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Original Research Article

Molecular Characterization of *Ichthyophthirius* spp. (Ciliophora: Ophryoglenidae) in Iraqi Freshwater Fishes Based on 18S rRNA Phylogenetic Analysis

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Abstract: ***Background*:** Members of the genus Ichthyophthirius are some of the most notable ectoparasitic ciliates affecting freshwater fishes globally. While infections have been documented in common carp (Cyprinus carpio) and goldfish (Carassius auratus) in Iraq, molecular confirmations and recent updates to host range data are lacking. Considering the recent expansion of aquaculture and the translocation of fish, the potential for greater dissemination of parasites among both native and introduced aquatic ecosystems is concerning. ***Aim*:** The primary goal of the current study was to molecularly identify and phylogenetically characterize Ichthyophthirius spp. affecting freshwater fishes in the Al-Diwaniyah River, Iraq, and examine new extensions of host range. ***Methods*:** A total of 720 fish specimens belonging to five species were collected and examined between October 2023 and October 2024. Skin and gill tissues were microscopically investigated for the presence of ectoparasitic infections. From these positive samples, DNA was extracted, and the 18S rRNA gene was amplified. Subsequently, the PCR products underwent sequencing, and the resulting data were analyzed using the BLAST and Maximum Likelihood (ML) methods for phylogenetic analysis. ***Results*:** Ichthyophthirius spp were found only in skin tissues, affirming strong predilection or specific tissue preference. In molecular amplification, clear 18S rRNA products were found within the range of 1500–3000 bp. In comparison to 18S rRNA sequences of Ichthyophthirius spp, analyses of the sequences showed 98–100% similarity to any of the global reference sequence of Ichthyophthirius in the GenBank. In the phylogenetic reconstruction, Iraqi isolates formed a distinct clade with considerable support of Ichthyophthirius, which is almost identical with Asian strains, albeit minor nucleotide variations which suggest lateral stability. Although the parasite has been documented in Cyprinus carpio and Carassius auratus, this study reports Ichthyophthirius spp. for the first time in Oreochromis aureus, Garra variabilis, and Oreochromis niloticus in Iraq. ***Conclusion*:** Molecular data in this study serve to confirm the presence of Ichthyophthirius spp. in the Iraqi freshwater system. This study also expands the number of documented hosts to several cyprinid and cichlid species. Findings of this study are important in the understanding of parasite ecology, transmission cycle, and the possible impacts of aquaculture of Iraq.

Keywords: Ichthyophthirius Spp, 18S rRNA, Host Expansion, Freshwater Fish, Phylogeny, Iraq.

INTRODUCTION

The Ichthyophthirius genus holds a reputation as one of the most destructive protozoan ectoparasites infesting freshwater fish around the globe. The ciliates cause white spot disease, a disease characterized by the overproduction of mucus and epithelial hyperplasia, and which affects osmoregulation. In aquaculture, outbreaks of white spot disease can cause very rapid and devastating financial losses. Recent studies show that infections by Ichthyophthirius multifiliis disrupt host immune responses and the normal microbial communities present on mucosal surfaces (Gong *et al.*, 2025; Bu *et al.,* 2025). Such disturbances may affect more than just local damage; they may cause systemic

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modulations of the immune system, which will affect the health and productivity of the fish.

The interaction between hosts and parasites has been better understood with the advent of omics and molecular tools. Among the freshwater fishes that were infected with I. multifiliis, metabolomic and metagenomic profiling revealed spceis-specific resistance. This profiling highlighted different immune and metabolic responses (Liu *et al*., 2025). Infection is shown to cause a complex regulation of genes, through multiple transcriptomic studies, ignited by inflammation, oxidative stress, and the epithelial barrier (Shen *et al*., 2022). Both hosts and parasites have a virulence that is influenced by the genetics and environment of the organisms. The quantitative polymerase chain reaction (PCR) has greatly enhanced monitoring of parasites in the environment and their detection within aquaculture systems (Guo *et al*., 2025). These molecular tools have become a first line of defense for epidemiological measurements and early intervention.

Ichthyophthirius is difficult to control because of its direct lifecycle and robustness throughout the aquatic ecosystem. Recently tested pharmaceuticals targeting the trophont and theront stages are showing promise for both in vivo and in vitro settings (Hu *et al*., 2025; Peng *et al.,* 2025). Vaccines targeting the protozoan have relied on unconventional methods such as DNA immunization and the use of cytokines as adjuvants to strengthen the immune response (Meira *et al*., 2024). In aquaculture, AI-based monitoring has been proposed for the early detection of disease outbreaks (Bonnichsen *et al.,* 2025). It shows the increasing awareness of the parasite and the need for a more integrative approach.

While there have been considerable numbers of studies focused on Ichthyophthirius infections on the more traditional hosts, i.e. the carp and the goldfish, they are have also been documented linked to more non-traditional hosts, exhibiting the possibility of greater adaptability to novel hosts. Infections on non-traditional hosts, e.g., amphibian species, demonstrate ecological plasticity and emerging patterns of host expansion (Poonlaphdecha *et al.,* 2025). Reporting infections on hosts from different species raises concern on the potential dissemination of infections among different freshwater ecosystems. Ichthyophthirius infections have been reported globally, however, the host spectrum or molecular studies of the Eastern Mediterranean region, particularly Iraq, have been neglected. It is imperative to conduct extensive molecular studies to determine the distribution, phylogeny, and emerging host association of the parasitic organism within the freshwater ecosystems of the region.

MATERIALS AND METHODS

Study Area and Fish Sampling

From October 1, 2023, to October 1, 2024, fish specimens were collected from five species of fish, including Cyprinus carpio, Carassius auratus, Oreochromis niloticus, Oreochromis aureus, and Garra variabilis, of which 720 specimens were collected from the Al-Diwaniyah River, which is a tributary of the Euphrates River in Al-Qadisiyah Province, Iraq. Fish were procured randomly from local fishermen from different sampling points. They were then transported in insulated polyethylene containers with crushed ice to the Parasitology Laboratory, College of Veterinary Medicine, University of Al-Qadisiyah, for analysis, which was done 4-6 hours after the fish were caught. Fish identification was done using the taxonomic keys for the identification of Iraqi freshwater fishes.

Parasitological Examination and Morphological Identification

All fish were checked for the presence of white cysts and lesions caused by Ichthyophthirius. Skin scrapings were taken from the laterals of the fish and later examined under a light microscope (Olympus CX23, Olympus Corporation, Tokyo, Japan) at 100 x and 400 x magnification. Furthermore, the gill arches were dissected to see the preference of the dissected gill arches. The presence of trophonts identified as Ichthyophthirius was determined based on size, ciliation, macronucleus shape and position, and behavior. Only Trophonts from the integumental tissues were selected for molecular analysis. In the positive PCR cases, no parasites were found in the gill tissues.

Extraction of DNA

Genomic DNA from single trophonts was extracted using a silica-based DNA extraction kit (Geneaid Genomic DNA Mini Kit, Tissue, Geneaid Biotech Ltd., New Taipei City, Taiwan; Cat. No. GS100). Prior to extraction, parasites were cleaned in sterile phosphate-buffered saline (PBS, pH 7.4) and removed from the debris. Each trophont was lysed in 200 µL of lysis buffer containing guanidine thiocyanate and 20 µL of proteinase K (20 mg/mL). This mixture was then incubated at 60 degrees for 30 minutes and mix intermittently.

After digestion, 200 µL of binding buffer and 200 µL of absolute ethanol were added. The mixture was put into GS spin columns and centrifuged at 14,000 × g for 1 minute. Wash steps were performed using W1 buffer and ethanol-based wash buffer as the manufacturer directed. DNA was stored at −20°C until analysed by PCR after it was eluted in 100 µL of preheated elution buffer. Concentration and purity (A260/A280) of the DNA sample was measured by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Only samples with a purity ratio of 1.8–2.0 were used for amplification.

PCR Amplification of 18S rRNA Gene

Molecular detection of Ichthyophthirius spp. was accomplished by amplifying the 18S rRNA gene. The used primers were genus-specific and were synthesized by Macrogen Inc. (Seoul, South Korea):Forward primer (18S-F):5′-AAC CTG GTT GAT CCT GCC AGT-3′ and Reverse primer (18S-R):5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′. The PCR reactions were performed in a total volume of 50 µL, which included:

- 25 µL of 2× Taq PCR Master Mix (Promega Corporation, Madison, WI, USA; Cat. No. M7502), 1 µL of each primer (10 pmol/µL), 3 µL of DNA template, and the rest was filled with nuclease-free water.The thermal cycler settings were as follows for the Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, USA):For the initial denaturation, the sample was heated for 5 minutes at 95°C;Then 35 cycles for denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 90 seconds;For the final extension, the sample was held at 72°C for 10 minutes.

Agarose Gel Electrophoresis

The PCR products were analyzed with the 1.5% agarose gel electrophoresis (1× TBE buffer) and (ethidium bromide 0.5 µg/mL) and with a 100 bp DNA ladder (GeneRuler™ 100 bp DNA Ladder, Thermo Fisher Scientific, USA) for size estimation. Electrophoresis was done at 100 volts for 45 minutes. The amplified fragments were between 1500–3000 bp and were illuminated with UV light and photographed with the Bio-Rad Gel Doc™ XR+ (USA) gel documentation system.

DNA Sequencing and Phylogenetic Analysis

The PCR products, which had a single band, were purified with a PCR purification kit (Qiagen QIAquick PCR Purification Kit, Hilden, Germany, Cat. No. 28104) and were sequenced bidirectionally with the Sanger method at Macrogen Inc. (Seoul, South Korea).

We used FinchTV software (Geospiza Inc., Seattle, USA) to view and edit chromatograms. Forward and reverse sequences were built into consensus sequences with BioEdit version 7.2. Sequence identity was checked with the Basic Local Alignment Search Tool (BLAST) to the NCBI GenBank database. Sequences that were ≥98% similar were validated as Ichthyophthirius spp. isolates.

Multiple sequence alignment was done with ClustalW embedded in MEGA11 (Tamura *et al.*, 2021). Phylogenetic trees were drawn using the Maximum Likelihood approach with the Tamura-Nei model and 1,000 bootstrap replicates. Robustness of the trees was supported at bootstrap values >70%.

Ethical Approval

All fish handling and samplings were done following institutional animal care and use guidance. Ethic’s committee approval was granted by the Scientific Research Ethics Committee, College of Veterinary Medicine, University of Al-Qadisiyah, Iraq (Approval No. 626 issued on 5 February 2026). Fish were treated with care and sampling was conducted to avoid excessive stress and suffering.

RESULTS

Detection and Tissue Localization of Ichthyophthirius spp

Among the 720 freshwater fish examined, only trophonts that were morphologically consistent with Ichthyophthirius spp. were found within the integumental tissues. Gill tissues of PCR-positive fish showed no signs of infection at the microscopic level. Infected fish exhibited characteristic whitish lesions and nodules scattered all over the body and fins.

Microscopic examination of skin scraping showed the presence of large, oval to spherical ciliates rotating slowly. The ciliates had a conspicuous macronucleus, and ciliates were evenlydistributed around the body, a distinguishing characteristic of the genus Ichthyophthirius. The intensity of infection varied with the host species with notably, Cyprinus carpio and Oreochromis niloticus having moderate to heavy infection.

Cyprinus carpio and Carassius auratus had previously identified the parasite in Iraq. The present study has confirmed Gourami, Garra, and Niloticus in the () to have infected for the first time species, which is a significant expansion in the host range in the freshwater ecosystem of Iraq.

Molecular Identification of Ichthyophthirius spp

PCR of the 18S rRNA gene produced clear amplifications in all samples that were positive microscopically. The amplified fragments of the ribosomal genes of Ichthyophthirius were in the expected range of 1500 - 3000 base pairs. Results from gel electrophoresis showed defined, single bands and no evidence of nonspecific amplification. The negative controls showed no evidence of amplification, supporting the conclusion that no contamination was present. The high rate of successful amplification suggests the chosen primer set is trustworthy for amplification at the genus level (Figure 1).

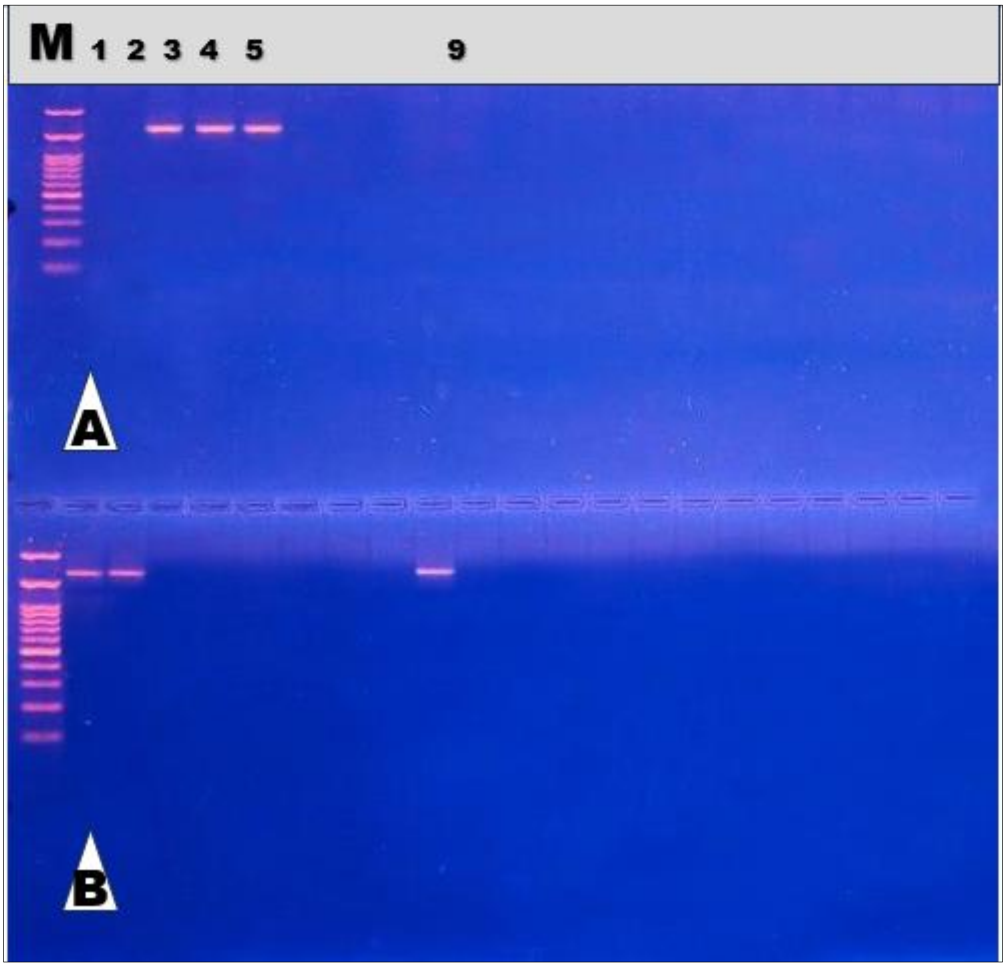

Figure 1: Agarose gel electrophoresis showing PCR amplification of 18s RNA products obtained for ectoparasite *Ichthyophthirius multifiliis* the amplicon about 1500-3000bp

Sequencing and BLAST Homology Analysis

Chromatograms for Sanger sequencing was obtained in high quality for both the forward and reverse directions. Once trimming and assembly were done, consensus sequences were obtained for the representative isolates.

When the consensus sequences were compared to the sequences contained in the NCBI GenBank database, the percentage of sequence similarity was found to be from 98% to 100% for sequences of Ichthyophthirius spp. from various locations in the world. It was noted that some Iraqi isolates had a few minor nucleotide changes, but nothing was found to be structurally different in the area that was amplified.

The isolates that were collected had the greatest similarity to those strains that were first described from Eastern and Southeastern Asia, suggesting that there is a high level of genetic conservation, even among populations that are widely spaced.

Phylogeny

When the Maximum Likelihood algorithm was applied to the phylogenetic analysis of the first 18 base pairs of the partial sequence of the 18 S rRNA for all the Iraqi isolates in the analysis, they all clustered together in a single Ithcyophthirius clade that was well supported. The support values for the major nodes were greater than 85%, which confirms phylogenetic validity.

Iraqi isolates constituted a compact sub-branch within the global clade, showing closest affinity to Chinese reference sequences. Although separated by large distances, the isolates showed little to no genetic divergence, indicating a lack of evolutionary change in the parasite at the ribosomal gene loci.

No distinct species-level divergence was found in the data, justifying the identification of the isolates at the genus level as Ichthyophthirius spp (Figure 2).

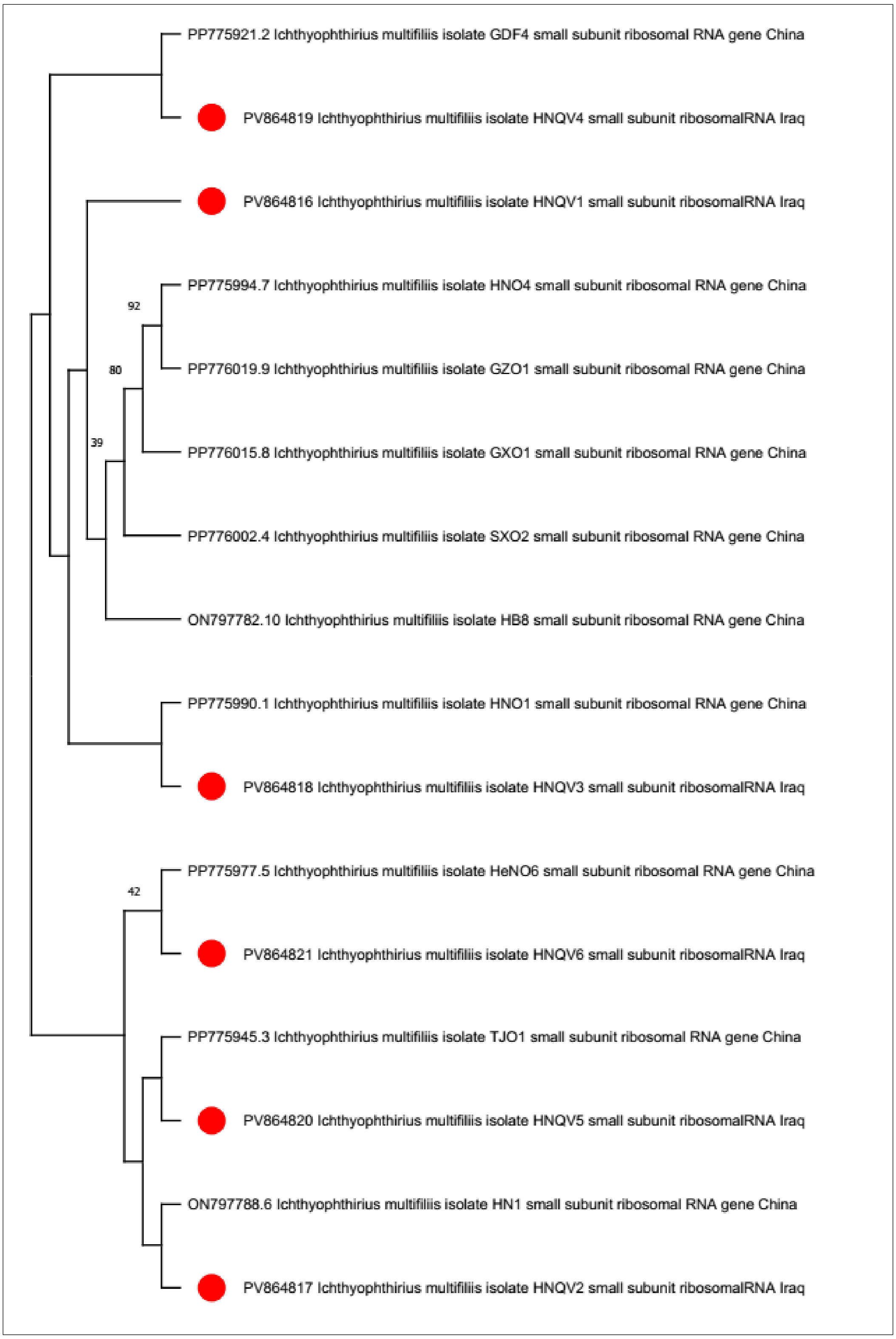

Figure 2: Phylogenetic analysis of *Ichthyophthirius multifiliis* based on the 18S rRNAgene.The partial 18S ribosomal RNA gene sequences inform the phylogenetic tree and the genetic associations of the different isolates of *Ichthyophthirius multifiliis*

DISCUSSION

The data obtained for this research is the first molecular examination of Ichthyophthirius spp. infesting freshwater fish in the Al-Diwaniyah River and provides evidence for a potential increase in parasitic host range in the freshwater systems of Iraq.

The finding that the parasite is confined to the tissue integument is in accordance with the epitheliophilic nature of Ichthyophthirius spp. as they exhibit an obvious tissue preference for the epidermis of fish hosts (de Lima Araú jo *et al.,* 2024). Inflammatory cell infiltration is predominant, and there is an increase in mucus cell production and epithelial cell hyperplasia due to the attachment of the trophont. The parasite is suggested to adopt an integumental immune evasion strategy by eliciting an obstructive dermal tissue response, as indicated by the heightened response of the proteomic and transcriptomic response and the dermal tissue response in inflammatory and epidermal barrier response (Zhao *et al*., 2025). This observation reinforces the concept that the primary infection niche of the parasite is the dermal tissue.

Molecular amplification of the 18S rRNA gene showed that Iraqi isolates were 98–100% similar to worldwide reference strains. Such conservation is consistent with previous molecular studies showing the absence of intraspecific divergence in the ribosomal gene regions of Ichthyophthirius spp. (Huang *et al*., 2024). The alignment of Iraqi isolates within a robust global clade indicates the parasite's genetic stability despite geographic disparities. The same degree of minimal divergence is also seen in host–microbiota–parasite interaction studies where isolates of different regions showed considerable genetic conservation (Li *et al*., 2025). Such a conserved genetic structure may indicate evolutionary stasis due to the obligate life cycle and direct transmission mechanism of the parasite.

Host expansion to Oreochromis aureus, Garra variabilis, and Oreochromis niloticus is an important epidemiological development. Ichthyophthirius spp. have traditionally been thought to only infect members of the family Cyprinidae, particularly, the carp and goldfish. However, their ecological plasticity appears to be rapidly increasing with the ability to infect multiple taxa within freshwater ecosystems. The parasite is also known to infect non-cyprinid ornamental fish (Banu *et al.,* 2025). Infection-induced changes in the microbiota and the modulation of the mucosal immune system have been observed in both Cyprinidae and non-Cyprinidae fish. Such changes show that the susceptibility of fish to infection is not confined to the so-called 'classic' hosts (Gong *et al*., 2025; Bu *et al*., 2025). The presence of Ichthyophthirius spp. in other fish species in Iraq might be a result of increased lateral movement of fish, climatic/environmental changes, or increased aquaculture activities that promote the transmission of the pathogen to new host species.

There is growing evidence of the complexity of immune responses of hosts to Ichthyophthirius. Liu *et al*., (2024) showed that dietary mannan-oligosaccharides induced the enhancement of mucosal immunity and antioxidant defense of goldfish, which increased goldfish resistance to the pathogen. Qu *et al*., (2024) reported that some catechol and its derivatives exhibited dual anti-parasitic and immunomodulatory functions. In another recent study, Wang *et al*., (2024) showed that some natural copepod predators can reduce theront abundance in aquaculture ponds. Ibrahim *et al*., (2024) showed that the presence of Ichthyophthirius along with some bacterial pathogens of fish can increase the severity of the disease, and this shows the need for early detection and the integrated management of fish disease. Expanding hosts in freshwater systems will increase the ecological and economic threat of this parasite.

CONCLUSION

Iraqi isolates demonstrate slight nucleotide variation. Phylogenetic coherence warrants conservative identification at genus level. Their slight divergence may suggest localized evolutionary adaptations, not species-level differentiation. Future studies with additional molecular analytic techniques may determine the possible presence of cryptic diversity within the regional populations. Overall, the findings confirm the molecular presence of Ichthyophthirius spp. in the freshwater fish in Iraq, demonstrate host range expansion beyond previously recorded species, and indicate there is an urgent need to monitor the freshwater fish in Iraq to enable studies of the epidemiological impact of the Ichthyophthirius spp. on aquaculture and wild fish.

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