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Morphological and Molecular Identification of *Limnodrilus* Claparede, 1862 Species (Clitellata: Naididae) IN Tigris River, Baghdad/Iraq

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Abstract

Sludge worm samples were collected from the Tigris River sediment during the period from November 2018 to June 2019 in Al Sarafiya District/ Baghdad- Iraq. Biometric morphological measurements focusing on the form of penis sheath and chaetal morphology were used for species identification, in addition to molecular analysis by amplification of conserved 18S rRNA encoding gene using ITS1 and ITS4 universal primers. According to the morphological measurement records, the results revealed the existence of *Limnodrilus hoffmeisteri* Claparede 1862, *L. claparedeianus* Ratzel, 1868 and *L. cervix* Brinkhurst 1963. Other two groups of specimens, with short penis sheath, were identified by molecular technology as *L. hoffmeisteri* complex, and *L. claparedeianus-cervix*. Both species were considered as new record for Iraqi fauna, and had been registered in NCBI under the accession number LC497073 and LC497074 respectively

Key words: *Limnodrilus cervix*, *L. claparedeianus*, *L. claparedeianus -cervix*, LC 497073, *L. hoffmeisteri*, *Limnodrilus hoffmeisteri* complex, Iraq, LC497073, NCBI

Introduction:

The cosmopolitan genus *Limnodrilus* Claparede, 1862 (Clitellata: Oligochaeta: Naididae) live in the sediment/water interface and serve as live food for fish and aquatic organisms, and playing a key role in freshwater ecosystems. The worms enhance sediment resuspension which is an important process in oxygen and nutrient recycling (1). They are tolerant species to contamination and live in polluted environments (2), and use as indicators of organic pollution in aquatic environments and for assessment (3,4). They are widely distributed in Iraqi surface water and well documented by many authors (5, 6, 7, 8, 9).

Species of genus *Limnodrilus* are characterized by the absence of hair chaetae in the dorsal bundles. All chaetae with teeth of about equal length, and are similar on dorsal and ventral bundles, except for *L. udekemianus*, which is differentiated from other species by their chaetal morphology, in which, the anterior chaetae with the upper tooth thicker, and at least twice as long as lower, and bent almost at right angle. Posterior chaetae have equal teeth. Other species are identified according to the morphology of their male ducts, which is terminated inside the body

with a tube-like chitinous penis sheath with variable length. The distal end of the tube is modified and showing great morphological differences (10, 11, 12).

Phylogeny of *Limnodrilus* spp. was analyzed by (13) based on multi-locus database. The molecular analysis including the sequences of three mitochondrial genes (COI, 12S and 16S rDNA), and four nuclear markers (18S and 28S rRNA, Histone 3, and ITS). The authors suggested that *Limnodrilus* is a monophyletic genus of Tubificinae prone to cryptic speciation, and divided into three groups, one of them contains morphospecies characterized by short cuticular penis sheaths and enlarged chaetae in anterior segments (*L. udekemianus*, *L. silvani* and *L. grandisetosus*). The second group is a small group of species with moderately long penis sheaths, (*L. sulphurensis* and *L. profundicola*). The third and largest group have long penis sheath which are exceptionally long in *L. claparedeianus*, *L. maumeensis*, in addition to the form, which is morphologically intermediate between *L. claparedeianus* and *L. cervix* (12).

Liu *et al.*, (14) studied the species boundaries among *L. hoffmeisteri*, by investigating a large samples of specimens from the northern hemisphere using a combination of mitochondrial and nuclear markers, and they observed that the congruence among the independent estimates in the genealogical history provides evidence of ten actual species in *L. hoffmeisteri* species complex with great morphological variation in the length / width ratio and distal shape of the penis sheath, with a large overlap across species. They designated an individuals from the type locality in Switzerland as a neotype of *L. hoffmeisteri sensu stricto*, which are belongs to one of these ten species.

The aim of this study is to identify *Limnodrilus* spp. in Tigris River, Baghdad- Iraq, and to insure the identification of confused species by molecular analysis data.

Materials and Methods:

Tubificid worms were collected monthly, from the sediment of Tigris River at Al-Sarafiya District/ Baghdad-Iraq during the period from November 2018 to June2019. The sorted worms were fixed in 80% ethanol. The anterior third part of the worm was fixed on a clean microscopic slide; mount with lactophenol for examination of the penis sheath found in the segment XI. The length of penis sheath; its basal width; the length of the anterior chaetae; and the length of distal & the proximal tooth of the chaetae were measured under compound microscope using ocular micrometer. Photos for each identification criterion were captured by microscope digital camera MC500. The specimen was identified to species level according to (10, 11, 12)

To ensure species identification, molecular technique was used by amplification of conserved 18s rRNA encoding gene using ITS1 and ITS4 universal primers. Genomic DNA was isolated from the worm's sample according to the protocol of ABIO pure Extraction (ABIO pure TM Total DNA) and the isolated DNA was subjected to PCR (OWL Electrophoresis System Thermo, USA). The PCR products were separated by 1% agarose gel electrophoresis and visualized using the Gel imaging system. A forward primer (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer (5'-TCCTCCGCTTATTGATATGC-3') were used; primers set provided by Macrogen, Korea.

Reaction to PCR amplification was performed in a total volume of 25µl containing 3ng/µl DNA, 1µM of each primer, (1 X) Master Mix (Promega, USA), and then a Nuclease Free Water were added in to tube. The thermal cycling setup

were performed as follows: Initial Denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55°C for 30s and 72°C for 30s with final extension at 72°C for 7 min using a thermal Cycler (BioRad, USA).The PCR products were separated by 1% agarose gel electrophoresis and visualized using Gel imaging system and The Ethidium bromide stained bands in gel (Promega, USA).The 18S rRNA gene sequencing was performed by Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation–Korea. The results were received by email then analyzed using genius software.

A search for homology was carried out using the Basic Local Alignment Search Tool (BLAST) program accessible online at the National Center for Biotechnology Information (NCBI) at (<http://www.ncbi.nih.gov>) and the BioEdit program. Statistical analysis system (15) was used to analyze any significant differences between species by calculating the least significant differences values (LDS).

Results and Discussion:

A total of 3255 individual tubificid worms were collected, and 2734 individuals of them were mature and suitable for identification, which represented 84% of the total worms collected. *Limnodrilus* spp. represented 77% of the total identified worms, in addition to *Branchiura sowerbyi* (15%); *Potamothrix hammoniensis* (3%); *Tubifex tubifex* (3%) and *Aulodrilus pigueti* (2%). Three different groups of *Limnodrilus* were morphologically identified as *Limnodrilus hoffmeisteri*; *L. claparedeianus*, and *L cervix* with percentages of 53% and 28% and 4% respectively. Two groups of individuals with short penis sheath were identified by molecular technique as *L. hoffmeisteri* complex, with a percentage of 12% and *L. claparedeianus –cervix* with a percentage of 3% (Fig.1).

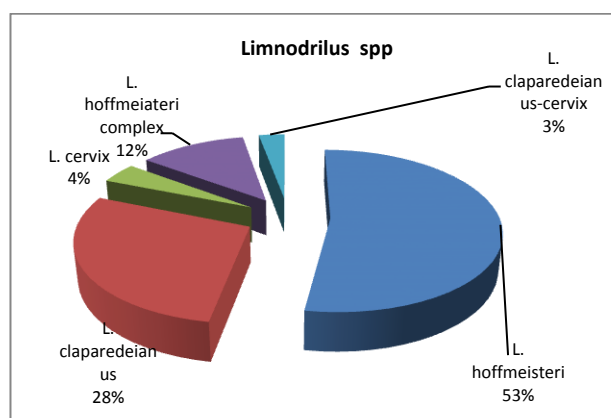


Figure 1. percentages of different *Limnodrilus* spp. from Tigris River /Baghdad-Iraq

Table 1 shows the biometric measurements of each species. *L. hoffmeisteri* had an average length of about 19 mm, with 66 segments; The penis sheath was 4.2-18.0 times as long as width (average 10.96 ±3.17) (Plate 1A and B) , and with two types of hood, one with symmetrical mushroom-like hood (plate 1E), or plate topped

hood (plate1D), and the other with typical asymmetrical hood (plate 1F). The latter form has longer penis sheath reach 570 µm. Some specimens which are not fully mature individuals have shorter, not fully developed penis sheaths. All forms have 5-9 chaetae in the anterior bundled, reduced to 2-5 in the posterior one, with about equal teeth (Plate 3 A and B), the ratio of distal tooth / ventral teeth is 38 /34 (Table 2).

Table 1. Biometric measurements of different *Limnodrilus* spp collected from Tigris River/ Baghdad-Iraq

species	body length (mm.)	No. of segments	penis sheath dimensions (µm)		
			length (L)	width(W)	L/W
<i>Limnodrilus hoffmeisteri</i>	14-45 26.58 ± 9.00 a	24-140 75.86±20.61 Ab	220-570 432.71±76.46 b	20-70 41.71±9.61 a	4.2-18 10.96±3.17 C
* <i>L. hoffmeisteri</i> complex	7.50-33.25 19.75±6.84 a	27-113 66.38±21.11 Ab	60-240 137.76±40.39 c	20-40 25.86±6.33 b	3.0-6.8 5.33±1.02 C
<i>L. claparedeianus</i>	14.00 - 45.6 30.03±10.18 a	50 - 120 76.62±18.41 Ab	600-1250 915.90±154.02 a	30-60 43.26± 7.51 a	15.0-29.0 21.38±3.31 B
<i>L. cervix</i>	21- 66 36.15±20.26 a	60-120 81.00±26.72 A	920-1030 950.00±57.16 a	30-34 31.00±2.00 b	30.0-30.6 30.60±0.71 A
* <i>L. claparedeianus-cervix</i>	11.- 20 17.46± 4.95 a	45-84 59.72 ± 13.42 A	100-330 215.71 ±75.47 c	20-40 28.57± 6.90 b	5.0-14.5 7.70 ±3.16 C
LDS values	23.73 NS	18.06 S	102.63 S	9.72 S	7.39 S

*- Identified by molecular techniques
means have different letters = with significant difference
NS= No significant differences; S= with significant differences

Table 2. Measurements of anterior chaetal length; chaetal number, and the ratio of distal tooth / proximal tooth of different *Limnodrilus* spp collected from Tigris River/ Baghdad-Iraq

species	number of chaetae in the anterior bundles	length of anterior chaetae (µm)		distal tooth/ proximal tooth
		Dorsal	ventral	
<i>Limnodrilus hoffmeisteri</i>	5-9 6.36±1.05 a	80-147 112.21±12.40 a	85-132.5 106.73± 11.32 ab	38/34
* <i>L. hoffmeisteri</i> Complex	4-8 6.04±0.91 a	80-110 100.09±10.23 a	73-107.5 94.43±11.19 b	32/29
<i>L. claparedeianus</i>	5-8 6.39±0.84 a	85-162.5 122.47±17.76 a	72.5-147.5 115.34±17.50 a	44-40
<i>L. cervix1</i>	6-7 6.50±0.58 a	105-130 117.50±10.41 a	100-125 110.00±11.37 ab	41/36
* <i>L. claparedeianus – cervix</i>	5-7 6±0.82 a	92.5-112.5 105.00± 6.77 a	80-107 98.57±5.40 ab	39/35
LDS values	1.263 NS	19.024 NS	17.662 S	

*- Identified by molecular techniques
means have different letters = with significant difference
NS= No significant differences; S= with significant differences

Small specimens with short penis sheaths of length ranged from 60-240 μm , and the base width 20-30 μm , with a ratio of L/W was 3.0-6.8 (Table 1). The distal end is concave with plate-topped hood, sometime indistinguishable (Plate 1C). They have less, but similar chaetae with about equal teeth in each bundle (Plate 3C). The number of chaetae in the anterior bundle ranged from 4-7, with average length of 80-147 μm , and the ratio of distal to proximal tooth length 29 -32 μm . This form was identified by molecular techniques as *Limnodrilus hoffmeisteri* complex, and was certain by sequence-based phylogenetic analysis using 18S ribosomal

RNA gene sequencing. The PCR product was subsequently sequenced to acquire DNA sequences and a 900 base pair product was acquired (Plate 4). This isolate was identified as *L. hoffmeisteri* complex, an 18S rRNA encoding genomic sequence was resented to the NCBI and recorded with the accession number LC497073.

The amplicon was alignment at NCBI with B LAST, the 18S rRNA isolated sequence worm showed 100% homology with the existing NCBI database sequence of *Limnodrilus hoffmeisteri* complex with accession number KY369413.1.

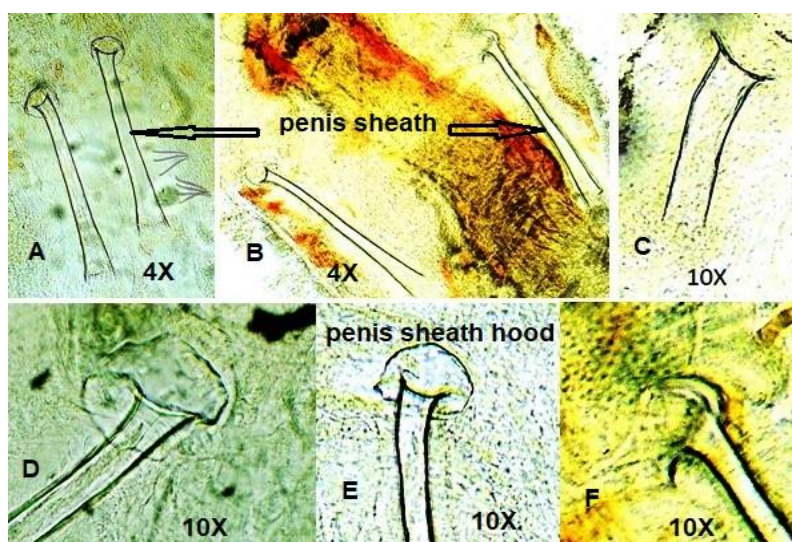


Plate 1. Types of penis sheath in *L. hoffmeisteri* complex, A&B- long penis sheaths; C- short penis sheath with indistinguishable hood; D- plate-topped hood; E- Mushroom – like hood; F- typical asymmetrical hood

L. hoffmeisteri is a species widely distributed in Iraq and recorded by many authors as the most abundant among the tubificid worms in the Iraqi water systems (5,8, 9,16 , 17). The forms with short penis sheath were misidentified as *L. profundicola* (9,18, 19) due to its short penis sheath end with rounded hood. van Haaren & Soors, (12) also indicated that less mature *L. hoffmeisteri* especially the form *L. hoffmeisteri* f. *spiralis* Eison 1879 can be confused with *L. profundicola*, but the authors referring to the widening of the alimentary canal and the presence of chloragogen tissue in *L. profundicola*, separate it from *L. hoffmeisteri*. They also indicated that length of penis sheath depends on the development stage of the individual, but the shape of the hood is constant in every stage, although, the hood may be missing in the immature specimens, and look like simple tube, as the shape which was noted in our specimens (Plate 1C).

L. claparedeianus have a penis sheath with 600-125 μm long (average 915 μm), and 30-50 μm width. The ratio of L/W ranged from 15-29 (Table 1), with a narrow pear-like hood (Plate 2, A & D),

and there are 5-8 chaetae in the anterior bundles, with slightly longer distal tooth (Table 2 and plate 3), while in *L. cervix* the penis sheath is longer, with length ranged from 920-1030 μm , and the width ranged from 30-34 μm , with the ratio (L/W) of 30.6. The wall of penis sheath in *L. cervix* with two layers, and the outer layer disappeared distally to leave a narrow neck. The distal head bears two triangle processes (Plate 2, B and C).

Few specimens with short penis sheath were identified by molecular techniques as *L. claparedeianus* - *cervix*, which has penis sheath with length ranged from 100-130 μm and the width at the base 20-40 μm . The distal end has a triangularly rounded hood (Plate 2E). The result suggests that this form represents an immature specimen of the species, while the mature individuals were mixed with the other two species and unable to recognize. This species was considered as a new record in Iraq, and has been registered in NCBI under the accession number LC497074.

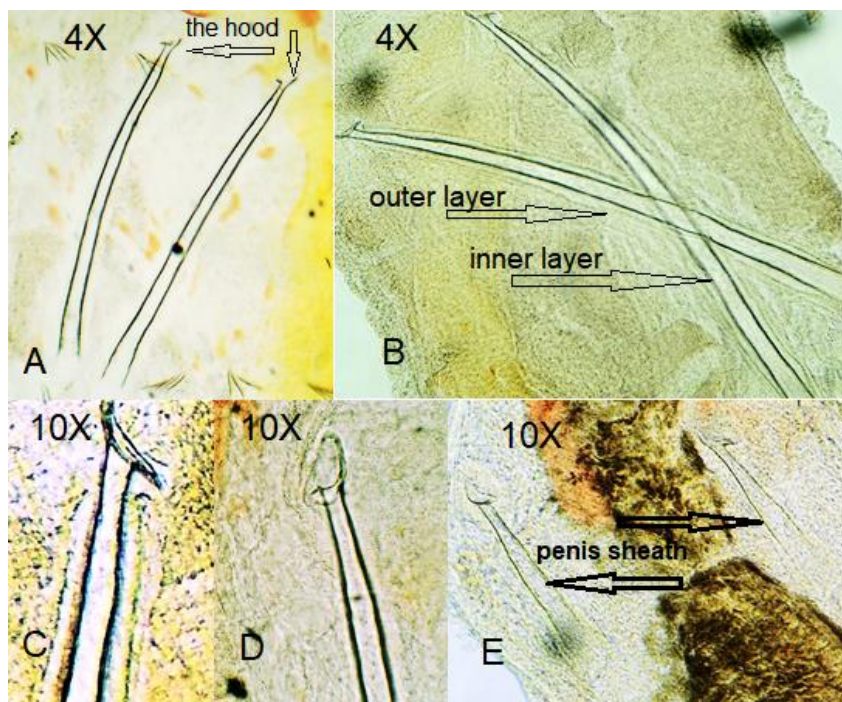


Plate 2. Types of penis sheaths in different *Limnodrilus* spp. A- *L. claparedeianus*; B- *L. cervix*; C- anterior end of penis sheath in *L. cervix* ; D- anterior end of penis sheath in *L. claparedeianus* C; E- *L. claparedeianus-cervix*

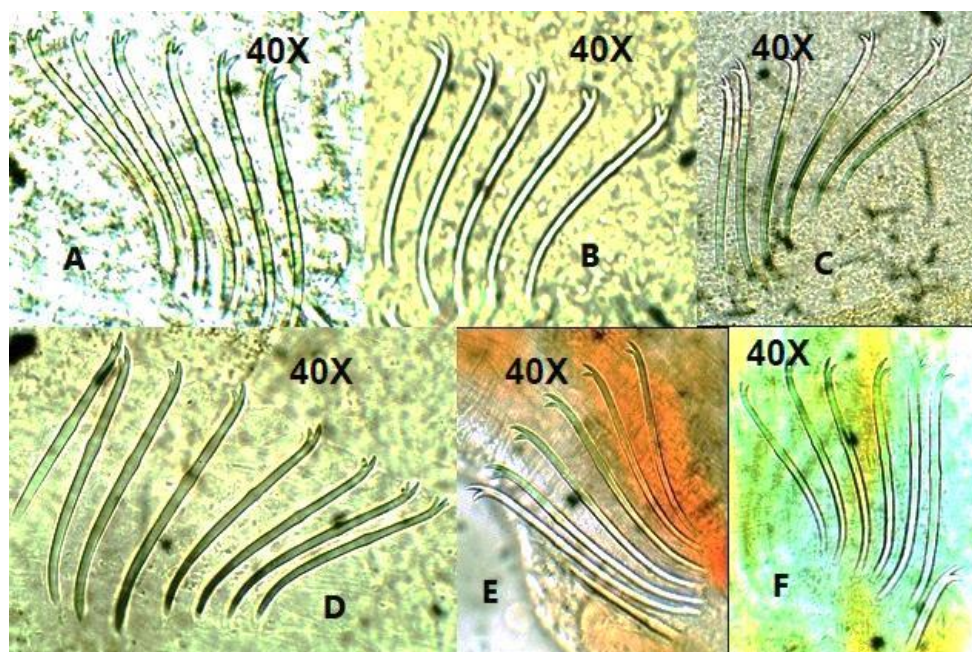


Plate 3. Types of anterior chaetae in different *Limnodrilus* species A, B & C, *L. hoffmeisteri*; D, *L. claparedianus*; E, *L. claparedeianus-cervix*; F, *L. cervix*

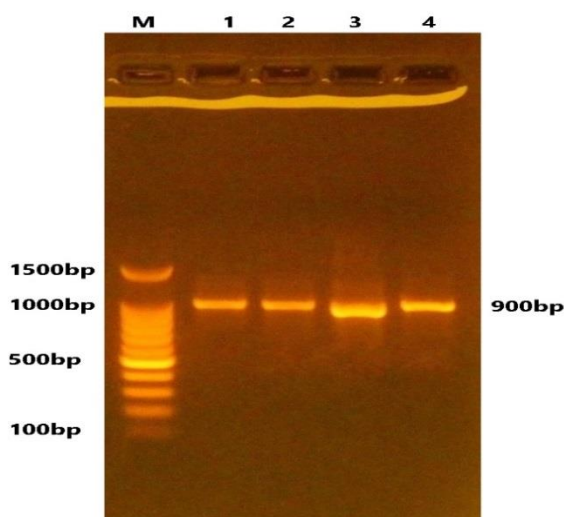


Plate 4. Amplified PCR product of band size 900 bp. Lane 1: the product was 1% agarose gel electrophoresis stained with Eth.Br. Lane1:100bp DNA marker at 1v/cm² for 1h, visualized using Gel imaging system

Conclusion:

Limnodrilus was the most frequent genus of tubificid worms in Tigris River, with *L. hoffmeisteri* as the dominant species, and its population consist of many forms, differ in their shape and size of penis sheathes. New form of *L. hoffmeisteri* with short penis sheath, and *L. claparedeianus-cervix* were recorded for the first time in Tigris River-Iraq. Therefore, Molecular analysis is important for the worm classification to promote the ambiguity of the classification of worm based on morphology features.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

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التشخيص المظهري والجزئي لأنواع الجنس *Limnodrilus* Claparede, 1862 (سرجيات – العائلة نايددي) في نهر دجلة / بغداد – العراق

هيفاء جواد جوير

ايناس عودة زائر

قسم علوم الحياة، كلية العلوم للنبات، جامعة بغداد، بغداد، العراق

الخلاصة:

جمعت عينات ديدان الوحل من رواسب نهر دجلة للفترة من تشرين ثاني 2018 ولغاية تموز 2019 في منطقة الصرافية / بغداد- العراق. اعتمدت في تشخيص الانواع القياسات الاحيائية الخاصة بغلاف العضو الذكري والاهلاب، اضافة الى التحليل الجزيئي باستخدام تضخيم الجين المشفر 18s rRNA , واعتمادا على البرايمر العالمي ITS1 و ITS4. اوضحت نتائج القياسات المظهرية تسجيل الانواع *Limnodrilus hoffmeisteri* Claparède 1862 و *L. claparedeianus* Ratzel, 1868 و *L. cervix* Brinkhurst, 1963، بينما شخضت مجموعتين تمتلك غلاف عضو تناسلي قصير بالتقنية الجزيئية على انها تابعة للنوع *Limnodrilus hoffmeisteri* complex والنوع *L. claparedeianus –cervix*. وعد النوعان تسجيلاً "جديداً" للفونا العراقية وسجلا في المركز الوطني للمعلومات الاحيائية NCBI تحت رقم الانضمام LC497073 و LC497074 على التوالي.

الكلمات المفتاحية: النوع *Limnodrilus cervi* و النوع *L. claparedeianus*، والنوع *Limnodrilus claparedeianus –cervix*، والنوع LC 497074 والنوع *Limnodrilus. Hoffmeisteri*، والنوع *Limnodrilus hoffmeisteri* complex, Iraq, LC497073، و المركز الوطني للمعلومات الاحيائية.