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Molecular Identification of Local Isolates *Aspergillus nidulans* from Erbil Province Using Internal Transcribed Spacers

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Abstract

Aspergillus nidulans recognized as unique cell factory to manufacture several of useful natural products for human beings. Also it considered as a rich reservoir for wide range of the unknown secondary metabolites. Due to the great capacity of *Aspergillus nidulans* for fabrication of valuable natural products and in order to have deeper insight for biosynthesis of these metabolites, precise identification is required. Accurate identification of *Aspergillus nidulans* from local isolates at the species level based on the multi-copies of internal transcribed regions of the rDNA unit (ITS1-5.8S-ITS2 rDNA), was the aim of current study. Thus, in this study, morphological features, molecular approach, sequence alignment, phylogenetic analysis and detection of the matrix diversification for amplified ITS regions, in combination with highest BLAST bit score, utilized for accurate identification of Aspergillus nidulans. Therefore, soil samples of Erbil province from different agricultural fields subjected for this study. Out of 106 samples only 12 of them successfully identified as Aspergillus nidulans. Similarity and uniformity of isolates determined by aligning sequencing results of amplified ITS1-5.8S-ITS2 regions with available sequences of close related species in NCBI GenBank database. Sequence alignment results demonstrate that, all isolates displayed (%100) similarity with sequence of at least 14 species previously identified as Aspergillus nidulans. Concurrently, they displaed (%100) similarity with other species in the same genus as well, such as with Aspergillus rugulosus, Aspergillus variecolor, Aspergillus rugulovalvus and Emericella dentate. Furthermore, Maximum likelihood method used for construction of phylogenetic tree for all isolates with referenced strain *Aspergillus nidulans* with accession number (AF138289) to ascertain phylogenetic position of isolates. Interpretation for outcome of phylogenetic analysis of isolates, determine that, isolates (MW228089, MW228090, MW228094, MW228095, MW228096, MW228097, MW228098, and MW228099) belonged to an ITS genotype corresponding to the reference strain Aspergillus nidulans.While due to the interspecies variation, profound changes, single and multiple substitutions in ITS1 region of isolates (MW228088, MW228091, MW228092 and MW228093), they displayed divergent phylogenetic position in dendrogram in compare with reference strain.

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In spite of that, taxonomic analysis results revealed that, all isolates be affiliated with class of Aspergillaceae (124 hits). Overall in present study we confirmed that, colony morphology, molecular approche, sequence alignment analysis for amplified regions ITS1 and ITS2 in combination with highest aligned bit score of species addressed in listed of BLAST search required for accurate identification of isolates at the species level and distinguishing them from other close related species

Introduction

Filamentous fungi such as *Aspergillus nidulans*, considered as well know biological model system to generate variety of complex secondary metabolites. These natural products are appraised as remarkably rich source of medically useful compounds [1, 2]. *Aspergillus nidulans* has substantial capacity of for manufacturing of valuable natural products. Essentially, due to the similarity and overlapping the morphological features of *Aspergillus nidulans* with close related species, accurate identification at te species level required. To have deeper insight for biosynthesis of these natural products and exploring for novel metabolites, unambiguous and reliable recognition methods are strongly recommended [3, 4]. Several techniques such as conventional microbiological methods and different molecular approaches should apply for authentic identification of Aspergillus species. Basically, morphological characteristics among close related species of Aspergillus species, are almost similar and instable. In the fact of that, long time and optimal standard culture procedure requested for discrimination of colony characteristics and adequate evaluation of morphological features of Aspergillus sp [5, 6]. Therefore, in compare with culture based methods, molecular approaches based on polymerase chain reaction are required to give sufficient devaluation in time for accurate diagnosis and identification of isolates at the level of species [7, 8]. Different molecular approaches have been used for detection of Aspergillus species [6, 7]. To delimit the species within the Aspergillus sp, most frequently, multiple gene loci such as protein coding regions (β-tubulin, calmodulin gene), and internal transcribed spacer (ITS) regions of the ribosomal RNA gene cluster (rDNA) applied for species identification [6,7, 8]. Among the DNA barcode markers, ITS regions has been chosen as admissible standard barcode marker for diagnosis of Aspergillus species, this is due to that, ITS region has highest expectation of successful in detection of fungal isolates [8]. Also, ITS regions are separates by 5.8S of rRNA genes and located in between the 18S and 28S rRNA genes [4, 9, 10]. Hereupon, this study was carried out to identify of *Aspergillus nidulans* from our local isolates at the species level by using internal transcribed spacer regions 1 and 2. Accordingly, to analyze the genetic diversity and construction of phylogenetic tree for isolates, sequence variation of ITS regions has been subjected for exploration [4, 7, 8].

Materials and Methods

Survey Sites

Soil composite were collected from the surface layer (0.0 -0.3 m) of the dominant cropped soils at different sites within Erbil Governorate. The sites encompassed for sampling, include soil of different agricultural fields which previously cultivated with different agriculture commodities. To remove impurities and stones, collected soil samples were passed through a sieve, then for further analysis samples putted inside the zip locked

cover and stored in refrigerator. All samples were collected in between (10th of Jan, 2019 - 10th of Feb 2020).

Fungal isolation

Isolation fungi from the Soil samples

Soil samples were subjected for fungal isolation within two days after of collection. Fungi were isolated by using soil dilution method on Potato-Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA). Both Potato-Dextrose Agar and Sabouraud Dextrose Agar, were prepared according to the instructions as indicated by the manufacture. After adding (100 mg\1L) chloramphenicol antibiotic for prevention of bacterial growth, they were sterilized by autoclaving at 121ºC for 15 min under 1.5 bar pressure. Tenfold dilution method used for inoculation of plates as described by [13, 18, 19]. The inoculated plates then incubated at 25º C. Morphological features examined after (5-7) days of incubation. The desired colonies were sub cultured onto test tubes (18 ×180mm) containing Sabouraud Dextrose Agar for working on fungal purification process [12, 13, 14].

Identification of *Aspergillus* species

Morphological identification

Morphological features such as growth rate, colony diameter, aerial mycelium, pigmentation of fungi, conidiophore, vesicle, metulae, phialides and conidia are generally basic essential tool for identification of Aspergillus species. Colony appearance were examined under the dissecting microscope after they have been grown on (PDA) at 25° C for seven days [7, 15].

Thereafter, for studying of microscopical feature, new subcultures of Aspergillus isolates were prepared on (PDA). Most microscopic characters such as (conidiophore, vesicle, metulae, phialides and conidia) were examined by (Slide Cultures Technique) as described by [17]. Slides were prepared and stained with lactophenol cotton blue, then examined under the light microscope. Criteria used for identifications of isolated fungi have been reported by [15, 17, 18].

Molecular identification

Culture preparation

For the commencement, 50 ml of Sabouraud broth has been prepared and inoculated with inoculum from previously cultivated culture, then incubated for 72 h at 28°C. Filters with 0.45-mm pore size used for recovering of hyphae from the grown media, subsequently filtrated hyphae washed with sterile saline [4].

DNA extraction

Afterward the genomic DNA from the all isolates were extracted and prepared by using DNA extraction kit (Bio Basic) as described by manufacturer's instruction and as reported by [19]. About 100-500 mg (wet weight) of hyphae has been grinded in liquid nitrogen by using a pestle. Then the grinded sample was transferred to a clean 1.5 ml microtube. The powder was suspended in 180 µl of Universal Digestion Buffer with 20 µl Proteinase K, then mixed thoroughly by vortexing and incubated at 56°C for 30-60 min. Following the steps, 100 µl of Universal Buffer, has been added and mixed by inverting, then incubated at -20°C for 5 minutes. After centrifugation at 12,000 x g for 5 minutes at room temperature, the supernatant was transferred to a new 1.5 ml tube which contained 200 µl

Universal Buffer BD. 200 µl of ethanol (96-100%) added to the tube and then mixed thoroughly by vortexing. After that, the mixture from the previous step (including any precipitate) was transferred into EZ-10 column and placed in a 2 ml collection tube. Subsequently centrifuged at 9,000 x g for 1 min. Flow through was discarded, then 500 µl of Universal PW solution has been added to the tube, and centrifuged for 1 min at 9,000 x g. Flow through was throw out again. 500 µl of Universal wash solution was added, and centrifuged for 1 min at 9,000 x g, flow through was throw away. Finally 50-100 µl Buffer TE was added directly onto the center part of EZ-10 membrane and incubated at room temperature for 1 min, then centrifuged for 1 min at 9,000 x g. Following the extraction, the eluted and purified DNA was stored at -20°C for the next step.

Universal primers ITS1 and ITS2

Universal fungal primers ITS1 (Forward primer, 5'-TCC GTA GGT GAA CCT GCG G- 3') and ITS4 (Reverse primer, 5'-TCC TCC GCT TAT TGA TAT G-3') as reported by [4, 20], used for amplification of ITS 1 and ITS 2 noncoding regions and intervening region 5.8S gene, of the ribosomal RNA (rRNA) gene complex.

PCR amplification

Amplification reactions were carried out in volumes of 50 µl containing 5 µl (10 pg–100 ng) of template DNA, 1 µl of each primers (20 mM), 5 µl of 2X PCR reaction buffer (HS Prime Taq DNA Polymerase 1 unit/10 µl, 20 mM Tris-HCl, 80 mM KCl, 4 mM MgCl2, enzyme stabilizer, sediment, loading dye, pH 9.0), 5 µl of (0.5 mM of each dATP, dCTP, dGTP, dTTP), supplied Prime Taq Premix GeNetBio (Korea). PCR reactions were performed in the Eppendorf Mastercycler Gradient. The PCR amplification protocol was as follow: after initial denaturation of DNA 1 cycle for 5 min at 95 °C, 40 cycles performed, each cycle consisted of a denaturation step at 95°C for 30 s, an annealing step at 50°C for 30 s, and an extension step at 72°C for 1 min, with a final extension at 72°C for 5 min following the last cycle. Amplified product stored at -20°C until used [4, 20, 21, 23]. PCR products were separated and detected on 2% agarose ethidium bromide gels in TAE 1X buffer (Tris–acetate 40mM and EDTA 1.0 mM), and visualized under UV light in gel documentation cabinet. The 100-bp DNA ladder GeNetbio (Korea) was used as the molecular size marker. Separated amplicons were purified from the gel by using the 96 PCR Kit (NucleoFast®, MACHEREY-NAGEL, Hoerdt, France), according to the manufacture's instructions.

Cloning procedure and insert amplification

Cloning for purified PCR products were performed by using the pGEM® -T Easy Vector System Kit (Promega, Lyon, France) as clarified by manufacturer's instructions and explained by [22]. Re-suspended 20 µl of bacteria suspension in a tube contain 80 µl of LB, then the suspension was plated onto the LB Agar plates supplemented with Ampicillin (100 µlg/mL), X-GAL (80 µlg/mL), IPTG (120 µg/mL) and the plates were incubated overnight at 37°C. Finally screening to confirm the presence of the inserts in white colonies fulfilled by using specified PCR amplification protocol as described above, with the M13 forward primer (5'-GTAAAACGACGGCCAG-3') and M13 reverse primer (5'-AGGAAACAGCTATGAC-3') as mentioned by [22, 23]. Correct sizes of the inserts were purified by using the 96 PCR Kit (NucleoFast®, MACHEREY-NAGEL, Hoerdt, France), according to the manufacturer's instructions.

Sequencing for Purified PCR Products

From previous steps purified cloned fragment of both strands of plasmid containing the fungal inserts subjected for sequencing with universal M13 forward and reverse sequence primers as reported by [22, 23]. Sequencing performed by Macrogen global biotechnology company for research and development of genome analysis. On other hand for direct sequencing of non-cloned amplicons, PCR products were directly sequenced using the ITS 1 and ITS 4 PCR primers. The outcome obtained from sequencing were deposited in GenBank database [4, 7. 20].

Sequence analysis

Acquired sequences were aligned with partial ITS sequences available in GenBank database by using MUSCLE algorithm [24]. Comparison alignment for obtained sequences from sequencing results have been performed with existing sequences in GenBank database by using BLASTN search alignment tool at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Through this way similarity and uniformity of isolates determined [22, 23].

Phylogenetic analysis

After alignment for sequencing results for all isolates based on the nucleotide sequence of ITS1 and ITS2 regions, and due to the great reliability of ITS region in distinguishing of close phylogenetic distance, Maximum likelihood method has been used for analysis and construction of phylogenetic tree with close related species [6-25-28-29]. Data from alignment process imported to the MEGA X 10.05, for generating Maximum likelihood tree. Program parameters setup by default [25-29].

Nucleotide sequences accession numbers

The ITS 1–5.8S–ITS 2 gene complex sequences used in this article were submitted in to the GenBank database with accession numbers from (MW228088 to MW228099).

Results and Discussion

Morphological identification of *Aspergillus nidulans*

Macroscopical identification

Based on morphological key features as reported by [26, 27, 28], macrospical characterstics of isolates determined. Typically colonies of *Aspergillus nidulans* after incubation for 5 days on PDA at 25o C, appeared granular in texture, and surface color dark green in center with pale olive green near the margin of colonies figure 1A. Revers color of colonies were hyaline or pale green, figure 1B. Usually exudate were present with light brown color.

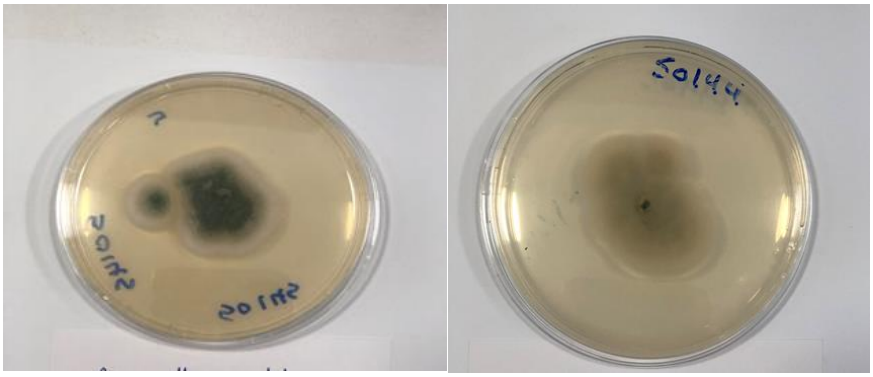


Figure 1.A Colony appearance of *Aspergillus nidulans* on PDA at 25° C after 5 days of incubation had, Granular texture, surface color dark green in center with pale olive green near the margins. B Revers color of colony was hyaline or olive pale green.

Microscopical identification

Among our isolates *Aspergillus nidulans* characterized microscopically, in such way that they had septate and hyaline hyphae. Conidial heads are short and columnar. Conidiophores commonly sinuous, near the foot cell and brown smooth-walled. Vesicles were hemispherical, with metulae and phialides occurring on the upper portion (Biseriate) figure 2 [26, 27, 28]. Summary of morphological features illustrated in table 1.

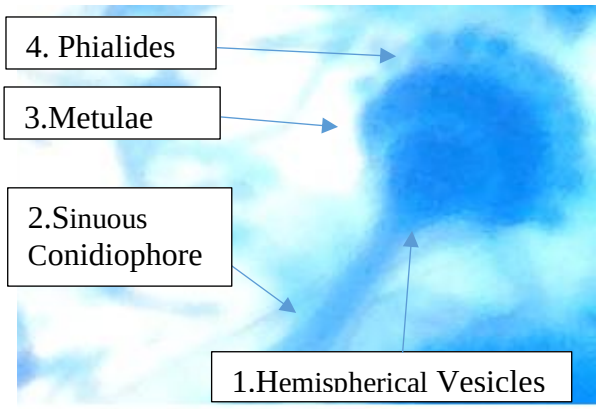


Figure.2 Microscopical features of *Aspergillus nidulans* 1) Hemispherical Vesicles bearing (Metulae and Phialides) biseriate conidial head. 2) Aspergillum like spore bearing sinuous conidiophore. 3) Metulae , 4) Phialides. Microscopic observation of the fungal isolate under 400x magnification, stained by (lactophenol cotton blue).

Table 1. Morphological features used to identify *Aspergillus nidulans* on PDA media.

Colony color\ Texture	Dark green \ Granular
Colony diameter (mm)	25-30
Colony reverse color	Hyaline, Pale olive
Seriation	Biseriate
Vesicle Shape	Hemispherical
Conidia Head Shape	Short

Molecular identification of *Aspergillus nidulans*

After morphological detection, based on the specific PCR amplification procedure, isolates in this study identified as *Aspergillus nidulans* with expected DNA size (561-565) base pairs as showed in figure 3. Out of 106 samples, only 12 of them successfully identified as *Aspergillus nidulans*.

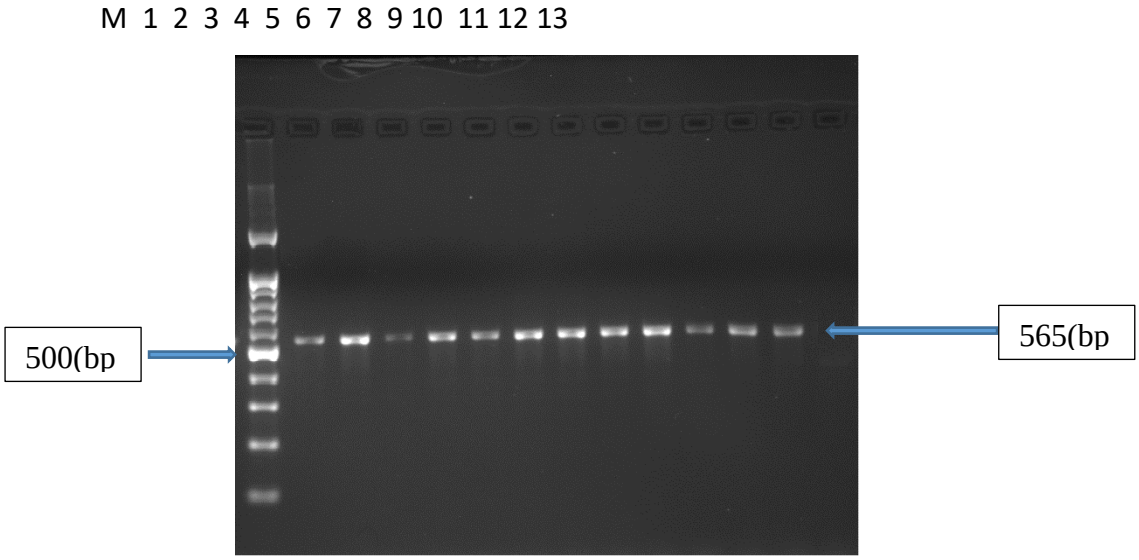


Figure 4. PCR-based detection of *Aspergillus nidulans*. Lanes (1–12) PCR products with (561-565 bp). PCR products include five regions of rDNA complex, IST1-5.8S-ITS2 and 3' end of the 18S ribosomal DNA with 5' end of the 28S rDNA gene. Lane (13) Non DNA template. M: (100) bp DNA molecular size marker. Amplification reactions were carried out in volumes of 50 µl containing 5 µl (10 pg–100 ng) of template DNA, 1 µl of each primers (20 mM), 5 µl of 2X PCR reaction buffer (HS Prime Taq DNA Polymerase 1 unit/10 µl, 20 mM Tris-HCl, 80 mM KCl, 4 mM $MgCl_2$, enzyme stabilizer, sediment, loading dye, pH 9.0), 5 µl of (0.5 mM of each dATP, dCTP, dGTP, dTTP), supplied Prime Taq Premix GeNetBio (Korea).

Analysis of ITS regions

Amplification of ITS1-5.8S-ITS2 regions for the 12 isolates which morphologically identified as *Aspergillus nidulans*, generate PCR products ranging in size from (561-565 bp) table 2. Sequencing carried out for both cloned amplicons and direct PCR products.

Table 2. PCR products sizes of *Aspergillus nidulans* isolates

Isolates accession number	PCR product size (bp)
MW228088	563
MW228089	564
MW228090	565
MW228091	561
MW228092	562
MW228093	564
MW228094	565
MW228095	565
MW228096	563
MW228097	565
MW228098	565
MW228099	565

Potential variations due to the random changes during the sequence amplification process examined for sequencing results of cloned amplicons and direct PCR products, by generating the amplicons with aid of *Taq* polymerase with proofreading capability. Copy of sequencing results from cloned amplicons and direct PCR products used for each isolates to determine the variation in their sequences. Variation in sequencing results of direct PCR products and cloned amplicons in ITS1-5.8S-ITS2 regions illustrated in in table 3.

Table 3. Number of nucleotide variations in sequencing results of ITS1-5.8S-ITS2 regions of direct PCR products in compare with sequencing results of cloned amplicons.

Isolates accession number	Number of Nucleotide Variations in sequencing results of ITS1 region of direct PCR products in compare with sequencing results of cloned amplicons	5.8 S	Number of Nucleotide Variations in sequencing results of ITS2 region of direct PCR products in compare with sequencing results of cloned amplicons
MW228088	2	0	0
MW228089	2	0	0
MW228090	0	0	0
MW228091	2	0	0
MW228092	3	0	0
MW228093	3	0	1
MW228094	0	0	0
MW228095	0	0	0
MW228096	2	0	0
MW228097	0	0	0
MW228098	0	0	0
MW228099	0	0	0

Maximum number of nucleotide sequence variations in between sequencing results of cloned PCR products with direct PCR products, observed in isolates with accession number (MW228092 and MW228093) with (3-4) varied nucleotides respectively. No more than (3) nucleotides seen varied in results of comparison of isolates with accession number (MW228088, MW228089 and MW228096). However, remaining isolates (MW228090, MW228094, MW228095, MW228097, MW228098, and MW228099), had the same sequencing results, with no nucleotide sequence variation.

Alignments for contiguous sequences of ITS1-5.8S-ITS2 regions for all isolates

Amplified sequence of ITS1-5.8S-ITS2 regions of all isolates subjected for BLASTn alignments with sequences of those closely related species in same taxa in GenBank database. Our alignments results demonstrated that, sequence of all isolates displayed (%100) similarity with sequence of at least 14 species previously identified as *Aspergillus nidulans*. Based on the highest aligned bit score of species addressed in listed of BLAST search, all isolates in this study identified as *Aspergillus nidulans*. Notwithstanding that, similarity with (%100) observed with other related strains as well, such as with (*Aspergillus rugulosus* KU866664.1*, Aspergillus variecolor* HQ674656.1*, Aspergillus rugulovalvus* AB248977.1 and *Emericella dentate* AB248999.1) respectively. Moreover, two strains of (*Emericella nidulans*) with accession number (HQ674655.1, and EU287942.1) and two

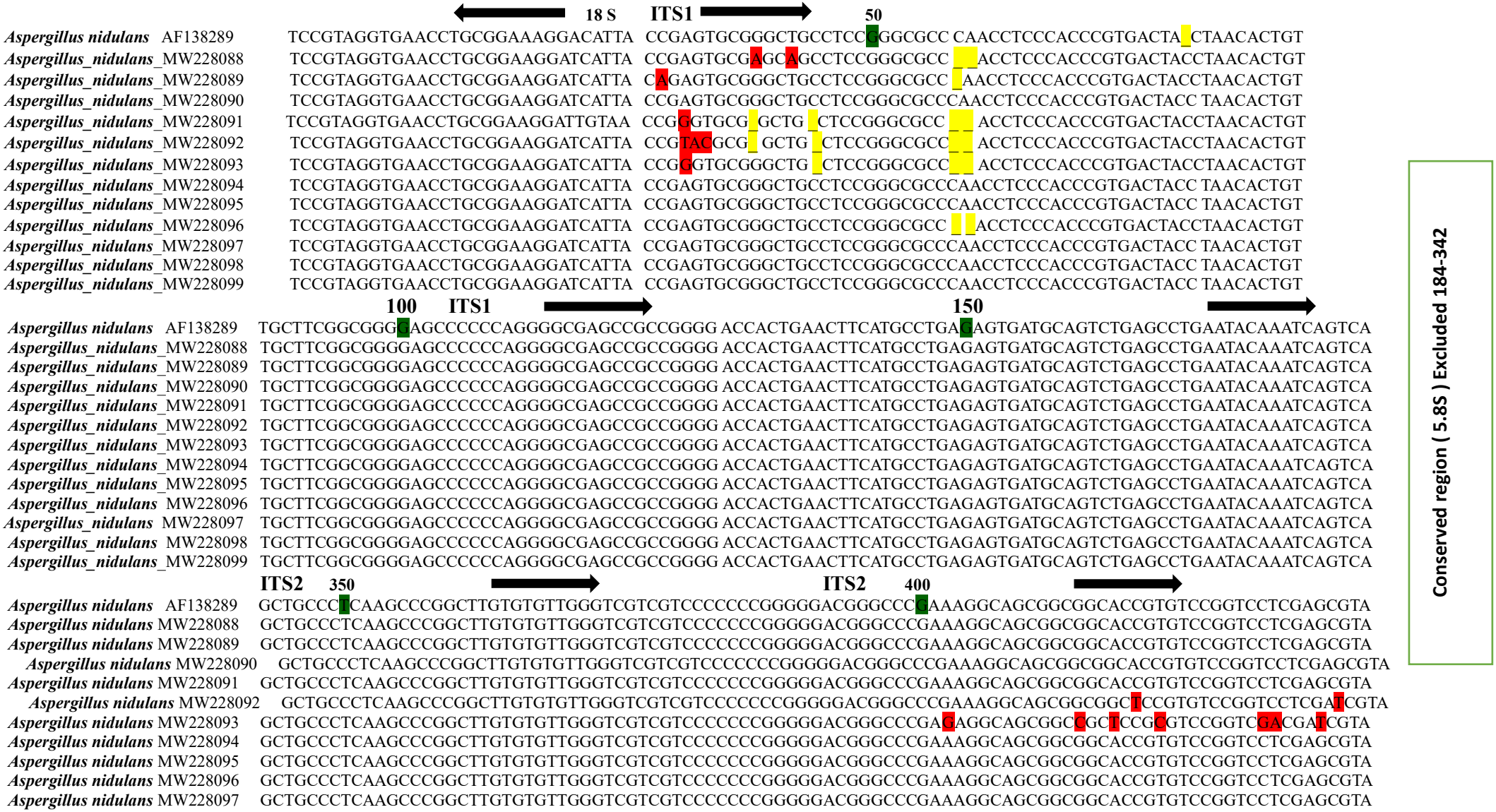
uncultured fungi with accession numbers (JF289097.1 and KF800548.1) present the same similar percentage identity with our isolates. Table 4 present the accession numbers, similarity with coverage percentage and size of those 14 strains which had (%100) similarity with our islates and identified as *Aspergillus nidulans*.

Table 4. Present the accession numbers, similarity with coverage percentage and size of those 14 strains which had (%100) similarity with our islates and identified as *Aspergillus nidulans*.

Accession No. of (%100) similar aligned shot sequences	Similarity percentage with isolates	Coverage%	Length in bp
MT316339.1	%100	100	846
MK806488.1	%100	100	571
MH858232.1	%100	100	857
MG991576.1	%100	100	569
KY074657.1	%100	100	1783
KY425597.1	%100	100	566
KP278174.1	%100	100	575
KP165435.1	%100	100	570
KP131596.1	%100	100	577
AY373888.1	%100	100	590
MW493182.1	%100	100	569
FJ878641.1	%100	100	590
FJ878647.1	%100	100	565
KP131594.1	%100	100	565

Determination of sequence diversity in matrix alignments of ITS1-5.8S-ITS2 regions for all isolates with referenced strain *Aspergillus nidulans* (ATCC 10074) accepted in GenBank database with accession number (AF138289)

Matrix diversification of ITS1-5.8S-ITS2 regions of isolates exhibited by comparing their alignment results with sequence of referenced strain *Aspergillus nidulans* (ATCC 10074) which accepted in GenBank database with accession number (AF138289). Variation in the lengths, and single or multiple nucleotide differences in sequence of rDNA complex regions of isolates observed as a results of deletion and insertion in the sequences of isolates. Figure 5 illustrate the sequence alignments results of all isolates with referenced strain of *Aspergillus nidulans* in GenBank database.



Conserved region (5.8S) Excluded 184-342

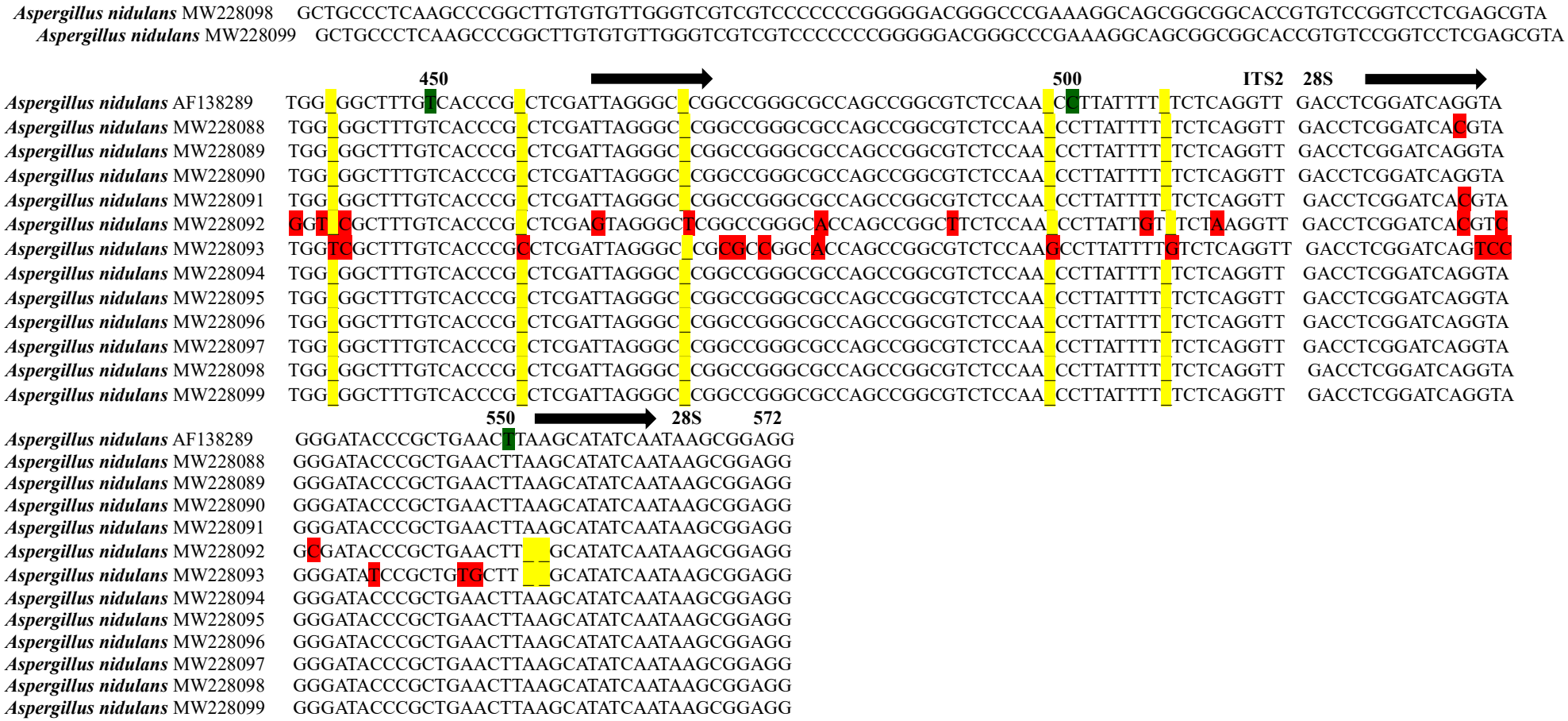


Figure 5. Sequence alignment of ITS regions of all isolates with referenced strain of *Aspergillus nidulans* in GenBank database with accession number (AF138289). Complete ITS1 and ITS2 regions with 3' end of the 18S ribosomal DNA (rDNA) gene which incorporate with ITS 1 primer site, and 5' end of the 28S rDNA gene which incorporate with ITS 4 primer site, present in this figure. Conserved region of 5.8S rDNA gene sequence has been excluded. Numbering of the sequence begins with the first nucleotide in the 3' end of the 18S ribosomal DNA and ended with 5' end of the 28S rDNA gene. Gap in a sequence is marked with hyphen and highlight with yellow (_). However any changes in nucleotide sequences highlighted with Red.

Comparison results of ITS1 region of isolates with referenced strain of *Aspergillus nidulans* with accession number (AF138289) reveal that, isolates with accession number (MW228088, MW228089, MW228091, MW228092, MW228093 and MW228096) had the most variations in nucleotide sequence of ITS1 region in contrast with ITS2 region. These variations occurred in area of ITS1 region with (27) nucleotide bases in length, as showed in figure 6. However, all remain isolates displayed (%100) identical similarity with ITS1 region of referenced strain of *Aspergillus nidulans*. The sequence of highly conserved region 5.8S rDNA of all isolates were identical with the referenced strain.

Except for isolates (MW228092 and MW228093), (%100) identical similarity at ITS2 region noticed in all isolates in compared with ITS2 region of referenced strain. In contrast with other, remarkable variations observed in nucleotide sequences of ITS2 region of isolates (MW228092 and MW228093) in compare with referenced strain of *Aspergillus nidulans*, in such way that (11-16) nucleotides varied in their sequence respectively. Nucleotide sequence variations of ITS2 region of isolate (MW228092 and MW228093) highlighted with red and illustrate in figure 5.

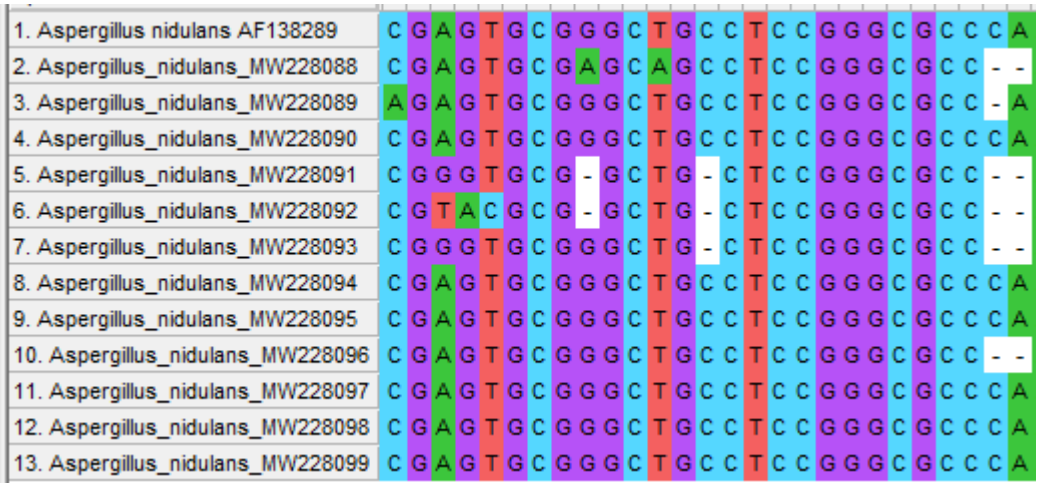


Figure 6. Nucleotide sequence variations of isolates (MW228088, MW228089, MW228091, MW228092, MW228093 and MW228096) in ITS1 region with referenced *Aspergillus nidulans* with accession number (AF138289). Variation changes observed only in (27) nucleotide bases of ITS1 region.

Construction of phylogenetic tree

Maximum likelihood method have been used for construction of phylogenetic tree and determination the phylogenetic distance with close related species [6, 25, 28, 29]. ITS1 and ITS2 regions subjected for construction and phylogenetic analysis, in view of the fact that, generally ITS regions displayed variations between close species. However, ITS regions present great reliability in distinguishing of close phylogenetic distance. Based on phylogenetic result figure 7, phylogenetic position of isolates within the genus and species level determined, regarding to the aligned region of ITS1 and ITS2. Our results elucidates that, isolates with accession number (MW228089, MW228090, MW228094, MW228095, MW228096, MW228097, MW228098, and MW228099) belonged to an ITS genotype corresponding to the reference strain *Aspergillus nidulans* (ATCC 10074) with accession number (AF138289). Furthermore, isolates (MW228088, MW228091, MW228092 and

MW228093), they displayed divergent phylogenetic position in dendrogram in compare with reference strain.

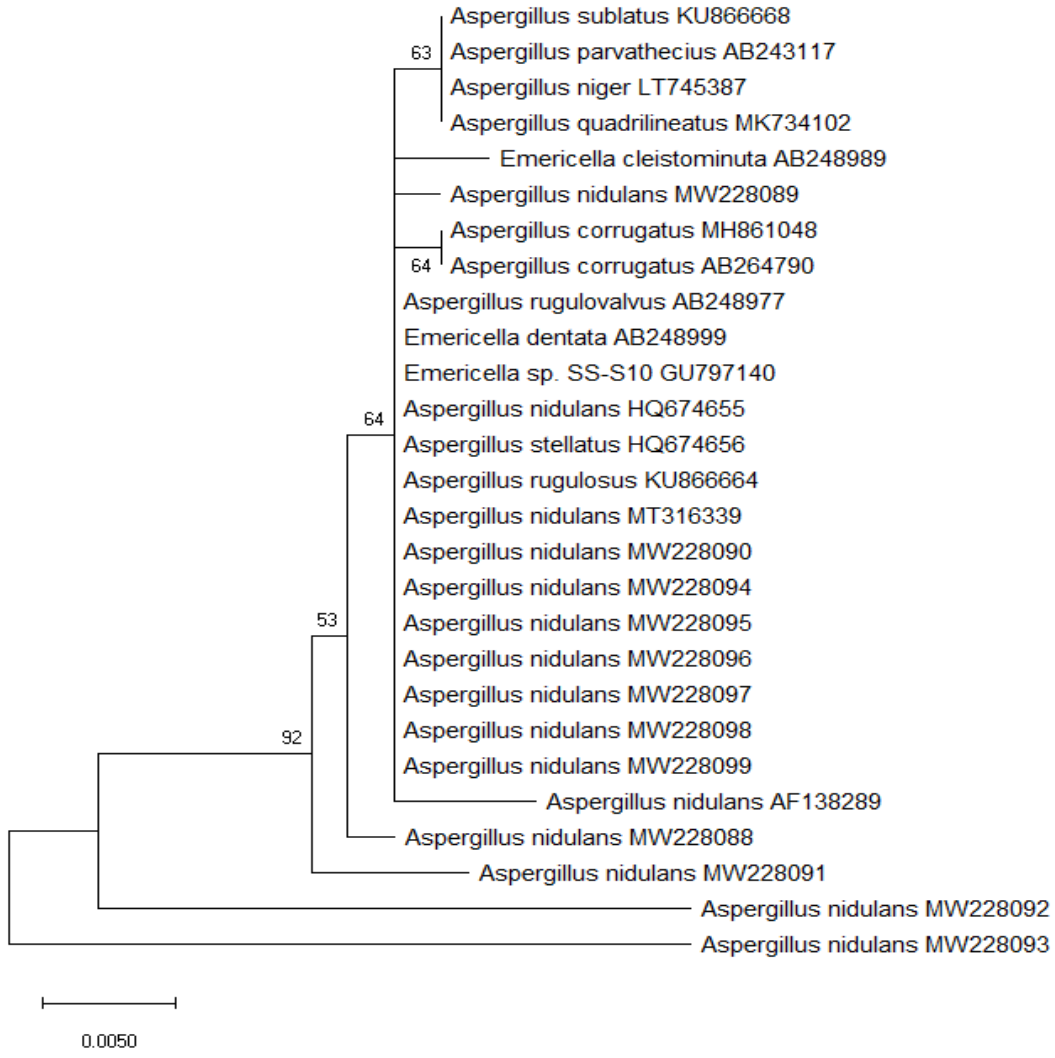


Figure 7. Genetic diversity of local isolates *Aspergillus nidulans* based on the sequence analysis of ITS1 and ITS2 region. Tree was constructed by maximum likelihood method with 1000 bootstrap replicates. Only bootstrap value > %50 are shown. Scale bar represents the genetic distance.

Results of taxonomic analysis for our isolates in GenBank database confirm that, our isolates are member of class Aspergillaceae (124 hits), and maximum similarity of sequences matched with *Aspergillus nidulans* (37 hits), table 5.

Table 5. Taxonomic analysis for isolates based on NCBI GenBank database confirmed that, our isolates belonged to class of *Ascomycetes* (124 hits) and maximum similarity of sequences matched with *Aspergillus nidulans* (37 hits).

Fungi	130	
. . Aspergillaceae	124	
. . . Aspergillus	101	
. . . . Aspergillus subgen. Nidulantes	38	
. . . . . Aspergillus nidulans	37	Aspergillus nidulans hits
. . . . Aspergillus rugulosus	9	Aspergillus rugulosus hits
. . . . Aspergillus pachycristatus	3	Aspergillus pachycristatus hits
. . . . Aspergillus spinulosporus	1	Aspergillus spinulosporus hits
. . . . Aspergillus stellatus	1	Aspergillus stellatus hits
. . . . Aspergillus rugulovalvus	1	Aspergillus rugulovalvus hits
. . . . Aspergillus quadrilineatus	13	Aspergillus quadrilineatus hits
. . . . unclassified Aspergillus	13	
. . . . Aspergillus corrugatus	11	Aspergillus corrugatus hits
. . . . Aspergillus niger	2	Aspergillus niger hits
. . . . Aspergillus sublatus	3	Aspergillus sublatus hits
. . . . Aspergillus parvathecius	1	Aspergillus parvathecius hits
. . . . Aspergillus montenegroi	1	Aspergillus montenegroi hits
. . . . Aspergillus miyajii	1	Aspergillus miyajii hits
. . . . Aspergillus fruticulosus	1	Aspergillus fruticulosus hits
. . . . Aspergillus foveolatus	2	Aspergillus foveolatus hits
. . . Emericella	23	
. . Acrophialophora sp. 4 YHY-2018	1	Acrophialophora sp. 4 YHY-2018 hits
. uncultured fungus	5	uncultured fungus hits

Discussion

Screening and mining for novel secondary metabolites among the filamentous fungi are challenging. Unambiguous identification of fungi require for precise exploration and characterization of novel metabolites. *Aspergillus nidulans* recognized as well know biological model for biosynthesis of variety of valuable natural products. Because of similarity and overlapping of morphological features of *Aspergillus nidulans* with close relate species and their alike ability for production of secondary metabolites as well, accurate discrimination for *Aspergillus nidulans* among the isolates strongly recommended.

Current study performed based on premise that authentic identification of *Aspergillus nidulans* at the species level, have great importance and profitable to us to manifest a clear vision for exploration of novel metabolites in *Aspergillus nidulans*. Thus, in this study,

molecular approach, sequence alignment ,phylogenetic analysis and determination the matrix diversification for amplified regions of ITS 1 and 2, in combination with highest BLAST bit score, utilized for accurate identification of *Aspergillus nidulans* at the species level.

Several methods, such as culture based methods and different molecular based techniques, recommended for identification of *Aspergillus nidulans*. Conventional microbiological methods, not quite enough to identify fungi at the species level, therefore molecular based technique is necessary for precise identification of fungi. ITS regions are most frequently utilized for identification broadest range of fungi, in the fact that, ITS region present highest probability of correct identification for wide number of fungal lineage [6, 7, 8, 9]. ITS1 and ITS2 regions have been chosen as a convenient universal DNA marker for detection of fungi at species level.

Identification of isolates as *Aspergillus nidulans*

Out of 106 sample, initially only 12 of them identified as *Aspergillus nidulans* based on colony characteristics. Concurrently, with aid of the molecular approach for amplification of ITS1 and ITS2, and results obtained from sequence alignment analysis for amplified regions, in compare with reference strain *Aspergillus nidulans* (ATCC 10074), identity of all isolates determined and confirmed as *Aspergillus nidulans*. Isolates in this study morphologically identical, in contrast they were varied in some nucleotide base pairs of ITS1 and ITS2 regions. Castañeda-Ramírez *et al*., (2016) noticed similar results in such way that, morphological features of their isolates were identical but variations seen in nucleotide sequence of ITS regions. Furthermore, they wrote that, changes in nucleotide sequences took place due to the evolution processes, which influenced by environmental factors.

Interspecies variations and similarity among the isolates based on ITS1 and ITS2 regions

In compare with ITS2 region, ITS1 area of our isolates with accession number (MW228088, MW228089, MW228091, MW228092, MW228093 and MW228096) displayed greatest interspecies variation. Henry, Iwen and Hinrichs, (2000), disclosed that, depend on alignment results for pathogenic species of *Aspergillus*, interspecies variation were common in ITS1 region rather than ITS2 region. Conversely, except for isolates (MW228092 and MW228093), (%100) identical similarity at ITS2 region noticed in all isolates in compared with ITS2 region of referenced strain. On other hand, frequency of multiple substitutions such as transition, transversion and deletion mutations in ITS1 region considerably greater than ITS2. The 5.8S rRNA gene sequence region highly conserved region with a little variations in all isolates. However, it had no remarkable impact on the overall the comparison process. Rely on our comprehension for comparison and alignments results, we declare that both ITS1 and ITS2 regions were necessary for identification of isolates at species level, concurrently this consequence confirmed by [4].

Comparing in reading of sequencing results of PCR products with cloned PCR products

Our results illustrated that, no more than four nucleotides were vary in reading between sequencing results of PCR products with cloned PCR products. Accordingly, it was not require to clone the PCR product to acquire the precise reading of the sequences.

Determination of isolates similarity with close relate species in GenBank database

Species similarity of all isolates determined rely on outcome of BLAST search in GenBank database and highest aligned bit score of species addressed in listed of BLAST search.

Isolates in this study exhibited (%100) similarity with *Aspergillus nidulans* species in GenBank database. Concurrently, all isolates displaed (%100) similarity with other species in the same genus as well such as with *Aspergillus rugulosus, Aspergillus variecolor, Aspergillus rugulovalvus* and *Emericella dentate*. Basically isolates of *Aspergillus nidulans* deffirentaites with mentioned closel related species rely on microscopical features such as, smooth walled ascospore with bearing two parallel, equatorial crests with 0.5 to 1.0 µ in width used for distingustion them.

Determination of taxonomic status and phylogenetic position of isolates with same loci of referenced strain

Taxonomic status and phylogenetic position of isolates in this study determined by construction of phylogenetic tree with using Maximum likelihood method rely on ITS1 and ITS2 regions in concordance with same loci of referenced strain. ITS regions have great reliability in distinguishing of close phylogenetic distance [6, 25, 28, 29]. Our results elucidates that, isolates with accession number (MW228089, MW228090, MW228094, MW228095, MW228096, MW228097, MW228098, and MW228099) belonged to an ITS genotype corresponding to the reference strain *Aspergillus nidulans* (ATCC 10074) with accession number (AF138289). In spite of that, taxonomic analysis results revealed that, all isolates be affiliated with class of Aspergillaceae (124 hits). Furthermore, due to the interspecies variation, profound changes, single and multiple substitutions in ITS1 region of isolates (MW228088, MW228091, MW228092 and MW228093), they displayed divergent phylogenetic position in dendrogram in compare with reference strain. Similar to our results, Berbee, *et al* (2000), described that, based on solely morphological features or single gene like rDNA, taxonomic status evaluation and identification at species level couldn't be processed. Moreover, Alshehri and Palanisamy, (2020), suggested that the use of protein coding regions such as b-tubulin and calmodulin genes more accurate for the identification of *Aspergillus* at species level.

Overall, in present study we confirmed that for accuate identification of isolates as a *Aspergillus nidulans* at the species level, colony morphology, molecular approche, sequence alignment analysis for amplified regions ITS1 and ITS2 in combination with highest aligned bit score of species addressed in listed of BLAST search required for distinguishing it from other close related species.

Conclusions

Approaches used in this study provide reliable accurate and efficient methods for identification at the species level. Furthermore, except the ITS regions, multiple gene loci such as protein coding regions (β-tubulin and calmodulin gene) highly recommended for identification of fungi at species level as well. Also, for determination of phylogenetic relationships among *Aspergillus* species, IGS regions from the nu-rRNA gene complex and two less often used mitochondrial gene sequences (rns and cox1) put forwarded.

Conflict of interest

There is no conflict of interest to declare.

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