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Original Article

Morphological and molecular identification of freshwater eutardigrade *Dactylobiotus parthenogeneticus* (Bertolani, 1982) in the Greater Zab River of Kurdistan Region-Iraq

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Abstract

Dactylobiotus parthenogeneticus is one of the widespread species of tardigrade all over the world. Tardigrades of this species were collected from the Greater Zab River in Erbil City-Iraq by filtering water of the river through a plankton net with a mesh of 45 µm pore. The samples were mounted on a slide with a cover slip and examined under the microscope to determine morphological characteristics and measurements. Based on these characters the species identified to be *D. parthenogeneticus.* To support this diagnosis, DNA barcoding techniques were applied to do molecular analysis and sequencing on the cytochrome oxidase subunit I (COI) gene. The sequence was subjected to the GenBank database of NCBI and recorded with the accession number PP140905. The result of the sequencing and molecular analysis of the cytochrome oxidase subunit I (COI) gene confirmed to be the same species diagnosed by relying upon morphological characters. This study represents one of the pioneer researches and documents on tardigrades and found *D. parthenogeneticus* for the first time in the Greater Zab River in Kurdistan, North of Iraq. Tardigrades play a magnificent role in different trophic levels and can be utilized as an indicator of ecosystem health.

Keywords: *Dactylobiotus parthenogeneticus*, Tardigrade, Morphological identification and molecular analysis, Greater Zab River, Erbil – Kurdistan

1. Introduction

Tardigrades or water bears are microscopic bilaterian metazoans in the phylum Tardigrada within the protostome superclade Ecdysozoa. The body is cylindrical with the dorsal part being convex and the ventral part being flattened. The length of their body ranges from (0.05 -1.2 mm) and is found in aquatic habitats all over the world. Their body is segmented forming from five somewhat indistinct segments namely: head or cephalic segment and trunk which bears four pairs of legs ending with claws. The anterior three pairs of legs are used mainly for locomotion and directed ventrolaterally while the rear fourth pair of legs is dedicated for grasping substrate and directed posteriorly. The body is surrounded by an integument formed from two parts: the outer layer is a cuticle containing chitin and the inner single layer of cuticle secreting epidermis [1–3].

The first description of tardigrades was by a German zoologist Johann August Ephraim Goeze in 1773 and called them little water bears which came from a German word (kleiner Wasserbär). Later on, in 1777 Lazzaro Spallanzani referred to them as “slow walker” (tardi - slow, grade - walker). Up to now, more than a thousand species have been described but it is probably the number of species is more many magnitude [1]. So far, all identified species placed in two classes: Heterotardigrada and Eutardigrada with two orders in each of them [4].

Many organisms including tardigrades do not possess specific structural adaptations for living as terrestrial organisms. When tardigrades face a dry condition they become dormant by entering cryptobiosis: anhydrobiosis or cryobiosis, respectively [5]. They are also able to resist many other unfavorable environmental conditions and physical extremes such as near complete desiccation, low and high temperatures (–253°C to +151°C), ionizing radiation (up to 6000Gy), osmotic shock, and extremes in pressure (up to 600MPa) as drastic as the vacuum of outer space. One of the reasons could be due to the presence of tardigrade-disordered proteins (TDPs) which do not exist in other organisms but the mechanism by which tardigrade resists these conditions through these proteins is not understood. Other mechanisms by which tardigrades survive such harsh conditions are encystment and cyclomorphosis [6,

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7].

The following morphological characters have been used as a base for the current taxonomy of clades with Tardigrada: claw size, shape, organization, and number; organization of the buccopharyngeal apparatus; length of stylets; size, shape, and number of placoids; cuticular patterns and ornamentation; and morphology of eggs and spermatozoa [8]. Tardigrade identification at the species level is often problematic due to the low number of taxonomic characters [5]. Even though morphological characters for identification are not sufficient to be reliable alone but an agreement of molecular and morphology-based phylogenies of tardigrades indicates that the current used morphological characters as ground rule for taxonomy is acceptable [8]. To overcome the taxonomic problem of tardigrades, DNA barcoding is a unique solution that merges with morphological characters (named MoDNA) to form an integrated taxonomy [9].

Dactylobiotus Schuster, 1980 is a freshwater genus of tardigrade with 18 species widespread throughout the world [10, 11]. *Dactylobiotus parthenogeneticus* has been recorded in many European countries such as Italy, Greece, Poland, and Spain, and in South American countries like Argentina, Bolivia, and Mexico [12, 13]. In Iraq, research and investigations on tardigrades are rare. So far, to our knowledge, three studies [14–16] indicated the presence of tardigrade species *Dactylobiotus dispar* in different locations. Another and the first study [17] in Smaquli Dam reported a new species of tardigrade *Dactylobiotus parthenogeneticus* based on merging both morphological and molecular identification. The purpose of this study was to look for and record the first-time tardigrade species in the Greater Zab River and examine them to identify them on the basis of their morphological characters and molecular analysis.

2. Material and Methods

2.1. Description of site and sample collection

The Tigris River has four main tributaries in Iraq. Greater Zab is located to the east of the Tigris River in the north of Iraq between 36˚-37˚ north latitudes and 43˚-44˚ east longitude and is the largest tributary in terms of its contribution to Tigris flow. Greater Zab River has two sources of origin: a mountain near the Iranian and Turkish boarder and the Springs Mountains of Kurdistan Region – Iraq. The entire length path from the sink to where it pours to Tigris which is located in Gwer district is about 392 Km [15, 18].

To obtain tardigrades, the water from the Greater Zab River in several sites was filtered through a zooplankton net (45 µm mesh size). Then specimens were preserved in two different concentrations of ethanol: 70% for morphological analysis and 100% for molecular analysis. For morphological identification, the specimens were observed and measured in the laboratory of the College of Education at Salahaddin University-Erbil/Iraq by using a compound microscope and ocular micrometer. The morphological characteristics were used to identify species by following the key [19] then several individuals of the same species separated and prepared for DNA extraction and further investigation of molecular analysis.

2.2. Genomic DNA extraction

Cytochrome oxidase subunit I (COI) gene consisting of 740 base pairs (bp) was chosen to do further molecular analysis for several reasons: mitochondrial DNA (mtDNA) does not go recombination because it is inherited maternally as a haploid genotype, availability of several copies of mtDNA per cell allows sufficient DNA extraction, evolving mtDNA faster than nuclear DNA [20]. The genomic DNA extraction from tardigrade samples was performed with the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's instructions (Qiagen, 2020), then extracted DNA genome DNA stored at a temperature of -20°C.

2.3. PCR amplification

To facilitated sequencing, isolated genomic DNA of targeted tardigrade was used to amplify Cytochrome oxidase subunit I (COI) gene through polymerase chain reaction (PCR) (Alpha-max, AC196-UK) with the help of universal primer formed from forward primer (5'GGTCAACAAATCATAAAGATATTGG3') and a reverse primer (5'GTAAATATATGRTGDGCTC3').

The PCR amplification of COI gene was carried out in 25 µL of reaction volume. The reaction mixture has the following components: 12.5 µL of 2× master mix (AMPLIQON, Denmark), 1.5 µL of each forward and reverse primer, 6 µL of genomic DNA template, and 3.5 µL of nuclease-free water. A step-by-step procedure of PCR described by the following protocol: 1) predenaturation at 94°C for 2 min; 2) denaturation at 94°C for 1 min; 3) annealing at 42 °C for 1.5 min; extension at 72 °C for 1.5 min (5 cycles); 4) denaturation at 94°C for 1 min; annealing at 50 °C for 1.5 min; extension at 72 °C for 1 min (35 cycles) and ended with the final extension of 5 min at 72 °C. Gel purification of amplified products was done by using the Gel and PCR cleaning kit from (Promega AddBio, KOREA), and then electrophoresis in 1.2% agarose gel was performed to determine 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.

2.4. DNA sequencing analysis

The obtained product from PCR was sent abroad for sequencing specifically to Macrogen, a commercial Korean company in sequencing. The obtained sequence data was examined to detect possible irregularities double peaks and noise...etc. The sequence of our sample aligned with other sequences available in NCBI-GenBank. It appeared that targeted sequence of our tardigrade has already been submitted to NCBI-GenBank from different parts of the world.

2.5. Phylogenetic inferences and statistical analysis

Before construction tree, several necessary steps were done such as converting file format from chromatogram to FASTA, modification of sequence with a special program and assessment of such modification to degree of homology among closely related species. Then, the sequence was used to construct tree by utilizing Molecular Evolutionary Genetic Analysis (MEGA5) software using maximum likelihood method. For assessment of node support 400 bootstrap iterations were employed [21].

3. Results

The collected organism was identified by using an integrated method, morphological examination under micros-

cope and later proceeding with molecular technique to get sequence. Based on these two approaches, the sample was found to be *Dactylobiotus parthenogeneticus.*

3.1. 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

3.2. Number of tardigrades

The total number of tardigrades is shown in Fig. 1. In February, tardigrade was found in four sites (sites 1, 3, 6 and 7) and the highest number was found in site 7. In March, number of tardigrades was found in all sites and was higher than in sites in February. The highest number in March was in site 3 while minimum number of tardigrades was in sites 1 and 4.

3.3. Morphological characteristics:

The length of the body of the collected samples ranged between 350-400 µm (Fig. 2A). Body is covered by cuticle. Several important morphological characteristics are exceptional for diagnosis and identification of this species which are: eyes are present, cuticle lacks pores and spines therefore it is smooth, possession of two dorsolateral papillae between posterior two pairs of legs. The species is bilaterally symmetrical with respect to its claw. Claws have a basal part followed by two primary branches which appear as V shape. Accessory points are quite clear on the primary branches of hind legs. The difference between hind leg and three anterior pairs of legs is worth mentioning, claws of hind leg are longer marginally if they are compared with claws of other legs (Fig 2C-D).

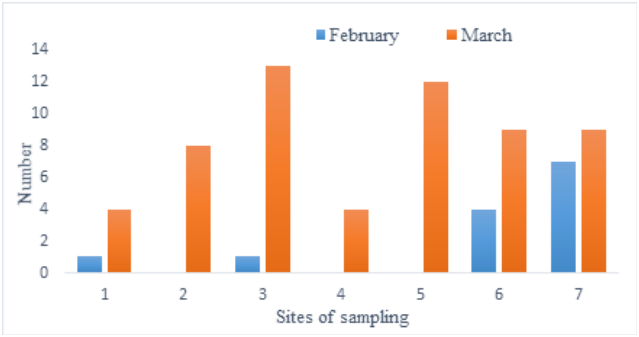

Fig. 1. Number of tardigrades found during sampling in February and March 2024.

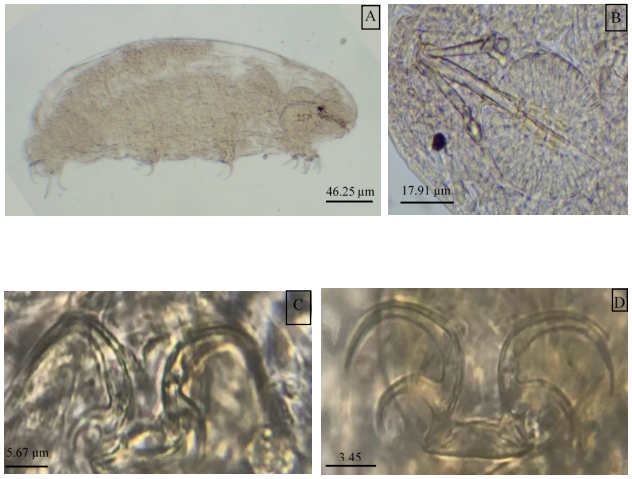

Fig. 2. Microscope image of *Dactylobiotus parthenogeneticus* (A) Whole body (B-C) Buccal apparatus with ventral lamina and ventral hook. (C) Claw I. (D) Claw IV.

Buccal-pharyngeal apparatus (Fig. 2B) is another highly valued taxonomic point of genus *Dactylobiotus*. This apparatus has four parts namely buccal ring, buccal tube, stylet system, and pharynx. Mouth is antero-ventral in position followed by the buccal ring. The buccal ring is surrounded by ten short peri buccal lamellae. The buccal tube is a stiff tube supported by ventral lamina and ends with Triangular apophyses. The ventral lamina possesses a hook which can be observed in lateral view. Stylet system exists on both sides of buccal tube which consists of two prominent stylets along with stylet sheaths and stylet supports. Each stylet ends with thickened basement known as furca which another point considered to have taxonomic diagnostic value in species identification. Pharynx is the last part of buccal-pharyngeal apparatus with the two rod-shaped macroplacoids which are sequential in arrangement. The macroplacoids differ in size with the first sequence being larger in size than other second sequence of macroplacoids.

3.4. Molecular analysis

The sequence of *Dactylobiotus parthenogeneticus* is deposited in GenBank under accession numbers: PP140905.

In regard to the molecular inquiry, the result of alignment of sequenced DNA of the specimen with other sequences of GenBank of NCBI showed a 100% similarity. More information on the result of alignment is shown in Table 1.

Table 1. Result of DNA sequenced specimen alignment with other sequences of GenBank of NCBI.

Accession Number of specimens	Identified as	Query Cover %	Identic Number %	GenBank Accession Number	Country
PP140905	*Dactylobiotus parthenogeneticus*	100	100	OR501571	Iraq
		100	100	MT373803	United Kingdom
		100	99.21	AY598771	Italy
		100	98.82	MT373805	Poland
		100	98.82	MT373804	France
		100	98.62	MT373806	Poland

4. Discussion

Our research contributes to providing data and information both morphologically and genetically on an aquatic species of tardigrade, *Dactylobiotus parthenogeneticus,* which was collected from Greater Zab River for the first time. Tardigrades are rarely studied in north of Iraq. All previous works have focused on searching for new species by describing morphological characters but few succeeded. Only two studies, including ours, attempted to achieve this goal by utilizing molecular approach in spite of morphological diagnosis. Even though both investigations succeeded and through their DNA sequences identified the same species of tardigrade they were conducted in two different locations confirming that this species may have a wider spread and probably very common in entire country. Such result can be used as a ground rule to pave the road for future works and encourage researchers to look after the same species in various places and different species.

Many organisms are identified and diagnosed based on the description of their morphological characters but the problem is inadequate description of morphological character might lead to validity issues which is the case for *Dactylobiotus aquatilis* and *Dactylobiotus henanensis.* They are described without any information on morphology of egg and cuticle, claws and buccal apparatus. Another species, *D. macronyx*, was reported to lay smooth unornamented eggs within exuviae which is inconsistent with genus *Dactylobiotus.* All of these species have been designated as *nomen dubium* [22]. Problems such as old, insufficient description and successive incomplete redescription of tardigrade have been major drawbacks in systematics of genus and species [23]. Our result of identification and morphological description of species under study is assured and supported by molecular analysis (Table 1).

Low nucleic acid concentrations in the cells of tardigrades are one of the factors and limitations in the study of tardigrades with respect to molecular analysis. The consequence of such low nucleic acid concentration appears in polymerase chain reaction (PCR) which requires sufficient amount of DNA to be extracted for further analysis but we were able to overcome this problem by sampling several individuals and examining them first for identification then doing molecular analysis. Low nucleic acid together with small size and only ten limited characters for the whole phylum are the main reasons in hindering tardigrade systematics [4].

Species of genus *Dactylobiotus* share similar and different morphological characteristics. The most commonly shared similar morphological features are macroplacoids with same number and claws with cuticular bars between them. An example of similar shared morphological traits among three species of *Dactylobiotus* is the presence of dorsolateral papillae. Dorsolateral papillae are located between the third and fourth pairs of legs and are used to separate apart three species *D. dispar, D. selenicus, and D. parthenogeneticus* from other species of the *Dactylobiotus*. Morphologically similar species of tardigrades can be differentiated through differences in their morphology of egg shell *i.e.* shape of the processes on the surface of their egg shell. On the other hand, there might be species with similar eggs but with different morphological traits. For instance, egg of *D. disper* is similar to that of *Murrayon nocentiniae* and *Murrayon pullari* with respect to their morphology therefore without animals it is impossible to distinguish these species with individuals being unavailable [11, 24]. An extraordinarily accepted universal tool to overcome all of these problems is DNA barcoding, using the mitochondrial COI gene as a tag to identify species probably due to robustness of the universal primers [25].

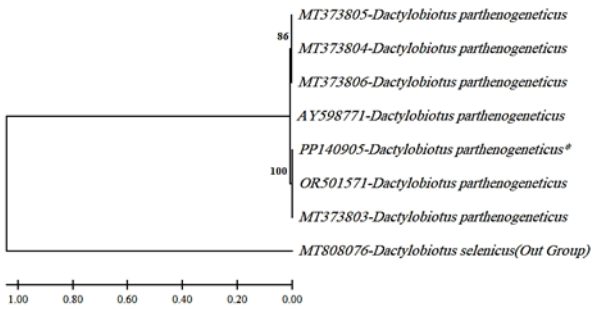

Fig. 3. Phylogenic tree of *Dactylobiotus parthenogeneticus* from Iraq. The phylogenic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 1000 re-samplings. Partial DNA sequences of concatenated partial COI gene were used as input data.

In regard to molecular analysis, the BLAST tool is used to further investigate and analyze gene sequences of targeted samples. With the BLAST tool, we matched side by side our amplified sequence of specimens with sequences that would have been stored in advance i.e. compare partial COI sequence with sequences of other species. The result of such analysis ranged from 0.00-1.38 of genetic variations as determined by nBLAST in GenBank (NCBI). In addition, a hundred percent similarity was revealed between our species and the first two sequences of (Table 1) accession numbered (OR501571) and (MT373803). By relying on the result of COI sequence alignment (Table 1) and phylogenetic tree (Fig. 3) we can comprehend that this species has been recorded in GenBank around the world.

5. Conclusion

Dactylobiotus parthenogeneticus has been recorded only two times in the North of Iraq indicating same and other species may exist in different parts of Iraq. Both morphological and more importantly molecular analysis confirmed the organism under study is *D. parthenogeneticus*.

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Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed Consent
The authors declare not used any patients in this research.

Availability of data and material
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Authors' contributions
The Authors contributed to this research work equally.

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