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MOLECULAR STUDIES AND SCANNING ELECTRON MICROSCOPE OF *Pomphorhynchus laevis* (ACANTHOCEPHALA: POMPHORHYNCHIDAE) FROM CYPRINID FISHES IN SULAIMANI GOVERNORATE

MUQDAD KAMAL ALI [a*]

[a] Department of Animal Science, College of Agricultural Engineering Science, University of Sulaimani, Sulaimani, Kurdistan Region, Iraq.

AUTHOR'S CONTRIBUTION

The sole author designed, analysed, interpreted and prepared the manuscript.

Article Information

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ABSTRACT

A total of 100 fishes (65 of *Squalius lepidus* and 35 of *Carasobarbus luteus*) have been collected in Sirwan River in Sulaimani city, Kurdistan region-Iraq. Light and scanning electron microscopy, as well as the sequencing and analysis of (18S rDNA), were used to describe *Pomphorhynchus laevis* in this study. Molecular analyses results indicate that the PCR product of *Pomphorhynchus laevis* was 528 bp recorded lowest genetic distance with *P. laevis* (AY218124.1, AY423346.1 and KF559309.1), *P. kashmirensis* (MZ381411.1, MZ381412.1 and MZ381413.1) and *P. tereticollis* (AY423347.1) is 0.000, recorded highest genetic distance with *Echinorhynchus gymnocyprii* (MT162047.1 and MT162051.1) is 0.017. Our phylogenetic analyses showed that taxonomic position of the species *P. laevis* is closely related with *P. kashmirensis* (MZ381411.1, MZ381412.1 and MZ381413.1) and *P. laevis* (AY218124.1, AY423346.1, KF559309.1 and JX014223.1) and *P. tereticollis* (AY423347.1) in the phylogenetic trees. The *P. laevis* identified in our study has the same morphological characteristics as the *P. laevis* which was found for the first time in Iraq.

Keywords: Acanthocephalan; *Pomphorhynchus laevis*; scanning electron microscope; 18S rDNA gene; DNA sequencing.

1. INTRODUCTION

There are currently 29 species of parasites on freshwater fish, marine fish, and amphibians in the Pomphorhynchus Monticelli, 1905 genus [1,2]. All fish acanthocephalans, including water amphipods as intermediary hosts, rely on tropic transfer to complete their life cycle [3]. Via eating the acanthocephalan

**Corresponding author: Email: muqdad.ali@univsul.edu.iq;*

eggs, the arthropod intermediate host is infected; in the host's digestive tract, the acanthor larva becomes free. The larva bores are developed from acanthella to cystacanth by the gut wall in the body cavity that may impact the vertebrate host [4].

Acanthocephalans's proboscis, or bulb, penetrates the fish host's gut walls deeply, causing significant harm to the digestive system [5]. *The first description of P. tereticollis was based on the morphological features of a specimen taken from a European flounder (Platichthys flesus) caught off the Baltic coast* [6]. *Since then, bar-coding techniques have been used to confirm P. tereticollis in a variety of gammarids, cyprinids, and perciform hosts in lowland rivers throughout Europe* [7,6]. There have been recent discoveries in Europe of two morphologically similar species, *P. laevis* Müller, 1776 and *P. tereticollis* [8,9,10,6], both of the genus *Pomphorhynchus*. This species, *P. tereticollis*, was formerly considered a synonym of *P. laevis*, but it was re-described and revived by [6] based on genetic and physical characteristics.

Pomphorhynchus kashmirensis Kaw, 1941 is one of 9 species of *Pomphorhynchus* Monticelli, 1905 known from the Jammu-Kashmir regions of the Northern Indian Subcontinent. The original description from *Triplophysa kashmirensis* Hora, 1922 (Nemacheilidae) was inadequate as much of its morphological features could not be adequately visualized or confirmed in text or in illustrations [11].

In moderate numbers, a fresh freshwater species, *P. spindletruncatus*, was discovered in two species of fish, one of them described herein [1] in a river system in northern Iraq and as taxonomic with key information was provided. In Iraq, just one species of *Pomphorhynchus, P. laevis* Zoega in Müller, 1776, was reported; it has been discovered in three different northern regions of the country, which are two Cyprinid species of fish [12,13].

One of the most frequently utilized molecular markers to ascertain the genetic diversity and phylogenetic relationships among acanthocephalans is the small subunit 18S rRNA gene. The main use of this gene is to infer phylogenetic analysis among the major classes of Acanthocephala. It is relatively conserved and evolves slowly [14]. In Pomphorhynchidae, previous taxonomic studies generally focused on classical morphological methods [1,15,16]. Today, a large number of Pomphorhynchidae have been identified and distinguished using ITS, 18S rDNA, and COX1 target sequences as genetic markers [17,18].

The aim of the study is identification of *P. laevis* by molecular technique such as 18S rDNA and morphological variability by Scanning Electron Microscope.

2. MATERIALS AND METHODS

2.1 Sample Collection

A total of 100 fishes belonging to cyprinid species (65 of *Squalius lepidus* and 35 of *Carasobarbus luteus*) (Fig. 1A; B) were collected from the Sirwan River in Sulaimani, Kurdistan region, Iraq. Fishes have been collected via gill-netting and cast-netting between May and August 2021. In the laboratory, fish were examined quickly just after capture. *Pomphorhynchus laevis* recovered is first washed with saline solution, refrigerated in cold water for 24 hours, and then fixed in ethanol 70% for SEM and %99 ethanol for molecular work.

2.1.1 Scanning Electron Microscope (SEM)

Specimens used for scanning electron microscopy (SEM) were completely rinsed of media (PBS is appropriate) and fixed at room temperature for 2 hours with SEM fixative (2.5% glutaraldehyde), rinsed three times with the same buffer for 5 minutes each rinse, and then post-fixed in 1% osmium tetroxide, dehydrated through a series of ethanol concentrations (50%, 70%, 80%, 95% and 100%). The samples were shaken for 24 hours to remove the acetone, then embedded on the targets and sputter-coated with gold before being studied with an FE-SEM TESCAN MIRAIII made in the Czech Republic.

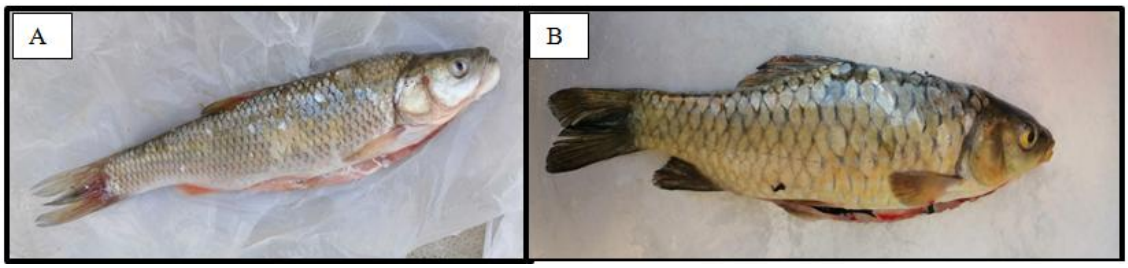

Fig. 1. A: *Squalius lepidus*; B: *Carasobarbus luteus*

2.2 Molecular study

2.2.1 DNA extraction

For DNA analysis of *Pomphorhynchus laevis*, which was isolated from (*Squalius lepidus* and *Carasobarbus luteus*) fish species collected from the Sirwan River. The fixed parasites in 99% ethanol were identified on the basis of morphological characteristics. Then the genomic DNA was extracted according to the AccuPrep® Genomic DNA Extraction Kit's methodology (Bioneer Corporation Cat. No.: K-3032 Korea).

The quantity of DNA was checked and quantification was done by a Nanodrop spectrophotometer. The quantity of DNA for *Pomphorhynchus laevis* was 1.75. Agarose gel electrophoresis 1% is used for identifying and assessing the quality of the extracted DNA.

2.2.2 DNA Amplification

For phylogenetic study, the 18S rDNA gene by PCR was amplified with forward primer (5'-GCGCGGTAATTCCAGCTC-3') and reverse primer (5'CTGGTGTGCCCCTCCGTC-3') [19] for *Pomphorhynchus laevis*.

Amplification of DNA was done via using (MultiGene OptiMax Thermal Cycler TC9610 /TC9610-230, Applied Bio systems, USA) thermal cycler and final reaction volume of 25 µl. All volume included taq prime premix (2X) Genet Bio PCR master mix (Taq DNA Polymerase 1 unit/10 µl, 80 mMKCl, MgCl2 4 mM, MTris-HCl 20m , sediment, enzyme stabilizer, , with loading dye, pH 9.0, 0.5 mM of dATP, dCTP, dGTP, dTTP), (10 pmoles/ µl) primers, 40 ng of DNA template and free DNase water. Gene was amplified using the following PCR condition: initial denaturation 94ºC for 10 min, then apply denaturation which was 35 cycles at 94ºC for 1 min; then annealing in 56ºC for 1 min and extension in 72ºC for 1 min with final extension in 72ºC for 10 min.

2.2.3 DNA Sequencing

The PCR product was checked by using 1.5% agarose gels and then purified by using Column Purification (Shanghai Sangon, China). The sequencing step has been done via using the Genetic Analyzer 3500. For discovering the nucleotide order of the 18S rDNA gene, Applied Bio Systems (USA) was used.

2.2.4 Phylogenetic analysis

Phylogenetic trees were constructed using neighbor joining trees and the Tamura parameter in MEGAX software [20]. Also, genetic distances were calculated with a pair-wise distance model. The bootstrap values with 1000 replications were used for the evaluation of the reliability of the tree.

3. RESULTS

The classification of *P. laevis* is shown in (Table 1).

Table 1. *Pomphorhynchus laevis* recorded in Sirwan River according to their classification status

Kingdom: Animalia
Phylum: Acanthocephala
Class: Palaeacanthocephla
Order: Echinorhynchida (Saouthwell and Macfie, 1925)
Family: Pomphorhynchidae (Yamagutii, 939)
Genus: Pomphorhynchus (Monticelii, 1905)
Species: Pomphorhynchus laevis (Müller, 1776)

3.1 General Description of *Pomphorhynchus laevis*

The morphological characteristics of *P. laevis* in *S. lepidus* and *C. luteus* fish species were the same, showing no differences between them (Fig. 2). The Proboscis of the acanthocephalan is cylindrical and has a very long neck and bulbous anterior; 9–11 hooks were detected (the longest hooks are the first four until five, but the fifth or sixth hooks are markedly shorter and sturdiest) (Fig. 3).

3.2 Molecular Study

Fig. 4 shows the 18S rDNA PCR result for *Pomphorhynchus laevis*, which were 528 base pairs long. We examined the 18S rDNA nucleotide sequences after they had been edited using the BioEdit program 7.2. It has been determined that the genetic distance between *Pomphorhynchus laevis* and other acanthocephalans recorded in GenBank. It was calculated that the genetic difference between *Pomphorhynchus laevis* and other acanthocephalans varied from 0.0000 to 0.017 Table 2.

Fig. 5 show the phylogenetic tree relationship of *Pomphorhynchus laevis* with other acanthocephalans species recorded in GenBank based on 18S rDNA.

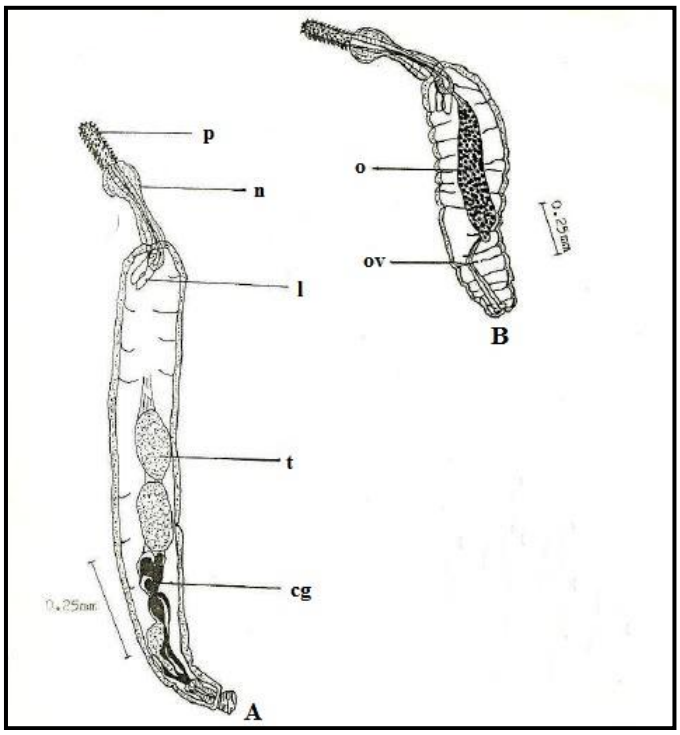

Fig. 2. ***Pomphorhynchus laevis*****: A- Male; B-Female [12]**
P: probpscis; n: neck; l: lemninous; t: tests; cg: cement gland; o: ovary; ov: oviduct

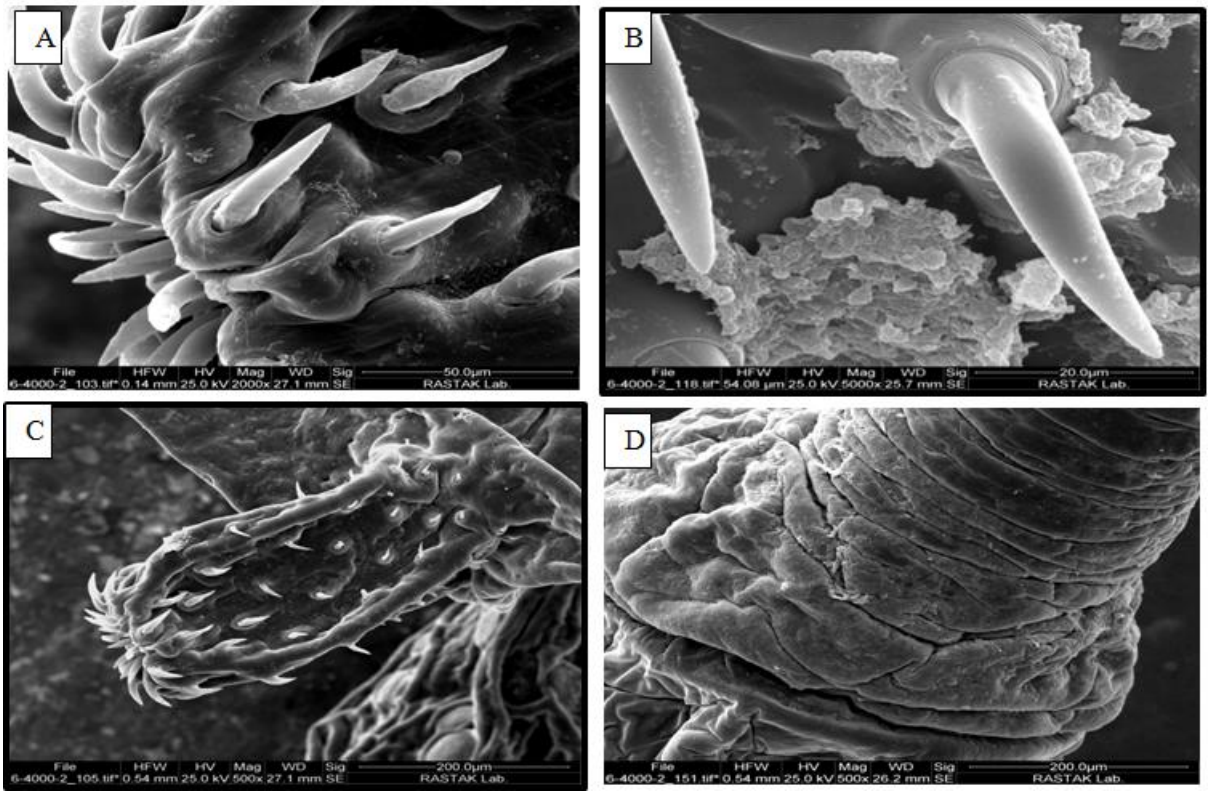

Fig. 3. SEM of specimens of *Pomphorhynchus laevis* from *S. lepidus* and *C. luteus. A: posterior hook; B: another hook; C:* Proboscis; D: Bulbless enlargement at the neck and anterior tip of the parasite

Table 2. Pairwise distance of *Pomphorhynchus laevis* with some acanthocephalans recorded in GenBank

P. laevis (sample)																		
B. balaenae MZ358109.1	0.101																	
E. gymnocyprii MT162047.1	0.017	0.093																
E. gymnocyprii MT162051.1	0.017	0.094	0.001															
L. pagrosomi LC195887.1	0.015	0.086	0.024	0.025														
L. pagrosomi KX641270.1	0.015	0.094	0.021	0.023	0.000													
P. zhoushanensis KY490050.1	0.015	0.107	0.034	0.035	0.008	0.000												
P. zhoushanensis KY490051.1	0.015	0.107	0.034	0.035	0.008	0.000	0.000											
R. lintoni JX014224.1	0.008	0.105	0.031	0.032	0.024	0.023	0.035	0.035										
A. anguillae LS991434.1	0.010	0.101	0.028	0.029	0.020	0.020	0.028	0.028	0.021									
R. pristis JX014226.1	0.004	0.104	0.030	0.031	0.025	0.023	0.034	0.034	0.008	0.020								
P. laevis AY218124.1 www	0.000	0.086	0.024	0.025	0.019	0.018	0.028	0.028	0.005	0.011	0.006							
P. laevis JX014223.1	0.002	0.098	0.025	0.026	0.019	0.017	0.029	0.029	0.007	0.014	0.006	0.002						
P. laevis AY423346.1	0.000	0.099	0.025	0.026	0.019	0.017	0.026	0.026	0.008	0.013	0.008	0.001	0.001					
P. tereticollis AY423347.1	0.000	0.100	0.025	0.026	0.019	0.018	0.027	0.027	0.007	0.015	0.006	0.000	0.003	0.002				
P. kashmirensis MZ381411.1	0.000	0.101	0.021	0.023	0.014	0.014	0.014	0.014	0.008	0.013	0.005	0.000	0.003	0.002	0.002			
P. kashmirensis MZ381412.1	0.000	0.101	0.021	0.023	0.014	0.014	0.014	0.014	0.008	0.013	0.005	0.000	0.003	0.002	0.002	0.000		
P. kashmirensis MZ381413.1	0.000	0.101	0.021	0.023	0.014	0.014	0.014	0.014	0.008	0.013	0.005	0.000	0.003	0.002	0.002	0.000	0.000	
P. laevis KF559309.1	0.000	0.098	0.025	0.026	0.019	0.017	0.025	0.025	0.008	0.013	0.007	0.001	0.001	0.000	0.002	0.002	0.002	0.002

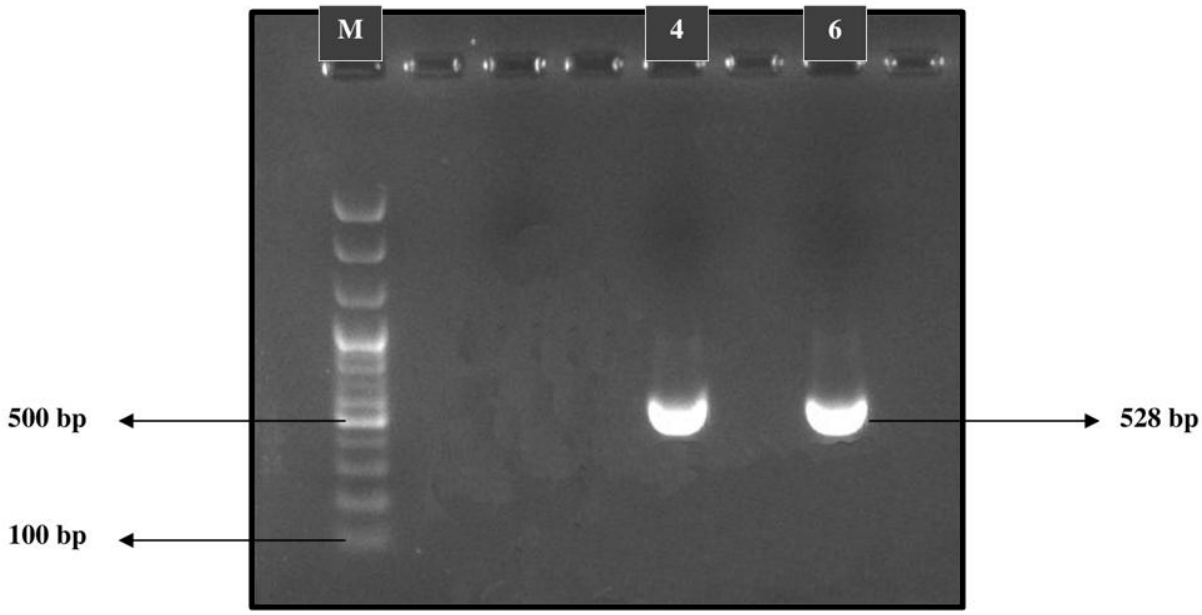

Fig. 4. PCR amplification of *Pomphorhynchus laevis*. Lane M= DNA ladder 100 bp, lane 4: *S. lepidus* and 6: *C. luteus* amplified nuclear 18S rDNA gene product size 528 bp.

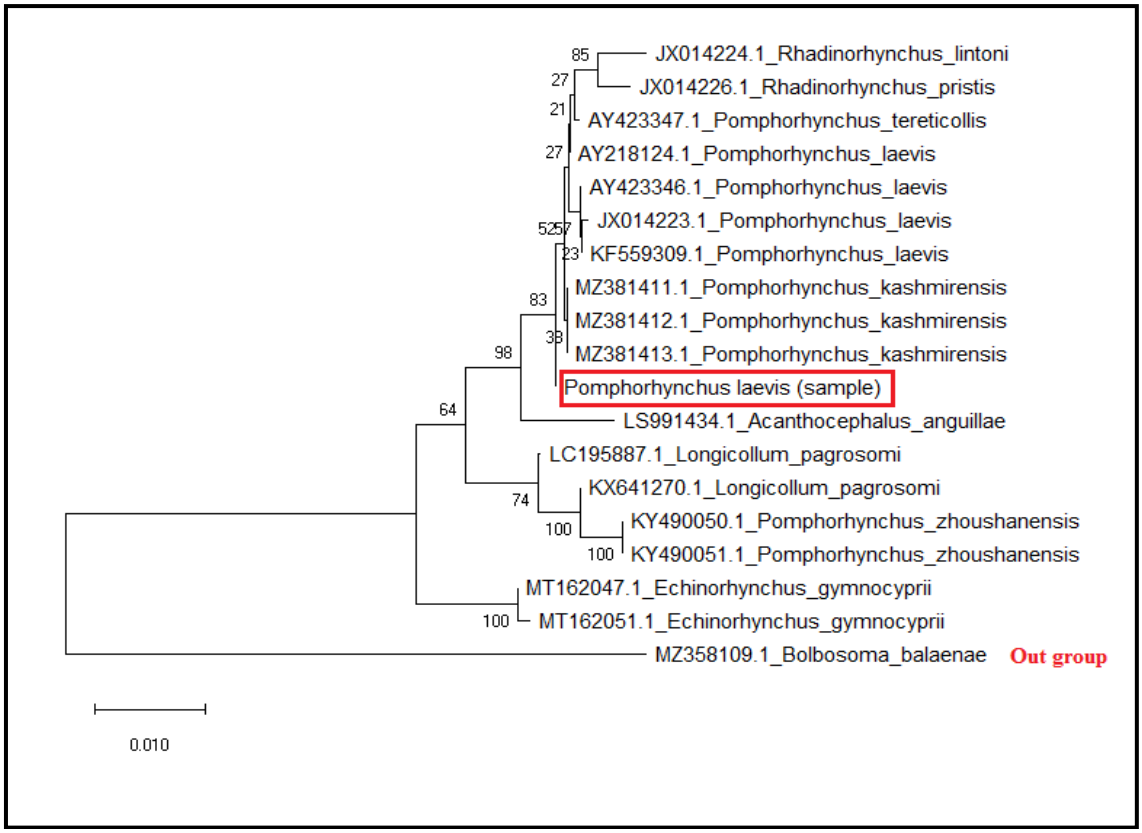

Fig. 5. Phylogenetic tree of specimens of the *Pomphorhynchus laevis* obtained for this study (sample) and other members of the families Acanthocephala were received from GenBank based on the partial 18S rDNA gene. The tree was created based on the neighbor joining tree and the Tamura parameter in MEGAX. *Bolbosoma balaenae* sequence was used as the out group

4. DISCUSSION

Presently, the genus Pomphorhynchus has 29 species of common parasitic worms that live in freshwater fishes' intestinal tracts, as well as occasionally in those of marine fishes and amphibians [2]. Only a few species from this genus have so far undergone molecular characterization studies [18,6,17,21–22]. In Europe, *P. laevis* is a typical fish parasite. Ecologists use acanthocephalans as a marker of environmental quality and the accumulation of heavy metal contamination [23,24]. The *P. laevis* identified in our study has the same morphological characteristics as the *P. laevis* described by [12], which was found for the first time in Iraq in *Barbus barbulus*. There are two novel hosts for this parasite in this study: *Squalius lepidus* and *Carasobarbus luteus. P. spindletruncatus*, a new species of *Barbus xanthopterus* and *Aspius vorax*, was discovered for the first time in Iraq by [1] in the Lesser Zab and Greater Zab rivers. Iran's Gamasiab River is home to the *P. laevis* of *Squalius cephalus* [25]. *P. laevis* was discovered in *Oncorhynchus mykiss* from Işkl Spring, Ivril, Turkey [26]. *P. spindletruncatus* was found in *Barbus xanthopterus* from the Lesser Zab River [27]. In Iraq, two new hosts for *P. spindletruncatus* have been discovered by [28].

There were no differences in morphometric parameters between the specimens of *P. laevis* examined using a scanning electron microscope in this investigation with *P. laevis* recorded by [12]. In our study, *P. laevis* has the smallest genetic distance with *P. laevis* (AY218124.1, AY423346.1 and KF559309.1), *P. kashmirensis* (MZ381411.1, MZ381412.1 and MZ381413.1) and *P. tereticollis* (AY423347.1) is 0.000 and the highest genetic distance with *Echinorhynchus gymnocyprii* (MT162047.1 and MT162051.1) is 0.017. The alignment sequence showed 100% identity with *P. laevis* (KF559309.1 and AY423346.1), *P. kashmirensis* (MZ381411.1, MZ381412.1 and MZ381413.1) and *P. tereticollis* (AY423347.1) in the GenBank database. The Phylogenetic tree analysis of the 18S rDNA gene (Fig. 5) showed that the sequence *P. laevis* obtained in our study grouped with *P. kashmirensis* (MZ381411.1, MZ381412.1 and MZ381413.1) and *P. laevis* (AY218124.1, AY423346.1, KF559309.1 and JX014223.1) and *P. tereticollis* (AY423347.1). Our 18S rDNA phylogenetic tree is similar to a previously published by [29]. A high level of sequence divergence at ITS1, ITS2 and cytochrome c oxidase subunit 1 (11%, 8% and 20%, respectively) has been discovered between the 'wrinkled type' (W) and the'smooth type' (S) of cystacanth *P. laevis* [9]. *P. tereticollis* and *P. laevis genetic analysis* reveals that some isolates previously identified as *P. laevis* were actually *P. tereticollis*, indicating that two distinct species exist. According to the most recent morphological and genetic evidence, both *Pomphorhynchus* species can infect the same freshwater fish hosts, such as chub and barbel, in addition to numerous isopods (Gammaridae) [6].

5. CONCLUSION

According to our phylogenetic study, *P. laevis* shares a taxonomic position with *P. kashmirensis*, *P. laevis*, and *P. tereticollis*. This study demonstrated that the 18S rDNA gene had higher taxonomic validity. Combining morphological characteristics with genetic analysis of the 18S rDNA region, we confirmed that the original specimens of *P. laevis* found in Iraq were the same.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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