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# Molecular identification of the entomopathogen *Aspergillus candidus* and its pathogenicity to the mealybug *Phenacoccus solenopsis* (Hemiptera: Pseudococcidae) in Egyptian sugar beet fields

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**Abstract**:

Several host plants are subject to infest with *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae), from which is sugar beet. The infested plants suffer from reduction in photosynthetic processes. Biological control is an essential approach to control. The fungus *Aspergillus candidus* is one of the entomopathogens attacking this mealybug. The current investigation was done at "Kafr Ascar" village, Kafr El-Sheikh Governorate, Egypt during 2016/2017 and 2017/2018 seasons. Obtained results indicated that the natural mortality of *P. solenopsis* adults due to the fungus *A. candidus* infection in the field ranged between 2-19 individuals/ 50 plants from 25 October to 10 May, 2016/2017 season and the total number of dead insects recorded throughout the season was 71 individuals/ 300 plants. According to statistical analysis, the results indicated that the temperature and humidity had a significant effect on the activity of the fungus and therefore on mortality rates.The entomopathogenic fungus was isolated and identified by GATC (Biotech Sequence Company, Germany), as *A. candidus* strain HNMF075 (Ascomycetes: Eurotiaceae) for the first time in Egypt. Suspension of this fungus was made, adjusted to $5\times 10^5$spores/ ml water, for using in laboratory and field tests. In a laboratory tests, the mortalities of adults were 55.00 and 95.00 two and four days after treatment, respectively. The mean of mortality was 75.00 %. Also, in a field tests, the reductions of adults were 53.00 and 67.42% two and four days after treatment, respectively, with 60.21% average reduction based on DNA sequences using rDNA, data showed that the *Aspergillus* isolate belonged to *A. candidus* with a high similarity of 94%. It is concluded that *A. candidus* strain HNMF075 is an effective biological agent against *P. solenops is* in sugar beet fields in Egypt.

## Introduction

The cotton mealybug *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) is a polyphagous insect feeding on more than 202 host plant species from 55 families (Fand and Suroshe, 2015). Large populations of mealybug cause general weakening, defoliation and death of susceptible plants. As well, the honeydew excreted by the mealybugs caused growth of sooty moulds and other secondary infections that reduce photosynthesis and impair the marketability of plant products (Ibrahim *et al.*, 2015). *P. solenopsis* has a wide geographical distribution, Central America, the Caribbean, Ecuador, Chile, Argentina and Brazil (Culik and Gullan 2005). In Egypt, the occurrence of *P. solenopsis* infestation was recorded on weed plants for the first time byAbd-Rabou *et al*. (2010). In sugar beet (*Beta vulgaris* L.) fields, Vennila *et al*. (2014) showed that pigweed *Chenopodium albam* L. and spinach *Spinacia oleracea* L. are host plants of *P. solenopsis*. Anonymous (2009) recorded that mealybug is one of the insect pests of sugar beet plants in Egypt. Also, Bazazo *et al*. (2017) identified the specimens of *P. solenopsis* at Plant Protection Research Institute, Agricutural Research Center. They estimated the numbers of *P. solenopsis* infested leaves as 15-18 symptoms/200 sugar beet plants at some fields at Kafr El-Sheikh Governorate. Mealybugs are difficult to control with insecticides, as they are able to rapidly develop resistance and exhibit cryptic behavior. Further, they are covered by waxes that protect their bodies from insecticide penetration (Franco *et al*., 2004). The low efficacy of insectcide use has been encouraged biocontrol strategies (Daane *et al*., 2004). The application of entomopathogenic fungus was a valuable method of biological control of mealybugs (Mustu *et al*., 2015). Fungal pathogens have certain advantages in pest control programms over other insect pathogens like bacteria and viruses. Mass production techniques of fungi are much simpler, easier and cheaper than those of bacteria and virus. Fungi unlike bacteria or viruses directly infect through insect cuticle and do not require ingestion for infection and also sucking insects are infected (Pucheta *et al*., 2016). Entomopathogenic fungi play an important role in the natural pest control in various crops through epizootics; more than 750 species are pathogenic against insects (EL-Husseini *et al*., 2003 and 2004 and Ramanujam *et al*., 2014). *Aspergillus candidus* Link as entomopathogen against different insect pests such as, *Thaumetopoea wilkinsoni* Tams (Lepidoptera: Notodontidae) (Kenneth and Olmert, 1975); *Coccus hesperidum* L. (Hemiptera: Coccidae) (Samsinakova and Kalalova, 1975)*;* *Indarbela* spp. (Lepidoptera: Metarbelidae) (Singh and Singh, 1982); *Carvalhoia arecae* Miler (Heteropetera: Miriidae) (Dhileepan *et al*., 1990). Finally, research on microbial pathogens of insects is increasing considerably in recent times to find out environmental friendly alternatives to hazardous chemical insecticides (Ramanujam *et al*., 2014).

*Aspergillus* spp. is commonly worldwide and includes industrially and biological control important members. It is very important to isolate and identify microorganisms to be used as biological control agents against insect pests. Moreover, morphological identification of microbial isolates could be insufficient for distinguishing the species. PCR and DNA sequences are speed, sensitive and specific way for identification and are proved extremely useful in assessing the changes in fungus such as *Aspergillus* and can also provide useful taxonomic information (Rasime, 2016). Abundant gene regions, such as rDNA regions could be used for sequencing and molecular identification

(Munusamy *et al*., 2010; Peterson, 2012 and Schoch *et al*., 2012) and there are precise analyses for rDNA for differentiation of *Aspergillus* (Sabreen *et al*., 2015). Moreover, internal transcribed spacer (ITS), as called, rDNA is commonly used for taxonomic studies, barcode gene for fungal identification and phylogenetic studies (Krijgsheld *et al*., 2013 and Rasime, 2016).

This study was carried out to molecularly identify *Aspergillus* from dead *P. solenopsis* picked up from the suger beet fields. Moreover, the virulence of *Aspergillus* against the insect pest was assessed under laboratory and field conditions.

**Materials and methods**

**1.Recording the dead *Phenacoccus solenopsis* adults with *Aspergillus candidus*:**

The dead individuals of *P. solenopsis* were recorded at the experimental field (about two feddans). Sugar beet, sultan cultivar sown on $15^{th}$ September, 2016 at Kafr Ascar village, Kafr El-Sheikh Governorate.They were picked up by a fine brush and preserved in small sterilized vials, as monthly samples beginning from $25^{th}$ October to $10^{th}$April 2017, for 50 plants per inspection.

**2.The effect of certain climatic factors on *Aspergillus candidus* activity:**

The daily records of temperature (˚C), relative humidity (RH%) and rain fall (mm/day) during the experimental season were obtained from Meteorological Department at Sakha, Agricultural Research Station. Monthly means of these factors were calculated. The relationship between these weather factors and the number of dead mealybug adults was statistically calculated. Linear correlations were fitted using IBM SPSS Statistics 19 software to reveal the correlation between mortality rates of *P. solenopsis* adults at respective weather factors.

**3. Isolation of entomopathogenic fungus:**

The dead insects were sterilized using 0.5% sodium hypochlorite and 75% ethyl alcohol and then rinsed in plenty of distilled water and dried with sterile filter paper. Then, the dead insects were individually kept in petri dishes (9 cm diameter) provided with moistened filter paper and incubated at 28ºC for three days to stimulate the growth of fungus.The pathogen was introduced into a petri dishes (9 cm diameter) having water agar medium and incubated for three days under 28 ºC. Finally , a piece of agar with mycelial growth was inoculated using a sterilized needle into petri dishes having Potato Dextrose Agar (PAD) medium, according to Dourou-Kpindou *et al*. (1995) (Figure,1).

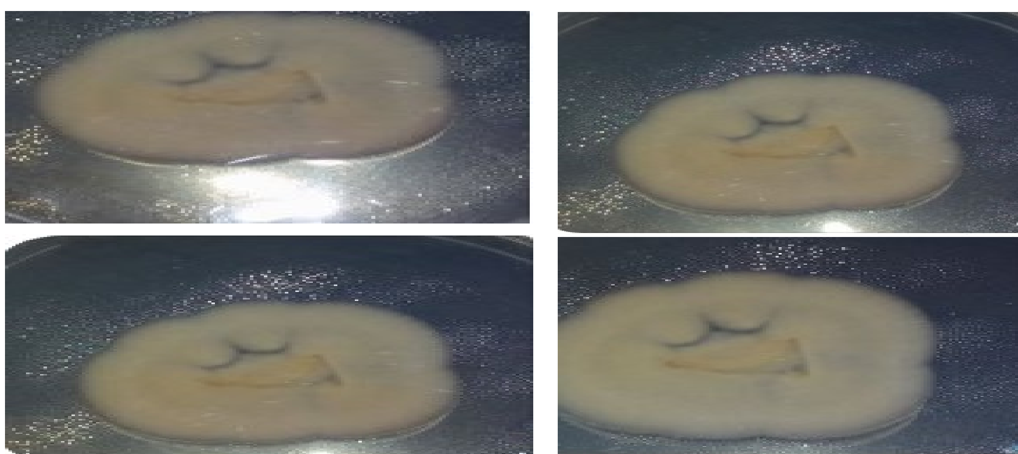

**Figure (1): *Phenacoccus solenopsis* adults with fungal hyphae of *Aspergillus candidus* strain HNMF075.**

**4. Spore isolation for using in laboratory and field tests:**

The petri dishes having sporulated fungi were washed with distilled water to exclude the fungus and formulate as a spore suspension that was adjusted to $5 \times 10^5$ spores/ml water, using the micrometer slide, to be used in tests against *P. solenopsis* adults.

**5. Laboratory tests:**

Forty *P. solenopsis* adults ($20\times 2$ petri dishes) for each sampling date, containing a piece of sugar beet leaf were treated with *A. candidus* spores suspension by hand sprayer (1 liter). The mortalities (%) were recorded two and four days after treatments according to Samsinakova and Kalalove (1975). The fungus was reisolated from the dead mealybug adults.

**6. Field tests:**

The experimental area (approximately $200\text{m}^2$) of sugar beet was divided into two plots. Each plot was divided into four replicates. One plot was sprayed with *A. candidus* spores suspension by hand sprayer (1 liter). The other one was left untreated (as a check).The number of dead mealybug individuals were recorded two and four days after treatment according to Samsinakova and Kalalova (1975). The spraying was done on 10$^{th}$January 2018. The reductions were calculated using Henderson and Tilton (1955) formula:

$$\text{Reduction (\%)} = 1 - \left(\frac{\text{No in control before spray}}{\text{No in control after spray}} \times \frac{\text{No in treated after spray}}{\text{No in treated before spray}}\right) \times 100$$

**7. DNA isolation, PCR and sequencing:**

Total DNA was isolated using CTAB method Doyle and Doyle (1990). Concentration of obtained DNA (ng/µl) and purity were determined on Nanodrop-photometer, and then kept at 20$^{o}$C till use. Polymerase Chain Reaction (PCR) was done in a volume of 50µl using 60ng genomic DNA, 0.2 mMdNTP, 1.5 mM $MgCl_2$, 5 pmol of primer and 0.5U Taq polymerase. PCR conditions were as follows: 95°C for 5 min, 35 cycles of 95 °C for 1.30 min, 55°C for 45 sec, 72°C for 1 min. then 72°C for 7 min. PCR products were separated by 1.2% agarose gels electrophoresis, which run with 1X TAE buffer. The 1Kbp DNA ladder was also run on each gel as a molecular weight standard. The amplification of 5.8S ribosomal RNA gene and internal transcribed spacer was done from fungal genomic DNA by PCR. PCR purified products of the rDNA of the strains were analyzed for nucleotide sequence determination by using ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems).

**8. Data analysis and phylogenetic analysis:**

A pairwise comparison among isolate and other sequences from GenBank database with the BlastN algorithm to determine relative phylogenetic positions was performed to produce a dendrogram using neighbor-joining (NJ) trees.

**Results and Discussion**

**1. Recording the natural mortalities of *Phenacoccus solenopsis* adults with *Aspergillus candidus*:**

Data in Table (1) showed that the number of dead adults was low on October 25$^{th}$ and November 16$^{th}$ (3 and 6 individuals/50 plants, respectively). The number jumped to 15 individuals/50 plants on December 18$^{th}$. The dead numbers reached 17 and 19 individuals/50 plants on January 19$^{th}$ and February 20$^{th}$, respectively. The numbers decreased sharply and reached to 9 and 2 individuals/50 plant on March 23$^{rd}$ and April 10$^{th}$, respectively.Throughout the season, the total number of dead adults was 71 individuals/ 300 sugar beet plants.

**2. The effect of certain weather factors on *Aspergillus candidus* activity:**

A fitted linear regression model showed that the mortality rates of *P. solenopsis* adults were positively correlated with the temperature (y = 0.7

x + 16, r2 = 0.825, p < 0.001) and (y = 0.6 x + 80, r2 = 0.516, p < 0.001). These results suggested that the high temperature and relative humidity enhanced the role of the entomopathogenic fugus in killing *P. solenopsis* adults.

**Table (1): Natural mortality of *Phenacoccus solenopsis* adults collected from 50 sugar beet plants /sampling date due to *Aspergillus candidus* during 2016/2017 season.**

| Date | No. of *Phenacoccus solenopsis* dead | Mortality% | Temperature (°c) | Humidity (RH %) | Rain (mm/day) |
|---|---|---|---|---|---|
| **October, 25** | 3 | 4.23 | 29.44 | 80.71 | - |
| **November,16** | 6 | 8.45 | 25.82 | 91.28 | - |
| **December,18** | 15 | 21.13 | 20.07 | 90.14 | 7.1 |
| **January, 19** | 17 | 24.94 | 18.82 | 91.71 | 8.1 |
| **Feburay, 20** | 19 | 26.76 | 17.01 | 90.00 | 8.7 |
| **March, 23** | 9 | 12.68 | 20.44 | 91.00 | - |
| **April, 10** | 2 | 2.82 | 25.08 | 81.00 | - |
| **Total** | 71 | - | - | - | - |

Pell *et al*., (2001) indicated that the environmental conditions particularly, humidity and temperature play an important role in the infection and sporulation of entomopathogenic fungus. Humidity (RH %) is required for spore germination and sporulation outside the host. Most of the entomopathogenic fungi in tropical and subtropical areas require an optimum temperature of 25-30 °C for successful control of insect pests. Samsinakova and Kalalova (1975) reported that the relatively high natural mortality of scale insects depends on temperature and humidity conditions. Also, Dhileepan *et al*. (1990) showed that the possible reason for the wide spread incidence of *A. candidus* against the spindle bug, *Carvalhoia arecae* in the field during the raining season.

**3. Pathogenicity of *Aspergillus candidus* to *Phenacoccus solenopsis* in laboratory tests:**

Table (2) showed that the mortalities of adults were 55.00 and 95.00% two and four days after treatment, respectively. The overall mortality recorded throughout the experimental period was 75.00%. Roberts and Yendel (1971) and Pierre (1985) indicated that *Aspergillus* spp. are imperfect fungi and are most frequently associated with insect diseases. Singh and Singh (1982) reported that *A.candidus* induced about 100% mortality under laboratory conditions against *Indarela* spp. Insects. Also, Dhileepan *et al*. (1990) showed that *A. candidus* is a virulent entomopathogen to *C. arecae* killing 50% within two days of inoculation and 100% in four days after inoculation. Finally, Samsinakova and Kalalova (1975) proved that *A. candidus* caused about 85% to 100% mortality against *Coccus hesperidum* L. (Hemiptera: Coccidae) insect, two days after treatment under laboratory conditions.

**Table (2): Mortality percentages of *Phenacoccus solenopsis* adult treated with *Aspergillus candidus* suspension ($5 \times 10^5$ ) /ml water in the laboratory, 2017/2018.**

| Duration after treatment (day) | No.of adults before treatment | No. of dead adults after treatment | Mortality (%) |
|---|---|---|---|
| 2 | 20 | 11 | 55.00 |
| 4 | 20 | 19 | 95.00 |
| Overall | 40 | 30 | 75.00 |

**4.Effect of *Aspergillus candidus* suspension on *Phenacoccus solenopsis*in the field tests:**

Table (3) indicate that the reduction of *P. solenopsis* opulations were 53.00 and 67.42% two and four days after treatment, respectively. Overall mean of reduction was 60.21%. El-Husseini (1981) proved that the entomopathogen fungi were effective microbial control agents in crops with vegetation contributes to the presence of high relative humidity in the microclimate within plants as occurring in sugar beet fields. Ramanujam *et al*. (2014) showed that entomopathogenic fungi play an important role in the natural pest control in various crops through epizootics.

**Table (3): Reduction of *Phenacoccus solenopsis* due to *Aspergillus candidus* suspension ($5 \times 10^5$ ) /ml water in the field, 2017/2018 season**.

| Treatment | | *Aspergillus candidus* | Control |
|---|---|---|---|
| **Before spray** | Total | 17 | 16 |
| | Mean | 4.25 | 4.00 |
| **After 2days** | Total | 11 | 22 |
| | Mean | 2.75 | 5.50 |
| | Red.(%) | 53.00 | - |
| **After 4days** | Total | 9 | 26 |
| | Mean | 2.25 | 6.50 |
| | Red.(%) | 67.42 | - |
| Overall mean of reductions | | 60.21 | - |

**5. Molecular identificaion:**

The method to identify isolated fungus at the species level was developed using the rRNA genes. The partial sequence with 600bp was amplified, sequenced and compared with other sequences in GeneBank. Based on BLST, the selected isolate was closely related to various species of *Aspergillus* and has 94% identical with *A. candidus* (Figures , 2 and 3).

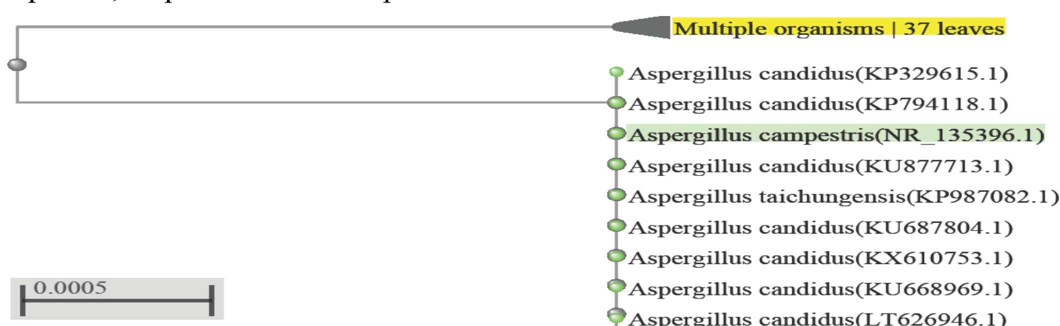


**Figure (2): Phylogenetic tree of the nucleotide sequences of the PCR product of rRNA gene from BLAST.**

Moreover, *Aspergillus* sp. taxonomy is complex, which makes its identification unreliable as a result of intraspecific similarities (Gontia-Mishra *et al*., 2013). Therefore, molecular characterization looks to be a confident tool in the identification isolated fungus based on the sequencing of the 18 S rRNA genes (Hunt *et al*., 2004). Sequences of rDNA regions and phylogenetic analysis have been used as targets for microbial isolates identification. It could be concluded that the use of DNA sequences gives a better picture of *Aspergillus* identification to be used as a biological control for insects.

| Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|
| Aspergillus candidus isolate HNMF075 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complet | 459 | 459 | 99% | 3e-125 | 94% | MH725571.1 |
| Aspergillus sp. strain Bdf-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trans | 459 | 459 | 99% | 3e-125 | 94% | MH681592.1 |
| Aspergillus tritici strain BXMA1-4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete seq | 459 | 459 | 99% | 3e-125 | 94% | MH634482.1 |
| Aspergillus candidus IPBCC.18.1399 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence | 459 | 459 | 99% | 3e-125 | 94% | LC387830.1 |
| Aspergillus candidus strain HDN15-152 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, comple | 459 | 459 | 99% | 3e-125 | 94% | MH430037.1 |
| Aspergillus candidus isolate SFC102207 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, | 459 | 459 | 99% | 3e-125 | 94% | MF186135.1 |
| Aspergillus candidus isolate 117 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequ | 459 | 459 | 99% | 3e-125 | 94% | MH345957.1 |
| Aspergillus sp. 4 JS-2017 genomic DNA sequence contains ITS1, 5.8S rRNA gene and ITS2, isolate FMR:15877 | 459 | 459 | 99% | 3e-125 | 94% | LT798907.1 |
| Aspergillus sp. 3 JS-2017 genomic DNA sequence contains ITS1, 5.8S rRNA gene and ITS2, isolate FMR:15736 | 459 | 459 | 99% | 3e-125 | 94% | LT798906.1 |
| Aspergillus sp. 3 JS-2017 genomic DNA sequence contains ITS1, 5.8S rRNA gene and ITS2, isolate FMR:15733 | 459 | 459 | 99% | 3e-125 | 94% | LT798905.1 |
| Aspergillus sp. 2 JS-2017 genomic DNA sequence contains ITS1, 5.8S rRNA gene and ITS2, isolate FMR:15444 | 459 | 459 | 99% | 3e-125 | 94% | LT798904.1 |
| Aspergillus sp. 1 JS-2017 genomic DNA sequence contains ITS1, 5.8S rRNA gene and ITS2, isolate FMR:15226 | 459 | 459 | 99% | 3e-125 | 94% | LT798903.1 |
| Aspergillus sp. 1 JS-2017 genomic DNA sequence contains ITS1, 5.8S rRNA gene and ITS2, isolate FMR:15224 | 459 | 459 | 99% | 3e-125 | 94% | LT798902.1 |
| Aspergillus candidus strain CHNSCLM-0393 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inter | 459 | 459 | 99% | 3e-125 | 94% | MF681708.1 |
| Aspergillus tritici isolate NB-DR-n internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete seq | 459 | 459 | 99% | 3e-125 | 94% | MG519717.1 |
| Aspergillus tritici genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, strain CCF 4653 | 459 | 459 | 99% | 3e-125 | 94% | HG915890.2 |
| Fungal endophyte isolate GZWMJZ-056 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal t | 459 | 459 | 99% | 3e-125 | 94% | KY038595.1 |
| Fungal endophyte isolate GZWMJZ-055 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal t | 459 | 459 | 99% | 3e-125 | 94% | KY038594.1 |
| Aspergillus candidus strain SW140 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete se | 459 | 459 | 99% | 3e-125 | 94% | KY260674.1 |
| Aspergillus candidus strain SW84 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcri | 459 | 459 | 99% | 3e-125 | 94% | KY260665.1 |
| Aspergillus tritici strain sp2-8-1 18S ribosomal RNA gene, partial sequence; and internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc | 459 | 459 | 99% | 3e-125 | 94% | MF716581.1 |
| Aspergillus tritici isolate ITS_4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spac | 459 | 459 | 99% | 3e-125 | 94% | MG022438.1 |

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Query  1    AGCGGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  456  AGCGGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGG  397

Query  61   GCCCGTCCCCGGGGGTACCGGGGACGGGGCCCAACACACAAGCCGTGCTTGAGGGCAGCA  120
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Query  121  ATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGAC  180
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Sbjct  156  AGACTGCACTT  146
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**Figure (3): *Aspergillus candidus* isolate HNMF075 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**

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