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RESEARCH ARTICLE

PHYLOGENETIC IDENTIFICATION OF ISOLATED SOIL FUNGI FROM SAUDI ARABIA USING 18S-RIBOSOMAL-DNA SEQUENCE ANALYSIS

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ABSTRACT

Fungi play an important role in the soil ecosystem, it decompose nutrients and elements to simple compounds for plants growth, also play a rolein biological control for suppression of plant pathogens. Isolation, purification of soil fungi using conventional microbiological techniques is hard but it needed for study the distribution of fungal community in soil rhizosphere. Molecular techniques using 18s rDNA based sequences analysis provide the taxonomic resolution for identification of fungal species and strains, also provide information on the diversity and dynamics of fungus in environmental samples. In the present study, isolation and purification of fungi near some medicinal plants soil rhizospher from Saudi Arabia regions, extraction of DNA from isolated fungal strains, 18S-ribosomal-DNA using universal primer sequences were used for amplification of 18S rDNA gene from each isolated fungus. Clear banding patterns were obtained with each isolated fungi by using primer sets. Sequencing of isolated 18S-ribosomal-DNA done by Macrogen Company in Korea and compared to known rDNA sequences using NCBI taxonomy were also done. Phylogenetic tree construction of each isolated fungus obtained and results confirmed that the 18S-rDNA PCR amplification are reproducible and confirming the fingerprints which indicate differences of the fungal community in used soil rhizosphere.

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INTRODUCTION

Soil Fungi are major diverse group of eukaryotic organisms in soil ecosystem and found in every habitats with huge number of species (Smit *et al*., 1999; Stajich *et al*., 2009 Hawks worth, 2012: Yarza *et al*., 2017). Soil fungi decompose nutrients and elements to simple compounds for plants growth, also play a rolein biological control for suppression of plant pathogens (Lumsden, 1981; Alabouvette, 1990; Jarosik *et al*., 1996). Due to identify fungus to the species level it is important to isolate and purify fungi, despite its difficulty, take a lot of time and also morphological transition for changing environmental conditions (Rayner and Coates, 1987; Thorn *et al*., 1996; Slepecky and Starmer, 2009). Molecular techniques using 18S rRNA gene region are widely used tofungal classification and assessing fungal diversity in the rhizosphere (Hang *et al*., 2001).

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Primer design target the 18S rRNA gene sequenceis important for fungal classification. The discovery of new fungal groups and taxonomy is depending on increasing the number of sequences in public databases (Lara *et al*., 2010;Jones, 2011; Banos *et al*., 2018).The sensitivity and specificity of 18S rRNA polymerase chain reaction (PCR) were useful tool for initial screening of fungal communities (Hunt*et al*., 2004; Embong *et al*., 2008).18S rRNA gene amplification techniques provide novel insights and significant advances in research on soil fungal (Hoshino *et al*., 2008).Phylogenetic analysis and database searches of each isolated fungi evidences that 18S rRNA gene sequence-based phylogenies are useful to reflect genealogy of Fungi at the levels of order and above, and justify their further usage and exploration (Möhlenhoff*et al*., 2001; Wu,*et al*., 2003; Banos,*et al*., 2018). This technique used to characterize fungal diversity (Anderson*et al*., 2003; Amutha and Godavar, 2014; Wagner *et al*., 2018; Tsai *et al*., 2019). In the present study, isolation and purification of fungi near some medicinal plants soil rhizospher from western region Saudi Arabia, extraction of DNA from isolated fungal strains, 18S-

ribosomal-DNA using universal primer sequences were used for amplification of 18S rDNA gene from each isolated fungus. Clear banding patterns were obtained with each isolated fungi by using primer sets. Sequencing of isolated 18S-ribosomal-DNA done by Macrogen Company in Korea and compared to known rDNA sequences using NCBI taxonomy were also done. Phylogenetic tree construction of each isolated fungus done and obtained results confirmed that the 18S-rDNA PCR amplification are reproducible and confirming the fingerprints which indicate differences of the fungal community in used soil rhizosphere.

MATERIALS AND METHODS

Sample collection and isolation of fungi: Soil samples were collected from different western region Saudi Arabia as indicated in Table (1)in sterile polythene bags. The soil samples were taken at a depth of 20 cm from the soil surface according to the method described by Johnson *et al*. (1959). Serial dilutions from 1 gram soil sample were prepared to 10-10. 100 µL of each diluted sample were taken, spread and purified onto two different sterilized media, (Rose Bengal Agar RBA, and Potato Dextrose BDA Agar, HIMEDIA Company) and incubated at room temperature for 4-7 days.

Purification of the isolated fungal species: The isolated fungal species were purified by transferring a very small part of each representative colony to new Petri dishes of the culture media by streak plate method. After incubation for 7 days at 30°C, a single colony was aseptically sub-cultured on a slant of modified Czapek Dox agar, HIMEDIA Company.

Classical identification of the isolated fungal species: The developing fungal colonies were identified up to species level by microscopic examination, using the following protocols (Barron 1972; Samson and Reenen-Koekstra 1988; Moubbasher 1993; Kern and Blevins 1997).

DNA isolation from fugal species: Fungi mycelium were harvested from culture PDA culture plates, and 50 mg of each mycelium was taken, transferred to new flasks contains potato dextrose broth (PDB) liquid culture with vortex. Incubated for 3 days, the mycelium was harvested by filtration, transferred to 1.5 Eppendorf tube and frozen at -80°C for 30 min. the collected mycelium transferred to sterile mortar contain liquid nitrogen to obtain mycelium powder, transferred to 1.5 Eppendorf tube and centrifuged at 10000 rpm for 5 min. After centrifugation the supernatant was discarded, mycelia was frozen dried (at -20°C) (Turzhanova *et al*., 2018). The DNA will be extracted from frozen dried mycelium powder using Qiagen DNeasy® plant mini kit. The DNA quantity and quality were checked by electrophoresis on a 1% agarose gel, visualized by UV trans-illuminator.

18s rDNA amplification: Standard PCR Protocol of for 18s rDNA amplification was done.The18S rDNA was amplified with primers that target highly conserved region of 18s rRNA. Forward Primer 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 and Revers Primer 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'.The total 25 µL PCR mix consisted of 8 µL PCR grade water (ddH20), 12.5 µL of 2x KAPA2G Fast Ready Mix, 1.25 µL of 10 µm Forward Primer, 1.25 µL 10 µm Reverse Primer and 2 µL of DNA template. The PCR running conditions were: Initial Denaturation at 94oC for 3 min, 35 cycles of denaturation at 94oC for 15 sec, annealing at 48oC for 15 sec, extension at 72oC for 5 sec and final extension at 72oC for 2 min (Hailu *et al*., 2017).

DNA electrophoresis and sequencing: PCR products were separated in 1.5% agarose gelelectrophoresis containing ethidium promide. The visualization of DNA bands done under Gel Documentation system. Amplified DNA bands was scored data not shown. Sequence results were performed at Macrogen company, Korea with universal sequencing primer 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'. Phylogenetic relationships were determined using MEGA 6.0.

RESULTS

The principal result of this work is a collection of soil from different locations as indicated in table (1).Soil materials were collected and used for fungal isolation and fungal systematics based on classical purification on appropriate plates as indicated in materials and methods. Isolation and purification of fungal isolates and macroscopic observation of the fungal colony showed different colony morphology of each isolated fungus, a total of 11 fungal isolates were obtained as shown in Fig. (1). Table (1) represent Location of each collected soil and also the type of fungi isolated from each soil. A total of 11 fungal isolates as shown in Figure (1) and table (1). Results indicated that different fungal isolation from each soil. Two *Aspergillus terreus* fungi were isolated from soils no. 1 and no.6, two *Mucor circinelloides* were isolated from soils No. 2 and no. 3, two *Alternaria sp*. isolated from soils no.2 and no.7, *Aspergillus unguis* isolated from soil 4 and 5, only one *Myrothecim verrucaria*, *Emericellanidulans*, *Aspergillus fumigatus* soils 4, 5 and 7 respectively. Results of classification of each fungal isolates were confirmed by a 18s rRNA sequencing.

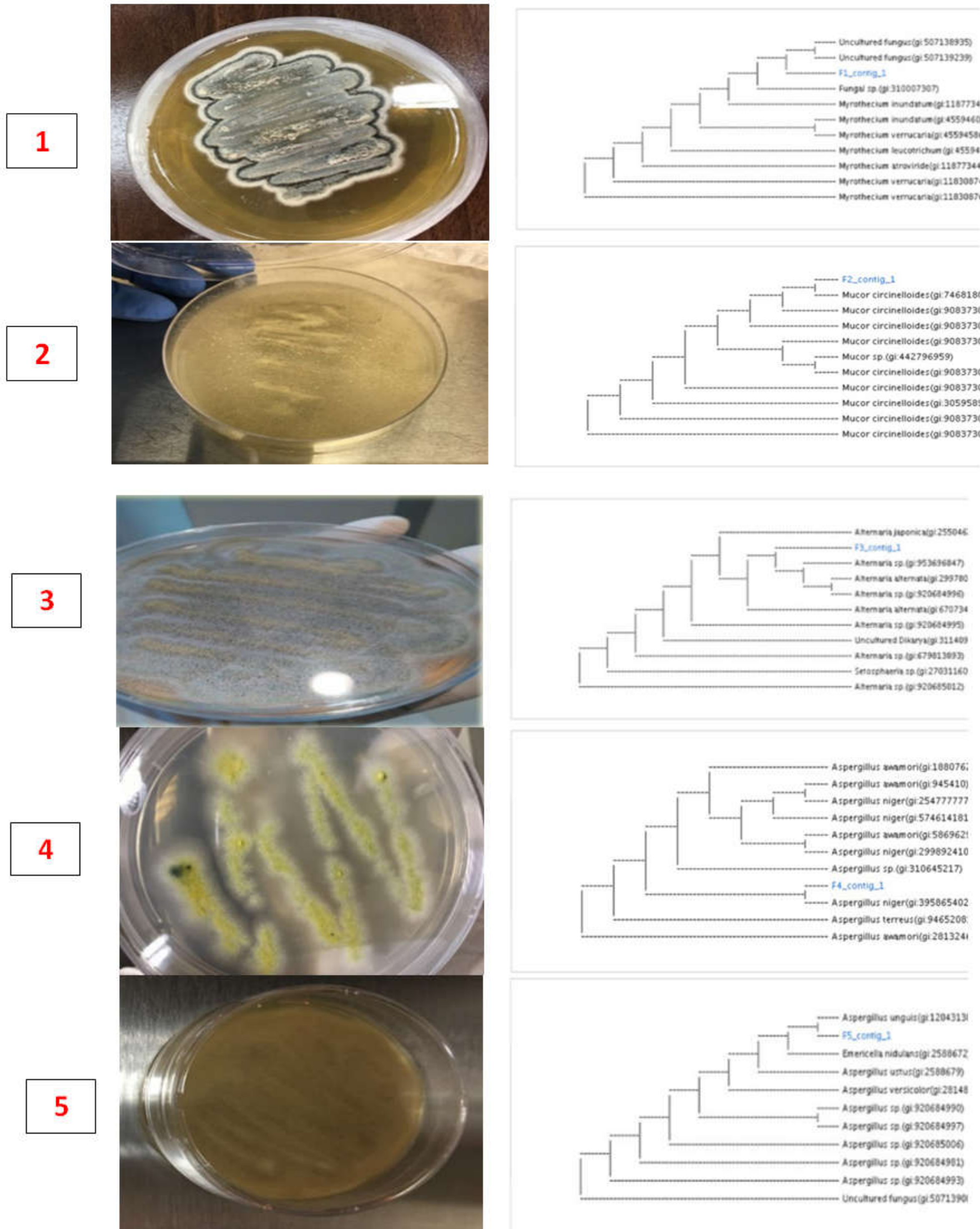
DNA was extracted from isolated fungal strains and the 18S rDNA gene isolation were done from each isolated fungus. The effectiveness of the DNA extraction method was evaluated by gel electrophoresis and spectrophotometric absorbance at (A260/A280 and A260/A230 ratios). Amplification of fungal 18S-ribosomal-DNA (rDNA) sequences was done. Results showed that The PCR product was visualized as thick bands in the Ethidium Bromide stained gel under UV trans-illumination at 700 bp. as shown in fig. (2). Sequencing from all 11 fungal 16s rRNA were done at Macrogen Company, Korea by using universal primer pairsas indicated in materials and methods. Aligned sequence entries of good quality, covering a significant portion of the fungal diversity, and a phylogenetic tree of all isolated fungus were constructed. As indicated by phylogenic tree of each isolated fungus, results indicated that successful in describing fungal taxonomy by 18s r RNA sequencing comparing with NCBIdata bases. The three independent phylogenetic constructions made, the phylogenetic tree had a strong correlation with the current fungal classification. The phylogenetic tree includes clade information of each isolated fungi Fig. (1). The observed data in this work help us to classify and taxonomy of isolated fungus

DISCUSSION

The present study aimed to isolation and identification of soil fungi and classification at species level. Our research based on

Table 1. Soil collected from different places in the Western Region

Soil samples	Soil Samples by GBS	Locations	Types of fungi isolated from soil
Soil 1	20°34'09.7"N 41°02'00.3"E	Bani Malik*	*F4 Aspergillus terreus*
Soil 2	20°35'13.8"N 41°02'42.0"E	Bani Malik*	*F2 Mucor circinelloides* *F7 Alternaria sp.*
Soil 3	20°35'40.0"N 41°02'42.2"E	Bani Malik*	*F11 Mucor circinelloides*
Soil 4	21°22'35.4"N 39°27'50.9"E	Makkah –Jeddah Road	*F1 Myrothecim verrucaria* *F9 Aspergillus unguis*
Soil 5	21°24'44.6"N 39°30'06.5"E	Bahra-Jeddah Road	*F5 Aspergillus unguis* *F6Emericellanidulans*
Soil 6	22°00'16.2"N 39°20'28.1"E	Usfaan-Jeddah Road	*F10 Aspergillus terreus*
Soil 7	23°04'38.4"N 39°57'13.5"E	Medina-Jeddah Road	*F3 Alternaria sp* *F12 Aspergillus fumigatus*



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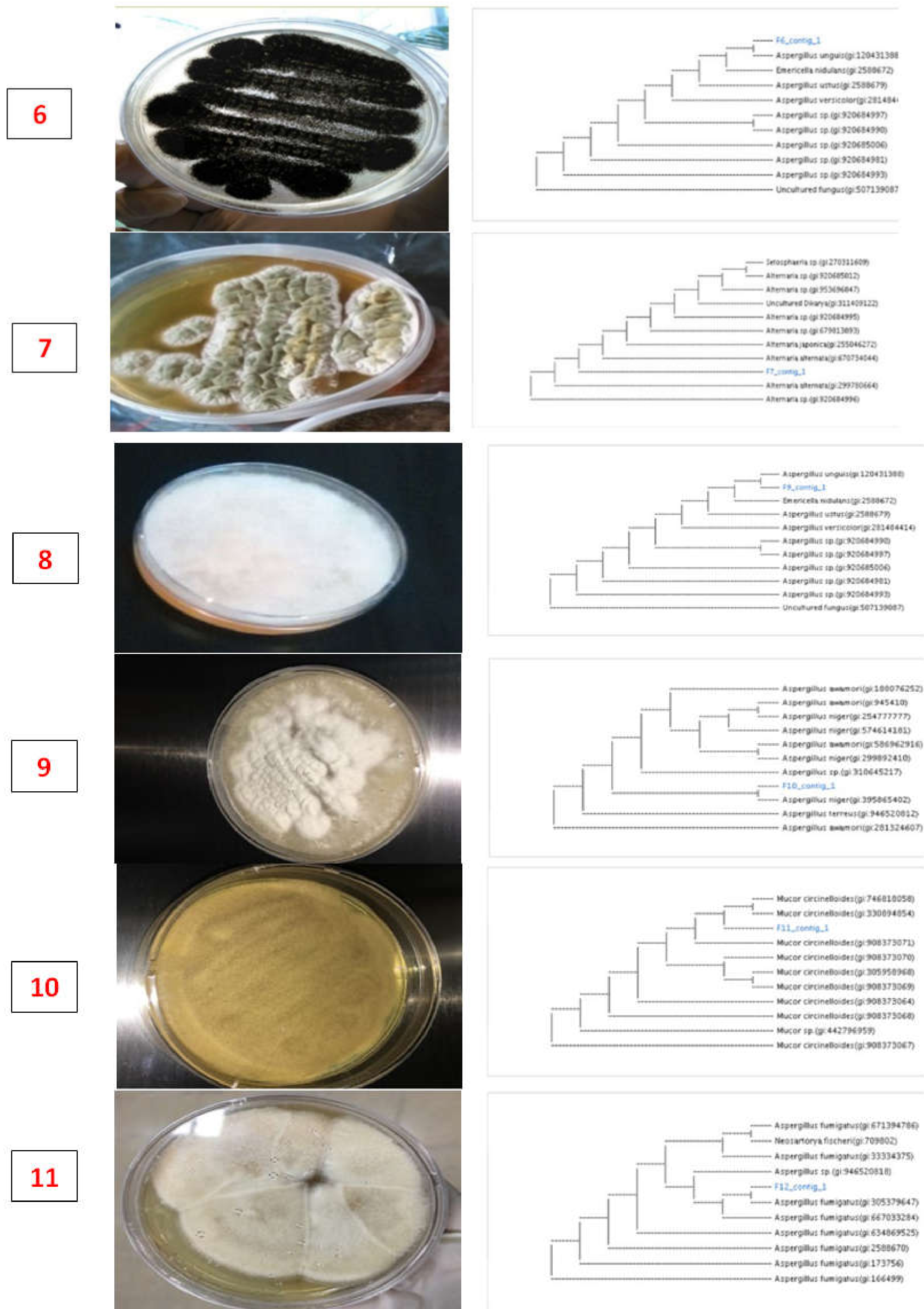

Fig. 1. F1, ***Myrothecium verrucaria*****, F2:** ***Mucor circinelloide*****, F3:** ***Alternaria s,*** **F4:** ***Aspergillus terreus*****, F5:** ***Aspergillus unguis*****, F6:** ***Emericellanidulans*****, F7:** ***Alternaria sp*****, F9:** ***Aspergillus unguis,*** **F10:** ***Aspergillus terreus*****, F11:** ***Mucor circinelloides,*** **F12:** ***Aspergillus fumigatus***

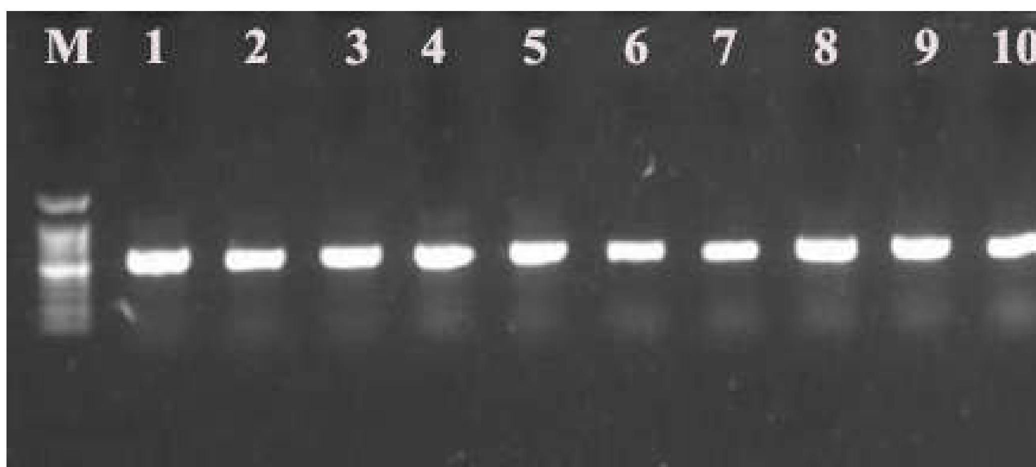

Fig. 2. PCR products of the amplified 18S rRNA gene region of all fungal strains. Lanes: M= 1Kb DNA marker; 1:F1, *Myrothecium verrucaria,* 2: F2: *Mucor circinelloide*,3:F3: *Alternaria sp*,4:F4: *Aspergillus terreus*, 5: F5: *Aspergillus unguis*, 6:F6: *Emericellanidulans*, 7:F7: *Alternaria sp,* 8:F9: *Aspergillus unguis*,9:F10: *Aspergillus terreus*, 10:F11: *Mucor circinelloides,*11:F12*: Aspergillus fumigatus*

isolation of fungal specific 18s rRNA gene and sequencing of 18s rRNA gene, comparing obtained sequences by alignment with a known sequence with NCBI databases to classify the isolated fungus species. Construction of phylogenic tree was done of each isolated fungus these results are in-agreement with all (Jones, 2011; Wijayawardene *et al*., 2014; Reblova *et al*., 2016). Phylogeny approaches help us to understand the evolution and diversity of fugal at the species level (Powell *et al*., 2009; Ghikas *et al*., 2010). Phylogenic tree topology could sometimes be better understood more about clades, these clades help us to understand more classes within a phylum. The obtained phylogenic trees proven that 18s rRNA sequencing and compare obtained sequences with reference database (BLAST) become a beneficial approach for fungal taxonomy (Ludwig and Klenk, 2001; Gryganskyi *et al*., 2013). This results also evidenced that 18s rRNA gene sequencing help scientists for fungal taxonomy to order level ((Yarza *et al*., 2017; Banos *et al*., 2018).

Conclusion

The 18s rRNA gene sequencing is fast and reliable techniques for fungal taxonomy. The obtained data revealed that the Saudi Arabia soil has enormous diversity of fungal

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