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Microscopical and molecular investigation of *Ichthyophthirius multifiliis* from fish in Mosul city, Iraq

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Abstract

The ectoparasite *Ichthyophthirius multifiliis* (*I. multifiliis*) is one of the most common external ciliate protozoa that infest freshwater fish and causes white spot disease, negatively affecting fish productivity. Two hundred of five species of fish (25 *Luciobarbus xanthopterus*, 30 *Chondrostoma regium*, 35 *Mesopotamichthys sharpeyi*, 54 *Cyprinus carpio*, and 56 *Arabibarbus grypus*) were collected from Mosul city markets from Aug. 2023 to Feb. 2024. Microscopically, fish were infected with the ectoparasite *I. multifiliis* (13/200) 6.5% total infestation rate by wet smears from skin, gills, and fins and based on the morphological features; the ectoparasite were pear-shaped, 0.03 - 1 mm with large horseshoe nuclei. Molecular phylogenetic analysis conducted with 18S rRNA gene confirming species identification of one positive isolate out of 13 belonging to the ectoparasite *I. multifiliis* under the accession number PQ012981 and according to the genetic tree analysis of *I. multifiliis* showed 100% match with each of the United States isolates for the same species and under the accession numbers KJ690571, KJ690572, KJ690570, KJ690568, KJ690567, KJ690566 and KJ690565 and isolates OM865867 in India and OM302501 in China. In contrast, it showed a 99.93% match with isolates MN372056 in the Philippines and ON797789 and ON797786 in China, as recorded in the GenBank. The current study is considered the first record of *Ichthyophthirius multifiliis* at the molecular level in Mosul City, Iraq.

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Introduction

Fish are considered a nutritional and economic source for food security worldwide and, like other animals, are vulnerable to infection with different internal and external parasites (1,2). The parasite *Ichthyophthirius multifilis* belongs to the Phylum Ciliophora, Order Hymenostomatida, Family Ichthyophthiriidae (3). The term (itch) is a scientific name that means fish louse with many children (4). The ectoparasite *Ichthyophthirius multifiliis* is an obligate ciliate protozoan that is widely distributed and capable of infecting most freshwater fish species, causing significant harm by infecting the epithelial tissues, especially the skin, causing notable white spots (5). This disease affects fish health and performance and directly increases fish susceptibility to other pathogens (6). The parasite *Ichthyophthirius multifiliis* has a direct lifestyle with four stages: theront, which attacks the epidermis and nurtures into a second stage trophont. After maturity, the parasitic trophont leaves the infected fish to become a protomont, the third stage, which forms a cyst wall called a reproductive moment, and the fourth stage, which contains large numbers of theronts (7-9). Infected fish with white-spot disease suffer from lack of feeding, weakness, decreased activity, and signs of irritation, thus rubbing their bodies against the rocks of the pond. When gills are highly infested, respiratory distress occurs, ulcers lead to bacterial infection, and infested fish are seen upside-down. They may die due to skin lesions within five days, and

mortality may reach 100% (10-12). Li *et al.* (13) found that *Ichthyophthirius multifiliis* infests more than a hundred ninety species of freshwater fish distributed in twenty-six countries (13). In Iraq, *I. multifiliis* was recorded in forty fish species (14).

Thus, this study aimed to investigate *Ichthyophthirius multifiliis* and conduct polymerase chain reaction analysis and phylogenetic tree to confirm the microscopical results.

Materials and methods

Ethical approval

The institutional animal care and use committee in the College of Veterinary Medicine, University of Mosul, ethically permitted this study (UM.VET.2023.119) on 23/7/2023.

Fish samples

About 200 of five fish species, 25 *Luciobarbus xanthopterus*, 30 *Chondrostoma regium*, 35 *Mesopotamichthys sharpeyi*, 54 *Cyprinus carpio*, and 56 *Arabibarbus grypus* were collected from local marketplaces in Mosul city from August 2023 to the end of February 2024.

Parasitological examination

Separated gills and fins were placed in Petri dishes, and wet smears were made from the skin. Gills and fins were examined with Lugol Iodine and Giemsa under light microscope power 40 and 100X (15). Depending on (16), the parasite's morphological characteristics were identified. Positive samples with microscopical examination were kept at -20ºC until PCR analysis.

DNA Extraction

The extraction of DNA was accomplished according to instructions by the manufacturer, and the extraction of DNA was done using the tissue mini kit (Korea, Add bio). 20 mg from each sample is put in a 1.5ml Eppendorf tube with 200µl of lysate buffer. The 20µl of K proteinase solution, 20 mg/ml, is added into each sample tube, incubated at 56◦C, and mixed by vortexing for 1 h. till tissues are lysed. Then, DNA was centrifugated at 13,000 rpm / 1 min. DNA was kept at -20 C° until further analysis. PCR analysis was done by using specific primers (Table 1). The primers are from Macrogen, Korea.

Table 1: Primers for *Ichthyophthirius multifiliis* (17)

Primers	Primer Sequence 5’ - 3’	bp
18S-F	ACCTGGTTGATCCTGCCAG	1700
18S-R	CTTCCGCAGGTTCACCTACGG	

Extraction of DNA

The PCR reaction mixtures were done on microscopically positive samples by (Prime 2x AddTaq Premix) (2X) (Korea Addbio). Materials were well mixed and distributed in 18 µl PCR tubes size 0.2 ml to perform the polymerase chain reaction procedure, then extracted DNA from samples in size 2 µl was added in the tube separately for each sample to reach the total volume of 20 µl in each tube as in (Table 2). The PCR reaction was carried through a thermocycler (USA, T100 BioRad), and the cycling conditions are shown in (Table 3). Then, tubes were removed from the device and put in the refrigerator at 4-8°C until electrophoresis was done. PCR amplified products were separated using 1.5% agarose gel, mixed with 3 µl of gel red stain, and examined under UV light.

Table 2: PCR reaction mixture for amplification of partial 18S rRNA gene

Components	Vol. (µl)	Final conc.
Master Mix 2X	12.5	1X
PCR grade water	8.5	---
Forward primer	1	1 µ
Reverse primer	1	1 µ
DNA	2	4 ng/µl
Total	25	

Table 3: PCR cycling conditions for amplification of partial 18S rRNA gene

Steps	Temp / °C	Time	Cycles
Initial denaturation	94	10 min	1X
Denaturation	94	45 sec	35X
Annealing	56	45 sec	
Extension	72	1 min	
Final extension	72	10 min	1X
Cooling	4	4 °C	∞

Sequencing DNA analysis

PCR products for infected samples with *I. multifiliis* were transferred to Macrogen, Korea, using Sanger sequencing analysis. The results were obtained as FASTA format text files and then analyzed for genetic affinity.

Results

The current study revealed that fish were infected with *I. multifiliis* with a total infection rate of 6.5% (13/200) by microscopic examination of a direct wet smear of skin, fins, and gills depending on the morphological characteristics, where the *I. multifiliis* appeared pear-shaped with a size of 0.03 - 1 mm with large horseshoe nuclei as shown in (Figures 1 and 2).

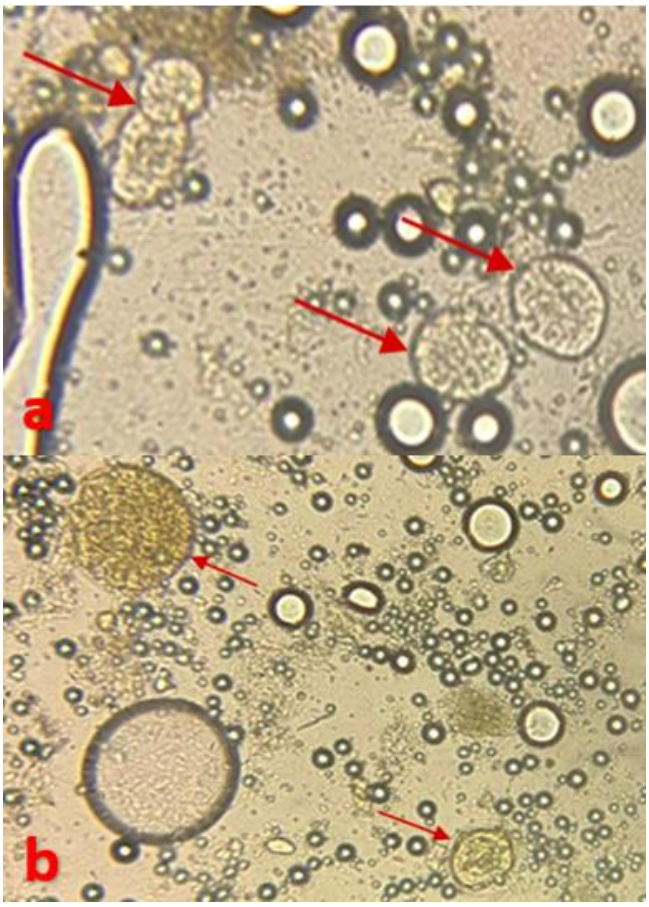

Figure 1: Tomont of *Ichthyophthirius multifiliis* (a, b) in wet smears 40X.

Molecular results

Molecular results of the current study using the polymerase chain reaction technique confirmed the morphological identification of *I. multifiliis* by amplifying the 18S rRNA gene, revealed that lanes 1, 2, 5, and 6 represent positive samples with the reaction product bp 1700 while 3, 4and 7 represent negative samples and lane 8 represents negative control as shown in (Figure 3). The gene sequencing results also confirmed the presence of *I. multifiliis* by using partial 18S rRNA gene in one sample out of 13 samples with the accession number PQ012981 matched with isolates recorded in the NCBI Genbank where the results showed 100% match with each of the isolates KJ690571, KJ690572, KJ690570, KJ690568, KJ690567, KJ690566 and KJ690565 in USA, OM865867 in India and OM302501 in China, 99.93% with the isolates MN372056 in Philippines and ON797789 and ON797786 in China as in (Table 4). The results of gene sequencing and PCR in the skin, fins, and gills of fish infected with *Ichthyophthirius multifiliis* were confirmed. According to the analysis of the parasite's genetic tree (Figure 4), positive isolation was recorded in NCBI under serial number PQ012981.

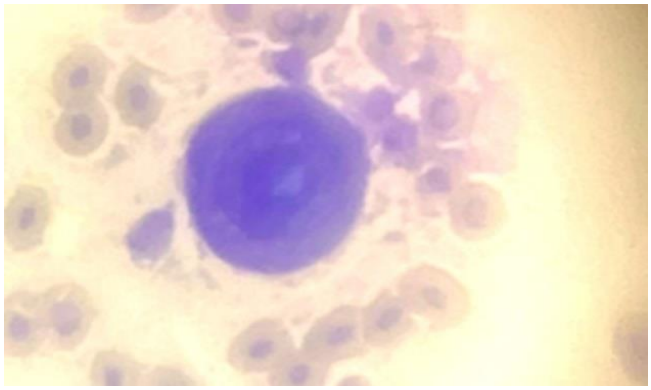

Figure 2: The Trophont of *Ichthyophthirius multifiliis* with a direct wet smear of gills stained with Giemsa 100X.

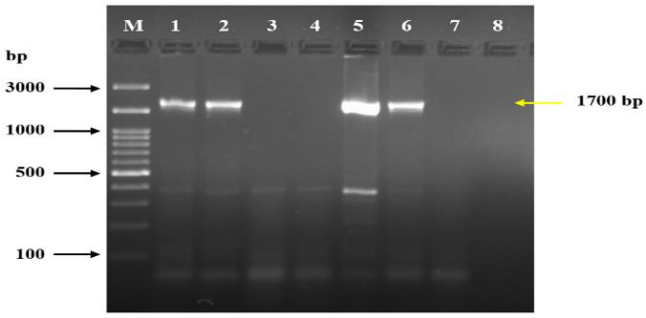

Figure 3: Agarose gel electrophoresis of PCR products to detect *I. multifiliis* Path M: The Marker represents a volume of 100 bp. Paths 1,2,5 and 6 represent positive samples with a product size of 1700 bp, Paths 3,4 and 7 represent negative samples, and lane 8 is negative control.

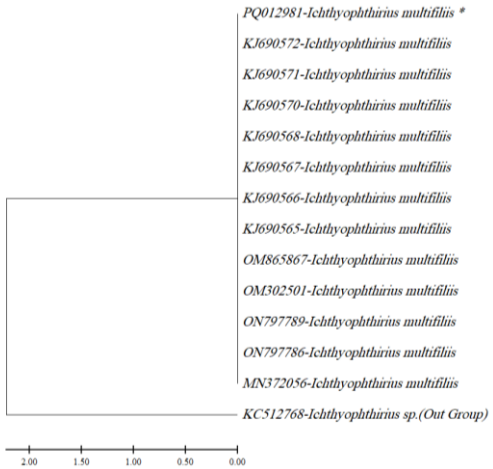

Figure 4: Phylogenetic tree analysis of partial DNA sequences of partial 18S rRNA gene of *Ichthyophthirius multifiliis* from Iraq (*), based on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 1000 re-samplings.

Table 4: Distribution of *I. multifiliis* on partial 18S rRNA based on nblast in Genbank of NCBI

Sample Accession No.	Query Cover %	Identic Number %	Genbank Accession Number	Country
PQ012981	100	100	KJ690571	USA
	100	100	KJ690572	USA
	100	100	KJ690570	USA
	100	100	KJ690568	USA
	100	100	KJ690567	USA
	100	100	KJ690566	USA
	100	100	KJ690565	USA
	100	100	OM865867	India
	100	100	OM302501	China
	100	99.93	MN372056	Philippines
	100	99.93	ON797789	China
	100	99.93	ON797786	China

Discussion

Fish meat is considered a primary source of animal protein for humans, and it has many beneficial components for human health, such as essential minerals, fatty acids, and lipid-soluble vitamins (18-20). External parasites are one of the major diseases affecting fish health in contrast to internal parasites, which are limited, and the skin surface is considered the preferable site for fourteen protozoans, while gills are eleven (21,22). Ich disease usually attacks the skin, gills, and fins of farmed fish and broad host specific (23).

In this study, the morphological characteristics of *Ichthyophthirius multifiliis* were identical to those previously described in many studies (20,24). Microscopical examination recorded an infestation rate of 6.5% with *I. multifiliis* in five species of fish (*Arabibarbus grypus*, *Cyprinus carpio*, *Mesopotamichthys sharpeyi*, *Chondrostoma regium*, and *Luciobarbus xanthopterus*) which considered higher than 1.6% in Damlaj Marsh in Diwaniyah in gills of *C. carpio* 3.5% in a study in Baghdad (25,26). In contrast, our result is lower than 13.97% in Babil (27), 16.9% in Darbandikhan Lake North of Iraq (28), 28.6% in Mosul (29), 29% in Erbil (30) and 50% in Erbil (31). These different infestation rates can be attributed to the environmental sites of fish collected (32,33). Other different factors: firstly, fish species, as mentioned by Abdullah and Abdullah (34) who recorded prevalence ranged between 2.89- 23.52%, in seven species of fishes in north or Iraq. The researchers Al-Awadi and Mhaisen (35) who recorded between 9-22% in gills in five fish species in Najaf. In Iraq, concerning the occurrence of Itch disease, documented 19 fish species, such as *C. carpio*, from many fish farms in mid and south of Iraq while recently until 2022 were showed increasing in fish hosts about 40 fish species are reported as hosts for *I. multifiliis* (14,36). Secondly, risk factors like genetic resistance, sex, age and length, weight, health state, external tissue characteristics, subcutaneous tissue scales and thickness, and several body exposures to parasites are essential in increasing susceptibility to infections (27). Thirdly, management factor effects on the biology of fish like a closed environment increased density of the fish population lead to the accumulation of external parasites, especially *I. multifiliis*, which has a short and direct lifecycle and high reproduction rate compared with Trematodes, Cestodes, and Acanthocephalans that have indirect life cycles (37,38). Fourthly, differences in environmental factors, physical conditions, chemical and physical properties of water and high-water temperatures, accompanied by many infected fish, make parasites more active in higher temperatures (1,27,39,40). Also, a high degree of water pollution caused by factories, wastewater, and the frequent use of pesticides create an environment suitable for suppressing fish immunity to infection (41-43). Fifthly, nutrition plays a role in causing many parasitic, bacterial, viral, or fungal diseases in fish, and good nutritional diets enhance the fish's development and improve fish's general health (1,44-47). Sixthly, factors related to the parasite's broad host specificity and the parasites become more pathogenic in mixed infestations; therefore, unwell fish are more susceptible to catching illnesses and spreading them to nearby species (48).

Molecular results using polymerase chain reaction tool by amplification of 18S rRNA gene with end product 1700 bp and positive isolate belong to the ectoparasite *I. multifiliis* in the current study correctly confirmed positive microscopical samples of skin, gills, and fins of infested fish that the causative agent infesting fish is *I. multifiliis*. Phylogenetic tree analysis reflected the close relationship between *I. multifiliis* in this study and *I. multifiliis* in other studies (17,49). Results of gene sequencing and phylogenetic tree analysis of the ectoparasite *I. multifiliis* isolated under the accession number PQ012981 showed 100% match with each of the United States isolates for the same species and under the accession numbers KJ690571, KJ690572, KJ690570, KJ690568, KJ690567, KJ690566 and KJ690565 and isolates OM865867 in India and OM302501 in China. In comparison, it showed a 99.93% match with isolates MN372056 in the Philippines and ON797789 and

ON797786 in China, as recorded in the Genbank; this match is due to submitting a partial sequence for NCBI for 1418bp. The current study is considered the first record of *Ichthyophthirius multifiliis* at the molecular level in Mosul City, Iraq.

Conclusion

The conducted study reported the presence of *Ichthyophthirius multifiliis* microscopically by wet mount smears of skin, gills, and fins of five fish species and the first molecular study for this parasite in Mosul, Iraq by amplification of 18S rRNA gene for identification of ectoparasite *I. multifiliis* isolated under the accession number PQ012981 showed 100% with many countries have.

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Conflict of interest

The authors announced that there are no matches of concern pertaining to the publication of this manuscript.

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التحري المجهري والجزيئي عن طفيلي قامِلَةُ السَّمَك في الأسماك في مدينة الموصل، العراق

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الخلاصة

يعد طفيلي قامِلَةُ السَّمَك من الأوالي الهدبية الخارجية الأكثر شيوعا التي تصيب اسماك المياه العذبة والمسبب لداء البقعة البيضاء والذي يؤثر سلبا على إنتاجية الأسماك حيث تم جمع ٢٠٠ عينة من خمسة أنواع من الأسماك (٢٥ الكطان، ٣٠ الزولي، ٣٥ البني، ٥٤ الكارب الاعتيادي و ٥٦ الشبوط) الأسواق المحلية في مدينة الموصل من شهر آب ٢٠٢٣ الى نهاية شهر شباط ٢٠٢٤. مجهريا، إن الأسماك كانت خمجة بالطفيلي الأولي الخارجي قامِلَةُ السَّمَك وبنسبة خمج كلية ٦,٥% (٣٠٠/١٣) وبالمسحات الرطبة للجلد والغلاصم والزعانف وبالاعتماد على الصفات الشكلية ظهر طفيلي قامِلَةُ السَّمَك كمثري وبحجم ٠,٠٣ - ١ ملم حاوي على نواة كبيرة الشكل على شكل حدوة حصان. في حين أثبتت تقنية تفاعل البلمرة المتسلسل و تعاقب الجينات من خلال تضخيم الجين 18S rRNA ناتجا تفاعليا١٧٠٠ زوجا قاعديا وان العزلة الموجبة من ١٣ عينة موجبة تعود للنوع قامِلَةُ السَّمَك وبالرقم التسلسلي PQ012981 ووفقا لتحليل الشجرة الجينية للنوع قامِلَةُ السَّمَك اظهر تطابقا بنسبة ١٠٠٪ مع كل من العزلات الولايات المتحدة الأمريكية لنفس النوع وبالأرقام التسلسلية KJ690571، KJ690572، KJ690570، KJ690568، KJ690567، KJ690566 و KJ690565 وللعزلات OM865867 في الهند و OM302501 في الصين ، في حين أظهرت تطابقا ٩٩,٩٣٪ مع العزلات MN372056 الفلبين في و ON797789 و ON797786 في الصين وكما مسجل في بنك الجينات العالمي. وتعد هذه الدراسة الأولى على المستوى الجزيئي في مدينة الموصل، العراق في تشخيص طفيلي قامِلَةُ السَّمَك.