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Finding Pathogenic Fungi in Immunocompromised Individuals in Al-Najaf City

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ABSTRACT: This research aimed to explore the identification of the Phenotype and genotype of Debaryomyces species isolated from patients with diabetic foot in Al-Najaf City. Samples were collected from the depths of wounds in diabetic foot patients, ensuring aseptic precautions were taken, and then cultured on Sabouraud's dextrose agar, followed by incubation at 30-35 °C for a duration of 5-7 days to promote growth. The identification of colonies was based on their microscopic characteristics. Subsequently, these yeasts were grown in chrome agar to isolate and identify Debaryomyces spp. A PCR technique was conducted to magnify the ITS1 and ITS4 sequences for the diagnosis of Debaryomyces spp., and the PCR products were exposed to sequencing using the Sanger method. All samples isolated were grown on SDA, and when utilizing CHROMagar differential agar. The findings indicated various molecular sizes of the ITS region for Debaryomyces spp., with the primer pair (ITS1-ITS4) targeting these ITS regions for the same sample. The products of PCR were dispatched to Macrogen Lab in the USA, where one sample of replicated products of PCR (both reverse and forward strands) was analyzed. The sequences in the current study were then matched with reference global sequences. It can be concluded that Debaryomyces species emerge as the most prevalent fungal pathogens associated with life-threatening invasive infections in highly immunocompromised individuals or those who have had major trauma and prolonged stay in the intensive care units. They occupy the fourth place as the most common agents of nosocomial-acquired bloodstream infections in hospitals.

INTRODUCTION

Fungal infections are significant diseases that have a profound effect on human life, primarily due to microorganisms (fungi) that share similarities in cellular and metabolism functions with infected cells (whether animal or human). This resemblance complicates both treatment and diagnosis, in addition to the identification of the specific fungi species by healthcare professionals [1]. The genus Debaryomyces, an ascomycetous group, reproduces sexually through jointure between the fungal cell and its bud or among independent cells. The available literature regarding the roles of Debaryomyces is often unclear, ambiguous, and at times even contradictory, likely because D. hansenii represents a great diverse species with significant variations in phenotype and instances of misidentification [2]. Debaryomyces hansenii is a hemiascomycetous yeast recognized for its undeniable biotechnological significance [3]. This yeast species exhibits heterogeneity and can thrive in extreme environments, including high salinity or relatively basic pH levels. It demonstrates increased respiratory activity coupled with low levels of fermentation [4]. Fermentation varies significantly based on the strain and the conditions under which it is cultivated [5]. In summary, some of its advantageous contributions include the formation of xylitol [6], lipases, and exopeptidases that are vital in the food production, as well as hemophilic glycosidase, which is essential for the formation of fuel alcohol [3]. Additionally, D. hansenii is regarded as a typical fungi for eukaryotic microorganisms in the exploration of osmotic adaptations and salt tolerance. This microorganism thrives in various carbon sources and is known to produce killer toxins [2]. Intriguingly, among yeasts, D. hansenii appears to possess the greatest coding capability [7]. It colonizes a wide range of specific microhabitats, suggesting the presence of yeast subpopulations that have exposed to adaptation to these environments and exhibit chromosomal polymorphism. Consequently, specific probes have been reported to aid in investigating the biodiversity within D. hansenii [8]. From the beginning of 1980s, D. hansenii has been grouped with other isolates such as Candida famata, which has been viewed as anamorphic counterpart in clinical practice. These classifications were primarily founded on imprecise phenotypic traits that are now under scrutiny. At present, a variety of biochemical and molecular methods are used for the precise identification of D. hansenii since it is difficult to differentiate this yeast from other similar species [9]. This particular yeast has been associated with several human infections, including but not limited to catheter-related mediastinitis, retinopathy occult zonal acute, peritonitis and infections bloodstream [10].

MATERIAL AND METHOD

Profound swabs were collected from the depths of the wounds of each patient with diabetic foot. These samples were collected in Al-Sadder Medical City, Al-Najaf City from January 2023 to December 2023. All swabs were investigated to the microbiology laboratory, at the College of Sciences, in Kufa University, for inoculation on Sabouraud's Dextrose Agar (SDA) at 30-35 °C for 5-7 days to aid in the growth of fungi. Fungi were diagnosed macroscopically and microscopically. Fungal isolates were inoculated in chrome agar for isolation and diagnosis of Debaryomyces spp. For DNA extraction, 1.5 ml culture broth was put into Eppendorf tubes, centrifuged at 4,300 x g for 5 minutes, and the supernatant was removed. Then, it was added 200 µl of TE buffer, mixed well, boiled in a water bath for 10 minutes, and then kept at ice for 1 minute. This was spun again for 10 minutes at 6,700 x g and the supernatant was kept aside, which contains the DNA template. PCR amplification was performed to produce ITS1 and ITS4 sequences in the identification of yeast species. In the current research, the primers used were ITS4-R (5'-TCCTCCGCTTATTGATATGC-3') and ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') [11]. For PCR reaction mixture set up, 5 µl of master mix prepared for a 100 µl PCR tube was added first; this was followed by adding 5 µl of template DNA extract, 2.5 µl of the 10 pmol/µl specific solution for the upstream (reverse) primer, and another 2.5 µl of the 10 pmol/µl specific solution for the downstream (forward) primer. Finally, Deionized Distilled Water was used to reach the total volume 20 µl. Vortex the tube to ensure the lyophilized blue pellet is completely dissolved and spin down briefly. The PCR amplification system was used with the following program: The reaction was initiated by a 4-minute denaturation step at 94°C, then 35 cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C, and a final extension at 72°C for 7 minutes. For the amplification products, agarose gel at 1.3% (w/v) was used for electrophoresis and subsequently stained with ethidium bromide. PCR product sequencing from fungal species was performed at Macrogen Lab, USA with obtained sequence data for each single fungal species. The processor kit of the company (Promega, Madison, USA) was used for PCR purification as described by the company in its protocols. Thereafter, the results of the sequencing were processed for multi-alignment by the BioEdit software when the product of PCR was exposed to sequencing by the Sanger method.

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RESULTS MORPHOLOGICAL IDENTIFICATION MICROSCOPIC IDENTIFICATION

A preliminary test for diagnosing Debaryomyces includes microscopic examination. This examination has been done at the advanced Mycology laboratory, Science Faculty, University of Kufa for both diagnosis and study purposes. Each sample is first stained with Lacto-phenol cotton blue stain before being examined microscopically. The diagnosis of pseudohyphae and chlamydospores was done by direct microscopic examination, figure (1).

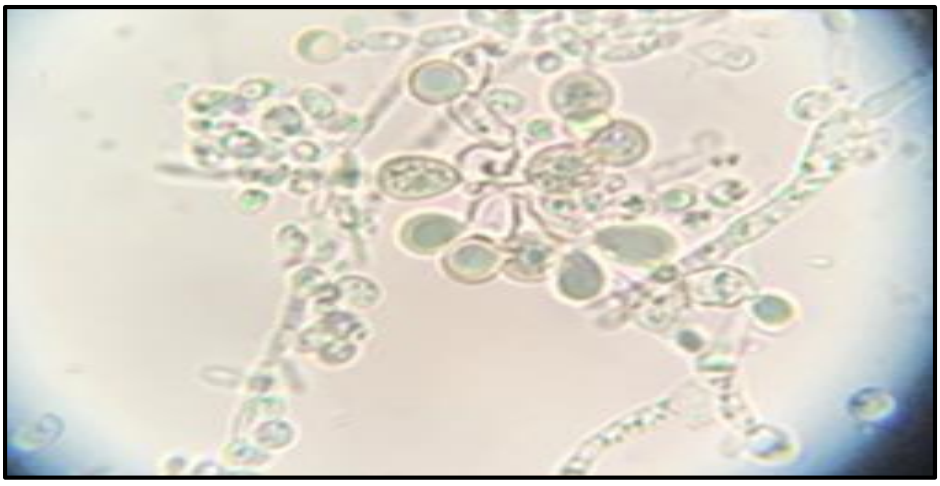


Figure 1: Micrographs about D. hansenii (chlamydospores and pseudohyphae) (all with magnification power 40x)

DETECTION ON SDA MEDIUM

All isolated samples were grown on SDA; the colonies of D. hansenii were characterized by wrinkled, butyrous and smooth margin, figure (2).

Figure 2: D. hanseniicolonies growing on SDA for 5-7 days at 30-35 Cº

IDENTIFICATION ON CHROMAGAR MEDIUM

This research has revealed that using CHROMagar Candida which is used as a differential medium the colonies appearD. Hansenii pink color with wrinkled peripheral, this result was disagree with [12] who appeared purple violet of D hansenii on CHROMagar, figure (3).

Figure 3: D. hanseniicolonies culture on CHROMagar for 5-7 days at 30-35 Cº

This is agar medium that has a rapid and efficient performance in the identification of C. neoformans at species level through the color reactions observed upon inoculation and incubation. This technique, which differs from conventional methods for culturing, is based on enzymes specific to the species that react with a chromogenic substrate unique in identifying samples with more than one yeast species. All yeast sample were cultured well on CHROMagar Candida after 5-7 days of incubation at 30-35°C; most yeasts were vigorous growers under these conditions, according to the provided leaflets [13].

Molecular identification PCR assay 3.2.1

The findings indicated variations in the molecular sizes of the ITS region among various yeast species. Additionally, figure (4) and table (1) presented the PCR products for these isolates. The primer pair utilized for the same sample, (ITS1ITS4), specifically targeted the ITS regions. The diagnosis of D. hansenii was conducted in accordance with the key provided in [14].

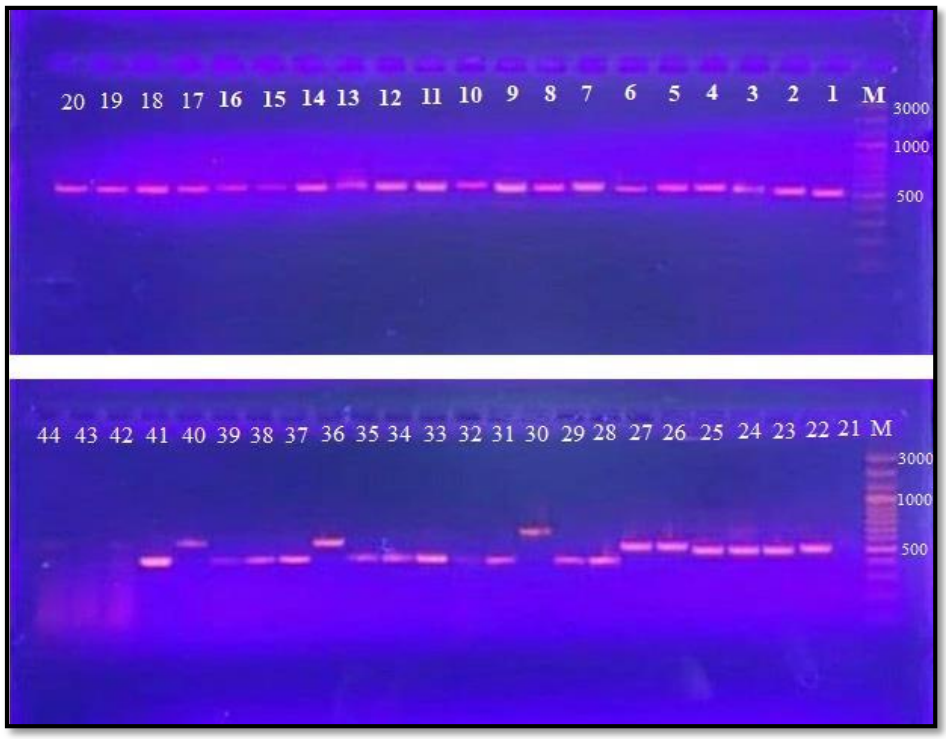


Figure 4: PCR products of ITS regions from various yeasts were analyzed using agarose gel electrophoresis (1.3g agarose gel, 80 volts for 1 hour) with the primer pair (ITS1-ITS4) (M: DNA ladder; lane 30: D. hansenii).

Table 1: Molecular weight of bands formed by replicated of ITS1 and ITS4 regions from fungal spp using UVIB and soft ware

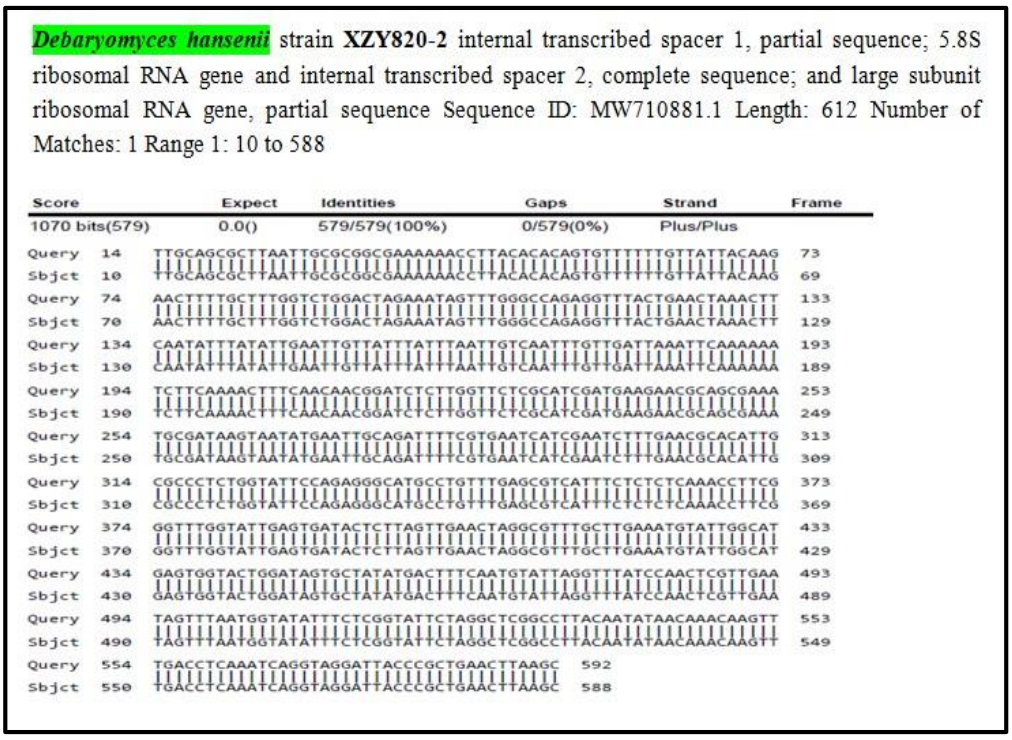
Isolates No.	Yeast identify	Molecular weight
30	D. hansenii	632-634

Sequences analysis

The rest of the PCR product was sent to Macrogen Lab, Inc in the USA for sequencing. One isolate of each of the forward and reverse strands of the locally replicated PCR products was sequenced. These sequences were matched with globally available reference sequences at the National Center for Biotechnology Information (NCBI) Gene Bank. Sequence data for the L30 strains were received by alignment to the NCBI database online using the BLAST tool and multiple alignments to each other using BioEdit software. The sequences in FASTA format were subsequently submitted to NCBI through Sequin software. The L30_LF strain was identified as being the nearest neighbor of D. hansenii strain XZY820-2 and had 100% identity (Table 2 and Figure 5). The L30_LR strain was closest to D. hansenii strain UOA/HCPF 10197B and showed a 99.66% identity (Table 3 and Figure 6).

Table 2: DNA sequences alignments of isolated L30_LF (D. hansenii) in comparing with database obtained from NCBI website

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len
Debaryomyces hansenii strain XZY820-2 internal transcribed spacer 1, partial sequence; 5.8S ri...	Debaryomyce...	1070	1070	94%	0.0	100.00%	612
Debaryomyces hansenii strain CBS 767 18S small subunit ribosomal RNA gene, partial sequenc...	Debaryomyce...	1064	1064	94%	0.0	99.83%	2917
Debaryomyces hansenii strain CBS 767 small subunit ribosomal RNA gene, partial sequence; int...	Debaryomyce...	1064	1064	94%	0.0	99.83%	2774
Debaryomyces vindobonensis AAN18 genes for ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and c...	Debaryomyce...	1064	1064	94%	0.0	99.83%	592
Debaryomyces hansenii isolate OUCMDZ-5157 small subunit ribosomal RNA gene, partial sequ...	Debaryomyce...	1064	1064	94%	0.0	99.83%	644



Debaryomyces hansenii strain **XZY820-2** 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 Sequence ID: MW710881.1 Length: 612 Number of Matches: 1 Range 1: 10 to 588

Score	Expect	Identities	Gaps	Strand	Frame
1070 bits(579)	0.0()	579/579(100%)	0/579(0%)	Plus/Plus	

```
Query  14   TTGCAGCGCTTAATTGCGCGGCGAAAAAACCTTACACACAGTGTTTTTTGTTATTACAAG  73
Sbjct  10   TTGCAGCGCTTAATTGCGCGGCGAAAAAACCTTACACACAGTGTTTTTTGTTATTACAAG  69
Query  74   AACTTTTGCTTTGGTCTGGACTAGAAATAGTTTGGGCCAGAGGTTTACTGAACTAAACTT  133
Sbjct  70   AACTTTTGCTTTGGTCTGGACTAGAAATAGTTTGGGCCAGAGGTTTACTGAACTAAACTT  129
Query  134  CAATATTTATATTGAATTGTTATTTATTTAATTGTCAATTTGTTGATTAAATTCAAAAAA  193
Sbjct  130  CAATATTTATATTGAATTGTTATTTATTTAATTGTCAATTTGTTGATTAAATTCAAAAAA  189
Query  194  TCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAA  253
Sbjct  190  TCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAA  249
Query  254  TGCGATAAGTAATATGAATTGCAGATTTTCGTGAATCATCGAATCTTTGAACGCACATTG  313
Sbjct  250  TGCGATAAGTAATATGAATTGCAGATTTTCGTGAATCATCGAATCTTTGAACGCACATTG  309
Query  314  CGCCCTCTGGTATTCCAGAGGGCATGCCTGTTTGAGCGTCATTTCTCTCTCAAACCTTCG  373
Sbjct  310  CGCCCTCTGGTATTCCAGAGGGCATGCCTGTTTGAGCGTCATTTCTCTCTCAAACCTTCG  369
Query  374  GGTTTGGTATTGAGTGATACTCTTAGTTGAACTAGGCGTTTGCTTGAAATGTATTGGCAT  433
Sbjct  370  GGTTTGGTATTGAGTGATACTCTTAGTTGAACTAGGCGTTTGCTTGAAATGTATTGGCAT  429
Query  434  GAGTGGTACTGGATAGTGCTATATGACTTTCAATGTATTAGGTTTATCCAACTCGTTGAA  493
Sbjct  430  GAGTGGTACTGGATAGTGCTATATGACTTTCAATGTATTAGGTTTATCCAACTCGTTGAA  489
Query  494  TAGTTTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTT  553
Sbjct  490  TAGTTTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTT  549
Query  554  TGACCTCAAATCAGGTAGGATTACCCGCTGAACTTAAGC  592
Sbjct  550  TGACCTCAAATCAGGTAGGATTACCCGCTGAACTTAAGC  588
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Figure 5: Pair wise alignment of ITS region of L30_LF (D. hansenii) strain XZY820-2using NCBI online blast

Table 3: DNA sequences alignments of isolated L30_LR (D. hansenii) in comparing with database obtained from NCBI website

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len
Debaryomyces hansenii var. hansenii strain UOA/HCPF 10197B isolate ISHAM-ITS_ID MITS360 1...	Debaryomyces...	1066	1066	94%	0.0	99.66%	639
Debaryomyces sp. isolate FTJA0004 internal transcribed spacer 1, partial sequence; 5.8S ribosom...	Debaryomyces...	1064	1064	94%	0.0	99.66%	588
Debaryomyces hansenii isolate URFM599 18S ribosomal RNA gene, partial sequence; internal tra...	Debaryomyces...	1064	1064	94%	0.0	99.66%	605
Debaryomyces hansenii isolate URFM605 18S ribosomal RNA gene, partial sequence; internal tra...	Debaryomyces...	1064	1064	94%	0.0	99.66%	604
Debaryomyces hansenii isolate URFM608 18S ribosomal RNA gene, partial sequence; internal tra...	Debaryomyces...	1064	1064	94%	0.0	99.66%	606

Debaryomyces hansenii var. hansenii strain **UOA/HCPF 10197B** isolate ISHAM-ITS_ID MITS360 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Sequence ID: GQ376085.1 Length: 639 Number of Matches: 1 Range 1: 14 to 596

Score	Expect	Identities	Gaps	Strand	Frame
1066 bits(577)	0.0()	581/583(99%)	0/583(0%)	Plus/Minus	

```
Query  11   ACTGATTTGAGGTCAACTTGTTTGTTATATTGTAAGGCCGAGCCTAGAATACCGAGAAAT  70
Sbjct  596  ACTGATTTGAGGTCAACTTGTTTGTTATATTGTAAGGCCGAGCCTAGAATACCGAGAAAT  537
Query  71   ATACCATTAAACTATTCAACGAGTTGGATAAACCTAATACATTGAAAGTCATATAGCACT  130
Sbjct  536  ATACCATTAAACTATTCAACGAGTTGGATAAACCTAATACATTGAAAGTCATATAGCACT  477
Query  131  ATCCAGTACCACTCATGCCAATACATTTCAAGCAAACGCCTAGTTCAACTAAGAGTATCA  190
Sbjct  476  ATCCAGTACCACTCATGCCAATACATTTCAAGCAAACGCCTAGTTCAACTAAGAGTATCA  417
Query  191  CTCAATACCAAACCCGAAGGTTTGAGAGAGAAATGACGCTCAAACAGGCATGCCCTCTGG  250
Sbjct  416  CTCAATACCAAACCCGAAGGTTTGAGAGAGAAATGACGCTCAAACAGGCATGCCCTCTGG  357
Query  251  AATACCAGAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACGAAAATCTGCAATTCA  310
Sbjct  356  AATACCAGAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACGAAAATCTGCAATTCA  297
Query  311  TATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAACCCAGAGATCCGTTGTT  370
Sbjct  296  TATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTT  237
Query  371  GAAAGTTTTGAAGATTTTTTGAATTTAATCAACAAATTGACAATTAAATAAATAACAATT  430
Sbjct  236  GAAAGTTTTGAAGATTTTTTGAATTTAATCAACAAATTGACAATTAAATAAATAACAATT  177
Query  431  CAATATAAATATTGAAGTTTAGTTCAGTAAACCTCTGGCCCAAACTATTTCTAGTCCAGA  490
Sbjct  176  CAATATAAATATTGAAGTTTAGTTCAGTAAACCTCTGGCCCAAACTATTTCTAGTCCAGA  117
Query  491  CCAAAGCAAAAGTTCTTGTAATAACAAAAAACACTGTGTGTAAGGTTTTTTCGCCGCGCA  550
Sbjct  116  CCAAAGCAAAAGTTCTTGTAATAACAAAAAACACTGTGTGTAAGGTTTTTTCGCCGCGCA  57
Query  551  ATTTAGCGCTGGCAAAAAGAATACTGTAATGATCCTTCCGCAG  593
Sbjct  56   ATTAAGCGCTGGCAAAAAGAATACTGTAATGATCCTTCCGCAG  14
```

Figure 6: Pair wise alignment of ITS region of L30_LR (D. hansenii) strain UOA/HCPF 10197BusingNCBI online blast

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