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Original Article

Molecular study of *Neoechinorhynchus iraqensis* parasite collected from some freshwater fish species in the southern region aquatic ecosystems of Iraq

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Abstract: The present study was conducted to examine the intestines of three freshwater fish species of *Planiliza abu, Aspius vorax,* and *Silurus triostegus*, collected from Thi-Qar Governorate, Iraq, for *Neoechinorhynchus iraqensis* parasitic infection. The large subunit 28S ribosomal DNA was used to identify this spiny-headed worm species. The results of the current study recorded ten isolates of *N. iraqensis* in Iraq. All the isolates were recorded in the International Gen Bank.

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Introduction

Acanthocephala (spiny-headed worms) parasitize the digestive tract of fish, causing either fish deaths or severe economic losses (Song et al., 2018). Therefore, their identification in the natural aquatic environments is essential. The large subunit 28S of ribosomal DNA (LSU) is a conserved fragment and among the most useful molecular markers for molecular identification and phylogenetic analyses in acanthocephalans (Martinez-Aquino et al., 2012; Rosas-Valdez et al., 2012; Pinacho-Pinacho et al., 2014). The current study aimed to molecularly diagnose *Neoechinorhynchus iraqensis,* a spiny-headed worm parasite, isolated from *Planiliza abu*, *Aspius vorax,* and *Silurus triostegus* from Iraqi inland waters, and to investigate its genetic variation using 28S rRNA in different fish hosts.

Materials and Methods

Samples collection: A total of 831 *P. abu*, 39 *S. triostegus*, and 17 *A. vorax* were collected from the local markets of Thi Qar Governorate, including Nasiriyah, Suq Ash-Shuyukh, Karmat Bani Said, and Chibayish (Euphrates River) and Basrah Governorate (Shatt Al-Arab) from 1st June to 30th December 2022. The collected fish were caught by local fishermen using special gill nets. The collected fish were transferred to the Parasitology Laboratory at the College of Education for Pure Sciences, University of Thi Qar, within iceboxes and stored in the refrigerator until examination. Fish identification was performed based on Eagderi et al. (2022) and Çiçek et al. (2022, 2023). In the laboratory, the intestines were opened longitudinally and then examined with the naked eye and a light stereomicroscope (Olympus) to search for parasites, as described by Ahmad et al. (2014).

Macroscopic examination: The alimentary canal of the studied fishes was examined internally using an anatomical stereomicroscope (Olympus) with a magnification ranging from 6.4 to 40. The detected parasites were removed and placed in a petri dish containing a physiological solution for washing. Some samples were preserved with a 70% ethanol solution for drawing the parasite with a Leica microscope. Some samples were kept by freezing for molecular examination.

Molecular study

DNA extraction: DNA extraction from parasite samples was performed in the Graduate Studies Laboratory, Department of Pharmacy, Mazaya University, using Geneaid extraction kit (USA) according to the following steps: (1) Parasite samples were centrifuged at 16,000 rpm for one minute to

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Table 1. The primer used in the current study.

28S ribosomal DNA	F	5ACCCGCTGAATTTAAGCAT-3
	R	5-CTCTTCAGAGATATTTCAAC-3

Table 2. The Components of the PCR mixture.

PCR master mix	**Volume**
DNA template	5 µL
forward primer	1.5 µL
reverse primer	1. 5 µL
PCR water	12 µL
Total	**20 µL**

Table 3. Thermocycler program for gene amplification used in the current study.

Step	Temperature (°C)	Time (Min)	The number of courses
Primary denaturation	95	9	1
Denaturation	94	45	35
Annealing	55	30	1
Extension	72	45	
Final extension	72	7	

remove distilled water, (2) 200 µL of GST buffer solution was added to the samples, followed by 20 µL of the protease K. The mixture was then well-shaken using the Votrerx shaker; the samples were placed inside the water bath for three hours, with the sample being shaken every five minutes, (3) The elution buffer was added to ir in the water bath at 60°C, (4) 200 µl of GSB buffer solution was added, then vigorously shaken for ten seconds using a shaker that was preheated to 60°C. 10 µl of protease K was added and shaken with a vibrating device. Then the samples were placed in a water bath for 15 minutes, with shaking every five minutes, (5) 200 µl of absolute ethanol was added to the decomposed sample and mixed 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 µl of the W1 buffer was added to the separation column and put in the centrifuge at 16000 rpm for 30 min. From the collection tube, the supernatant was discarded. The separation column is placed in a 2 mL collection tube, and 500 µL of wash buffer is added after 100 µL of absolute ethanol has been added to the tube. A 25-µl washing solution was added to the washing column and placed in the centrifuge at 16,000 rpm for 30 seconds. The flowing substance was discarded, and the separation tube was placed in a 2 ml collection tube. It was then centrifuged at 16,000 rpm for 3-5 minutes until it dried in the column template, and (8) the separation column after drying was transferred to a 1.5 ml tube, and 100 µl of pre-heated elution buffer was added to the center of the column template and left vertically for at least 3 minutes to allow the solution to fully absorb and put in a centrifuge for 16000 C for 30 seconds to separate the pure DNA.

DNA amplification: The polymerase chain reaction (PCR) was used to amplify the 28S ribosomal DNA (Mallaret et al., 2000) (Table 1). The components of the reaction mixture were placed in 0.2 mL tubes in the PCR (Table 2). The samples were mixed using a Vortex, then centrifuged at 3000 rpm for three minutes, and then mixed inside the tube. After that, they were placed in a thermocycler, and the device's program was set as listed in Table 3.

Agarose gel electrophoresis: The electrophoresis method used to detect DNA samples was as described by Sambrook et al. (1989). To 25 mL of electrophoresis buffer in a 100 mL beaker, 0.25 mg of agarose was added to achieve a final concentration of 1%. The hot plate was used until the solution was completely dissolved, stirring the beaker, and then left to cool for 3 minutes. After that 0.2 of bromide dye

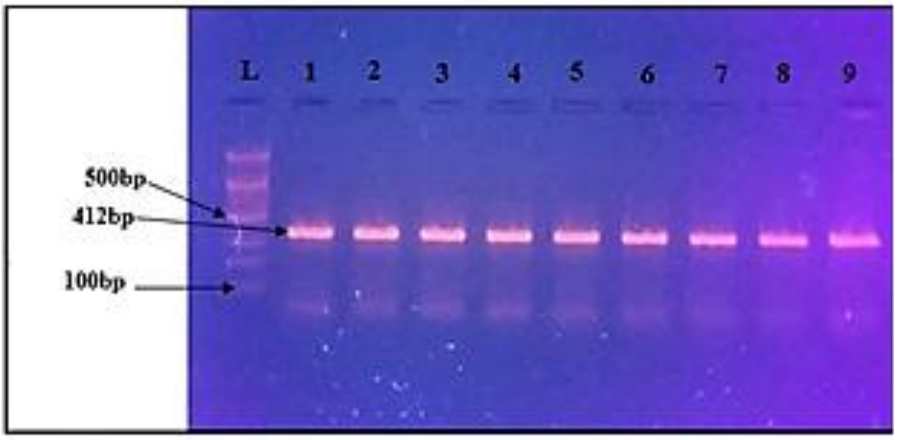

Figure 1. Electrophoresis of DNA metabolites of *Neoechinorhynchus iraqensis* on agarose gel (1%, L = ladder, while numbers 1, 2, 3, and 4 represent samples of *N. iraqensis* parasite isolated from *Planiliza abu*, 5, 6, and 7 represent samples isolated from *Silurus triostegus*, and 8, 9, and 10 represent samples isolated from *Aspius vorax*.

was added and the ethidium prepared the mold for casting the agarose, The comb fixed at one end to make holes inside the agarose, put the template on a flat surface to ensure that the agarose is distributed evenly, then, it was poured the thawed and cooled agarose into the mold and leave it to solidify at room temperature after the agarose hardened, the comb was lifted, the mold was returned to its proper place, and the electrophoresis solution was added until the agarose was covered entirely. Seven µl of DNA were added to three microliters of ethidium bromide dye and mixed well. The mixture was then added to the wells in the agarose. The positive and negative electrodes of the device were connected in the mold. Voltage was set to 80 volts. After completing the electrophoresis process, the agarose template was transferred and examined with UV to ensure the presence of DNA bundles, then photographed.

Phylogenetic tree: A tree was reconstructed using the maximum likelihood method based on the Tamura-Nei model using MAGA software (Version 11). The molecular distance of the local sequences compared to those of global sequences is also analyzed in the Mega software.

Results and Discussions

Neoechinorhynchus iraqensis in the present study was isolated from *P. abu*, *S. triostegus*, and *A. vorax.* PCR using the primer 28S ribosomal DNA revealed the bands of 412 bp in all DNA samples (Fig. 1). The sequence of ten local *N. iraqensis* isolates from 3 *P. bu*, 4 *S. triostegus* and 3 *A. vorax*, showed closeness with the sequence of the Iraqi isolate registered in the Gene Bank with serial number *N. iraqensis* MZ061666 from Amara, Iraq with a 100% match (Fig. 2). The distances between the local isolates in the current study and those of the gene bank isolates showed a degree of similarity of 0-0.452592 Iraqi *N. iraqensis* isolate registered in the Gen Bank.

Mhaisen (2014) indicated that there are eight species of *Neoechinorhynchus* in Iraq *N. agilis, N. rutili, N. critatus N. macronucleatus N. dimorphospinus, N. australis, N. iraqensis,* and *N. zabensis. Neoechinorhynchus* was described for the first time in *P. abu* from the Euphrates River in the Fallujah region, Anbar Province (Amin et al., 2001). This species was referred to as *N. agilis* in many Iraqi works. The species *N. zabensis* was also described in Iraqi waters for the first time from the intestines of *Capoeta trutta* in the Great and Little Zab rivers, northern Iraq (Amin et al., 2003a).

Hashim (2014) used the PCR technique to amplify DNA extracted from *Neoechinorhynchus* using the 5.8S rDNA gene. Two species of *Neoechinorhynchus* were recorded, including *N. iraqensis,* from *P. abu,* and *S. triostegus, N. zabensis* from *Capoeta trutta.* Al-Masaidi (2020) employed PCR and gene sequencing techniques on ribosomal and mitochondrial genes (28S and Cox1), yielding 412 bp and 446 bp, respectively, for all PCR products in Misan Province.

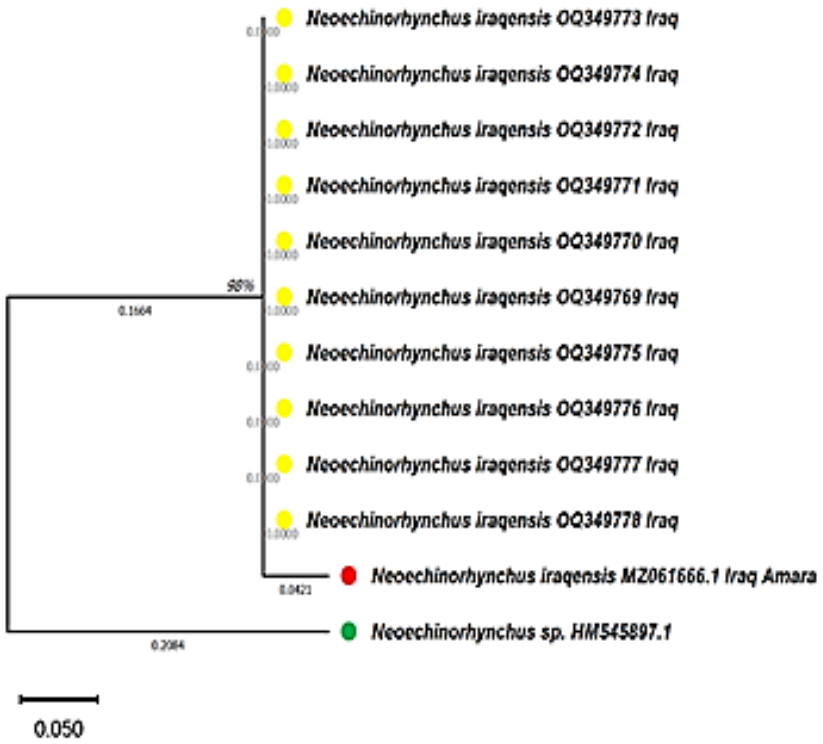

Figure 2. The ML tree of *Neoechinorhynchus iraqensis* in the current study based on the Tamura-Nei model. The yellow circles indicate the sequence of local isolates, and the red circle indicates the Iraqi sequence of *N. iraqensis* from the Gene Bank.

Ali (2022), using 18S rRNA, described Acanthocephala *Pomphorhynchus laevis* (Pomphorhynchidae) from the cyprinid fishes of *Squalius lepidus* and *Carasobarbus luteus* in Salaimani Province, Iraq.

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