Tikrit Journal of Pur Science Vol. 28 (3) 2023

https://doi.org/10.25130/tjps.v28i3.1421

TJPS

IASJ

المجلات الاكاديمية العراقية

Tikrit Journal of Pure Science

ISSN: 1813 – 1662 (Print) --- E-ISSN: 2415 – 1726 (Online)

Journal Homepage: http://tjps.tu.edu.iq/index.php/j

Ecology, Morphology and Molecular Confirmation of *Chaetogaster limnaei* (Annelida: Naididae) retrieved from freshwater snail *Physa acuta* from Greater Zab River, Iraq.

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ARTICLE INFO.

Article history:

-Received: 15 / 9 / 2022

-Received in revised form: 17 / 10 / 2022

-Accepted: 22 / 11 / 2022

-Final Proofreading: 11 / 6 / 2023

-Available online: 25 / 6 / 2023

Keywords: *Chaetogaster limnaei*, *Physa acuta*, Greater Zab, Iraq.

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ABSTRACT

The present study is concerned with the *Chaetogaster limnaei* retrieved from the freshwater snail *Physa acuta* collected from (March-October 2021) from various locations near Chama-dubz village on the Greater Zab River, Erbil province, Iraq. Some Physicochemical variables, like water temperature, pH, Dissolved Oxygen, and Calcium ion experiment on a freshwater snail. The result showed statistically significant differences among Monthly variations with variables (number of snails, water temperature, pH and DO, and Calcium ion). In addition, isolated the *Chaetogaster limnaei* from *Physa acuta* with a prevalence was 5.75% (33/574), which has no dorsal and ventral chaeta approximately 14 to 20 per bundle, lack in segments 3 to 5, and chaetae and a vestige of a prostomium with sharply bent teeth. The identified morphologically and molecularly confirmed by using a COI sequence marker confirmed a 100% match to species with accession number (KF952336.1). As a result, the current study is the first report of *Chaetogaster limnaei* in Iraq, supported by phylogenetic analysis using Maximum Likelihood and Maximum Parsimony Methods.

البيئة، المظهر الخارجي والتأكيد الجزيئي لـChaetogaster limnaei (Annelida: Naididae) المسترجعة من حلزون المياه العذبة *Physa acuta* من نهر الزاب الكبير ، العراق

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الملخص

تتناول الدراسة الحالية *Chaetogaster limnaei* المستخرج من حلزون المياه العذبة *Physa acuta* الذي تم جمعه من (مارس – أكتوبر 2021) من مواقع مختلفة بالقرب من قرية Chama-dubz على نهر الزاب الكبير ، محافظة أربيل، العراق. بعض المتغيرات الفيزيائية والكيميائية، مثل درجة حرارة الماء، ودرجة الحموضة، والأكسجين المذاب،و أيون الكالسيوم تجرب على حلزون المياه العذبة. أظهرت النتائج وجود فروق ذات دلالة إحصائية بين المتغيرات الشهرية مع المتغيرات (عدد القواقع ، درجة حرارة الماء ، الأس الهيدروجيني و DO ، وأيون الكالسيوم). بالإضافة إلى ذلك ، تم عزل *Chaetogaster limnaei* من *Physa acuta* بنسبة انتشار بلغت 5.75 ٪ (574/33). تم تأكيد التحديد شكليًا وجزيئيًا باستخدام علامة تسلسل الجينيCOI. ونتيجة لذلك ، فإن الدراسة الحالية هي أول تقرير لـ *Chaetogaster limnaei* في العراق ، مدعومًا بتحليل النشوء والتطور باستخدام طرق الحد الأقصى من الاحتمالية Maximum Likelihood والحد الأقصى من Maximum Parsimony.

https://doi.org/10.25130/tjps.v28i3.1421

1. Introduction

Chaetogaster limnaei is an oligochaete considered ectosymbiont and cosmopolitan species belonging to the family Naididae, its close commensal relationship with more than 40 species of freshwater snails, mostly memberships of Physidae, Planorbidae and Lymnaeidae [1-3], infesting the mantle, pulmonary cavities, or foot mucus [4]. *C. limnaei* has been reported in association with a variety of host taxa across the world including Africa, Asia, Australia, Europe, North America, and South America [5].

External morphological characteristics including chaetae, proboscis, and gills serve to distinguish the genus *Chaetohaster*. The chaetae, which include the segment on which dorsal chaetae originate, the number of chaetae per bundle, the relative sizes of bifid chaetae teeth, as well as the existence or lack of hair chaetae, are regarded particularly essential features for the identification of genera and species of Naidinae [6, 7]. Furthermore, the difficult taxonomic based on morphological characteristics compromises, then using DNA barcodes like mitochondrial Cytochrome Oxidase I (COI) primer serves as an efficient barcode for oligochaetes, making it easier to use them in biomonitoring and advancing ecological diagnostics [8, 9].

In Iraq, various species of *Chaetogaster* were recorded, including *C. limnaei* by authors in different localities [7, 10-12]. Previously, not recorded in the Greater Zab river only one paper by Ali [13] reported another species *Chaetogaster langi*.

In this research, we studied the impacts of environmental variables on snails and their *C. limnaei*, then considered to be the first report of *C. limnaei* invasive *Physa acuta* in Greater Zab river, Iraq, which were supported by morphology and molecular (COI) primer, added to Iraqi Oligochaeta fauna.

2. Material and method

Study area

Species of living freshwater snail (*Physa acuta*) collected from various sites in Iraqi Greater Zab River, Erbil Province close to Chama-dubz Village, during (March-October/ 2021) (36°13'56.2"N 43°37'30.6"E), were transferred to the Advanced Invertebrate Lab, Education College, Salahaddin University, in a container filled with river water.

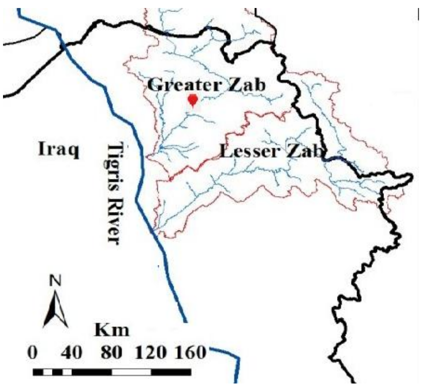

Fig. 1: Studied area on Greater Zab river (North Iraq) [14].

In the field, water temperature and pH conductivity were directly evaluated utilizing portable HANNA instruments, while dissolved oxygen and calcium ions content were measured in the laboratory, all variables performed depend on [15]. The species identified morphology by using the key for identification [16]. A traditional method was employed to isolate the naididae species from snails, which involved dissection of the snails (crushing infected snails) [3] and preserving in (90%) ethanol for morphology study and molecular identification.

DNA Extraction, COI Amplification, and Sequencing

The Oligochaetean DNA fixed in “Ethanol %90”, centrifuged at 8,000 RPM/3min., and the solution “ethanol” eliminated. A commercially available kit (GeneAll® Exgene™ Clinic SV, Korea) was used to extract the DNA following the manufacturer’s protocol. The DNA barcoding for oligocahaetian was achieved based on amplification and sequencing of the mitochondrial DNA Cytochrome Oxidase subunit I (COI) primer (680-720 bp long) Folmer [17] by using forward primer LCO1490 (GGTCAACAAATCATAAAGATATTGG) and reverse primer HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA). The PCR Coctail (40 µl) comprised of: 20 µl master mix, 4 µl “F & R primers”, 3 µl DNA template, and 13 µl (ddH2O). PCR program running under 94 C°/3 min; 35 cycles of 94 C°/60 sec; 50 C°/45 sec; 72 C°/45 sec, and 72 C°/7 min. in MJ Research, Applied Biosystem (AB) thermocycler. PCR results were separated in a 1.3% agarose gel and dyed with a UV-safe dye to detect DNA [14]. DNA sequencing was performed by utilizing a Macrogen Inc. ABI 3730XLs nucleotide sequencing analyzer (Korea). All the DNA sequences were edited and aligned with (ClustalW algorithm), available in MUSCLE program within EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/muscle/). All sequences were subjected to the Basic Local Alignment Search Tool for nucleotides “Blastn” implemented in the NCBI GenBank database. Further estimates of COI variation and relationships among species use the Maximum Parsimony (MP) analysis, Maximum Likelihood (ML) method , and Kimura 2-parameter model [18]. The index of consistency is (0.867188), the index of retention is (0.892405), and the index of composite is (0.773883) for all locals and parsimony-informative sites. In the bootstrap test (100 replicates), the proportion of duplicate trees in which the linked taxa clustered together is displayed next to the branches [19]. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm [20], and in the ML method, Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. All positions with less than 100% site coverage were eliminated. Evolutionary analyses were

Tikrit Journal of Pur Science Vol. 28 (3) 2023

TJPS

https://doi.org/10.25130/tjps.v28i3.1421

conducted in MEGA X [21] including a sequence representative from species currently available on GenBank.

Statistical analysis

The data collected were subjected to using the one-way (ANOVA) to analyze the impact of months and Physico-chemical parameters, and Tukey's multiple comparison test to compare environmental between them.

3. Results and Discussion

3.1. Results

3.1.1. Ecology description

Several Physico-chemical variables have been researched on *P. acuta* (Table 1). Monthly variation statistically showed significant differences ($p< 0.05$) with all parameters (water temperature, DO, and Ca^{+2}) (Fig. 3). A total of (574) freshwater snails *P. acuta* were collected, 33 of them were infected with *C. limnaei*, with a prevalence (5.75 %). The maximum number of snails examined recorded in July and minium in March (Fig. 4). While the highest snail infected during both months June and July and the lowest in March was zero (Fig. 4).

3.1.2. Morphology description

It has no dorsal and ventral chaeta, approximately 14 to 20 per bundle are lack in segments 3 to 5, and the existence of an inconspicuous prostomium. A chaetae and a vestige of a prostomium with sharply bent teeth are further characteristics of the species (Fig. 2).

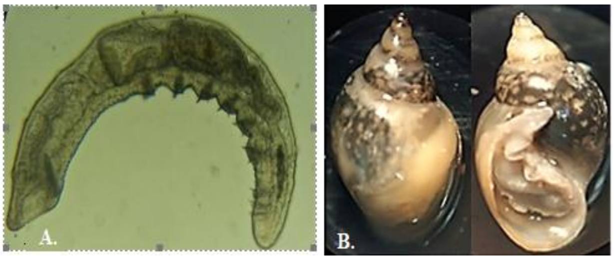

Fig. 2: A. *Chaetogaster limnei*, lateral view (200X); B. *Physa acuta*, dorsal and ventral view (20 X).

3.1.3.Molecular result

The GenBank Blasted DNA (COI) sequence of *C. limnaei* confirmed a 100% match to species with accession number (KF952336.1) (Fig. 6).

Table 1: Number of snails examined (574) versus parameters of Greater Zab River, representes as Mean ± SE, and P-value from March to Septmember2021

Parameters	Mean ± SE Min.-Max.	P-value	Significant
No. snail infected	4.12 ± 1.2 0-9	<0.0001	****
Water Temp. (°C)	25.99 ± 2.06 17-32	0.0219	*
pH	7.83 ± 0.05 7.6-8.1	0.9709	NS
DO (mg/L)	8.42 ± 0.71 6.1-11.1	0.0075	**
Calcium Con. (mg/L)	42.79 ± 1.21 39.75-49	0.080	**

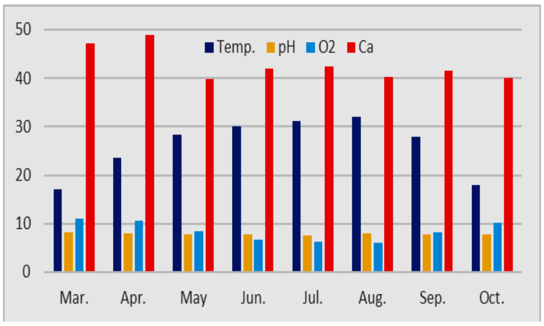

Fig. 3: Monthly variation vs. Water Temperature, pH, O^{2}, and Ca^{+2} of Greater Zab river in the studied site

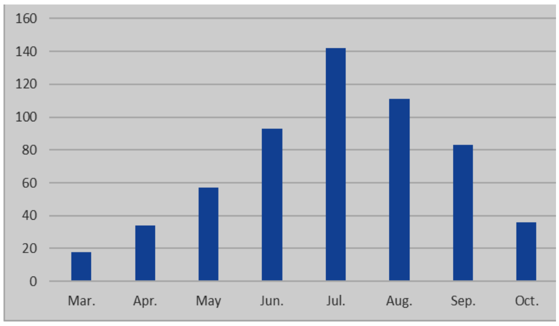

Fig. 4: Monthly variation vs. Number of snails examined

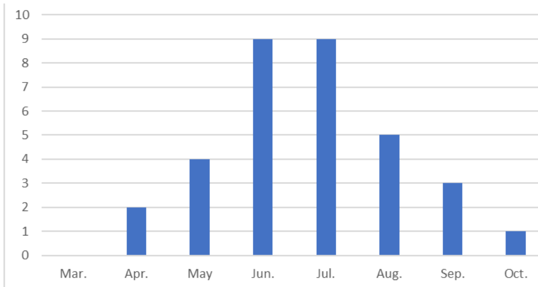

Fig. 5: Monthly variation vs. Number of snail infected

3.2. Discussion

The current investigation identified *Chaetogaster limnaei* isolated from *Physa acuta* in Greater Zab River (north Iraq). Monthly variations show significantly with snails *Physa acuta* (Table 1), the minimum and maximum numbers examined ranged (18-142) during March and July (Fig. 4), because this snail invades all freshwaters in the world and is present in a moderate amount of aquatic vegetation and organic debris [22, 23]. Another explanation is that it is hermaphrodite, which means it can self-fertilize and generate a high number of offspring throughout the year [24]. Also, the highest prevalence of infected snails with oligochaetes was (9.36 %) observed in July, and the lowest in March (0%). Statistically, shows significant differences ($p<0.05$) between *Physa acuta* with water temperature, DO, and Ca^{+2} and non-significant with pH (Table 1). In Figure (4), shows increasing the number of snails gradually, this result agrees with Sahin [25] claimed that the high adaptation capabilities of gastropods show a wide distribution of areas, common in organically-rich polluted waters can tolerate low levels of oxygen values. Furthermore, it is found in warm water and huge numbers throughout the summer, which is likely due to the appropriate temperatures for breeding and

https://doi.org/10.25130/tjps.v28i3.1421

reproduction during these months., these results are in line with [26-28]. High temperatures in the snail vectors cause thermal stress [29]. The pH value ranges (7.3-8.1) during the most studied period (Fig. 3), and monthly variation agree with Iraqi inland water is considered neutral to be alkaline, reflecting the geologic region [30]. The Dissolved oxygen content of water, which is critical for all living creatures [31]. It ranged from 6.5-12.3 mg / L (Fig. 3), and this outcome was comparable to Ali [32], and greater than that recorded by Shekha [33] at the same location. The lower DO level was detected during warm months, due to an increase in water temperature (32 C°) due to rasing in organic waste disposal, as well as an increase in decomposer bacteria activity [13]. The high DO level was recorded in March, this was probably linked to water temperature and the dilution effect of rainfall [34]. The calcium ion is one of the most significant components in the snail shell [35], the ranges (30.80-50.02 80 mg/l) were noted in the spring season, and this phenomenon came by the results [13, 36]. Dalesman and Lukowiak [37] claimed that snails exposed to lower level (20 mg/l) had significantly slower locomotion than snails exposed to a higher levels (80 mg/l). In addition, various environmental factors have a collective impact on organisms, therefore it is challenging to separate the effects of different factors [38]. Additionally, it has been demonstrated that aquatic vegetation affects snails' habitat as a result of elements like food availability, competition, and interactions between predators and prey [39].

The molecular technique proved effective in verifying the precision with which a species was identified depend on morphology. A fragment of 597 base pairs produced by COI gene amplification in the *C. limnaei* specimen. Analysis of BLASTn revealed that 100% identical with *C. limnaei* (KF952336.1) from New York, USA [1] (Fig. 6). In both trees our sequence of *C. limnaei* formed a monophyletic group (100% MP and 99% ML), together with *C. limnaei* from New York, USA, within a clade (66% MP and 63% MP) comprised of further *Chaetogaster* species, and other species *Chaetogaster diaphanous* **(**LN810268.1**)** by Vivien et. al, [9] in Switzerland and (JQ519897.1) by Envall et. al, [40] in from Europe, North America, and China were located in another clade, for which relationship supported with *Chaetogaster cf. diastrophus* (MK837023.1) by Klinth et. al, [41] in Iceland, the out-group is *Lumbriculus variegatus* (FJ639308.1) by Gustafsson et. al, [42].

In the current investigation, detected that *C. limnaei* and *P. acuta* were commensal, and an assessment of the freshwater snails revealed any destruction to the paleal tissues, suggesting that this is a theory that needs additional research. In contrast, assumed that an epizoic antibiosis relationship between organisms at high infestation levels proposed by the decreased growth levels of the infected snails. *C. limnaei* is an omnivorous commensal that consumes a wide range of prey depend on particle size, includes: rotifers, foraminiferans, ciliates, flagellates, diatoms, filamentous algae, and trematode cercariae [5].

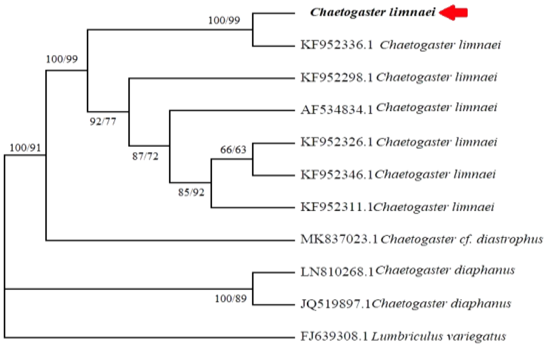

Fig. 6: The history of evolutionary by using the Maximum Parsimony analysis and Maximum Likelihood method and Kimura 2-parameter model "Numbers above nodes represent MP probabilities, and numbers below are ML bootstrap values".

Conclusion

It can be concluded, based on the outcomes of this investigation, considered to be the first report of *C. limnaei* invasive *Physa acuta* in Greater Zab river, Iraq, which were supported by morphology and molecular (COI) primer, added to Iraqi Oligochaeta fauna.

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