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ORIGINAL ARTICLE

# First molecular evidence for two new associate copepods of genus *Clausidium* Kossmann, 1874 (Copepoda: Cyclopoida: Clausidiidae) from the Persian Gulf and Gulf of Oman

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## ABSTRACT

*Clausidium* Kossman, 1874 is a genus of copepods that is found in subtropical to temperate coastal areas. All species of the genus occur on the bodies of mud shrimp of the families Callianassidae and Upogebiidae. Based on morphological data from light scanning and confocal laser scanning microscopy, there are four species of *Clausidium* copepod in Iran. In this study we address *Clausidium iranensis* Sepahvand, Kihara and Boxshall, 2019 and *Clausidium persiaensis* Sepahvand and Kihara, 2017 that were reported on the body of the burrowing shrimps *Neocallichirus jousseaumei* (Nobili, 1904) and *Callianidea typa* Milne Edwards, 1837, respectively. We undertook analyses of mitochondrial DNA gene sequences (CO1) to evaluate taxonomic status and taxonomic relationships of the *Clausidium* species. The result demonstrates that two major clades, with strong support, can be identified within the *Clausidium* copepods in the southern waters of Iran, representing distinct taxonomic entities at the species rank. Our data indicate that CO1 can be a powerful tool for species identification and delimitation. In the case of *Clausidium* copepods, the general utility of CO1 for taxonomic relationship inferences within a genus or a family is still under investigated. Our study adds the first genetic data from these copepods from the Persian Gulf and Gulf of Oman.

## KEYWORDS

CO1, Copepoda, DNA barcoding, north-west Indian Ocean

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## Introduction

Biological research, including biodiversity and ocean monitoring, require accurate species records and continuous observation (DeSalle and Amato, 2004). Traditionally, the biodiversity of marine habitats has been examined using morphological identification, needing specialized taxonomic expertise. Furthermore, morphological approaches lack the resolution to identify cryptic taxa or deal with morphological plasticity (Hebert *et al.,* 2004; Pfenninger *et al.*, 2006). Integrating data from multiple lines of evidence will support a more reliable species delineation as the baseline for subsequent biodiversity evaluations (Dayrat, 2005). Molecular approaches have been broadly used to provide a faster and more precise species identification for many taxa (Tautz *et al.,* 2003; Vogler and Monaghan 2007; Pereira *et al.*, 2008).

In spite of the recent progress in other diverse genetic and genomic indicators, the barcode region of the mitochondrial cytochrome c oxidase subunit I (CO1) gene remains a useful, and in some cases unique, diagnostic character for species-level identification of copepods (Blanco-Bercial *et al.,* 2014).

Ghost shrimps or burrowing shrimps, representatives of the infraorders Axiidea and Gebiidea, are among the most common benthic macro-invertebrates in coastal regions of the Persian Gulf and Gulf of Oman (Sepahvand *et al.*, 2013). These cryptic shrimps are adapted to a burrowing lifestyle and their burrows can also be occupied by a variety of organisms, including copepods (Dworschak *et al.*, 2012). *Callianidea typa* Milne Edwards, 1837 and *Neocallichirus jousseaumei* (Nobili, 1904) are two widely distributed burrowing shrimps in the southern waters of Iran that are reported as hosts for *Clausidium* Kossman, 1874 copepods (Sepahvand *et al.*, 2017; 2019). Copepods of the genus *Clausidium* are external associates of burrowing decapods of the families Callianassidae and Upogebiidae (Kihara and Rocha, 2013).

Most clausidiid copepods live in loose association with marine invertebrate hosts (Huys and Boxshall, 1991), and species of *Clausidium* are recorded exclusively living in association with ghost shrimps (Boxshall and Halsey, 2004). Although it has been suggested that members of *Clausidium* are parasitic on their hosts (Wilson, 1935; Pillai, 1959), this relationship has yet to be conclusively demonstrated (Hayes, 1976). There is very scarce available documentation on the biology of these copepods, or their interactions with their host, or with the environment. Although *Clausidium* species are rarely recorded because of the cryptic lifestyle of their hosts, a total of 18 species of *Clausidium* have been described to date and it is hypothesized that each species shows a preference for a specific host Sepahvand *et al.* (2019).

The only available molecular study on *Clausidium* was carried out by Huys *et al.* (2012). In that study, the 18S rRNA gene, in combination with morphological features, were investigated in an integrative approach to study a Clausidiiform complex (Huys *et al.,* 2012). Here, we use CO1 gene sequences to delineate species boundaries and find cryptic diversity within *Clausidium* in the Persian Gulf and the Gulf of Oman.

## Materials and Methods

### *Sample collection*

Copepods specimens were obtained from the body of *N. jousseaumei* and *C. typa* (Fig. 1) from the Iranian coast of the Persian Gulf and Gulf of Oman (Fig. 2). Specimens were preserved in 96 % ethanol at the sampling site and after 24 h, these specimens were transferred to fresh 96 % ethanol at the Iranian National Institute for Oceanography and Atmospheric Science (INIOAS). *Clausidium* copepods were later sorted using a microscope-mounted camera at the German Center for Marine Biodiversity Research (DZMB) in Wilhelmshaven, Germany. Photographs of 96 selected specimens in ethanol were obtained using a camera for further morphological identification. Individuals were preserved in 96 % ethanol and stored at -20 °C, as morphological vouchers for future reference. Specimens were identified to species level using diagnostic morphological characters based on the identification key of Kihara and Rocha (2013).

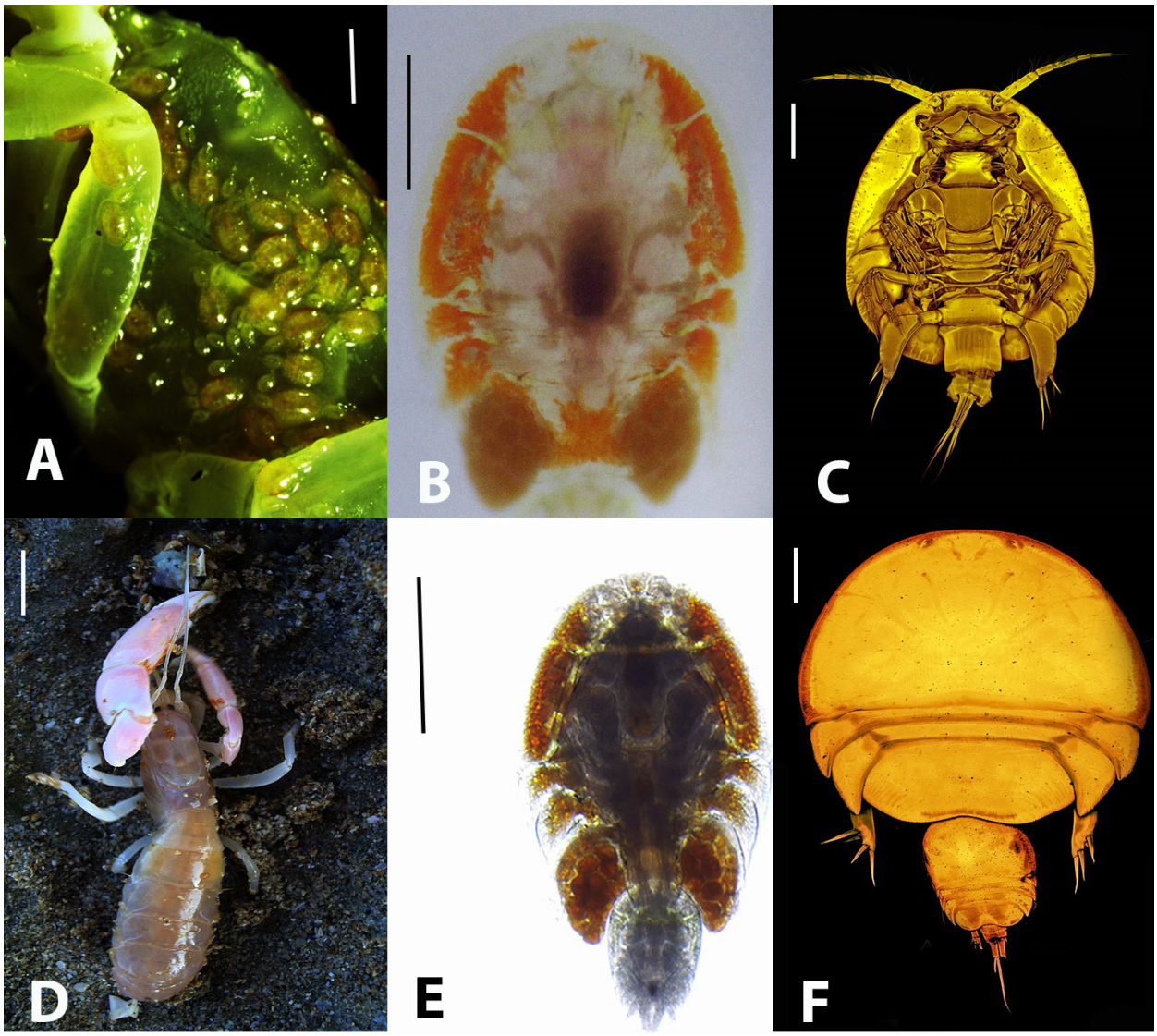


**Figure 1.** **A**, *Callianidea typa*, lateral view of cephalothorax with copepods; **B**, *Clausidium persiaensis*, habitus, ventral view, female; **C**, *C. persiaensis*, confocal laser scanning microscopy maximum projections; **D**, *Neocallichirus jousseaumei*, habitus, dorsal view; **E**, *Clausidium persiaensis*, habitus, ventral view; **F**, *C. iranensis*, confocal laser scanning microscopy maximum projections, dorsal view. Scale bars: **A**, 1 mm; **B**, 0.5 mm; **C**, 100 µm; **D**, 1 cm; **E**, 1 mm; **F**, 100 µm.

*DNA extraction and gene amplification*

DNA extractions from 63 selected morphologically identified specimens stored at -20 °C before use, were carried out using the 30–40 µl Chelex (InstaGene Matrix, Bio–Rad) protocol. The primers LCO1490: 5' -GGTCAACAAATCATAAAGATATTGG-3' and HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al.*,1994) were used to amplify the CO1 gene. PCR cycles consisted of an initial denaturation at 95 °C for 5 min, followed by a denaturation at 95 °C for 30 s, annealing at 45 °C for 1 min, and extension at 72 °C for 1 min, for 60 cycles and a final elongation 72 °C for 7 min. The number of cycles was decreased if the concentration of PCR product reached an optimum. The PCR was performed using IllustraPuReTaq Ready–To–Go PCR Beads (GE Healthcare) in 25 µL volume containing 22 µL H2O, 0.5 µL of each primer (10 pmol/µL) and 2 µL of DNA templates. All PCR products were checked by electrophoresis on a 1 % agarose/TBE gel containing 1 % GelRed. PCR product purifications and sequencing was carried out by Macrogen (Amsterdam, Netherlands). All the COI sequences are deposited in GenBank and accession numbers are provided in Tab. 1.

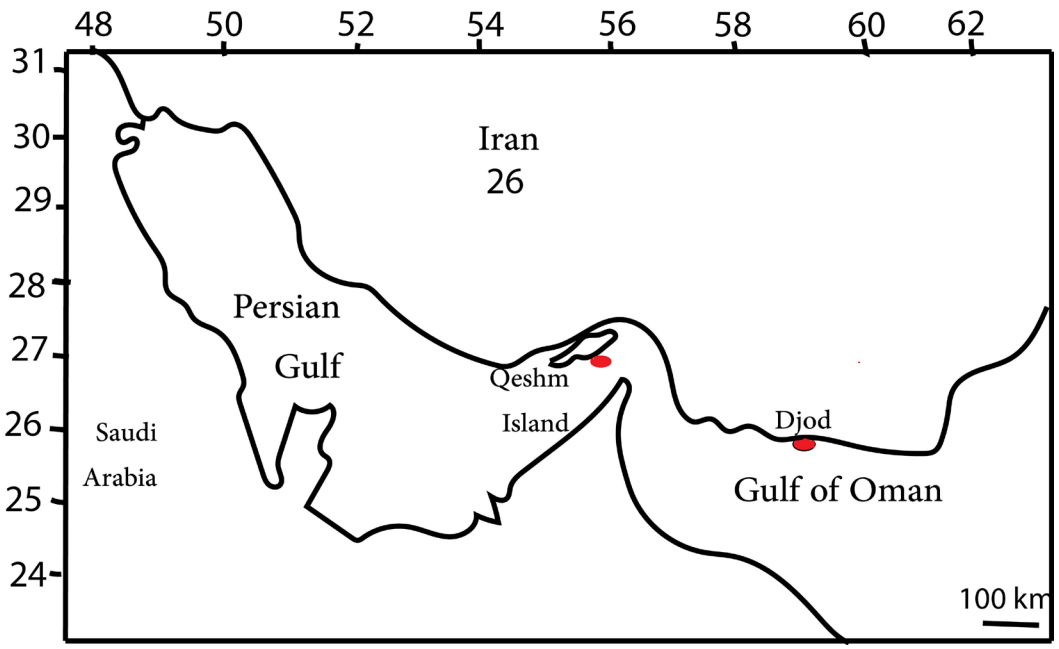


**Figure 2.** Map of sampling stations in the Persian Gulf and the Gulf of Oman.

**Table 1.** Sequence accession numbers in NCBI, host of *Clausidium* copepods, and locality of sample collection.

| Species | Sample code | Accession numbers in GenBank | Host | Locality |
|---|---|---|---|---|
| *C. persiaensis* | q25 | SUB8380602 q25 MW175444 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q4 | SUB8380602 q4 MW175445 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q2 | SUB8380602 q2 MW175446 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q27 | SUB8380602 q27 MW175447 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q8 | SUB8380602 q8 MW175448 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q10 | SUB8380602 q10 MW175449 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q7 | SUB8380602 q7 MW175450 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q14 | SUB8380602 q14 MW175451 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q24 | SUB8380602 q24 MW175452 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q26 | SUB8380602 q26 MW175453 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q1 | SUB8380602 q1 MW175454 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q21 | SUB8380602 q21 MW175455 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q12 | SUB8380602 q12 MW175456 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q5 | SUB8380602 q5 MW175457 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. persiaensis* | q22 | SUB8380602 q22 MW175458 | *Callianidea typa* | Persian Gulf, Qeshm Island |
| *C. iranensis* | J10 | SUB8380602 J10 MW175459 | *Neocallichirus jousseaumei* | Gulf of Oman, Djod |
| *C. iranensis* | J12 | SUB8380602 J12 MW175460 | *Neocallichirus jousseaumei* | Gulf of Oman, Djod |
| *C. iranensis* | J4 | SUB8380602 J4 MW175461 | *Neocallichirus jousseaumei* | Gulf of Oman, Djod |
| *C. iranensis* | J2 | SUB8380602 J2 MW175462 | *Neocallichirus jousseaumei* | Gulf of Oman, Djod |
| *C. iranensis* | J8 | SUB8380602 J8 MW175463 | *Neocallichirus jousseaumei* | Gulf of Oman, Djod |
| *C. iranensis* | T24 | SUB8380602 T24 MW175464 | *Neocallichirus jousseaumei* | Gulf of Oman, Djod |
| *C. iranensis* | J6 | SUB8380602 J6 MW175465 | *Neocallichirus jousseaumei* | Gulf of Oman, Djod |

*Genetic analyses*

Sequences of CO1 were aligned using Clustal W, as implemented in the Bioedit software version 7.0.5.3 (Hall, 1999). The DNA substitution model of TIM1+I+G (AIC = 6054.89, –lnL = 2967.44, k = 60, p-inv = 0.43, gamma shape = 0.73) was estimated using the Akaike information criterion run in jModelTest, version 0.1.1 (Posada, 2008). Taxonomic

relationships were reconstructed using Maximum Parsimony method in MEGAX (Kumar *et al.*, 2018), the Maximum Likelihood method implemented in PHYML version 2.4.4 (Guindon and Gascuel, 2003) and a Bayesian Inference tree in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Bayesian Inference was performed with two simultaneous runs and four search chains within each run (three heated chains and one cold chain) for 10,000,000 generations, sampling trees every 1000 generations using the Markov chain Monte Carlo method. Reliability of nodes was assessed using 1000 bootstrap replications for all methods (Felsenstein, 1985). Genetic distances were calculated to quantify sequence divergence between species using Kimura's (Kimura, 1980) two-parameter (K2P) model by MEGAX (Kumar *et al.*, 2018). Genetic diversity was measured for each species based on haplotype diversity (Hd) and nucleotide diversity ($\pi$). Values for the number of polymorphic sites, parsimony informative sites, haplotype frequencies, and the average number of nucleotide differences between sequences were estimated. These genetic diversity values were computed using the software DnaSP v5.0 (Librado and Rozas, 2009). Taxonomic relationship networks were calculated using the software PopArt (Leigh and Bryant, 2015; http://popart.otago.ac.nz).

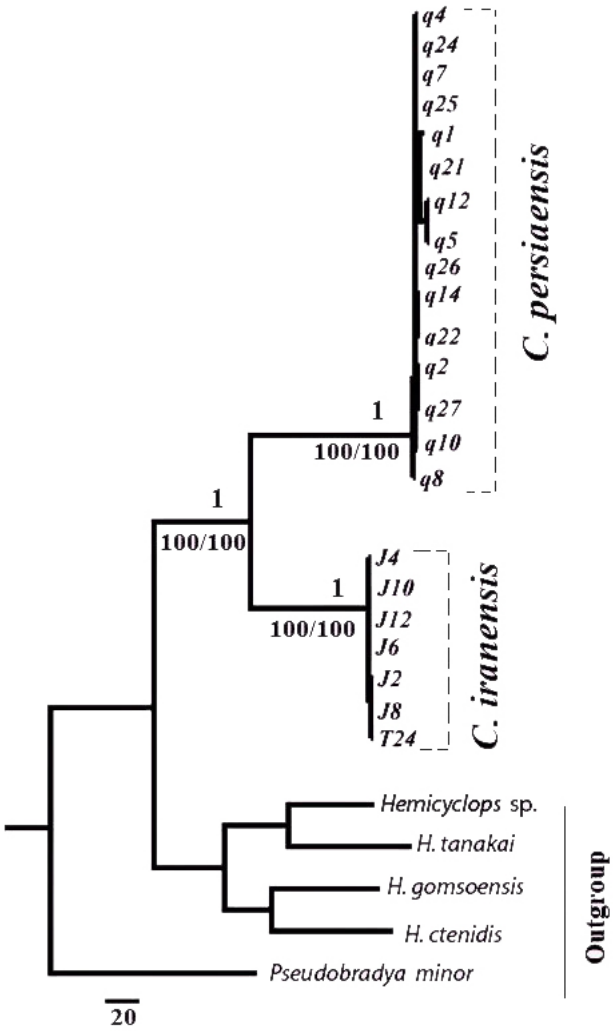


**Figure 3.** Maximum Likelihood (ML) gene tree based on partial CO1 gene sequences of the *Clausidium* species from the Persian Gulf and the Oman Gulf. The average branch lengths are proportional to the number of substitutions per site. Numbers above the nodes indicate Bayesian posterior probabilities and numbers below the nodes represent bootstrap support values for MP/ML (1000 replicates).

## Results

Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian Inference (BI) based on CO1 sequences obtained trees with similar topologies (with minor changes), each confirming the taxonomic status of two recently described morphospecies of *C. persiaensis* and *C. iranensis* as distinct lineages. The species were represented by monophyletic clades on all the taxonomic relationship analyses. Moreover, in all trees, *Clausidium* grouped into two major clades: one clade included sequences from Qeshm Island in the Persian Gulf as *C. iranensis*, while the other included samples from the Oman Gulf as *C. persiaensis*. Monophyly of these clades was strongly supported by BI posterior probability, MP, and ML bootstrap values (1/100/100 respectively for all the *Clausidium* clades) (Fig. 3).

The rate of variation between sites was modeled with a gamma distribution (shape parameter = 1). The analysis used 27 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding and all positions containing gaps and missing data were eliminated. There was a total of 645 positions in the final dataset. The mean genetic divergence (%) in the CO1 gene sequences between the species of the genera *Hemicyclops* Boeck,1872, *Clausidium*, and *Pseudobradya* Sars G.O., 1904 are shown in Tab. 2.

**Table 2.** The percentage of mean genetic divergence in the CO1 gene between the species of the genera *Hemicyclops*, *Clausidium*, and *Pseudobradya*. Numbers in upper right (Bold) are P-distances and in lower left are Kimura's two-parameter (K2P) distances.

| Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| *1-Hemicyclops ctenidis* | | 14.7 | 18.1 | 15.3 | 20 | 22.9 | 22.2 |
| *2-Hemicyclops gomsoensis* | 18.8 | | 16.2 | 15.9 | 20.2 | 23.5 | 21.8 |
| *3-Hemicyclops tanakai* | 24.5 | 21.4 | | 13.4 | 22.3 | 24.1 | 24.5 |
| *4-Hemicyclops* sp. | 19.6 | 20.7 | 16.6 | | 18.9 | 23.6 | 21.1 |
| *5-Clausidium iranensis* | 28.4 | 32.8 | 32.8 | 26 | | 24.2 | 18.2 |
| *6-Pseudobradya minor* | 34 | 35.5 | 36.3 | 35.7 | 36.6 | | 27.1 |
| *7-Clausidium persiaensis* | 32.6 | 31.8 | 37.7 | 30 | 24.8 | 43.5 | |

The Kimura's (1980) two-parameter (K2P) genetic distance between *C. persiaensis and C. iranensis* was 24.6 % and between these two species and outgroups ranged from 26 to 43.5 %. The mean genetic p- distance between the two species was 18.2 %. The mean genetic divergence (%) within *C. persiaensis* and *C. iranensis* were 0.44 % and 0.13 %, respectively. K2P interspecies genetic distances within genus *Clausidium* (between *C. persiaensis* and *C. iranensis*) were 99.2 times higher than the mean intraspecific genetic divergence.

Based on the 648 bp of CO1 examined in *C. persiaensis* (n = 15), 12 sites were variable (polymorphic) and 8 sites were parsimony informative, resulting in the identification of 9 haplotypes (Fig. 4). Haplotype diversity (Hd) and nucleotide diversity ($\pi$) were 0.914 and 0.0044 for this species, respectively. Furthermore, based on the 648 bp of CO1 examined in *C. iranensis* (n = 7), 3 sites were variable (polymorphic) and 2 sites were parsimony informative, resulting in the identification of 3 haplotypes. Haplotype diversity (Hd) and nucleotide diversity ($\pi$) were 0.714 and 0.0013 for *C. iranensis,* respectively.

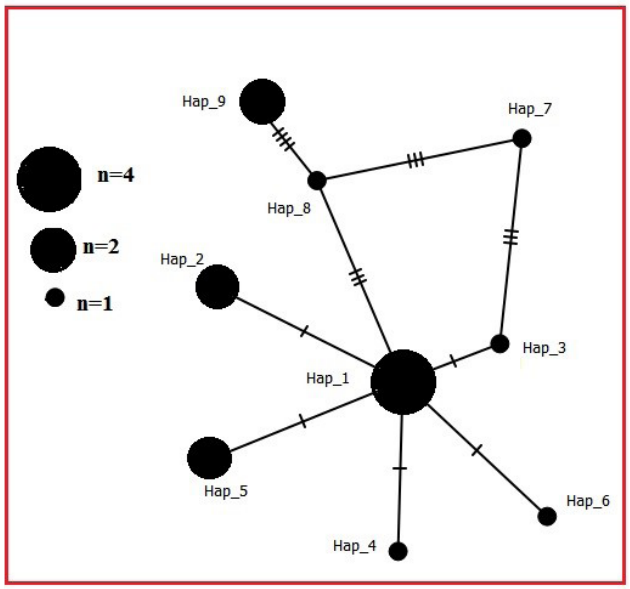


**Figure 4.** 95 % minimum spanning haplotype network of COI haplotypes of *Clausidium persiaensis* from the Persian Gulf, Qeshm Island. The size of the circle is proportional to the frequency of that haplotype.

## Discussion

This study is the first to assess the species delimitation of burrowing shrimp-associated copepods via molecular markers in the Persian Gulf and Gulf of Oman. The results extracted by molecular analysis in the present study absolutely confirmed morphospecies: *C. persiaensis* and *C. iranensis*.

The morphological species concept is most commonly applied in *Clausidium* copepod taxonomy. The molecular sequence data available for *Clausidium* copepods is still very scarce and the relationships within the genera of the Clausidiidae remain elusive, because of the wide host range they utilize and the different morphologies of this group. Copepods of the family Clausidiidae represent an early offshoot of the Poecilostome lineage within the order Cyclopoida Burmeister 1834 (see Khodami *et al.*, 2017). Most clausidiids live in loose association with marine invertebrate hosts (Huys and Boxshall, 1991) and species of *Clausidium* are recorded exclusively living in association with ghost or burrowing shrimps (Boxshall and Halsey, 2004).

*Clausidium persiaensis* and *C. iranensis* can be readily identified based on their morphological characteristics (Sepahvand *et al.*, 2017; 2019). *Clausidium iranensis* shares the armature formula of swimming legs 2 to 4 with *C. persiaensis* but can be easily distinguished by unique characteristics of the

females: the prominent spine on endopodal segment 1 of the antenna, the armature of the maxilliped, and the elongated basis of the swimming legs (Sepahvand *et al.*, 2019). In this study, we were able to show that they are genetically distinct lineages based on the CO1 gene. Moreover, the mean genetic distance between *C. persiaensis* and *C. iranensis* was 99.2-fold higher than the mean intraspecific genetic variation for each species. Our findings, furthermore, emphasize the effectiveness of molecular data, such as CO1 gene sequences, in species delimitation and identification for marine metazoans. However, some drawbacks of using CO1, and mtDNA in general, for species identification, include the possible co-amplification of nuclear mitochondrial pseudogenes (numts) (Song *et al.*, 2008; Hazkani-Covo, 2010), introgression through hybridization or incomplete lineage sorting, and heteroplasmy (Hoeh *et al.*, 1991).

Our taxonomic relationship analyses demonstrated that, two major clades strongly supported by BI posterior probability, MP, and ML bootstrap values (1/100/100 respectively for all *Clausidium* clades) can be identified within *Clausidium* copepods in the southern waters of Iran. Each clade comprises individuals of one of the two morphospecies with substantial genetic divergence between the clades. The degree of genetic divergence between *C. persiaensis and C. iranensis* was 24.8 % and between these two species and outgroups ranged from 26 to 43.5 %. This large genetic divergence taxonomically supports placing the two distinct taxa at the rank of species. Here, we found congruency between morphological and molecular species delimitation of the genus *Clausidium* in Iran.

Despite tremendous effort, we were only able to obtain sequences from two species of four recognized species of *Clausidium* copepods recorded in the Persian Gulf and the Gulf of Oman. This highlights the challenges of collecting *Clausidium* copepods that are only associated with ghost or burrowing shrimps because they have such a cryptic lifestyle and are hard to find. Sampling these copepods is usually very time consuming and expensive. Moreover, extracting high quality DNA from the collected specimens can be daunting.

The lack of a complete DNA barcode library is the most limiting parameter for precise and trustworthy discrimination and documentation of species of copepods. In fact, DNA barcodes are currently available for only ~ 400 copepod species, including many parasitic and freshwater taxa (Blanco-Bercial *et al.*, 2014). In addition, extensive coverage of species diversity is especially critical for efficient resolution in large datasets using automated methods.

In conclusion, our data indicate that CO1 may be a powerful tool for species identification and delimitation. In the case of *Clausidium* copepods, the general utility of CO1 for taxonomic relationship inferences within a genus, or a family, is still under investigation. Hence, our study adds a few, but highly important, data points for future comparative studies.

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