Morphological and Molecular Characterization of *Heterocypris incongruens* (Ramdohr 1808), A New Species Ostracod in Kurdistan Region-Iraq

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Abstract

The present paper is intensive on screening new Ostracod’s species obtained from four selected sites in different areas, Erbil Governorate, Kurdistan Region, Iraq. Samples have been collected during October 2021, March and June 2022. *Heterocypris incongruens* (Ramdohr 1808) was observed in water after taking some algae (*Chlorella vulgaris*, *Anabaena*, etc., and aquatic plants (*Polygonum* sp., *Nasturtium officinale* and *Cynodon dactylon*) with its residue near-shore water. Later separated the sample and fixed in 70% ethanol. After the morphological study, the PCR product of 28s rRNA was sequenced and deposited in the GenBank database with accession numbers OP600471-OP600474. According to phylogenetic analysis, the examined *Heterocypris incongruens* were identified and are being reported from Iraq.

Keywords: Ostracod, Molecular analysis, Phylogenetic tree

Introduction

Global climate is significantly affected by global biodiversity, which in turn affects all aspects of life (Sulieman *et al.*, 2022). The species *Heterocypris incongruens* (Ramdohr 1808) is a member of the phylum Arthropod's Cyprididae family. It’s a tiny aquatic crustacean that ranges in size from 0.3 to 5 mm. Small calcite and tough carapace component of mineralized skeletal tissue with two dorsally linked valves. The hinged shell that can entirely cover and defend the non-mineralized body parts and appendages, makes them most recognizable. Mostly have rounded shells that are
embellished with pits, and ridges, with other features. It has a complex body inside their carapace that normally have eight pairs of appendages. These appendages serve as variety of purposes during the shell is open such as swimming, sensing, crawling, feeding, and mating.

It can be found in most aquatic ecosystems, including marine and freshwater (lake, river and pond). It is also occasionally found as a parasite species in fishes' digestive systems. This community's species vary according to location because they are impacted by several water properties, including temperature, base geography, saltiness, pH, alkalinity, DO (Bellin et al., 2021; Rossetti et al., 2020). Furthermore, it has been vital to decades of academic and applied palaeontology research in a variety of areas, including evolutionary and environmental studies (Czajkowska, 2022). Additionally, it provides precise details on the dynamics of permafrost and the climate throughout the late Quaternary (Machado et al., 2020; Tan et al., 2021). First discovered Chlamydotheca unispinosa (Baird, 1862) was in the Caribbean island of Montserrat by (Schmidt et al., 2018).

Karanovic et al, (2020) conducted a research for molecular identification of every specimen were analyzed. In order to harvest DNA, genetic markers were used are (ITS, 28S, 18S, COI). They report two new species, *Ishizakiella occidentalis* and *I. miurensis*, whose shell shapes match the molecular phylogeny, according to the findings.

In an investigation in the perennial freshwater lake of Singanallur, India. The *Cypretta campechensis* was the first record of the ostracod species, based on the molecular examination of the mitochondrial cytochrome oxidase subunit (mt-COI). LCO1490 and HCO2198 universal primers were used to amplify the gene, which was subsequently sequenced and verified using NCBI GenBank (MN641913). (Kalpana et al., 2021).

Through the use of a molecular approach, Huyen and Karanovic (2022) discovered four new species of Parasterope, including *P. busanensis, P. single, P. sohi*, and *P. sagami*. Based on the partial 16S rRNA. The following primers were used: forward and reverse, 16S MYOF1, 16S MYOF2, 16S MYOR1 and 16S MYOR2.

On the other hand, two new species were listed. *Vestalenula gravata* of the Darwinulidae family and *Microloxoconcha Semicircularis* in the Cytheroidea family. (Smith & Chang, 2022).

Geometric morphometric techniques are effective tools for distinguishing between closely related Ostracod species and for investigating the relationship among their morphological variability, taxonomy, and paleoecology. In this work, valve outline analysis enables differentiation between
Riocypris whatleyi and Cypridopsis silvestrii juvenile non-marine ostracods by highlighting variations in the posterior valve area and surface ornamentation (Ramos et al., 2021).

Arthropods are ecologically importance because of their sheer numbers and extreme diversity. They play extremely important roles in maintaining the ecosystem and can also be beneficial for humans (Schapheer et al., 2021). Ostracods were found in small numbers in Iraq (Hassan, 2018) collected specimens from ten locations in the Baghdad and wasit governorate. He revealed thirteen species of ostracods among them Ilyocypris forester, Stenocypris hislopi, Cyclocypris sp, Eucypri sp, etc. with indicate that the population of Stenocypris hislopi reached up to 3000. Also, (Zwair, 2021) introduced Pseudocandona sp for the first time in Iraq. Generally, the study of the Ostracodes fauna poorly documented in the Kurdistan region. Five ostracode species have been described from Baluti Formation (Upper Triassic) near Sarki Village situated about (11 km) Southeastern Amadiya City, Dohuk province, Northern Iraq, there are Fabanella sp., Hungarella moorei, Ogmoconcha sp., Ogmoconcha bristolensis and Cytherella acuta (Al-Khashab & Al-Halawachi, 2018). Prior to this study, Al-Meshleb (2012) was the only researcher who discovered a species of Heterocypris giesbrechtii in Iraq. No study has yet been conducted on the prevalence of Heterocypris in Iraq. The aim of this study was to investigate the ostracods species living in the Kurdistan region. We did this in the hope of obtaining different types of ostracods in a watery environment.

Materials and method

Sample collection

The Ostracod samples were collected from 4 different sites which include Altun kupri about 55km, Xalan site 100km, Bridge Hafiz 94 km and Xanaqa 119 km distance from Erbil city respectively (Figure 1). Specimens were gathered by taking algae which include Chlorella vulgaris, Anabaena, cylindrica and Oscillatori and plants belong to Polygonum sp., Nasturtium sp. and Cynodon dactylon with its sediment near shore water, during October 2021, March and June 2022. The samples were transferred to the laboratory and placed under an oxygen instrument for about one week, then ostracod species began to grow and it was removed from the bottle and then fixed in 70% ethanol. Identification of ostracods sample has performed in the laboratory of Education College, Salahaddin University- Erbil, by using compound microscope (OPTIKA B-350) and a small amount of glycerin. Characteristics were examined using identification keys (Fernando & Biology, 1981; Karanovic, 2012).
PCR Technique and genomic extraction

_Heterocypris incongruens_ was discovered and sequenced using PCR technology. By utilizing the GeneAll® ExgeneTM for Clinic Cell SV small kit, which was acquired from GeneAll® (Songpa-gu, Seoul, KOREA), in accordance with the manufacturer's instructions, and 50 mL of elution buffer, total genomic DNA was extracted from the specimens. Using a nanodrop spectrophotometer for calculate concentrations of isolated DNA. Before starting a PCR, the extracted genomes were kept at -20°C.

PCR amplification

A region of 28SrRNA gene was amplified by PCR. The universal primers were including forward primer C1 (5ACC CGCTGAATTTAAGCAT) at 5'-3' position and reverse primer C3 (CTCTTCAGATACTTTTCAAC) at 5'-3' position. PCR cocktail was run in a total volume 50 µL reaction mixture containing 25 µL of 2× PCR master mix (AMPLIQON, Denmark), 1.0 µL of each primer (10 pmol), 1.5 µL of genomic DNA template. The volume was completed to 50 µL with free nuclease water. Each gene was independently amplified. The subsequent program was used to continue the PCR after that: the thermal cycling conditions comprised of initial
denaturation at 94c for 5 min, 35 cycles of each denaturation at 94c for 45 sec, annealing temperatures at 51 sec for 45 sec and extention at 72c for 45 sec, and final extention at 72c for 5 min. the PCR product was confirmed by using agaros gel electrophoresis. The samples prepared and run in 2% gel of agaros verify the size of the fragment after which the DNA is dyed with ethedium bromide to make it visible under ultraviolet light. Having a PCR product predicted size of 365bp.

**DNA sequencing analysis**

PCR product of DNA samples were organized for sequencing by using forward primers of 28s rRNA in Zheen international hospital, Erbil, Kurdistan region, Iraq. The National Center for Biotechnology Information (NCBI) Gen Bank was used to compare the alignment findings of the sequences with previously deposited sequences. The sequences of *Heterocypris incongruens* in this study were submitted in NCBI-Gen Bank.

**Results**

There does not appear to be any preceding literature on this species in Iraq. Specimens of four revealed sites that pursuant to the microscopic inspection and ordering keys with molecular sequences discovery they have been diagnosed as *Heterocypris incongruens* as a new species in Iraq (Fig. 2 and 3).
Figure 2. A photomicrograph of *Heterocypris incongruense* A-Whole amount, B- Antennula, C- Antenna, D- Mandibula, E- Maxillula, F- Branchial plate, G- First limb, H- second limb, I- Third limb, J- Furca
**Figure 3.** Lucida drawing of *Heterocypris incongruense* A-Whole amount, B- Antennula, C- Antenna, D-Mandibula, E- Maxillula, F- Branchial plate, G- First limb, H- second limb, I- Third limb, J- Furca

*Heterocypris incongruens*

The anterior region of the shell of this species is repeatedly covered in tiny tubercles that are arranged along the margin in a regular row of twenty to thirty, only on the right or smaller valve. Also along the margin of the valves, a series of regularly spaced denticles can be seen. The carapace, which encases the entire body, is made up of two valves joined dorsally. It passes through moulting, until it reaches adulthood. The carapace's two valves close with extreme precision, with one valve being slightly larger than the other and therefore covering it. By letting go of the adductor muscles that protrude from the shell and using the appendages to force the valves apart, the carapace can be opened.

The outer lamella and the inner lamella are the two primary components of the valve. The first one creates the outermost surface and makes up the majority of the valves. Numerous tiny pores, which are openings in the carapace wall through which sensory setae emerge, are present in this layer. Has a radial (marginal) pore canals on the borders of the valves as well. The valve layer inside the carapace is the second one. They both cross the area known as the fused zone.
Their length varies from 0.8 to 1.1 mm, and the upper portions of the shell are orange-yellow, fading to brown. It has a complicated soft body that has been shrunk such that the carapace may completely encapsulate the body's non-calcified regions. This makes it difficult to distinguish between the head, thorax, and belly. The mandibles, maxilla, antennae, and antennules are the four pairs of appendages that the head supports. Because there isn’t a distinct line separating the head and thorax, it has been argued on occasion that the first pair of thoracic appendages should be considered the fifth appendage. Typically have eight pairs of appendages that serve a variety of purposes, including sensing, movement, cleaning, mating, and eating.

The first appendage, known as the antennule or first antenna, is situated close to the hinge's anterior end. It has six segments and is uniramous. Are long and slender, and together with the antennae, they are flexible enough to be employed for swimming. The lengthy setae are spread out when the ostracods is at rest in order to detect movement in the water. The antennae, which are biramous and have two branches, are the second set of limbs (the endopod and exopod). Utilized to swimming, crawling and drag food to the mouth region. The third appendages at either side of the mouth are the mandibles. Consist of two primary components: the coxa, which bears teeth on the inside to masticate food, and the palp, which sweeps food into the mouth. The fourth limb is the maxillula. There are two primary sections. The endopod and endites, which are utilized for eating, are in the anterior parts. The huge branchial plate in the posterior portion of it aids in respiration by circulating water through the carapace cavity.

The first pair of thoracic legs altered to a masticatory appendage. , it is usually the smallest of the walking legs. These appendages consist of protopod which is the proximal portion, and endopodite with exopodiet at the inner and outer side of protopodite respectively. The second pair of thoracic legs five interconnected. The first piece of this limb is the protopod, while the other segments are the endopod. Limb is positioned in the posterior section of the carapace. It is a walking limb and sometimes used as a clasping appendages. Usually ends with a claw. The third thoracic legs Five jointed. Located in the posterior region of the carapace. It is either a cleaning limb bent upwards into the carapace, or a walking leg, analogous in shape to the second limb, the first segment of its limb is the protopod, while the other segments are the endopod.

The final appendage, a rod-like structure located at the back of the body, is known as the caudal ramus (also known as the furca or uropodal ramus). Typically, it has a rigid ramus or base with several setae and claws sticking out of it. Both the extremely long terminal claw and the sub-
terminal claw have hair at their ventral ends. Additionally, there are two setae: one on the proximal ventral side of the sub terminal claw and another one at the distal end of the terminal claw. The caudal ramus and furcal attachment are joined internally. This attachment has two branches that are positioned at an acute angle at the proximal end. Long, wide, and straight define the furcas attach's median region. Arched and tapering at its terminal end, the articulation extremity is rounded. In some populations, the caudal ramus is employed to move the male sexual organs during copulation, also used for movement. Regarding the molecular investigation, the blast result in the DNA (28srRNA) sequence of *Heterocypris incongruence* in GenBank indicated 99% similarity to this species with accession No. (op600471-op600474) (Table.1).

**Table 1:** Pairwise alignment of 28srRNA sequence of *Heterocypris incongruens*. Query is the study or sample sequence and Subject is the GenBank sequence

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**Phylogenetic inferences**

According to phylogenetic analysis based on the 28S rRNA nucleotide sequence, the examined *Heterocypris incongruens* were grouped along predicted lines. Sequence divergence similarity data and a phylogeny were used to determine how closely related species within different genera to one another (Fig. 4).
Figure 4. Phylogenetic tree of Heterocypris incongruens samples from Iraq: Kurdistan region-Iraq. The phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 100 re-samplings. Partial DNA sequences of concatenated partial 28S rRNA gene were used as input data.

Discussion

For the first time, *Heterocypris incongruens* was recorded by (Ramdor 1808). Then, additional researchers throughout the globe work on it in diverse contexts. Considering that Iraq had no prior knowledge of it. The current record is the first to be set in this nation. The morphological analysis of the species in the illustrations is consistent with (Karanovic, 2012; Purper & Würdig-Macieł, 1974; Rossetti et al., 2020). Otherwise, the reason of presence one species in an area and absent in other are likely to be caused by ecological traits such as base geography, saltiness, pH, alkalinity and DO. In some studies, (Parra & Espinoza-Villalobos, 2020; Szlauer-Łukaszewska & Pešić, 2020) indicated that various ecological conditions and calcification mechanisms are believed to be the root of the different outcomes for different species. According to certain experts who studied 'aquatic creatures' calcification or development was accelerated by low pH levels. Also, in an investigation done by (Hassan, 2018) with the drop in water temperatures and the decline in the abundance of other crustacean groups came the appearance of an expanding ostracods colony. The highest number was recorded in the January, March and April.

Concerning the molecular study, The BLAST tool from (http://blast.ncbi.nlm.nih.gov/) is used to analyze gene sequence samples with a size of 700–800 bp to compare our amplified sequences to other *Heterocypris incongruens* sequences that had been saved. The BLAST results showed that 4 *Heterocypris incongruens* had the highest identity number query sequence with 100% identity.
This aliment suggest that we submit our query sequences to NCBI. According to the alignment of 28srRNA sequence of *Heterocypris incongruens* that shown in Table (1) and Figure 4 Suggested that this species recorded in Gen Bank three times only. The first time in Japan (AB675003), then in Germany (EU370438) and the last one current record in Iraq (op600471-op600474). There are other Heterocypris species described in Japan and recorded in Gen Bank such as *H. spadix* (LC557033). However, further research is needed to investigate the other species of Ostracoda in our freshwater environments.

**Conclusion**

The present paper is intensive on screened new Ostracods species morphologically and molecular analysis. Otherwise, the result indicated that various ecological situations and calcification mechanisms are believed to be the origin of the different outcomes for different species.

**References**


Huyen, Ph. T. and Karanovic, I. (2022) Four new Parasterope (Ostracoda, Myodocopina) from the Northwest Pacific and their phylogeny based on 16S rRNA. ZooKeys, 1095: 13–42. https://doi.org/10.3897/zookeys.1095.77996


