

## Molecular study of *Diplostomum spathaceum* (Rud.1819) (Digenea) parasitic the fish eyes of *Carasobarbus luteus* and *Cyprinus carpio* Southern of Iraq

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### Abstract

**Background:** The larval stage (metacercariae) of *Diplostomum* spp. is regarded as a causative agent of the worm cataract diplostomiasis in many freshwater fishes of Iraq. The aim of the current paper was to study the molecular diagnosis of *Diplostomum* Parasite isolate from the eyes of the *Carasobarbus luteus* and *Cyprinus carpio* using ribosomal 28S rDNA gene amplified by PCR technique. **Methods:** A total of (236) *Carasobarbus luteus* and (92) *Cyprinus carpio* fishes were collected from Thi Qar and Basrah Governorates during the period from 1/6 to 30/12/2022.. The live fish were transferred in a cork containing a quantity of river water to the Laboratory College of Education for Pure Sciences at Thi Qar and Basrah Universities. PCR technique, specific gene and primers was used. **Results:** The polymerase chain reaction (PCR) technique, using the gene R 1500, showed the appearance of bands at the 1300 bp site in all DNA samples. The sequence of the two local isolates Lc739726.1 and Lc739727.1 of the *D. spathaceum* parasite isolated from the eyes of the *C. luteus* fish showed closeness with the sequence of the two Czech isolates KR269765.1 and the Ukrainian isolate MZ314171 with a 100% match. **Conclusion:** Two local of *Diplostomum spathaceum* isolates LC739726.1, LC739727.1 and one local isolate of *Diplostomum* SP.AA1 gene LC768865 found in the present study from *C. luteus* and *C. carpio* respectively.

**Keywords:** *Diplostomum spathaceum*, *Diplostomum* SP, PCR, *Carasobarbus luteus*, *Cyprinus carpio* Phylogenetic tree. R 1500 gene

### Introduction

Fish are very important source for human, which involve as basic of protein, fat, phosphate, iron, calcium, amino acids and vitamins which include soluble in fat and soluble in water (1). *Diplostomum Spathaceum* was first recorded in Iraqi waters by (2) who found it in the eyes of fresh water fish at Great Zab, Isk- Kalak, Iraq. Both wild and aquaculture fish suffer from a problem of eye *Diplostomum metacercaria* causing fish mortalities particularly in fingerlings. A definitive bird hosts normally get infected when consuming metacercaria in fish hosts (3). Identification of members of the genus *Diplostomum* is difficult because of morphological similarity with in and among species (4). Molecular methods have been proven as an alternative solution. There are no Iraqi studies on the molecular diagnosis of *Diplostomum* parasite infected fish eyes. but globally some studies have been conducted on the subject, (5) in Denmark determined the genetic differences of metacercariae species belonging to *Diplostomum pseudospathaceum* and *D. paracaudum*. The sequence of the barcode region of cytochrome C oxidase subunit1 (CO1) was used to distinguish *Diplostomoids*, in fish collected from the Saint Lawrence River - Canada. The genetic of *Diplostomum* species infecting Nile tilapia

in western Kenya. was studied using the genes ITS&18S rDNA (ITS1-5.8S-ITS2 (6).

**Materials and methods**

A total of (236) *C.luteus* and (92) *C.carpio* fishes were collected from Thi Qar Province (Euphrates River – Suq Ash-Shuyukh , Karmat Bani Said -Chabayish) and Basrah Province (Karmat Ali) during the period from 1/6 to 30/12/2022.. These fishes were caught by a fisherman using special nets. The live fish were transferred in a cork containing a quantity of river water to the Laboratory College of Education for Pure Sciences at Thi Qar and Basrah Universities while dead fish were transported with ice to be kept in refrigerator until they were examined .. All the molecular analyses concerned the current study were carried out in the Graduate Studies Laboratory \ Department of Pharmacy \ Mazaya University and Thi Qar University College of Pharmacy, Molecular Genetics Laboratory and Al-Qadisiyah University, College of Veterinary Medicine, Genetics Laboratory, DNA isolation, DNA extraction from parasite samples were done using American company Geneaid kit .

A fragment of nuclear ribosomal 28s rRNA gene was amplified by PCR using forward primer dig 12(F-5 AAG CAT ATC ACT AAG CGG3) and reverse primer 1500R (R-5 GCT ATC CTG AGG GAA ACT TCG3) (7). The components of the reaction mixture were placed in white opaque tubes with a volume of 0.2mL (Table 1). The samples were mixed using Vortex, then centrifuged using a centrifuge at a speed of 3000 rpm for three minutes, and the components were mixed inside the tube, placed in a thermocycler and set the program of the device (Table 2).

PCR master mix	Volum
DNA template	5µL
forward primer	1.5µL
reverse primer	1.5µL
PCR water	12µL
<b>Total</b>	<b>20µL</b>

Table 1: Components of the PCR mixture

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

Table (2) Thermocycler program for gene amplification of Diplostomum parasite

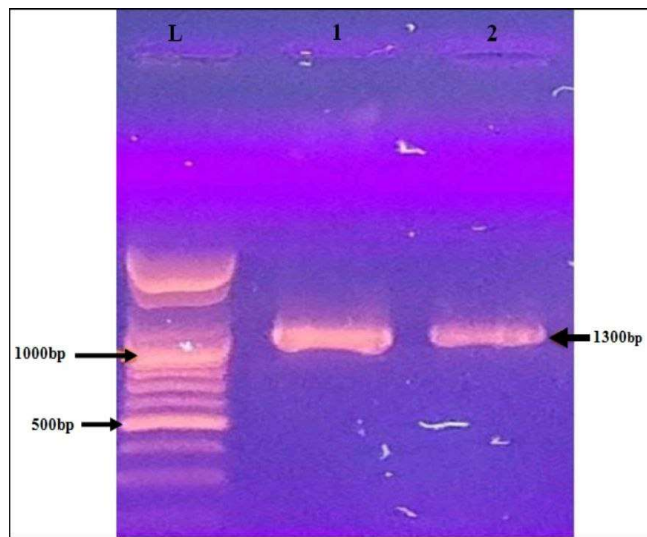
The electrophoresis method was used to detect DNA samples was according to the method of (8).

## Results

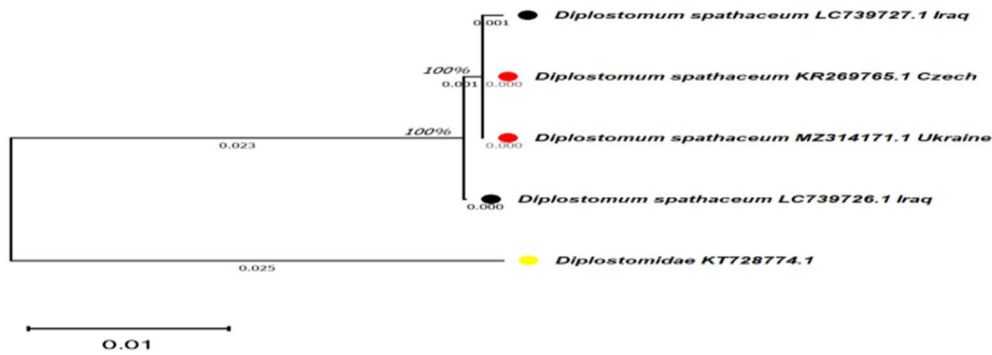
### Molecular Study

#### 1-Diplostomum.spathaceum from C.luteus:

The polymerase chain reaction (PCR) technique, using the gene R 1500, showed the appearance of bands at the 1300 bp site in all DNA samples that were extracted according to the instructions of the kit provided by the American company Jeneaid DNA Figure (1). The sequence of the two local isolates Lc739726.1 and Lc739727.1 of the *D. spathaceum* parasite isolated from the eyes of the *C.luteus* showed closeness with the sequence of the two Czech isolates KR269765.1 and the Ukrainian isolate MZ314171 with a 100% match. The evolutionary tree (Fig.2) of *D. spathaceum* parasite in the current study was drawn using the maximum likelihood method and the Tamura-Nei model (version MAGA11). The black circles indicate the sequence of the two local isolates, and the red circles indicate the sequence of the global (Ukrainian and Czech. The analyses were carried out using the combined maximum likelihood model. This analysis included 5 nucleotide sequences. Evolutionary analyzes were carried out using MEGAX 11.(Table 3).



**Figure 1:** Electrophoresis of DNA products of *D.spathaceum* parasite on agarose gel (1%), L = ladder..1,2 positive samples of the parasite



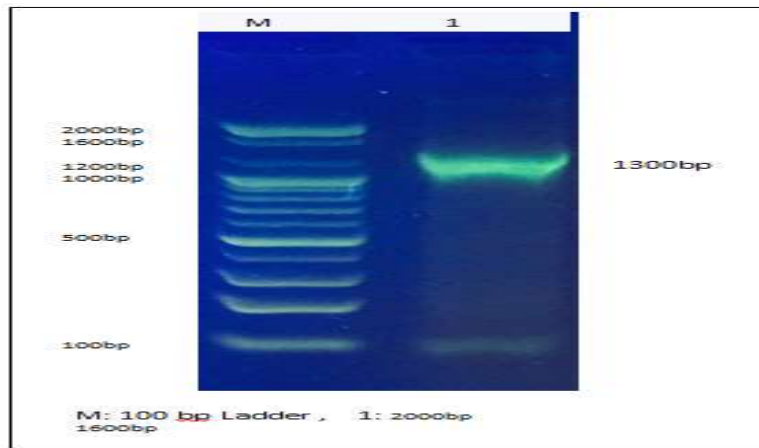
**Figure 2:** The evolutionary tree of *D. spathaceum* parasite in the current study

**Table 3:** The nucleotide sequence differences of the local and global isolates

	LC739726.1_Iraq	LC739727.1_Iraq	MZ314171.1_Ukraine	KR269765.1_Czech
LC739726.1_Iraq				
LC739727.1_Iraq	0.0010894909			
MZ314171.1_Ukraine	0.0005296189	0.0005442566		
KR269765.1_Czech	0.0005296189	0.0005442566	0.0000000000	
Diplostomidae_KT728774.1	0.0266368573	0.0280167813	0.0272296760	0.0272296760

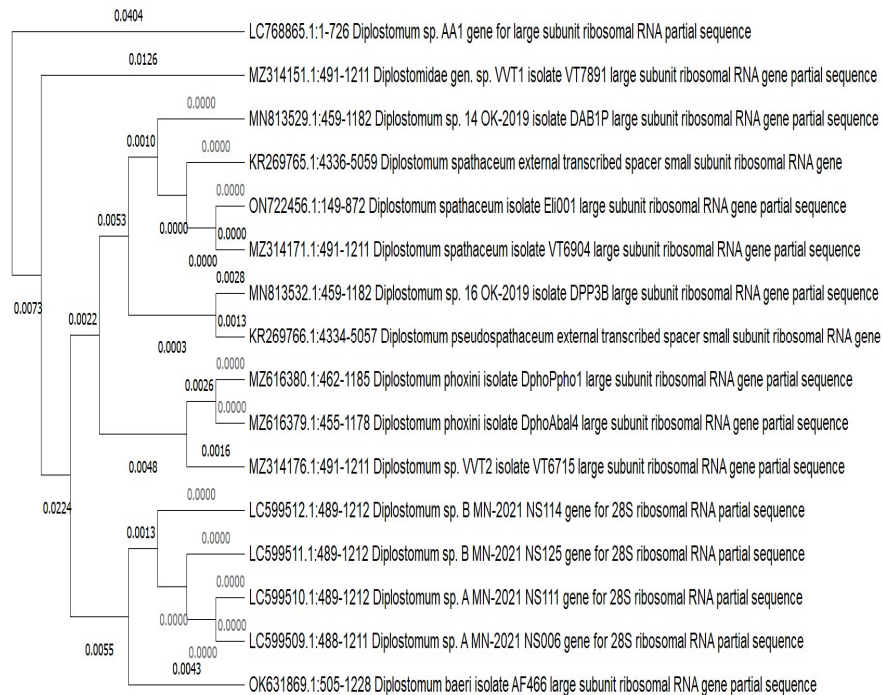
**Diplostomum sp.AA1gene from C.carpio**

Using the same R 1500 gene with *C.carpio* fish showed the appearance 1300bp band site in DNA samples, that were extracted according to the instructions kit of American Geneaid company USA . Figure (3)



**Figure 3:** Electrophoresis of DNA products of *Diplostomum* sp. AA1 gene parasite on agarose gel (1%), L = ladder..1, positive sample of the parasite from *Cyprinus carpio*

The evolutionary tree of *Diplostomum* SP. for *C. carpio* was shown in Figure (4).



**Figure 4:** The evolutionary tree of *Diplostomum* SP. AA1 gene for *C. carpio* was drawn using Neighbor joining method . The evolutionary distance were computed using maximum composition likelihood method. Evolutionary analyses were done using MEG11

## Discussion

In the current study, the polymerase chain reaction (PCR) technique with 1500R initiator, which is located within 28S rRNA region, showed the appearance of all molecular bands at the 1300pb site (according to the instructions of the Jeneaid company). The sequences of this gene is used to detect the quantities DNA due to high copy number and high reliability. During the present study the *D. spathaceum* species from the eyes of, *Carasobarbus luteus*, was registered in the Global Gene Bank with the serial number LC739726 for the first sample and the serial number LC739727 for the second sample. The above two local isolates were recorded in the Global Gene Bank for the first time in Iraq. They showed closeness with the sequence of Czech isolate KR269765.1 and the sequence of Ukraine MZ314171. with a 100% match. The current study also recorded *Diplostomum* sp. AA1 gene from the eyes of the carp fish *Cyprinus carpio*, which was registered in the International Gene Bank with serial number LC76886. There are no real studies in Iraq on the use of molecular techniques in diagnosing the *Diplostomum* parasite. The previous studies relied on describing the phenotype and morphology of this parasite. *D. spathaceum* and *Diplostomum* sp. From the eyes of *C. luteus* fish and carp referred by (9) in Basrah using the Randomly Amplified Polymorphic DNA (RAPD), which used to characterize some similar species. (10) used mitochondrial and nuclear ribosomal RNA (rRNA) and recorded the two species *D. spathaceum* and *Diplostomum* sp. In Ukraine, in which the rRNA molecular unit and the COX1 genes were used, recorded six similar species and from different hosts, namely birds and turtles. The above two local isolates were recorded in the Global Gene Bank for the first time in Iraq. (12) in Turkey using the COI gene and examining 150 eyes of *Copoeeta umble* fish, where *D. spathaceum* species were recorded. This species was compared with the analyzes of the global gene bank, and the match rate was found to be 100%. (13) Russia shed light on a wide range of diverse strains of fish-eye flukes and referred to the data recorded in the gene bank of three species of flukes, namely *D. spathaceum*, *D. indistinctum*, and *D. paracaudum*. The emergence of these species may be attributed to the differences between the same species. There is some difficulty in diagnosing the genus *Diplostomum* in the metacercaria stage due to the similarity in shape and difference between the species depends on the host, age and density of metacercariae (14). (15) Found three species of digenic flukes, including *D. spathaceum*, from fish collected from the Karmat Ali and the Salihiya rivers Basrah province. The study showed that the infection with the parasite is higher in the Karmat Ali river than the Salihiya due to the presence of snails on which the parasite depends as an intermediate host. A phenotypic study conducted by (16) in which *Diplostomum* from the Great Zab river (northern Iraq) was recorded. *Diplostomum* SP. of all kinds of carp fish collected from fish farms local markets in the city of Shamiya - Al-Qadisiyah was recorded (17). *Diplostomum* sp. was recorded from common and Beninese bigmouth carp in Mosul province, thus the parasite was recorded in the northern and southern regions of Iraq the same host. (18).

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