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Molecular identification of *Candida glabrata* and *C. parapsilosis* based on sequencing analysis of rDNA

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Abstract:
The taxonomy of the most Candida species especially C. parapsilosis and C. glabrata have undergone changes due to confuse in some taxonomic characters such as their color on CHROMagar and biochemical properties compare with accurate molecular method. The aim of this study was to evaluate the feasibility of using sequencing of the ribosomal internal transcribed spacer (ITS) region for identification of 18 Candida isolates. Preliminary CHROMagar assay and amplified ITS regions by primer pair ITS1/ITS4 and sequenced . Species identification performed based on the results of sequences which comparison by BLAST searches for homologous sequences in public databases. The CHROMagar assay results showed confuse in colonies colors of both species revealed white-pink color for both species .The results of PCR assay , rDNA sequences analysis and phylogenetic analyses showed accurate methods for identification of both species, Significant variation was observed among PCR products for 23 Candida species :511bp for C.parapsilosis 720 bp for C.kyfer and 780bp for C.glabrata .Sequence analysis show variation between C.glabrata, and C.parapsilosis and phylogenetic analyses produced different trees relative to analyses with consensus sequences. In conclusion, the CHROMagar candida medium unreliable test, showed high similarity in their color (white –pink color)characters for both C. parapsilosis and C. glabrata ,while the results of ITS sequencing provides a very accurate and useful method for the identification of Candida species in general trend , and C. parapsilosis and C. glabrata were showed distinct species based on sequences of ITS (rDNA).

Keywords :*Candida glabrata, Candida parapsilosis* ,PCR ,rDNA sequencing .phylogenetic analyses

INTRODUCTION

The genus *Candida* contains heterogeneous anamorphic yeasts and comprises about 196-200 species (Murray et al.1995). The more important pathogenic species, *Candida albicans*, *C. parapsilosis*, *C. krusei*, and *C. glabrata*, are phylogenetically related to the Ascomycetes.

CHROMagar medium have been manufactured to distinguish common *Candida* species on the basis of the colonies color (Murray et al**.,**2005) . its support relatively specific colony colors for *Candida albicans*, *C. tropicalis*, and *C. krusei* but not with other *Candida* species such as *C,glabrata* and *C.parapsilosis*, many investigators exaggerated the important of CHROMagar Candida media as comparably high sensitivity medium (Nadeem et al.,2010).Recently many reports showed that CHROMagar Candida media insufficient test for identification of *C.albicans* and subspecies *C.dublinensis* isolates (Imran, 2015). And also couldn't identify *C. glabrata* or *C. parapsilosis* (Baumgartner,et al., 1996; Pfaller, et al., 1996 ;Powell, et al., 1998). Many *Candida* species produce white to pink colonies on CHROMagar (Imran and Al-Asadi 2014).Recently observed that *C. glabrata* clade included more than species were defined molecularly (Alcoba-Florez, et al**.,**. 2005, Correia et al**.,** 2006**).**

Using the CHROMagar Candida in which *C. glabrata* colonies appear pink to purple although rare, some *C. glabrata* strains that formed white colonies on this medium have also been described (Bishop et al., 2008). In fact, in 2005, a group of Spanish researchers isolated three fungal strains from different clinical specimens and although these yeasts were initially identified as *C. glabrata* by the ID 32C kit, subsequent molecular analysis demonstrated that they belonged to a new *Candida* species (called *Candida nivariensis*) phenotypically and genetically related to *C. glabrata* (Alcoba-Flórez et al., 2005). However, the lack of a wide range of colors limits the expansion to more yeast species, and the increased cost of CHROMagar beside confuses results. All these justifications made CHROMagar Candida medium unreadable test has been widely used for initial screening of large *C. glabrata* culture collections on the basis of the white color of their colonies (Bishop et al., 2008; Borman et al., 2008). Moreover, colonies of these

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three species (*C. parapsilosis, C. orthopsilosis* and *C. metapsilosis*) typically appear white on CHROMagar Candida (Tavanti et al., 2005) but, a very recent report described the environmental isolation of two *C. orthopsilosis* strains that showed a pink pigmentation on this medium (Robll et al., 2014) and they may, therefore, be confused with *C. glabrata* (Silva et al., 2012) .

The phenotypic analysis methods currently used for diagnosis *C. parapsilosis* cannot discriminate it at species level among the "psilosis complex", since *C. parapsilosis, C. orthopsilosis* and *C. metapsilosis* show the same cellular morphologies, growth characteristics, biochemical assimilation and fermentation profiles (Tavanti et al., 2005). Owing to the importance of *C. glabrata* and the need for better understanding of its epidemiology, new molecular techniques have been developed to provide strain characteristics (Bretagne et al., 1997; Soll, 2000).

Given the increase in *C. glabrata* infections and their public health importance In particular, *C. glabrata* has emerged as a major cause of mucosal and invasive fungal infection in the United States, second only to *C. albicans* (Wisplinghoff et al.,2004), there is a need to develop reliable molecular tools for species delineation(Guarro et al.,1999). The present study is the first to utilize this approach to identify close related *Candida* species with *C. glabrata*, the results showed that ITS region polymorphisms were useful for *Candida* species identification(Aanen et al.,2001).
Amplified of rDNA is being used for typing of many fungal pathogens (Tamura et al., 2001;Imran and Al-Asadi 2015). Therefore, the right time to assess the potential contribution that other molecular techniques could make towards the identification of the relationships between *C.glabrata* and C. *parapsilosis*.

The aim of this study was evaluate the CHROMagar assay for differentiation between *C. glabrata* and C. *parapsilosis*, and achieve a detailed and unequivocal understanding of the evolutionary relation-ships between them, by performing simple PCR, and emphasizing the identification of genotype variations for both *C. glabrata* and *C. parapsilosis* by using the sequencing analysis and supporting BioEdit software and constructed Neighboring Joining(NJ) dendrogram .

MATERIALS AND METHODS

1-Sapmles collection

A total of 150 swabs samples were collected from different areas of the patient's bodies viz., eye swabs, skin swabs, buckle swabs , bloody santé pads , vaginal swabs and soil samples. Clinical samples using a sterile cotton swabs were taken from body sites and infection sites of different patients hospitalized in Al Surgery hospital Hilla city in Iraq ,samples collection followed Imran and Al. shukry (2014).

2-Identification of *Candida* isolates

Each swabs samples were submerged in 600 µl sterile water with Tween 20 for made suspension ,a loop full of suspension were streaked on both CHROMagar plates and Sabouraud's Dextrose Agar (SDA).. Incubated under standard condition (Tavanti et al., 2005) **,** and then selected only single colonies showed pink and white-pink, purple color only (in this study was excluded all *C. albicans C.tropicales* and *C.krusea* based on colonies color on CHROMagar) The *C.glabrata* and *C.parasilosis* were selected because no visible variation was observed in the CHROMagar assay ,they showed white to white pink colors.

2-DNA extraction:

The DNA template extraction was performed according to Imran
(2015) . Loop full from single colony of *Candida* spp growing on SDA for 24 h was pick up into 600µl of cell lysis buffer and the vortex for two minutes ,added 200 µl nuclear lysis buffer with 20 µl protinase K and incubated for one hour at 55ºC ,then added 100 µl of protein precipitate, mixed gently and incubate in refrigerator for ten minutes .centrifuged for 1mint at 5000 rpm. Discharged the supernatant into new tube and added 500 µl phenol-chlorophorm and vortexed for one minute. and centrifuged 5000rpm for 2 minutes. Discharged upper layer into new tube ,500 µl isopropanol was added, mixed gently for 2 minute and centrifuged for10 minute at 12000 rpm. discharged the isopropanol and rinse the pellet containing DNA with 70% ethanol and dry the DNA pelt .70 µl of TE for dissolved the DNA pellet It was air dried; pellets were re-suspended with 100 µL TE and frozen at -20°C until use (Imran ,2015).

3-PCR and sequencing assays

PCR assay was performed the following condition : The primer pair that targeted the sequences site of the ITS1-5.8S-ITS2 gene was ITS1/ITS4(White et al.,1990) for amplify the rDNA of isolates under our interest . The PCR mixture (25 µl) consisted of 12.5 µl of 20x Master Mix (Promega), 2 µl (10 pemole) of each primer and 1 µl template DNA, made up to 25 µl with molecular-grade water. The PCR mixture was amplified by the thermal cycler PCR System (Labnet, USA) .initial denaturation temperature 95ºC for 5 min, , 30 cycles, 95ºC for 30 sec , annealing temperature of 58ºC for 1.5 min , extension temperature of 72ºC for 1 min ,final extension temperature 72ºC for 10 min ,cool step by 4 ºC (Imran, 2015).
23 PCR products for *Candida* species were sent to Macrogen Laboratory in UAS and the received the sequences data for different *Candida* species . Prior to the sequencing reaction. The sequencing results were subjected

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for alignment based on blast NCBI database .The sequence alignment were performed by BioEdit software.

4-Phylogeny tree

The phylogeny tree of eighteen *Candida* spp species was instructed based on Mega6 software.

Results and Discussion

1- Isolation and Identification

A total of 1150 *Candida* colonies they showed white to white pink colors on CHROMagar out of 3354 yeast colonies were isolated from clinical samples and 25 Candida colonies out of 70 yeast colonies collected from soil samples .Others yeasts included *C.albicans,C.krusei* and *Rhodotorula* were excluded . The distribution of these isolates according to their sites of recovery was summarized in Table 1. Our results was coincidence with previous studies of Momani, (2000) .

Table 1: Distribution of these isolates according to their sites of recovery

Source of sample	No. Of sample	No. of *Candida* spp	Type of *Candida* spp.
Eye swab	20	41	CG,CP*
Vaginal swab	15	283	CG, CK, CP
Buckle swab	35	266	CG, CP, CK
Skin swab	30	370	CG, CK, CP
Blood of pads	15	165	CG, CP, CK
Soil samples	35	25	CG, CK
Total	150	1150	

**CG : Candida glabrata ;CP : Candida parapsilosis ;CK : Candida kefyr*

2-CHROMagar assay

The results of this test showed different colonies colors(*C. albicans* colony showed green color, *C. tropicalis* blue colony color , *C. krusei* showed pink color) on the CHROMagar medium ,but on the SDA these *Candida* spp showed white-creamy to brown –creamy color. Black arrow refer to *C.albicans* (CA) While the blue arrow refer to *C.kefyr* (CK) that are creamy on SDA medium(Figure 1) . Our results agree with those of Paritpokee et al. (2005) and Nadeem et al. (2010).

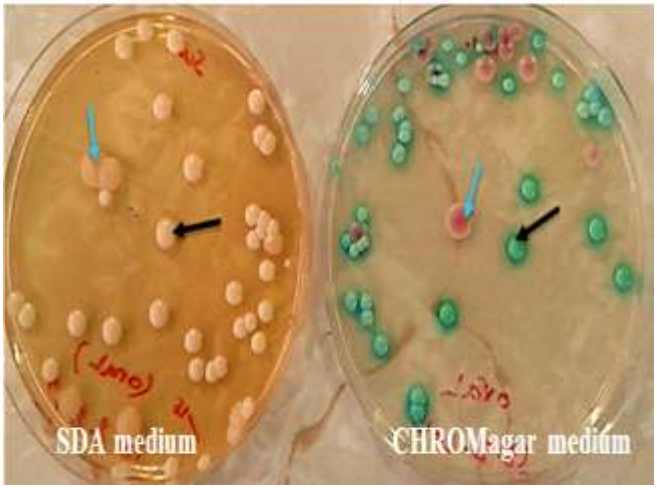

Figure (1): Appearance of colonies of *Candida* spp. on both of SDA and CHROMagar media. Black arrow refer to *C.albicans* (CA) While the blue arrow refer to *K. marxianus* (anamorph *C.kefyr*) that are creamy on SDA medium.

K. marxianus (anamorph *C.kefyr*) exhibited a pale pink on CHROMagar medium and this result is similar to those reported by Rodrigues et al. (2001). *C.glabrata* and *C. parapsilosis* exhibited variety of colors on CHROMagar medium ranged from white to whitish purple or pale pink (Figure 2) . Our result coincidence with those of Madhavan et al.(2011) .

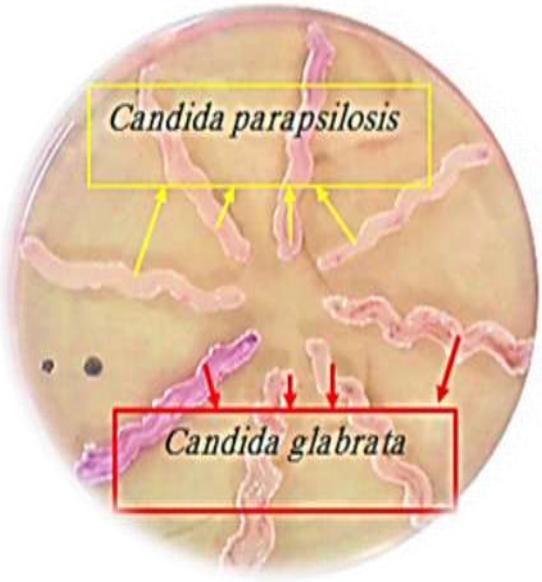

Figure (2): Appearance of *C.parapsilosis* and *C.glabrata* colonies on CHROMagar incubation period for 24 h at 30ºC.

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Both *C.parapsilosis* (CP) and *C.glabrata* (CG) showed ranged of color from white to white-pink , Our results agree with those of Agrawal et al.(2008). Elongation of incubation period led to change of colonies colors were became more bold with the time *Candida* colonies color development on CHROMagar medium under different incubation periods(24 h, 48 h and 72h), Figure(4) these color changes reduces the efficiency of this medium for diagnosis and made it not a reliable for diagnosis 100% for *Candida* spp. Our results coincidence with Imran ,(2015).

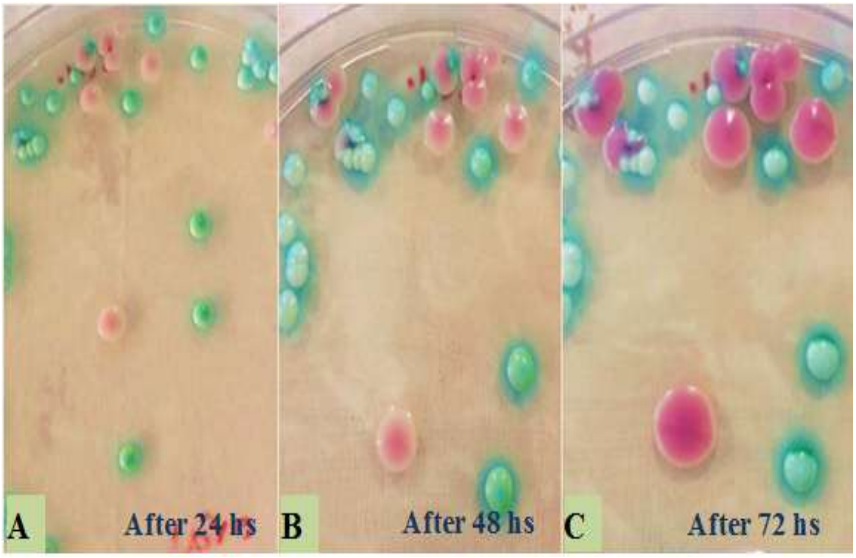

Figure (3):Appearance of *Candida* spp colonies on CHROMagar under different incubation periods (for 24 h ,48 h and 72 h.).

A-PCR assay

The results showed different molecular sizes of PCR products of *Candida* spp. Amplified the ITS region of 23 isolates (Table 1) by universal primers ITS1 /ITS4. These isolates were between 511-780 bp. Figure(4).

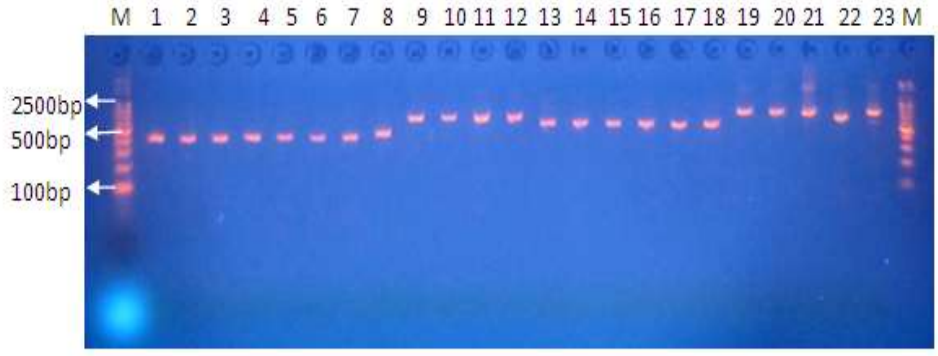

Figure (4).**:** Agarose gel electrophoresis of PCR products for *Candida* species isolates amplified by pair primers 1TS1 /ITS4. Lane M= Molecular marker100 bp; lanes 1- 8 isolates of *C.parapsilosis* isolates (511 bp). lanes 9- 12.19-21,23 isolates of *C.glabrata* (879 bp). lanes 13-18,22 *K. marxianus* isolates(720 bp).molecular weight key based on Fujita et al. (2001).

B-Sequence analysis

A total of 18 PCR products out of 23 PCR products of *Candida* under interest were succeeded in sequencing analysis. High sequence similarities were observed in the ITS between *C.glabrata* (99–100% similarities) and (94–100% similarities) among isolates of *C.prapsilosis* few intraspecific variation in the ITS region was found in all *Candida* spp under our interest that were sequenced .about 1-5 nucleotide (Nts) similar between *C.glabrata* and *C.prapsilosis* labeled be rectangles (Figure 5). High sequence similarities were observed in the ITS region between *Candida* spp (97% similarities) and distancing itself from the species *C.parapsilosis* (93% similarities) (Figure 5) .

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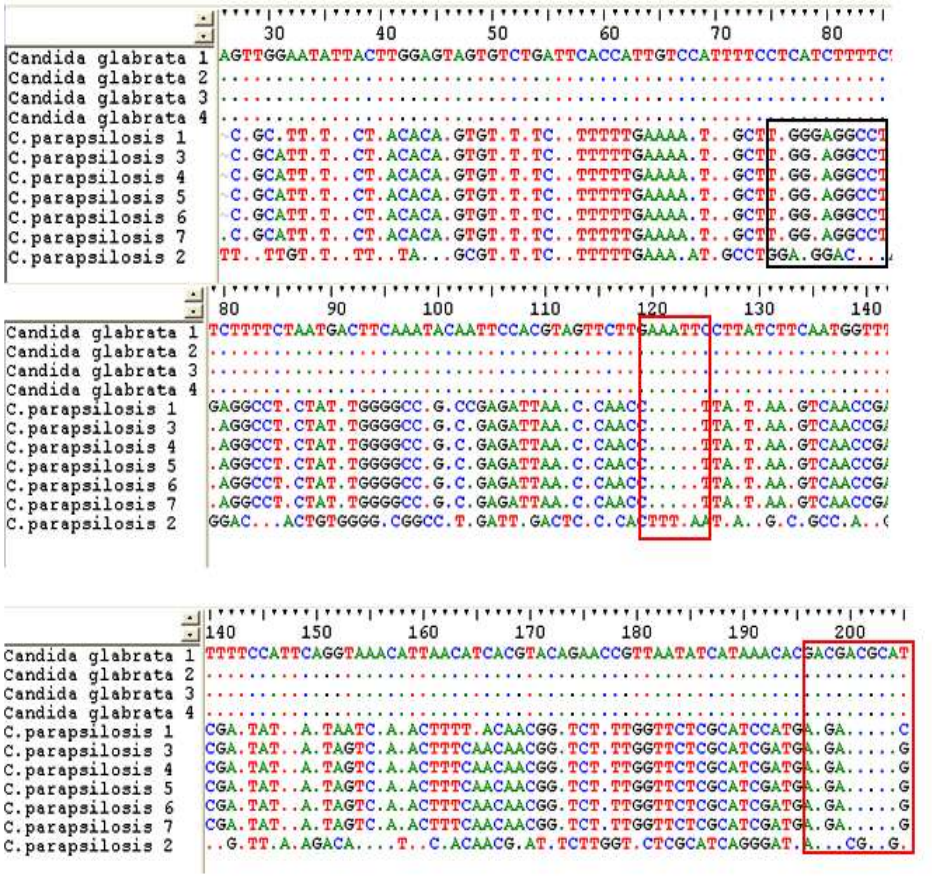

Figure (5):Selected of partial sequence alignment of ITS regions. The sequences of two species of *Candida* spp were aligned by using the Clustal W computer programs. alignment. *C.glabrata* (4 isolates) *C.prapsilosis (*7 isolates) respectively. .about 1-5 of the nucleotide sequence similar between *C.glabrata* and *C.prapsilosis* labeled be rectangles.

C-Phylogeny tree:

Neighboring Joining(NJ) tree of 18 *Candida* spp on the basis of their ITS1 sequences was constructed with data for standard strains of *Candida* species four clusters of *Candida* spp. Were observed *C.parapsilosis* showed in cluster 1 ,*C.intermedia* in cluster2, *Kluyveromyces marxianus* in. cluster 3 and *C.glabrata* in cluster 4 while *C.pararugosa* showed out of the other clusters .

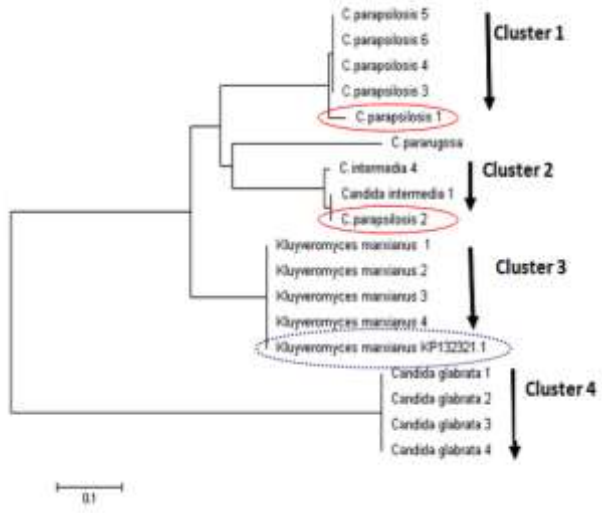

Figure (6): Phylogeny tree for 18 *Candida* species constructed by Neighbor -joining (NJ) tree based only on ITS1-5.8S-ITS2 gene sequences, identification of for 18 *Candida* isolates species into four clusters.

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Recently, sequencing analysis based on the ITS1-5.8S-ITS2 gene region showed a high taxonomic cohesiveness for 18 *Candida* isolates commonly found within clinical specimens,this results agree with Broge et al.(2005). In the current study, the dendogram of ITS1-5.8S-ITS2 of 18 isolates of *Candida* isolates from different clinical specimens (e.g., eye swabs, skin swabs, buckle swabs , bloody santé pads , vaginal swabs) generated 4 clusters, which were identified as in (Figure 6). Although the NJ tree based on ITS1-5.8S-ITS2 observation suggests that isolates of *Candida* isolates did not yield a single genotype but very close no identical populations. In our study, the maximum intraspecific evolutionary divergence between *C. parapsilosis* isolates 1 and 2.

Table 1**.** Distance matrix between the ITS rDNA sequences of 18 isolates of clinical swabs. Below the diagonal are the absolute distances corresponding to the number of divergent bases. Above the diagonal are the percentages of different bases in relation to the total number of bases sequenced.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. Candida glabrata 1																		
2. Candida glabrata 2	0.0																	
3. Candida glabrata 3	0.0	0.0																
4. Candida glabrata 4	0.0	0.0	0.0															
5. Candida intermedia 1	1.0	1.0	1.0	1.0														
6. C.parapsilosis 2	1.0	1.0	1.0	1.0	0.0													
7. C.intermedia 4	1.0	1.0	1.0	1.0	0.0	0.0												
8. C.pararugosa	1.0	1.0	1.0	1.0	0.4	0.4	0.4											
9. C.parapsilosis 1	1.1	1.1	1.1	1.1	0.4	0.4	0.3	0.5										
10. C.parapsilosis 3	1.1	1.1	1.1	1.1	0.3	0.3	0.3	0.4	0.0									
11. C.parapsilosis 4	1.1	1.1	1.1	1.1	0.3	0.3	0.3	0.4	0.0	0.0								
12. C.parapsilosis 5	1.1	1.1	1.1	1.1	0.3	0.3	0.3	0.4	0.0	0.0	0.0							
13. C.parapsilosis 6	1.1	1.1	1.1	1.1	0.3	0.3	0.3	0.4	0.0	0.0	0.0	0.0						
14. Kluyveromyces marxianus 1	1.0	1.0	1.0	1.0	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3					
15. Kluyveromyces marxianus 2	1.0	1.0	1.0	1.0	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.0				
16. Kluyveromyces marxianus 3	1.0	1.0	1.0	1.0	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.0	0.0			
17. Kluyveromyces marxianus 4	1.0	1.0	1.0	1.0	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.0	0.0	0.0		
18. Kluyveromyces marxianus KP132321.1	1.0	1.0	1.0	1.0	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.0	0.0	0.0	0.0	

Most of *Candida* species showed similar size PCR products of amplified ITS region ,sometimes may not easily to observed the variation in the size of PCR products ,in this case we in need for sequence analysis of these PCR products for detects the intraspesiphic variation or microevolution in the genome under interest . Sequence similarity appears to remove all PCR method-based ITS variation. However some of this microvariation in fact might be real, a result observed by Simon and Weiss (2008) in four Ascomycota species to determine whether the microvariation observed in this study. Some of difference sequences were observed in this study were significantly greater than 5% different from the consensus. Our study Agree with Lindner and Banik. (2011).

In conclusion, the CHROMagar candida medium unreliable test, showed high similarity in their color (white –pink color)characters for both *C. parapsilosis* and *C. glabrata* ,while the results of ITS sequencing provides a very accurate and useful method for the identification of *Candida* species in general trend , and *C. parapsilosis* and *C. glabrata* were showed distinct species based on sequences of ITS (rDNA).

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