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Morphological and molecular diagnosis of fungi associated with two species of carp (*Cyprinus carpio*) and shank (*Tilapia* sp.) in Karbala / Iraq.

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Abstract: The aimed of study was identifying the fungi associated two types of fish, carp (Cyprinus carpio) and the shank (Tilapia sp.) from freshwater fish in Karbala / Iraq. The isolates from all body regions of the fish revealed three types of fungi, Lichtheimia corymbifera with a percentage of The frequency was 20% and Aspergillus terreus and Aspergillus niger had a frequency of 50 and 40% respectively .The morphologically isolated fungi were diagnosed using the approved taxonomic keys, the diagnosis was confirmed by polymerase chain reaction (PCR) technique.

Key words: Cyprinus carpio, Tilapia sp , *Lichtheimia corymbifera*, PCR, Aspergillus sp.

Introduction

Aquatic environments are a good home for several biological communities, including Aquatic Fungi (Water molds), which are of economic and environmental importance, especially those belonging to the order of water molds (Rafique and Khan, 2012). Freshwater fish live in Asia, numbering at least 193 species. Only 31 are commercially important species that play an important role as a source of food and livelihood in Asia (Webster and weber, 2007).

Freshwater fish are infected with at least one type of fungus during their lifetime (Neish et al., 1997) Fungal diseases are one of the causes of fish losses (Meyer et al., 1991) Fungi attack fish from egg stage to adult fish (Bangyeekhun and Sylvie, 2001) have reported several dangerous pathogenic fungi to freshwater fish in Australia, Japan and throughout southern Asia (Koeypudsa et al., 2005).

Stressed and weak fish are exposed as a result of infection with various diseases, including bacterial septicemia (Iqbal et al., 2000 and Iqbal et al., 2001), which makes them more susceptible to infection with fungi (Siddique, 2009).

The carp *Cyprinus carpio*, is a freshwater fish and the shank *Tilapia* sp. Which is widely popular in Iraq as an important food source (Soranganba and Saxena, 2007).

The genus *Lichtheimia* belongs to the order Mucorales, and the fungus *Lichtheimiad corymbifera* is the main pathogen that causes infection in humans and animals (Alastruey-Izquierdo, 2010).

The fungus was isolated from decomposing organic matter such as leaves, rotting wood and plants, and from animal waste (Hassan and Voigt, 2019).

Infection of marine mammals with this type of fungus is rare, as it has been recorded on free-living whales or those raised in private ponds (Huckabone et al., 2015). Only two cases were recorded after more than 7000 autopsies of marine mammals were conducted in California, and a case of marine mammals has been documented. One after 444 examinations in the Baltic Sea (Wunschmann et al., 1999).

It was recorded by Huggins et al. (2020) on mammals causing mortality in many areas of the Pacific Northwest. A number of researchers were able to isolate the fungus *Aspergillus* from freshwater fish (Iqbal et al., 2012; Iqbal and Mumtaz, 2013; Chauhan, 2013).). Since more than 60 species of *Aspergillus* fungi are of medical importance and cause infection to humans and animals and belong to the order Eurotials, a class of Ascomycota fungi, Bhattacharya and Bhattacharya et al. (1988) mentioned that both *A. niger* and *A. terreus* caused disease in fish, and it was also possible Shrivastava (1996) isolated *A. terreus* from freshwater fish and tested its pathogenicity on the same species of fish.

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The aim of this research is to identify the fungi associated with pathogenic fish in fish ponds by diagnosing the fungi under study according to the classification keys and then by adopting the PCR technique.

Materials and Methods:

Isolation of Diagnosis the pathogen from fish samples:

Samples were collected for two types of fish carp and shank fish that were brought from the fish breeding lakes.Fish were washed with running water, then 5.0 cm pieces were taken from the areas adjacent to the ulcer. The pieces were superficially sterilized with 1% sodium hypochlorite, then washed with sterile distilled water and dried between two sterile Whatman No.10 filter papers. Pieces were transfer in Petri dishes containing Potato Dextrose Agar (PDA) with five pieces per dish. The plates were incubated at 25 ± 2 °C for five to seven days.The growing colonies were purified by using the Hyphal Tip technique. Fungi were morphologically diagnosed using taxonomic keys (Willoughby, 1994 and Ellis et al., 2007)

The frequency of each isolated fungus was calculated.

$$\text{The percentage of frequency} = \frac{\text{The number of times fungi appear}}{\text{The number of total colonies}} \times 100$$

The diagnosis was confirmed by polymerase chain reaction (PCR) technique

Molecular Identification

DNA extraction:

The MiniPrep fungal/yeast/bacterial ZR DNA kit (ZYMO, USA) was used to extract and purify DNA using beads for DNA analysis (Schloss et.al; 2005). As per manufacturer recommendations. DNA purity was examined by nanodrop ND-3000 (Fermentas Scientific, Inc.). Extracted DNA was kept at -20° until use.

Polymerase Chain Reaction (PCR)

Perform a PCR reaction using a Gene Amp and PCR system 9700 Thermal cycler (Applied Biosystem, USA). The ITS region was amplified using the ITS1 (forward) 5′- TCCGTAGGTGAACCTGCGG -3′ and ITS4 (reverse) 5′TCCTCCGCTTATTGATATGC-3′primer pair provided by (IDT Corporation, Canada), as described in (White et.al; 1990). Taq PCR PreMix (Intron, Korea) was used in the optimized PCR recipe

Approximately 550 bp of the ITS region was amplified using the following software: pre-denaturation at 95 °C for 3 min; (denaturation at 94°C for 45 seconds, annealing at 52°C for 1 minute and extension at 72°C for 1 minute) for 35 cycles; Final extension at 72 °C for 7 min and then compression at 4 °C. The amplicons were separated using a 1.5% agarose gel.

Results and Discussion :

Isolation, purification and Morphology Diagnosis:

The results of isolation from all the anterior and posterior body regions of the fish showed the presence of *L. corymbifera* with a frequency of 20%, and *A. terreus* and *A. niger* at a frequency of 50% and 40%, respectively.The fungal colonies of *L. corymbifera* appeared in white color at the beginning of their growth and with age they turned into a fast-growing pale gray color. Upon microscopic examination, the spores appeared in rectangular to oval shape, their average dimensions ranged from 3-7 x 2.5-4.5 µm, their color was transparent to light gray with walls soft These results are in agreement with Alastruey-Izquierdo et al (2010) and Vitale et al (2012).

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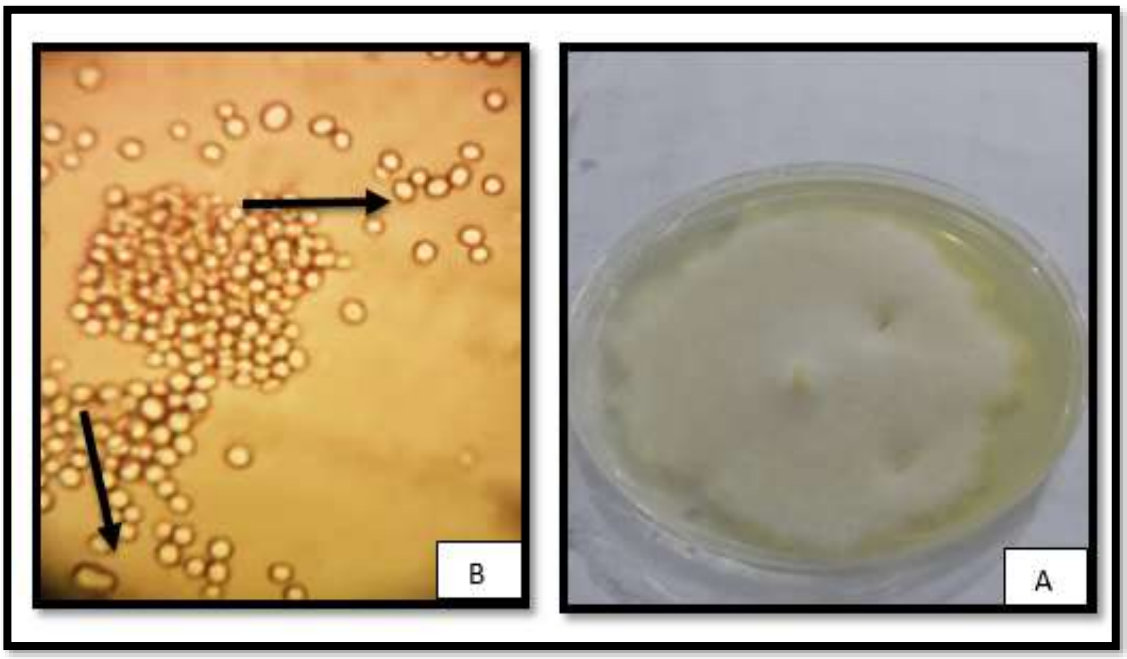

Figure (1): *L. corymbifera*, A: Colony appearance on PDA , B: spores

While the colonies of *A. terreus* appeared velvety, tanned skin-like, in a dark pink color that increased, turning from pale yellow to bright yellow to dark brown, growth was moderate to fast. Fungi spinning divided and branched. Small conidia spherical to ellipsoidal shape (1.5-2.5 µm in diameter) with smooth walls (Dismukes et al., 2003 and Samson et al., 2014).

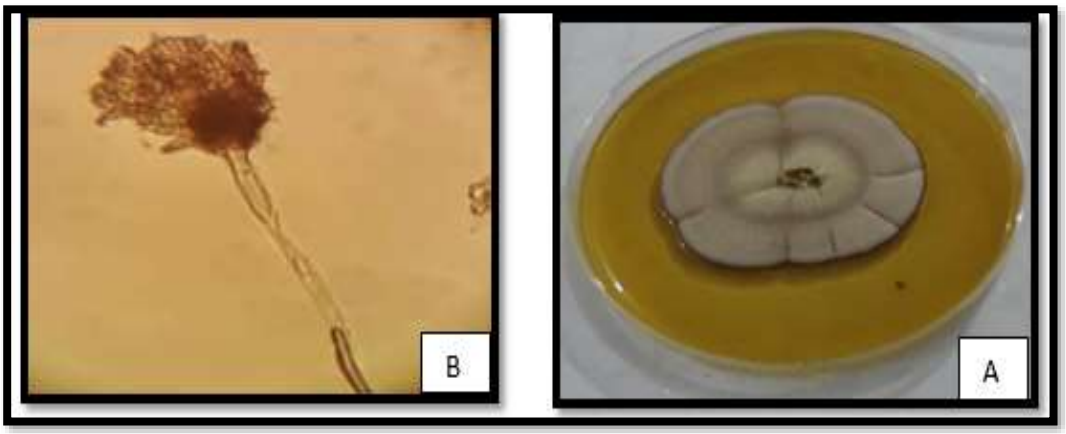

Figure 2 : *A. terreus* , A: colony appearance on the PDA . B : An undivided, unbranched conidial bearing that ends with a head carrying the conidia in chains.

The fungus *A. niger*, it grows quickly 3-4 days, the colony is cottony in appearance at first white to yellow and then turns green or black The mycelium is characterized by abundant growth and is branched and divided and the conidia is spherical in shape and single-celled, rough-walled on the outside, diameter 3.5-5 Dark brown to black micrometers (Someren et al., 1990).

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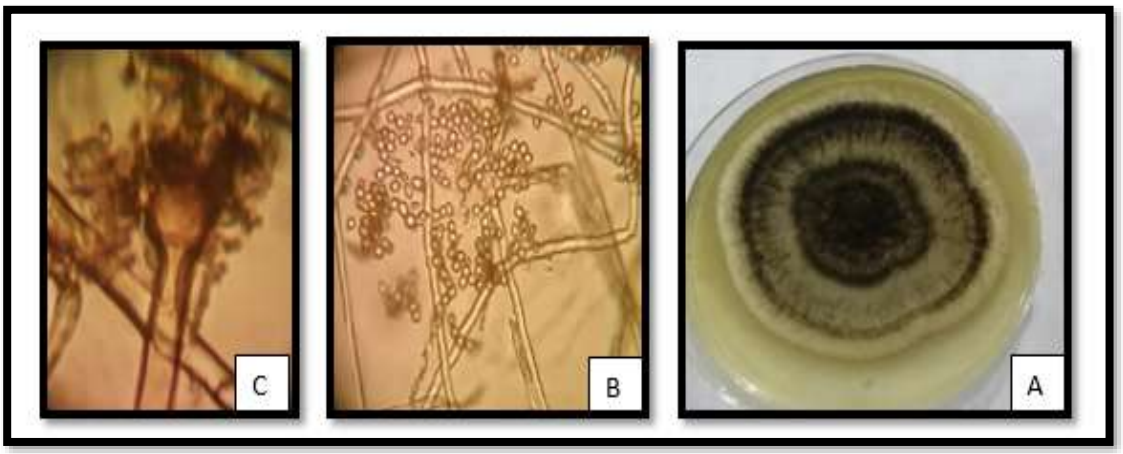

Figure 3. *A.niger* , A: Colony on the PDA B- conidia, C: conidia bearing that ends with a swollen head.

Molecular Diagnosis :

The results of extracting DNA from fungi after subjecting them to a polymerase chain reaction (PCR) showed the possibility of doubling PCR-amplified products, each with a size of about 600 bp).

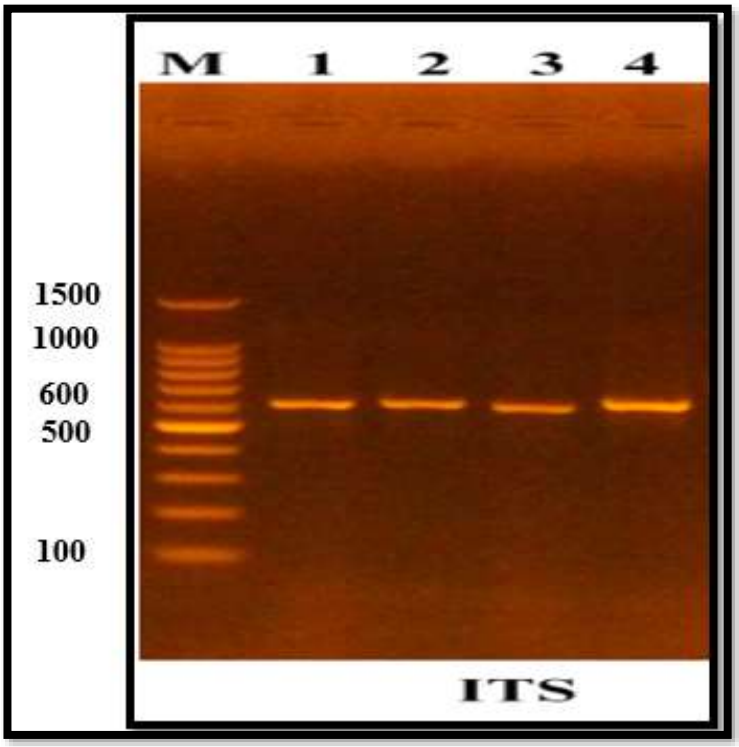

Figure 4: Agarose gel electrophoresis of amplified PCR product (550 bp).

The results of the analysis of sequencing showed that isolate No. 1 and 3 belong to *A. niger*, isolate No. 2 is *A. terrues*, and isolate No. 4 *L. corymbifera* Figures 5, 6 and 7 represent the Neighbor-Joining tree that shows the genetic relationship between the fungi isolated in this study and other isolates of the same fungi that were previously registered at the National Center for Biotechnology Information (NCBI).

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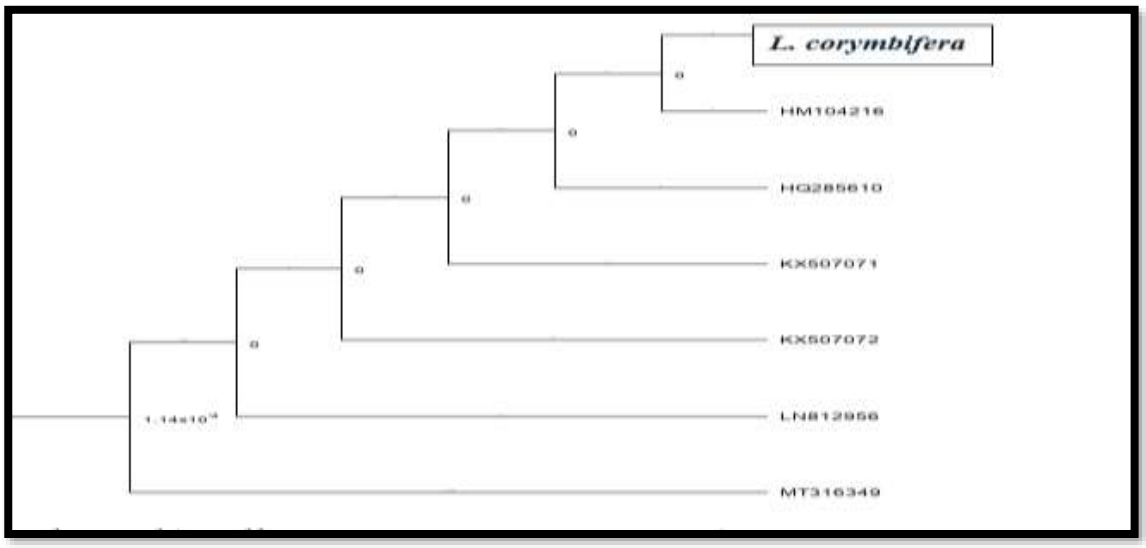

Figure (5) :The Neighboor-Joining tree of *L. corymbifera*

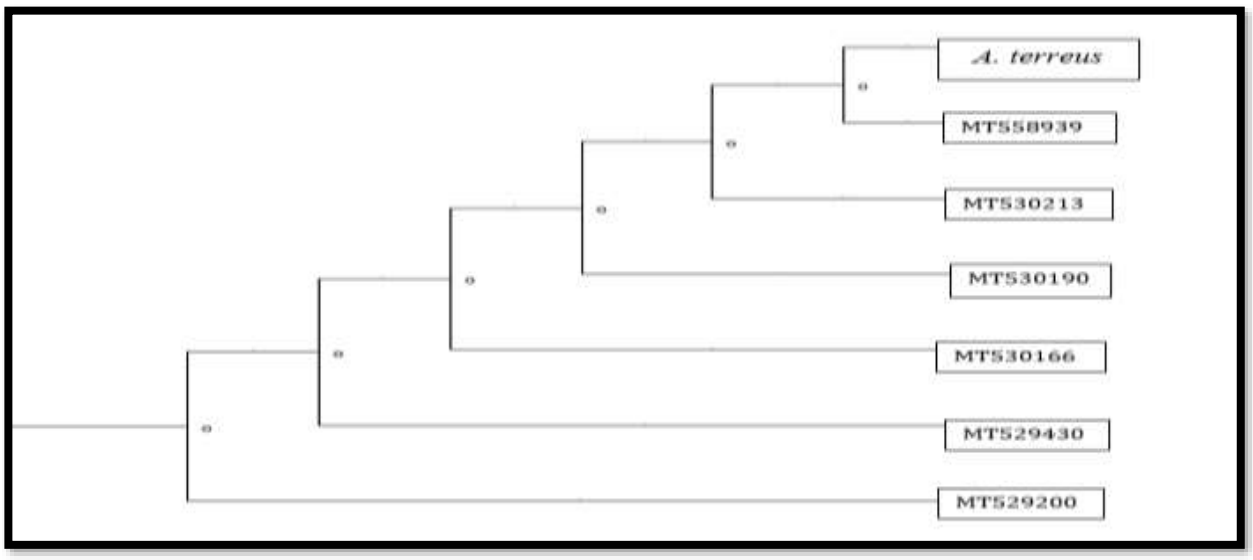

Figure (6) :The Neighboor-Joining tree of *A. terreus*

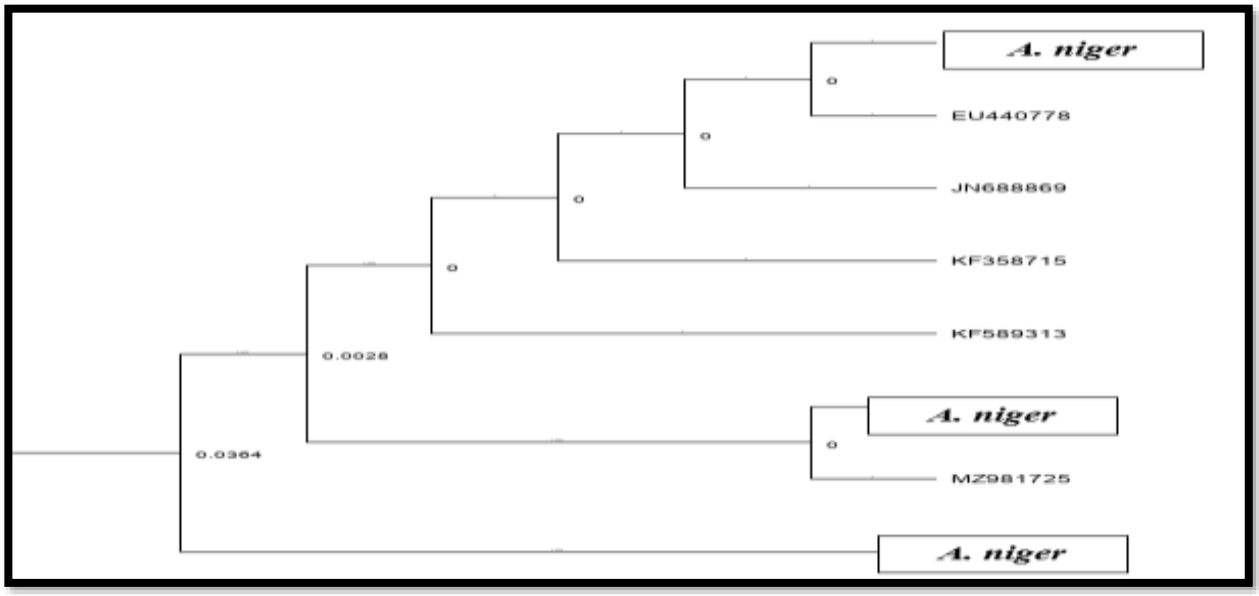

Figure (7) :The Neighboor-Joining tree of *A. niger*

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The similarity percentage reached 100% for each of *A.terreus, A. niger*, and *R. stolonifer*, while *L. corymbifera* reached to 99.86 ,the neighboor joining tree which indicate that the first recorded of it on fish in Iraq.

Conclusion:

The isolation results indicate the presence of three fungi associated with fish, *L. corymbifera, A. terreus* and *A. niger*. morphology diagnosed and confirmed using Polymearase chain reaction (PCR) technique, which is the first recorded in Iraq on carp and Shank fish.

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