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Morphological & Molecular Identification of Some Oligochaete Species (Annelida – Clitellata) as a New Record for Iraq

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Abstract

Megadrili oligochaete samples were collected from different sites on the middle sector of Euphrates River sediment/Iraq. Sediment texture, pH and sediment organic matter percentage were also measured. The sorted worms were identified morphologically and by molecular analyses. For molecular identification, 18srRNA gene was amplified via PCR using the universal primer, and the gene sequences were obtained from pheretimoid species and compared for similarity using BLAST at NCBI site and deposited in Gen Bank with assigned accession numbers. The generated and alternate homologous sequences adapted from the Gen Bank database were uploaded in MEGA-X 10.1 version 39 for alignment. Morphologically the worms were identified according to specialized keys, described and the taxonomic criteria for each species were photographed. The result revealed that the sediment texture range between loam to clay loam with 3-13 % organic matters and pH 7.3-7.8. Three species of megadrile oligochaete were identified including, three individuals of species *Bimastos eiseni* Levinsen, 1884. The gene sequence of this species was uploaded to Gen Bank with accession number LC807036. The second identified species was *Haplotaxis gorioides* Hartman, 1821 five individuals were collected, its gene sequences was uploaded to the gene bank with accession number of LC807034. Finally, *Metaphire houlleti* Perrier, 1872. Two individuals were collected; its gene sequences were uploaded to the gene bank with accession number of LC807033 This is the first record of these species in Iraq.

Keywords: Oligochaete, Phylogenetic tree, Lumbricidae, Megascolecidae, Haplotaxidae,

1. Introduction

Aquatic oligochaetes have been identified as an important group of benthic invertebrates in freshwater (Atanacković et al., 2011), as well as in marine waters (Jeounghee & Jongwoo, 2021). They are known for their contributions to organic pollution and water quality assessment, as well as their roles in recycling organic matter in freshwater ecosystems and as a reliable food source for aquatic animals (Rodrigues et al., 2011, Timm et al. 2001).

The Oligochaeta group is a subclass of clitellated annelids that encompasses all annelids, regardless of whether they reside in freshwater, on land, or in the marine water. (Erséus *et al.*, 2020). Approximately 20,200 species inhabit aquatic habitats worldwide (Wagner *et al.*, 2021). There are two super orders of aquatic oligochaetes, the Megadrili, which comprises earthworms and other robust terrestrial worms. In contrast, the other is the Microdrili, which comprises aquatic worms that are frequently smaller and thinner and lack any near relatives with terrestrial worms (van Haaren & Soors, 2011). Three orders of worms are present "in freshwater, including the Naididae, the Lumbriculidae, and the Enchytraeidae, in addition to lesser families such as Pavidrilidae, Dorydrillidae, Phreodrilidae, andpropappidae. Lumbricine" oligochaetes, the well-known earthworms, and Haplotaxid oligochaetes, the groundwater residents' order, are among the oligochaetes that freshwater ecologists occasionally encounter (Timm, 2009).

Numerous authors have documented over 60 freshwater species in Iraq (Jaweir, 2014). Jaweir and Al-Sarai (2016) noted the presence of 11 Oligochaeta species in Al-Delmage Marsh, Iraq. (Zaar & Jaweir, 2020) employed molecular analysis techniques to analyse *Limnodrilus spp*. by amplifying the conserved 18s rRNA encoding gene with the universal forward primer ITS1 and reverse primer ITS4. The objective of the current investigation is to ensure the identification of specific species of oligochaetes worms that were collected from various freshwater sources in the middle Euphrates region of Iraq. This will be achieved through molecular analysis, as well as morphological descriptions and photographic illustrations.

2. Material and Methods

Samples of oligochaete were collected from three sites chosen on freshwater systems in the middle Euphrates region / Iraq as well as its branches and small streams. S1 is the main Euphrates river in Fallujah city. The area was characterized by being mixed residential and agricultural. S2 is the Al-Husseiniyah River in Karbala city, which branches off the Euphrates River. The surrounding area was agricultural, the third site is S3 Site, Al- Abbasiya River, in the holy city of Najaf, before the Al-Abbasiya dam. The area was characterized by being agricultural (Figur1).

Hand shovel was used to collect sediment about 50 cm from the riverbed and at a depth of 10 to 20. Sediment samples were sieved using a 0.25 mm meshed sieve. Then, they were spread on a white tray with a thin water layer. Oligochaete worms were hand-sorted using fine forceps. Collected species were left in a wide glass petri dish with water for about overnight to expel their intestinal material. Then, they were washed thoroughly with distilled water. After that, they were cut in two. The anterior parts were fixed and preserved in 85% alcohol for morphological identification. In contrast, the posterior parts were preserved frozen at -20 ^{0} C for molecular identification.

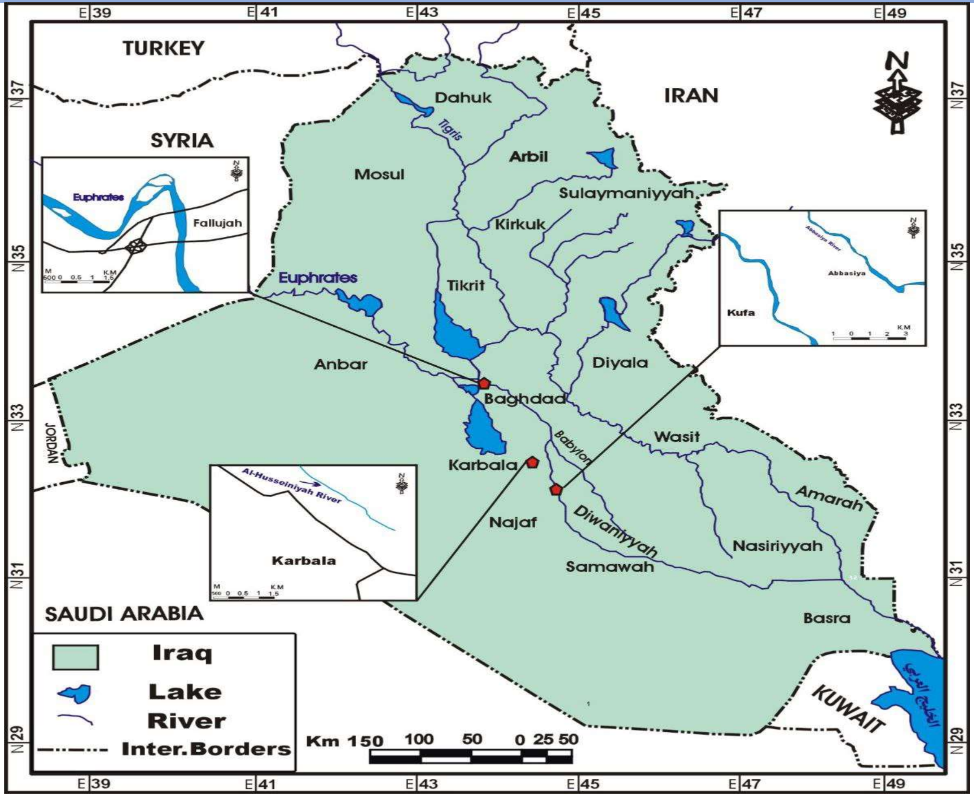
We placed the front half of the worm on sterile glass for morphological identification. Additionally, a small amount of lactophenol mounting solution (Brinkhurst, 1971) was placed under a cover slip with mild pressure and let rest for the night. The next day, it was inspected using a compound microscope at 4X, 10X, 40X, and maybe 100X power. To capture images of the exterior taxonomic criteria, a sony A7riv camera was attached to the microscope. Sources consulted for specimen identification include (Brinkhurst, 1971), (Timm, 2009), and (van Haaren & Soors, 2012).

For molecular identification, the genomic DNA was isolated from worm's sample according to progene amplication and sequencing. 18srRNA gene was amplified via PCR using the universal primers (Table 1), 18s gene sequences were obtained from pheretimoid species and compared for similarity using BLAST at NCBI site before being deposited in Gen Bank and assigned accession numbers. The generated and alternate homologous sequences adapted from the Gen Bank database were uploaded in MEGA-X 10.1 version 39 for alignment.

Table (1): primers designed for the current study

Primer	Sequence	Number	Anneal	Produc

Name			**ing temp. (0C)**	**t size**
Forward 18s gene	GGTCTGTGATGCCCTTAGATGT	22	60	**375**
Reward 18s gene	GATCCTTCCGCAGGTTCACCTAC	23	60	**375**



Coordinates Latitudes (North) Longitudes (East)	
33°.33'.84" N	43°.76'.09" E
32°.62'.19" N	44°.03'.56" E
32°.07'.81" N	44°.45'.07" E

Figure 1. Map of Iraq showing the oligochaete sampling location within Euphrates River in the middle of Iraq with Latitudes and Longitudes.

3. Result and Discussion

3.1.Sediment texture

It is noted from (Table 2) The texture of the sediment which was loam type in S1 with low percentage of organic matter. In S2 the soil texture was clay loam, also showed that the highest percentage of organic matter was recorded at site S3 with clay loam texture. This may be due to the agricultural area adjacent to the river, which specializes in rice cultivation; also, the value of neutral PH indicated in the table below.

Table (2) Bottom texture of the study sites, pH and organic matter are shown.

Site	PH	% Organic matter	Sediment texture
S1	7.8	2%	**Loam**
S2	7.6	8%	**Clay**

			loam
S3-	7.3	11%	**Clay loam**

3.2. *Bimastos eiseni* (Levinsen, 1884)

Three individuals belonging to this species were collected from Site S1 in Euphrates River in Fallujah city. The body color of the living individuals appeared light brown to reddish. It has one simple pointed dorsal and ventral chaetae in all bundles. The length of the worms ranged from 4.5 to 6.2cm. The number of segments ranged between 179 to 212. The length of chaetae ranged from 118.19 to 195.40 µm (Table 3), (Figure2).

Molecular analysis of a partial and complete sequence of 18s rRNA gene is indicated in the National Center for Biotechnology Information (NCBI). It was uploaded to Gen Bank with accession number LC807036. This species belongs to the genus *Bimastos,* which has twelve valid species that are intensified "in the Midwestern and eastern United States. Some species have become cosmopolitan throughout the world (Csuzdi et al., 2017). Species belonging to this genus are sometimes classified as subgenus status within *Allobophora* or *Helodrilus* (smith, 1917) or placed variously in the genera *Eisenia, Dendrobaena, Dendrodrilus Allolobophoridella* Mršić,1990,1991). However, it differs from *Eisenia* and *Dendrobaena* in having U-shaped nephridial bladders. At the same time, *Allolobophoridella* and *Dendodrilus* have been synonymised with *Bimastos"* (Csuzdi *et al.* 2017).

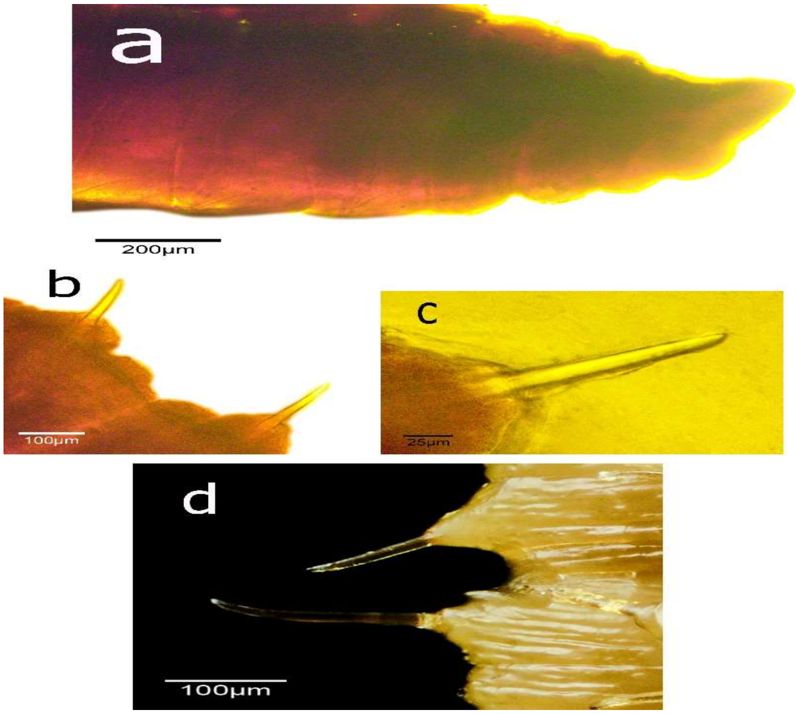


Figure2. ***Bimastos eiseni*** **a- anterior end b -dorsal chaetae c&d -ventral chaetae**

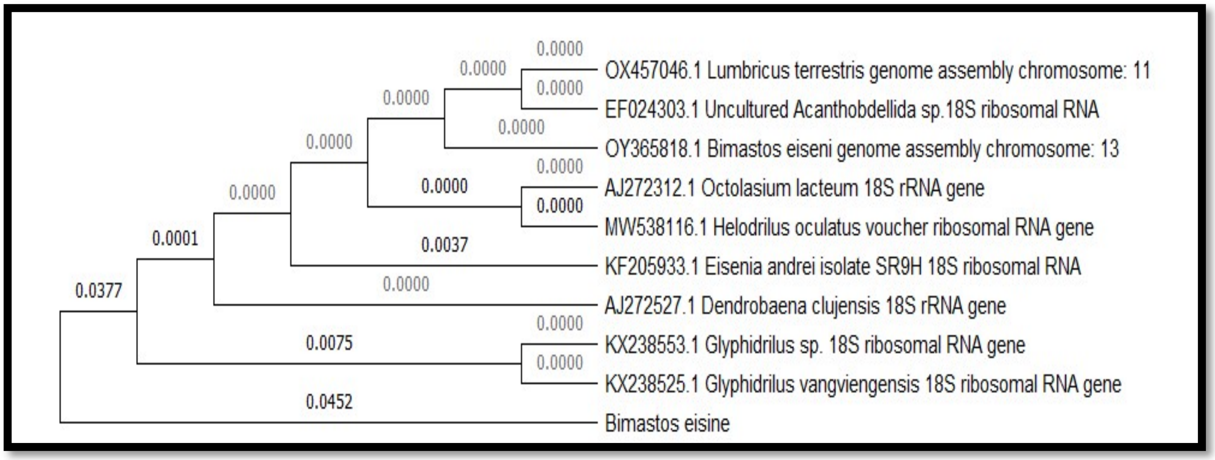


Figure 3. Phylogenetic tree of the species *Bimastos eiseni* based on the 18srRNA gene sequence, analysis and matching in the BLAST program at the National Center for Biotechnology Information (NCBI).

3.3. *Haplotaxis gordioides* (Hartman, 1821)

From a morphological standpoint, this species is readily identifiable based on its size, which ranges between 7 to 12.5 cm. The number of segments ranges between 178 to 232. The species have a single pair of thick ventral chaetae with simple-pointed end and a single pair of thinner dorsal chaetae with simple-pointed end (Figure3). Each anterior segment has four chaetae, with one chaeta per bundle. The chaetae had a length ranging from 137.66 to 285.00 µm, as shown in (Table 3), (figure 4).

In most cases, segments after XI do not have dorsal chaetae. There was persistent prostomium in the specimens. The sample size for this species was five individuals. An individual's colour might vary from a light brown to a reddish brown when they are living. The clitellum in the segment XI–XXVIII. Anterior to the ventral setae of XI and XII are male pores. Occurring on the ventral setae line are female pores in 12/13 and 13/14 (Table 3).

Molecular analysis of a partial and complete sequence of 18s rRNA gene is indicated in the National Center for Biotechnology Information (NCBI). It was uploaded to Gen Bank with accession number LC807034. *Haplotaxis gordioides* is one of the longest oligochaete worms known from Europe, but it is uncommon. It can reach up to 40cm long. Moreover, it preys on macro-and micro invertebrates, based on the discovery of *Limnodrilus* species inside the intestines of some individuals. Sexual reproduction does not exist in this species, so it was classified as reproducing asexually by architomy (van Haaren & Soors, 2012). This species has been recorded in Türkiye (Yildiz *et al*., 2012). (Pietro *et al*., 1987) indicate that there are two species belonging to the genus *Haplotaxis,* including *H. gordioides* and *H.villiersi*.

The results of a DNA-barcoding study using the mitochondrial cytochrome oxidase I (COI) from 46 Haplotaxis specimens collected from groundwater in Switzerland revealed that *H. gordioides* is actually a complex of at least six different species. This finding has significant implications for our understanding of the type species' precise identity as well as for the synonymy of many species described in the 19th century (Patric & Martin, 2023).

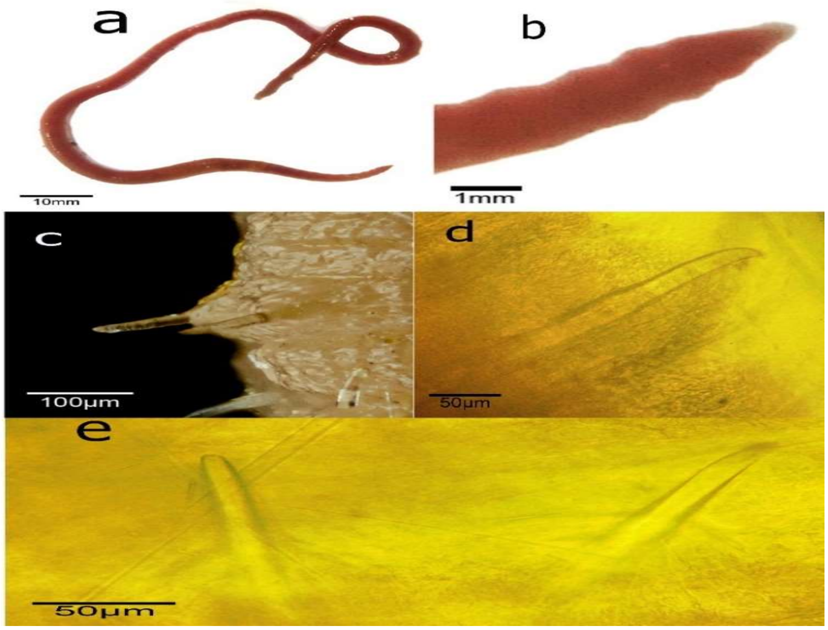


Figure 4. *Haplotaxis gordioides* a- Whole worm- b- anterior end c & e -ventral chaetae d - dorsal chaetae

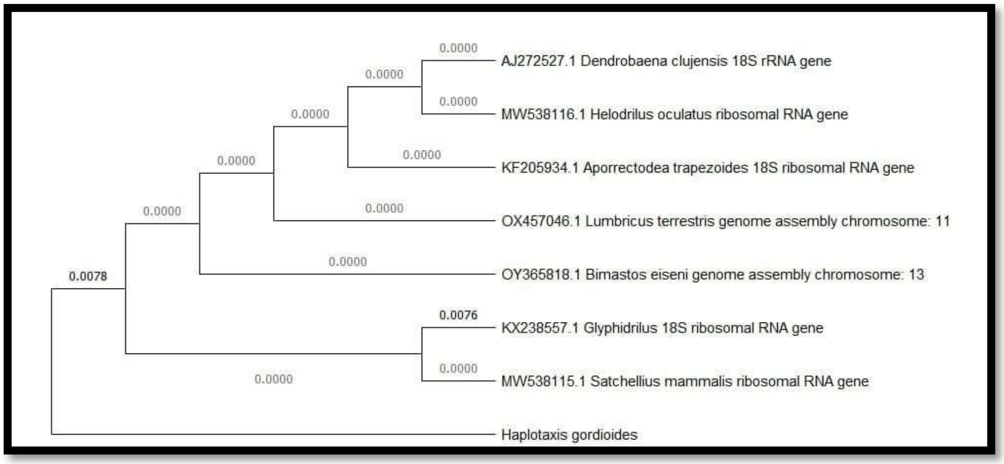


Figure 5, phylogenetic tree of the species *Haplotaxis gordioides* based on the 18srRNA gene sequence, analysis and matching in the BLAST program at the National Center for Biotechnology Information (NCBI).

3.4. ***Metaphire houlleti*** (Perrier, 1872)

Two individuals of this species were discovered by (Sim & Easton in 1972). The clay loam sediments of S3 were collected from the Abbasiya River. It branches off the Euphrates River in the holy city of Najaf in September 2023. The four bundles of chaetae in this species are characterized by the presence of two simple pointed perichaetial chaetae in each of the two dorsal and two bundles. Their dorsal side is predominantly purplish brown, while the ventral side is paler. Clitellum-shaped annular in segments 14 to 16 and characterized by an epilobic prostomium. The segment length varied from 7.5 to 8.6 cm, and the segment number ranged from 189 to 211. The chaetae's length is between 137.66 and 285.00 µm (Table 3), (Figure6).

Molecular analysis of a partial and complete sequence of 18SrRNA gene is indicated in the National Center for Biotechnology Information (NCBI) and was uploaded to Gen Bank with accession number LC807033. The genus *Metaphire* is widely distributed as many species of it have been recorded in China, including *M. planata, M. jianfengensis, M. dadingmontis, M. saxicolous,* and *M. remanens sp. nov*., which is a new species (Qing J. *et al*., 2022) depending on sequenced the mitochondrial DNA. *Metaphire balhli* (Gates, 1945), was registered from India as a new record (Narayanan *et al*., 2019). In a diagnostic and ecological study of oligochaetes in western Meghalaya in India, the species *M. houlleti* was mentioned, as the length of the worms according to this study ranged between 63-109 mm and the number of segments ranged between 98-105 segments. These values are not consistent with the results of the current study. The same source indicated that this species is widespread in the United States of America, Australia, the Philippines, China, Sri Lanka, Madagascar, Indonesia and the Bahamas (Kharkongor, 2018).

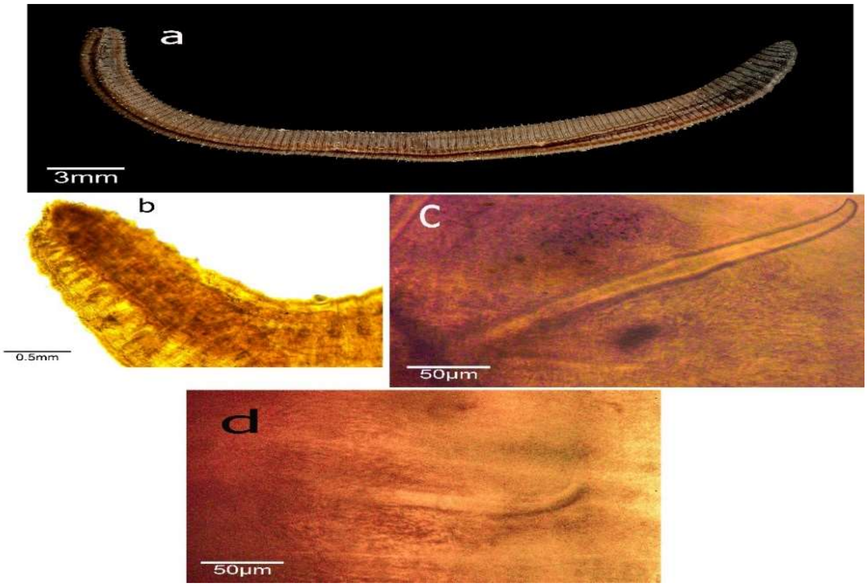


Figure 6. *Metaphire houlleti* a- Whole worm- b- anterior end c -dorsal chaetae d -ventral chaetae

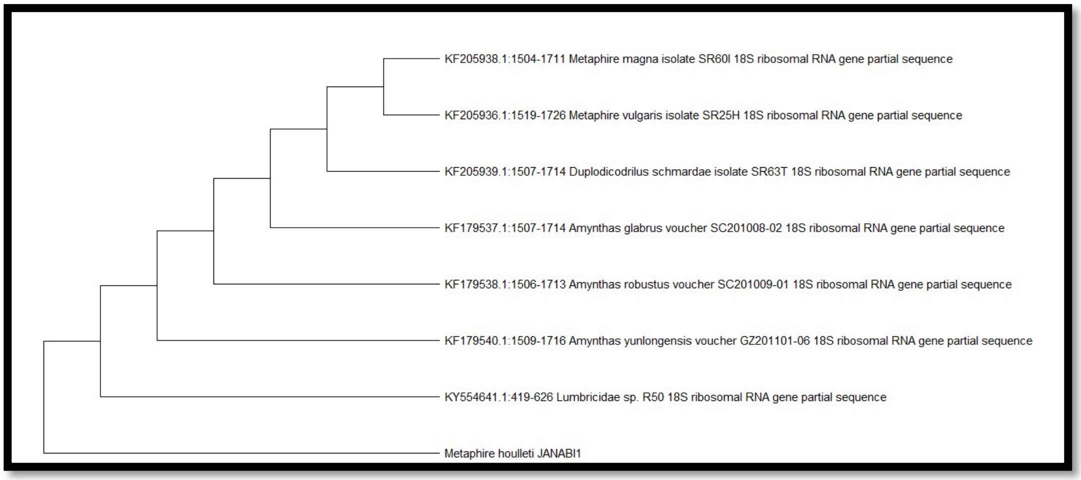


Figure 7. Phylogenetic tree of the species *Metaphire houlleti* based on the 18srRNA gene sequence, analysis and matching in the BLAST program at the National Center for Biotechnology Information (NCBI).

Table 3. The morphological characteristics of the species in the current study.

	species	**Family**		**No. of segments**	**Pigmentation**	**Clitellum segments no.**	**Renge of chaetae llenght** µ*n*
	Bimastoseiseni	Lumbricidae		179-212	light brown to reddish	24-33	156.795
	Haplotaxisgo	Haplotaxidae		178-232	reddish brown	11-28	227.204

	rdioides				to light brown		
	Metaphire houlleti	Megascolecidae		189 to 211	publish brown to pale on the ventral side	14-16	200.313

4. Conclusion

The results of molecular identification revealed that the collection samples contain three species of megadrile oligochaetes including *Bimastos eiseni* **(**Levinsen, 1884) and *Haplotaxis gordioides* (Hartman, 1821) and *Metaphire houlleti.* (Perrier, 1872). All these species were considered as new record for the Iraqi fauna. Morphologically, these species were distinguished from each other by their length, number of chaetae in each bundle, shape of chaetae and shape of prostomium. The result also showed that these species inhabit loam-clay loam sediments with lower percentage of organic matter, and can tolerate pH slightly above the neutral level.

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