Full Length Research Paper

**Candida albicans ssp. dubliniensis stat.et comb. nov., a new combination for Candida dubliniensis based on genetic criteria**

Zaidan Khlaif Imran

Biology Department, All Women College of Science, Babylon University, Babylon Province, Hilla, Iraq.

Received 30 November, 2014; Accepted 13 April, 2015

One accredited species, *Candida albicans* subspecies *dubliniensis*, has been proposed to replace the existing designations of *Candida dubliniensis*. The study of the genetic diversity among the clinical isolates of *C. albicans* and *C. dubliniensis* was performed based on the amplified transposable intron region in the 25S rRNA gene. This study attempts to verify the unequivocal understanding of the genetic relationship between *C. albicans* and *C. dubliniensis*. Twenty (20) isolates of *C. albicans* and *C. dubliniensis* were studied using the method of typing by rDNA, restriction fragment length polymorphism (RFLP), random amplification polymorphism DNA-polymerase chain reaction (RAPD-PCR) and intron sequencing in the 25S gene. The results reveal that the specific primer pair CABF59F and CADBR125R was the successfully amplified target for all the *C. albicans* isolates and three isolate of *C. dubliniensis*. The *Candida* isolates revealed a genetic pattern based on the analysis of the RAPD-DNA fingerprinting pattern. The RFLPs generated by Hhal and Hae 111 enzymes elucidated similar recognition sites for both the *C. albicans* and *C. dubliniensis* isolates. Analysis of the intron sequence in the 25S gene region of the genotype *C. albicans* and *C. dubliniensis* showed identical with only a few differences in the base substitution. The sequence variations appear among the same isolates in each species. In all the cases, the clinical isolates of both species showed a percentage sequence similarity of >99.5%. This result emphasizes a high indication of similarity between *C. dubliniensis* and *C. albicans*. It was concluded that the taxonomic position of *C. dubliniensis* was puzzled due to insufficient genetic and phenotypic characters to warrant species status. Variations were occasionally observed to occur among the same isolates, within the same species; however, this indication is applied to other taxonomic criteria between them, with no credibility for the great differences observed between *C. dubliniensis* and *C. albicans*. This is the final taxonomic decision for *C. dubliniensis* to merit an amendment in order to be included as *C. albicans* subspecies *dubliniensis* stat. et comb. nov. *C. dubliniensis* with a revised synonymy for *C. albicans*.

Key words: Amendment, *Candida albicans* ssp. *dubliniensis* stat. et comb. nov, restriction fragment length polymorphism (RFLP), random amplification polymorphism DNA (RAPD), polymerase chain reaction (PCR), sequence, phylogenetic tree.

INTRODUCTION

Over the past 19 years, several studies have been done to evaluate the relationship between *Candida dubliniensis* and *Candida albicans*, a typical *Candida* strain difficult to identify up to species level, because of the heterogeneous morphological, biochemical and genetic characteristics they exhibit (Sullivan et al., 1995; Pujol et al., 1997; Sullivan and Coleman, 1997). *C. dubliniensis* had been described as a separate
species in 1995 by Sullivan et al. (1995). Retrospective studies revealed that it had been earlier commonly identified as C. albicans, to which C. dubliniensis is closely related, and with which it shares several characteristics including those of growth conditions, germ tube formation, chlamydospore formation and color interaction on CHROMagar (Tamura et al., 2001). Systematic studies of Candida spp. based on phenotypic criteria alone have been revealed to be unreliable markers, although they enabled us to elucidate some taxonomic complications between C. albicans and C. dubliniensis, such as similarities in the phenotypic characters, especially in their color on chromogenic agar, as well as following other conventional criteria, although not the perfect solution for the differences between them (Ahmed et al., 2002; Marot-Leblond et al., 2006; Imran and Al Asadi, 2014). In fact, misidentifications between some Candida spp., particularly between C. dubliniensis and C. albicans have frequently been observed (Tamura et al., 2001; Abaci et al., 2008). Coronado-Castellote and Jiménez-Soriano (2013) referred to C. dubliniensis as exhibiting similarity with C. albicans in their germ tubes and chlamydoconidia, as well as the high probability for mating between them and the similarity in some of their sequences at different loci (Pujol et al., 2004). Most of these reasons were critical in the identification of the taxonomic position of C. dubliniensis. Ribosomal DNA, considered an essential marker in Candida and other fungi, is ideally suited for the development of molecular studies. The high discriminatory power of the molecular tools like polymerase chain reaction (PCR) PCR, PCR-RFLP, RAPD-PCR, as well as sequencing, have provided change barter fast, relatively simple to perform, precise and reliable methods for the diagnosis of the Candida spp. (Mirhendi et al., 2005; Santos et al., 2010; Mijiti et al., 2010; Shokohi et al., 2010).

McCullough et al. (1999) utilized CABF59F and CADBR125R primers designed to span the region that includes the site of the transposable intron of the 25S rRNA gene (rDNA). The molecular target, the transposable intron and the design CA-INT primer were the highly reproducible markers for typing the C. albicans subgenotypes and differentiated the C. dubliniensis from the closely related isolates of the C. albicans compartment with a selection of the ITS or other regions (Tamura et al., 2001). The simple PCR attached to the inserted intron in 25S rDNA classified the strains of C. albicans into three or four subgroups as the given genotype A, genotype B, genotype C and sometimes even genotype D (McCullough et al., 1999). A special genotype was also assigned to C. dubliniensis (Tamura et al., 2001). However, Tamura et al. (2001) showed that no group I intron was observed in the other Candida species tested, including those of Candida glabrata, Candida krusei, Candida parapsilosis and Candida tropicalis. Imran and Al Asadi (2014) revealed the presence of introns in most non albicans species. In contrast, if reproducible markers like the restriction enzyme are utilized, they could facilitate solving the difficult cases. By analyzing the restriction fragment length polymorphisms (RFLPs), the unique polymorphism in the monomorphic PCR bands can be identified (Mirhendi et al., 2005). From this perspective, the intron inserted in the region of the 25S rRNA gene sequences offers several advantages to the Candida spp. genotypes (McCullough et al., 1999). The intron in the 25S rRNA gene has been shown to have a high heterogeneity within the Candida species (Hanafy and Morsy, 2012). The contribution of the intron inserted in the region of the 25S rRNA in clinical diagnosis remains to be determined, due to the lack of a complete molecular database that could enable the systematic comparison of the inter- and intra-species variations in the different isolates among the Candida spp.

Most of the regions of the large ribosomal subunit genes (LRSg) of yeasts are reproducible markers, which provided very useful information concerning the phylogenetic relationships among the various marine yeasts (Fell and Kurtzman, 1990).

It is not surprising that some irregularities are seen in the taxonomy of Candida. Several recent studies have described the Candida isolates, whose properties do not correspond precisely with the descriptions of the classical species, leading to further confusion (Mahrous et al., 1990; McCullough et al., 1994, 1995; Boerlin et al., 1995). It is, therefore, the right time to assess the potential contribution that other techniques could make towards the identification of the relationships between C. albicans and C. dubliniensis. Sullivan and Coleman (1997) indicated the necessity of further confirmation, which can be obtained by conducting any of the several DNA fingerprint techniques available, as well as by RFLP and RAPD analysis. These are also effective, as well as quicker and easier to perform in order to discriminate between C. albicans and C. dubliniensis.

Indeed, the comparative nucleotide sequence analysis of the rDNA has been used extensively to study the evolutionary relationships among a wide variety of fungi. Most of these studies have been performed on small ribosomal subunit gene sequences (Hendriks et al., 1991; Fleischmann et al., 2004). A search conducted in the Gene Bank nucleotide sequence databases over the past few decades revealed that the sequence data on the rDNA genes have been reported only for a large number of Candida spp. However, these studies indicate that the
25S gene sequences can be used to confirm the natural relationships within the genus, such as the close evolutionary relationship between *C. albicans* and *C. dubliniensis*, based on biochemical and phenotypic criteria (Kumar et al., 2006; Nawrot et al., 2010).

The aim of this study was to achieve a detailed and unequivocally understanding of the evolutionary relationships between *C. albicans* and *C. dubliniensis*, by performing rapid genotyping based on simple PCR, RFLP-PCR, RAPD-PCR and emphasizing the identification of genotype patterns for both *C. dubliniensis* and *C. albicans* by using the sequencing tools.

**MATERIALS AND METHODS**

**Yeast collection and cultural characters**

A total of 60 clinical vaginal swabs were collected from the clinics in the province of Babylon, Iraq, during the study conducted in 2013-2014. Clinical samples using a sterile cotton swab were taken from the vagina of patients exhibiting clinical signs of the vaginal candidiasis based on Imran and Al. shukry (2014). They were transferred to the biotechnical laboratory where they were directly streaked on Sabouraud agar medium (SDA) supplemented with chloramphenicol and streptomycin (50:50 μg/ml). After inoculation, the Petri plates were incubated for 24-48 h at 37°C (Sivakumar et al., 2009; Baveja, 2010). All the sixty isolates in this study were subjected to preliminary identification was done based on CHROMagar Candida (Ghelardi et al., 2008; Marsh and Martin, 2009; Nadeem et al., 2010).

**Extraction of genomic DNA**

Twenty isolates out of 60 isolates of *Candida* spp. were subjected to DNA extraction and PCR assays. A loop full of *Candida* colony was suspended in the lysis buffer (200 mM Tris-HCl, 20 mM EDTA, 150 mM NaCl and 0.5% SDS) and heated in water bath at 95°C for 2 min. The suspension was centrifuged at 5000 rpm for 2 min and the supernatants were decanted into new sterile tubes, and precipitated with an absolute alcohol and then, washed DNA pellet by 70% ethyl alcohol, dried pellet of DNA dissolved in elution buffer and preserved at -20°C until use (Fredricks et al., 2005).

**PCR assays**

The phenotypic results were confirmed by simple PCR by specific primer pair for *C. albicans*: CAF59F: 5'-TTGAAACATCTCCAGTTTCAAGGT-3' and CADBR125R: 5' -AGCTAAATTCATAGCAGAAAGC-3'. ampliﬁed target 665 bp (Kane et al., 2002). Genotypes and subgenotypes of Candida isolates were determined by PCR based on method of Tamura et al. (2001). The primer pairs whose sequences span the site of the transposable intron in the 25S rDNA were those described by McCullough et al. (1999). The PCR primer pairs used were CA-INT-L (5’-ATAGGGAATGCGCAAATAGACCTGTA-3’) and CA-INT-R (5’-CTTTGGCCTGTTTCCTAGTAGTATG-3’).

1 μL of DNA (20 μg/µl) from each of 20 Candida isolates were mixed with PCR mixture ( ﬁnal reaction volume 25 µL) consisted of 12 μL of 2x Master Mix (Promega), 2 μL of primers (10 pmole) and rest molecular-grade water. The PCR conditions for CA-INT-L and CA-INT-R primers were 95°C for 3 min followed by 30 cycles 94°C for 1 min, annealing temperature 65°C for 1 min. Extensions temperature 72°C for 2.5 min followed by final extension temperature 72°C for 7 min. The PCR conditions for primer pairs CAF59F and CADBR125R was similar to previous cycle except annealing temperature which was 55°C in place of 65°C. The PCR mixture was ampliﬁed by thermal cycler PCR System (Labnet, USA).

The PCR products for each target region were run on 1.2% agarose gel (Bio Basic Canada Inc.). Electrophoresis was performed at 100 V. in TBE buffer. The gel was pre-stained with 0.05% ethidium bromide. The DNA bands were detected by Desktop Gel imager scope 21 ultraviolet transilluminator (Korea Com.).

**PCR-RFLP assay**

The PCR-RFLP assay was performed as described earlier by Mirhendi et al. (2006). In brief, the incubation of a 10 µL aliquot of the PCR products consisted of 12 µL of 2x Master Mix (Promega), 2 µL of primers (10 pmole) and rest molecular-grade water of amplified intron region of 25S rRNA gene with 10 µL of HaeIII and HhaI cocktail restriction enzymes (Promega, USA) was performed in single reaction, at 37°C for 3 h, using both enzymes. Next, 8 µL of the RFLP-PCR products were run on 1.5% agarose gel at 70 V for 60 min. The gel was pre-stained using 0.05% ethidium bromide and visualized under UV light and photographed by the Desk Gel imager scope 21 ultraviolet transilluminator (Korea Com).

**RAPD-PCR assay**

RAPD-PCR was accomplished by utilizing a total volume of 34 µL consisting of 0.7 µL (20 µg/ml) genomic DNA, 18 µL of 2x master mix (Promega USA) 12 µL molecular-grade water and 1.5 µL (50 pmole) of random primer GGTTGATTGT. The mixture was ampliﬁed under the following conditions: 95°C for 4 min; 38 cycles at 94°C for 1 min; 36°C for 1.5 min; 72°C for 1.30 min and 72°C for 8 min (Labnet PCR System). Further, 8 µL of the PCR products were run on 1.5% agarose gel at 70 V for 60 min. The gel was pre-stained with 0.05% ethidium bromide.

**Sequencing assay**

To study the relationship and similarity at morphological and molecular level that are sometimes exhibited between the *C. albicans* and *C. dubliniensis* isolates particularly because *C. albicans* and *C. dubliniensis* also possess the transposable intron in the 25S rDNA, the genomic DNA of the representative isolates for both *C. albicans* and *C. dubliniensis* were ampliﬁed with the CA-INT primers. After PCR ampliﬁcation, the puriﬁed products for 8 isolates were sequenced. The PCR primers CA-INT-L was used for DNA sequencing of transposable intron in the 25S rDNA of Candida isolates. Sequence analysis was performed using the Macro Gene Company, USA. The sequence alignment of *C. albicans* and *C. dubliniensis* was compared with the BLAST database and were aligned with sequences from the BLAST database derived from the following reference strains: (*C. dubliniensis* sequence ID: embl FM992695.1 United Kingdom isolate; *C. albicans* sequence ID: gbl DQ465844.1 New Zealand isolate).

**Phylogenetic analysis**

The phylogenetic tree dendrograms of RFLP and RAPD-PCR products for isolates of *C. albicans* and *C. dubliniensis* was created by clustering methods applied on distance matrix unweighted pair
group method with arithmetic mean (UPGM) which offers automatic lane/band detection, band matching and molecular weight computation. The phylogeny tree computation was analyzed based on UVI band software GD/45230 for gel image analysis. The software is able to analyze gel image patterns of bands for different isolates or species and generates phylogenetic tree based on the information available in gel image. It also evaluated the similarity coefficient factor according to Mackenstedt et al. (1994) and Ute et al. (1994). The phylogenetic tree based on sequencing and sequence table were constructed employing the Mega 6 software.

RESULTS

Phenotypic and molecular diagnosis for *Candida* spp.

All the sixty isolates in this study showed up in green color on the CHROMagar Candida. The results of molecular assay showed that 20 isolates of *Candida* were identified as *C. albicans*. The amplification of the targeted region produced an amplicon of size 665 bp (Figure 1). The target regions of three isolates for *Candida* showed faint bands as seen in Figure 1, lanes B, J and Q.

Genotyping of *Candida* spp. by CA-INT primer pair

The specific primer CA-INT was designed to flank the transposable intron region of the 25S rRNA gene. PCR was successfully amplified the target region of the genomic DNA of the 20 isolates. The amplification result designated five isolates as *C. dubliniensis*, which had a high PCR product (1080 bp). Thus, 16 isolates of *C. albicans*, with low PCR products could be classified and three genotypes could be designated viz., (i) genotype C, (ii) (450 and ~840 bp), (iii) A genotype of (~840 bp) of *C. albicans* (450 bp) (Figure 2).

RFLP-PCR assay

Both restriction enzymes (Hhal and Hae 111 enzyme) have an equal chance of making a cut anywhere in the PCR product. However, the restriction banding patterns by using the Hhal enzyme showed large fragments (500 bp) of *C. dubliniensis*, as in Figure 3a (lanes B, H, J, K and Q).

This enzyme also revealed a similar basal band with the PCR fragments of average length approximately 380 bp in all the isolates for *C. albicans* and *C. dubliniensis*. However, the use of the Hhal enzyme cut PCR products into many short PCR fragments (<100 bp), as seen in Figure 3a. The restriction banding patterns by using the Hae 111 enzyme showed characteristic cleavage profile (350, 300, 180 and 60 bp fragments) for *C. dubliniensis*.

However, the PCR products of *C. albicans* also showed variation in their RFLP patterns. The first pattern revealed two fragments such as C, E-G, I and M, whereas the second pattern was composed of four fragments such as D, L, N, O, PT and U (Figure 3a). The isolates of *C. dubliniensis* B, H, J, K and Q showed variation in their RFLP-PCR patterns, in which the H, J and K appeared closely related, while the B and Q showed differences (Figure 3b). The Hae 111 enzyme resulted in a large fragment of the PCR, of *C. dubliniensis*, of about 350 bp as in the B, H, J, K and Q lanes. The PCR product of the albicans isolates showed fragments of about 300 bp, as in C, E-G, I, M, R and R-S, as well as a fragment of 270 bp in D, L, N-P, T-U; This enzyme also revealed a similar basal band of average length of the PCR fragments, approximately 100 bp, in all the isolates for *C. albicans* and *C. dubliniensis* (Figure 4a).

RAPD-PCR assay

The results show that the oligo primer GGTGTAGTGT successfully genotyped the 20 isolates of *C. albicans* and *C. dubliniensis* into 7 genotypes: *C. dubliniensis* revealed three genotypes, while *C. albicans* showed four genotypes (Figure 5). RAPD-PCR produced multiple bands, the main band of which was consistently present in all the
isolates (constant basal band 450-550 bp); the greatest variation occurred among the upper bands of the constant single bands at 600-800 bp (Figure 5). *C. dubliniensis* revealed three genotypes: (B-Q 70%, K-H 45% and J). and *C. albicans* showed 10 genotypes (M-N, O-U, P-S, F-G, R-T, J, C, L, I and E).

**Sequence analysis**

The results of sequence analysis for eight isolates of *Candida* spp. showed a similarity with the entry in the percentage sequence of >99.5% with the intron region of the 25S rRNA gene. Genotypes A and C of *C. albicans* isolates (D, T and E) showed high similarity of about 99.98% in their sequence at the same time, the genotype of *C. dubliniensis* isolates (B and Q) showed high similarity of about 99.97% with the A and C genotypes of *C. albicans* isolates (D, T and E), genotype B of *C. albicans* isolate (R) showed similarity of 99.96 when compare with the genotypes C and A of *C. albicans* isolates (C and L) (Figure 6).
**DISCUSSION**

Our results concurred with several recent studies and demonstrated a wide degree of genetic homogeneity between *C. dubliniensis* and *C. albicans* (Jackson et al., 2009). The results of Boucher et al. (1996) found that both the intron and ribosomal RNA nucleotide sequences were almost perfectly identical between the different *C. albicans* strains, as well as between the *C. albicans* and *C. dubliniensis*. Although it is difficult to distinguish between the *C. albicans* and *C. dubliniensis* colonies formed on CHROMagar which are green in color, the CHROMagar medium can be unstable following subculture or storage (Schoofs et al., 1997; Sullivan and Coleman, 1998). This result indicated that the specific primer pair (CABF59F and CADBR125R) for *C. albicans* amplified its target in *C. dubliniensis* as well as in 16 isolates of *C. albicans*. Despite the concurrence in the results for *C. albicans* with those of Costa et al. (2010), the result with *C. dubliniensis* indicated the similarity of the sequence target region for *C. dubliniensis* and *C. albicans*. This result concurred with the report of Pujol.
Figure 5. A- Agarose gel electrophoresis of the amplified RAPD-PCR products for *Candida* spp. Detection polymorphism of 20 clinical isolates of *Candida* spp. using the oligo primer GGTGTAGTGT. B- Phylogenetic relationships (homologous coefficient %) between different isolates of *Candida*. The consensus tree was based on data of RAPD-PCR generated via UPGMA cluster analysis. A = Molecular size marker 100 bp; B-T, clinical isolates of *Candida*.

Figure 6. Phylogenetic relationships (homologous coefficient %) between different isolates of *Candida* based on sequence data were constructed employing the Mega 6 software.

et al. (2004) as far as the similarity in some of the loci sequences between the two species. The specific primer CA-INT designed to flank the transposable intron region of the 25S rRNA gene successfully typed 5 isolates out of the 20 isolates designated as *C. dubliniensis* (1080 bp), while the other isolates were designated as three genotypes of *C. albicans* (genotype C = 450 and ~840 bp; genotype A = ~840 bp and genotype B = 450 bp). This result concurred with the earlier studies of Tamura et al. (2001), Kumar et al. (2006) and Nawrot et al. (2010).
Both the restriction enzymes, Hhal and Hae 111, revealed a high degree of polymorphisms with respect to RFLP-fingerprinting in both C. albicans and C. dubliniensis. In total, 16 isolates of C. albicans showed polymorphic RFLP-pattems. However, the use of the Hhal enzyme revealed a similar basal band with an average length of the PCR fragments at approximately 380 bp in all the isolates for C. albicans and C. dubliniensis, the phylogenetic tree highlighting the degree of homogeneity in the sequence of recognition site of enzyme between C. albi cans and C. dubliniensis.

The Hae111 enzyme elucidated polymorphic RFLP-pattems in both C. albicans and C. dubliniensis and yielded different fragment sizes with C. dubliniensis having other short PCR fragments. On the other hand, the PCR products of C. albicans also showed variation in their RFLP-pattems. This enzyme also revealed a similar basal band of average length of the PCR fragments, approximately 100 bp in all the isolates for C. albicans and C. dubliniensis. This implied a similarity in the recognition site and the same sequence in this site for both species. The phylogenetic tree supported the natural variation such as mating, mutations and recombination which may occurred in many isolates related to the same species. Our result coincidence with many studies (Mirhendi et al., 2005; Mirhendi et al., 2006; Shokohi et al., 2010).

The sequence introns of 25S from eight isolates of C. albicans and C. dubliniensis were retrieved through BLAST analysis, the sequences in C. albicans and C. dubliniensis were found to differ by approximately 0.02%. This difference is not similar to the differences in the sequences found between C. tropicalis and C. maltosa (2.8%) and between C. parapsilosis and the ascosporic species, Lodderomyces elongisporus (3.2%). Based on this, results were contrary to those of Peterson and Kurtzman (1991), in which they earlier suggested that strong evidence for a separate species exists when this region contains a substitution of greater than 1% of the nucleotide between the two organisms. Most of the phenotypic characters of C. dubliniensis did not serve to confirm the taxonomic status of a distinct species.

Amendment of C. dubliniensis: subspecies novus

In the light of these results, despite the C. dubliniensis had been described as a separate species over the past decade (Sullivan et al., 1995; Coleman et al., 1997; Tamura et al., 2001), the understanding that prevailed was that a few variations occasionally occurred in many strains due to natural selection, which were, however, insufficient to justify the emergence of a new species. Besides strong confirmation from many studies including the work of Coleman et al. (1997) who revealed the very close similarity among the isolates of C. dubliniensis to those of C. albicans. Particularly, because C. dubliniensis and C. albicans are phenotypically very similar, it is highly likely that the isolates of C. dubliniensis had been misidentified as C. albicans or C. stellatoidea in the past (Anthony et al., 1995; Boerlin et al., 1995; Coleman et al., 1997). On the other hand, it is shown that it is impossible to consider any variation in the phenotypic and genetic properties, which were not contingent upon their definitive identification as C. albicans, based on the views of Coleman et al. (1997) to be the emergence of a new species.

In spite of the C. albicans and C. dubliniensis, isolates produced chlamydospores on the TOC agar medium based on Tamura et al. (2001). Our results showed the density of the chlamydospores produced by both species were not a good taxonomic character from which to draw any conclusion.

Our results demonstrate that C. dubliniensis shares a very close relationship with C. albicans based on the results of both the RFLP, RAPD-PCR patterns and the sequence marker shown in Figures 6 and 7, this result coincides with prior studies that suggested that C. dubliniensis isolates were not merely mutant derivatives
of \textit{C. albicans}; in the same trend, we do not observe sufficient differences to separate \textit{C. dubliniensis} from \textit{C. albicans} to warrant a species status. Therefore, we provided further support for its designation and confirmed that \textit{C. dubliniensis} should be considered as a subspecies of \textit{C. albicans}. This judgment, based on the molecular RFLP patterns, such as the Hhal and Hae 111 enzymes revealed a similar basal band, this indicated the presence of the same sequence and recognized the region in both species. Sequence also confirmed part of this truth based on sequence analysis. These results are in agreement with those of Jackson et al. (2009) and Sullivan Coleman (1997) where they refer to the requirement for further confirmation, which can be obtained by performing any of the several DNA fingerprint techniques available. The phylogenetic tree, based on the sequencing of the introns on the 25S gene, showed close similarity (99.5\%) between \textit{C. dubliniensis} and \textit{C. albicans} as shown in Figure 7, with only subtle differences in sequence between the two species.

The amendment of \textit{C. dubliniensis} taxonomic state agrees with early and recent studies that are closely related \textit{C. dubliniensis} to \textit{C. albicans} which was routinely misidentified as \textit{C. albicans} (Sullivan et al., 1995; 2004; Moran et al., 2004; Jones et al., 2004). Based on the results of Tamura et al. (2001), the genotype 1080 bp was elucidated only as \textit{C. dubliniensis} on typing a transposable intron region in the 25S rRNA gene from four other genotype strains viz.: genotypes of \textit{C. albicans} (genotype C = 450 and 840 bp, genotype A = \~{} 840bp, genotype B = 450 bp and genotype E = 1400 bp), these genotypes continued to remain as different strains of \textit{C. albicans}. Tamura et al. (2001) referred to the genotype E strain which showed a high degree of similarity to \textit{C. dubliniensis} when compared with the degree of similarity of the strains of the other \textit{C. albicans} genotypes, in which the similarity was determined based on the group I intron sequence; however, from his results, he neglected to include this genotype within \textit{C. dubliniensis}, as was expected. From our view, with his erroneous taxonomic judgment along with his tempera ment and individuality, based on the trend of Tamura et al. (2001), each one of the all the genotypes of \textit{Candida} (450, \~{} 840, 450+850 and 1400 bp) was merited to be included as a new species at the same time. We think it is insufficient to justify the emergence and support genotype 1080 bp of a new species by Tamura et al. (2001). Therefore, the differentiation of the two taxa was based on the color of the colony on CHROMagar. \textit{Candida}, thus, may not be as reliable as was considered earlier, to utilize CHROMagar to differentiate between \textit{C. albicans} and \textit{C. dubliniensis}. Tamura et al. (2001) revealed a dark blue color of the colonies on CHROMagar, which could not confirm the differentiation of four of the \textit{C. dubliniensis} isolates out of five. They also used the growth at 45\textdegree{}C as a criterion for the differentiation between the two species. This was confuted by Tamura et al. (2001) when he referred to all the \textit{C. albicans} genotypes, including the five \textit{C. dubliniensis} strains, which grew well at 45\textdegree{}C on a culture media such as PDA and Sabouraud dextrose agar.

We concluded that, it is impossible to consider any few variations in the phenotypic and genetic properties of the \textit{Candida} strains, and showed that a few variations occasionally occurred in many strains due to natural selection, which were however, insufficient to justify the emergence of a new species. Besides strong con firmation from previous studies which revealed the very close similarity among the isolates of \textit{C. dubliniensis} to those of \textit{C. albicans}, \textit{C. dubliniensis} is not an emerging new species. We provided further support for its designation and confirmed that \textit{C. dubliniensis} do not merit being included as a new species and should be considered as \textit{C. albicans} subspecies \textit{dubliniensis} stat. et.comb. nov.

Ethical approval

Author hereby declare that all actions have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Conflict of interest

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

This study was conducted in the Biotechnology Laboratory, The Department of Biology, All Women College of Science, Babylon University, Iraq. The author would like to thank his postgraduate students for samples collection, Dr. Mohamad Al-Rufaei and thank Hilla hospital staff for their logistic support and their help in sampling.

REFERENCES

Abaci \Ö, Halki-Huztan A, Ates M (2008). Specific identification of \textit{Candida albicans} and \textit{Candida dubliniensis} by PCR using species-specific primers. Ann Microbiol. 58: 325–331.

Ahmed S, Khan Z, Mustafa AS, Khan ZU (2002). Semi-nested PCR for diagnosis of candidemia : comparison with culture, antigen detection, and biochemical methods for species identification. J. Clin. Microbiol. 40:2483-2489.

Anthony RM, Midgley J, Sweet SP, Howell SA (1995). Multiple strains of \textit{Candida albicans} in the oral cavity of HIV positive and HIV negative patients. Microbial Ecol. Health Dis. 8:23–30.

Baveja C (2010). Medical mycology, in Text Book of Microbiology for Dental Students, Arya Publications, Delhi, India, 3rd edition. pp. 322-323.

Boerlin P, Boerlin-Petzold F, Durussel C, Addo M, Pagani JL, Chave JP, Bille J (1995). Cluster of oral atypical \textit{Candida} isolates in a group
of human immunodeficiency virus-positive drug users. J. Clin. Microbiol. 33:1129-1135.
Boucher H, Mercure S, Montplaisir S, Lemay G (1996). A novel group I intron in Candida dubliniensis homologous to a Candida albicans intron. Gene 180: 189-196.
Coleman DC, Sullivan DJ, Bennett DE, Moran GP, Barry HJ, Shanley DB (1997). Candidiasis: the emergence of a novel species, Candida dubliniensis. AIDS. 11(5):557-567.
Coronado-Castellote L, Jiménez-Soriano Y (2013). Clinical and microbiological diagnosis of oral candidiasis. J. Clin. Exp. Dent. 5(5):e279-288.
Costa JM, García-Hermoso D, Olivi M, Cabaret O, Farrugia C, Lecellier G, Dromer F, Bretagne S (2010). Genotyping of Candida albicans using length fragment and high-resolution melting analyses together with minisequencing of a polymorphic microsatellite locus. J. Microbiol. Methods. 80(3):306-309.
Fell JW, Kurtzman CP (1990). Nucleotide sequence analysis of the large subunit rRNA for identification of marine occurring yeasts. Curr. Microbiol. 21:295-300.
Fleischmann R, Liu H, Wu, Chieh-Pin (2004). Polyadenylation of ribosomal RNA by Candida albicans also involves the small subunit. BMC Molecular Biology 5:17.
Fredricks DN, Smith C, Meier A (2005). Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J. Clin. Microbiol. 43: 5122-5128.
Ghelardini C, Chiovato L, Barletti S, Barresi S, Tavanti A, Campa M (2008). Efficacy of Chromogenic Candida agar for isolation and presumptive identification of pathogenic yeast species. Clin. Microbiol. Infect. 14:141-147.
Hanafy AMM, Morsy MRA (2012). Molecular analysis and in vitro susceptibility profiling of some clinically important Candida spp. to the common antifungal drugs. Afr. J. Microbiol. Res. 6(42): 6978-6986.
Hendriks GJ, Goris A, van Peperen Y, Barresi S, Tavanti A, Campa M, Kersters K, Hennebert GL, De Wachter R (1991). Phylogenetic analysis of five medically important Candida species as deduced on the basis of small ribosomal subunit RNA sequences. J. Gen. Microbiol. 137(5):1223-1230.
Imran ZK, Al Asadi YF (2014). Multiple molecular markers for diagnosis of conjunctivitis caused by Candida spp. in Iraq. Afr. J. Microbiol. Res. 8(38):3482-3488.
Imran ZK, Al Shukry HN (2014). Molecular diagnosis of vaginal candidiasis by polymerase chain reaction (PCR) and random amplification polymorphism DNA (RAPD-PCR) in Babylonia Province, Iraq. Afr. J. Microbiol. Reach. 8(6):496-502.
Jackson AP, Gamble JA, Yeomans T, Moran GP, Saunders D, Harris D, Aslett M, Barrell JF, Butler G, Citalto F, Coleman DC, et al. (2009). Comparative genomics of the fungal pathogens Candida albicans and Candida dubliniensis. Genome Biol. 10(2):2231-2244.
Jones T, Federspiel NA, Chibana H, Dungan J, Kalman S, Magee BB, Newport G, Thorstenson YR, Agabian N, Magee PT, et al. (2004). The diploid genome sequence of Candida albicans. Proc. Natl. Acad. Sci. 101:7329-7334.
Kanbe T, Horii T, Arishima T, Ozeki M, Kikuchi A (2002). PCR-based identification of pathogenic Candida species using primer mixes specific to Candida DNA topoisomerase II genes. Yeast 19: 973-989.
Kumar GCP, Hanafy AM, Katsu M, Mikami Y, Menon T (2006). Molecular analysis and susceptibility profiling of Candida albicans isolates from immunocompromised patients in South India. Mycopathologia 161:153-159.
Mackenstedt U, Luton K, Baverstok PR, Johnson AM (1994). Phylogenetic relationships of Babesia divergens as determined from comparison of small subunit ribosomal RNA gene sequences. Mol. Biochem. Parasitol. 68: 161-165.
Mahrous M, Lott T, Meyer SAY Sawant AD, Ahearn DG (1990). Electrophoretic karyotyping of typical and atypical Candida albicans. J. Clin. Microbiol. 28: 876-881.
Marot-Leblond A, Beucher B, David S, Nail-Billaud S, Robert R (2006). Development and evaluation of a rapid latex agglutination test using a comparison of small subunit ribosomal DNA reactivity to identify Candida dubliniensis colonies. J. Clin. Microbiol. 44:138-142.
Marsh PD, Martin M (2009). "Oral fungal infections," in Oral Microbiology, pp. 166-179, Churchill Livingstone, Edinburgh, UK.
McCullough M, Ross B, Reade P (1995). Characterization of genetically distinct subgroup of Candida albicans strains isolated from oral cavities of patients infected with human immunodeficiency virus. J. Clin. Microbiol. 33:696-700.
McCullough MJ, Clemons KV, Stevens DA (1999). Molecular and phenotypic characterization of genotype C. albicans subgroups and comparison with Candida dubliniensis and Candida stellatoidea. J. Clin. Microbiol. 37:417-421.
McCullough MJ, Ross BC, Dwyer BD, Reade PC (1994). Genotype and phenotype of oral Candida albicans from patients infected with the human immunodeficiency virus. Microbiology 140:1195-1202.
Mijit J, Pu XM, Erfan A, Yaguchi T, Chibana H, Tanaka R (2010). Genotyping of fluconazole-resistant Candida albicans isolated from Uighurian people in Xinjing (China) using ALTS/RFLP and micro-TGGE method. Nippon Ishinkin Gakkai Zasshi. 51:165-168.
Mirhendi H, Makimura K, Khoramizadeh M, Yamaguchi H (2006). A One-Enzyme PCR-RFLP assay for identification of six medically important Candida species. Nippon Ishinkin Gakkai Zasshi. 47:225-229.
Mirhendi H, Makimura K, Zomorodian K, Maeda N, Otshtima T, Yamaguchi H (2005). Differentiation of Candida albiican from Candida dubliniensis using a single enzyme PCR-RFLP method. Jpn. J. Infect. Dis. 58:235-237.
Moran G, Stokes C, Thewes S, Hube B, Coleman DC, Sullivan D (2004). Comparative genomics using Candida albicans DNA microarrays reveals absence and divergence of virulence-associated genes in Candida dubliniensis. Microbiology 150:3366-3382.
Nadeem SG, Hakim ST, Kazm SU (2010). Use chromoagar candida medium for the presumptive identification of Candida species from clinical specimens in resource –limited setting. Libyan J. Med. 5:1-6.
Nawrot U, Pajaczkowska M, Wlodarczyk K, Mecler I (2010). rDNA-based genotyping of clinical isolates of Candida albicans. Pol. J. Microbiol. 59:213-216.
Petersen SW, Kurtzman CP (1991). Ribosomal RNA sequence divergence among sibling species of yeasts. Syst. Appl. Microbiol. 14:124-129.
Pujol C, Daniels KJ, Shaw RL, Srikantha T, Joshua BR, Geiger J, Soll DR (2004). The closely related species Candida albicans and Candida dubliniensis can mate. Eukaryot. Cell. 3(4):1015-1027.
Pujol C, Renaud F, Mallie M, de Mees T, Bastide JM (1997). Atypical strains of Candida albicans recovered from AIDS patients. J. Med. Vet. Mycol. 35:115-121.
Santos MS, Souza ES, S Junior RM, Talhari S, Souza JV (2010). Identification of fungemia agents using the polymerase chain reaction and restriction fragment length polymorphism analysis. Braz. J. Med. Biol. Res. 43:712-716.
Schoofs A, Odds FC, Colebunders R, Ieven M, Goossens H (1997). Use of specialised isolation media for recognition and identification of Candida dubliniensis isolates from HIV-infected patients. Eur. J. Clin. Infect. Dis. 16:296-300.
Shokohi T, Hashemi Soteh MB, Pouri ZS, Hedayat MT, Mayahi S (2010). Identification of Candida species using PCR-RFLP in cancer patients in Iran. Indian J. Med. Microbiol. 28:147-151.
Sivakumar VG, Shankar P, Nalina K, Menon T (2009). Use of CHROMagar in the differentiation of common species of Candida. Mycopathologia 167:47-49.
Sullivan D, Coleman D (1997). Candida dubliniensis: Characteristics and Identification. J. Clin. Microbiol. 36(2):329-334.
Sullivan DJ, Westerneng Tj, Haynes KA, Bennett DE, Coleman DC (1995). "Candida dubliniensis sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals". Microbiology 141(7): 1507-1521.
Tamura M, Watanabe K, Mikami Y, Yasawas K, Nishimura K (2001). Molecular characterization of new clinical isolates of Candida albicans and C. dubliniensis in Japan: analysis reveals a new genotype of C. albicans with group 1 Intron. J. Clin. Microbiol. 39(12):4309-4315.
Utton M, Kim L, Peter B, Alan J (1994). Phylogenetic relationships of Babesia divergens as determined from comparison of small subunit ribosomal RNA gene sequences. Mol. Biochem. Parasit. 68:161-165.