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RESEARCH ARTICLE

Determine the genotype of the local fungal isolation *Aspergillus niger* 5 producers of citric acid

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Abstract

The current study included determining the genotype of local fungal isolation *Aspergillus niger* 5 producer of citric acid and preserved in the laboratory of fungi in the Department of Biology/College of Science/ University of AL-Qadisiya by using PCR-RFLP technique and get the sequence of DNA bases for region ITS1-5.8S rRNA gene-ITS2 and determining variation areas, genetic distances and Phylogenetic tree analysis for a local fungal isolation and compare it with some of the world fungal isolates registered in the National Centre for Information biotechnology database (NCBI) by using the MEGA 6 program.

The results of the extraction of DNA of five replicates of *A. niger* 5 contains a single and clear DNA bands and after the measured by Nanodrop spectrophotometer was concentrations of DNA ranging from (12.6-15.0) ng/µL and purity ranged between (1.44-1.69). and after amplification the DNA by using the PCR technique was the result of amplification a molecular size 546 base pairs.

The results of enzymatic cutting of the result of amplification by using *RsaI* restriction enzyme showed a single two bands with a molecular size (480,66) base pairs and that the genotype of the fungus under study is of the type (A). also found 10 variation areas after A lineup sequences of nucleotides for isolate under study and compare it with some of the world fungal isolates of *A. niger* registered in the (NCBI) and the Phylogenetic tree analysis showed the presence of *A. niger* 5 with six world isolates (HQ392477.1 Malaysia, JX291197.1 Malaysia, JX036483.1 China, HQ392475.1 Malaysia, KJ881376.1 India, JX501376.1 Malaysia) in one secondary group.

INTRODUCTION

Citric acid (2-hydroxy propane 1,2,3- tricarboxylic acid) is the most important organic acids, derived its name from the Latin word which means Citrus, the Swedish chemist Scheele Carl Wilhelm is the first isolation of citric acid from lemon juice in 1784, and also the citric acid produced naturally by metabolic pathways that take place in a living cell by tri carboxylic acid cycle (Swain *et al*., 2011).

Citric acid has many uses in the areas of food, chemical and pharmaceutical industries as it uses 70% of the citric acid in various food industries and 12% in the chemical, pharmaceutical, medical, and 18% in other industries (Soccol *et al*., 2003). and in spite of the possibility of producing citric acid from plant, animal sources and chemical

methods, but that commercial production has been mainly by microbial fermentation that characterized by this fermentation of the possibility of increasing production by improving the environmental conditions and genetic control for microorganism (Demain & Dana, 2007).

Factories producing citric acid used many strains of *A. niger* for the production of citric acid in large quantities with some improvements on the various production stages from time to time in order to increase production of citric acid (Hossain *et al*., 1984). The most important advantages of using *A. niger* in the production of citric acid is the ease of isolation and high susceptibility to fermentation of a large quantity of cheap raw material price and thus get high productivity as characterized fungus ability to convert existing sugars in the fermentation media to the citric acid with high efficiency ranging from (70-90)% (Meers & Milsom, 1987).

Consequent nuclear rDNA general in fungi in units repeated each unit containing three genes rRNA in the small secondary units are rRNA 18S and 5.8 S rRNA and large are 28 S rRNA and separates the genes in a single unit two areas called Internal Transcribed Spacer (ITS) that include (ITS1) and (ITS2) and separating every two units of the rDNA region called Intergenic Spacer (IGS) (White *et al*., 1990). and these Spacer regions more heterogeneous than sequences of secondary units have been used widely in study relations between species within the same-genus or individuals within the same species (Buscot *et al*., 1996).

The development of Polymerase Chain Reaction and design primers to amplify different regions of the rDNA had a great merit in taxonomic-fungal studies especially primers designed depending on the region ITS and by which was detected many areas of the ITS to various fungi, which used to investigate, classify and study relations genetic among the various types within the genus or the physiological forms of the same species (Bridge *et al*., 1998).

The present study aims to determining the genotype of local fungal isolation *A. niger* 5 which have a high ability to the production of citric acid by using the PCR-RFLP technique and get the sequence of DNA bases for region ITS1-5.8S rRNA gene-ITS2 and determining the variation areas, genetic distances and Phylogenetic tree analysis for a local fungal isolation and compare it with some of the world fungal isolates registered in the (NCBI).

Materials & Methods

Microorganism Used

Citric acid producer strain *A. niger* 5 obtained from the laboratory of fungi in the Department of Biology/College of Science/University of AL-Qadisiya and this isolation has a high capability to produce citric acid has been activated isolation by growing on PDA and incubated in the incubator at a temperature of 25 ° C for 7 days.

DNA Extraction

Extracted DNA from isolated *A. niger* 5 by using the (EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit) processed from the South Korean company Bioneer as follows:

1. Transferred about 200 mg of fungal colonies by 5 replicates to ceramic container on sterile all alone and using liquid nitrogen with -169 ° C was crushed fungal colonies and then to a sterile 1.5 ml tubes capacity.
2. After it was added 180 µL of Universal Digestion Buffer solution and 20 µL of enzyme Proteinase K to each sample and then mix well by vortex and then the samples were incubated 56 ° C in the water bath for 30 minutes.
3. Added 100 µL of Universal Buffer PF solution by mixing and stirring pipes and incubated at -20 ° C in the refrigerator for 30 minutes.
4. Put the samples in the centrifuge speeds of 10000 r/min for 5 minutes and then transfer to a new floating liquid tube capacity 1.5 ml.
5. Added 100 µL of Universal Buffer BD solution and mix well by vortex.
6. After that was added absolute ethyl alcohol 96% to all samples and mix well by vortex.
7. The mix was transferred to a private pipe containing a filter to extract DNA equipped with the kit EZ-10 column placed in Tube collection with a capacity 2 ml and then placed in a centrifuge speeds of 12000 r/min for one minute and then neglect the precipitate.
8. Added 500 µL of Universal PW Solution and then put tubes in the centrifuge speeds of 12000 r/min for one minute and then neglect the precipitate.
9. After that add 500 µL of Universal Wash Solution and then put the tubes in the centrifuge speeds of 12000 r/min for one minute and then neglect the precipitate.

10. Place the EZ-10 column containing the DNA in sterile tubes capacity of 1.5 ml and then put the tubes in a centrifuge speeds of 12000 r/min for two minutes to dry the EZ-10 column membrane of alcohol and then neglect the precipitate.
11. Added 50 µL of the solution TE Buffer to melt the DNA into the EZ-10 filter column and then incubated at room temperature for one minute and then put all the tubes in a centrifuge and speeds of 12000 r/min for one minute to collect DNA and then transferred to refrigerator in -20 ° C until use.

Measuring the Concentration and Purity of DNA

It was detected the DNA extracted from the isolation *A. niger* 5 by use Nanodrop spectrophotometer in wavelength (280/260) nm.

Primers

The use of Primers ITS 5 and ITS 4 for area ITS1-5.8S rRNA gene-ITS2 it has been processing by South Korean Bioneer company, as shown in below:

Primers		Sequence	Amplicon
ITS1-5.8S rRNA gene-ITS2	F (ITS5)	5´-GGAAGTAAAAGTCGTAACAAGG-´3	546 bp
	R (ITS4)	5´-TCCTCCGCTTATTGATATGC-´3	

Prepare the PCR Components

The preparation of the PCR components by using of Accupower ® PCR PerMIX processed by the South Korean company Bioneer and followed the manufacturer's instructions in preparation, as shown in the below:

PCR master mix	Volume
DNA template	5 µL
Forward primer (10pmol)	1.5 µL
Reverse primer (10pmol)	1.5 µL
Free nuclease water	12 µL
Total volume	20 µL

After that, put a combination of the PCR reaction components in the pipe size 0.2 ml especially for the (Accupower ® PCR Premix) and containing the other components of the PCR reaction and then moved all the pipes to the Vortex centrifuge (Exispin) speeds of 3000 r/min for three minutes and then transferred to the Thermocycler to amplification process.

Thermocycling System Bracing

Amplify the DNA was extracted from samples using PCR Thermocycler and by the way of Spadaro *et al.*, (2012) as shown in below:

PCR Step	Temperature	Time	Repeat cycle
Initial denaturation	**95 ° C**	**5 min**	**1**
Denaturation	**95 ° C**	**1 min**	**35**
Annealing	**52 ° C**	**1 min**	
Extension	**72 ° C**	**2 min**	
Final extension	**72 ° C**	**10 min**	**1**
Hold	**4 ° C**	**Forever**	**-**

Electrophoresis of the PCR Products

Electrophoresis was made by using Agarose gel 1% as follows:

1. The melt 1 g of Agarose gel in 100 ml of the buffer solution TBE buffer concentration of 1X by using Microwave for 5 minutes.
2. Leave to cool the gel at 50 ° C and then added 3 µL of DNA Ethidium bromide radioactive dye and mix well with the gel.
3. Pour the Agarose gel in Tray template containing the Comb to locate the samples, and then it left for solidifies at room temperature for 15 minutes and then removed from the comb carefully.
4. Loaded the PCR samples (5 replicates) and place in the drilling gel at 10 µL each hole.
5. Scale was used measurement Marker ladder (2000-100 base pairs) to measure the product of the PCR and 5 µL put from it in the first hole, and then added TBE Buffer solution concentration of 1X and close the cover and run the device using the current 100 volts and 80 amps for one hour.
6. After the end of the process were examined the gel containing the PCR products by using UV light source to determine the measurement and images of the gel by using a digital camera.

PCR-RFLP Assay

The test included the preparation of a combination PCR-RFLP using the *RsaI* restriction enzyme obtained from the British company Biolab as shown in the below:

RFLP-PCR Master mix	Volume
PCR product	**5 µL**
1XCutSmart™ Buffer	**2 µL**
RsaI Restriction enzyme (10 unit)	**0.5 µL**
Free nuclease water	**12.5 µL**

Total volume **20 µL**

After that, put a combination PCR-RFLP reaction in the Vortex centrifuge (Exispin) speeds of 3000 r/min for three minutes and then placed in Thermocycler device to incubated at a of 37 ° C for two hours, then was Loaded the samples (5 replicates) and place in the drilling gel (Agarose gel 2%) at 10 µL each hole, Scale was used measurement Marker ladder (2000-100 base pairs) to measure the product of the PCR-RFLP and 5 µL put from it in the first hole, and then added TBE Buffer solution concentration of 1X and close the cover and run the device using the current 100 volts and 80 amps for one hour. After the end of the process were examined the gel containing the PCR-RFLP products by using UV light source to determine the measurement and images of the gel by using a digital camera.

DNA Sequencer Method

It was obtained DNA sequencing for region ITS1-5.8S rRNA gene-ITS2 by sending the product of the PCR reaction, to Macrogen company in South Korea in order to determining the DNA sequence by using AB DNA sequencing system and after that was determining variation areas and genetic distances and made Phylogenetic tree analysis by using the program MEGA 6 by comparing the sequence of DNA of *A. niger* 5 with some of the world fungal isolates for *A. niger* that registered in the (NCBI).

Results & Discussion

Extraction of DNA from *A. niger* 5

The results of the extraction of DNA of five replicates for *A. niger* 5 by using the kit contains a single and clear DNA bands and after the measured by Nanodrop spectrophotometer was concentrations of DNA ranging from (12.6-15.0) ng/µL and purity ranged between (1.44-1.69). and after amplification the DNA by using the PCR technique was the result of amplification a molecular size 546 base pairs as shown in the table (1).

Table (1): Concentration of the DNA and purity.

Replicates	Concentration (ng/µL	Purity
1	**14.1**	**1.69**
2	**13.4**	**1.54**
3	**15.0**	**1.51**
4	**12.6**	**1.49**
5	**13.8**	**1.44**

Polymerase Chain Reaction (PCR)

This processes made by using Specific Primers for region ITS1-5.8S rRNA gene-ITS2, as observed after the Electrophoresis by Agarose gel 1% and the test under ultraviolet light the presence Single bands of DNA resulting from amplification of the five replicates for fungal isolation process,. Was estimated molecular sizes of the bands by

compared with the molecular size standard for Marker ladder (2000-100 base pairs) it is found that the bands with molecular size 546 base pairs (Fig. 1).

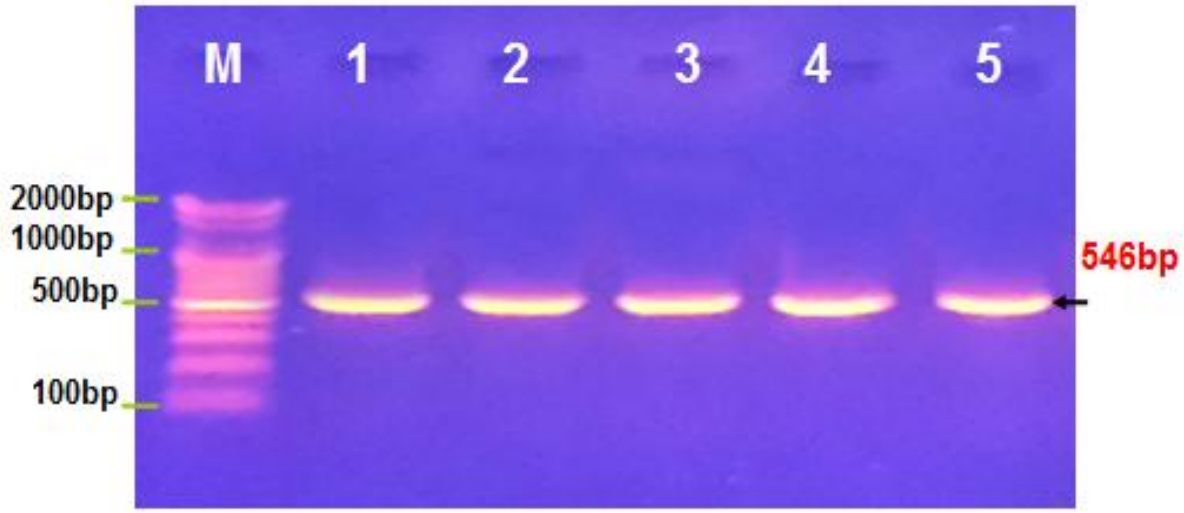

Figure 1: Amplified products for region ITS1-5.8S rRNA gene-ITS2 for *A. niger* 5 isolated from the soil and electrophoresis on Agarose gel (1%), as it represents the first column M handed measurement (Marker ladder 2000-100base pairs) and columns (1-5) represent replicates for isolation *A. niger* 5 positive results an product length of 546 base pairs and did not show negative results (non-appearance bands).

Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP)

The results of used *RsaI* restriction enzyme showed outputs two bands with a single molecular size (480,66) base pairs (Fig. 2). and the Genotype for *A. niger* 5 was (A) according to Spadaro *et al*., (2012) who was able to Determining two Genotype (A) and (B) of the fungus *A. niger* when using *RsaI* restriction enzyme.

Used PCR-RFLP technique in a number of studies to determine the Genotype and distinguish between different types of fungi as used Accensi *et al*., (1999) *RsaI* restriction enzyme to distinguish between the two fungi *A. niger* and *A. tubingensis* as two bands for *A. niger* with molecular size of 519 and 76 base pairs and one bands for the *A. tubingensis* with molecular size of 595 base pairs. Kizis *et al*., (2014) as well as he can to distinguish between some of the species of the fungus *Aspergillus* sp. By Using PCR-RFLP technique by three enzymes a *RsaI*, *HinfI* and *HhaI*.

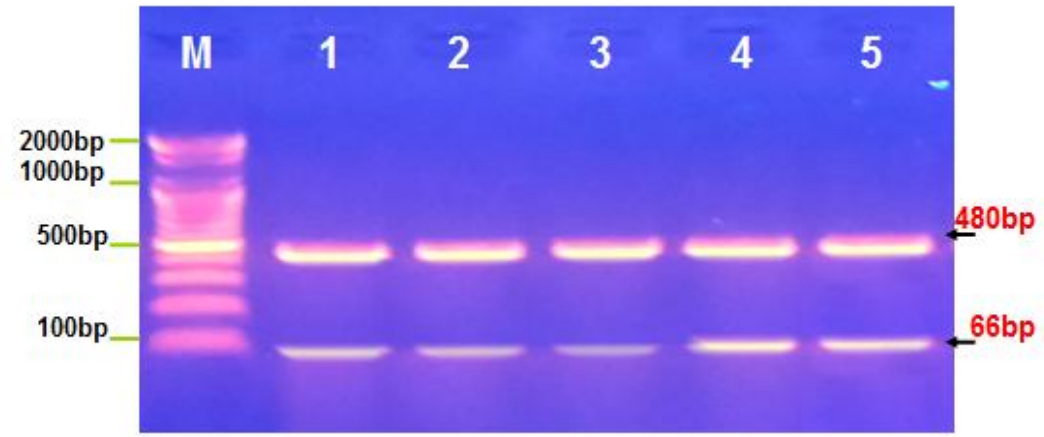

Figure 2: Products of the using *RsaI* restriction enzyme for PCR amplified region ITS1-5.8S rRNA gene-ITS2 for *A. niger* 5 isolated from soil and electrophoresis on Agarose gel (2%), as it represents the first column M ladder measurement (Marker ladder 2000-100 base pairs) and the columns of (1-5) represent replicates for isolation *A. niger* 5 positive results and which refers to the Genotype (A) A length of an product cut of 480 and 66 base pairs.

DNA sequencer

The sequence of DNA bases for region ITS1-5.8S rRNA gene-ITS2 were determining after sending the products of the PCR reaction to Macrogen company in South Korea in order to determining DNA sequencing by using AB DNA sequencing system then was determining variation areas, the results of nucleotides sequence alignment for region ITS1-5.8S rRNA gene-ITS2 for *A. niger* 5 and compare it with some of the world fungal isolates of *A. niger* registered in the (NCBI)) showed presence 10 variation areas, the results also showed the presence of a 100% match in sequences of nucleotides for region ITS1-5.8S rRNA gene-ITS2 for *A. niger* 5 with some of the world fungal isolates of *A. niger* registered in the (NCBI)) which include (JX501376.1 Malaysia, KJ881376.1 India, HQ392475.1 Malaysia, JX036483.1 China, HQ392477.1 Malaysia, JX291197.1 Malaysia) (Table 2).

Table (2): Variation areas between isolates of the fungus *A. niger* sequences for region ITS1-5.8S rRNA gene-ITS2.

species	23	25	60	63	65	116	160	403	404	523
Aspergillus niger 5_soil isolate_Iraq	T	C	A	G	A	C	T	C	T	C
Aspergillus niger_soil_isolate_KC602371.1 Canada	.	.	.	.	.	.	.	T	C	.
Aspergillus niger_soil_isolate_JX501376.1 Malaysia	.	.	.	.	.	.	.	.	.	.
Aspergillus niger_soil_isolate_KJ881376.1 India	.	.	.	.	.	.	.	.	.	.
Aspergillus niger_soil_isolate_KM103363.1 Nepal	.	.	.	A	.	T	.	.	.	T
Aspergillus niger_soil_isolate_HQ392475.1 Malaysia	.	.	.	.	.	.	.	.	.	.
Aspergillus niger_soil_isolate_JX036483.1 China	.	.	.	.	.	.	.	.	.	.
Aspergillus niger_soil_isolate_JX501365.1 Malaysia	G	G	.	.	.	.	.	.	.	.
Aspergillus niger_soil_isolate_HQ392477.1 Malaysia	.	.	.	.	.	.	.	.	.	.
Aspergillus niger_soil_isolate_JX291197.1 Malaysia	.	.	.	.	.	.	.	.	.	.
Aspergillus niger_soil_isolate_JQ660373.1 India	.	.	.	A	.	T	.	.	.	T
Aspergillus niger_soil_isolate_JN246057.1 Australia	.	.	.	A	.	T	.	.	.	.
Aspergillus niger_soil_isolate_JX291201.1 Malaysia	.	.	T	.	T	.	.	.	.	.
Aspergillus niger_soil_isolate_HQ710538.1 India	.	.	.	.	.	.	G	.	.	.

It was also compared the Genetic distance for the region ITS1-5.8S rRNA gene-ITS2 for *A. niger* 5 with some of the world fungal isolates of *A. niger* registered in the (NCBI) by using the program MEGA 6 the results shown in the Table (3) match the genetic distances for *A. niger* 5 with some of the world fungal isolates of *A. niger* which include (JX501376.1 Malaysia, KJ881376.1 India, HQ392475.1 Malaysia, JX036483.1 China, HQ392477.1 Malaysia, JX291197.1 Malaysia) while some differences appeared in the genetic distances with other *A. niger* fungus World It ranged from (0.2-0.6). %

Also Phylogenetic tree analysis made based on the sequences for the region ITS1-5.8S rRNA gene-ITS2 for *A. niger* 5 and comparing them with the same region for some of the world fungal isolates of *A. niger*, analysis showed the presence of two main groups first divided into five secondary groups, a one of these containing *A. niger* 5 with six world isolates which corresponds with it 100% and the second is divided into two secondary groups includes three world isolates (Figure 3).

Many studies have focused on the design primers to amplify different regions of the rDNA, which have the great merit of the taxonomic-fungal studies especially primers designed based on the ITS region and by means of which was detected many region of the ITS to various fungi, used primarily to study the genetic relationships between different species within genus or physiological forms of the same type (Mitchell *et al*., 1995).

Gonzalez-Salgado *et al*., (2005) able to distinguish between some of the genus *Aspergillus* sp species. These species are *A. niger*, *A. tubingensis*, *A. japonicus*, *A. ellipticus*, *A. heteromorphus* and *A. carbonarius* by

amplification region ITS1-5.8S rRNA gene-ITS2 for this fungal species and detection of variation areas. As he studied Ferracin *et al*., (2009) Genetic relationships among 51 fungal isolates belonging to the fungus *A. niger* and 7 isolates belonging to the fungus *A. tubingensis* and was able to made Phylogenetic tree analysis and determining of the main and secondary groups.

Table 3: Genetic distances between isolates fungus *A. niger* sequences for region ITS1-5.8S rRNA gene-ITS2.

***Aspergillus niger* 5_Iraq**													
***Aspergillus niger*_KC602371.1 Canada**	**0.00 4**												
***Aspergillus niger* _JX501376.1 Malaysia**	**0.00 0**	**0.00 4**											
***Aspergillus niger*_KJ881376.1 India**	**0.00 0**	**0.00 4**	**0.00 0**										
***Aspergillus niger*_KM103363.1 Nepal**	**0.00 6**	**0.00 9**	**0.00 6**	**0.00 6**									
***Aspergillus niger*_HQ392475.1 Malaysia**	**0.00 0**	**0.00 4**	**0.00 0**	**0.00 0**	**0.00 6**								
***Aspergillus niger*_JX036483.1 China**	**0.00 0**	**0.00 4**	**0.00 0**	**0.00 0**	**0.00 6**	**0.00 0**							
***Aspergillus niger*_JX501365.1 Malaysia**	**0.00 4**	**0.00 7**	**0.00 4**	**0.00 4**	**0.00 9**	**0.00 4**	**0.00 4**						
***Aspergillus niger*_HQ392477.1 Malaysia**	**0.00 0**	**0.00 4**	**0.00 0**	**0.00 0**	**0.00 6**	**0.00 0**	**0.00 0**	**0.00 4**					
***Aspergillus niger*_JX291197.1 Malaysia**	**0.00 0**	**0.00 4**	**0.00 0**	**0.00 0**	**0.00 6**	**0.00 0**	**0.00 0**	**0.00 4**	**0.00 0**				
***Aspergillus niger*_JQ660373.1 India**	**0.00 6**	**0.00 9**	**0.00 6**	**0.00 6**	**0.00 0**	**0.00 6**	**0.00 6**	**0.00 9**	**0.00 6**	**0.00 6**			
***Aspergillus niger*_JN246057.1 Australia**	**0.00 4**	**0.00 7**	**0.00 4**	**0.00 4**	**0.00 2**	**0.00 4**	**0.00 4**	**0.00 7**	**0.00 4**	**0.00 4**	**0.00 2**		
***Aspergillus niger*_JX291201.1 Malaysia**	**0.00 4**	**0.00 7**	**0.00 4**	**0.00 4**	**0.00 9**	**0.00 4**	**0.00 4**	**0.00 7**	**0.00 4**	**0.00 4**	**0.00 9**	**0.00 7**	
***Aspergillus niger*_HQ710538.1 India**	**0.00 2**	**0.00 6**	**0.00 2**	**0.00 2**	**0.00 7**	**0.00 2**	**0.00 2**	**0.00 6**	**0.00 2**	**0.00 2**	**0.00 7**	**0.00 6**	**0.00 6**

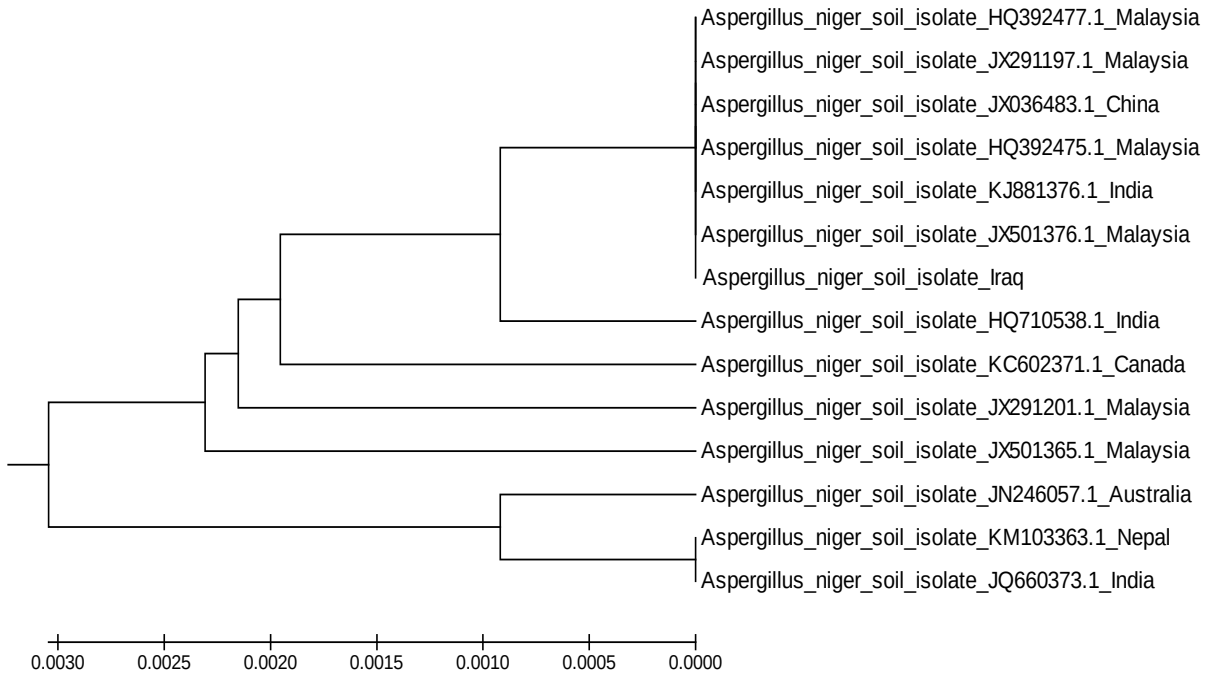

Figure 3: Phylogenetic tree analysis of type (Test UPGMA tree) (Unweighted Pair Group Method with Arithmetic Mean) using MEGA 6 program.

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