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# MOLECULAR STUDY OF TWO FUNGI *MILLEROZYMA FARINOSA* AND *CANDIDA ORTHOPSILOSIS* BY PCR USING ITS GENE AND PHYLOGENETIC STRUCTURING TREE ISOLATED FROM DIABETIC PATIENT IN BAGHDAD

**Sama Mustafa Mohammed* and Israa Ahmed Ali*[1]**

Department of Biology, Collage of Science, University of Baghdad, Iraq.

## Abstract

Diabetic mellitus is one of the main risk factors of fungal infections because poor glycemic control is associated with a high level of glucose in blood and saliva which could be treated as nutrient to fungi. This study aimed to isolate and identification of pathogenic fungi from diabetic patient. 140 samples were taken from different places of human body from the national center of diabetic patients that related to Mustansiriyah University / college of medicine and Al-yarmuk Hospital in Baghdad. 84 sample (60%) tested positive to fungi and 56 sample (40%) tested negative to fungi. The most frequented fungi isolated have been chosen for molecular identification by PCR (*Millerozyma farinosa* and *Candida orthopsilosis*) using specific primers (ITS1 and ITS 4) and phylogenetic structuring tree. Analysis was done by sequences and confirmation of microorganism's homogenic data using database (NCBI) after amplification of Fungi's ribosomal RNA. Result showed clinical isolate *Milerozyma farinosa* showed 100% compatibility and score (1112) and clinical isolate *Candida orthpopsilosis* showed 100% compatibility and score (893) with wild type of ITS gene from gene bank.

***Key words :*** *Millerozyma farinose, Candida orthopsilosis*, gene.

## Introduction

Diabetes mellitus is a group of metabolic diseases characterized by high blood glucose level (American Diabetes Association., 2010). Diabetes mellitus is being increasing globally worldwide. Fungal infection is more common in diabetic because derangement of glucose metabolism, relative insulin availability, defect in collagen production and poor wound healing affects all organ systems in the body of diabetic patients (Nern, 2002).

Infections of fungi may occur on the surface of the skin, in skin folds, and in worm areas which kept moist by clothes and shoes. They may occur at the site of an injury, in mucous membranes, the sinuses, and the lungs. (Santhosh *et al.,* 2011). Many of causative fungi are opportunistic and usually effected immunocompromised individuals the most common fungal diseases in humans is the Superficial infection of the skin and mucosal infection of oral and genital tract(Abu-Elteen *et al.,* 2006). *Candida* is the most common pathogen that cause invasive fungal disease in immunocompromised persons (Puebla, 2012), it affects various sectors of the world population, colonization with *Candida* species occurs more frequently in diabetic patients compared with non-diabetic individuals (Bohannon, 1998). There is also relationship between smoking and higher rate of fungal infections by diabetics. *Millerozyma farinosa* is another opportunistic pathogen. It has been identified as a cause of fungemia (Anaissie *et al.,* 1989). And colonization of oral mucosa was reported in immunocompromised patients (Alder *et al.,* 2007).

Identification of fungi using conventional method are often problematic require a great deal of skill and time consuming (Sugita *et al*. 2003). DNA and RNA have been used in classification and identification of fungi using Polymerase Chain Reactions (PCR) and sequencing. The diagnosis by PCR technique depends on amplifying small molecules of DNA using primers (a small nucleotide that able to bind to the target DNA) to generate large

***Author for correspondence :** E-mail : sama_mustafa@yahoo.com

quantities of a specific DNA from a complex DNA template in a simple enzymatic reaction in only few hours (Aswad, 2018).

In this study *Millerozyma farinosa* and *Candida orthopsilosis* were isolated from diabetic patients that the first molecular study in Baghdad.

## Materials and Methods

### Culture media

In this study sabourauds dextrose agar (SDA) (Oxoid/ England) was used as fungal medium prepared according to the instructions of the Manufacture Company. Then Chloramphenicol solution of 0.05 g/L added to the agar solution to inhibit the growth of bacteria.

### Sample collection

This study carried out on diabetic patient from national center of diabetic patients that related to Al Mustansiriyah university/College of Medicine and Al-yarmuk Hospital in Baghdad. Swaps were taken from different places of human body such as (skin, mouth, ear, eye, nail and vagina) using sterile transport media swabs.

### Direct microscope examination

Swaps were subjected for direct examination by taking rubbing the swaps on a clean slides mounted with a drop of lactophenol cotton blue and covered with a covers slip. Then examined under the microscope looking for fungal elements (Ali, 2017).

### Identification and diagnosis of fungi

The diagnosis of fungi was depend on the colony morphology and color on pertri dishes from both sides, in addition to the shape of sporangium if found and spores according to Kidd *et al*., (2016).

### Identification of fungi by PCR using ITS gene

The most frequented fungi isolated have been chosen for molecular identification by PCR.

### DNA Extraction

Genomic DNA was isolated from fungal growth according to the protocol of ABIOpure Extraction, at first loopful of grown hypha was suspended in 500µl of Buffer YL in 1.5 ml micro-centrifuge for pellet cells. Then 7.5µl of 20mg/ml lyticase was added and mix gently. Then mixes were incubated at 37°C for 30 - 60 min to digest the cell wall. After incubation, cell pelleted using centrifuge at 5,000 x g for 5 min. The supernatant then removed and cell pellet was re-suspended completely in 200µl of Buffer CL. And 20µl of Proteinase K solution (20 mg/ml) was added to the tube and mixed vigorously using vortex and Incubated at 56°C for 15 min for protein digestion. After complete lysis, the lysis mixture will turn to clear from turbid. After that 200µl of Buffer BL was added to the tube then the tube mixed thoroughly using vortex and Incubated at $70^0$C for 10 min also 200µl from absolute ethanol was added to the sample this help DNA associated with mini column, pulse-vortex to mix the sample thoroughly. After addition of ethanol, All of the mixture was transferred to the mini column carefully, then centrifuge for 1 min at 6,000 x g above (>8,000 rpm), and the collection tube was replace with new one. If the mixture has not passed completely through the membrane, centrifuge again a full speed (>13,000 x g) until all of the solution has passed through. Then 600µl From Buffer BW was Added to the mini column, and centrifuge for 1 min at 6,000 x g above (>8,000 rpm) and the collection tube was replaced with a new one. After that 700µl From Buffer TW 700µl was applied. Centrifuge for 1 min at 6,000 x g above (>8,000 rpm). The pass-through was discarded and the mini column was reinserted back into the collection tube. The mini column was Centrifuge at full speed (>13,000 x g) for 1 min to remove residual wash buffer, then the mini column was placed into a fresh 1.5 ml tube. And 200µl From Buffer AE was added and Incubated for 1 min at room temperature, then Centrifuge at full speed (>13,000 xg) for 1min.

### Quantitation of DNA

Quantus Florometer was used to detect the concentration of extracted DNA. For 1 µl of DNA, 199 µl of diluted Quanty Flour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

### Reaction Setup and Thermal Cycling

After extraction of DNA the PCR components was performed in a total volume 25 µl as shown in the table (1.1):

### PCR program

The thermal cycling condition was done as follow (1.2)

### Agarose Gel Electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria.

Preparation of agarose :

100 ml of 1X TAE was taken in a beaker.

1. 1 gm (for 1%) agarose was added to the buffer.
2. The solution was heated to boiling (using water bath) until all the gel particles were dissolved.

**Table (1.1):** Showed mixture Component required for PCR reaction.

| Master mix components | Stock | Unit | Final | Unit | Volume 1 Sample |
|---|---|---|---|---|---|
| Master Mix | 2 | X | 1 | X | 12.5 |
| Forward primer | 10 | µM | 1 | µM | 1 |
| Reverse primer | 10 | µM | 1 | µM | 1 |
| Nuclease Free Water | | | | | 7.5 |
| DNA | | ng/µl | | ng/µl | 3 |
| Total volume | | | | | 25 |
| Aliquot per single rxn | 22µl of Master mix per tube and add 3 µl of Template | | | | |

**Table (1.2):** Showed amplification reaction program of PCR.

| Steps | ℃ | m:s | Cycle |
|---|---|---|---|
| Initial Denaturation | 95 | 05:00 | 1 |
| Denaturation | 95 | 00:30 | 30 |
| Annealing | 55 | 00:30 | |
| Extension | 72 | 00:30 | |
| Final extension | 72 | 07:00 | 1 |
| Hold | 10 | 10:00 | |

3. 1µl of Ethidium Bromide (10mg/ml) was added to the agarose.
4. The agarose was stirred in order to get mixed and to avoid bubbles.
5. The solution was allowed to cool down at 50-60C°.

**Casting of the horizontal agarose gel**

The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to solidfy at room temperature for 30 minutes. The comb was carefully removed and the gel was placed in the gel tray. The tray was filled with 1X TBE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

**DNA loading**

PCR products were loaded directly. For PCR product, 5µl was directly loaded to well. Electrical power was turned on at 1v/cm$^2$ for 1h. DNA moves from Cathode to plus Anode poles. The Ethidium bromide stained bands in gel were visualized using Gel imaging system.

**Standard Sequencing**

PCR product were send for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received by email then analyzed using genious software.

## Results

**Samples were examined**

In this study 140 specimen were collected from different places of diabetic patients diagnosed clinically by a specialist doctor and blood tests which include fasting blood sugar values (FBS) and other tests from national center of diabetic patients that related to Mustansiriyah University / college of medicine and Al-yarmuk Hospital in Baghdad. Using sterile transport media swabs.

The study was carried out on 140 patients there age were ranging from 20 to 84 years with mean 52 years. The fungal infection recorded among females 71 (50.71%) which were higher than males 69 (49.29%). Identification of fungal infection in this study was based on direct microscopic examination using lacto phenol blue smears which were showed the fungal element in 30 (21%) samples of the total cases, while in culture fungal growth were positive in 84 (60%) samples of the total cases and 56 sample (40%) were negative to fungi. From the total positive cases 84 (60%) *Milerozyma farinosa* and *Candida orthopsilosis* represented the most frequently isolated yeast that showed 10 isolates (6.6 %) and 9 isolates (6.0 %) respectively.

**Identification of fungi by PCR using ITS gene**

Two of fungi isolated have been chosen for molecular identification by PCR. Using specific primers (ITS1 and ITS4) in two clinical isolates (*Millerozyma farinosa and Candida orthopsilosis*).

**Extraction Genomic DNA**

Genomic DNA was extracted successfully from 2 isolates using ABIOpure Extraction kit after the activation of isolates from clinical samples, the kit provide a simple and convenient technique to isolates high quality and purity DNA from fungi. Quantus Florometer was used to detect the concentration of extracted DNA. Concentration of *Millerozyma farinosa* (16 ng/ml) *and Candida orthopsilosis* (8.4 ng/ml).

**Detection of ITS region in *Millerozyma farinosa and Candida orthopsilosis***

Polymerase chain reaction ( PCR) preformed for detection of ITS gene region in *Millerozyma farinosa and Candida orthopsilosis*, samples show positive result for ITS region with PCR product > 500 pb. Genomic DNA of isolates was recognized and complementary to ITS primer sequence and represented by the presence of a single band in molecular weight *Millerozyma farinosa* (669bp) and *Candida orthopsilosis* (512bp). The results demonstrated that ITS1 and ITS4 primer based

PCR method had high sensitivity and specificity in detection of fungi. The figure show agarose gel electrophoresis for amplification of ITS region.

**Sequencing and alignment of NCBI**

PCR product samples were sent for sequence analysis of *Millerozyma farinosa and Candida orthopsilosis*. The samples were treated with ABI3730XL Applied biosystem machine, automated DNA sequencer, by Macrogen Corporation– Korea Company. The result of sequence analysis was analyzed by blast in the National centre biotechnology information (NCBI) online.

Clinical isolate *Milerozyma farinosa* showed 100% compatibility and score (1112) and clinical isolate *Candida orthpopsilosis* showed 100% compatibility and score (893) as shown in the table.

The table represent description and identities of sequences in fungi *Millerozyma farinose* and *Candida orthopsilosis,* which analysed by the blast in the National centre Biotechnology Information (NCBI).

**Submission of local Iraq isolate in NCBI**

The 18S Rrna gene of *Millerozyma farinosa* were registered after the correspondence of the National Centre Biotechnology Information (NCBI) and obtained accession number and became a reference to Iraq and Middle East. It is available for download at https:// www.ncbi.nlm.nih.gov/nuccore/1682061222/ MN062264.

**Phylogenetic tree structuring**

The phylogenetic tree diagrammatic by Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 sequences that showed neighbor-joining species and reflect the actual evolutionary pathways.

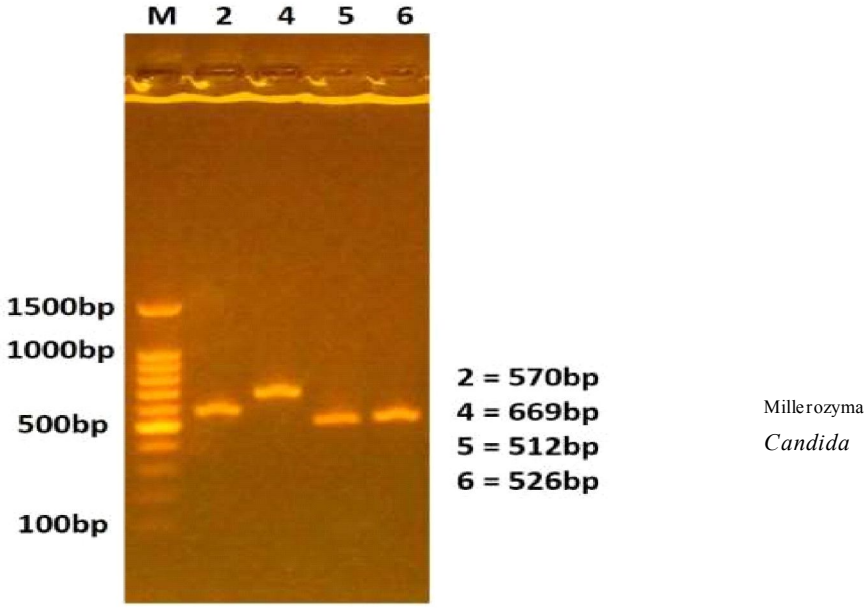


**Fig (1.1):** Represented Agarose gel electrophoresis for amplification of ITS region to *Millerozyma farinosa and Candida orthopsilosis.*

**Table (1.3):** Represent description and identities of four fungi.

| Description | Maxscore | Total score | Querycover | Evalue | Identities | Accession |
|---|---|---|---|---|---|---|
| *Millerozyma farinosa* culture CBS:2768 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence ; and large subunit ribosomal RNA gene, partial sequence. | 1112 | 1112 | 100% | 0 | 100% | KY104529.1 |
| *Candida orthopsilosis* strain SLDY-266 small subunit ribosomal RNA gene, partial sequence, internal transcribed spacer 1, ; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence. | 893 | 893 | 100% | 0 | 100% | KY104529.1 |

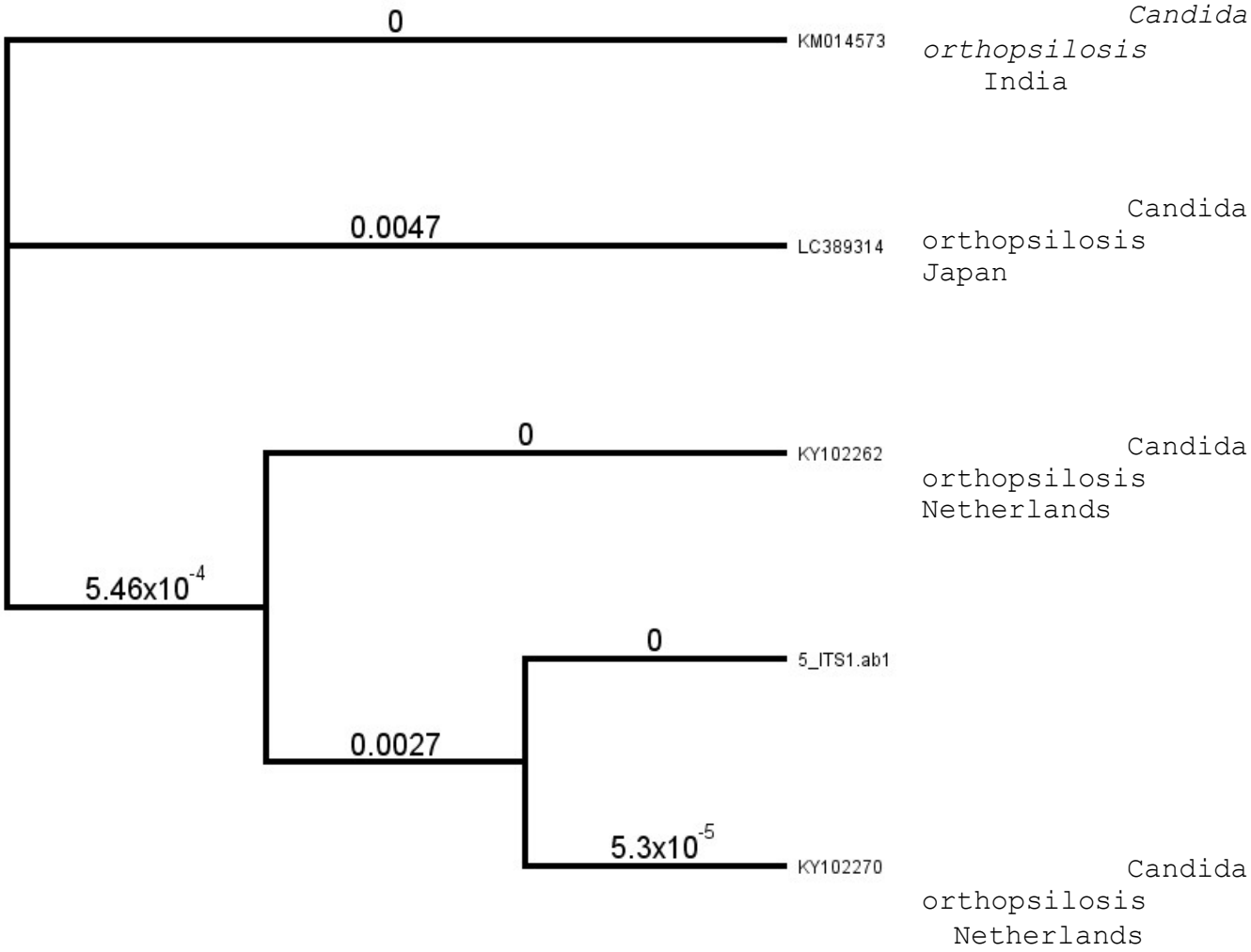


**Fig (1.2):** *Candida orthopsilosis* neighbor-joining tree of 18S rRNA gene.

***Candida orthopsilosis* sequencing:**

**_ITS1:**

CCTGCGGAAGGATCATTACAGAATGAAAGTGCTTAACTGCATTTTTTTACACATGTGTTTTTCTTTTTTTGAAAACTTTGCTTT

GGTGGGCCCACGGCCTGCCAGAGATTAAACTCAACCAAATTTTATTTAAAGTCAACTGATTAACTAATAGTCAAAACTTTCA
ACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATATTCGTGAAT
CATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAAACCTTC

GGGTTTGGTGTTGAGCGATACGCTGGGTTTGCTTGAAAGAAAGGCGGAGTATAAACTAATGGATAGGTTTTTTTCCACTCA
TTGGTACAAACTCCAAAATTCTTCCAAATTCGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATA

**5_ITS4:**

TATGCTTAAGTTCAGCGGGTAGTCCTACCTGATTTGAGGTCGAATTTGGAAGAATTTTGGAGTTTGTACCAATGAGTGGAAA

AAAACCTATCCATTAGTTTATACTCCGCCTTTCTTTCAAGCAAACCCAGCGTATCGCTCAACACCAAACCCGAAGGTTTGAGG

GAGAAATGACGCTCAAACAGGCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACGAAT

ATCTGCAATTCATATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAGTTTTGA

CTATTAGTTAATCAGTTGACTTTAAATAAAATTTGGTTGAGTTTAATCTCTGGCAGGCCGTGGGCCCACCAAAGCAAAGTTTT
CAAAAAAAGAAAAACACATGTGTAAAAAAATGCAGTTAAGCACTTTCATTCTGTAATGATCCTTCCGCAGG

**ITS1:**

TGGTATCTTGCCAGCGCTTAATTGCGCGGCGAGTGCTATTAGAAGTCCATAAGTTCTTACACACAGTGTTTTTTGTTTGTGAA

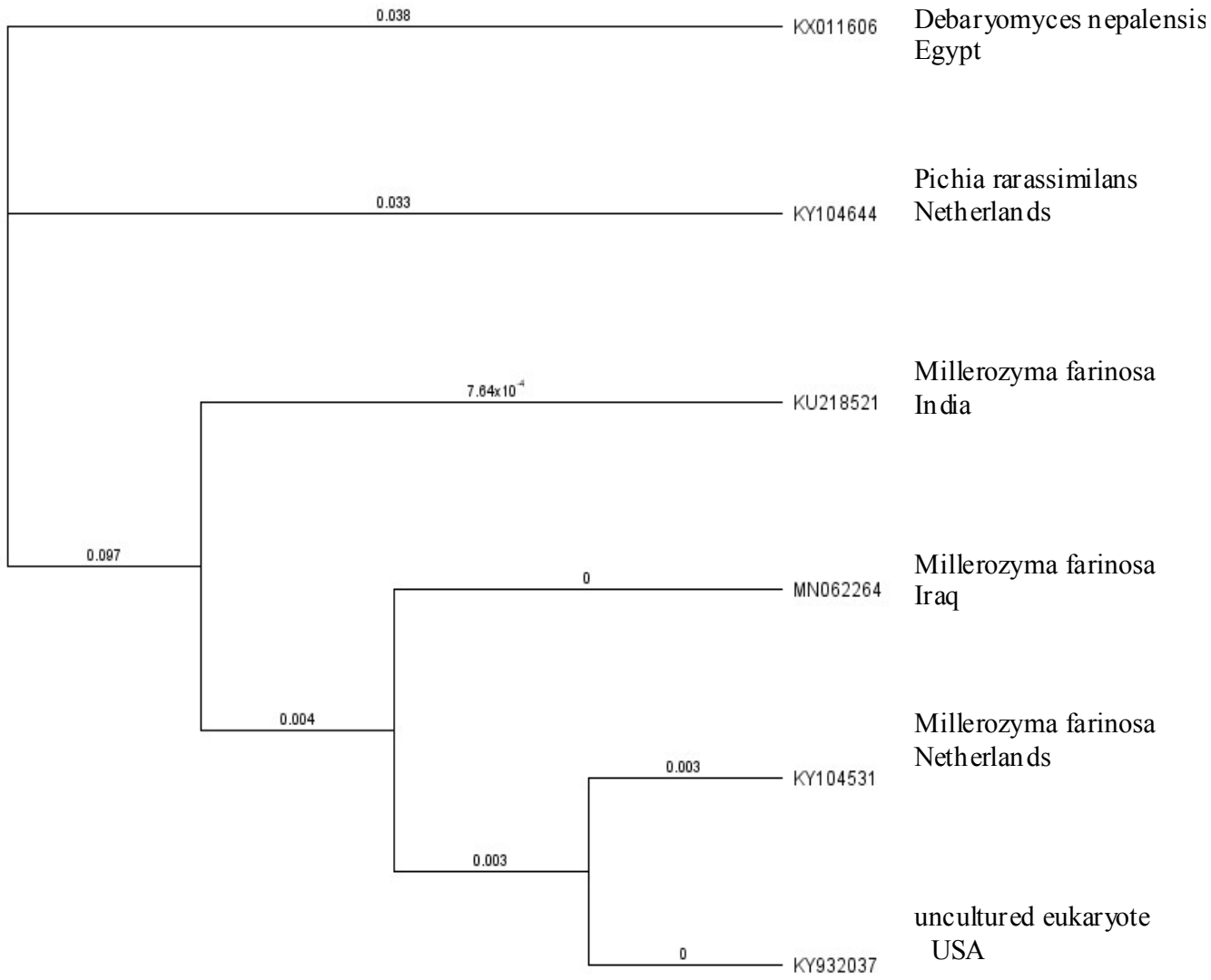


**Fig (1.3):** *Millerozyma farinosa* neighbor-joining tree of 18S rRNA gene.

AAAAAATTACTTTGGTCTGGAGCTAGAAATAGTTTTGGGCCAGAGGACAACCTAACTTCAATTTATATTGAATTGTTTTTAAA
TTTATTTGTCAAATTATTGATATTAATCAAAAATCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAAC
GCAGCGAAATGCGATAAGTAATATGAATTGCAGATTTTCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTAT
TCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCTCTCAAACCGCAAGGTTTGGTGTTGAGCGATATAGATATTCAGTATCTA
TTTGCTTGAAATGGATTGGCATGAGTATTTACAGTAGATAAATGCCGTTTGACTCTTCAATGTATTAGGTCTAACCAACTCGT
TGAAACAGTTAGCGGTAGTATCTGTGTAAAAGAGGCTCGGCCTTACAACAATCTACAAAGTTTGACCTCAAATCAGGTAGG
AATACCCGCTGAACTTAAGCATA

**ITS4:**

TATGCTTAAGTTCAGCGGGTATTCCTACCTGATTTGAGGTCAAACTTTGTAGATTGTTGTAAGGCCGAGCCTCTTTTACACAG
ATACTACCGCTAACTGTTTCAACGAGTTGGTTAGACCTAATACATTGAAGAGTCAAACGGCATTTATCTACTGTAAATACTCA
TGCCAATCCATTTCAAGCAAATAGATACTGAATATCTATATCGCTCAACACCAAACCTTGCGGTTTGAGAGAGAAATGACGC
TCAAACAGGCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACGAAAATCTGCAATTCAT
ATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAGTTTTGAAGATTTTTGATTA
ATATCAATAATTTGACAAATAAATTTAAAAACAATTCAATATAAATTGAAGTTAGGTTGTCCTCTGGCCCAAAACTATTTCTA
GCTCCAGACCAAAGTAATTTTTTTTCACAAACAAAAAACACTGTGTGTAAGAACTTATGGACTTCTAATAGCACTCGCCGCGC
AATTAAGCGCTGGCAAGATACCA

## Discussion

In this study focus on fungal infections collected from diabetic patients because they are very often prone to fungal infections, Diabetes is one of the leading causes of morbidity and mortality across the globe, and this disease is projected to increase from 425 to 629 million adults between 2017 and 2045. The association between diabetes and infections has been linked to a number of causal pathways, including impaired immune responses due to high blood sugar which lead to suppression of certain immune proteins that move toward infections and kill microbes. Also extra sugars make it easier for fungi to colonize the skin cells and mucus gland (Cary *et al*., 2018).

Different colonies of fungi isolated from diabetic patient were observed on (SDA) at 35±2 C° for 3-7 days incubation. The most frequented fungi isolated have been chosen for molecular identification by PCR using ITS gene which has been widely used as genetic marker during the last 15 years for exploring fungal diversity (Bellemian *et al*., 2010). The result of this study demonstrated that rDNA-based PCR method had high sensitivity and specificity for wide range of medically important fungi. However identification using conventional method is difficult or fails (Kim *et al*., 1999).

The fungal pathogen was identified by PCR and DNA sequencing to amplify ITS region of the rDNA gene of *Millerozyma farinosa* and *Candida orthopsilosis,* then sequences were genetically analysed to distinguish fungi at species level and determining phylogenetic relationship between neighbor-joining fungal isolates. The result agrees with many studies such as Luo and Mitchal. (2002), Turenne *et al*., (1999) and Lau et al. (2007) who concluded that the use of PCR automated DNA sequencing permits the identification of clinical isolates to the species level. This procedure provides a molecular tool for the diagnosis of mycological infections, because of its relative simplicity and rapidity.

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