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# Molecular Study of *Neoechinorhynchus roseum* Parasitic in Some Freshwater Fish in the Southern of Iraq

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## ABSTRACT

This study aimed to conduct a molecular diagnosis of the *N. roseum* parasite isolated from *Planiliza abu*, *Aspius vorax*, and *Silurus triostegus*, analyze genetic variation using specific primers, document new parasite species from various fish hosts, and construct an evolutionary tree. This study involved the examination of 86 intestines from 30 *Planiliza abu*, 17 *Aspius vorax*, and 39 *Silurus triostegus*, collected from Thi-Qar Governorate between June 1 and December 30, 2022. The 5.8S ribosomal DNA primer was utilized. This study documented the species *Neoechinorhynchus roseum* in Iraq for the first time. The International GenBank documented it under the serial number OQ194064.

## INTRODUCTION

Most research indicated that parasitic infections are one of the major pathogenic factors affecting fish, often leading to fish deaths and subsequently severe economic damage **(Song *et al.*, 2018)**. *Acanthocephala* spiny-headed worms include a group of worms that parasitize the digestive tract of fish. The current study aimed to conduct a molecular diagnosis of *N. rosum* parasite isolated from *planiliza abu*, *Aspius vorax*, *Silurus triostegus*, analyze the genetic variation using specialized primers, record new parasite species from different fish hosts in the gene bank, as well as drawing the evolutionary tree **(Pinacho-Pinacho *et al.*, 2014)**. *Neoechinorhynchus roseum* was isolated from the intestine of *Planiliza abu* and *Silurus triostegu*. It had two major bodies regions. One region, at the anterior end of the body, is a proboscis. The second region, posteriorly, is the trunk. The trunk is long and consisted of the main body and it is generally cylindrical in shape. Sexes are separated, male and female gonads, and they generally float within the body cavity. The proboscis is small compared to the rest of the body, it is spherical in shape, covered with three spiny rings, each one has six spines, attached to the trunk by a neck. These hooks have a characteristic shape in number and

arrangement for each species, and enable the worm to attach securely to the intestine of its host. The proboscis retracts into a proboscis sheath (also called a receptacle). The sheath is attached to the internal wall of the trunk **(AL-musaedi, 2020)**. The purpose of this study was to perform a molecular diagnosis of the *N. roseum* parasite extracted from *Planiliza abu*, *Aspius vorax*, and *Silurus triostegus*, evaluate genetic variat ion with specific primers, identify new parasite species from diverse fish hosts and develop an evolutionary tree.

## MATERIALS AND METHODS

### 1-Sample collection

A total of 30 *planiliza abu*, 39 *Silurus triostegus* and 17 *Aspius vorax* were collected from the local markets of Thi Qar Governorate (Nasiriyah - Euphrates River - Al-Shuyoukh Market - Karmat Bani Said) and Basrah Governorate (Shatt al-Arab) during the period from 1/6 to 30/12/2022. Some fishes were caught by a fisherman using a special net.

The fish were transferred to the Parasitology Laboratory \ College of Education for Pure Sciences \ University of Thi Qar, while the dead fish were transported with ice to be kept in the refrigerator until they were examined in the laboratory. The intestines were opened longitudinally, put in physiological solution and then examined with the naked eye. Moreover, a light microscope (Olympus) was used to search for parasites **(Ahmad *et al.*, 2014)**.

### 2-Macroscopic examination

The alimentary canal of the fish was examined internally using a anatomical microscope with a magnification power ranging from 6.4- 40. The parasites were isolated and placed in a petri dish containing a physiological solution for the purpose of washing. Some samples were kept frozen to perform Polymerase Chain Reaction (PCR) tests.

### 3- Molecular study

#### *3-1 Isolation of Neoechinorhynchus parasite*

DNA extraction from parasite samples was carried out in the Graduate Studies Laboratory \ Department of Pharmacy \ Mazaya University, using American company Geneaid kit according to the following steps:

1- Parasite samples were centrifuged at 16,000 cycles for one minute to get rid of distilled water and to obtain a precipitate containing the sample.

2- 200 Microliters of GST Buffer solution was added, and 20 microliters of enzyme protanase K were added. Then, it was well shaken by the Votrerx shaker. The samples were placed inside the water bath for three hours, with the sample being shaken every five minutes.

3- Elution buffer was placed in the water bath at 60°C

4- 200µl of GSB buffer solution was added, and then vigorously shaken for ten seconds using a shaker that was preheated to 60°C.

10- 10 Microliters of enzyme proteinase K was added and shaken with a vibrating device, and the samples were placed inside the water bath for fifteen minutes, with the sample being shaken every five minutes.

5- 200 Microliters of absolute ethanol solution was added to the decomposed sample and mixed directly with vigorous shaking.

6- The separation column GD was placed in the collection tube with a capacity of two ml, and the mixture was transferred to the separation column and placed in the cooled centrifuge at a speed of 16000 rpm.

7 - 400 microliters of the W1 buffer solution were added to the separation column and were put in the centrifuge for 16000 cycles for 30min. The collection tube containing the material flowing from it was discarded. The separation column was placed in a collection tube of two ml and 500 wash buffer was added after making sure that 100ml absolute ethanol was added into a tube.

The washing solution, with a capacity of 25ml, was added to the washing column and placed in the centrifuge at a speed of 16,000 cycles for 30 seconds. The flowing substance was discarded and the separation tube was placed in a collection tube of two ml, after which it was placed in the centrifuge, again at a speed of 16,000 and for a period of three to five minutes until it dried.

8- The separation column after drying was transfered to a tube with a capacity of 1.5ml and 100 microliters of pre-heated Elution buffer were added to the center of the column template and left vertically for at least three minutes to allow the solution to fully absorb and put in a centrifuge for 16000 for 30 seconds to separate the pure DNA.

***3-2 Parasite DNA amplification***

The polymerase chain reaction (PCR) technique was used to amplify the 5.8S ribosomal DNA

F-5 AAG CAT ATC ACT AAG CGG3

R-5 GCT ATC CTG AGG GAA ACT TCG3

**Table 1.** The primer used in the current study

| | | | |
|---|---|---|---|
| 5.8S ribosomal DNA | F | GTCGTAACAACAAGGTTTCCGTA-3 | 450bp |
| | R | 5-ATACGAATTTAAGTCGCCCA-3 | |

**Table 2.** Components of the PCR mixture

| PCR master mix | Size |
|---|---|
| DNA template | 5µL |
| forward primer | 1.5µL |
| reverse primer | 1. 5µL |
| PCR water | 12µL |
| Total | 20µL |

**Table 3.** Thermo cycler program for gene amplification of parasite

| The number of courses | Time Min | Temperature ℃ | Step |
|---|---|---|---|
| **1** | 9 m | 95 | Primary denaturation |
| **35** | 45 m | 94 | Denaturation |
| **1** | 30 m | 55 | Annealing |
| | 45 m | 72 | Extension |
| | 7 m | 72 | Final Extension |

The components of the reaction mixture were placed in white opaque tubes with a volume of 0.2mL of the PCR apparatus (Table 2). The samples were mixed using Vortex, then centrifuged using a centrifuge at a speed of 3000 revolutions/min for three minutes, and the components were mixed inside the tube. After that, they were placed in a thermo cycler and the device was programmed according to the settings shown in Table (3).

***3-3 Electrophoresis agarose gel***

The electrophoresis method was used to detect DNA samples according to the method of **Sambrok *et al*. (1989)**. 25ml of electrophoresis buffer were added in a 100 ml beaker, followed by the addition of 0.25 mg of acarose to make the final concentration 1%. Hot plate was used until the solution was completely dissolved, stirring the beaker from time to time then leaving it to cool for three minutes, then 0.2 of bromide dye was added. In order to cast the agarose gel, ethidium bromide was synthesized. To make wells in the agarose, a comb was attached to one end of the mold. To guarantee that the gel was distributed evenly, the casting tray was set up on a level surface. The agarose was then poured into the mold after being frozen and allowed to cool to room temperature before solidifying. After the agarose solidified, the mold was put into the electrophoresis chamber and the comb was gently taken out. Until the gel was fully submerged, electrophoresis buffer was added. Three microliters of ethidium bromide dye were combined with seven microliters of DNA. After that, the mixture was put into the agarose gel's wells. The positive and negative electrodes were linked to the electrophoresis chamber, and the voltage was set to 80 volts. After the completion of the electrophoresis process, the agarose template was transferred, and it was examined with ultraviolet rays to ensure the presence of DNA bundles, and photographed with a camera.

***3-4 Phylogenetic tree***

The evolutionary tree was drawn in the current study using the maximum likelihood method and the Tamura-Nei model (MAGA11 version). A table was made showing the nucleotide sequence differences for the local study compared to the global sequence, Figs. (1, 2) show the degrees of similarity and DNA multiple alignment for the current and global study samples.

## RESULTS

**Table 4.** Nucleotide sequence differences of the local isolate *N. roseum* and the global isolates

| | OQ194064_ Iraq | MG870701.1_ USA | FJ388981.1_ Mexico |
|---|---|---|---|
| OQ194064_Iraq | | | |
| MG870701.1_USA | 0.0148989399 | | |
| FJ388981.1_Mexico | 0.0148989399 | 0.0000000000 | |
| Acanthocephala_JF803289.1 | 0.5366685884 | 0.5351359696 | 0.5351359696 |

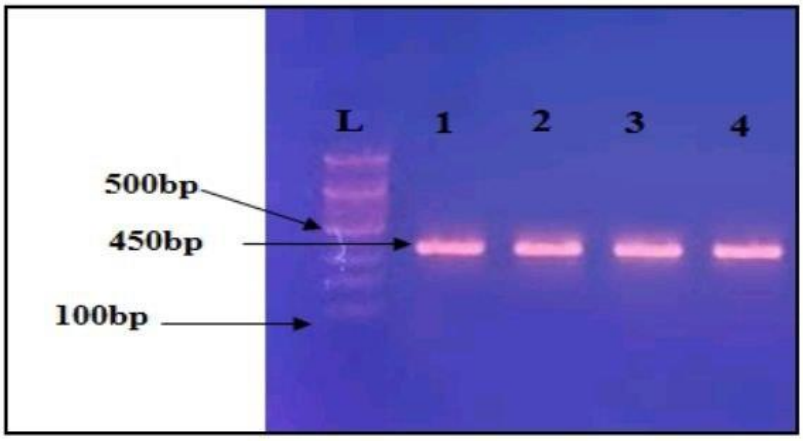



**Fig. 1.** Electrophoresis of DNA metabolites of *N. roseum* on agarose gel

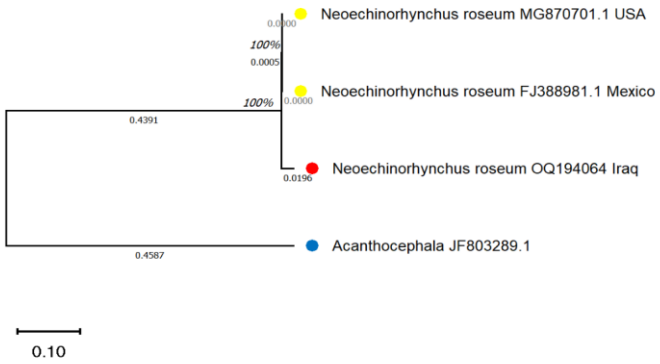
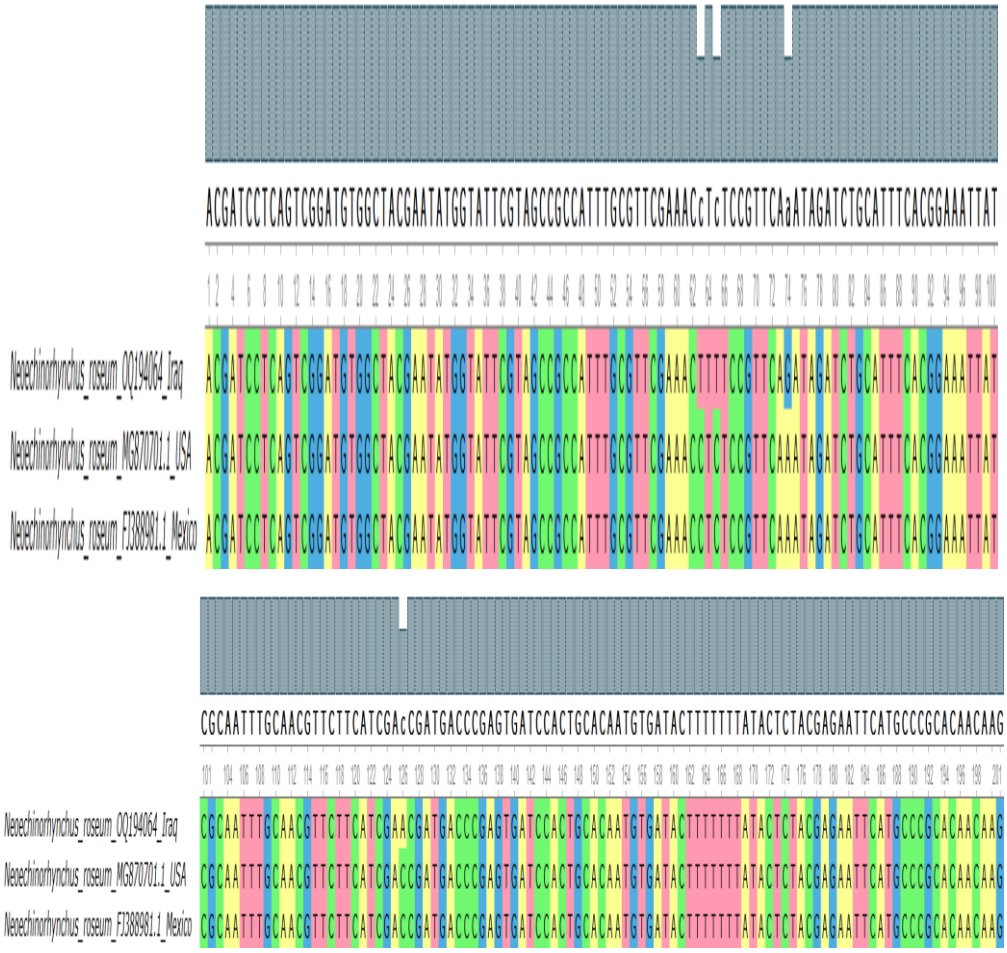



**Fig. 2.** The evolutionary tree of *N. roseum*




## DISCUSSION

*Neoechinorhychus roseum* is recorded for the first time in the provinces of Thi Qar and Basra from several hosts. In the present study, it was isolated from *Aspius vorax Silurus, triostegus* and *Planiliza Abu*, and *Leucisaus vorax*. **Mhaisen (2014)** indicated that there are eight species of *Neoechinorhynchus* in Iraq, including *N. agilis, N. rutili, N. critatus, N. macronucleatus, N. dimorphospinus, N. australis, N. iraqensis,* and *N. zabensis. Neoechinorhynchus* was classified for the first time as a new species in *Planiliza abu* fish in the Euphrates River in the Fallujah region, Anbar province, by **Amin *et al*. (2001)**. This species was referred to as *N. agilis* in a large number of Iraqi sources. The species *N. zabensis* was also classified in Iraqi waters for the first time in Iraq, from the intestines of *Varicorhinus trutt* fish from the Great and Small Zab rivers in northern Iraq **(Amin *et al.*, 2003)**. **Hashim (2014)** used the PCR technique to amplify DNA extracted from *Neoechinorhynchus* using special primers designed for the 5.8S rDNA gene. Two isolates of *Neoechinorhynchus* were recorded, *N. iraqensis* from *Planiliza abu* and *Silurus triostegus,* and N. *zabensis* from *Capoeda* fish. **Al-Masaidi**

**(2020)** used polymerase chain reaction PCR and gene sequencing technique of ribosomal and mitochondrial gene (28S and Cox1) and he got 412 bp and 446bp, respectively ,with all PCR product in Misan Province. On the other hand, **Ali (2022)** conducted a phylogenetic study (18sr DNA) and described Acanthocephalan *pomphorhynchus laevis* family pomphorhynchidae from cyprinid fish *Squalius lepidus* and *Carasorbarbus luteus* in Salaimani Province.

## CONCLUSION

The study concluded that *Planiliza abu* and *Silurus triostegus* serve as the intermediate hosts for this parasite. It was notable that molecular diagnosis is the most reliable approach for identifying new *Acanthocephala* species. It is worthnoting that *Neoechinorhynchus roseum* was documented in Iraq for the first time.

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