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RESEARCH ARTICLE

MOLECULAR ISOLATION, SCREENING AND IDENTIFICATION OF HYDROCARBON DEGRADING FUNGI FROM OIL CONTAMINATED SOILS, KRI, IRAQ

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ABSTRACT

This study aims to isolate and recognize (native/indigenous) hydrocarbon degrading fungal strains from oil-laden soils, at different geographical locations in KRI oilfields, Iraq. The identification was mainly based on macroscopic and microscopic examinations, but due to morphological similarities, which make the identification at species level doubtful in some cases, an attempt was made to use molecular techniques as well, as confirmatory/comparative analysis. The extracted DNA from hypha was used for polymerase chain reaction (PCR) amplification, using specific internal transcribed spacer primer (ITS1/ITS2). PCR product sequencing analyses were compared with the other related sequences in GenBank (NCBI) for molecular evolutionary analyses. Results showed that; out of 68 screened fungal strains; only two genera (i.e. *Aspergillus* spp. and *Penicillium* sp.) were identified as the most potent hydrocarbon degrading fungi. DNA sequence analysis confirmed the validity of the species as; *Aspergillus fumigatus*-KU321562.1; *A. flavus*-MH270609.1; *A. niger*-MK452260.1 and *Penicillium chrysogenum*-MK696383.1.

KEYWORDS

Hydrocarbon degrading fungi, molecular identification, oil-laden soil, KRI oilfields, Iraq

1. INTRODUCTION

Numerous studies have demonstrated that petroleum hydrocarbons are thought to be biodegraded in the natural environment, primarily by a variety of bacterial and fungal species (Wang et al., 2016; Varjani, 2017; Alsohaili and Bani-Hasan, 2018; Ite and Ibok, 2019; Ahmad and Ganjo, 2020a).

According eukaryotic fungus organisms are among the best organisms capable of breaking down oil hydrocarbon compounds to (Ahmad and Ganjo 2020a). Numerous fungal species, including Trichoderma, Mortiecerella, Aspergillus spp., Alternaria, Talaromyces, Cephalosporium, Penicillium, Geotrichum, Fusarium, and Cladosporium, have been found through various studies to be capable of using crude oil as their primary source of energy and carbon. As a result, Aspergillus and Penicillium derived from oil-polluted sites would be very impressive in decomposing drilling wastes and oil spills (Ite et al., 2013).

stated that the identification and isolation of fungi from various environmental sources is still crucial for the observation and identification of additional species, revision of scientific classification, assessment of their effects in the natural world, and provision of strains for biological control, ecological remediation, and industrial applications (Udoh et al., 2015). However, intentional introductions of fungi that break down or use hydrocarbons have probably been rare (Soni and Sharma, 2014; Alsohaili and Bani-Hasan, 2018).

There has been much discussion and ongoing research on the molecular identification of isolated hydrocarbon-degrading fungi at the species level (Ite and Ibok, 2019). According to molecular identification methods based on the extraction of total fungal DNA offer a distinctive barcode that can be used to determine and identify various fungal isolates up to the species level (Gherbawy and Voigt, 2010). For mycologists researching fungal taxonomy, molecular evolution, population genetics, or fungus-plant interactions, molecular identification using this barcode has become a crucial tool (Möller et al., 1992; Landeweert et al., 2003). The sequencing of PCR-amplified 18S rRNA gene segments using universal primers for fungal species allows for a more accurate molecular identification of fungi. (Hensel and Holden, 1996; Monod et al., 2006; Wang et al., 2016; Alsohaili and Bani-Hasan, 2018).

It is admirable that strong native fungi that degrade hydrocarbons have been identified and isolated for the purpose of restoring hydrocarbon-contaminated soil sites through the use of bioremediation techniques. In order to identify and isolate haydrocarbon-degrading fungal isolats (confirmed at the species level, using molecular strategies) from oil-laden soils at various geographical locations in the oilfields of the Kurdistan Region of Iraq (KRI), Iraq, this study will be conducted.

2. MATERIALS AND METHODS

2.1 Sample Collection

Samples of oil-laden soil, also known as oil-contaminated soils, were taken for this study from 41 drilling waste pits. The pits were spaced out across five significant oil and gas fields in KRI, Iraq, each with a distinct geographic location. A tiny hand auger was used to gather the sample collection. 100 g representative samples that ranged in depth from 0 to more than 15 cm were extracted. Three to four samples collected over several square meters were homogenized to create each sample, which was then put into sterile nylon bags.

2.2 Isolation, Macroscopic and Microscopic Identification of Fungi

After isolating the fungi using the soil enrichment technique with Potato Dextrose Agar (PDA), pure isolates were examined to see if they could

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grow on Bushnell Haas Media (BHS), as stated in (Dawoodi et al., 2015). With reference to the Manual of Fungi Atlas the gross morphology of the fungal growth on plates was screened for their morphological features (i.e., color, shape, size, hyphae, conidia, conidiophores, arrangement of spores, etc.) (Watanabe, 2002). By comparing the fungi's identities with verified representative reference text keys and employing the cultural method, their identities were verified. (Booth, 1977; Okigbo, 2009). The incidence and occurrence (%) of each single fungus were calculated using the formula given by (Sepic et al., 1995).

2.3 Molecular Identification

A tube containing only pure fungal isolates was used to extract about 0.5 g of fungal hyphae. For sixty minutes, hyphae were incubated in a 100 µl lyticase solution at 30°C. To break down the protein in the raw sample, 20 µl of proteinase K was added and incubated for 90 minutes at 55°C. At 65°C, the sample was incubated for two hours. Ultimately, approximately 10 µl of these specimens were employed for amplification via polymerase chain reaction (PCR). The software was configured in compliance with the requirements outlined by (Standley and Katoh, 2013). In summary, Ayotamunoa et al. reported that the universal primers ITS1 (5′-TCC GTA GGT GAA CCT TGC GG-3′) and ITS2 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were utilized for the 25 µl PCR reactions. The amplification process was run under the following conditions: 94°C for 1 minute, 55.5°C for 2 minutes of annealing, 72°C for 2 minutes of extension, and 4°C incubation at the conclusion of the final cycle. The PCR was set up for 35 cycles. Quantification was carried out using the Quant-iTTM HS ds-DNA assay kit (Invitrogen, Paisley, United Kingdom) in conjunction with the QuBitTM Fluorometer, while electrophoresis on a 0.8% agarose gel was used to verify the purity of the DNA.

2.4 PCR Product Sequencing And BLAST Analysis

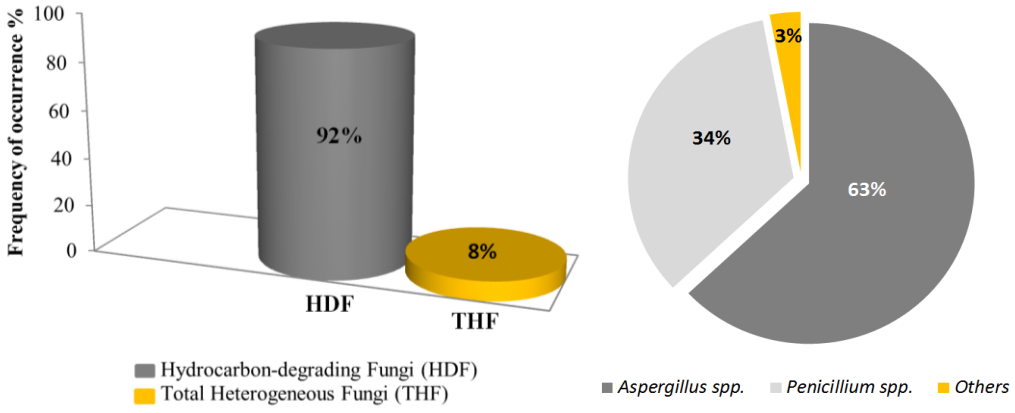
Strong strains (determined by ITS1 and ITS2 primers) were compared to related sequences from GenBank, such as Aspergillus and Penicillium commune. Using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) program, a homology search was conducted on the sequences. Using the NCBI, a phylogenetic tree was constructed and the fungi were identified based on the homology index. The most extreme Likelihood method was used to calculate the evolutionary distances, which are expressed in base substitutions per site. MEGA Version-4 was used to perform the phylogenetic tree and evolutionary analyses, as stated by (Ling et al., 2019).

3. RESULTS

3.1 Isolation and Identification of Fungi

It was determined through the screening of 68 fungal isolates that were collected from various geographical locations within KRI oilfields that the frequency of occurrence of hydrocarbon degrading fungi (HDF) was significantly higher than that of total heterogeneous fungi (THF) in the oil-laden soil samples, with 92% of the samples showing the presence of HDF and only 8% of the samples showing the presence of THF (Figure 1A).

In contrast, the mean percentage occurrence of the 92% frequency occurrence of (HDF) isolates was only 3% for other isolates, 34% for Penicillium sp., and 63% for Aspergillus spp. (Figure 1B).



(A) (B)

Figure 1: Frequency of occurrence % (A), along mean percentage occurrence (B) of HDF and THF isolates in the oil-laden soils.

3.2 Morphology (macroscopic and microscopic) Features of Isolated Fungi

The morphological, microscopic, and cultural traits of the isolated fungi were investigated. Only four (4) fungal species—Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, and Penicillium chrysogenum—were found to be the most potent isolates that degraded or utilized hydrocarbons out of 68 fungal strains. These isolates were chosen for additional identification. To prevent duplication, the self-explanatory Plates (1, 2, 3, and 4) display the microscopic morphological characteristics of the recognized species, respectively.

3.3 Molecular Identification and DNA Extraction From Isolated Fungi

Using particular primers, the internal transcribed spacers (ITS1) and (ITS2) were amplified. The PCR result was examined on a 2% agarose gel that had been ethidium bromide-stained. Using ITS1 and ITS2 primers, these isolates were molecularly identified as Penicillium (i.e., Penicillium chrysogenum) and Aspergillus (i.e., A. fumigatus, A. flavus, and A. niger). Figures 2 (a and b), 3 (a and b), 4 (a and b), and 5 (a and b) show the partial sequencing results and pairwise alignment partial genes for the A. fumigatus, A. flavus, A. niger, and Penicillium chrysogenum species, respectively. The corresponding author can provide full details on the tabular overview of the BLASTN result, including hits identifiers and scoring 28SrRNA sequences for the four fungi of concern.

Using MEGA Version-4 Figure (3), the phylogenetic tree of these four sequences was built against nine distinct other related sequences from GenData Bank. The ideal tree is displayed with the branch length sum equal to 36.19313893. According to the data, many of the Aspergillus and Penicillium species from the GenData Bank and those displayed in Table (1) belong to the same groupings as these fungal species.

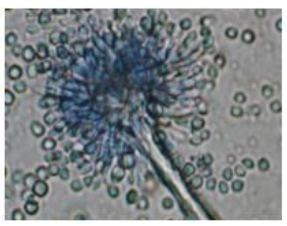


Plate (1): ***Aspergillus fumigatus***
Size: 200-400. Stipe's color: grayish near apex. Surface: smooth walled. Vesicle serration: uni-seriate pyriform. Metula covering: 2/3. Shape: globuse small in columns. Conidia surface: smooth or spiny.

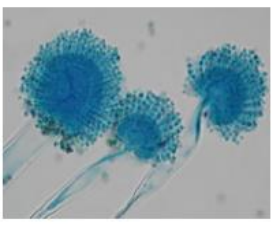
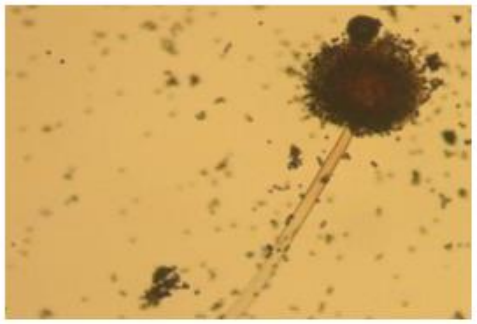


Plate (2): ***Aspergillus flavus***
Size: 400-800. Stipe's color: pale brown roughened. Surface: quietly spherical. Vesicle serration: bi-seriate. Metula covering: 3/4. Shape: glubose ellipsoid. Conidia surface: smooth finely roughened.



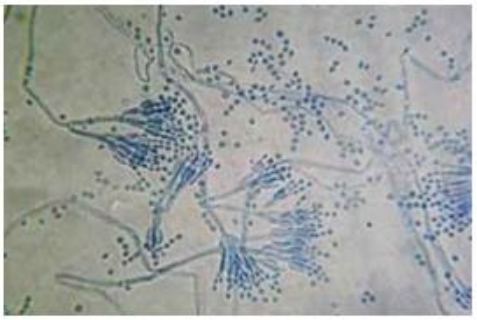
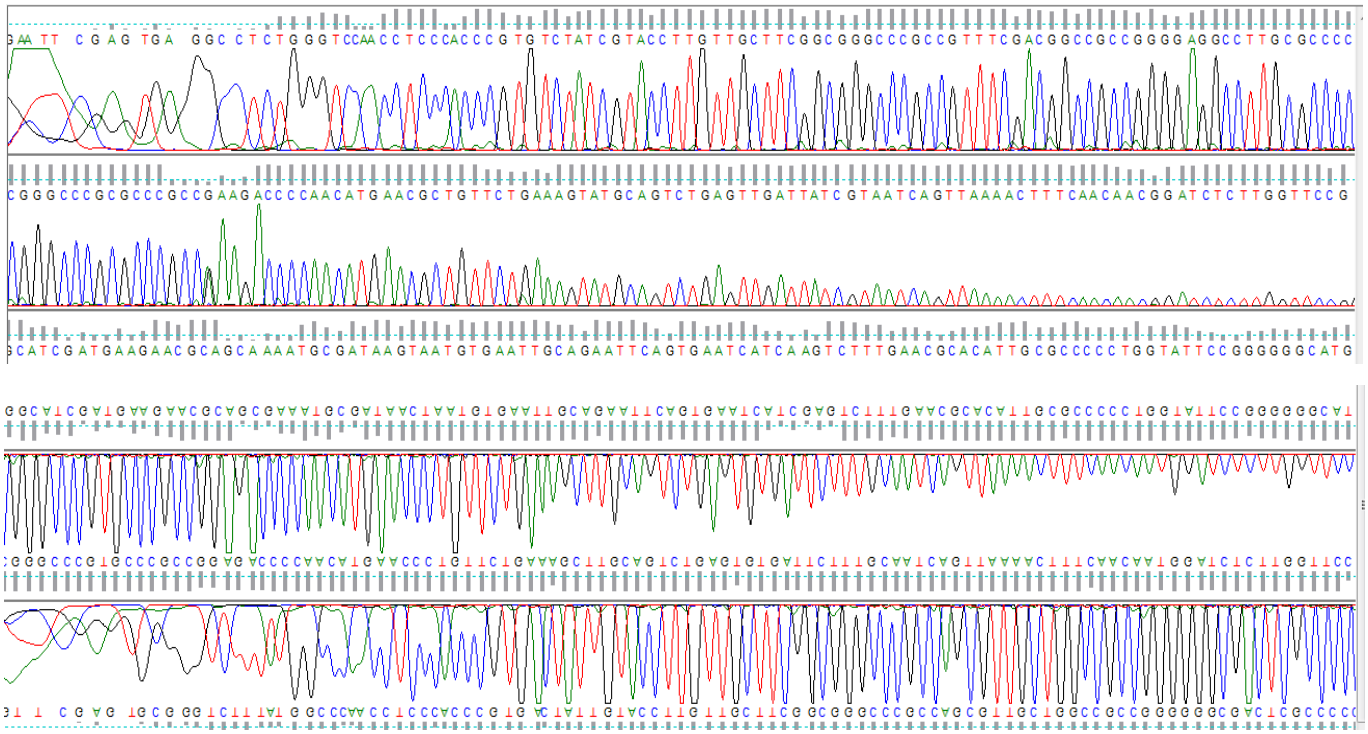


Plate (3): ***Aspergillus niger***
Size: 400-3000. Stipe's color: slightly brown. Surface: smooth walled. Vesicle serration: bi-seriate large size. Metula covering: entirely. Shape: glubose. Conidia surface: very rough irregular.

Plate (4): ***Penicillium chrysogenum***
Stipe's: short, smooth. Penicilli: treverticillate. Phialides: ampulliform. Collula: short. Conidia: spherical to ellipsoidal smooth, greenish.



(a)

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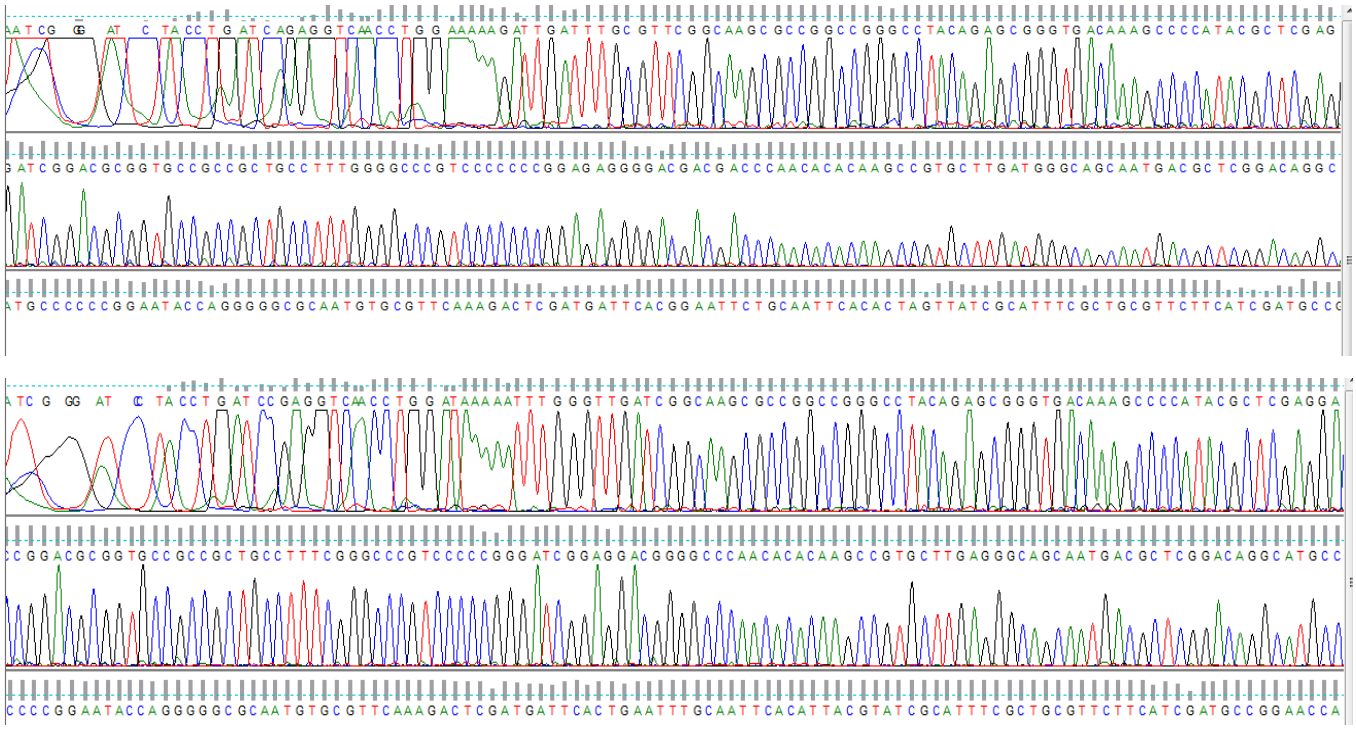
Sbjct  361  GTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCC  401
```

(b) NCBI blasting pairwise alignment of 28SrRNA query sequence

Figure 2: The partial sequencing (a) and pair wise alignment partial genes (b) results for *Aspergillus fumigatus* Strain 004



(a)

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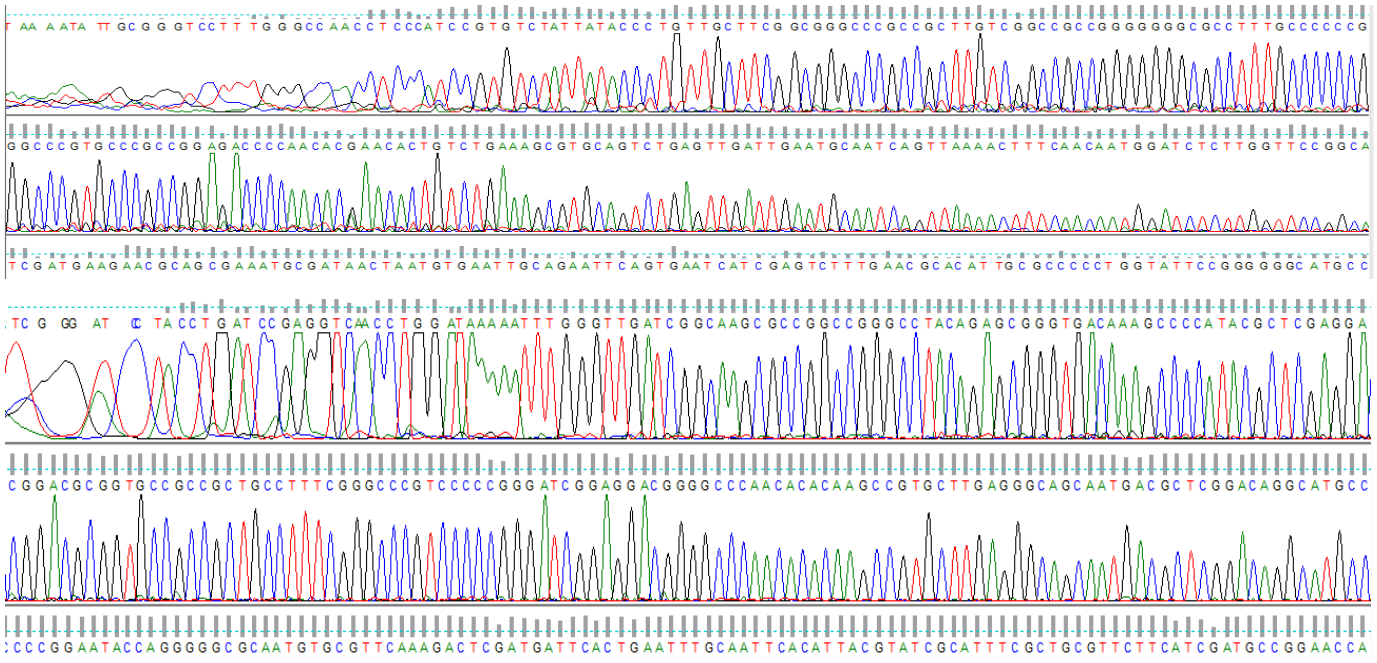
(b) NCBI blasting pairwise alignment of 28SrRNA query sequence

Figure 3: The partial sequencing (a) and pair wise alignment partial genes (b) results for *Aspergillus flavus* ND103



(a)

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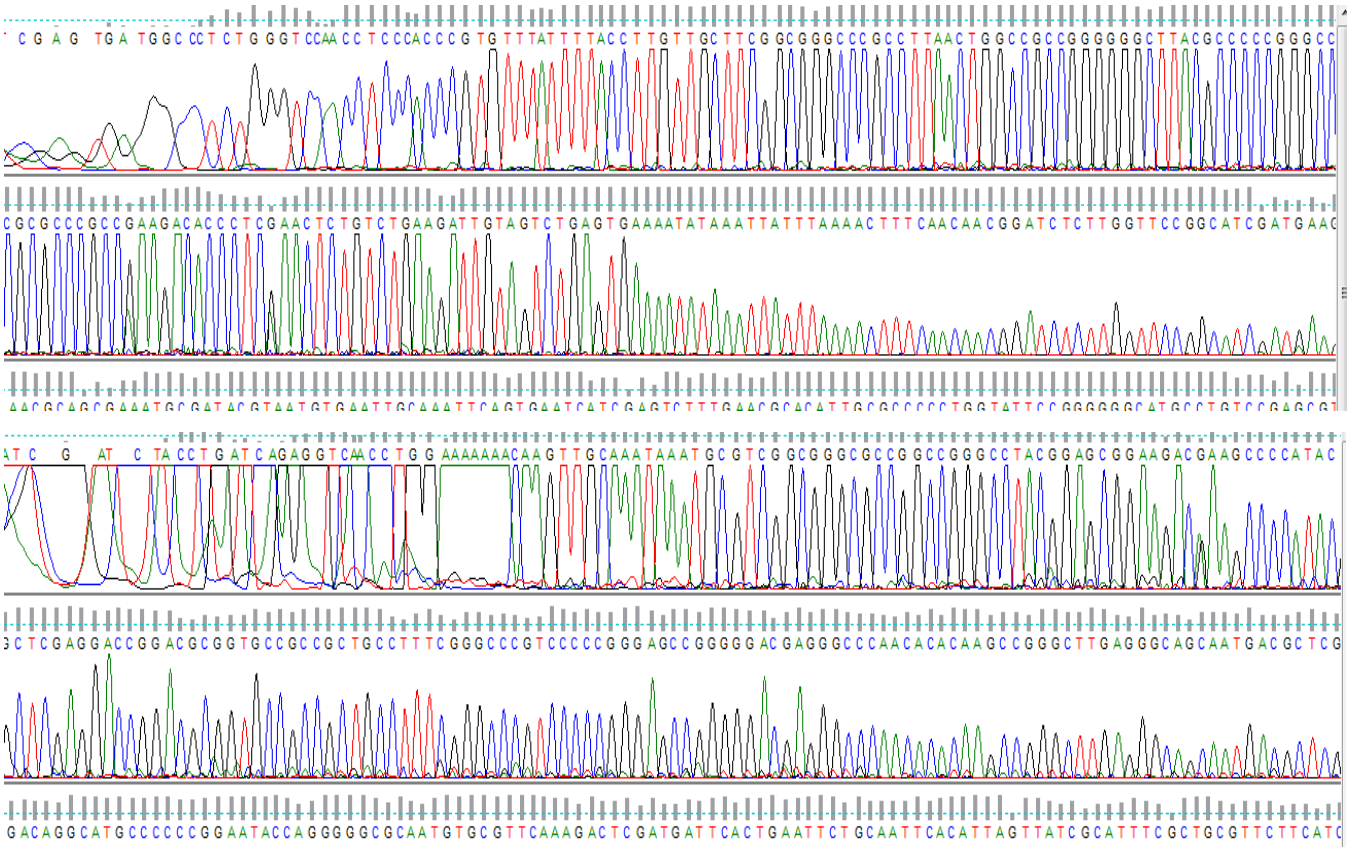
(b) NCBI blasting pairwise alignment of 28SrRNA query sequence

Figure 4: The partial sequencing (a) and pair wise alignment partial genes (b) results for *Aspergillus niger* Strain F3



(a)

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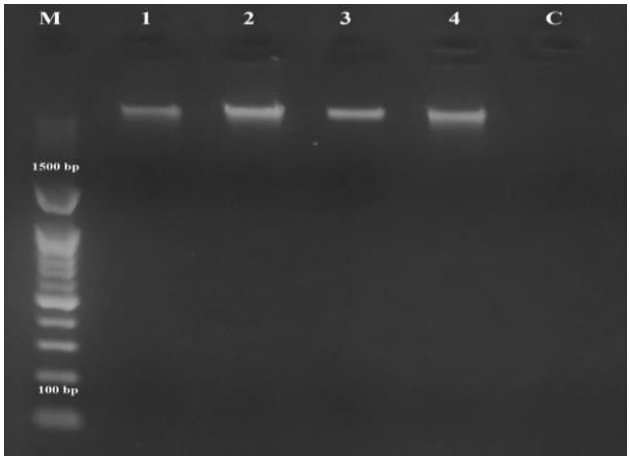
(b) NCBI blasting pairwise alignment of 28SrRNA query sequence

Figure 5: The partial sequencing (a) and pair wise alignment partial genes (b) results for *Penicillium chrysogenum* Strain CBS132208

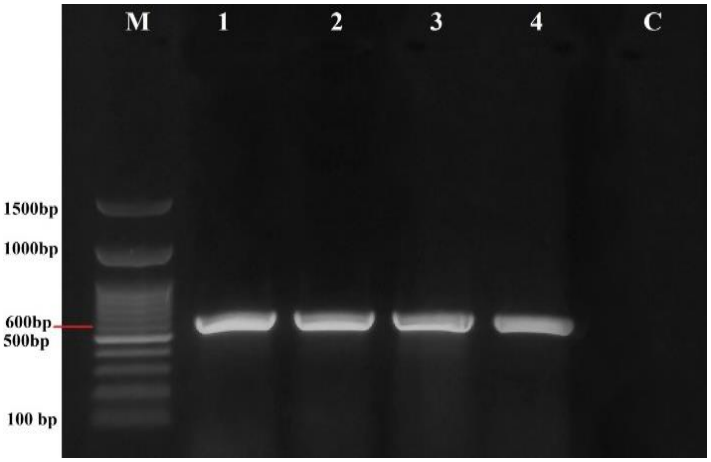
Table 1: Identification of fungus, along GenBank accession number and GenBank species identification.

Query Cover (%)	Identity Number (%)	GenBank Accession Number	GenBank Species Identification	Country Identification
97	98.86	MK452260.1	*Aspergillus niger* strain F3	Egypt
97	99.00	KU321562.1	*Aspergillus fumigatus* Strain 004	China
97	99.82	MK696383.1	*Penicillium chrysogenum* Strain CBS132208	China
97	98.07	MH270609.1	*Aspergillus flavus* ND103	Zimbabwe

Figure 6(A*)* The internal transcribed spacer (ITS) gene control and 600–100 bp marker are shown in Figure 6(B), which also shows the DNA extraction product from fungal isolates. Aspergillus niger, Aspergillus fumigatus, Penicillium chrysogenum, and Aspergillus flavus are among the fungi from which the PCR amplified product is shown.



A: DNA extraction product from fungal isolates and control for ITS gene along with 600-100 bp marker. Where; M = markers; 1, 2, 3 & 4 = isolated fungi and C = control.



B: Polymerase chain reaction (PCR) amplified product from various isolated fungi. M: markers; Lane 1: *Aspergillus niger*; Lane 2: *Aspergillus fumigatus*; Lane 3: *Penicillium chrysogenum*; Lane 4: *Aspergillus flavus* and Cis the control.

Figure 6: Using ITS primers, agarose gel electrophoresis was used to analyze the fungal isolates' PCR amplification product.

3.4 Dendrogram Method

The outcome of dendrogram method made for identification of the isolated most potent species of fungi is; *A. niger*-MK452260.1; *A. fumigatus*-KU321562.1; *Penicillium chrysogenum*-MK696383.1 and *A. flavus*-MH270609.1 (Figure 7).

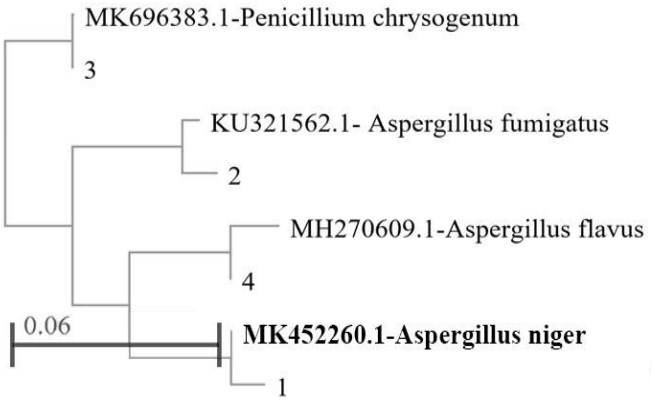


Figure 7: Evolutionary tree for the isolated fungi based on the sequence of internal transcribed spacer (ITS).

4. Discussion

Based on Figures 1A and 1B, it was determined that HDF isolates could use crude oil as their only source of energy and carbon more effectively than THF strains. Similar findings were previously reached in this context by (Samuel et al., 2017; Bensch et al., 2012). Additionally, it was evident that the four most potent HDF isolates were identified in the following order based on their ability to utilize or degrade total petroleum hydrocarbons (TPH) in oil-laden soil samples: Aspergillus fumigatus, Penicillium chrysogenum, A. niger, and A. flavus (data not provided). They were also arranged in an equal order based on the percentage of time they were found in each of the four studied geographical locations within the KRI oilfields. According to taxonomy, the four fungi isolates are members of the Trichocomaceae class, Ascomycota division, and Eurotiomycetes class. Numerous writers have examined a range of variables that frequently impact the rate at which distinct fungal species in the soil environment break down petroleum hydrocarbons (Das and Chandran, 2011; Das and Adholeya, 2012; Ite and Semple, 2012; Srivastava et al., 2014). However, using both conventional and contemporary methods, a wide variety of fungi were isolated from various geographical locations and substrates and identified (Möller et al., 1992; Landeweert et al., 2003; Soni and Sharma, 2014; Udoh et al., 2015; Alsohaili and Bani-Hasan, 2018). These might hold true for the study's target fungi.

For the purpose of this study, native or indigenous fungal strains that degrade hydrocarbons were isolated and identified from oil-laden soils using both macroscopic and microscopic analyses as well as molecular techniques. According a combination of multiple methods is always recommended for the characterization and identification of unknown fungal isolates, as relying solely on morphological characters can be highly error-prone in identifying the isolates to their correct genera and even phyla to (Gherbawy and Voigt, 2010).

The majority of taxonomy researchers concur that DNA coding is a helpful method for identifying and classifying fungal species (Gherbawy and Voigt 2010). It has been demonstrated that molecular methods, like PCR and DNA sequencing, are extremely sensitive and specific for identifying different species of organisms (Madigan et al., 2012). Molecular methods, specifically the polymerase chain reaction (PCR) technology, have transformed both molecular biology and fungal molecular diagnostics.

To date, no single paper has been investigated that uses molecular techniques for diagnosis and classification of Penicillium spp. and Aspergillus spp. in Iraq. Results from studies using extraction of genomic DNA and PCR amplification of marker genes for identification of considerable number of soil and air-borne fungi, may confirm DNA sequence and PCR amplification analyses results in the present work by (Gherbawy and Voigt, 2010; Hong et al., 2005; Einax and Voigt, 2003). However, they concluded that;

- GenBank Accession numbers GQ221107, GQ221161, and GQ221087: the fungal material was morphologically identified as Aspergillus fumigatus. The fungal material was further identified by sequence BLAST (BLAST identity), revealing the following: ITS (6405): 100% A. fumigatus, Btub (6264): 100% A. fumigatus, Cmd (6264): 99% A. fumigatus, and finally, A. fumigatus.

- Using morphological analysis, the fungus was identified as Aspergillus flavus var. oryzae (GenBank Acc.no., GQ221106, GQ221160). When identification was confirmed by sequence BLAST (BLAST identity), the fungus was identified as ITS: A. flavus var. oryzae (99%), Btub: A. flavus var. oryzae (99%), and A. flavus var. oryzae (final identification).

- Aspergillus niger var. niger was identified morphologically by the fungus (GenBank Acc.no., GQ221164–GQ221165, GQ221089–GQ221090). When the identification was confirmed by sequence BLAST (BLAST identity), the fungus was identified as Btub: A. niger var. niger (99%), Cmd: A. niger var. niger, A. awamori (98%), and finally as A. niger var. niger.

- GenBank Accession number: GQ221143–GQ221146, GQ221178–GQ221181: the fungus was identified as Penicillium chrysogenum based on its morphology. This identification was confirmed by the BLAST sequence, which revealed that the fungus was identified as ITS: P. chrysogenum (100%), Btub: P. chrysogenum (98–100%), and finally, Penicillium chrysogenum

For the current fungal species in this work, the same coordination steps can be observed. However, the primer subsequence of Aspergillas fumigatus and partially of Penicillium chrysogenum and two other fungi is approximately 600 bp DNA fragment of PCR, as Figure (6) illustrates. Aspergillas fumigatus has been presented as the primer in the fungus-specific system. The 18SrDNA sequences of Aspergillus fumigatus and Penicillium chrysogenum, which were aligned for Aspergillus niger (Lane 1), Aspergillus fumigatus (Lane 2), Penicillium chrysogenum (Lane 3), and Aspergillus flavus (Lane 4), could be powerfully amplified by these primers. However, Figure (7) demonstrates that the BUILD was used to perform alignment and phylogenetic reconstructions.

The environment's role in facilitating tree exploration(ETE) V3.0.0b32 as performed on the GenomeNet (Ali and Abdullah, 2019). The alignment process was completed by applying the fast Fourier transform (MAFFT) program at the default settings for multiple alignments (Bensch et al., 2012). Furthermore, Fast Tree V2.1.8's default parameters were applied to create the tree. The filamentous fungal strains' internal transcribed spacer (ITS) region was arranged and subsequently added to the GenBank sequence database, bearing the entrance number MK452260.1.

This was evident from the optimal evolutionary tree of the main strains, which displays the evolutionary tree of the powerful fungal isolates according to the ITS sequence. The findings demonstrated that the morphometric information and molecular techniques were KU321562.1 for Aspergillus fumigatus, MH270609.1 for A. flavus, MK452260.1 for A. niger, and MK696383.1 for Penicillium chrysogenum were successfully identified. A total of 277 fungal species were recovered from 1,403 endophytic strains isolated from common plants in arctic, boreal, temperate, and tropical localities, which represent phylo-genetically diverse plant taxa, based on ITS rDNA sequence similarity (95%) to operationally designate species boundaries (Arnold and Lutzoni, 2007).

5. Conclusions

We can distinguish between different fungal species and varieties thanks to the application of molecular techniques to the taxonomy of fungi that was previously thought to be based solely on morphology. As a result, identifying genes that, without prior cultivation, characterize fungal organisms at various taxonomic levels becomes increasingly crucial. However, a wealth of information that is independent of cultivation and does not rely on physiological irregularities can be obtained from DNA sequences and other genetic markers. Genetic markers enable regulating the degree of resolution by selecting the right genes and continuously reflect the identification treasure concealed in the genetic information.

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