

REVIEW ARTICLE

Molecular genetic approaches to identification, epidemiology and taxonomy of non-*albicans* *Candida* species

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The reported incidence of fungal infections associated with non-*albicans* species from the *Candida* genus is increasing. Most of these infections occur in immunocompromised patients, particularly those infected with HIV. The role of molecular genetic techniques alongside the existing techniques for the identification and typing of these organisms is discussed. Species-specific genomic DNA fragments cloned from *C. tropicalis* and *C. krusei* have been developed for identification and strain typing. Analysis of tRNA profiles has been shown to be effective for the identification of *C. glabrata*, *C. guilliermondii*, *C. parapsilosis* and *C. tropicalis*. A PCR method employing primers complimentary to large ribosomal subunit genes and the lanosterol- α -demethylase gene has been applied for several species, including *C. glabrata*, *C. krusei* and *C. tropicalis*. Strain typing by comparison of genomic DNA fingerprints has been demonstrated for *C. tropicalis* and *C. krusei* following hybridisation analysis with species-specific probes. Synthetic oligonucleotide probes—which do not have to be species-specific and which can detect minor polymorphisms—have also been used for strain typing of isolates of several non-*albicans* species. Random amplification of polymorphic DNA (RAPD) has also been used for analysis of *C. glabrata*, *C. lusitaniae* and *C. tropicalis* isolates. The potential for the application of these and other techniques to *Candida* spp. taxonomy—and the example of a recently discovered novel species, *C. dubliniensis*—is discussed.

Introduction

The increased interest of the scientific and medical communities in *Candida* spp. reflects directly a steady rise in the number of reports on the incidence of *Candida* infections in man over the past decade. This is related largely to a dramatic increase in the number of individuals with deficient cellular immunity, in particular those infected with HIV and individuals receiving immunosuppressive treatments, both in organ transplantation and in anti-cancer therapy. *C. albicans* is the most pathogenic member of the genus *Candida* and the most frequent cause of candida infection [1]. However, in recent years, species of *Candida* other than *C. albicans* have been implicated more frequently in human disease, although many reports describing cases of candida infection by non-*albicans* *Candida* spp. provide insufficient data to establish a causal relationship between the species isolated and the infection.

Whether the widespread use of antifungal drugs has contributed to an increased incidence of non-*albicans* *Candida* organisms being isolated from HIV-positive and AIDS patients remains an open question. In the few reports that have commented on this possibility, some [2–4], but not all [5, 6], consider that antifungal drug therapy makes a significant contribution. However, only a few studies have used molecular techniques to identify or type the organisms involved, and given that there are many other clinical and methodological differences, it is beyond the scope of this review to carry out a detailed comparison of these studies. However, the data from our collection of isolates suggest that, with long-term treatment, changes in the prevalence of *C. albicans* strains and other *Candida* spp. occur more frequently, and that many of these organisms are less susceptible to azole antifungal agents [3, 7]. Whether this is of relevance to the patient's clinical condition has yet to be answered.

need for detailed epidemiological investigations of the organisms responsible [7, 8]. The purpose of this review is to describe the development and application of molecular techniques for the analysis of non-*albicans* *Candida* spp. involved in clinical disease. After a brief description of the clinically relevant species, the processes of culture, species identification and strain typing are discussed, followed by consideration of the taxonomy of *Candida* spp. since, at present, there are a number of confusing anomalies that molecular techniques could resolve.

Clinically important non-*albicans* *Candida* species

According to Barnett *et al.* [9], there are 166 species in the genus *Candida* (Berkhout), but only a small proportion of these is found in man, and of these only a handful pose clinical problems. Several non-*albicans* *Candida* spp. are known to be pathogenic and are responsible for disease in man, while several others cause disease occasionally (Table 1). These organisms are responsible most commonly for chronic superficial infections of skin, nails and mucosal surfaces. Less frequently, they can disseminate and cause life-threatening systemic (or deep-seated) disease, and it is these infections that present the greatest challenge to clinicians. Of the superficial forms of infection, oral candidosis is the most important and frequent form encountered in HIV-infected individuals [7, 10, 20, 21], whereas vulvovaginal candidosis is often encountered in otherwise apparently healthy women [1]. Systemic candida infections, although relatively rare, show the versatility of the genus in the range of internal organs that can be infected, and there has been a dramatic increase in the incidence of disseminated candidosis in

the last two decades [11]. However, the clinical manifestations of diseases caused by different pathogenic *Candida* spp. are usually indistinguishable. A complex interplay between multiple strains and species can occur in both superficial and systemic infections, thus making it difficult to determine the contribution, if any, of individual organisms to infection [3, 4, 12, 13].

Identification of non-*albicans* *Candida* species from clinical specimens

Culture and identification

It is important to be able to distinguish candida infections from those caused by other yeasts and fungi. Furthermore, since several non-*albicans* *Candida* spp. are inherently less susceptible to commonly used antifungal drugs [6, 22], their rapid identification is essential if the most appropriate therapeutic approach is to be used. The selection of media for the growth and isolation of *Candida* spp. from clinical specimens is of critical importance [7]. Ideally the medium used should sustain the growth of all the *Candida* spp. found commonly in clinical specimens and should prohibit the growth of bacteria. The medium should not interfere with the viability of the organisms and should facilitate the identification of clinical specimens harbouring mixtures of yeast species. Traditionally, various preparations of Sabouraud-dextrose agar, often supplemented with antibiotics to further suppress bacterial growth, are used for this purpose [7, 23]. However, this medium is not differential and is not useful for distinguishing between different *Candida* spp. present in the same clinical specimen [24]. Four different media have been described which distinguish *C. albicans* from other yeast species on the basis of colony colour or morphology, or both, including Pagano-Levin agar [25] (a medium that contains triphenyltetrazolium chloride incorporated into Sabouraud agar), phosphomolybdate agar [26, 27], Nickerson's medium [28] and CHROMagar Candida [29]. All these media are useful for identifying clinical specimens containing mixtures of *C. albicans* and other yeast species [24, 29]. CHROMagar Candida—a newly developed commercially available medium—has an additional advantage in that it allows the presumptive identification of *C. albicans*, *C. tropicalis* and *C. krusei* [29]. We use potato dextrose agar as the standard culture medium for the isolation of *Candida* spp. from clinical specimens and have found that it is particularly useful for distinguishing between colonies of different yeast species from the same clinical specimen [7].

After primary isolation, the number and relative abundance of distinct yeast colony types, including colonies with distinct morphologies or colours, depending on the media employed, should be recorded. Representatives of each colony type should then be purified and the species to which they belong identified [7]. *C. albicans* isolates can be differen-

Table 1. Non-*albicans* *Candida* spp. responsible for opportunist infections

Species*	References
Common opportunist pathogens	
<i>C. dubliniensis</i> †	
<i>C. guilliermondii</i>	
<i>C. kefyr</i>	1, 2, 4, 10, 11, 12, 13,
<i>C. krusei</i>	14, 15, 16, 17, 18
<i>C. lusitanae</i>	
<i>C. parapsilosis</i>	
<i>C. tropicalis</i>	
Rare opportunist pathogens	
<i>C. famata</i>	
<i>C. holmii</i>	
<i>C. inconspicua</i>	
<i>C. norvegensis</i>	1, 4, 16, 19
<i>C. rugosa</i>	
<i>C. utilis</i>	
<i>C. zeylanoides</i>	

*Listed in alphabetical order.

†*C. dubliniensis* is a novel species of *Candida* that is present in around one-third of oral isolates from HIV-infected patients in our population and has been isolated from other subjects and in other countries; see text for details.

tiated from other yeast isolates by the ability of the former to form germ tubes and chlamydo-spores [7].

Isolates of individual species of the genus *Candida* are identified classically on the basis of physiological and biochemical reactions and a limited number of morphological features [1]. Commercially available yeast identification kits record the ability of isolates to assimilate a variety of compounds that can be used as sole sources of carbon or nitrogen [1]. However, in practice there are a number of problems associated with the interpretation of these results [30, 31]. Particular assimilation test results may not be consistent because the same isolate could yield a positive test result on one occasion and a negative test result on another. Phenotypic switching or the existence of a mixture of metabolic variants are possible reasons for this [7, 32]. Furthermore, genetically diverse species of yeast can yield similar substrate utilisation profiles, resulting in poor discrimination between unrelated yeast species if the profile is the sole means of identification. The pattern of results from such a procedure provides a list of species in order of their probability. When these results are taken together with morphological and serological test results, the likelihood of an accurate identification is increased greatly.

An excellent example of the difficulties that can be encountered in identification was found while investigating populations of oral *Candida* isolates from HIV-infected individuals with the API ID 32C yeast identification kit [33]. According to the API APILAB database, the ID 32C profiles of these isolates corresponded to poor identification, in decreasing order of probability, of *C. sake*, *C. albicans* 2 (i.e., *C. stellatoidea*) and *C. albicans* 1. These organisms formed germ tubes and chlamydo-spores, and were therefore thought initially to be unusual isolates of *C. albicans* or *C. stellatoidea* [7]. Preliminary phenotypic and genetic characterisation of several of these isolates in Dublin suggested that they could also constitute a novel species of *Candida* [33]. Subsequent detailed analysis demonstrated that this was likely to be the case (see below).

Molecular identification of Candida spp.: use of molecular techniques

It takes a minimum of 24–48 h to culture and identify a species of *Candida* from a clinical specimen by conventional mycological procedures [7] and, even then, there may be some difficulty in identifying some isolates definitively (see above). Therefore, there is a compelling need for rapid, sensitive and specific tests to aid in the diagnosis of candida infections, especially disseminated infections. Several molecular procedures have been applied to a small number of *Candida* spp., and the majority of these have been performed *in vitro* with DNA extracted from cultured organisms, with the

ultimate intention of further development for application directly to clinical specimens, thus obviating the necessity for culture [34–45].

One of the first approaches involved restriction fragment length polymorphism (RFLP) analysis of fragments obtained by restriction endonuclease digestion of genomic DNA from *Candida* spp. and subsequent separation of the fragments by agarose gel electrophoresis [7, 34, 46, 47]. However, RFLP analysis of total genomic DNA from both bacteria and fungi can result in the generation of complex patterns of fragments that are ambiguous and difficult to interpret objectively [7]. RFLP patterns obtained from *Candida* spp. with the restriction endonuclease *EcoR1* (used in the majority of studies) contain only a limited number of bands that are suitable for analysis [7]. The low precision of RFLP analysis, coupled with concern over its reliability, means that it is not suitable for species identification [7].

A second molecular approach to the identification of *Candida* spp. involves Southern hybridisation analysis. Several *C. albicans*-specific DNA sequences and genes have been used as molecular probes to identify this organism *in vitro* [7, 48, 49], and similar species-specific DNA probes have been developed for the molecular identification of *C. tropicalis* and *C. krusei* [13, 42, 50]. Unfortunately, hybridisation analysis involves several time-consuming steps and can take ≥ 24 h to yield a result. Furthermore, detection of non-amplified *C. albicans* target DNA by Southern hybridisation has a detection limit of (5×10^2) – (1×10^5) blastoconidia [51, 52].

A third molecular technique involves tRNA profile analysis [53]. This technique is based on the electrophoretic pattern of total cellular tRNA samples from cultured organisms. The profiles generated are analysed by high-resolution semi-denaturing urea-PAGE, followed by methylene blue staining. It has been demonstrated that reproducible and strain-independent species-specific tRNA profiles can be obtained for the species *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii* and *C. glabrata*. Again, this technique takes at least one working day to yield results after the test organisms have been cultured in the laboratory.

The approach that offers the greatest potential in precision, sensitivity and speed involves the application of PCR technology [54] to amplify target DNA sequences by thermostable DNA polymerase-mediated extension of specific oligonucleotide primers. Theoretically, it should be possible to design PCR tests that are capable of detecting any of the clinically important pathogenic species of *Candida* in a few hours by employing primers complementary to DNA sequences that are common but unique to these organisms as a group. Subsequently, it should be possible to identify

individual species with species-specific primers. PCR has been used in several recent studies to facilitate the detection of particular *Candida* spp. *in vitro* and in clinical specimens with primers complimentary to various genes and multicopy DNA elements, including the *C. albicans* heat shock protein 90 gene (HSP 90) [39], the cytochrome P-450 lanosterol- α -demethylase gene [41], 5S rDNA sequences [44] and the *C. albicans* EO3 element [38]. Primers generated from the HSP 90, 5S rDNA and EO3 sequences have been used only in the detection of *C. albicans*. Other studies, employing primers complimentary to large ribosomal subunit sequences, have demonstrated that PCR can be applied successfully to the rapid *in-vitro* identification (several hours) of the non-*albicans* *Candida* spp. encountered most frequently, including *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. parapsilosis* (K. A. Haynes and T. J. Westerneng, personal communication). Species-specific primers have also been developed to detect purified DNA from *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei*, based on a highly variable region of the lanosterol- α -demethylase gene from these organisms [41]. These primers were found to be specific for their target species, with the exception of the *C. glabrata* primers which also gave a weak positive reaction with *C. parapsilosis* DNA. Primers complimentary to sequences from a conserved region of the lanosterol- α -demethylase gene yielded positive PCR reactions with purified DNA from various *Candida* spp. and also allowed the detection of purified DNA from *Cryptococcus neoformans* and *Trichosporon beigelii* [41]. However, when the primers for *C. albicans* and *C. glabrata* were used to detect cells in clinical samples, it was found that PCR was less sensitive than culture-based techniques. Other problems associated with the technique include false-negative results with particular samples, such as thick pus, where DNA extraction can be difficult, and false-positive results caused by contamination of specimens with the normal flora of patients [41, 44, 45], thereby emphasising that PCR technology is limited currently to the detection of *Candida* organisms in samples from normally sterile sites such as blood and peritoneal fluid. Despite these limitations, the application of PCR technology to the detection and identification of *Candida* spp. in clinical material is at an early stage of development, and the potential for the technique is still tremendous given its rapidity. Further research in this area is required, including the development of quantitative PCR techniques for sensitive, efficient and specific tests for routine clinical application.

Another potential method for species identification involves the synthesis of oligonucleotide probes complimentary to portions of the internal transcribed spacer regions of rDNA. This has been shown to be effective for *C. albicans* identification and would be equally applicable to non-*albicans* spp. [55]. A disadvantage of the technique for routine use is that

the sequence of the internal spacer regions must be determined before a species-specific probe can be developed.

***Candida* strain typing**

An ability to distinguish between separate isolates of the same *Candida* spp. is essential for epidemiological investigations. Accurate typing of *Candida* isolates can provide valuable information regarding the source of a particular infection, it can facilitate the detection of multiple strains in a clinical specimen or the detection of the same or different strains from recurrent episodes of infection in individual patients. Furthermore, strain typing can provide valuable population information about isolates of the same *Candida* spp. from separate individuals and patient groups. Over the last 20 years, a wide variety of typing methods has been applied to *C. albicans* isolates and, to a lesser extent, isolates of non-*albicans* *Candida* spp. [7]. Most of these typing methods are based on phenotypic characters, many of which are not very discriminatory or exhibit poor reproducibility, or both. The ability of many *Candida* spp. to exhibit phenotypic variation can limit the usefulness of these techniques [7, 56]. In an attempt to overcome these shortcomings, several of the molecular approaches used for species identification have also been applied to non-*albicans* *Candida* spp.

Molecular approaches for typing non-albicans Candida species

The same limitations that restrict the usefulness of RFLP analysis for identification of *Candida* spp. also pertain to strain discrimination by this technique. However, a number of studies have shown that RFLP patterns generated by the restriction endonuclease *Hinf*I can contain several well-resolved fragments of high mol. wt that can be used effectively for strain discrimination [34, 42, 57]. A variant of this technique is the digestion of intact chromosomal DNA by restriction endonucleases that cleave DNA infrequently, thereby giving rise to large DNA fragments that can be separated by pulsed-field gel electrophoresis and subjected to RFLP analysis. Studies with isolates of *C. tropicalis*, *C. parapsilosis* and *C. lusitaniae* have shown that this improves the efficiency of discrimination between isolates [58–60]. This technique is not suitable for routine application to clinical specimens because of its complexity.

Another molecular approach that has been used extensively to type isolates of *C. albicans* [7, 48, 61, 62] and, to a lesser degree, isolates of individual non-*albicans* *Candida* spp., involves fingerprint analysis with species-specific DNA probes. Restriction endonuclease-digested total cellular DNA is separated by electrophoresis and transferred to a membrane filter support, followed by Southern

hybridisation with a labelled species-specific probe homologous to repetitive DNA sequences which are non-contiguous or dispersed throughout the genome. The use of such probes ensures the generation of hybridisation patterns consisting of multiple bands for improved isolate discrimination. Comparison of the patterns obtained with DNA from different isolates can then be used as a basis for differentiating between individual strains. This type of approach has been used successfully to discriminate between isolates of *C. tropicalis* with a cloned *C. tropicalis*-specific repeat sequence probe termed Ct13-8 [13], and between isolates of *C. krusei* with a cloned *C. krusei*-specific probe termed CkF1,2 [42]. The technique has excellent specificity and precision, and the profiles obtained are suitable for computerised storage and analysis. However, a disadvantage of the technique is that a different DNA probe is required for each species of *Candida*.

The use of DNA fingerprinting analysis of genomic DNA for typing isolates of non-*albicans* *Candida* spp. has been extended by the application of synthetic oligonucleotides composed of short repetitive sequence motifs as molecular fingerprinting probes [33]. All eukaryotic genomes contain multiple copies of short tandemly repeated microsatellite sequences which are excellent markers for the detection of genomic variation [7, 63–66]. By using short oligonucleotide probes complementary to these sequences, including (GGAT)₄, (GACA)₄, (GATA)₄, (GTG)₅ and (GT)₈, informative DNA fingerprint profiles consisting of multiple hybridisation bands—ranging in size from c.100 bp to c.20 kb for isolates of *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. dubliniensis* and *Cryptococcus neoformans*—can be obtained [18, 33, 67]. This approach has several major advantages over conventional DNA fingerprint analysis with species-specific probes. Firstly, a set of probes can be used to fingerprint isolates from a range of different *Candida* and other fungal spp. without the necessity for species-specific probes. The optimal combination of oligonucleotide probe and restriction endonuclease for the most informative and discriminatory fingerprints with particular species are best determined empirically [33]. Secondly, several distinct fingerprints of the same isolate can be obtained by sequential hybridisation of the same restriction endonuclease-digested genomic DNA sample, thereby increasing the sensitivity and hence the discriminatory powers of the procedure greatly [33]. Furthermore, the technique is sufficiently sensitive to allow detection of minor polymorphisms among populations of closely related isolates from the same clinical specimen [33].

Karyotype analysis has also been used to type isolates belonging to several non-*albicans* species of *Candida* [7, 15, 18, 36, 68–73]. This procedure compares patterns of chromosomal DNAs obtained following their electrophoretic separation according to size in agarose

gels by pulsed-field electrophoresis. This approach has been used extensively with isolates of *C. albicans*, *C. glabrata* and *C. parapsilosis* and has been shown to be of benefit. However, there are significant disadvantages, in comparison with other procedures, that render it unsuitable for routine analysis of large numbers of clinical isolates. The method requires expensive and highly specialised equipment; individual experiments can involve electrophoretic separation times of several days; and the preparation of yeast chromosomal DNA samples before electrophoresis is both tedious and time consuming.

PCR technology has also been applied to strain discrimination within several species of *Candida* [18, 33, 37, 74]. Potentially, one of the most useful PCR approaches is random amplification of polymorphic DNA (RAPD) analysis [75–78]. RAPD involves PCR amplification of target genomic DNA sequences with one or more short oligonucleotide primers, followed by separation of the amplicons by agarose gel electrophoresis. The primer sequence is selected at random and the most suitable primers for a particular species have to be determined empirically. In a number of recent studies, distinctive PCR profiles consisting of multiple amplified fragments were obtained by RAPD with isolates of *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. lusitanae* and *C. dubliniensis* [18, 37]. Furthermore, in one recent study a physiologically homogeneous collection of *C. parapsilosis* isolates was differentiated into three distinct groups by RAPD analysis [37]. In several cases, RAPD profiles of separate isolates of the same species demonstrated intra-species DNA amplicon size polymorphisms. However, these were more similar to the patterns obtained with other isolates of the same species than RAPD profiles derived from a different species. RAPD has many advantages over other molecular procedures for typing isolates of *Candida*, including simplicity, speed and large sample volume throughput. However, the reproducibility of RAPD depends on a number of factors, each of which must be controlled carefully if accurate and consistent results are to be obtained [79–81]. Furthermore, although nucleotide sequence information on the organisms being examined is not required, RAPD primers that yield informative profiles with isolates of one *Candida* spp. may not do so with another. RAPD primer(s) that yield informative fingerprint profiles with all the *Candida* spp. isolated regularly from clinical specimens have yet to be described. It remains to be seen whether such a universal primer(s) will be discovered.

Several recent studies have described the use of PCR–restriction fragment length polymorphism (PCR–RFLP) analysis as a typing system for various bacterial species [82, 83]. This process involves RFLP analysis of PCR amplicons obtained with primers complementary to specific genomic DNA sequences.

This approach may be useful for differentiating between isolates of *Candida* spp. but, as far as we are aware, no such studies have yet been reported.

Application of molecular techniques to the taxonomy of *Candida* spp.

While most *Candida* spp. that cause disease in man are being well-studied, little is understood of the natural relationships within the genus. The single major factor unifying the species of the genus is the absence of a detectable sexual stage. This is hardly a satisfactory foundation on which to base relationships between species which may be closely or more distantly related. Therefore, *Candida* taxonomy has relied largely on analysis of physical traits such as cell-wall properties, the ability to produce germ tubes, chlamydo-spores, pseudohyphae and true hyphae, and on physiological traits such as the ability to utilise a range of compounds as the sole source of carbon or nitrogen [1]. However, as phenotypic characteristics can vary considerably within some species, it is not surprising that there have been irregularities in the taxonomy of *Candida* [1, 7, 33, 56, 84–91]. Several recent studies have described isolates of *Candida* whose properties do not correspond precisely with classical species descriptions, thereby leading to further confusion [18, 88, 92–94]. All of the above considerations make the concept of species within the genus rather ambiguous. It is therefore timely to assess the potential contribution that other techniques could make to the elucidation of relationships between species of the genus *Candida*.

Application of molecular techniques to resolving anomalies in *Candida* taxonomy

Despite the absence of a sexual phase being a prerequisite for a species to be classified within the genus *Candida*, a few *Candida* spp., including *C. krusei*, *C. guilliermondii*, *C. lusitaniae* and *C. kefyr* have been found to be the asexual (anamorphic) forms of sexually reproductive (teleomorphic) yeasts [1]. However, these organisms are still classified within the genus. Although the relationships between these *Candida* spp. and their respective teleomorphic forms have been determined by classical chemotaxonomic techniques, the synonymy of *C. krusei* and *Issatchenkia orientalis* has been confirmed by comparing karyotype profiles and by hybridisation of a *C. krusei*-specific DNA probe to isolates of *I. orientalis* [50]. Clearly, the application of similar and other molecular techniques could also be used to confirm the relationships between other *Candida* spp. and their respective teleomorphic counterparts, including *C. kefyr* and *Kluyveromyces marxianus*, *C. lusitaniae* and *Clavispora lusitaniae*, and *C. guilliermondii* and *Pichia guilliermondii*.

The precise relationship between *C. albicans* and two other closely related proposed species—*C. clausenii*

(considered to be a germ-tube-negative mutant of *C. albicans*) and *C. langeronii* (considered to be a chlamydo-spore-negative mutant of *C. albicans*)—has long been questioned. However, this situation has been resolved by studies that compared karyotype profiles and DNA hybridisation patterns obtained with the *C. albicans*-specific fingerprinting probe 27A, which demonstrated conclusively that *C. clausenii* and *C. langeronii* are synonymous with *C. albicans* [50].

In contrast, the relationship between *C. albicans* and *C. stellatoidea* is more complicated. Karyotype and fingerprinting data suggest that there are two kinds of *C. stellatoidea*, termed types I and II [87]. Type II strains differ from *C. albicans* only in their inability to assimilate sucrose—and so are considered to be sucrose-negative variants of *C. albicans*—while type I strains, as well as being sucrose-negative also have karyotypes distinct from that of *C. albicans* and appear to be less virulent [86, 95]. Although these data suggest that type I *C. stellatoidea* strains are not just simply mutant derivatives of *C. albicans*, they do not show sufficient differences from *C. albicans* to warrant species status. Therefore, it has been suggested that type I *C. stellatoidea* should be considered as a subspecies of *C. albicans* [1]. Further evidence in support of this designation is the very close homology between the sequence of the V3 region of the genes encoding the large ribosomal subunit of reference *C. albicans* and *C. stellatoidea* strains, in comparison with other *Candida* spp. [18].

We recently reported the isolation from HIV-infected individuals of atypical oral *Candida* isolates that produced chlamydo-spores and germ tubes. These features are usually diagnostic for *C. albicans* and *C. stellatoidea*, but the atypical isolates were not identifiable as either of these organisms on the basis of carbohydrate assimilation profiles [18, 33]. Furthermore, these organisms were unable to grow at 42°C and often produced chlamydo-spores that occurred in contiguous pairs or various multiples suspended from single suspensor cells, a feature that is quite unlike *C. albicans* [18]. Subsequent detailed molecular investigations—including karyotype analysis and DNA fingerprinting analysis of genomic DNA with: (i) the *C. albicans*-specific probe 27A which hybridises efficiently to multiple fragments of *C. albicans* and *C. stellatoidea* genomic DNA; (ii) five separate oligonucleotide probes homologous to eukaryotic microsatellite sequences; and (iii) RAPD analysis with random sequence primers—demonstrated that the atypical *Candida* isolates had a genomic structure distinctly different from those of *C. albicans* and *C. stellatoidea* [18]. Furthermore, comparison of the nucleotide sequences of the V3 variable region of the large ribosomal subunit genes of several atypical isolates with the corresponding sequences of *C. albicans*, *C. stellatoidea* and several other non-*albicans* *Candida* spp. demonstrated that the atypical isolates were

distinct phylogenetically and constituted a discrete taxon within the genus *Candida* for which the novel species name *C. dubliniensis* was proposed [18]. A particularly interesting feature of this proposed new species was its apparent relative genetic homogeneity in comparison to *C. albicans* isolates [18, 33]. The isolates studied to date come from three sources—Dublin (Ireland), Melbourne (Australia) and the UK—and this degree of homogeneity suggests strongly that this species may be the result of relatively recent evolutionary divergence.

In contrast with *C. dubliniensis*, several recent studies have demonstrated a wide degree of genetic heterogeneity within *C. parapsilosis* [37, 59, 70, 96, 97]. Two such studies—employing RAPD analysis, isoenzyme profile analysis and nucleotide sequence analysis of the ITS1 internally transcribed spacer sequences flanking the 5.8S RNA gene—have detected three distinct groups among *C. parapsilosis* clinical isolates [37, 97]. Although further research is required, two of these groups show significant genetic dissimilarity from the type strain of *C. parapsilosis* and it is possible that they may represent distinct species or sub-species.

One of the most controversial aspects of the taxonomy of the genus *Candida* is its relationship with the genus *Torulopsis*. As is the case with *Candida*, *Torulopsis* is known as a 'form' genus comprising yeasts that are difficult to classify [1]. Yarrow and Meyer [98] proposed that the inability of *Torulopsis* spp. to form pseudohyphae was not by itself a sufficient criterion for the two genera to be separate, and that both should be considered to be a single genus with the name *Candida* (despite the historical precedence of *Torulopsis*). This determination has been accepted by most mycologists, although some still consider *C. glabrata* to be *T. glabrata*, and *C. famata* to be *T. candida*, preferring to adhere to the provisions of the International Code of Botanical Nomenclature [99, 100]. 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 (see below).

Molecular phylogenetic analysis

Phylogenetic studies based on phenotypic characteristics alone have been shown to be unreliable [101]. Ribosomal DNA occurs in all living organisms and is ideally suited to the development of molecular phylogenies [102]. Indeed, comparative nucleotide sequence analysis of rDNA has been used extensively to study the evolutionary relationships among a wide variety of bacteria and, to a lesser extent, fungi [102–107]. Most of these studies have been performed

on small ribosomal subunit gene sequences. A search of the EMBL and GenBank nucleotide sequence databases performed during August 1995 revealed that sequence data on small ribosomal subunit genes have been reported only for a limited number of *Candida* spp. and other ascomycetous yeasts, and not always for type strains. However, these studies indicate that small ribosomal subunit gene sequences can be used to confirm natural relationships within the genus, such as the close evolutionary relationship between *C. albicans* and *C. tropicalis* determined on the basis of biochemical and phenotypic criteria [107]. However, in order to achieve a detailed and unequivocal understanding of the evolutionary relationships between the species of the genus, additional extensive studies need to be performed with type strains from a wide range of *Candida* spp., including all species that have been isolated from human samples or have been reported as having the ability to cause disease in man.

Nucleotide sequence analysis of the V3 variable region of the large ribosomal subunit genes has provided very useful information concerning the phylogenetic relationships between various marine yeasts [108, 109]. Similarly, the phylogenetic analysis performed in the study of *C. dubliniensis* isolates highlighted the potential of this approach to develop an unequivocal phylogeny for members of the genus *Candida* that cause infection in man [18]. Comparison of 500 bp of the V3 variable region of the large ribosomal subunit genes from nine *C. dubliniensis* isolates—from Dublin, Australia and the UK—and the corresponding sequences determined from *C. albicans*, *C. stellatoidea*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. kefir* and *C. krusei* demonstrated clearly that the *C. dubliniensis* isolates formed a homogenous cluster (100% similarity) that differed significantly from the isolates of the other *Candida* spp. analysed. It also demonstrated that type I *C. stellatoidea* shares very close homology with *C. albicans* in comparison with the other *Candida* spp., thereby providing further support for its designation as a subspecies of *C. albicans* [18].

Conclusions

With the use of suitable culture media and careful analysis of phenotypic and metabolic tests, it is feasible to obtain with reasonable precision an identification of most non-*albicans* *Candida* spp. Molecular techniques will soon be sufficiently simple, rapid and specific to be considered as first line tests. *C. dubliniensis* provides a cautionary lesson in this respect because examination of an isolate listed as *C. stellatoidea* from a culture collection (strain NCPF3108) by the traditional techniques indicated that it was similar to *C. dubliniensis*. Only when other *C. stellatoidea* type strains were examined by traditional and molecular techniques was it possible to differentiate the three

organisms. However well maintained they are, *Candida* isolates from culture collections need to have their identity verified as stringently as any clinical isolate. The majority of our *C. dubliniensis* isolates came from HIV-infected intravenous drug users, but as the study of *C. dubliniensis* progressed, additional isolates were found among stored collections from different groups of HIV-infected patients and from other sources, such as a vaginal specimen from a woman presenting with vaginitis, an oral specimen from a diabetic patient and several oral isolates from normal healthy individuals. Atypical *Candida* isolates from HIV-infected Australian subjects were included in the characterisation of the novel species *C. dubliniensis*, and similar isolates have been obtained from HIV-infected intravenous drug users in Switzerland and from AIDS patients in the UK. It seems that this organism has been present in several different clinical settings and in a number of countries for some time, and that it has been classed as an unusual *C. albicans* or *C. sake*. It is hoped that this example will provide the stimulus for a systematic reassessment of the methods for identifying organisms isolated from cases of candidosis. However, the precise combination of features—genetic and phenotypic—that will be acceptable in the future to distinguish the genus *Candida* from other asporogenic yeasts, and to distinguish one species of *Candida* from another, remains to be determined.

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