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MOLECULAR DIAGNOSTICS OF ISOLATES OF FOREST CANKER FUNGI USING POLYMERASE CHAIN REACTION

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Abstract:
Accurate classification of fungi is important in the diagnosis of pathogenic fungi Several studies have indicated that phenotypic traits cannot be relied upon the diagnosis of fungi, as it requires sufficient experience in the field of classification of fungi, especially when dealing with fungal groups that are very similar to each other in addition to that it requires a great deal of time and effort, as well as the influence of the sizes, shapes and colors of spores by various environmental factors (Huang et al. 2016).

Keywords: Polymerase Chain Reaction

INTRODUCTION:

Accurate classification of fungi is important in the diagnosis of pathogenic fungi Several studies have indicated that phenotypic traits cannot be relied upon the diagnosis of fungi, as it requires sufficient experience in the field of classification of fungi, especially when dealing with fungal groups that are very similar to each other in addition to that it requires a great deal of time and effort, as well as the influence of the sizes, shapes and colors of spores by various environmental factors (Huang et al. 2016). With the rapid development of molecular biology techniques and the emergence of the Polymerase Chain Reaction (PCR) technology and the use of Molecular Markers, including Random Amplified Polymorphic (DNA) (RAPD) indicators, which depend on PCR technology in (DNA) replication. Obtaining (DNA) packets, and from the results of this replication, it is possible to study many types of organisms that are difficult to accurately diagnose based on their phenotypic characteristics or when using traditional indicators, determining the genetic dimension and revealing the genetic relationship between species and races (Kämpfer, 2006). The rapid development that took place in the field of biotechnology led to the discovery of the polymerase chain reaction (PCR), which was first described by the American researcher Saiki and others in year 1985. Millions of copies of them are outside the cell in vitro, and this process is a simulation of what happens in nature in all living organisms whose genetic material is duplicated during division, and this discovery made a great revolution in the world of Molecular Biology comparable to the revolution brought about by the discovery of the double structure of (DNA) and thus deserved it Mullis Nobel Prize in 1993 (Kavya, 2015). Currently a technolog PCR is the most popular technology in molecular genetics laboratories all over the world and the basis on which many studies at the level of (DNA) depend, as it is characterized by strength, effectiveness and high sensitivity and is not used in amplifying (DNA) only, but also RNA (Park, 2012), as well as It is simple, fast and inexpensive, requires a small amount of (DNA) to perform the reaction and does not require that the (DNA) be pure in order to replicate it, and it helps in identifying the nature and properties of many cognate genes (Schuller et al. 2010; Ichida and Abe, 2019).

MATERIALS AND METHODS :

(DNA) Extraction:

(DNA) was extracted from the isolates of the fungi under study using the extraction kit supplied by (Geneaid Taiwan) company, following the working method provided by the company, and it was as follows

Table (1): Solutions used for the extraction process

Volume / ml	solution
75	GT
15	PR
60	GB
45	W1
25	Wash
30	Elution
100	Beadbeating tubes type B

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1- Sample preparation:
Place 100 mg of fungal colonies incubated 24 hours into 1.5 ml Eppendrof tubes.

2- The sample was analyzed by adding 600 µl of GT solution, mixed using a Vortexer electric mixer, transferring the mixture to tubes for analyzing cell walls (Beadbeahing). The tubes were incubated at a temperature of 70°C for 10 minutes, stirring every 3 minutes. during the work, the PR solution was mixed and mixed to get rid of foam on the sample. The tubes were incubated with ice for 5 minutes and centrifuged for 3 minutes, then 450 µl of the suspension was transferred to a 1.5 capacity Eppendorf tube. during this time, 200 µl of Elution Buffer was incubated for the purpose of using it. In the (DNA) thawing step.

3- (DNA) stabilization:
Added 450 µL of GB solution and 450 µL of absolute ethyl alcohol were to the sample and mixed immediately for 10 seconds, then a GD filter was placed in a 2 ml collection tube. The mixture was transferred in two stages to a GD filter tube with a capacity of 700 µL each time and centrifuged at 16,000 cycles. /min Xg and each time the stuck is cleared.

4- Washing:
400 µl of wash solution W1 (Wish Buffer) was added to the spin column filter tube and centrifuged for 30 seconds, the suspension was discarded and the filter tube was returned to the 2 ml collection tube. Then 600 µl of the final wash solution W2 Wish Buffer was added to the filter tube and centrifuged at 16,000 rpm Xg. To ensure the filter tube was dry, further centrifugation was performed under the same conditions.

5- (DNA) dissolving
Transfer the filter tube to a 1.5 ml Eppendrof tube and keep the thawed extracted (DNA) at -20°C until use.

Determination of concentration and efficiency of extracted (DNA)

1- The (DNA) was migrated within the agarose gel at a concentration of 0.7% to reveal the ability of the (DNA) molecules to penetrate the pores of the agarose gel.

2- A Spectronanometer was used to determine the concentration of extracted (DNA), caibration of the device was done by placing a drop of the dissolved (DNA)-free solution over the lower lens and the (DNA) concentration was determined at zero concentration, then 5 µl of the dissolved extracted (DNA) mixture was placed over the lens Lower (DNA) concentration to be read. All samples showed that the (DNA) concentration was between 25-35 ng/ml, as the reading was determined at a wavelength of 260 nm.

3- Determination of the purity of the extracted (DNA)
The purity of (DNA) extracted from isolates of fungi was determined using a micro-drop spectrophotometer at a wavelength between 260-280 nm, and it was found that the purity of the (DNA) of the samples under study ranged between 1.7-1.9.

Electrical relay

1- Electrophoresis solution:
The electrophoresis solution was prepared by Bioneer South Korrea at a concentration of 10X as the base concentration. The solution was diluted with a mixture of 10 ml (TBE) Tris Boratc EDTA (15X) with 90 ml of distilled ion-free water to form a TBE solution (1X used in the electrophoresis). During the preparation of agarose gel.

2- Dye loading:
A loading dye was prepared by Bioneer South Korrea. This dye helps detect electrophoresis with the naked eye, in addition to its role in increasing the specific weight of (DNA), which leads to its stability in the migration pits until it penetrates the pores of the agarose gel during migration and the investigation of the integrity of the (DNA) Then mix 1 µL of the loading dye with 4 µL of the (DNA) to be migrated and then injected into the migration pits within the agarose gel.

3- Gveenstar dye brilliant:
It is that dye that has a positive charge that enables it to bind with (DNA) molecules with a negative charge that leads to the appearance of fluorescent bands when exposing the agarose gel to the irradiator.

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(DNA) amplification of the ITS1 and ITS4 gene

1- Prepare the amplification mix

The amplification mixture was prepared as shown below:

Table (2): The volume and concentration of the ingredients

Ingredient	Volume µl	Concentration Nano g/ml
(DNA) extracted	6	35-25
front initiator	1	Pmol10
Reverse starter	1	Pmol10
Distilled deionized water	12	-

2- Amplification of ITS1 and ITS4 genes using thermo polymer:
The amplification products of the samples under study (9) were sent to the Korean Macrogin Company for the purpose of revealing the sequence of nitrogenous bases representing the isolates under study.

Table (3): Isolation steps for the samples under study

Steps	Temperature (C)	Time (sec)	Number of cycles
Fusion	94	40	35
Link	50	50	
Elongation	72	90	
Primary fusion	72	600	1
Final elongation	4	180	___

DIAGNOSIS OF ISOLATES ACCORDING TO THE SEQUENCE OF NITROGENOUS BASES:

The alignment of the ITS1 and ITS4 gene base sequences was done within the BLAST algorithm (Basic Local Alignment Search Tool) within the National Center for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION:

Molecular diagnosis of ulcerative fungi isolates:

1-1 Diagnosis using polymerase chain reaction (PCR) technology:

Isolates of ulcer-causing fungi *A. brassicicola*, *F. solani, F. oxysporum, N. mangiferae, R. solani, T. harzianum,* using primer-based polymerase chain reaction (PCR) designed to amplify gene segments (ITS). The results of the electrical conductivity of the (DNA) amplification products of the parts ITS1 and ITS4 showed the presence of eight bands of 559 base pairs within the agarose gel as shown in Figure (1)

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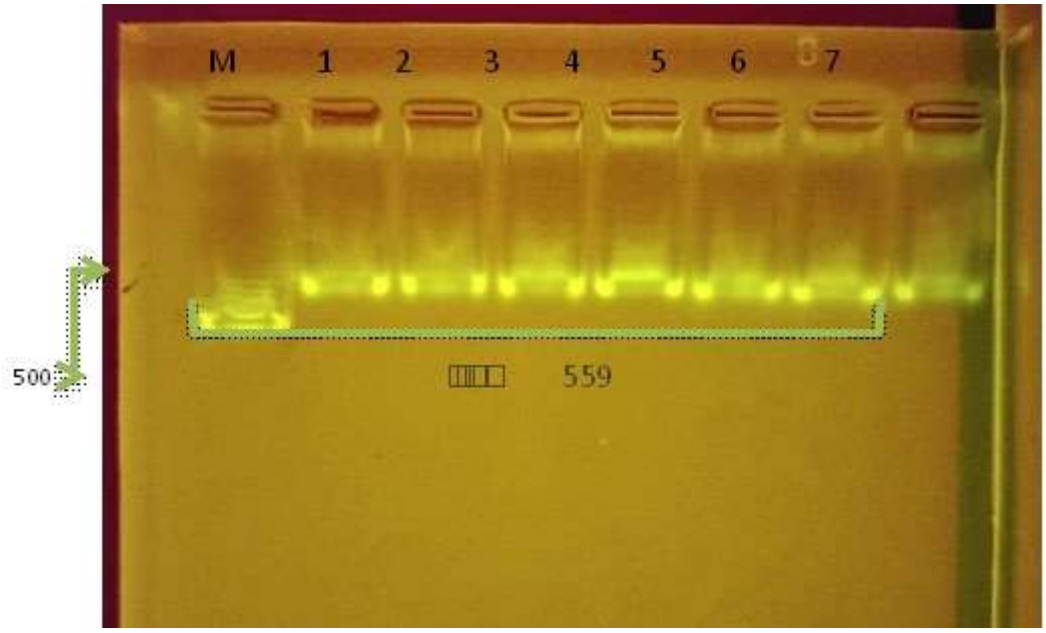

Fig.1 : Electrical conduction of PCR amplification products of fungal isolates using the ITS1 initiator.

Path (M): Molecular scale (100-2000) base pairs.
Routes (1-8): Positive result of 559 base pair amplification.
Path 1: *F.oxysporium.* Path 2: *F.oxysporium.*
Path (3): *F.solani*. Path (4): *A.brassicicola.*
Path 5: *R.solani.* Path (6) *T.harzianum*.
Path (7): *N.mangiferae*. Path (8): *N.mangiferae.*

The appearance of the bundles at the mentioned size represents a match in the size of the amplified portion according to the designed primer, and therefore this is a preliminary diagnosis for isolates of fungi (Baker, 2021). This is consistent with what was indicated by studies that dealt with the diagnosis of fungi using PCR technology. Diagnosed the fungi *D.avenae* and *Aureobasidium* by using a pair of specialized primers ITS1 and ITS2. In another study carried out by the researcher (Zarin et al. 2016), 50 isolates belonging to the genus Fusarium isolated from other environments were identified using the same technology and based on the ITS1 and ITS4 regions of rRNA, while (Osman, 2021) the causative fungus *F. verticilloides* was identified. Knife-cutting disease of sugarcane plants using ITS1 and ITS4 nitrogen base sequences.

1-2 Determination of the nitrogenous base sequence of ITS1 and ITS4 gene amplifiers of the fungi isolates under study:

The results of aligning the nitrogenous base sequences of the isolates of the fungi under study in the NCBI GenBank showed the identification of two isolates of *F.oxysporium* spores, an isolate representing *F.solani*, an isolate representing *A.brassicicola*, an isolate representing *R.solani*, an isolate representing *T.harianum* and two isolates representing *N.mangiferae*.

1-3 Molecular analysis of genetic relationship between isolates of fungi:

1- Fungi *Fusarium spp*:

The results of the genetic relationship analysis included the presence of genetic affinity for the two isolates of *F.oxysporium* under study within the subgroup (A) at a level of closeness of 96%, and this corresponds to what was stated in the source of the aforementioned fungus isolate from the two types of fungus and gogh trees, which shows the ability of the aforementioned fungus to cause infection in the fire The Gogh as members of the Platancea and Salicaccae families. On the other hand, the results of genetic analysis showed a clear divergence of *F. solani* from individuals of *F. oxysporium* to be found within subgroup (B) with isolate *F.solani* A5252.

There are many studies that have been completed in this field, such as the study , which classified the species of the mentioned genus according to the alignment of the sequence of nitrogenous bases with their counterparts in the National Center for Biological Information (NCBI)

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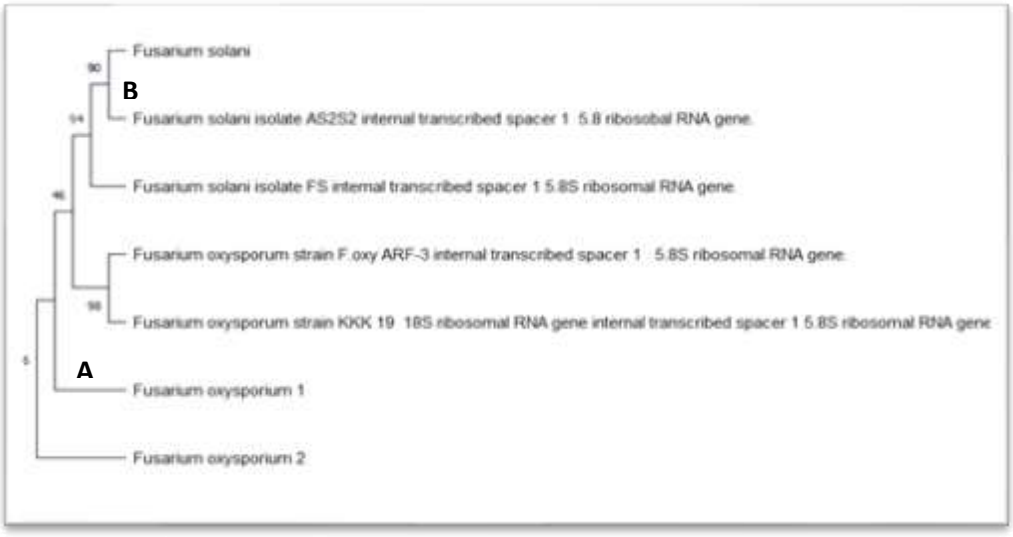

Fig.2: Genetic relationship analysis of Fusarium isolates under group (A) included two isolates *F.oxysporium* 1 and *F.oxysporium* 2 with a level of approximately 96%, and *F.solani* A5252 was within subgroup B with a level of approximately 90%

2- Fungi *A.brassicicola*:

The results of the molecular analysis of the genetic affinity and divergence of the aforementioned fungus showed a clear affinity of 94% with *A. brassicicola*, while it was close to the rest of the isolates in different proportions. This is consistent with what has been indicated by several studies (Blagojevic et al. 2020; Fontains et al. 2021) where the results of these studies are consistent with the results of the current studies and by determining the affinity between individuals of the fungus *A. brassicicola* based on the determination of the sequence of nitrogenous bases of the target gene.

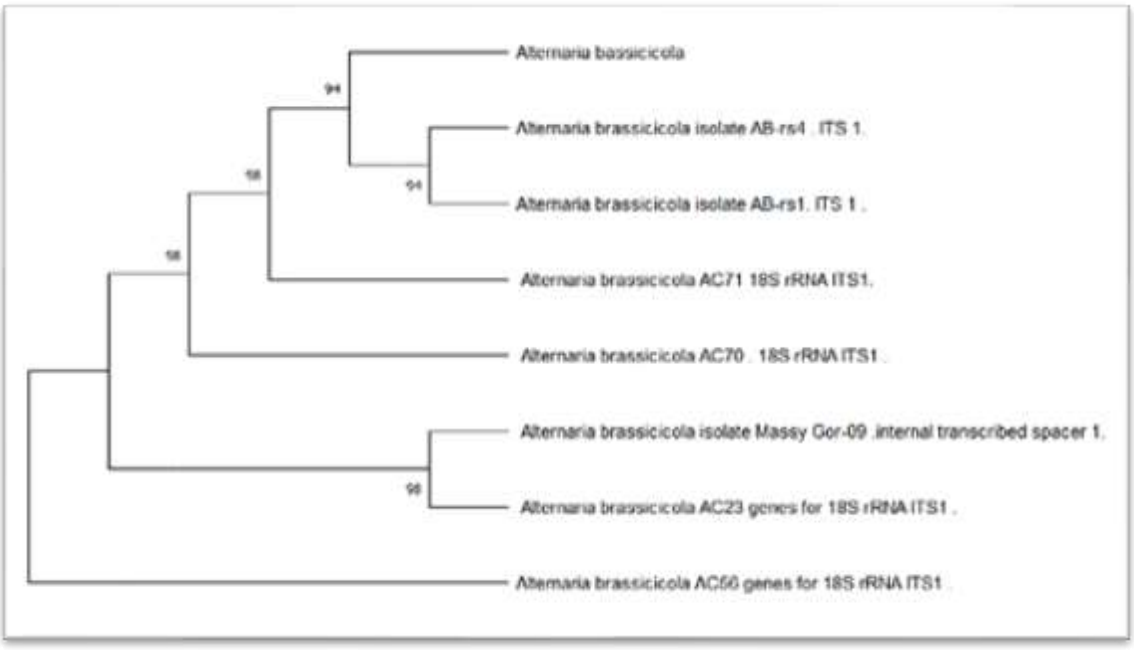

Fig.3: Analysis of the genetic relationship of *A. brassicicola* with a level of about 94%

3- Fungi *R.solani*:

The results of the molecular analysis of the genetic affinity and divergence of the fungus

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R.solani showed a clear affinity of 96% with the isolate *R.solani* (FX1AG4-HG-L) while it was close to the rest of the isolates at different rates and this is consistent with what was indicated by the study (Tziros et al. 2022). The results of these studies are consistent with what was mentioned during the current study (Keshavarz-Tohid et al. 2017).

4- Fungi *T.harzianum*:

The results of the molecular analysis of genetic affinity for *T.harzianum* isolate showed a high affinity of 99% with isolate *T.harzianum* T.h64697, while this group was close to the rest of the isolates in the genetic tree by 80%. Several studies have reached similar results in the diagnosis of fungi of this species (Tseng et al. 2020) and (Haouhach et al. 2020).

5- Fungi *N.mangiferae*

The results of the diagnosis of *N.mangiferae* were similar to that of *N.mangiferae* within subgroup (A) by about 92% and under group (B) by about 95% and 90% with the rest of the isolates in the genetic tree. These results were similar to the study of (Mogal et al. 2022) and (Solanki et al. 2016).

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