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Research Article

Molecular identification, phylogenetic analysis and histopathological evaluation of gill fungal infection in some ornamental fish: First report and new species

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Abstract

Among infectious pathogens, fungal and fungal like organisms cause the remarkable social and economic impacts on ornamental farmers. Some freshwater ornamental fishes including the cardinal *Carassius auratus,* Green Terror Cichlids *(Andinoacara rivulatus),* the guppy (*Poecilia reticulata*), the Koi (*Cyprinus rubrofuscus*) with typical signs of severe respiratory symptoms were prepared from a large commercial breeding centers ornamental fish shops in Tehran, Iran. The gill samples were taken and laboratory evaluations carried out. Accordingly, Culturing of fungal isolates, sequencing and molecular identification, pathogenicity as well as gross and microscopic evaluations were also carried out. Four isolated with NCBI-accession numbers of OL891804, OL912959, OL990026 and OL912961, respectivelyas Fungal sp., *Penicillium* sp., Fungal sp. and *Candida* sp. were reported and registered in Genebank. Eventually, the Ornamental fish diseases of Tehran ornamental fish centers where a few fish including the cardinal (*C. auratus*), Green Terror Cichlids *(A. rivulatus),* the guppy (*P. reticulata*) and the Koi (*C. rubrofuscus*), were suffering from gill disorders had been infected with *Penicillium crustosum, Alternaria alternata, Filobasidium* like organism, *Candida zeylanoides,* respectively.

Keywords: Ornamental fish, Fungal infection, *Penicillium crustosum, Alternaria alternata, Candida zeylanoides*

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Introduction

In aquaculture, diseases cause tremendous economic losses, and fungal infections have become increasingly important in the last 20 years. Fish farmers are continuously facing a number of pathogenic diseases. Among pathogens, fungal and fungal like mortalities lead to tremendous social and economic impacts on stakeholders. The Iranian fish farmers are sometimes facing challenge from fungal pathogens, leading to terrific losses particularly in rainbow trout hatcheries (Magray *et al.*, 2021). Freshwater fish has dominated the cultured species throughout the world which in 2016 surpassed 171 million tons with 47% in aquaculture contribution (FAO, 2016b). aquaculture production participated in 54.1 million tons of different groups of fish (US$ 138.5 billion) (FAO, 2016a). Nowadays, aquaculture are considers as a key solution for food security due to exponentially increasing human population (FAO, 2016a). However, mortality of cultured fish causes the most significant economic losses (Magray *et al.*, 2021). Among these diseases, fungal ones are the second most serious reasons of outbreaks in aquaculture (Gozlan *et al.*, 2014). The most common infections recorded in temperate regions are fungal diseases due to easily spread of spores through pond water, colonies and cause mortality (Sarkar *et al.*, 2022). Aquaculture mortality due to waterborne fungal infections (saprolegniasis) can be occurred by Aspergillus, Saprolegnia, Achlya, Aphanomyces, Branchiomyces, Ichthyophonus and Fusarium species (Zhang *et al.*, 2021). Virulence factors of aquatic pathogenic fungi causing infection through iron acquisition siderophores. Mitochondrial protein secreting kinases and kinase inhibitors; secretary proteins secret fungal binding domain (FBD), proteolytic enzymes and hydrolytic enzymes act as immunosuppressive compounds for the host immune system are virulent factors of fungi (Clemons *et al.*, 2002). These factors can damage the tissues of the susceptible host. However, the overview of the emergence of diseases associated with fungal pathogens remains limited. Researchers are trying to provide different possibilities for developing new treatments by understanding these pathogenic molecules (Sarkar *et al.*, 2022). The range of life of fungi is impressive and diverse, (Zhdanova *et al.*, 2000; Joseph *et al.*, 2021). Fungi can convert from opportunistic to pathogenic in population with weak immunity or unfavorable environmental conditions (Meyers *et al.*, 2019). *Cladosporium, Alternaria*, Aspergillus, and Penicillium can grow on the pollen grains of plants, and their spores are transported along with the pollen in the form of aerosols (Magyar *et al.*, 2022). Several cases of fungi have been introduced as opportunistic agents of fish. They can be present in the skin, fins, and internal organs of fish and become pathogenic under certain conditions (Meyers *et al.*, 2019; Younis *et al.*, 2020).

Cladosporium and other black fungi have been isolated from sturgeon suffering from phaeohyphomycosystemic disease, a significant disease in this fish species, as a pathogenic agent (Yazdi *et al.*, 2021). New diseases are constantly being studied and identified in aquatic animals. In the investigation of tilapia brown rust disease, which caused many casualties in Ghana, several fungal agents were identified and responsible for the losses occurred by *Aspergillus niger* and *Flavobacterium fungi* (Deho, 2019). It is customary to use ITS regions for molecular identification of fungi (Törün *et al.*, 2022). Pathogenic fungi spores are scattered everywhere, and depending on environment fish are grown, and there is a possibility of contracting all kinds of diseases. Therefore, considering the existing problems in treating fish, the lack of access to the necessary advances in fish treatment services, the scarcity of required drugs, and the high cost of treatment, breeders should observe health tips regarding fish breeding and growth (Kristiansen *et al.*, 2020).

Due to the aforementioned clarification, we try to histopathologically and molecularly identify the gill fungus of some freshwater ornamental fishes in Iran, which showed gill dysfunction with expansion of operculum movement and increasing beat rate.

Material and methods

Fish

Approximate 90 freshwater ornamental fishes including the cardinal *C. auratus*, Green Terror Cichlids *(A. rivulatus)*, the guppy (*P. reticulata*), the Koi (*C. rubrofuscus*) with typical signs of severe respiratory symptoms were prepared from a large commercial breeding centers ornamental fish shops in Tehran, Iran, where the centers were shown well in management. The information and characteristics of the symptoms were recorded and fish transferred to the veterinary laboratory immediately.

Sampling distribution

The samples were initially studied by the wet mount, a part was placed in formalin solution for microbial culture and the remaining dedicated for histopathological procedures. Nutrient agar and Saburo dextrose agar (SDA) containing chloramphenicol were used for the cultivation method (Kovalevska *et al.*, 2019).

Culturing of fungal isolates

For microbial culture, surface of infected gill samples was sanitized to inhibit secondary contamination with airborne spores with immersing in 0.5% formaldehyde. The gill tissues were placed in hygienic sucrose lysis buffer and kept at -20^{o}C until needed examination following the method of Iqbal and Saleemi (2013) with minor modification. The swabs from infected gill samples were spread on SDA and placed in a refrigerated incubator at a temperature of 21±2^{o}C for three weeks.

The fungi growth rate was then checked for one day. In the preliminary examination of the different characteristics of the fungi that were grown in the environments used, things like growth speed, colony color, colony changes, type of colony, differences in growth, reproductive structures, spore types, mycelium color, the presence of a transverse wall, and the purity of the colony were among the most important criteria that were taken into consideration.

Gross and microscopic evaluation

First, the gross appearance of lesions was checked. In the direct evaluation, physiological serum, calcofluor white which binds to chitin in the fungal cell wall, and several staining methods were used. At first microscopic observation was carried out to identify fungal components such as hyphae and/or conidia using wet mount without or with staining so that the gill samples were taken and stained using the Lactophenol Cotton Blue (LPCB) mounts (in addition to 10% potassium hydroxide, which dissolve excess and surrounding material). Specimens were labelled as "fungus positive" when these fungal components were identified (Khalid *et al.*, 2021). The fish were anesthetized and sampled in compliance with hygiene and animal rights principles. A drop of fish environment was then dripped on the slide, the gills lesions were removed by a sterile scalpel and photographed using a Nikon Y-THR optical microscope (Japan).

Pathogenicity evaluation

Histopathological features of the infected gill samples were carried out to clarify the pathogenicity criteria of fungal infected fish. Gills were fixed in neutral buffered formalin solution (10%) and the gill samples were then routinely processed and placed into paraffin blocks (Suliman *et al.*, 2021). The blocks of the gill samples were cut at 5 μm thickness and stained with Haematoxylin and Eosin (H&E). Some of the sections were stained with Periodic Acid Schiff (PAS) to present the hyphae. The tissue sections were examined by a light microscope and photographed using Nikon Y-THR optical microscope (Japan).

Molecular identification

Fungal hyphae are washed with physiological serum to extract the DNA of filamentous fungi with liquid nitrogen. They were then placed between two sterile filter papers to dry. After drying, the hyphae were put in a clean and sterile porcelain mortar, then pour some liquid nitrogen on them and immediately start crushing them with the handle of the mortar, continued until the hyphae turned into a soft powder. One gram of hyphae powder was slowly mixed with 0.2 mL of phenolic extraction buffer named hyphae-buffer (Karakousis *et al.*, 2006) in a 1.5 mL tube for one minute. This buffer contains 1.5 parts of phenol balanced with Tris buffer (Tris pH 8.0), one part of Isopropyl naphthalene sulphonic acid, and one part of P-amino-salicylic acid solution (with a concentration of 0.12

g/mL) and half a part of 5 times buffer. It is RNB buffer contains 121.1 g Tris, 73.04 g NaCl and 95.1 g EDTA, pH 8.5 in one liter of water following the method of Karakousis and Langridge (2003) with some modification. After making the extraction buffer, it was heated to 55ºC. The 0.5 mL of chloroform was added to hyphae-buffer tube and remixed for one minute. The tube was centrifuged at 10,000 rpm for 10 min. The supernatant was then separated. Again, 1 mL of chloroform was added to the supernatant and mixed for 1 min. Excess chloroform is removed by centrifugation and two phases were performed. The DNA existed in the liquid was precipitated using isopropanol. For this purpose, the same volume of liquid, isopropanol, was added and then preserved at -20 for 30 min. The solution was then centrifuged for 20 min in a refrigerated centrifuge (4ºC). The supernatant solution was removed, and the plate was washed twice with ethanol. Once with 100% ethanol and the second time with 70%, however, the centrifugation is repeated after each wash. It was then left open in the tubes at room temperature until the alcohol evaporates and dried. Eventually, the resulting pellet was re-suspended and dissolved using TE buffer. TE buffer contains 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. The desired ITS sequences were made and used by Arin Gene Gostar based on universal primers ITS1 (5'TCC GTA GGT GAA CCT GCGG) and ITS4 (5'TCC TCC GCT TAT TGA TAT GC) (Aşgın and Değerli, 2019) to amplify of the ITS including the ITS1, 5.8S and ITS2 region.

Preparation of PCR mix

one microliter of the solution containing DNA 1:10 was diluted. Two microliters were then taken and poured into special PCR micro tubes. Then 2.5 microliters from the 10x PCR master mix containing all the materials needed for PCR (except primer and enzyme) were taken and added to the tube. If the master mix or buffer does not contain dNTPs, they must be added separately). Then, 1.5 microliters of MgCl2 solution with a concentration of 1.5 mM was added to the tube. One microliter of each primer (each type of fungi has a pair of primer; the same pair is used in each PCR) was added to the tube. The 16 microliters of DNase-RNase-free water and one microliter of the enzyme were added and centrifuged for a few seconds and put the microtube in the PCR machine (Huang *et al.*, 2018).

PCR machine program

Initial denaturation was at 95ºCfor 30 seconds. The number of cycles was 35, denaturation at 95ºC for 15 seconds/annealing depending on the primer, 55 to 65ºCfor 20 seconds/extension at 68ºCfor 30 seconds, and the final extension at 68ºCfor 5 min. Finally, storage was done at 4 to 10ºC(Foster *et al.*, 1993).

Electrophoresis method

The 1% agarose was prepared in TBA buffer. Ten microliters of DNA were added to the agar wells, and two

microliters of colored loading buffer solution according to the size of the wells in the Sinacloon ledger. First, it was added, and electrophoresis was performed at 140 volts for 70 min. The extracted DNA was stained using ethidium bromide solution and the performed bands photographed.

Sequencing

After performing PCR and ensuring the amplification of the desired regions, 15 µl of each product were placed in vials with lids separately. Accordingly, ten microliters of the desired primer (forward or reverse) for each product were prepared and sent to the Codon Genetics Group Laboratory. The edited with MEGA X software (Kumar *et al.*, 2018) obtained raw nucleotide sequence was and thus the final sequence was determined using evolutionary analysis by maximum likelihood method. The evolutionary history was inferred by the Maximum Likelihood method and the model was presented by Tamura *et al.* (2004). Determining the sequences was compared and analyzed with BLAST software with similar sequences in the Gen Bank. The desired isolates were identified using sequence similarity in the NCBI database (França *et al.*, 2002).

Results

Pathology

The most lesions found in the skin and gills. Gills were partially infected with fungal hyphae. The gills' lesions were leukocytes infiltration within trabecula. (Fig. 4 A, B, C). Acute dermatitis and leukocyte infiltration (Figs. 1 and 2).

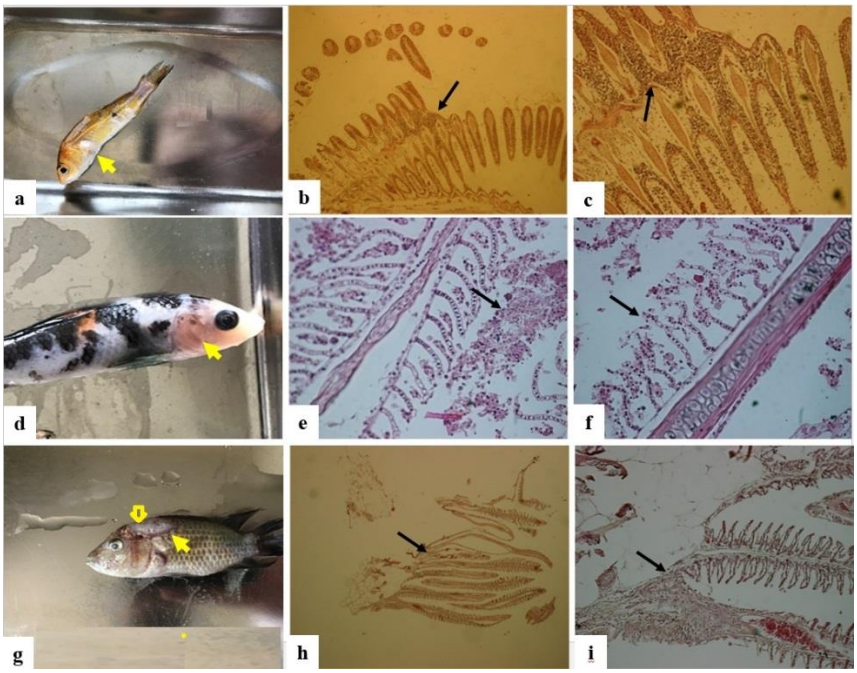

Figure 1: Macroscopic (a: *Carassius auratus*, d: *Cyprinus rubrofuscus*, g: Green terror and Microscopic appearance of the fish affected with the fungi. The leukocyte infiltration and edema are shown in the gills trabecula. Yellow arrow: gross lesion; Black arrow: leukocyte infiltration (H&E, b, e, h × 4; c, f, i × 10).

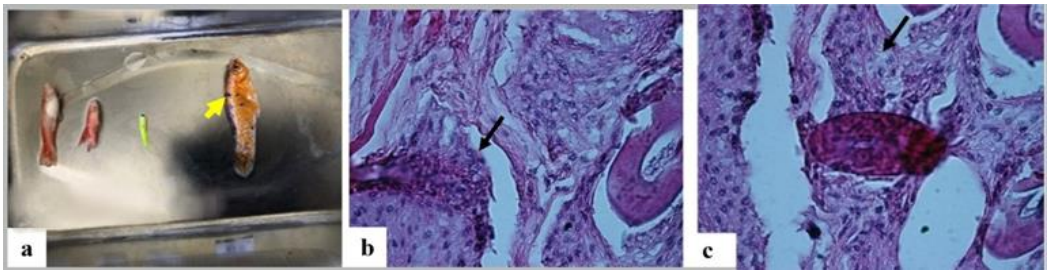

Figure 2: Macroscopic (a) and Microscopic appearance of the fish affected with the fungi. The leukocyte infiltration and edema are shown in the skin trabecula. Yellow arrow: gross skin lesion; Black arrow: leukocyte infiltration (H&E, b × 4; c × 10).

Gene sequencing

Gene sequencing was performed for the four following fungi, and the results were recorded in the NCBI database.

In the fish case of *C. rubrofuscus* with *Penicillium* sp. involvement, The LOCUS was OL912959 543 bp DNA linear PLN 24-DEC-2021 defined as fungal sp. Eukaryota; Fungi. This is demonstrated as follows:

ORIGIN: Sampe 1

1 gaaaggggtg gagtaccctg gttcacctcc cacccgatga ttattttacc ttgtggtttt
61 gttggccccc ttatcgggcc gcctggggga ttacaccccc cggcccggac ccgtccaaaa
121 acttcactaa ctctgtctga tgatatttaa tagatagaaa atattattta tttaaaactt
181 tcaaccctgg atctcttgca tccgggatcc attaggaatc cccttaattg cgaaccccaa
241 tgaaaattgc aaattctatg aatcatcgaa tttttgggaa tcattgcgcc ccctggtatt
301 ctggggcctt gcctgtccca aaggcatggc tgccctcacg ccgttccccc cccagggccc
361 cggccccga tctccggagg accctaccca aaggaagcgg ttggtttaaa ttcggtcctc
421 tagagggcgg ggctttgtca cccgctctgt aggcccgtgt cggcgcttgc cgatcaaccc
481 aaatttttat ccaggtggac attacatcag gtagggattc ccgctgaaaa caaccatata
541 cct

In the fish case of Koi (*C. auratus*) with *Penicillium* sp. involvement, The LOCUS was OL891804 602 bp DNA linear PLN 22-DEC-2021 defined as *Penicillium* sp. Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Eurotiales; Aspergillaceae; Penicillium. This is demonstrated in the follow:

ORIGIN: Sample 2

1 tgttcaagag gaggacgggc tggacctctc ggggttacag ccttgctgaa ttattcaccc
61 ttgtcttttg cgtacttctt gtttccttgg tgggttcgcc caccactagg acaaacataa
121 acctttttgta attgcaatca gcgacagtaa caaattaata attacaactt tcaacaacgg
181 atctcttggt tctggcatcg atgaagaacg cagcgaaatg cgataagtag tgtgaattgc
241 agaattcagt gaatcatcga atctttgaac gcacattgcg ccctttggta ttccaaaggg
301 catgcctgtt cgagcgtcat ttgtaccctc aagctttgct tggtgttggg cgtcttgtct
361 ctagctttgc tggagactcg ccttaaagta attggcagcc ggcctactgg tttcggagcg
421 cagcacaagt cgcactctct atcagcaaag gtctagcatc cattaagcct tttttcaact
481 tttgacctcg gatcaggtag ggatacccgc tgaacttaag catatcaata agcggaggaa
541 cac

In the fish case of Green terror (*A. rivulatus*) with *Candida* sp. involvement, The LOCUS was OL990026 603 bp DNA linear PLN 28-DEC-2021 defined as fungal sp. Eukaryota; Fungi.

ORIGIN: Sample 3
61 gccccccttg accgggggggg cttcctgcgt tttttcccca tgttgttttt ctttattttt
121 gaaaactgtg ctttgttgcc cccttggcct gcttagattt aactctacca aattttattt
181 aatcatctga ttaactaata aaacaaaact ttcaacaacg cttttcttgc ttctctcatc
241 gatgaacaac gcaacgaaat gcaataaatg agatgaattg aatatattca agaatcatat
301 aatctttgaa cgctcgttgc gccctttgtt cttccgaagg tgatgcctgg ttgggcgtga
361 tttctccctc aacccttttgg gtatggtgat cagcgattca ctggggattt ttgaaataaa
421 ggcggatgat acacttcagg ataggttttt ttccactcat tggtacaaac tccaaaatcc
481 ttccaaattc gacctcaaat caggtaagac taccctctga acttaaggat atcaataact
541 ggaggaaaac taaaccatca ctttaagtgc atctttggat gttactcatt gtgtaacttt
601 gacatctggc ctcaaatcaa gtacgactac ccgctgaact taagcatatc aataagcgga ggaA

In the fish case of *P. reticulata* with *Candida* sp. involvement, The LOCUS was OL912961 609 bp DNA linear PLN 24-DEC-2021 characterized as *Candida* sp. (in: Saccharomycetales) Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; *Saccharomycetales incertae sedis*; Candida; unclassified Candida (in: Saccharomycetales).

ORIGIN: Sample 4
1 ttgccccttg gccccgtgtg tccccgtgct ttaattgcgc ggcgaaaaac cttacacact
61 atgttttttt gatttgaaac ttcctcgctt tggtctgact tagaaatgag ttgggccaaa
121 ggttttatac taaaacttca attttattat tgaattgtta attaattata ttgtcaattt
181 gttgattaaa ttcaaaaatc ttcaaaactt tcaacaacgg atctcttggt tctcgcatcg
241 atgaagaacg cagcgaaatg cgataagtaa tatgaattgc agattttcgt gaatcatcga
301 atctttgaac gcacattgcg ccctatggta ttccataggg catgcctgtt tgagcgtcat
361 ttctctctca aatcttcgga tttggttttg agtgatactc ttagtcagac taagcgtttg
421 cttgaaatgt attggcatga gtggtactag atagtgctga actgtttcaa tgtattaggt
481 ttatccaact cgttgaccag tatagtattt gtttattaca caggctcggc cttacaacaa
541 caaacaaagt ttgacctcac atcaggtagg actacccgct gaacttaagc atatcaataa
601 gcggaggaa

Phylogenetic tree

After registering the genetic sequences in the NCBI system and matching the genetic sequences of the samples with the samples registered in the system, the phylogenetic tree of the samples was drawn using the Mega X software.

As shown in Figure 3, the optimal tree with the sum of branch length = 2.10835040 is shown for *C. rubrofuscus*

infected with *Penicillium* sp involvement. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 22 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 639 positions in the final dataset.

A neighbor-joining phylogenetic tree was constructed for all the close strains (Fig. 3). The phylogenetic tree showed that 26 strains were mainly clustered into one distinct groups, namely Cluster 1. All strains had at least 96.0% match and 97% coverage with GenBank reference sequences (unpublished data), which were identified as true fungi. According to the result given in Figure 3 and based to the close strains such as Accession numbers, MF564101.1 and KY945041, our strain belonged to *Penicillium crustosum.*

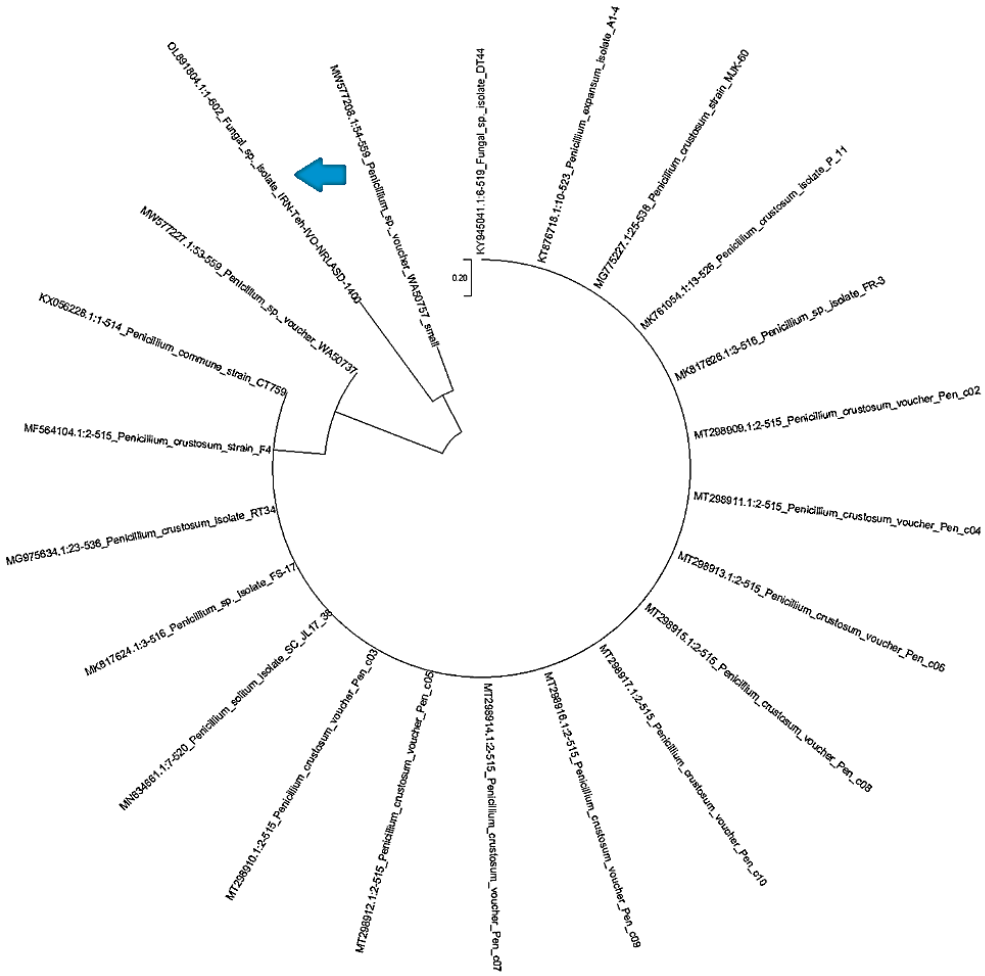

Figure 3: Evolutionary relationships of taxa for *Fungal* sp. Isolate. IRN.Teh.IVO NRLASD 1400.

As shown in Figure 4, the evolutionary history was inferred by using the Maximum Likelihood. The tree with the highest log likelihood (-944.12) is shown for *C. auratus* with *Penicillium* sp involvement. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 23 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 546 positions in the final dataset. A neighbor-joining phylogenetic tree was constructed for all the strains (Fig. 4). The phylogenetic tree showed that 23 strains were mainly clustered into one distinct groups. All strains had at least 96% match and 99% coverage with GenBank reference sequences (unpublished data), which were identified as true fungi. According to the result given in Figure 4 and based to the close strains such as Accession numbers, MH728994.1 and KY703400.1, our strain belonged to *Alternaria alternata*.

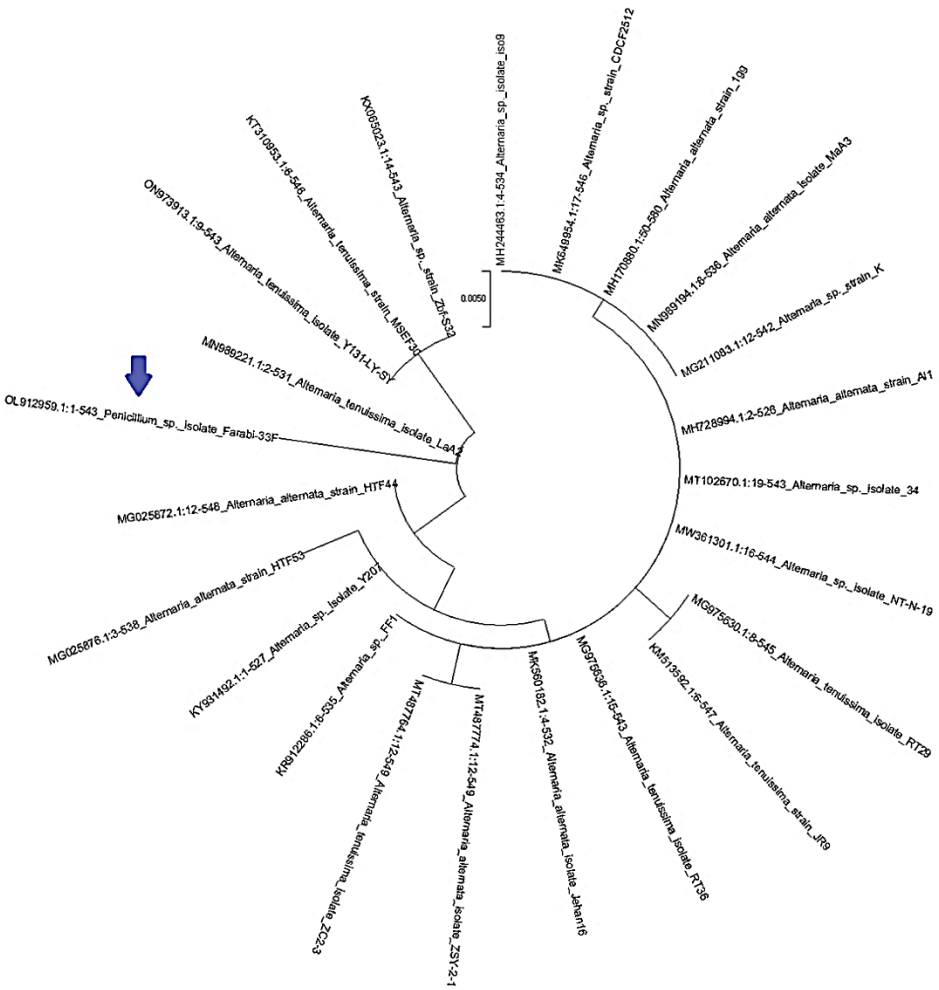

Figure 4: Evolutionary analysis by Maximum Likelihood method for *Penicillium* sp. isolated Farabi-F33.

The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length=8.26681312 is shown in Figure 6 for Green terror (*A. rivulatus*) with *Candida* sp. involvement. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree. This analysis involved 32 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 603 positions in the final dataset. A neighbor-joining phylogenetic tree was constructed for all the strains (Fig. 5). All strains had at least 99.15% identification and 19% coverage with GenBank reference sequences (unpublished data), which were identified as true fungi.

According to the results given in Figures 5 and 6 (Based on the BLAST analysis), our strain was approximately 19% assigned to *Filobasidium magnum*, therefore, this isolate seems to be a new one that its sound will be hear more in future.

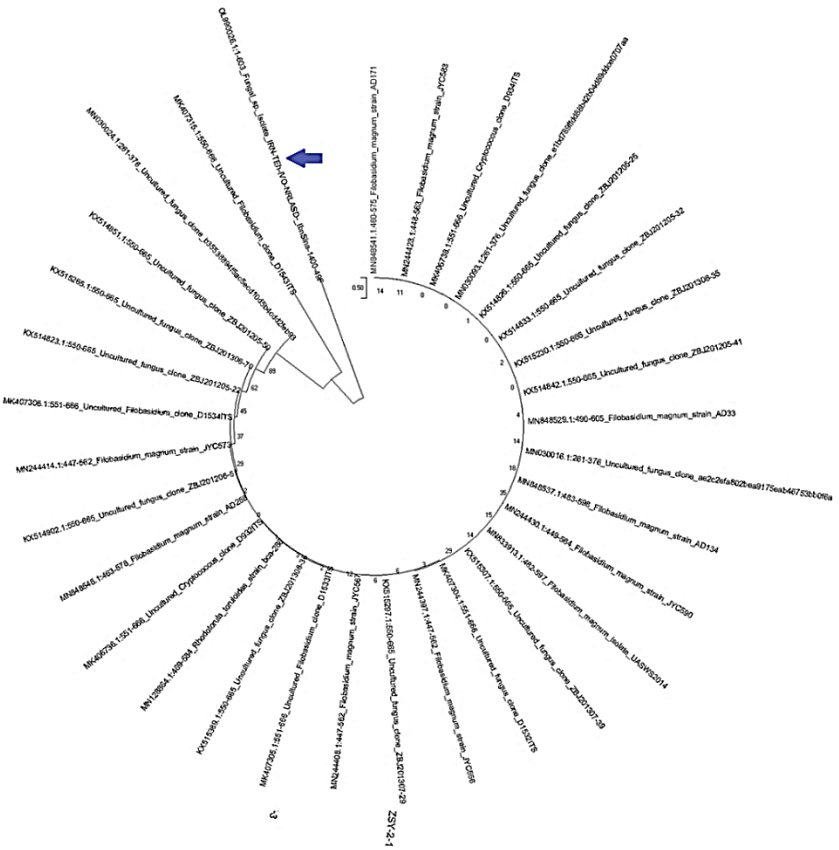

Figure 5: Evolutionary relationships of taxa for Fungal sp. isolated OL990026.1-IRN.Teh.IVO-NRLASD-IbnSina 1400-49F.

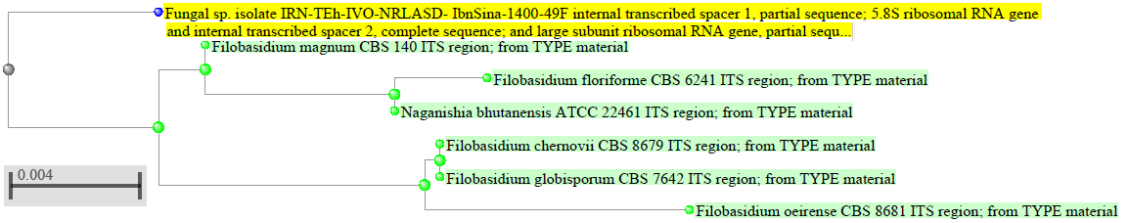

Figure 6: Distance tree of taxa for isolate OL990026 and the closest species .

As shown in the Figure 7, the evolutionary history was inferred using the Minimum Evolution method for *Poecilia reticulata* with *Candida* sp. involvement. The optimal tree with the sum of branch length=4.20962922 is shown in Figure 7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 632 positions in the final dataset.

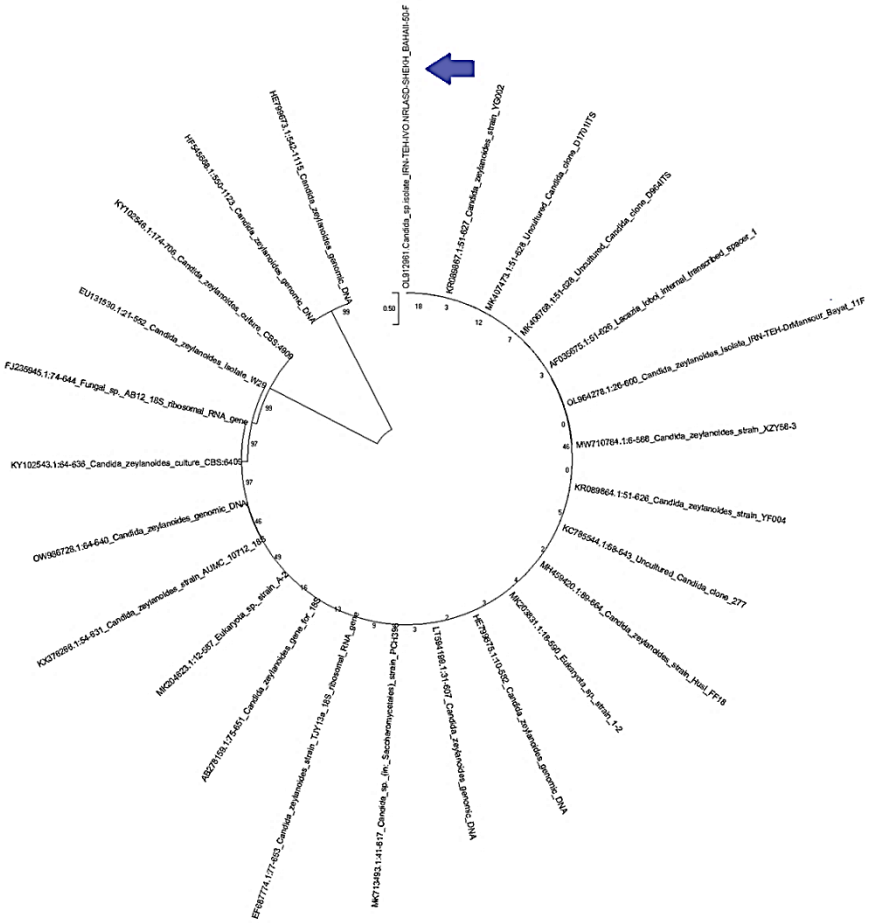

Figure 7: Evolutionary relationships of taxa for *Candida* sp. isolate IRN.Teh.IVO-NRLASD-Shekh-Bahaii 1400-50F.

A neighbor-joining phylogenetic tree was constructed for all the strains (Fig. 7). All strains had at least 98% match and 97% coverage with GenBank reference sequences (unpublished data), which were identified as true fungi. According to the result given in Figure 3, our strain belonged to *Candida zeylanoides.*

Discussion

The fundamental predisposing factors of recent growth in pathogenic fungi of ornamental fish industry are due to opportunistic, hardy environmental stages as well as resistant spores, and considerable rise in global trade and spread of invasive species (Andreou *et al.*, 2009). The managerial and environmental conditions of some ornamental fish farms of Tehran have also increased the epidemiological aspects and disease outbreaks. Thus we hypothesized that the understanding the cause of mortality, taxonomic diversity, and host-parasite associations of fish fungal outbreaks is critical for its operational management. In this study, the fungal species obtained by molecular and culture-dependent attitudes belonged to Ascomycetes. Among the Ascomycetes, the most variety was observed for genus *Penicillium* and *Candida*. These fungi are either reported earlier from the similar or different fish hosts or as new cases. (Gozlan *et al.*, 2014; Cutuli *et al.*, 2015) These are not considered as direct pathogens but, are as opportunists fungus of fish (Shin *et al.*, 2017). The fungal contamination has been imposed to the ornamental fish industry and those who reared fish as pets. In a study conducted in Gorgan fish shopping centers, *P. reticulata* is more sensitive to Mucor and Yeast particularly in time of spring and summer (Gharavy *et al.*, 2020). In this study, the clinical signs observed in the infected fish caused by *P. crustosum* were sever respiratory symptoms, These clinical observations state that the pathogens could have invaded the unplanned wounds after initial abnormalities or accidental injuries (Seyedmousavi *et al.*, 2013; Kotob *et al.*, 2017). Similar to this study, the clinical sign of *P. reticulata* showed that gills became pale due to the presence of fungal pathogens (Haroon *et al.*, 2014). *Clarias gariepinus* was infected to *Penicillum* spp., *Alternaria* spp. (10.8% each), and *C. albicans* (5.4%) (Younis *et al.*, 2020). In a study (EM *et al.*, 2017), *P. crustosum*, and *P. echinulatum* naturally isolated from coral colonies but had no effect on. *Aspergillus* sp. was the most invasive fungus contaminated all the organs of *Catla catla*, was followed by *Blastomyces* sp. and *Penicillium* sp. The infection observed on eyes and gills led to serious disease with respiratory dysfunction of the fish (Iqbal and Saleemi, 2013). Jalilpoor *et al.* (2006) also claimed that *Penicillium* sp. and *Mucor* sp. were reported in the eggs of *Acipenser persicus.*

Alternaria alternata, which found in the second sample of this study OL912959.1, is a fungus which has been recorded causing leaf spot and other diseases on over 380 host species of plant (Marin *et al.*, 2022). The major

allergen, *A. alternata*, has been documented as the main causative agent of airborne allergies in patients affected by a mold allergy in Europe, and can be considered as a marker of primary sensitization to *A. alternata* and homologue Pleosporaceae species. To our knowledge, this is the first report of *A. alternata* infection in fish (*C. rubrofuscus*). In this study, *A. alternata* was an invasive pathogen to gill of the fish (Fig. 1) with leukocyte infiltration and edema features. On the other hands, it is known to have excellent potential for anticancer and inhibition of hepatitis C virus property (Hawas *et al.*, 2015). *Alternaria alternata* was isolated using enrichment method (Patidar *et al*., 2005) from polluted soil at the market of fishery (Khaled *et al.*, 2011), fish sale to present this fact that it was previously isolated from fish. Some fungal genera viz. *Penicillium* spp. (22.2%); and *Alternaria* spp. (27.7%) were isolated from head and gill of *C. auratus*, respectively. Two genera *Aspergillus* spp. and *Alternaria* spp. were isolated from *Hypophthalmichthys molitrix* (Iqbal *et al.*, 2012). A lethal behavior characterized with abnormal swimming of carp spp. was attributed to cerebral infection with *Alternaria* spp. probably following suddenly changes of water attributes (Sharaburin and Bazderkina, 1990).

Filobasidium is a genus of fungi in the Filobasidiaceae was the closest species to our finding. Most *Filobasidium* spp. are only familiar due to their yeast criteria, but some of them produce hyphae with haustorial cells, representing that they are parasites of other fungi. Basidia are tubular with terminal and sessile basidiospores (Aboutalebian *et al.*, 2020). *Filobasidium magnum*, which is widely spread in the environment, is stated as a colonizer of the nasal cavity of pediatric patients suffering from cancer and cases of animal infections (Ghajari *et al.*, 2018). Third sample of this study is 19% close to *F. magnum* (Figs. 5 and 6) therefore it is claimed to be a new species named *Filobasidium* like organism. In this study, gill dysfunction with expansion of opercula and increasing beat rate as well as skin lesions/necrosis were observed.

In this study, the findings showed that gills of the fourth fish samples *P. reticulata* were infected with *Candida zeylanoides.* Clinical signs of the fish showed sever respiratory symptoms with pale filaments. The diseased freshwater fish species may harbor potential clinically relevant yeast species, including *C. albicans* and non albicans *Candida* species, *Trichosporon*, *Cryptococcus* and *Rhodotorula* species. In the fishes *Clarias gariepinus* and *Mugil cephalus*, the most prevalent cultivable yeasts was associated to Ascomycota particularly Candida spp. (65.12%) was higher than Basidiomycota phyla including Cryptococcus, Rhodotorula and Trichosporon with infection of skin and gill tissues. *Candida zeylanoides* was morphologically and molecularly identified as biofilm forming fungi from fish farms and fish benches (Tartor *et al.*, 2018). A noticeable upsurge in yeast

value, mainly by those species associated to human and animals, such as different *Candida* species are related with contaminated waters and plankton over-growth (Andlid *et al.*, 1995). Moreover, *Candida* spp. have been documented to cause infection in salmon, and gilthead sea bream (Galuppi *et al.*, 2001; Gatesoupe, 2007).

It is concluded that the Ornamental fish diseases of Tehran ornamental fish centers where a few fish including the cardinal (*Carassius auratus*), Green Terror Cichlids *(Andinoacara rivulatus)*, the guppy (*Poecilia reticulata*) and the Koi (*Cyprinus rubrofuscus*), *C. carpio* were suffering from gill disorders had been infected with *Penicillium crustosum ,Alternaria alternata, Filobasidium* like organism, *Candida zeylanoides*, respectively.

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