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

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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 reveals 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

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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 unequivocal 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*: CABF59F: 5'-TTGAACATCTCCAGTTTCAAAGGT-3' and CADBR125R: 5 '-AGCTAAATTCATAGCAGAAAGC-3'. amplified target 665 bp (Kanbe et al., 2002). Genotypes and subgenotypes of *Candida* isolates determination 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′-ATAAGGGAAGTCGGCAAAATAGATCCGTAA-3′) and CA-INT-R (5′-CCTTGGCTGTGGTTTCGCTAGATAGTAGAT-3′).

1 µL of DNA (20 µg/ml) 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 CABF59F 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 t h e 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 Hae111 and Hhal 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 GGTGTAGTGT. 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

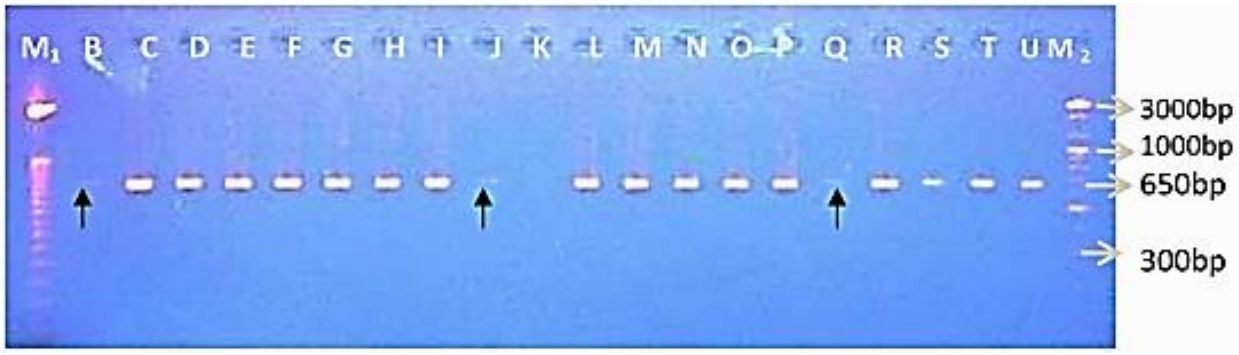

Figure 1. PCR products of 20 *Candida* isolates were amplified by the primer pair CABF59F and CADBR125R. (M_1 = molecular size marker one step 50 bp ladder; M_2 = molecular size marker 100 bp ladder); all positive lanes are *C. albicans* and faint or negative bands are *C. dubliniensis* (labeled by arrows).

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 (HhaI 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 HhaI 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 HhaI 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

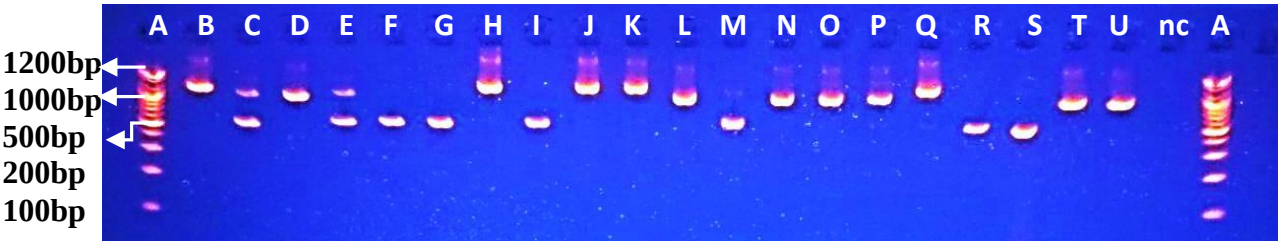

Figure 2. Gel electrophoresis of the PCR product amplified by the primer pair CA-INT. Bands in lanes B, H, J-K and Q: *C. dubliniensis* (1080 bp), lanes, C and E = genotype C (450 and 840 bp), lanes D, L, N-P and T-U. A genotype of *C. albicans* (~840bp), lanes F-G, I, M and R-S = B genotype of *C. albicans* (450bp) A= molecular marker 100 bp.

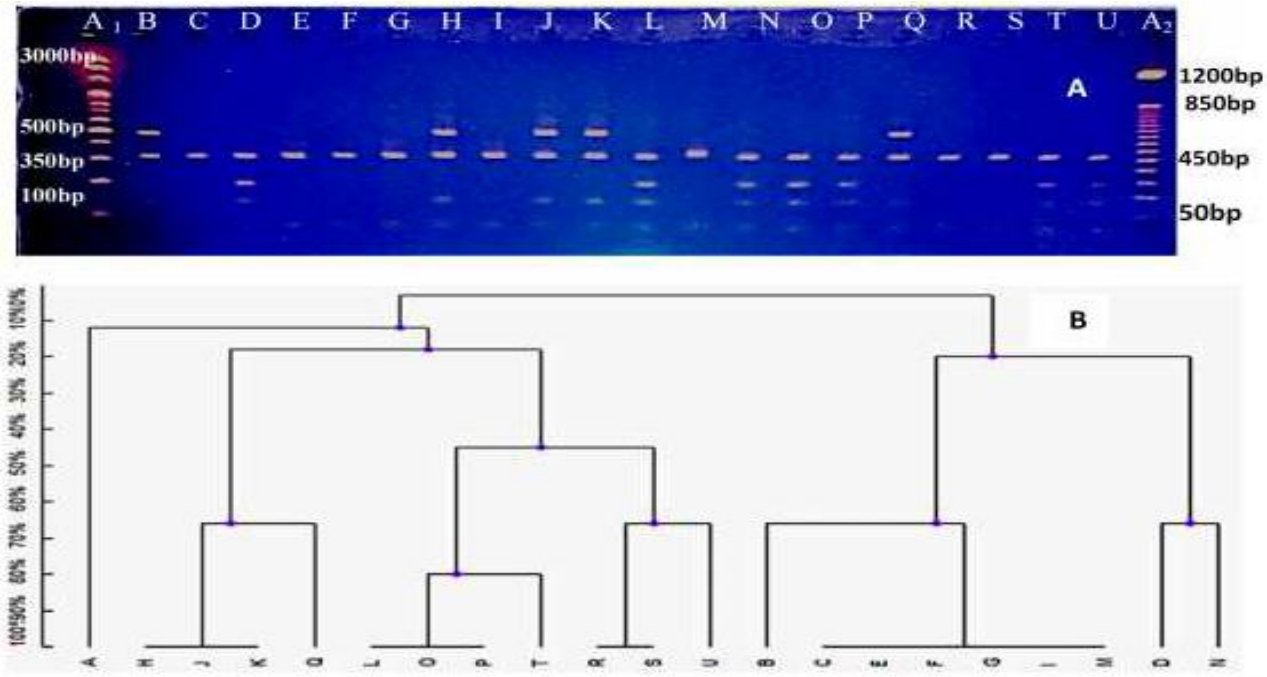

Figure 3. A- Restriction digestion patterns of the transposable intron region of the 25S rRNA gene PCR products with the HhaI enzyme from 20 randomly selected representative isolates of the *Candida* species. Lane A: DNA molecular size marker (A_1 =One step 100 bp ladder; A_2 = one step 50 bp), lanes B, H, J-K and Q: *C. dubliniensis*, lanes C and E = genotype C lanes: D, L, N-P and T-U. A genotype of *C. albicans*, lanes F-G, I, M and R-S = B genotype of *C. albicans*. B- Phylogenetic relationships (homologous coefficient %) between different isolates of *Candida.* The consensus tree was based on data of RFLP-PCR by HhaI enzyme generated via UPGMA cluster analysis. A = molecular size marker 100 bp; B-T, clinical isolates of *Candida***.**

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).

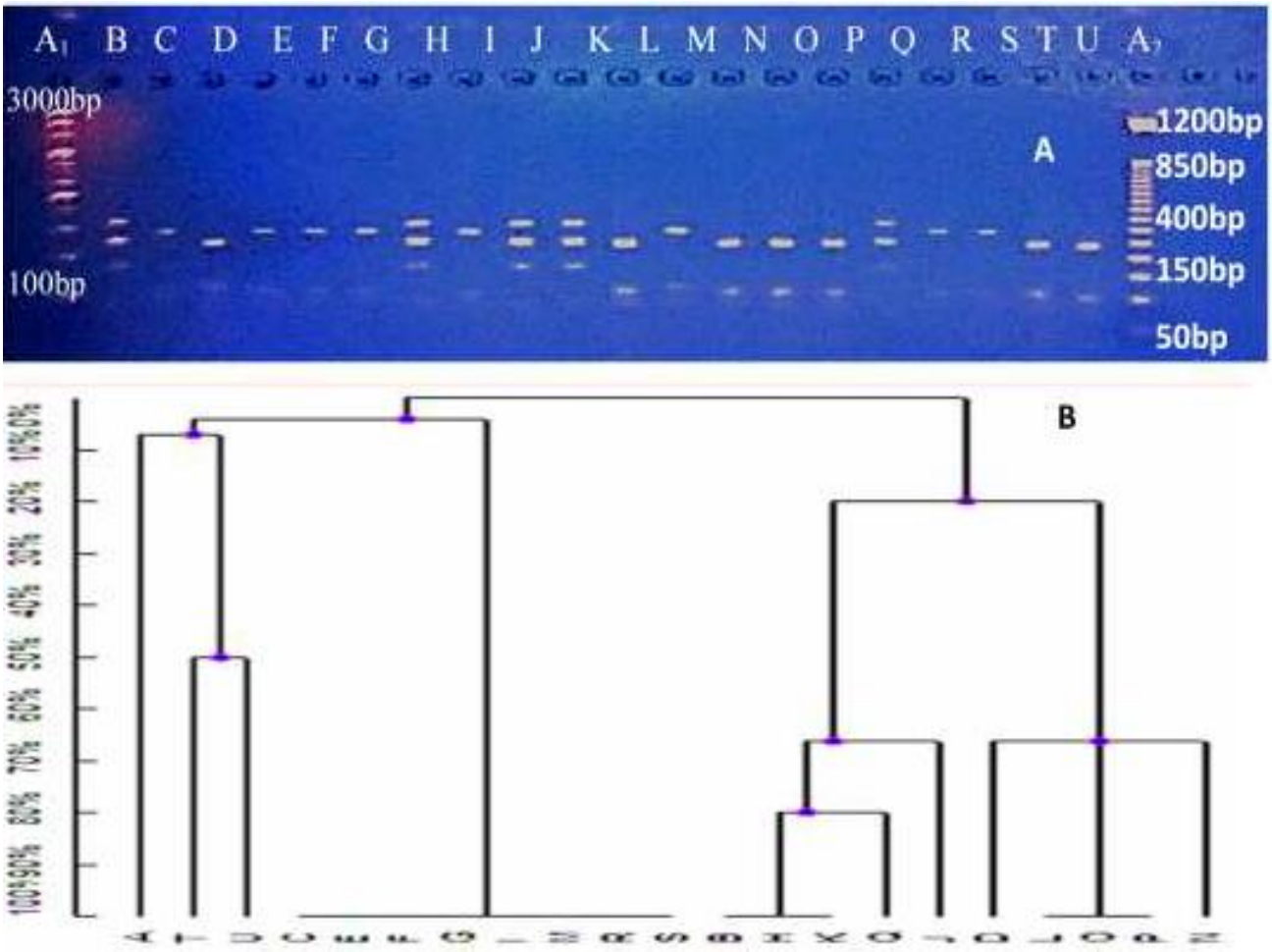

Figure 4. A-Restriction digestion patterns of the transposable intron region of the 25S rRNA gene PCR products with Hae 111 enzyme from 20 randomly selected representative isolates of the *Candida* species. Lane M: DNA molecular size marker (One step 100 bp ladder), lanes B, H, J-K and Q: *C. dubliniensis*, lanes: C and E=genotype C, lanes: D, L, N-P and 19-20) A genotype of *C. albicans*, lanes F-G, I, M and R-S= B genotype of *C. albicans*. B- Phylogenetic relationships (homologous coefficient (%)) between different isolates of *Candida.* The consensus tree was based on data of RFLP-PCR by Hae 111 enzyme generated via UPGMA cluster analysis. A, Molecular size marker 100 bp; B-T, clinical isolates of *Candida*.

Figure 7 showed sequence analysis for eight isolates of *Candida* spp. *C. albicans* isolates D, T and E showed high similarity in their sequence with *C. dubliniensis* isolates B and Q except difference in two adenine nucleotides at the same time, the *C. albicans* isolates C, L and R showed high difference in their sequence with *C. albicans* isolates D, T and E *and C. dubliniensis* isolates B and Q.

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

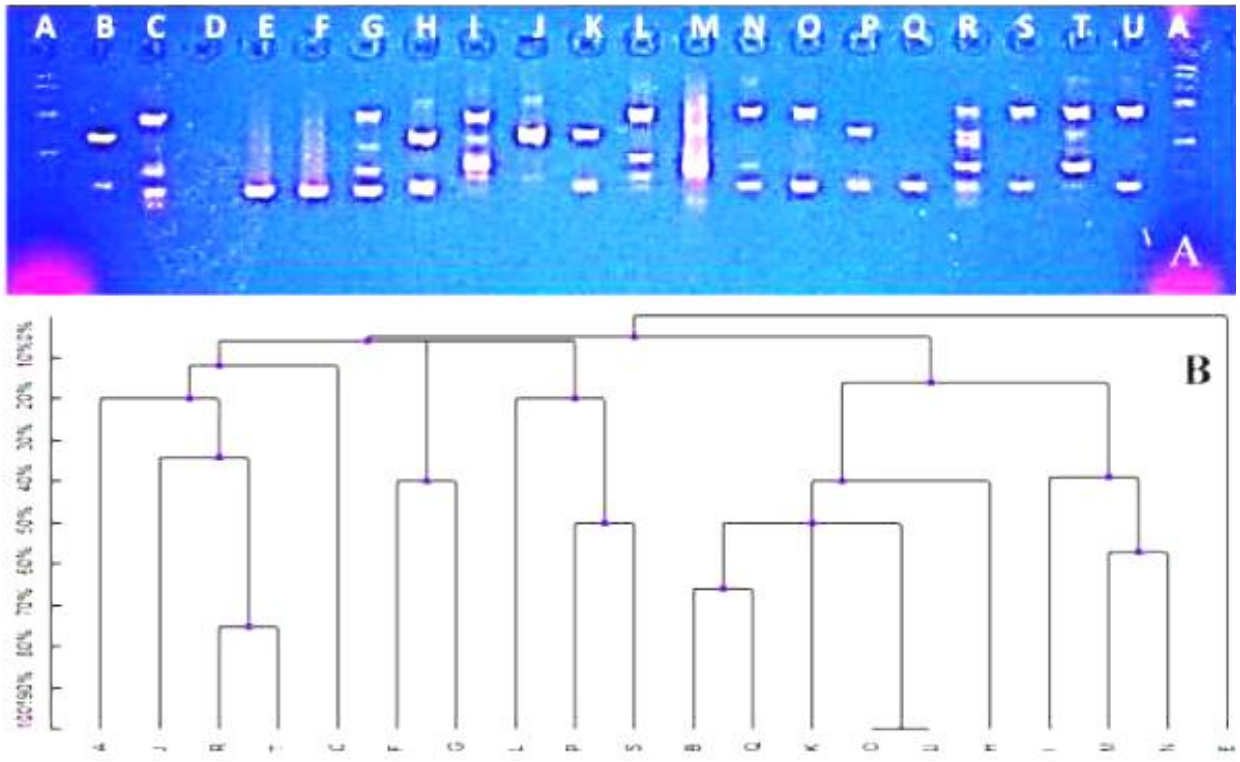

Figure 5. A- Agarose gel electrophoresis of the amplified RAPD-PCR products for *Candida* spp. Detection polymorphism of 20 clinical isolates of *Candida* spp. using the oligo primer GGTGTAGTGT. B- Phylogenetic relationships (homologous coefficient %) between different isolates of *Candida.* The consensus 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*.

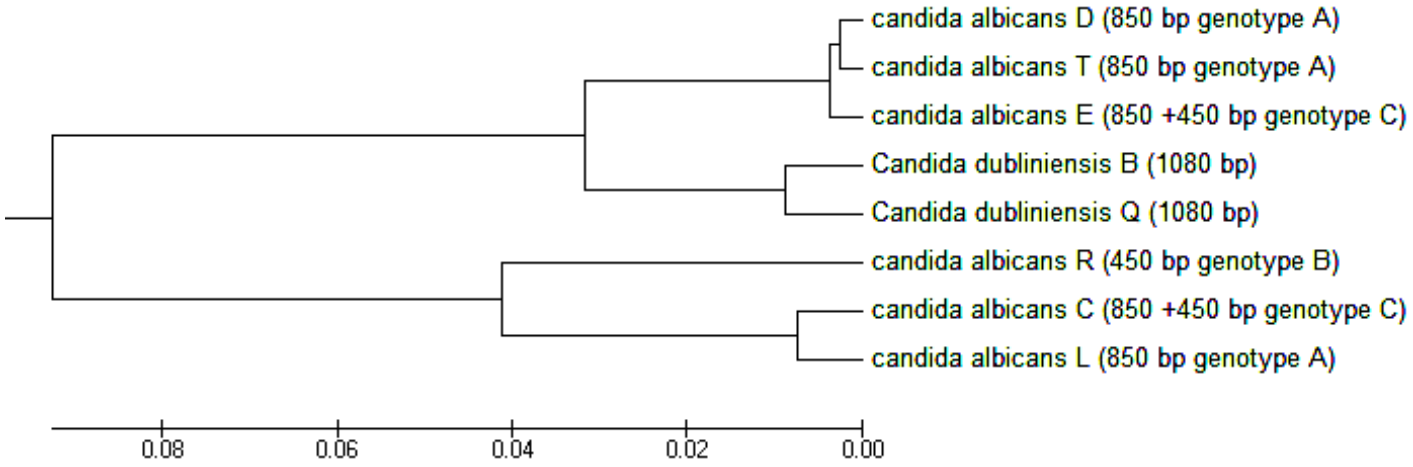

Figure 6. Phylogenetic relationships (homologous coefficient %) between different isolates of *Candida* based on sequence data were constructed employing the Mega 6 software.

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).

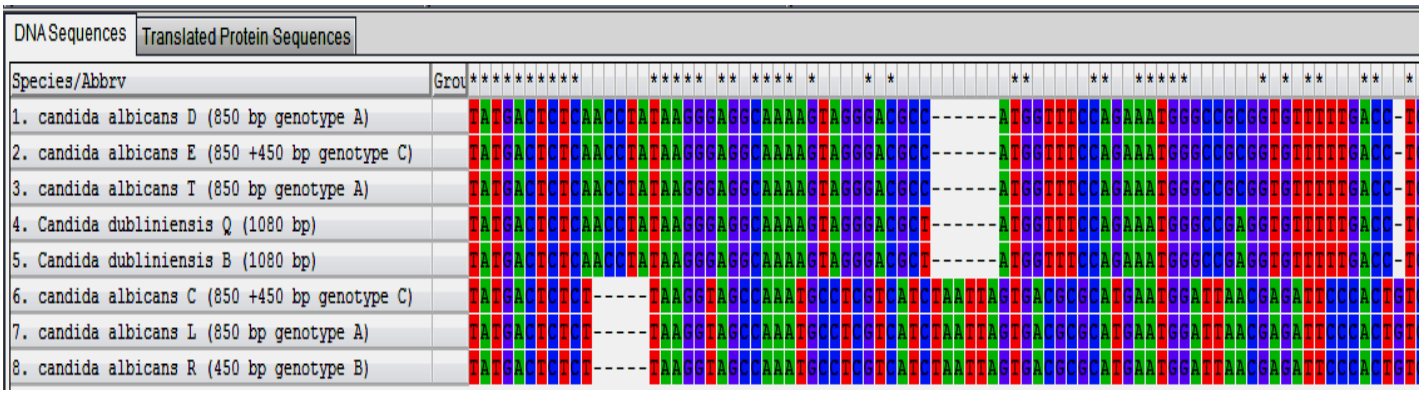

Figure 7. Sequence data were constructed employing the Mega 6 software.

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-patterns. 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. albicans* and *C. dubliniensis.*

The Hae111 enzyme elucidated polymorphic RFLP-patterns 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-patterns. 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 *C. albicans;* in the same trend, we do not observe sufficient differences to separate C. *dubliniensis* from *C. albicans* to warrant a species status. Therefore, we provided further support for its designation and confirmed that C. *dubliniensis* should be considered as a subspecies of *C. albicans.* This judgment, based on the molecular RFLP patterns, such as the HhaI 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 *C. dubliniensis* and *C. albicans* as shown in Figure 7, with only subtle differences in sequence between the two species.

The amendment of *C. dubliniensis* taxonomic state agrees with early and recent studies that are closely related *C. dubliniensis* to *C. albicans* which was routinely misidentified as *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 *C. dubliniensis* on typing a transposable intron region in the 25S rRNA gene from other four genotype strains viz.: genotypes of *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 *C. albicans.* Tamura et al. (2001) referred to the genotype E strain which showed a high degree of similarity to *C. dubliniensis* when compared with the degree of similarity of the strains of the other *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 *C. dubliniensis*, as was expected. From our view, with his erroneous taxonomic judgment along with his temperament and individuality, based on the trend of Tamura et al. (2001), each one of the all the genotypes of *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. *Candida,* thus, may not be as reliable as was considered earlier, to utilize CHROMagar to differentiate between *C. albicans* and *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 *C. dubliniensis* isolates out of five. They also used the growth at 45°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 *C. albicans* genotypes, including the five *C. dubliniensis* strains, which grew well at 45°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 *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 confirmation from previous studies which revealed the very close similarity among the isolates of *C. dubliniensis* to those of *C. albicans, C. dubliniensis* is not an emerging new species. We provided further support for its designation and confirmed that C. *dubliniensis* do not merit being included as a new species and should be considered as *C. albicans* subspecies *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.

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