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Research Article

Morphological and Molecular Study of *Dactylobiotus parthenogeneticus (Bertolani, 1982);* A new Species of Freshwater Eutardigrada in Kurdistan Region-Iraq

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Abstract

Dactylobiotus parthenogeneticus was obtained from the outflow stream of Smaquli Dam, located in Erbil Governorate, within the Kurdistan Region of Iraq. During July 2022, a sample collection was conducted at the study site. The collected samples consisted of populations of *D. parthenogeneticus*, which were found in conjunction with green algae. The sampling process involved passing a volume of 50 liters of water through a hand net equipped with mesh components measuring 0.45 µm. The initial characterization of this species was conducted through the examination of its morphological features and measurements, alongside the utilization of DNA barcoding techniques. The acquisition of molecular data was accomplished through the process of sequencing the cytochrome oxidase subunit I (COI) gene. These data were subsequently archived in the GenBank database, where they were assigned the unique accession numbers OR501571. The specimens that were collected were determined to belong to *D. parthenogeneticus* based on the analysis of morphological and molecular analysis. The present observation represents the first documented occurrence of this species within the geographical boundaries of Iraq. Tardigrades offer valuable perspectives on the dynamics of ecosystems, and the interactions between trophic levels, and can serve as indicators of the overall health of an ecosystem and maintaining its balance.

Keywords: *Dactylobiotus parthenogeneticus*, Tardigrade, Molecular analysis, Smaquli Dam, Erbil

Introduction

Tardigrades, also known as "water bears," are a collection of microinvertebrates that typically measure between 100 and 1,000 µm in length. Tardigrades exhibit a body plan consisting of five segments, namely a head, three trunk segments, each possessing a pair of lobe-like legs, and a caudal segment housing a fourth pair of legs. The appendages of freshwater aquatic and limno-terrestrial tardigrades culminate in claw-like structures. The organism's external surface is encased in a chitinous cuticle (Marley et al., 2011; Nelson, Guidetti & Rebecchi, 2015).

Tardigrades exhibit remarkable resilience to a variety of environmental stressors, including but not limited to near-complete desiccation, freezing, high temperatures, ionizing radiation, osmotic shock, and extreme pressure akin to the vacuum conditions encountered in outer space. The organisms are currently engaged in significant survival tactics known as encystment and cyclomorphosis, which enable them to endure adverse environmental circumstances (Carrero et al., 2019; Guidetti & Møbjerg, 2018).

At present, there exists a recognition of more than 1400 nominal taxa across two classes, namely Heterotardigrada and Eutardigrada, which are found in marine, freshwater, and terrestrial environments (Degma et al.,2022; Hesgrove & Boothby, 2020; Nelson et al., 2015). Tardigrades exhibit a survival capability within the ecological niches of lichens and mosses. According to (Devasurmutt & Arpitha, 2016), milder environments such as meadows, ponds, and lakes tend to support a greater diversity of species. In the context of food webs, they can assume the roles of predators, prey, or primary consumers. Certain species exhibit predation behavior towards micrometazoan, particularly nematodes, rotifers, and other tardigrades. On the other hand, certain species adopt an herbivorous diet. Alternatively, some species derive sustenance from bacteria and detritus (Nelson et al., 2010).

The taxonomy of tardigrades is established through the examination of various morphological characteristics. These characteristics encompass the size, shape, organization, and number of their claws, as well as the arrangement of their Bucco-pharyngeal apparatus. Additionally, the length of their stylets, the size, shape, and number of their placoids, the patterns and ornamentation on their cuticles, and the morphology of their eggs are also considered (Nichols, 2005). The species-level diagnosis of tardigrades is frequently challenging due to the limited number of taxonomic characters available. Recently the DNA barcoding approach exhibits the highest potential in

species identification besides the morphological characteristics. (Cesari et al., 2009; Jørgensen et al., 2018).

The genus Dactylobiotus Schuster, 1980, was established by (Schuster et al., 1980). Currently, this genus consists of 18 species, all of which are known to inhabit aquatic environments (Kihm et al., 2020). *D. parthenogeneticus*, a cosmopolitan species, which was documented in several countries including Mexico (Moreno-Talamantes et al., 2015), in Italy (Guidetti, Gandolfi, Rossi, & Bertolani, 2005). Morphological and molecular analyses, utilizing sequencing of the COI gene conducted by (Pogwizd & Stec, 2020) in United Kingdom, France, and Poland. Tardigrade studies in Iraq were poorly documented. In previous studies only a single species of tardigrade was recorded *Dactylobiotus dispar*, from various locations in Kurdistan Region of Iraq (Ali, 2012; Ali &Latif, 2017; Dhahir & Ali, 2017). The present investigation focuses on *D. parthenogeneticus*, a recently discovered tardigrade species found in Iraq. The aim of this research is to examine and perform a molecular analysis on the freshwater tardigrade species found in the Kurdistan Region of Iraq. There is limited information available regarding this particular group of invertebrates that is known to exist in Iraq.

Materials and methods

Sample collection

Samples of tardigrades were collected from the outflow stream of Smaquli Dam. The Dam is situated at a celestial coordinate of 36°10'18.89" N and 44°22'51.28" E. It is positioned to the east of Erbil city in the Kurdistan region of Iraq, as indicated by (Namiq & Hewrami, 2020) (Fig.1). During the month of July 2022, samples were collected from the shore of the designated study sites by filtering 50 liters of water through a hand net with mesh components measuring 45 µm. The dominant algal species in the catchment area were *Chara sp*., *Spirogyra sp*., and *Pediastrum sp*. The specimens were transported to the laboratory and subsequently positioned within an oxygen apparatus. The morphological identification of a Tardigrade sample was conducted in the laboratory of the College of Education at Salahaddin University- Erbil, utilizing a compound microscope. The examination of characteristics was conducted by utilizing identification keys as described in previous studies (Guidetti et al., 2012; Nelson et al., 2019; Nelson et al., 2010). In accordance with the methodology outlined by (Cesari et al., 2009) individuals of the same species

who are astronauts were freshly subjected to isolation in order to extract DNA with the objective of obtaining a substantial quantity.

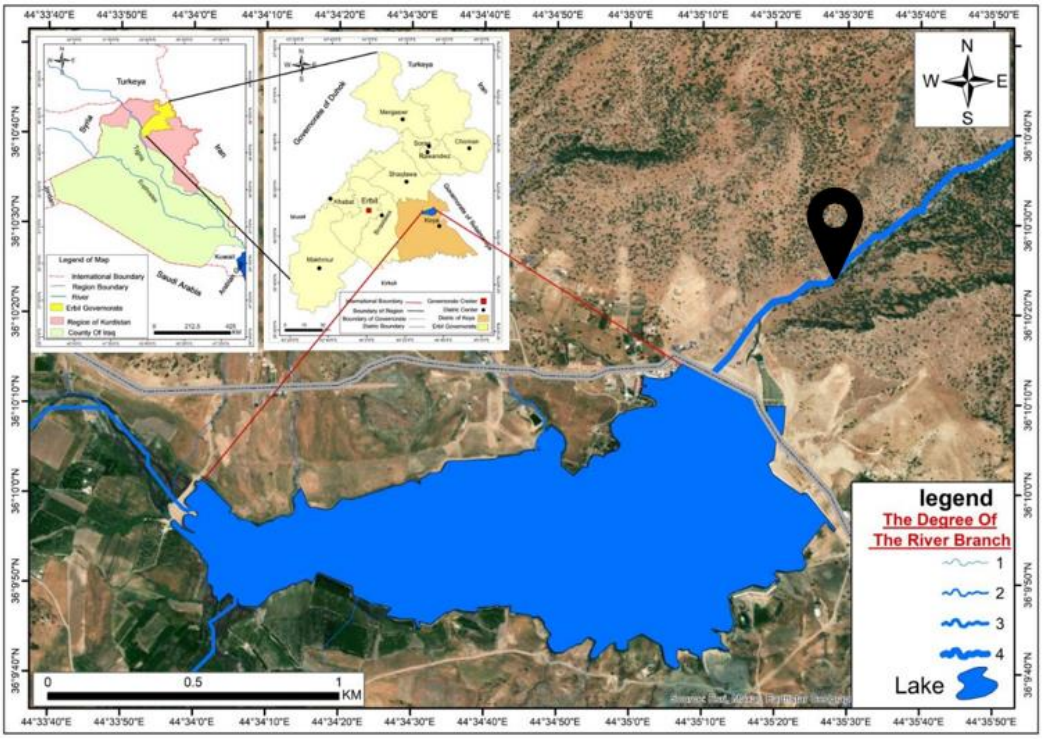

Figure 1. Map of Iraq and Kurdistan region show the studied site (Smaquli Dam outflow stream).

Genomic DNA extraction

A molecular analysis was conducted on a segment of the cytochrome oxidase subunit I (COI) gene, specifically consisting of 740 base pairs (bp). The sequencing of specimen was facilitated through the utilization of polymerase chain reaction (PCR) technology. The genomic DNA from the sample was performed by employing the Qiagen DNeasy Blood & Tissue Kit following the instructions provided by the manufacturer (Qiagen, 2020), the extracted genome DNA was stored at a temperature of -20°C.

PCR amplification

The PCR (Alpha-max, AC196-UK) was employed to amplify a specific segment of the COI gene, and the universal primers consisted of a forward primer (5'GGTCAACAAATCATAAAGATATTGG3'), and a reverse primer (5'GTAAATATATGRTGDGCTC3').

The PCR reaction was prepared using a reaction mixture with a total volume of 25 µL. This mixture consisted of 12.5 µL of 2× master mix (AMPLIQON, Denmark), 1.5 µL of each primer, 6 µL of genomic DNA template, and 3.5 µL of nuclease-free water. The tuchdown cycling conditions consisted of predenaturation 2 minutes at 94°C and an initial set of 5 cycles, with each cycle comprising of denaturation 1 minute at 94 °C, followed by annealing 1.5 minutes at 42 °C, and finally extension 1.5 minutes at 72 °C. This was then followed by 35 cycles, each consisting of denaturation 1 minute at 94 °C, annealing 1.5 minutes at 50 °C, and extension 1 minute at 72 °C. The final extension step was performed at 72 °C for a duration of 5 minutes (Cesari et al., 2009). The amplified products underwent gel purification (Consort-EV243, Biotic Ficher-Germany) using the Gel and PCR cleaning kit from (Promega-USA). The samples were prepared and subjected to electrophoresis in a 1.2% agarose gel to determine the size of the DNA fragments. Subsequently, the DNA fragments were stained with Red Safe dye to enhance their visibility under ultraviolet light. The PCR product is anticipated to have a size of 740 bp.

DNA sequencing analysis

The DNA sample was subjected to PCR amplification using forward and reverse primers specific to the COI gene, to prepare them for sequencing. The PCR products were submitted to Macrogen, a commercial sequencing company based in Korea, for further analysis. The alignment finding of the sequence was compared with previously deposited sequences using the NCBI GenBank. The sequence obtained from *Dactylobiotus parthenogeneticus* in this study was deposited in the NCBI-GenBank.

Phylogenetic inferences

The chromatograms were transformed into FASTA format utilizing the Finch TV chromatogram viewer software. The DNA sequences contained in the ABI file were manually modified utilizing Bio Edit v.7.0.5. The outcomes of sequence editing were assessed by employing the BLAST (Basic Local Alignment Search Tool) NCBI, which was utilized to determine the degree of homology with the most closely related species. The construction of a phylogenetic tree was performed using the maximum likelihood method, employing the Bootstrap calculation with 400 iterations in the Molecular Evolutionary Genetic Analysis (MEGA) software program version X (Tamura et al., 2021).

Results

No previous literature regarding this species in Iraq has been found. An organism that collected in Iraqi Kurdistan region was examined under a microscope and analyzed using molecular sequencing techniques. Based on this analysis, this specimen has been identified as a new species called *Dactylobiotus parthenogeneticus*.

Taxonomic account

Phylum Tardigrada Doyère, 1840
Class Eutardigrada Richters, 1926
Order Parachela Schuster, Nelson, Grigarick & Christenberry, 1980
Family Murrayidae Guidetti, Rebecchi & Bertolani, 2000
Genus Dactylobiotus Schuster, 1980
Dactylobiotus parthenogeneticus Bertolani, 1982

Morphological characteristics:

The typical body length of the studied species was approximately 410 μm. The body color of *D. parthenogeneticus* is characterized by transparency and a slight opaqueness in live specimens. This species is characterized by the presence of eyes, a smooth cuticle without spines and pores, and the presence of two dorso-lateral papillae located between the third and fourth limbs. These morphological features serve as important distinguishing characteristics for this species.

D. parthenogeneticus exhibits bilateral symmetry in its double-claws, wherein the two branches diverge immediately after the basal tract in a V-shaped manner. The arrangement of the claw branches follows a lateral-medial sequence, specifically characterized as "2-1-1-2 claws." The morphology of the claws exhibits a consistent shape across all limbs. The hind legs possess claws that are marginally longer in comparison to the three anterior claws. The development of accessory points is prominent in the primary branches of claws, particularly in the hind limbs. (Fig. 2 and 3 (d,e)).

The taxonomic significance of the buccal-pharyngeal apparatus is observed in the Dactylobiotus. This apparatus is composed of four distinct components, namely the buccal ring, buccal tube, stylet system, and pharynx. The mouth is in the antero-ventral region and is followed by the buccal ring. *D. parthenogeneticus* exhibits the presence of ten peri buccal lamellae, which are short in length, surrounding the buccal ring. The buccal tube is a structure that exhibits a cylindrical and rigid

morphology. It is upheld by a ventral lamina. The presence of a hook can be observed in the ventral lamina of the lateral. (Fig. 2b, 3b). The presence of the stylet system can be observed in both sides of the buccal tube, as depicted in Fig.2c, 3c. This system comprises two protrusible stylets, along with stylet sheaths and stylet supports. The posterior end of each stylet exhibits a thickening at its base, known as the furca, which can potentially serve as a valuable characteristic for taxonomic identification purposes.

The pharynx is comprised of two rod-shaped macroplacoids that are arranged in a sequential manner. In the taxa that were examined, it was observed that the macroplacoids in the first sequence were larger in size compared to those in the second sequence. Triangular apophyses are observed in the posterior region of the buccal tube. The eggs of the current species *D. parthenogeneticus* exhibit a spherical morphology, displaying a whitish or slightly yellowish. These eggs possess a diameter of approximately 85 µm. The apices are characterized by the presence of micro granulations in the shape of short cones (Fig. 2f, 3f). In relation to the molecular inquiry, the findings from the DNA sequence analysis of *D. parthenogeneticus* in GenBank revealed a complete match of 100% similarity to this species, as indicated by the accession number (MT373803) (Table 1).

Figure 2. A microscope image of *Dactylobiotus parthenogeneticus* (a) Whole amount. (b) Lateral view of buccal apparatus with ventral lamina and ventral hook. (c) dorso-ventral view of the buccal apparatus. (d) Claw I. (e) Claw IV. (f) Egg of *Dactylobiotus parthenogeneticus.*

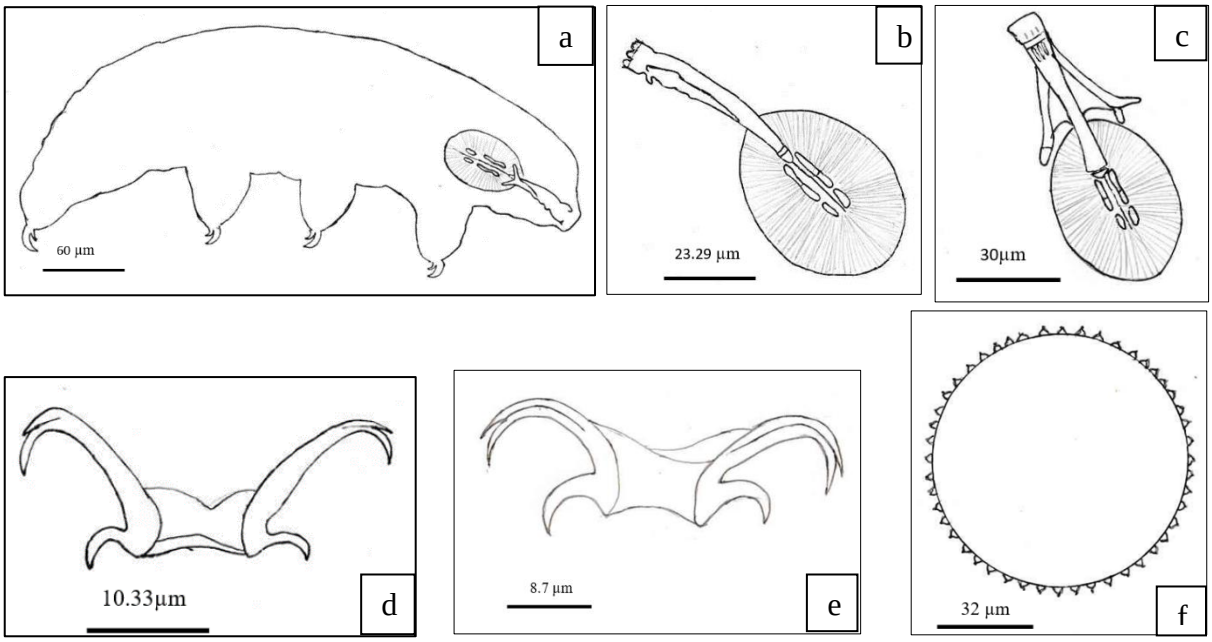

Figure 3. Lucida drowing of *Dactylobiotus parthenogeneticus* (a) Whole amount. (b) Lateral view of buccal apparatus with ventral lamina and ventral hook. (c) dorso-ventral view of the buccal apparatus. (d) Claw I. (e) Claw IV. (f) Egg of *Dactylobiotus parthenogeneticus.*

Table 1. Percentage distribution of *Dactylobiotus parthenogeneticus* based on CO1 gene according to nBLAST in GenBank of NCBI

Sample Accession Number	Identified	Query Cover %	Identic Number %	Genbank Accession Number	Country
OR501571	*Dactylobiotus parthenogeneticus*	100	100	MT373803	France
		100	98.75	MT373804	France
		100	98.75	MT373805	Poland
		100	98.60	MT373806	Poland
		98	98.73	AY598771	Italy

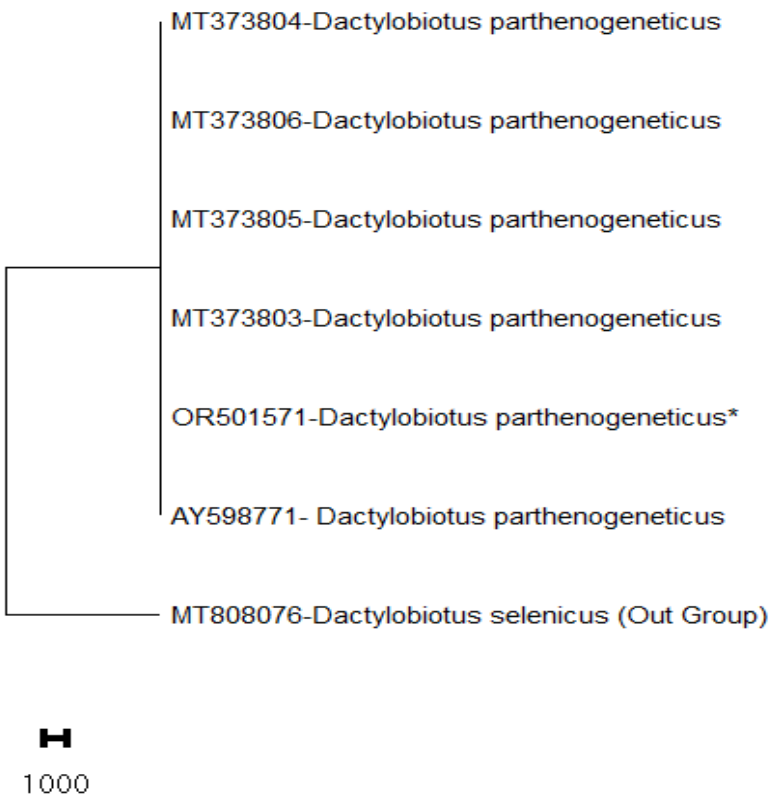

Figure 4. Molecular phylogenetic analysis by maximum likelihood method. Evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model.

Discussion

The current study aims to examine the abundance of the species under investigation in the water of Smaquli Dam, and its potential correlation with the biodiversity of other aquatic organisms, including mosses, algae, and aquatic invertebrates. These organisms are known to consume plant cells, algae, and other small organisms as part of their diet. Tardigrades play diverse ecological roles within ecosystems. The dietary preferences of organisms within a given species and their corresponding ecological niche can vary, encompassing herbivory, carnivory, or decomposition, contingent upon the specific species and its habitat. In their role as decomposers, they actively engage in maintaining aeration and nutrient cycling processes, while also occupying a significant position within the food web (Geross et al., 2005). The spatial and temporal distribution of tardigrades is influenced by various factors, as indicated by (Kaczmarek et al., 2011; Nelson et al.,

2018). These factors include altitude, the type of vegetation substrate (such as algae or lichen), environmental conditions (such as precipitation and temperature), and the presence and abundance of other invertebrates, such as rotifers and nematodes.

Belonging to the genus Dactylobiotus exhibit the distinguishing morphological feature of possessing an equal number of macroplacoids and cuticular bars between their claws. The distinguishing morphological characteristic that sets apart three species, namely *D. dispar, D. selenicus, and D. parthenogeneticus*, from other species within the genus is the presence of dorso-lateral papillae located between the third and fourth limbs (Kihm et al., 2020). The dimensions of structure of buccal tube are subject to variation across different species. The position of hook is visible in the ventral lamina in lateral view of the specimen that indicates the studied organism belongs to one species of genus Dactylobiotus. On the other hand, ornamented eggs were crucial for the accurate identification of species of freshwater eutardigrades, particularly in genera where the organisms share similar morphological traits. The morphology and diagram of studied organism and eggs were accordance with (Moreno-Talamantes et al., 2015; Pogwizd & Stec, 2020).

Under the examination of both morphological characteristics and based on the molecular data, our study provides explicit evidence that the organism in question is *D. parthenogeneticus*. The initial documentation of this event occurred in Italy by Bertolani in 1982. Since then, the occurrence of this phenomenon has been documented in various European nations, including Greece, Poland, Spain, Argentina, Bolivia, Mexico, France, and Great Britain (Pogwizd & Stec, 2020). Given the absence of prior knowledge on the part of Iraq. The present record represents the inaugural achievement within this country. The morphological examination of the depicted species aligns with the findings reported by (Guidetti et al., 2012; Hesgrove & Boothby, 2020; Nelson et al., 2015; Pogwizd & Stec, 2020).

In the context of molecular research, the BLAST tool available at (http://blast.ncbi.nlm.nih.gov/) is employed to conduct an analysis of gene sequence samples. This analysis aims to compare our amplified sequences with a set of previously stored sequences. The present study focuses on the comparative analysis of *D. parthenogeneticus*, specifically examining partial CO1 sequences, in relation to other related species. The analysis of COI sequences from GenBank reveals minimal genetic variations, ranging from 0.00% to 1.4%, as determined by nBLAST in GenBank of NCBI.

Furthermore, a complete match of 100% similarity is observed between this species and the sequence with Accession No. (MT373803) as documented in Table (1).

Based on the COI sequence alignment of *D. parthenogeneticus*, as presented in (Table 1) and (Fig. 4), it is evident that this species has been recorded in GenBank on six occasions, originating from four distinct countries. Specifically, the initial occurrence was reported in Italy (AY598771) (Guidetti et al., 2005) followed by subsequent records in France (MT373803 and MT373804), Poland (MT373805 and MT373806) (Pogwizd & Stec, 2020) , and most recently, Iraq (OR501571). Additionally, in Finland, several other species of Dactylobiotus have been documented and cataloged in Gen Bank. One such species is D. *selenicus* (MT808076), which exhibits notable differences from the haplotypes of D. parthenogeneticus (Stec, Vecchi, Maciejowski, & Michalczyk, 2020).

Conclusion

Dactylobiotus parthenogeneticus was found in the Kurdistan Region of Iraq, specifically from the outflow stream of Smaquli Dam. The species was characterized through morphological features, DNA barcoding, and molecular data acquisition. The specimens, collected, were found alongside green algae, marking the first documented occurrence of this species within Iraq's geographical boundaries.

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