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## Morphological and Molecular Identification of Three Genera of the Family Heptageniidae (Ephemeroptera) from Ava Sheen Branch/ Greater Zab Tributary, North of Iraq

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### Abstract

The aquatic Heptageniidae family of Ava-sheen branch (Greater Zab Tributary) in Duhok Governorate/ Iraq was studied. Samples were collected using Surber stream sampler to study their diagnostic morphological characteristics and molecular phylogenetic profile using a nuclear gene 16s ribosomal RNA. The morphological and molecular identification supported that the three species, *Epeorus longimanus*, *Heptagenia elegantula* and *Ephemerella cornutus* belong to Heptageniidae family, while the molecular results also confirmed the monophyletic origin of these three genera.

**Keywords:** Molecular, morphology, Heptageniidae and Iraq.

### التعرف المظهري والجزيئي لثلاثة أجناس من عائلة: هيبتاغينيديا (Ephemeroptera) من فرع آفا شين/ نهر الزاب الكبير شمال العراق

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### الخلاصة

تمت دراسة مجتمع الحشرات المائية من ضمنها عائلة Heptageniidae في فرع آفا شين (نهر الزاب الكبير) في محافظة دهوك / العراق. تم جمع حوريات ذبابة مايو باستخدام شبكة Surber لدراسة صفات التشخيصية المورفولوجية وتحليلها الجزيئي باستخدام الجين النووي 16s rRNA. التعرف المورفولوجي والجزيئي المدعوم بأن الأنواع الثلاثة *Epeorus longimanus* و *Heptagenia elegantula* و *Ephemerella cornutus* بانها تنتمي إلى عائلة Heptageniidae واعتمادا على النتائج الجزيئي التي تم الحصول عليها تؤكد النتائج الأحادية لهذه الأنواع الثلاثة بانها انواع جديدة وغير مسجلة في قائمة حشرات العراقية.

### Introduction

Aquatic insects are among the most commonly used benthic macroinvertebrates which, as part of their life stages, are linked with an aquatic or semi-aquatic environment and have an important role in the functioning of the ecosystem, nutrient cycling, primary production, decomposition and translocation of materials [1, 2]. These organisms can also be used as bio-indicators for freshwater monitoring[3]. The physical, chemical and biological characteristics of the river affect aquatic insects

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because each has distinct environmental tolerance values where only few species can survive in polluted ecosystems [4, 5].

Mayflies constitute an insect group that make up the Ephemeroptera order [6] and play a significant role in nearly all undisturbed freshwater populations [7]. They also function as key taxonomic insects for biomonitoring of water bodies and form an essential part of standardized running water assessment systems [3, 7]. The world's fauna of Ephemeroptera is comprised of 42 families, 400 genera and 3000 species [8]. Heptageniidae is one of the most abundant and significant family that belong to the order Ephemeroptera. Nymphs live as benthic communities in distinct kinds of lotic freshwater habitats such as rivers, streams, lakes, brooks, ponds, and marshes in most parts of the globe, while they can also be found under the rocks, tree trunks, plants, and leaf debris. The distribution of nymphs depends upon water current and substrate type [9]. Many species of heptageniida were targeted in aquatic research because of their important role in biomonitoring studies of anthropogenic disturbance [10].

It is particularly difficult to identify mayflies at the larval stage on a morphological basis [10, 11]. Thus, it is often impossible to assign species names in research in which immatures constitute a big percentage of the sample [10]. Recently, many efforts were made to identify species using modern and non-traditional techniques. In most instances, two distinct methods are used, which are DNA taxonomy and contemporary photo processing and classification [11]. DNA barcoding solves this issue by connecting freshly gathered samples to a reference library of authoritative samples [12]. Hence, DNA barcoding in insects has proven to be a fast, reliable and cost-effective strategy for finding new species [3]. This method holds much promise as a tool for taxonomic research and as a basis for phylogenetic analysis and biomonitoring of aquatic ecosystems [13, 14]. Genetic diversity, however, is important in identifying unique populations for conservation purposes. Aspects of DNA barcoding, loss of genetic diversity in cryptic species, and examples of molecular phylogenetic and molecular phylogeographic research of headwaters aquatic insects from significant biogeographic areas are commonly examined in the context of taxa and habitats prioritized for preservation [15].

The Great Zab River catchment is a major left bank tributary of the Tigris River and drains a significant portion of Northern Iraq's Kurdistan Region [16]. There have been a limited number of studies [17- 19] on the faunistic and environmental aspects of aquatic insects in Northern Iraq. The present study intends to help fill this gap by providing an overview of the literature published to date on the aquatic insects of Iraq especially in Kurdistan region. This is the first comprehensive faunistic study of aquatic insects from the county and may provide basic data for further taxonomic and ecological studies of aquatic insects in the north of Iraq. A further aim of this study was to consider the obtained results from the ecological aspect as bio-indicators of Greater Zab Tributary.

## Materials and method

### 1. Description of study sites

In this study, Ava sheen River was selected as site for sampling and collecting of aquatic insects, which is one of the of the Greater Zab Tributary Basin branches that is located about 32 km south east Amadiya town and about 83 km north east Duhok Governorate in the north of Iraq ( $37^{\circ}01'05''$  N,  $43^{\circ}50'09''$  E) (Figure-1).

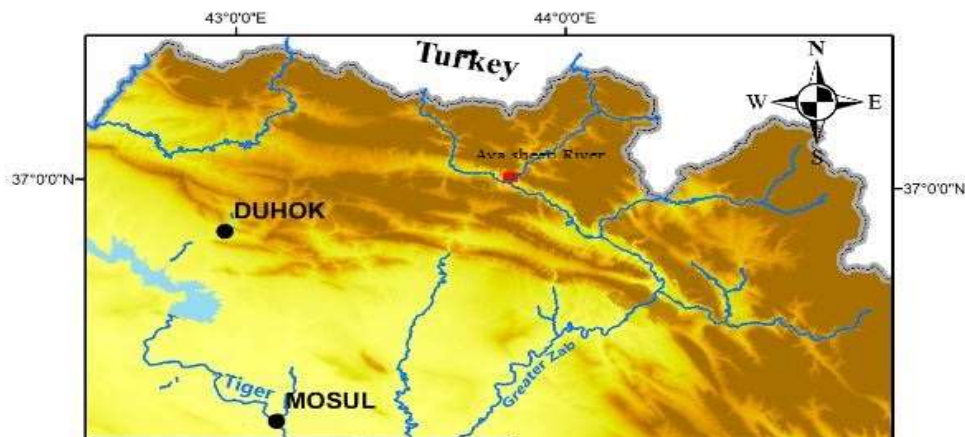


Figure 1- A map of northern Iraq showing the studied site.

## 2. Specimen sampling

A total of twenty mayfly nymphs were collected by Surber sampler (500 µm mesh) from water as well as sediments, rocks and fallen leaves during three-month period from (October, November and December 2016). Samples were identified with date, place and co-ordinates and first kept in the field in ethyl alcohol. After being transported to the laboratory, benthic macroinvertebrates and insect samples were isolated from the substratum materials, washed with water and then preserved in 95% ethyl alcohol solution for further studies [20].

## 3. Morphological examination

The morphology and aquatic nymph determination were carried out using the keys and descriptions available, based on taxonomically significant structures for the species such as mouth parts, dorsal surface, posterior margin of terga, denticles on tarsi, tibiae, and femora, in addition to tarsal claws and abdominal gills [21]. Specimens were examined using an Olympus BX51 light microscope equipped with a digital camera.

## 4. Molecular evaluation

After the morphological identification, a total of ten samples (2 replications for each) from the collection site at Sheen River were processed at the Molecular Biology Laboratory of the Department of Biological Sciences of Ankara University (Ankara, Turkey).

Tissue samples from the thorax and abdomen were obtained according to a previously described procedure [22]. Genomic DNA was extracted using the CTAB method [23], following the standard protocol indicated by the manufacturer. DNA quality and quantity were measured using a NanoVue™ Plus spectrophotometer.

PCR amplification was conducted using universal primers. The 16S ribosomal RNA gene was amplified using the primer pair 16Sa (F) 5'- GCCTGTTTATCAAAAACAT-30 and 16Sb (R) 5'-CTCCGGTTTGAAGCTCAGATCA-30 [24].

The amplifications were performed on a Techne TCPLUS thermocycler according to the following conditions: initial denaturation at 94°C for 2 min, followed by 7 cycles at 94°C for 30 sec., 45°C for 30 sec., and 72°C for 45 sec., followed by 28 cycles at 94°C for 30 sec., 48°C for 30 sec., and 72°C for 45 sec., with completing the reaction with a final extension cycle at 72°C for 7 min for the 16S rRNA gene [22].

PCR products were tested using agarose gel electrophoresis (Invitrogen). PCR products were purified using the QIA quick PCR purification kit (QIAGEN), according the manufacturer's instructions, and sequenced at Macrogen Advancing Through Genomics— Netherlands. Sequences obtained were evaluated and edited using the MEGA software.

## 5. Data analysis

The phylogenetic characteristics were evaluated using MVSP software, whereas the forward and reverse sequences were edited and assembled using Sequencher © 2011. All the sequences were aligned in MEGA v 6.0.6 [25], using the Clustal W algorithm with default parameters.

## Results and Discussion

The present study's combined molecular and morphological analyses clearly support the assignment of *Epeorus longimanus*, *Heptagenia elegantula* and *Ephemerella cornutus* to Heptageniidae family. The results also demonstrated that the three species have common characteristics such as distinctly flattened body; flattened head; dorsal eyes and antennae; invisible mandibles in dorsal view; much shorter claws than tarsi; and straight tibiae and tarsi (Heptageniidae) [21]. Some species of the *Epeorus sp.* were identified here depending on the larvae and reared imagos, in line with the taxonomic research of the mayflies [26- 31], whereas *Heptagenia sp.* was studied by other articles [31-35] and *Ephemerella sp.* was described another group of authors [36-39].

### *Epeorus longimanus* (Eaton, 1885)

Figure-2 (A) shows the whole body of *E. longimanus* which has the following characteristics: Body and head distinctly flattened, gills lateral in position at least on abdominal segments 4 to 6, lamellae usually broad, gill filaments variable, with only two well developed caudal filaments, gills inserted laterally on segments 2-6, possible extension ventrally on segments 1 and 7, claws without a tooth but with three or more subapical denticles, abdominal tubercles present or absent. Some of these characters were previously described [40]. *E. longimanus* is usually found in clear and cool streams in areas of moderate to fast flow, particularly on rocks in riffles. This was confirmed by a previous publication [41], which stated that *Epeorus longimanus* was the most common aquatic invertebrate

inhabiting the riffles and an intolerant species in the Heptageniidae family which has developed the ability to use their gills to adhere to substrates in rapid currents in the sampled riffle.

***Heptagenia elegantula*** (Eaton, 1885)

There are small differences between *Heptagenia* species [21, 31]. Some of the diagnostic morphological characteristics are shown in Figure-2 (B), as follows. Brown colour with pale markings present on head, thorax and abdomen; distinctly flattened body and head; dorsal eyes and antennae; front of head entire or only slightly incised medially; pronotum widest at anterior margin; straight or slightly emarginated posterior margin; invisible mandibles in dorsal view; apex of outer incisor of planate mandible; fore femora are usually brown dorsally with pale undulate median band and large basal pale spot, often extending to median band; dorsal surface of each femur is with an oval or paddle – shaped setae; claws without denticles but with a basal tooth; gills lateral in position at least on abdominal segments 4 to 6; lamellae usually broad; gill filaments variable, with three well developed caudal filaments; maxillary palpi rarely protrude at sides of head; gills on segment 7 similar to preceding pairs but smaller; trachea of gill 7 with lateral branches; gill lamellae on segment 1 are two-thirds as long as those on segment 2; fibrilliform portion of gill 1 usually subequal to or shorter than lamella; labrum never narrower at apex than at base; a widespread and relatively common genus; can be found year round inconspicuous; in lotic waters and in slow moving waters under stones or debris near banks or in pools. *Heptagenia elegantula* larvae are found in warmer silted streams [32, 33]

***Ephemerella cornutus*** (Gose, 1980)

Genus diagnosis is shown in Figure-2 (C). The most reliable characteristics for recognizing *E. cornutus* are its large size with specimens larger than 9 mm.; generally, brown in colour; wings smoky in colour [42]; abdominal gills exposed; gills on segment (2-7) variable; long setae absent; gills on segment 2 non operculate; gills absent on abdominal segment 2, absent or rudimentary on segment 1, present in segment 3; gills on segments 3-7 or 4-7 consist of anterior oval lamellae and posterior lamella with lobes; thorax, head, and abdomen without large paired tubercles; maxillary palps well developed; leading margin of fore femora with tubercles; apical tibial projection usually straight and moderately sharp; tarsal claws with two to two to four denticles; caudal filaments brown; caudal filaments sub-equal in length; caudal filaments with or without whorls of spines at apex of each segment [36, 43, 44]. They inhabit primarily lotic waters, in areas of fast flow in crevasses of hard substrates, but some species are found in vegetation and root mats in edge areas where there is slow flow.

**Phylogenetic analysis**

The present study applied the Bayesian phylogenetic reconstruction (Figure-3) which clearly supports the monophylogeny of the *Ephemerella* but not the *Epeorus* and *Heptagenia* species. *Epeorus* and *Heptagenia* are form similar monophyletic groups (Gower General Similarity Coefficient= 0.679) and the taxonomic integrity of all sampled species within the two genera is also validated. The focal species, *Epeorus longimanus* and *Heptagenia elegantula*, clustered together to form a distinct and highly supported monophyletic clade. The *Ephemerella cornutus* clade is further divided into separate clade. The maximum-likelihood analysis yielded similar topology (PP = 0.462) but with lower support values (indicated on the Gower General Similarity Coefficient).

The analysis of phylogenetic relations between individual Caucasion lineages would require a broader spectrum of genetic markers [45] and we refrain from making phylogenetic assumptions from our data. They suggested for mayflies in general [10]. Additionally, every species for which we had more than one specimen was strongly supported as monophyletic. The only species pair that showed lower than expected divergence was *E. cornutus* vs. *E. longimanus* and *H. elegantula*. Ephemeroptera was strongly supported as monophyletic. Ephemeroidea constituted a monophyletic group and some of the relationships between the different families were not always strongly supported [46].

**Molecular analysis**

The percentage distribution of three insect species BLAST from Gene bank NCBI of partial 16s ribosomal RNA gene is shown in table 1. We found that the sequences of 16s rRNA successfully distinguished between genera and species of *E. longimanus*, *H. elegantula* and *E. cornutus* because intra- and intraspecific sequences differed by at least an order of magnitude in nearly every species comparison.

### Phylogenetic inferences

Phylogenetic analysis based on ribosomal RNA nucleotide sequence revealed the grouping of the three investigated species of *Epeorus longimanus*, *Ephemerella cornutus* and *Heptagenia elegantula* on expected lines, as shown in Figure-4. From sequence divergence similarity data and the constructed phylogeny, it was revealed that the species belonging to the respective genera were close to each other. The three samples species grouped in three clusters with low distances and high similarity. In the first cluster, *Epeorus longimanus* is in the same cluster with *Epeorus longimanus* by Gene bank Accession Number (GAN) AY749757.1, with a distance of 0.08. In the second, *Ephemerella cornutus* is in the same cluster with *Ephemerella cornutus* by GAN of FJ443041.1, with a distance of 0.05. In the third cluster, *Heptagenia elegantula* is in the same cluster with *Heptagenia elegantula* by GAN of MH456926.1, with a distance of 0.03. All the three species were identified by NCBI nblast.

This result was also supported by several previous studies [36, 44] which clarified that some species of Ephemerella have a monophyletic clade. Also, another study [46] supports our result, as it showed that some families such as Heptageniidae, Isonychiidae, Baetidae, Acanthametropodidae, Metretopodidae, Ameletidae and Nesameletidae are monophyletic. It was recently determined that the monophyly of the families Heptageniidae, Baetidae, and Ephemerellidae is highly supported [47]. *E. herklotsi* is a sister clade to *Epeorus* sp2. (KJ493406).

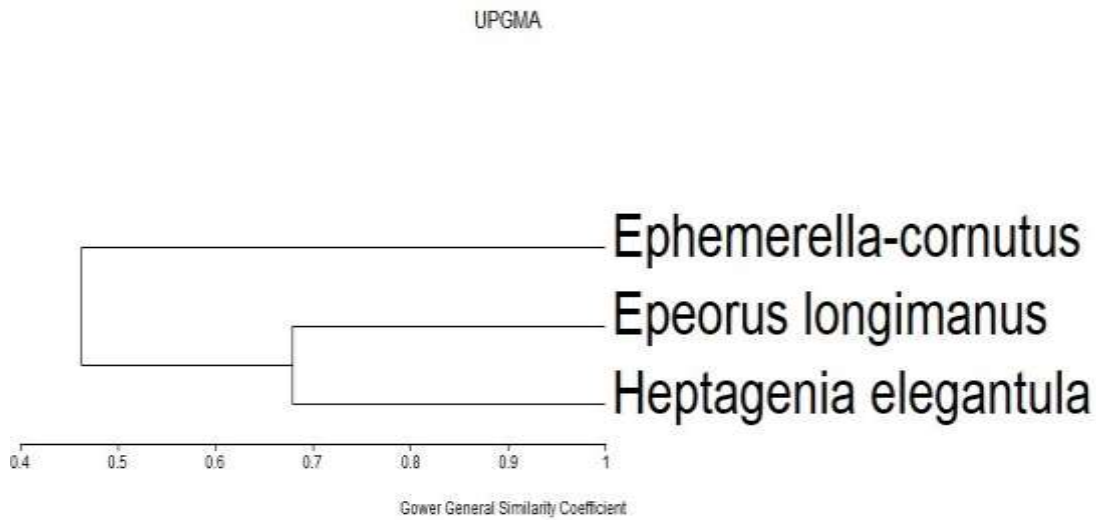
It was also endorsed by relatively high genetic distances to other species. Several studies of the DNA barcoding highlighted the cases where this approach was found unsuitable for species identification [29, 48]. However, the results obtained in the studies on mayflies show a close correspondence between the morphologically defined species and the barcode clusters [10, 12, 14, 33, 44]. The relatively low interspecific divergences were also previously observed in other genera within mayflies, e.g. *Baetisca* Walsh, 1862, *Caenis* Stephens, 1835 [10], and *Siphonurus* Eaton, 1868[14]. These results might be explained by the young age of the lineages or may reflect the lower substitution rate in these taxa [10, 49]. If we assume *E. (C.) sinitshenkovae* and *E. (C.) longimaculatus* to be separate species, the intraspecific variation within our dataset ranged between 0.00 and 4.72% and interspecific between 5.79 and 18.51%. The barcode gap width formed was 1.07%. The previous studies on mayflies showed variable ranges of barcode gap, from much higher values [33, 50] to no gap observed [10, 14, 44]. The mean interspecific divergence (14.33% with *E. (C.) sinitshenkovae* and *E. (C.) longimaculatus* constrained as separate species) obtained in our results showed similar value to other reported divergences for congeneric species within mayflies [14, 33, 44] or within Heptageniidae in particular [10]. Therefore, our results confirmed the generally high level of 16S rRNA sequence divergence within Ephemeroptera [14].



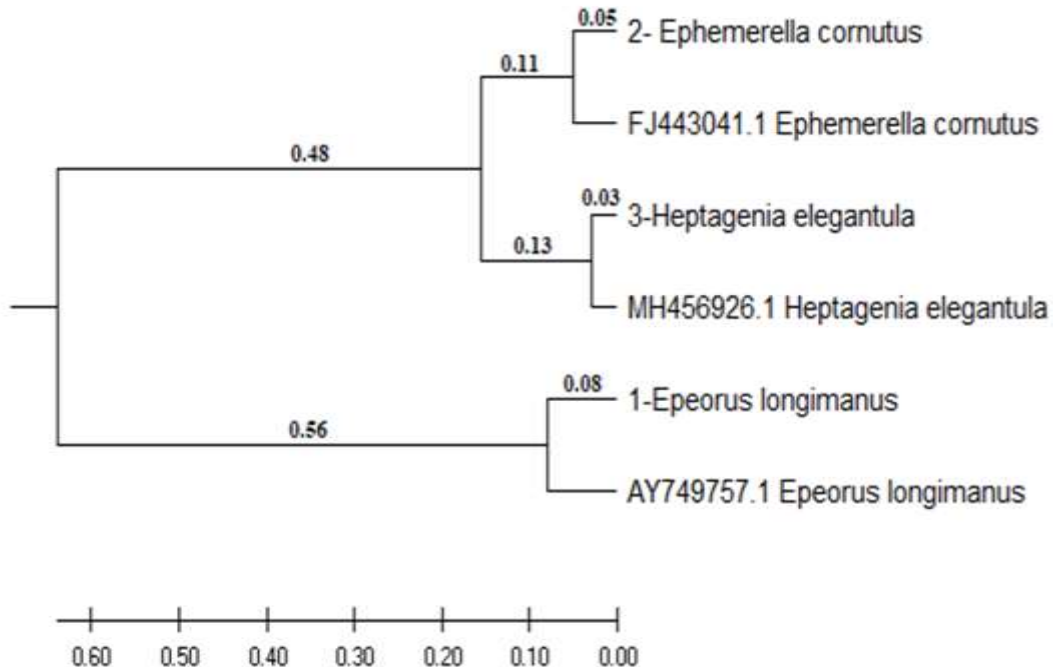
**Figure 2-** Whole body of the three studied genera, (A) *Epeorus longimanus*; (B) *Heptagenia elegantula*; (C) *Ephemerella cornutus*

**Table 1-**Percentage distribution of three samples insect species blast from Gene bank NCBI of partial 16s ribosomal RNA gene

Insect Samples	Query Cover %	Identic Number %	Genebank Accession Number	Genebank Insect Species Identification	Country Identification
1	99%	87.68%	AY749757.1	Epeorus longimanus	USA
2	100%	100%	FJ443041.1	Ephemerella cornutus	USA
3	100%	100%	MH456926.1	Heptagenia elegantula	USA



**Figure 3-**Dendrogram of morphological characters of the three Heptageniidae species



**Figure 4-**Mega X program showing phylogenetic positioning and identification of the three difference species of *Epeorus longimanus*, *Heptagenia elegantula* and *Ephemerella cornutus* according to partial sequences of ribosomal RNA gene, 1-*Epeorus longimanus*, 2-*Ephemerella cornutus* and 3-*Heptagenia elegantula*.

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