the probe in dot blot hybridisation

As species-specific probe, CkF1,2 (Carlotti *et al.* 1996) for *C. krusei* was previously introduced, the sensitivity of CKP was compared to CkF 1,2 in order to examine whether our probe has a better sensitivity for the identification of *C. krusei*. Previously, CkF1,2 was known to detect up to 60 ng of *C. krusei* DNA. In this study, the hybridisation signal with the lowest intensity was detectable as little as 0.43 ng of *C. krusei* (ATCC 6258) (Figure 9.2). A strong signal was recorded up to 10^{-2} which is equivalent to about 4.3 ng, and this data suggests that up to 4 ng of *C. krusei* DNA is detectable. Another *C. krusei* strain (18949 from bioMérieux) was also tested to determine sensitivity of the CKP probe, and the same result was obtained (data not shown).

9.2.2 Comparisons of probes for the detection of *C. krusei*

For the medical use of the *C. krusei* species-specific probe, the specificity was assessed. Both newly designed probes, CKP and CIP were tested against medically important fungal isolates including procaryotes. The species-specific sites for these probes are described in Figure 8.2.

In particular, the CKP probe sequence was then compared with another species-specific probe for *C. krusei*, CK (5' GG CCC GAG CGA ACT AGA CTT TT 3'; Fujita *et al.* 1995), which was designed from the same ITS 2 region (Figure 5.16). The sequence alignment of the ITS 2 region where two probes, CKP and CK, were designed is described in Figure 5.16 (Chapter 5), and there is over 88% similarity between two sequences. Interestingly, 1 base difference where CK probe was designed was found between two sequences. Fujita *et al.* (1995) mentioned that the CK probe reacted poorly with *C. krusei* DNA after 1 h hybridisation but that positive results were recorded after 24 h hybridisation. In contrast, the site where our CKP was designed showed 100% homology between two sequences.

9.2.3 Specificity test of species-specific probes against other genomic DNA

To determine the usefulness of these probes for the identification of all strains of *C. inconspicua* and *C. krusei* against other species, a total of 52 genomic DNA samples were included in this study.

In the first test against 41 isolates, ten *C. inconspicua* and ten *C. krusei* isolates were included. Various combinations of hybridisation and washing conditions were tried. First,

with a 50°C hybridisation temperature for 1 h with CKP probe with washing twice in 2 X SSC for 15 min at 50°C (low stringency washing conditions), several cross-hybridisation signals between isolates appeared (Figure 9.3.A). In particular, *T. denticola* and *E. coli* cross hybridised with this probe strongly. There was also very faint cross hybridisation of *C. norvegensis* isolates with CKP (data not shown). Second, hybridisation of genomic DNA with both CKP and CIP probes at 50°C for 1 h, followed by washing the membranes in 2 X SSC twice for 15 min at 50°C, and in 0.1 X SSC twice for 15 min at 50°C yielded faint hybridisation signals with *T. denticola* (Figure 9.3.B).

However, after hybridisation at 52°C followed by high stringency washing conditions at 52°C, only *C. inconspicua* and *C. krusei* strains were detected with CIP and CKP respectively (Figure 9.4.A and B). None of the other isolates was cross-hybridised with these probes. Another experiment against eleven *Candida* isolates (ten *C. norvegensis* and one *C. glabrata* isolate) was also performed under the same conditions as described above, only the ten *C. inconspicua* and ten *C. krusei* isolates were detected (data not shown) with CIP and CKP, respectively, and no cross-hybridisation was found between them. This result indicates that these probes can be useful for the identification of these isolates.

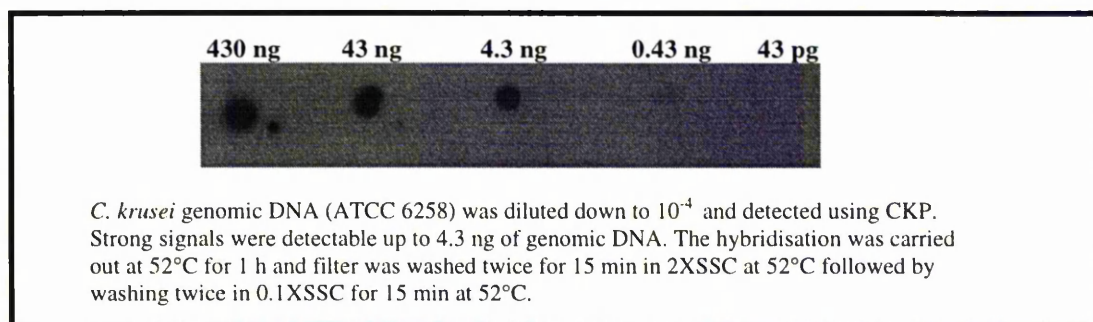
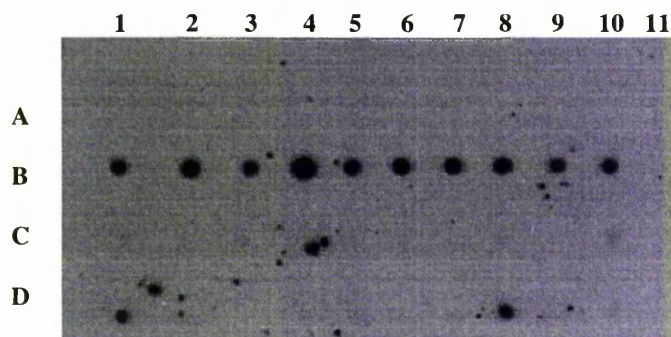


Figure 9.2 Sensitivity test for the detection of genomic DNA using species-specific probe, CKP.



A. Detection of *C. krusei* isolates at 50°C^a with CKP probe under low stringency washing conditions.

Row A, lanes : 1 to 9, *C. inconspicua* strains from bioMérieux^b; 10, *C. inconspicua* type strain ATCC 16783

Row B, lanes : 1 to 10, *C. krusei* strains from bioMérieux^c

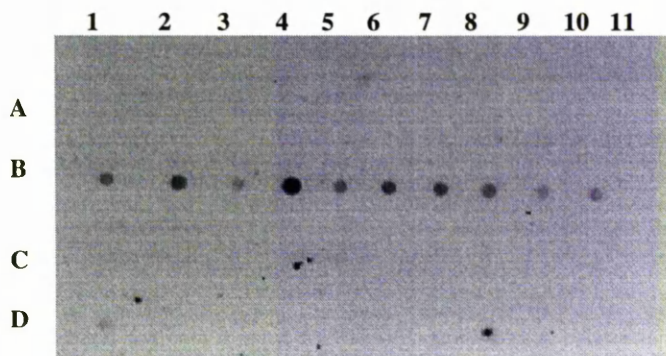
Row C, lanes : 1 to 3, *C. glabrata* FA/2867, FA/3309 (large), FA/2823, respectively; 4, *Rhodotorula rubra* FA/3081; 5, *C. tropicalis* FA/3504; 6, *C. kefir* FA/1727; 7, *Aspergillus nidulans* G15; 8, *C. parapsilosis* FA/351; 9, *Trichosporon beigelii* FA/2441; 10, *Saccharomyces cerevisiae* FA/3388.

Row D, lanes : 1, *Treponema denticola* ATCC 35405; 2, *C. albicans* Hope strain; 3, *C. dubliniensis* FA/448; 4, *Cryptococcus neoformans* FA/3239; 5, *C. kefir* FA/1727; 6, *C. albicans* FA/1470; 7, *Aspergillus fumigatus* AF 210; 8, *Escherichia coli* PA 360 ; 9, *Staphylococcus epidermidis* F337; 10, *Staphylococcus anginosus* MS12a ; 11, Human DNA.

^a : hybridisation temperature.

^{b,c} : The strain numbers and lane order are corresponding to the list in Figure 6.8 and 6.9.

Other isolate numbers are corresponding to the numbers in Table 2.1. Membrane was washed twice at 50°C in 2XSSC.

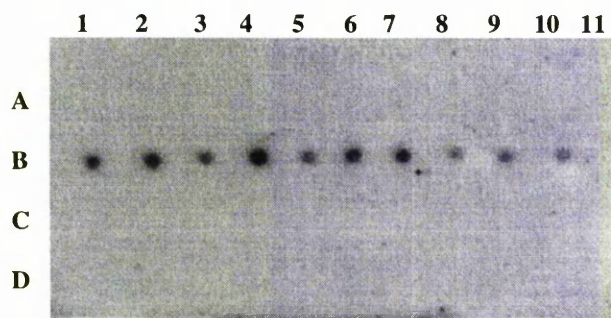


B. Detection of *C. krusei* isolates at 50°C with CKP probe under high stringency washing conditions.

Lanes and row order are same as Figure 9.3.A.

Membrane was washed in 2X SSC for 15 min twice followed by washing in 0.1XSSC twice for 15 min at 50°C.

Figure 9.3 Detection of *C. krusei* strains using species-specific probe, CKP by dot hybridisation.



A. Detection of *C. krusei* isolates at 52°C^a with CKP probe under high stringency washing conditions.

Row A, lanes : 1 to 9, *C. inconspicua* strains from bioMérieux^b; 10, *C. inconspicua* type strain ATCC 16783

Row B, lanes : 1 to 10, *C. krusei* strains from bioMérieux^c

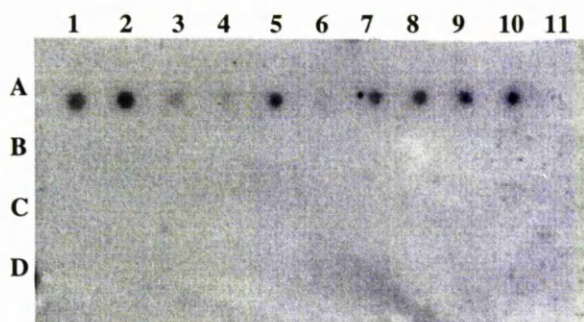
Row C, lanes : 1 to 3, *C. glabrata* FA/2867, FA/3309 (large), FA/2823, respectively; 4, *Rhodotorula rubra* FA/3081; 5, *C. tropicalis* FA/3504; 6, *C. kefyr* FA/1727; 7, *Aspergillus nidulans* G15; 8, *C. parapsilosis* FA/3511; 9, *Trichosporon beigeli* FA/2441; 10, *Saccharomyces cerevisiae* FA/3388.

Row D, lanes : 1, *Treponema denticola* ATCC 35405; 2, *C. albicans* Hope strain; 3, *C. dubliniensis* FA/448; 4, *Cryptococcus neoformans* FA/3239; 5, *C. kefyr* FA/1727; 6, *C. albicans* FA/1470; 7, *Aspergillus fumigatus* AF210; 8, *Escherichia coli* PA360; 9, *Staphylococcus epidermidis* F337; 10, *Staphylococcus anginosus* MS12a; 11, Human DNA

^a : hybridisation temperature

^{b,c} : The strain numbers and lane order are corresponding to the list in Figure 6.8 and 6.9.

Other isolate numbers are corresponding to the numbers in Table 2.1.



B. Detection of *C. inconspicua* isolates at 52°C with CIP probe under high stringency washing conditions.

The isolates and lane order are corresponding to Figure 9.4.A.

Figure 9.4 Detection of *C. krusei* and *C. inconspicua* isolates using species-specific probes by dot hybridisation.

9.3 Discussion

A further refinement from PCR-based identification or Southern blotting is the use of dot hybridisation. In this procedure, DNA is fixed on a membrane and hybridised with species-specific probes. Therefore dot hybridisation was employed as a rapid, reliable, and sensitive molecular approach for the identification of *C. krusei* and *C. inconspicua*.

As several molecular approaches using genus-specific or species-specific oligonucleotides derived from tandemly repeated areas, i.e. ribosomal RNA for the detection of some clinically important pathogenic yeasts, have been described (Lischewski *et al.* 1996; Montone *et al.* 1994; Botelho *et al.* 1994), the ITS (Internal Transcribed Spacer) regions have been chosen as sites for useful probe preparations. Botelho *et al.* (1994), for instance, used oligonucleotide probes derived from the ITS 1 and 2 fragments of ribosomal DNA of the *Candida* species and demonstrated their clinical usefulness as a diagnostic tool for the detection of *C. albicans*. They also tested probe specificity against other yeast species.

To confirm the faithfulness of the probes designed, dot hybridisation against 10 *C. krusei* and 10 *C. inconspicua* isolates was performed. The dot blotting technique has been widely used for the detection of isolates using species-specific probes labelled radioisotope or non radioisotope materials. For the genomic DNA analysis in yeasts, several areas including the large ribosomal DNA, small subunit rRNA(ssu), the V3 region in large subunit rRNA and other areas have been chosen for the preparation of species-specific probes. Previously, Niesters *et al.* (1993) used PCR-based methods using primers derived from the ssu rRNA sequence. They also used the dot blotting technique with species-specific probes against *Candida* species.

In this study, the identification of all isolates of *C. krusei* and *C. inconspicua* with just 1 h hybridisation was successful, suggesting that the sequence of probes corresponding to the target isolates' DNA enhances successful identification. In particular, in clinical laboratories, time is of the essence. All isolates of *C. krusei* genomic DNA could be detected in less than a total of 5 h (2 h for prehybridisation, 1 h hybridisation, 1 h for washing and 30 min for detection), and this results implies that our probe, CKP, is correctly designed for the *C. krusei* species. Carlotti *et al.* (1996) used CkF1,2 probes for identifying this species and carried out the dot blotting test in overnight hybridisation. In contrast, our approach showed that 1 h hybridisation with CKP was sufficient to identify these species without conducting 24 h hybridisation. Previously, the difficulty in identifying *C. krusei* using dot hybridisation was reported (Fujita *et al.* 1995; Carlotti *et al.* 1996), and 24 h hybridisation was required for the identification of *C. krusei* previously. Identical conditions as for *C. krusei* were employed to detect *C. inconspicua*, showing that all isolates (10 isolates from bioMérieux) were detectable in 1 h hybridisation although three isolates showed a slightly weak signal compared with the others. Perhaps the conditions for *C. inconspicua* identification should be further refined.

Before conducting dot hybridisation, overall two factors were considered, i.e. hybridisation and washing conditions, in order to improve the efficacy of the identification of these species. Dot hybridisation was performed first at 50°C under low stringent washing conditions and the hybridisation and washing conditions were then gradually increased to establish optimum conditions to identify these species. Using hybridisation at 50°C under low stringency washing conditions, several cross-hybridisation were recorded. However, after hybridisation at 52°C followed by high stringency washing

conditions, all 10 *C. krusei* isolates were clearly detectable, suggesting that this condition can be applicable as a diagnostic tool for the detection of these species.

Another merit of this approach is that the detection limit is superior to that in other similar approaches employed. Sensitivity may also be important in the clinical setting. Botelho *et al.* (1994) reported that up to 1 µg of *C. albicans* target DNA was detectable using probes, ANAB2 and ANAB3, derived from the ITS region. Likewise, Carlotti *et al.* (1996) used the CkF1,2 DNA probe and the lowest range of *C. krusei* DNA was recorded as 60 ng in dot hybridisation. In contrast, the CKP probe used in this study enabled the detection of amounts of DNA as small as 0.43 ng, and a strong signal was recorded at about 4 ng, indicating a high degree of sensitivity. However, it is possible that the sensitivity and specificity of this technique can be inconsistent between experiments and influenced by several factors, i.e. oligonucleotide labelling methods, length of probe, the quality of genomic DNA, washing and hybridisation temperatures, etc. In particular, different oligonucleotide labelling methods with digoxigenin, i.e. end labelling, end tailing, etc. can generate different sensitivity results so it is thought that dot hybridisation alone can not be used for the identification of target species. Although CKP and CIP probes were thought to be species-specific in this study, a variety of isolates from different sources, particularly from patients with candidiasis should be tested to establish whether these probes can detect target species. Therefore, dot hybridisation should be used in conjunction with other techniques to provide more reliable results.

In conclusion, several molecular methods such as PCR using ITS 1 primer and CKP or CIP combination, *HhaI* digestion from amplified products using ITS 1 and 4, or dot

hybridisation using CKP or CIP probes were used in this study to identify *C. krusei* and *C. inconspicua* in order to suggest more reliable diagnostic technique. It is thought that dot hybridisation using CKP and CIP proved to be a rapid diagnostic technique. In addition, it was also found that the lowest quantity of target DNA might be detectable if this method is employed, so that it can possibly be applicable in the clinical laboratory. Finally, because digoxigenin labelled probes can not only eliminate the handling of hazardous materials, but are also reusable several times, they also satisfy both safety and economy requirements.

In order to confirm target *Candida* species, other identification techniques such as biochemical and /or colonial morphologies, PCR-RFLP, etc., should be employed in conjunction with dot hybridisation. Probably, more isolates from a variety of sources should be required to test the utility of these probes in the future.

Conclusions

Conclusions

- 1) The amplified products of *C. inconspicua*, *C. krusei* and *C. norvegensis* using ITS 1 and 4 universal primers were 455, 509 and 490 bp, respectively. Therefore, additional techniques are required as a confirmatory step for these species identification. In contrast, *HhaI* digestion of amplified fragments from ITS 1 and 4 universal primers showed species-specific band patterns for the differentiation of *C. krusei*, *C. inconspicua* and *C. norvegensis*.
- 2) Direct PCR with ITS primers followed by restriction digestion with *HhaI* without preparation of genomic DNA proved to be a possible approach for the presumptive identification of these *Candida* species from single colonies on agar plates.
- 3) *C. krusei* and *C. inconspicua* are closely related to each other based on the highly conserved 5.8S rDNA with only 2 bp differences (99% identity). These species also have similar sequences in the ITS 1 and 2 regions (81 and 62% identity, respectively).
- 4) RAPD using primer R108 distinguished between these three species, and genetic differences at the species level were recorded.
- 5) Specially designed oligonucleotides, CIP and CKP, showed good reliability in the identification of *C. inconspicua* and *C. krusei*. In particular, CKP showed enhanced sensitivity compared with a previously published probe, CK which were designed from the same ITS 2 region. These oligos could be used either in a PCR as primers or as hybridisation probes to detect *C. krusei* and *C. inconspicua*. In addition, these oligos

should provide a rapid identification approach for heterogeneous collections of isolates or colonies.

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Appendix

Publication

Species differentiation by internally transcribed spacer PCR and *Hha*I digestion of fluconazole-resistant *Candida krusei*, *Candida inconspicua*, and *Candida norvegensis* strains

Journal of Clinical Microbiology

Species Differentiation by Internally Transcribed Spacer PCR and *Hha*I Digestion of Fluconazole-Resistant *Candida krusei*, *Candida inconspicua*, and *Candida norvegensis* Strains

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PCR amplification of the regions containing the internally transcribed spacers and 5.8S rRNA gene of *Candida krusei*, *C. inconspicua*, and *C. norvegensis* yielded fragments of 510, 460, and 500 bp, respectively. *Hha*I digestion of these fragments yielded species-specific bands. Random amplification of polymorphic DNA with primer R108 showed interspecific discriminatory band patterns. Susceptibilities to fluconazole and amphotericin B were determined.

With the rise in the number of *Candida* infections (14) and the proportion of non-*albicans* species implicated (6, 8, 9, 10), accurate identification of *Candida* to species level is increasingly important. *C. krusei*, *C. inconspicua*, and *C. norvegensis* have recently emerged as fluconazole-resistant pathogens (2, 3, 8, 17). Standard methods for the differentiation of *Candida* species are based on assimilation and fermentation reactions together with morphology (4). However, assimilation tests based on API 32C strips (bioMérieux, Marcy l'Etoile, France) can occasionally fail to distinguish *C. inconspicua* from *C. norvegensis* or *C. krusei*.

Molecular techniques developed for the identification of yeast species have included species-specific oligonucleotides for use in hybridizations and PCR of regions of the rRNA gene (rDNA) repeat (5, 7, 15, 18). Species can be distinguished simply on the basis of the size of the amplified fragment (22) or after digestion with a set of restriction enzymes, which results in distinctive species-specific patterns (11, 13, 22). Here we present an analysis based on PCR and a single restriction enzyme digestion to distinguish three *Candida* species that may be resistant to fluconazole. In addition, random amplification of polymorphic DNA (RAPD) was used to confirm that the three species were genetically distinct. These data were used to confirm the identifications based on classical taxonomy of 32 culture collection strains.

A total of 30 strains of *Candida* were donated by bioMérieux. Two strains, including the type strain of *C. krusei*, were from the American Type Culture Collection, and one strain, SM 038, was a clinical isolate from an AIDS patient in the Monsall Unit (Table 1). All strains were identified with the ID 32C identification strip (bioMérieux). An esculin test is included in the ID 32C strip, and results were read visually as positive or negative. Rice-Agar-Tween (RAT) medium (bioMérieux) was used to determine whether the isolates had the ability to produce pseudophages. The strain of *C. glabrata* (NCPF 3309) was from the National Collection of Pathogenic Fungi (Bristol, United Kingdom) and was used only in the

internally transcribed spacer (ITS) PCR and RAPD experiments.

Susceptibility to amphotericin B was determined by a microbroth method with antibiotic medium 3 (Difco, Surrey, United Kingdom) with 2% glucose at pH 7.0 (16). Concentrations of the drug ranged from 0.031 to 32 µg/ml, and a final inoculum of 10⁴ CFU/ml was used. The MIC was taken as the lowest drug concentration to inhibit ≥80% of growth, although clear endpoints were always obtained. MICs of ≥0.25 µg/ml were (provisionally) regarded as intermediate or resistant. MICs of fluconazole were also determined by a microbroth method with high-resolution medium (Unipath, Basingstoke, United Kingdom). Drug concentrations ranged from 0.0977 to 100 µg/ml, with a final inoculum of 10⁴ CFU/ml. Inhibition of growth by ≥50% compared with growth of the drug-free control was used to determine the MICs (20). MICs of ≥25 µg/ml were regarded as resistant, and those of 6.25 to 12.5 µg/ml were regarded as of intermediate susceptibility based on clinical correlation studies (2).

Cultures were grown in Sabouraud dextrose broth for 16 h at 37°C and pelleted. The cells were washed with 1 ml of 1 M sorbitol and were resuspended in 1 ml of buffer (1.2 M sorbitol, 10 mM Tris-Cl, 50 mM EDTA [pH 7.5]) with 0.6 mg of lyticase (Sigma, Dorset, United Kingdom). After incubation for 75 min at 37°C, the spheroplasts were resuspended in 500 µl of buffer (0.15 M NaCl, 0.1 M EDTA) with 0.25 mg of proteinase K (Boehringer Mannheim, East Sussex, United Kingdom) and 1% (wt/vol) sodium dodecyl sulfate. After incubation for 45 min at 55°C, two phenol-chloroform-isoamyl alcohol (25:24:1) extractions and one chloroform extraction were performed. The DNA was precipitated with 1 volume of isopropanol before dissolving in 50 µl of Tris-EDTA buffer.

The ITS primers used were ITS 1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS 4 (5' TCC TCC GCT TAT TGA TAT GC 3') (21). The primer R108 (5' GTA TTG CCC T 3') was used for RAPD (1). The reactions were performed in a volume of 50 µl under mineral oil. The reaction mixtures with primers ITS 1 and 4 consisted of 0.25 µM each primer, buffer IV, 1.5 mM MgCl₂, 200 µM (each) the four deoxynucleoside triphosphates, 100 ng of genomic DNA, and 2.5 U of *Taq* DNA polymerase (Advanced Biotechnologies Ltd., Surrey, United Kingdom). PCRs were performed as follows: 94°C for 1 min,

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TABLE 1. *Candida* strains tested in this study

Species	Lane no. in Fig. 2-5	Strain no.	API 32C Code	Very good or good id. ^a	ESC ^b	RAP ^c	MIC (µg/ml)	
							Amphotericin B	Fluconazole
<i>C. inconspicua</i>	1	85 07 247	0200010005	—	—	—	0.12	3.12
	2	85 09 110	0200010005	—	—	—	0.062	6.25
	3	85 09 109	0200010005	—	—	—	0.062	12.5
	4	85 03 199	0200010005	—	—	—	0.12	12.5
	5	85 09 111	0200010005	—	—	—	0.12	3.12
	6	85 10 102	0200010005	—	—	—	0.062	50
	7	93 12 113	0200010005	—	—	—	0.031	6.25
	8	85 02 122	0200010005	—	—	—	0.062	6.25
	9	85 02 119	0200010005	—	—	—	0.062	3.12
	10	85 03 197	0200010005	—	—	—	≤0.031	3.12
	11	SM 038	0200010005	—	—	—	≤0.031	12.5
<i>C. krusei</i>	1	89 08 008	0300000001	—	—	—	0.25	100
	2	88 08 110	0300010001	+	—	—	0.50	50
	3	91 05 030	0300010001	—	—	+	0.25	100
	4	89 08 006	0300000001	—	—	+	0.25	100
	5	85 10 089	0300010401	+	—	—	0.25	50
	6	91 12 022	0300010001	+	—	—	0.25	100
	7	91 12 020	0300010001	+	—	+	0.25	50
	8	91 12 021	0300010001	—	—	—	0.25	25
	9	89 08 007	0200000001	—	—	—	0.25	50
	10	90 03 063	0200010001	—	—	+	0.12	100
	12	ATCC 6258	0300010401	—	—	—	0.12	50
<i>C. norvegensis</i>	1	88 08 100	0200010005	—	+	—	0.062	12.5
	2	89 04 072	0200010005	—	+	—	0.12	12.5
	3	85 03 050	0200010005	—	—	—	≤0.031	12.5
	4	95 02 007	0200010005	—	+	—	0.062	6.25
	5	85 03 051	0200010005	—	—	—	0.12	12.5
	6	89 03 079	0200010005	—	—	—	≤0.031	6.25
	7	95 02 006	0200010005	—	—	—	0.062	6.25
	8	85 04 237	0200010005	—	—	—	0.062	12.5
	9	85 03 195	0200010005	—	—	—	≤0.031	25
	10	88 07 095	0200010005	—	+	—	0.12	12.5
	13	ATCC 20686	0200010005	—	—	—	0.062	12.5

^a Quality of identification (id.) proposed by bioMérieux with the API system: +, very good or good id.; —, less-than-good id. to species level.^b ESC, esculin test result.^c RAT medium was used for the determination of pseudohypha production.

56°C for 1 min, and 72°C for 1 min for 35 cycles. The reactions with primer R108 were as described above except that 0.5 µM primer and 2.5 mM MgCl₂ were used and the temperature profile was 1 min at 94°C, 1 min at 36°C, and 1 min at 72°C for 30 cycles. The PCR products were analyzed in 1.8% standard agarose gels. The ITS PCR products were extracted with chloroform followed by ethanol precipitation. The DNA was resuspended in 50 µl of Tris-EDTA. Ten microliters was used in 20-µl digests containing 2 µl of bovine serum albumin (1 mg/ml), 1× buffer 4 (New England Biolabs, Hertfordshire, United Kingdom), and 20 U of *Hha*I. After incubation at 37°C for 3 h, the digested DNA was analyzed in 3% 3:1 NuSieve agarose (FMC BioProducts, Kent, United Kingdom) gels in 1× Tris phosphate-EDTA buffer.

Thirty-three strains of three *Candida* species were identified by standard methods (Table 1). Only 7 of the 11 *C. krusei* strains gave better than good identifications on the basis of the API 32C codes. The *C. inconspicua* and *C. norvegensis* strains had identical codings but could be differentiated on the basis of the esculin test (integral to the API 32C test kit), with which the *C. norvegensis* strains gave a positive result. In addition, apart from *C. norvegensis* ATCC 20686, only the *C. krusei* strains were able to produce pseudohyphae. All the *C. krusei* strains were resistant to fluconazole (MICs, 25 to 100 µg/ml).

and in addition, they all had relatively high MICs of amphotericin B, with one isolate (MIC, 0.50 µg/ml) in the intermediate to resistant range (16). The *C. inconspicua* and *C. norvegensis* strains were in the middle range of fluconazole susceptibility, with many intermediate MICs and one resistant strain in each group (3.12 to 50 µg/ml). All these strains were susceptible to amphotericin B.

The region of the rDNA repeat covering the internally transcribed spacers and the 5.8S rDNA was amplified from each strain with universal primers. The three species gave specifically sized fragments of similar sizes which were not readily resolvable on agarose gels (Fig. 1). The amplified fragments were of the following sizes: *C. inconspicua*, 460 bp; *C. krusei*, 510 bp; and *C. norvegensis*, 500 bp. After digestion of the amplified fragments with the enzyme *Hha*I and electrophoresis in high-resolution agarose gels, all of the 30 bioMérieux culture collection strains gave the appropriate distinctive pattern for each of the three species (Fig. 2). It was possible to amplify this region directly from a colony and restrict it with *Hha*I (data not shown) and so to speed up the process by eliminating the need to extract DNA.

All the strains of each species were shown to be genetically similar by RAPD with a single primer (Fig. 3 to 5). Strains of a species gave similar patterns with several bands in common.



FIG. 1. PCR products with primers ITS 1 and 4. Lanes 1 to 10, *C. inconspicua* strains from bioMérieux; lane 11, *C. inconspicua* SM 038; lane 12, *C. krusei* ATCC 6258; lane 13, *C. norvegicus* ATCC 20686; lane 14, *C. glabrata* NCPE 3309. The molecular size markers (lanes M) are fragments increasing in size by 100 bp and ranging from 100 bp to 1 kb.

The banding pattern of each of the three species is distinguishable by visual comparison. For instance, all the *C. krusei* strains have a strong band at 900 bp and all the *C. norvegicus* strains have a distinctive band at 800 bp and a doublet between the 2,560- and 2,838-bp size markers.

Conventional identification of *C. krusei* relies on both the production of pseudohyphae on suitable media and various biochemical reactions (API 32C codes) which together comprise the species identification but with differing degrees of confidence (Table 1). Some isolates of *C. krusei* are especially problematic (12). Isolates of *C. inconspicua* and *C. norvegicus* often generate identical API 32C codes. They are usually separable by the esculin test, an integral part of the API 32C kit.

Several studies using a combination of PCR of regions of the rDNA repeat followed by restriction digestion as a means of



FIG. 3. RAPD profiles of the *C. inconspicua* strains with primer R108. The lane order of the *C. inconspicua* strains is given in Table 1, and the molecular size markers (lanes M) are digested with *Pst*I. Lane 11, *C. inconspicua* SM 038; lane 12, *C. krusei* ATCC 6258; lane 13, *C. norvegicus* ATCC 20686; lane 14, *C. glabrata* NCPE 3309.

species-specific identification have been published. Niesters et al. (13) amplified the small-subunit rDNA and, using a combination of five enzymes, could distinguish four *Candida* species. Maiwald et al. (11) used a similar procedure to identify presumptively eight *Candida* species with PCR products of the small-subunit rDNA and digestion with six restriction enzymes. Williams et al. (22) used PCR to amplify the internally transcribed spacer region of the rDNA repeat and digested the amplified fragments with three restriction enzymes. They concluded that eight *Candida* species could be identified on the basis of size and sequence variation. We have applied this approach to the species *C. krusei*, *C. inconspicua*, and *C. norvegicus* and have been able to differentiate them using a single restriction enzyme digestion of the amplified product. The *Hha*I digestion profiles of the three species are readily distinguishable on high-resolution agarose gels. The use of PCR-restriction fragment length polymorphism has advantages over other genetically based tests, such as restriction fragment length polymorphism with genomic DNA and electrophoretic karyotyping (12), in being simple and quick, especially if the PCR is done directly from a colony.

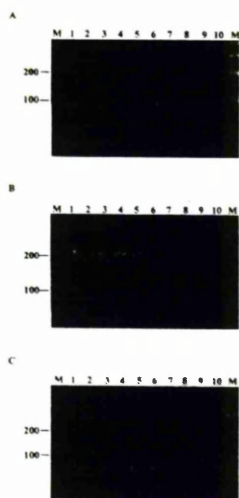


FIG. 2. PCR products of the bioMérieux strains digested with *Hha*I. The lane order of the strains is given in Table 1, and the molecular size markers (lanes M) are a 100-bp ladder. (A) *C. inconspicua*. The sizes of the *Hha*I-digested fragments as determined by sequencing are 82, 63, 69, 82, 87, and 102 bp. (B) *C. krusei*. The sizes of the *Hha*I-digested fragments as determined by sequencing are 82, 69, 178, and 203 bp. (C) *C. norvegicus*. The sizes of the *Hha*I-digested fragments as determined by image analysis are 80, 70 (two times), 90 (two times), and 110 bp.

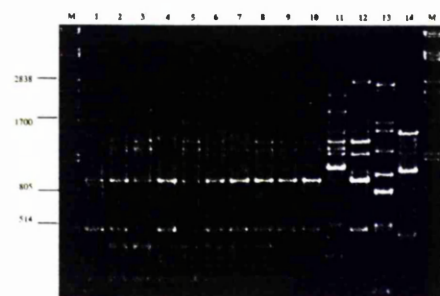


FIG. 4. RAPD profiles of the *C. krusei* strains with primer R108. The lane order of the *C. krusei* strains is given in Table 1, and the molecular size markers (lanes M) are digested with *Pst*I. Lane 11, *C. inconspicua* SM 038; lane 12, *C. krusei* ATCC 6258; lane 13, *C. norvegicus* ATCC 20686; lane 14, *C. glabrata* NCPE 3309.

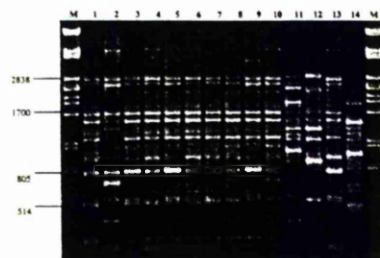


FIG. 5. RAPD profiles of the *C. norvegicus* strains with primer R108. The lane order of the *C. norvegicus* strains is given in Table 1, and the molecular size markers (lanes M) are λ digested with *Pst*I. Lane 11, *C. inconspicua* SM 038; lane 12, *C. krusei* ATCC 6258; lane 13, *C. norvegicus* ATCC 20686; lane 14, *C. glabrata* NCPF 3309.

Although RAPD has mainly been used for the typing of isolates of the same species, it has been used to investigate genetic differences at the species level. For instance, in the work of Sullivan et al. (19), the *C. dubliniensis* isolates gave RAPD patterns noticeably different from those of the *C. albicans* isolates. Likewise, in the results of our study, the RAPD patterns distinguish the three species with potential species-specific bands present in all the strains of a single species.

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