

Scanning Electron Microscope and Molecular Identification of *Cypris pubera* Female from AlUla, Kingdom of Saudi Arabia

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ABSTRACT

The *Cypris* is thought to be the most ancient ostracod generic name. The first species of ostracod to be called by the Linnean binomial classification was *Cypris pubera*. Our studied species *Cypris pubera* was collected from AlUla, Saudi Arabia and has geographical parthenogenesis. The right valve with an anter-ventral edge that resembles a lip, the left and right valves overlap and the second thoracic leg composed of 4-segments. *C. pubera* has a set of noticeable denticles in the frontal portion of the valve and several bigger spines on the posterior edge. The natatory setae of An2 of *C. pubera* not reaching the tips of the terminal claws. The third segment of the mandibular palp of *C. pubera*, bears four unequal smooth sub-distal setae. The molecular identification of the current studied species *Cypris pubera* is confirmed by DNA barcoding using COX1. COX1 sequencing is a good marker for molecular identification of *Cypris pubera*. This study adds additional species to the list of biodiversity found in the aquatic environment of Saudi Arabia.

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Authors' Contribution

RMA and AAS collected and identified the samples. EAY participated in the morphological identification of samples. All authors participate in the writing, review, editing, discussion and methodology and approved the final version of manuscript for publication.

Key words

Cypris pubera, Molecular identification of invertebrates, AlUla, Cypridinae, Ostracods

INTRODUCTION

Ostracods are tiny crustaceans with two calcified valves that surround the entire body. Ostracods can be found in a wide range of aquatic settings. They can be found in both marine and freshwater interstitial waters, as well as in temporary and permanent freshwater bodies, large and small lakes, springs, streams, rivers, and pools, as well as semi-terrestrial environments and deep underground waters. The Cyprididae is the most diverse family of freshwater Ostracods, which includes half of all freshwater Ostracod species presently documented (Smith *et al.*, 2011). Many Ostracod species of family Cyprididae inhabit temporary waters, and they can swim and produce eggs that can withstand droughts. The subfamily Cypridinae Baird, 1845

of Ostracods includes the genus *Cypris*. *Cypris* is certainly the oldest and type genus of the Cypridinae subfamily, with *Cypris pubera* O.F. Müller, 1776 serving as its type species (Meisch *et al.*, 2019). The species of this genus *cypris* are found in a wide range of freshwater bodies, and its distribution is practically entirely global (Karanovic, 2012). According to Daday (1892, 1900) *Cypris pubera* was described in the following five diversities; *C. anacantha*, *C. monacantha*, *C. diacantha*, *C. triacantha* and *C. polyacantha*.

The present study contributes to our knowledge of Saudi Arabia's little-known temporary ostracod species and offers the first detailed description of *Cypris pubera*, complete with images of its valves and appendages.

MATERIALS AND METHODS

A handheld-net with a 50 µm net size was used to collect samples from temporary waterways in Al-Ward rainwater area, about 100 km from AlUla Governorate (Fig. 1), Kingdom of Saudi Arabia (Alansari *et al.*, 2024). Samples were sorted in the lab, preserved in 70% ethanol, and then counted. After that, the specimens were dissected under a binocular stereomicroscope with the assistance of two tungsten needles and mounted in Hoyer's. A camera lucida and Olympus microscope were used to draw appendages

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Fig. 1. Shows location of the collection samples at Al-Ward Dam, AlUla Governorate, Kingdom of Saudi Arabia.

of the specimens. For scanning electron microscope studies, a blend of 1% osmium tetroxide and 4% glutaraldehyde was used to fix several individuals of *Cypris pubera*. Ostracod samples were dehydrated in a progressed sequence of ethyl alcohol. In Assiut University, carapaces and a few appendages of the ostracod species under study being gold-coated and analyzed using a JSM 5400 LV SEM set to an accelerating voltage of 15 kV. Prof. Francesc Mesquita-Joanes of the Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, Paterna, Spain, assisted in the identification of the current ostracod species and by using the keys in McKenzie and Keij (1977), Meisch (2000), Park and Martens, (2001), Mesquita-Joanes (2020), and Peng *et al.* (2021).

DNA extraction and DNA barcoding using COX1

Cypris pubera samples were saved in 90% ethanol for molecular identification. The samples were sent to Macrogen Genome Center (Seoul, Republic of Korea) for DNA extraction, polymerase chain reaction (PCR), and sequencing. DNA extraction was done by MG Tissue SV (Doctor Protein INC, Korea). The primers used for cytochrome c oxidase subunit I (COX1) gene is COX1-OligoF: 5'-GRYTTTWYTCYACRAAT-

CAYAARGAYATTGG-3' and COX1-OligoR:5'-AAT-TAARATRTADACTTCWGGRTGACC-3' (Mesquita-Joanes *et al.*, 2020). Amplification of COX1 fragment was done by a PCR machine (DNA Engine Tetrad II Peltier Thermal Cycler, BIO-RAD). PCR reaction includes 30 cycles; the denaturation step (94°C for 30 sec.), annealing temperatures (50°C for 30 sec.), and extension step (72°C for 30 sec), and final step at 72°C for 4 min. PCR products were electrophoresed by 1.5% agarose gel to check on the bands. PCR product was purified by ExoSAP-IT™ Express PCR Product Cleanup (ThermoFisher). The sequencing was done by ABI PRISM 3730XL Analyzer Sequencer, BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences of *COX1* gene of the *Cypris pubera* under study have been uploaded to the GenBank/EMBL/DBJ genetic databases and taken accession number (PQ533181) which were compared with others *Cypris pubera* by BLAST algorithm (<https://www.ncbi.nlm.nih.gov/>) (Madden, 2002). Evolutionary analyses (phylogenetic tree, the pairwise genetic distances, and alignment) were conducted in MEGA12 software package (Kumar *et al.*, 2024).

TAXONOMIC DESCRIPTION

Class: Ostracoda Latreille, 1806
Subclass: Podocopa G.W. Müller, 1894
Order: Podocopida Sars, 1866
Suborder: Podocopina Sars, 1866
Superfamily: Cypridoidea Baird, 1845
Family: Cyprididae Baird, 1845
Subfamily: Cypridinae Baird, 1845
Genus: *Cypris* O. F. Müller, 1776
Cypris pubera O. F. Müller, 1776

**Cypris pubera* O.F. Müller, 1776: AU, NA, NT, PA
 Syn.: *C. pubera* var. *triaculeata* Daday, 1892 syn. nov.
 Syn.: *C. pubera* var. *polyacantha* Daday, 1892 syn. nov.
 Syn.: *C. pubera* var. *anacantha* Daday, 1900 syn. nov.
 Syn.: *C. pubera* var. *monacantha* Daday, 1900 syn. nov.
 Syn.: *C. pubera* var. *diacantha* Daday, 1900 syn. nov.
 Syn.: *C. pubera* var. *triacantha* Daday, 1900 syn. nov.
 Syn.: *C. triaculeata* (Daday, 1892; Fuhrmann and Pietrzeniuk, 1990) syn. nov.

DESCRIPTION OF ADULT FEMALE (MALES NOT KNOWN)

Carapace

On the exterior: The large carapace has a sub-triangular form (Table I). The dorsal border (Fig. 2A) is strongly arched, with the greatest height considerably in obverse of mid-length and the ventral edge is obviously

winding (Fig. 2A, B). The anterior margin is wider and more rounded than the posterior one (Fig. 2C). In lateral view, the shell surface is pitted and covered with many sensillae. Furthermore, there are a few little bumps on the anterior and posterior regions (Fig. 2D, E). Additionally, the anterior, posterior, and ventral edges of the left valve overlap the right one, which is smaller (Fig. 2C, F).

Table I. Measurements of right and left valves of Carapace (Females in mm, n = 25) of *Cypris pubera*.

	Length	Height
Right valve	1.90-2.20	1.02-1.20
Left valve	2.10- 2.30	1.05-1.30

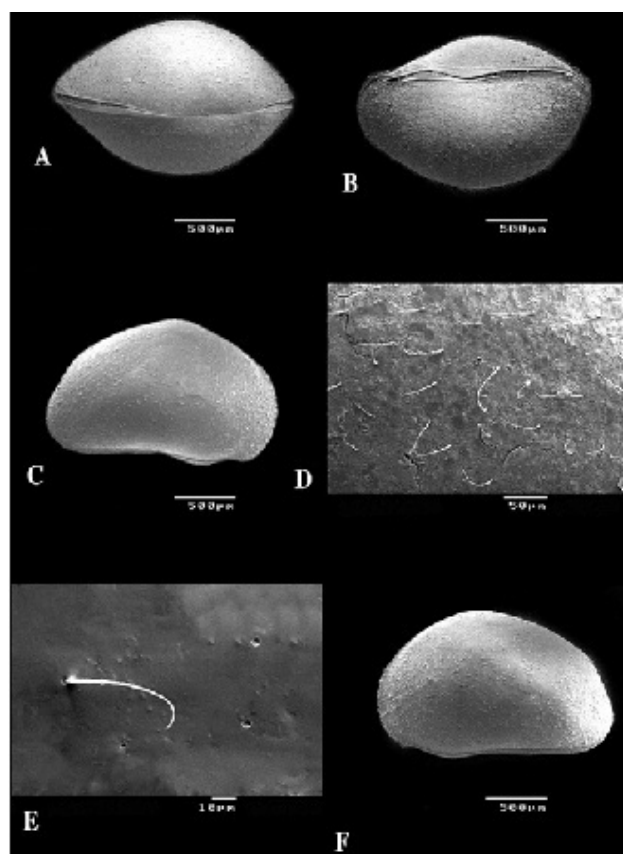


Fig. 2. Scanning electron microscope of *Cypris pubera* female displaying; (A) the dorsal margin of the carapace, (B) the ventral margin of carapace, (C) the external view of right valve, (D) the pore canals with sensillae, (E) enlargement of (D) and (F) the external view of left valve.

Internally: In the right valve (RV), the selvage suggestively banished inward along anterior antero-ventral and postero-ventral margins (Fig. 3A). The majority part

of anterior and postero-ventral margins with valve margin defined by a flange (Fig. 3A). The inner list is present and readily seen at the antero-ventral and postero-ventral portion of the shell. There are a few tiny denticles on the right valve's posterior margin, and the valve's postero-ventral region has a longer, blunt spine (Fig. 3B).

The selvage in the left valve (LV) is outlying along the remaining valve borders and shifted inward at the anterior and posterior margins. Well-developed inner list that is near the inner margins (Fig. 3C).

First antenna (A1) (Figs. 3D, 5A) composed of eight podomeres; the first two of which fused to form a large base with pilose ventral margin. The broad base bearing one pilose dorso-median seta, and two uneeven extended ventral setae (one of them is plumose). The Rome organ (RO), which developed from lobe-like structure on the ventral side of the third segment, is short and bears a short seta and dorso-distal smooth. There are smooth ventro-distal setae and pilose dorso-distal setae in the fourth segment. The fifth segment has two smooth, long dorso-distal setae and two smooth, short, ventro-distal setae. Three very long, plumose setae and one medium-length, smooth seta are present in the sixth podomere. The elongated seventh segment features four long, plumose setae and a very short, sinuous seta α . A smooth ventro-distal seta, two lengthy plumose setae, and an aesthetasc ya are all present on the eighth segment, which has a slender shape.

Second antenna A2 (Figs. 3D,E, 5B): The first segment has three unequal setae among them a pilose seta. The next segment has a long ventro-distal seta. Exopodite has three unequal setae, the elongate one is attainment almost half of penultimate segment. The first endopodal podomere carries many developed tiny setae on the dorsal and ventral margins. Five plumose dorso-distal setae on the first segment of endopodite, which extend well beyond the ends of the distal claws, and one short natatory setae are also present. Besides, has aesthetasc Y short spoon-like seta situated behind mid-length of the ventral margin of the first endopodal segment and plumose long ventro-distal seta extending to basal part of terminal segment. The second endopodal (penultimate segment) segment with two unequal smooth dorso-median setae, very fleeting aesthetic y1 is located behind the t-setae. Also, the penultimate segment carries four plumose ventro-median t-setae, one of them is short. The distal margin of the second podomere of endopododite bears three smooth z-setae, three long, strong serrated claws (G1, G2, G3) and short, smooth aesthetic y2. The distal podomere is dumpy and narrow, it bearing two distal serrated claws (GM, Gm) and a smooth long aesthetic y2.

Table II. Estimates of evolutionary divergence between sequences of COX1 based pairwise genetic distances for *Cypris pubera* Saudi Arabia (PQ533181.1).

	<i>Cypris pubera</i> (Saudi Arabia) (PQ533181.1)	<i>Cypris pubera</i> (Spain) (MT024244.1)	<i>Cypris pubera</i> (China) (PP999775.1)	<i>Cypris pubera</i> (Canada) (HM425332.1)	<i>Cypris pubera</i> (China) (PP999776.1)	<i>Cypris pubera</i> (Canada) (MG320478.1)	<i>Cypris pubera</i> (China) (PP999823.1)
<i>Cypris pubera</i> (Saudi Arabia) (PQ533181.1)							
<i>Cypris pubera</i> (Spain) (MT024244.1)	0.0020						
<i>Cypris pubera</i> (China) (PP999775.1)	0.0000	0.0020					
<i>Cypris pubera</i> (Canada) (HM425332.1)	0.0020	0.0041	0.0020				
<i>Cypris pubera</i> (China) (PP999776.1)	0.0010	0.0030	0.0010	0.0030			
<i>Cypris pubera</i> (Canada) (MG320478.1)	0.0030	0.0051	0.0030	0.0030	0.0040		
<i>Cypris pubera</i> (China) (PP999823.1)	0.0505	0.0530	0.0505	0.0506	0.0517	0.0492	

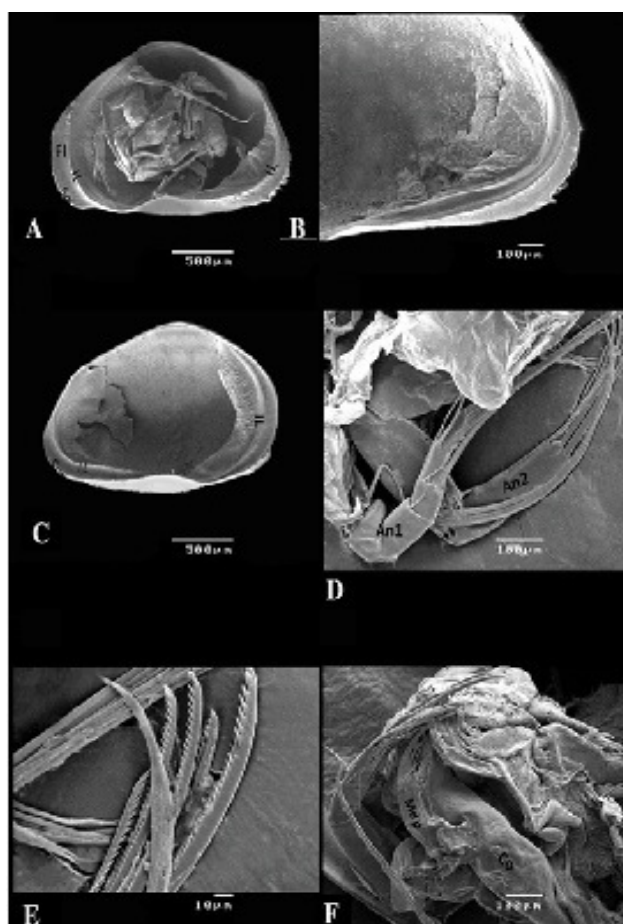


Fig. 3. Scanning electron microscope of *Cypris pubera* female showing: The internal view of right valve (A), enlargement of posterior margin of right valve (B), the internal view of left valve (C), first and second antennae (D), enlargement of distal claws of second antenna (E), and mandible (F).

Mandibula (Fig. 3F, 4A,B, 5C-E) consists of mandibular palp and mandibular coxa; the mandibular palp composes of four segments. The first segment bears dorso-proximal vibratory plate with 6+1 wide rays. Furthermore, the ventro-distal margin of the first segment carries robust, plumose S1 and S2 setae, a smooth seta and setae α fine and long, reaching to nearly mid-length of S1. The second segment of mandibular palp has three unequal dorso-distal setae. The ventro-distal margin of the second segment bears four smooth setae extending to about end of terminal segment, associated with a long pilose seta and dumpy pilose seta β . The third podomere covered with many minute setae on dorsal and interior margins. It carries four long dorso-distal setae, strong, serrated seta γ located on central surface. Moreover, five uneven smooth ventro-distal setae. The distal podomere bears four smooth claws. Mandibular coxa provided with twelve terminal strong teeth arranged in two rows and seven smooth setae between them. Besides, coxa bears dorsal pilose seta and four ventro-distal setae (one of them is strong, long and serrated at one side).

Maxillula (Figs. 4C, D; 5A, B) consists of three endites and maxillary palpation. Maxillary palpation composes of two-segmented; the first segment has 7 setae, one of them is short and smooth. The second segment is cylindrical in shape with terminal four smooth setae. The first endite bears two smooth dorso-median setae and one pilose ventro-median. Also, the terminal margin of this endite bears three smooth setae and two tooth bristles strongly serrated distally. The second and third endites bear many of terminal, smooth setae and two ventro- proximal pilose seta on the third endite.

First thoracic leg (Fig. 4D, 6C) composed of endopod, respiratory platter and protopd. The endopod bears three uneven terminal setae (1 pilose and 1 long,

smooth), respiratory platter has five long plumose rays, and a short pilose seta, protopodite has two stout a-setae, b and d pilose setae, apart from one of the apical brushes of c. Above a shorter one closer to the d-seta, there are thirteen almost equal setae.

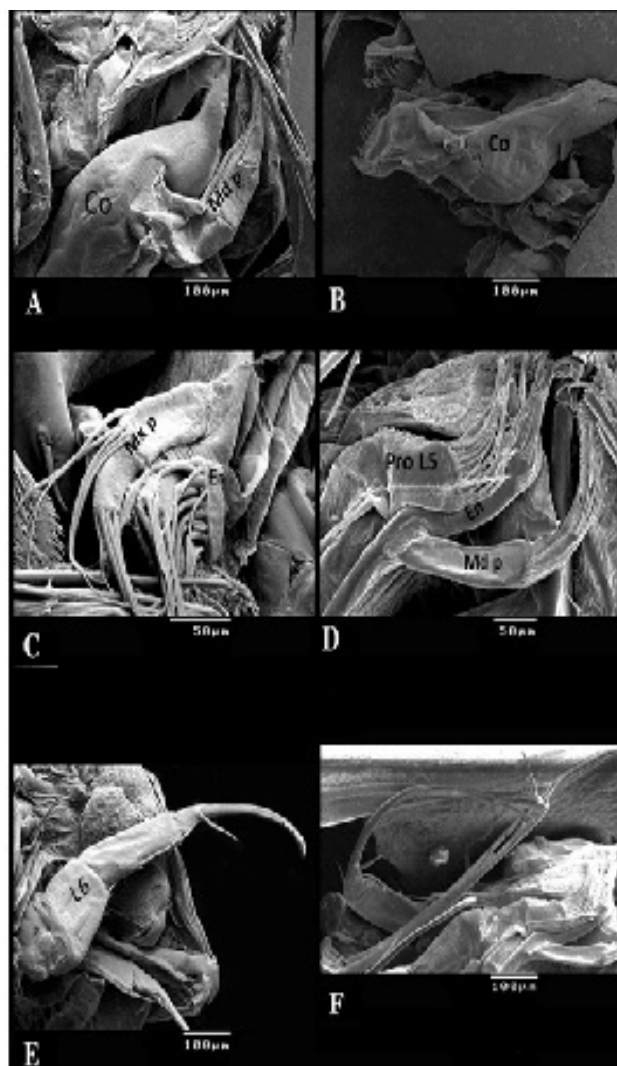


Fig. 4. Scanning electron microscope of *Cypris pubera* female showing enlargement of mandible (A), enlargement of coxa of mandible (B), maxillary palp and endites (C), mandibular palp, endites and protopodite of L5 (D), enlargement of L6 (E), and caudal ramus (F).

Second thoracic leg (Figs. 4E, F, 6D) L6 is tough and consisting of four segments. The first segment is short, narrow and anchoring structure with sclerotization. It has both short pilose seta d2 and long pilose seta d1 on it. The second segment is strong carrying long many dorsal and ventral tiny setae and ventro-distal pilose seta e. The third

segment is elongate and subdivided bearing many tiny dorsal and ventral setae. Also, it is carrying ventro-median pilose seta f and ventro-distal short pilose seta g. The fourth segment is stout and sub-triangular in shape, with a pilose seta h1, toughly saw-like claw h2, and smooth seta h3.

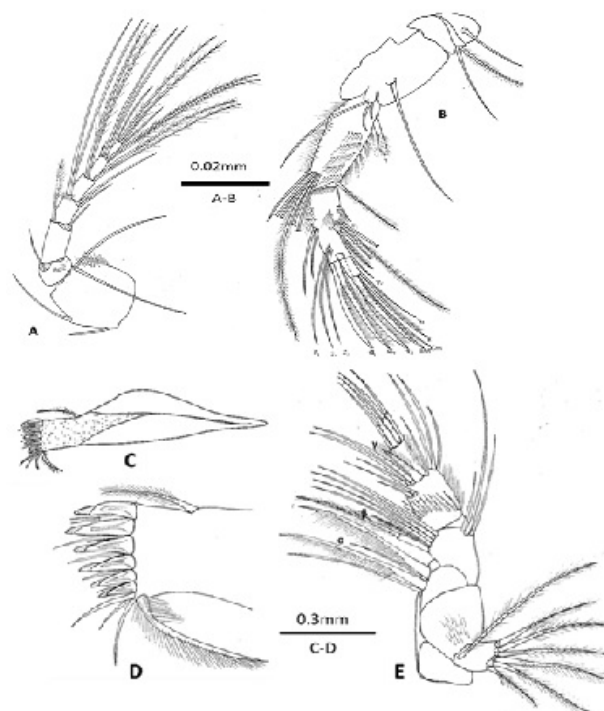


Fig. 5. Camera lucida diagram of *Cypris pubera* showing; (A), first antenna, (B) second antenna, (C) coxa of mandible, (D) enlargement of coxa, (E) mandibular palp.

Third thoracic leg (Fig. 7A, B) composed of three podomeres. The first one has unequal smooth, elongate three setae dp, d1 and d2. The most long segment is the second one, which has dorso-distal plumose seta that reaches the terminal segment's near distal end. The third segment carries short, pilose seta and advanced pincer organ.

Ur ramus (Figs. 4F, 7C) is thin and lengthen, has many tiny setae on its ventral margin. It bears unequal long serrated two laws Ga and Gp. Moreover, it bears apical smooth Sa seta and pilose Sp seta located adjacent to claws Ga and Gp. Curved main Ur attachment branch with short, two-thirds-length ramus-originating dorsal branch.

Molecular analysis

Gel electrophoresis showed the band of PCR product for COX1 was around 800 bp. The Blast analysis showed that the obtained sequence of COX1 for *Cypris pubera* with accession number (PQ533181) in Saudi Arabia

showed similarity with sex species of *Cypris pubera* which previously recorded in Spain (MT024244.1) (99.8%), China (PP999775.1) (99.65%), Canada (HM425332.1) (99.52 %), China (PP999776.1) (99.48 %), Canada (MG320478.1) (99.22 %), and China (PP999823.1) (91.8%). The genetic distances between *Cypris pubera* and the references that are available in the GenBank database were determined using COX1 pairwise (Table II). The phylogenetic tree was employed to estimate the degree of similarity between *Cypris pubera* (PQ533181) in Saudi Arabia and its GenBank references (Fig. 8). The phylogenetic tree (Fig. 8) showed a cluster which appeared as a single taxon and contain *Cypris pubera* in Saudi Arabia (PQ533181)(0.0000), Spain (MT024244.1)(0.0037), China (PP999775.1)(0.0000), and China (PP999776.1)(0.0019). Two separated clusters which contain *Cypris pubera* from Canada (HM425332.1)(0.0019) and (MG320478.1) (0.0037). The last cluster which is faraway from other clusters and contain *Cypris pubera* (China) (PP999823.1). The phylogenetic tree's findings support the molecular identification of *Cypris pubera* in the sample under study.

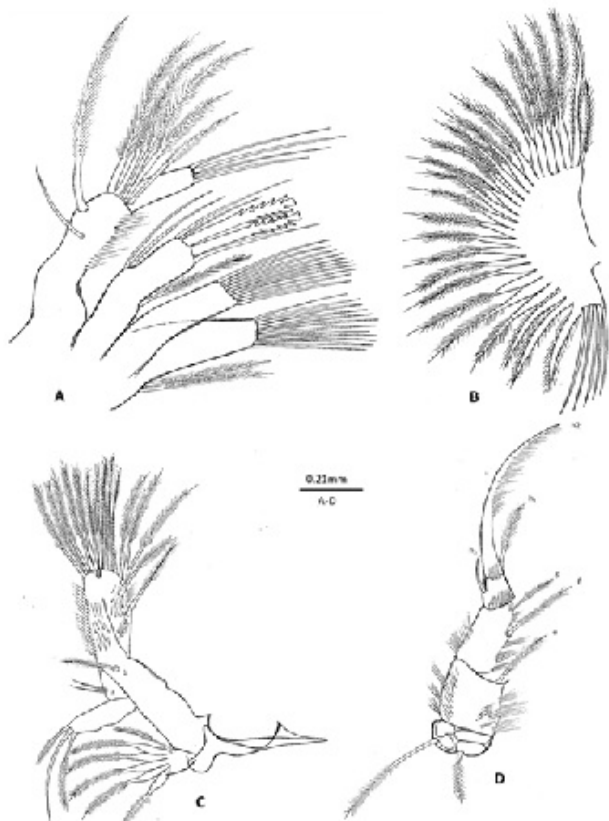


Fig. 6. Camera lucida diagram of *Cypris pubera* showing; (A) maxillary palp and three endites, (B) branchial plate, (C) first thoracic leg L5, (D) second thoracic leg L6.

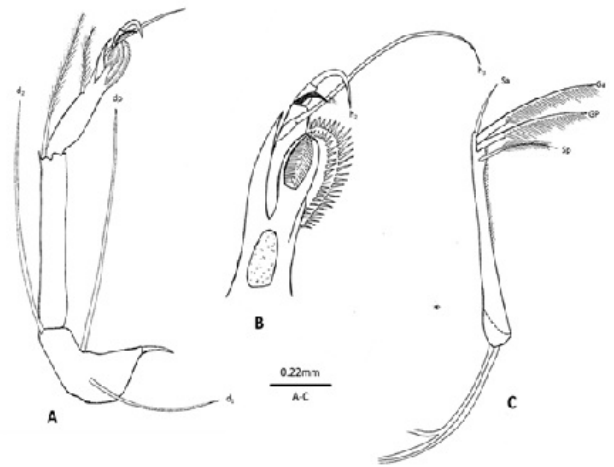


Fig. 7. Camera lucida diagram of *Cypris pubera* showing; (A) third thoracic leg L7, (B) enlargement of distal portion of L7, (C) caudal ramus.

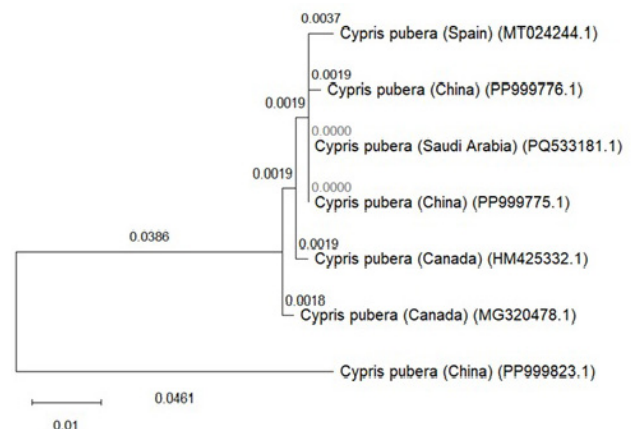


Fig. 8. Phylogenetic tree of *COX1* gene sequences for *Cypris pubera* in the Saudi Arabia (PQ533181.1) and its related species obtained from the GenBank/NCBI database.

DISCUSSION

According to the Linnean system, the *Cypris* is thought to be the most ancient ostracod generic name. O. F. Müller initiated the genus *Cypris* to embrace the bivalved *Monoculus* species that Linnaeus had described (Meisch, 2000). *Cypris pubera*, originally known as *Monoculus concha pedata* (later changed to *Monoculus conchaceus*), was the first ostracod species to be named using the Linnean binomial system of classification (Meisch, 2000). The highest diversity of genus *Cypris* in afrotropical and Asian areas (Meisch *et al.*, 2019). Our studied species *Cypris pubera* was collected from AlUla,

Saudi Arabia and has geographical parthenogenesis. This is consistent with (Meisch, 2000; Karanovic, 2012; Martens *et al.*, 2013), who studied the parthenogenesis ostracods *C. pubera* and *C. decaryi* in afrotropical and Oriental regions. Also, central, northern, and eastern Europe have a wider distribution of the most prevalent *C. pubera*. Additionally, it has been found in China (Yu *et al.*, 2009), New Zealand (Eagar, 1994), and South America (Ramón-Mercau *et al.*, 2012). In Canada, it is highly prevalent (Delorme, 1970).

Numerous ostracod species in the *Cypris* were eventually dismissed as juvenile equivalents of other species or as outdated or undefined (Meisch *et al.*, 2019). For example, *Cypris triaculeata* (Daday, 1892) which is thought to be a synonym for *C. pubera*. Furthermore, Fischer (1851) had already concluded that *C. striata*, as reported in Jurine (1820), truly matched *C. pubera* juveniles. Therefore, in terms of developing a comprehensive knowledge of its diversity, *Cypris* remains a dynamic genus, and its taxonomy appears to be far from finalised.

According to Mesquita-Joane *et al.* (2020), the type species *C. pretusi* sp. of the genus *Cypris* is the most similar to *C. pubera*. They are similar in the following: The two valves with a significant inward displacement of the anterior selvage, the left valve anteriorly with an inner list that is well developed, the left valve plus an extra inner list. Also, the right valve with an anteroventral edge that resembles a lip, the left valve overlaps the right valve and the second thoracic leg (walking leg (L6) composed of 4-segments. However, they differ in that *C. pubera* has a row of obvious denticles in the frontal portion of the valves and several bigger bristles on the posterior border. Also, the denticles near the posterior border of *C. pretusi* are all small and subequal, while the anterior portion lacks any visible denticles or only possesses very little ones. In contrast, the valves of *C. pretusi* are asymmetrical and more obviously beak-like. The dorsal seta on the third podomere of first antenna of *C. pretusi* is hirsute, while it was smooth in *C. pubera*. The natatory setae of An2 of *C. pretusi* and *C. pubera* not getting to the terminal claw tips.

Approving to Karanovic (2012) the New Zealand species, *C. kaiapoinensis* Chapman (1963), exhibits the same outer margin denticles as *C. pubera* of Müller 1776. *Cypris maculosa* is regarded as a synonym for *Cypris pubera*, according to Martens *et al.* (2013), however other publications recognised them as two separate species (Meisch *et al.*, 2019; Mesquita-Joane *et al.*, 2020). Because *C. maculosa*'s carapace lacks spines, we generally believe that they are not the same species. The sister species of *C. pubera*, *C. triaculeata*, is primarily found in central Europe.

At the present study, the current species *Cypris*

pubera live in rainwater from Al-Ward rainwater area (tropical region), nearly 100 km from Al-Ula Governorate, Kingdom of Saudi Arabia. According to Meisch (2000) and Martens *et al.* (2013) the Oriental and Afrotropical regions seem to be habitat to the majority of *Cypris* species. Most *Cypris pubera* are widely distributed in eastern, northern and central Europe and Canada (Delorme, 1970). Also, Ramón Mercau *et al.* (2012) observed *Cypris pubera* in South America.

Daday (1892, 1900) identified five forms of *Cypris pubera*, primarily based on the development and presence of spines on the the posteroventral margine of right valve as follow: There are no spines on anacantha, one on monacantha, two on diacantha, three on triacantha, and more than three on polyacantha. We think these variations reflect the morphological variations of the main species. We hypothesised that these variation were due to fully parthenogenetically.

On the third podomere of the mandibular palpation of *C. pubera*, we observed four sub-distal setae, which is consistent with Mesquita-Joane *et al.* (2020) in *C. pretusi* sp. nov., *C. decaryi*, *C. bispinosa*, and *C. pubera*. Also, Neale (1976a, b) founded four setae in *C. granulata*. Whears, Martens(1990) noted only three in *C. pubera* and Neale (1976a, b) observed three setae in *C. decaryi*. Adult species of closely related genera, including *Eucypris virens* (Aguilar-Alberola and Mesquita-Joanes, 2013), *Heterocypris bosniaca* (Petkowski *et al.*, 2000), and *Heterocypris salina* (Kubanc *et al.*, 2007), possess four setae on the third podomere of the mandibular palp's sub-distal border. It's suspected that all adult stages species of *Cypris* have four subapical setae of the third podomere of the mandibular palp; as a result, the literature that only display three setae may represent juvenile stages. To support that, Yousef *et al.* (2024) studied the ontogeny of *C. pubera* and noticed three sub-distal setae on the third podomere of the mandibular palp in the eighth and seventh juveniles and four setae in adult stage. In our results, the dorso-distal margin of the third segment of the mandibular palp bears four unequal smooth setae. Agree with, Mesquita-Joanes (2020) who noted four setae in *Cypris pretusi* and *Cypris pubera*. Although, Martens (1990) recorded only three setae in *Cypris pubera*. Besides Neale (1976) observed three setae in *Cypris decaryi*. Moreover, Aguilar-Alberola and Mesquita-Joanes (2013) and Ali *et al.* (2018) figured four setae in *Heterocypris bosniaca* and *Heterocypris salina*, in that order. The molecular identification of the current studied species *Cypris pubera* is confirmed by DNA barcoding using COX1. Prvious work confirms the use of DNA barcoding for molecular identification of ostracoda (Alansari *et al.*, 2024). The phylogenetic tree based on the COX1 showed low reflecting the decreased

genetic distances between *Cypris pubera* from Saudi Arabia and from Spain or from China. The current study used DNA barcoding and scanning electron microscope to identify for the first time *Cypris pubera* from non-marine water of Saudi Arabia. COX1 sequencing is a good marker for molecular identification of *Cypris pubera*. This study contributes new species to the Saudi Arabian water environment's biodiversity list.

DECLARATIONS

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Ethical statement

The experiment was conducted in accordance with the ethical guidelines (approval number: CSRE-12-23) by the Faculty of Science at Sohag University in Egypt.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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