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New record of six *Brachionus* species from the South of Iraq

Hala F. Hassan[1*] iD , Malik H. Ali[1] iD, Adnan I. Al-Badran[2] iD

[1]Department of Marine Biology, Marine Science Centre, University of Basrah, Basrah, Iraq

[2]Department of Biology, College of Science, University of Basrah, Basrah, Iraq

*[1]Corresponding Author e-mail: hala.hassan@uobasrah.edu.iq

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Abstract

In this survey, six species of *Brachionus* genus are indexed as new record in the south Iraqi water resource at the first time in Iraq depending on the sequence of 18S rRNA gene, these species were registered as a new record in Iraq and the other two species which are HH1 and HH2 were registered in the National Center for Biotechnology Information (NCBI), The European Nucleotide Archive (ENA) and DNA Data Bank of Japan (DDBJ) sequentially.

Keywords: 18S rRNA gene, *Brachionus*, Rotifers, biotechnology.

Introduction

The phylum Rotifera is belong to Brachionidae which is a family of rotifers and relative to the order Ploima that have relatively small group of microscopic aquatic or semi-aquatic invertebrates, encompassing about 2000 species of un segmented, bilaterally symmetrical pseudocoelomatest they increase in marine fishes larvae rearing around the world has been noticed partly due to availability of rotifers (*Brachionus* spp.). The Rotifera of Iraq are mostly unknown (Ahmed and Ghazi, 2009). Sabri (1988) studied the ecology of rotifera in the Tigris river. Investigations in 1989 indicated the presence of 11 species of Brachionid rotifers (Abdul-Hussein *et al*., 1989). The quality of Rotifer cultures are evaluated not only by reproduction rate and density, but also essential nutrients and associated microbiota for the larvae predators (Dhert, 1996). *Brachionus* are widely used in aquaculture systems as the first living food to the larvae of fishes, and considered as the main food source of the marine finfish industry (Lubzens *et al*., 2001). Because of

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their high economic value, extensive research had been carried out on the ecophysiology of Brachionus strains, types, or species (King, 1972; Gallardo *et al*., 2000). Methods of identification are essential to a better understanding of *Brachionus* rotifers both in research and aquaculture. In some cases and studies when the morphological identification getting difficulty to distinguish, the molecular identification can dissolve this issue. Much progress has been made in the development of DNA markers in aquaculture species (Liu and Cordes, 2004; Sato *et al*., 2005). In Brachionus, a high number of sequences have been published for different markers (Go´mez *et al*., 2002; Papakostas *et al*., 2005). The genetic composition of the cultures was recorded and it revealed that a single Brachionus biotype was prevalent in all cultures. Further analyses even suggest the existence of more species, up to 14. Mainly for phylogenetic purposes, these sequences can be used for the genetic identification of the various Brachionus species and/or biotypes. In addition, it had been shown that the freshwater rotifer *Brachionus calyciflorus* also comprises a species complex (Gilbert and Walsh, 2005).

The genetic identification methods of *Brachionus* rotifers, need to be incorporated in the rotifer culturing industry to uncover possible species interactions which were not yet described. All these findings suggest that strain discrimination on the basis of rotifer body size is currently unreliable. Since cryptic speciation seems to be widespread in Brachionus Different *Brachionus* species or biotypes may have different optima with respect to culture conditions. (Ortells *et al*., 2003).

Materials and Methods

Samples collection

Ten samples of *Brachionus* species were collected from Shatt Al-Arab river by conical net of 1 meter length and 40 cm diameter with mesh size of 50 µm. The conical net was thrown into the water and pulled to a distance of 3 meters by tide rope and then the collected amount of water were poured in a Plastic bottles. (Hammadi, 2010).

Morphological Identification

Brachionus are multicellular animals with body cavities that are partially lined by mesoderm. These organisms are valuable live food for

larval fish and crustacean culture (Akter *et al*., 2013). Brachionus rotifers are morphologically well adapted to the aquatic habitats and acquire different characteristics suitable to different biotopes they inhabit. most species of rotifers inhabit fresh water, some species also occur in brackish (estuarine) and marine habitats. Descriptions related to essential organs, necessary for the taxonomic study and identification are given. The rotifer body is generally of elongated form and divisible into the broad or narrowed or lobed anterior end, usually provided with a ciliary apparatus, called corona an elongated trunk and a slender terminal region, the tail or foot. In some species the bodies are covered by thorough structure called "lorica". Such forms are generally known as loricates. Other forms which do not have lorica, but soft, thin and transparent skins are known as illoricate forms (Tayade and Dabhade, 2011).

Corona: Rotifers possess two distinctive features, first, at the apical end (head) is a ciliated region called the corona, which is used in locomotion and food collection. In the adults of some forms, ciliation is lacking and the corona is a funnel or bowl-shaped structure at the bottom of which is the mouth. The corona may be with anterior and posterior lines of cilia known as trochus and cingulum respectively. The structure of the corona is of basic importance in the classification of rotifers. There is much more variation in the structure of corona among different families of rotifers.

Lorica: In many rotifers, the cuticle is thin and flexible throughout, the body is very mobile. In many other rotifers, the cuticle may be stiffened in places forming relatively inflexible plates, such a structure is called a "lorica".The lorica may consist of several pieces with or without longitudinal sutures. The margins of the lorica may project as teeth or spines and these are subject to much variation within a single species. compared length and width of the hardened and thickened epidermis,named lorica, and distances between the anterior spines, and found that these measures had a wide overlap . These lorical spines help to escape from the attack of predators. In some genera, the body has characteristic appendages as skipping spines, paddles and arms with bristles which help in locomotion and have taxonomic value. The part of the lorica covering the neck region may be marked off from the general

trunk lorica by a grove and is then termed as head shield (Tayade and Dabhade, 2011).

Foot and Toes: The foot is a prolongation of the body posterior or ventral to the anus and is not properly called a tail; that term is being restricted to a fold or prolongation dorsal or anterior to the anus. The foot bears at its end conical toes, and contains within two cement glands which secrete a sticky material which helps in the attachment. Foot and toes are useful for various functions as locomotion and attachment. They are with drawn into the body in a contracted condition and become obscure. The foot of rotifer in a wrinkled or jointed form with toes or a disc at the posterior end. The toes may be elongated, shortened or reduced (Tayade and Dabhade, 2011). There may be only one toe, representing a fusion of the two.The foot is very small, insignificant structure in many rotifers, and in is some, it is absent altogether.

Mastax: The muscular pharynx, possessing a complex set of hard jaws, called trophi, is present in all rotifers. Mastax is characteristic of and peculiar to rotifers, rounded, trilobed, or elongated organ of complicated form and structure, whose inner wall bears the masticatary apparatus. The trophy consist of seven main pieces, the unpaired fulcrum, and the parried rami, unci and manubria (Tayade and Dabhade, 2011). Trophi of brachionidae have a species-specific shape and size, and are often used as a reliable taxonomic character. Mastax is an important feature used as criteria at all taxonomic levels. Several types of trophy can be recognized. They are named according to the relative development of the parts. The development of the various parts and the associated muscles are related to the feeding habits (Tayade and Dabhade, 2011).

Molecular Identification

Include DNA extraction purification, PCR amplification and Sequencing analysis.

DNA Extraction

The whole DNA was extracted from ten samples of the *Brachionus* species according to the method of Wen and He (2003). 30µl STE buffer (10 mM/L NaCl,1mM/L EDTA pH 8.0, 10 mM/L Tris-HCl, pH 8.0) and 2µl proteinase K (10mg/ml) were added to the homogenate sample.

To reduce the loss, the grinding tip was washed with 30µl STE buffer.

The samples were incubated at 56°C for 2 hours and then incubated at 95 C° for 45 seconds centrifuged at 8000-10000 rpm for 30 seconds. The supernatant being used as amplification template in PCR reaction, was stored in the refrigerator.

Agrose Gel Electrophoresis

The study involve DNA immigration which needs many of materials such as agarose, Tris Borate EDTA buffer, Ethidium bromide, Bromo phenol blue, DNA marker (100bp) and DNA sample.

Procedure

The procedure for electrophoresis consisted of three steps.

1- Preparation of agarose gel :

A- 25 ml of 1x TBE was taken in a beaker.

B- 0.2 gm agarose was added to the buffer.

C- The solution was heated to boiling (using hot plate) until all the gel particles were dissolved.

D- The solution was allowed to cool down at 50-60 C° .

2- Casting of the horizontal agarose gel.

A- The gel was assembled to casting tray and the comb was positioned at one end of the tray.

B-The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to gel at room temperature for 30 minutes.

C-The comb was carefully removed and the gel replaced in electrophoresis chamber. The chamber was filled with TBE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

3- Loading and running DNA in agarose gel.

A- DNA (9 µL) was mixed with bromophenol blue in the ratio of 3:1 and loaded in the wells of the 0.8% agarose gel .

B- The cathode was connected to the well side of the unit and the anode to the other side.

C- The gel was run at 60 V and 2mA until the bromophenol blue tracking dye migrated to the end of the gel.

D- The DNA was observed by staining the gel with ethidum bromid and viewed under UV transilluminator.

Identification of *Brachionus* spp. using specific 18S rRNA amplification

The 18S rRNA gene was amplified using primers corresponding to conserved regions as 200bp (winnepenninckx *et al*., 1995) which showed in table 1,2, and 3, were designed on the basis of 18S rRNA published sequence data (Genbank: U29235).

Table 1: Oligonucleotide primer sequences used for PCR amplification of 18S rRNA gene

Primer	Sequence	Number
Forward primer	5-AGATTAAGCCATGCATGCGTAAG-3	23
Reverse primer	5-TGATCCTTCTGCAGGTTCACCTAC-3	24

Table 2: Reagents of PCR amplification (50 µl) for 18S rRNA

No	Reagent	Volume
1	DNA	10µl
2	Forward Primer	2µl
3	Reverse Primer	2µl
4	Master Mix .2x	11µl
5	Nuclease-free water	25µl
	Total	50µl

Table 3: PCR amplification protocol for (50 µl) of 18S rRNA

No	Stage	No. of Cycle	Time in min
1	DNA Denaturation	1 Cycle	10 min
2	DNA Annealing	35 Cycle	1 min
3	DNA Extension	1 Cycle	10 min

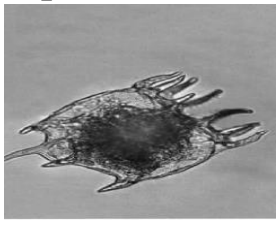
Sequencing preparation and sending

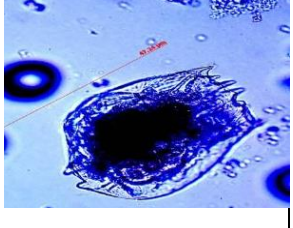
Ten samples of purified PCR products with 20 µl were send to Bioneer company in Korea for nucleotides data base analysis

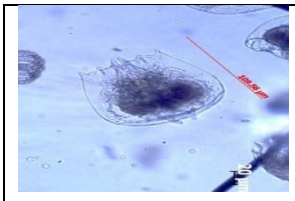
Results and Discussion

The results of DNA extraction , purification , PCR amplification and sequencing analysis of six *Brachionus* species which were different from their reference strains in several positions of nucleotide sequences. Current study approves the results of the previous studies which showed the same domains of these species like (Garey *et al*., 1998 and Papakostas *et al*., 2006). The present study results are in agreement with the results of (Winnepenninckx *et al*., 1995). So we recorded these six species as a new species and as the first time in Iraq, these species were published by The National Center for Biotechnology Information (NCBI), The European Nucleotide Archive (ENA) and DNA Data Bank of Japan (DDBJ) (Hala *et al*., 2021).The databases of these strains were recorded in the GenBank for DNA sequences) as shown in table (4).

Table 4: New record of six *Brachionus* species in the south of Iraq.

B. patulus 	GTGCTAAGGTAGCGTGATTCTTGTTTTCTTAATTAGAAATCT GTTCAAAGAATGATATGTAGAAAATTTTTTGTTTTTATAGAA AATGAATTTAAATTGTTAGTGAAAATTCTAACTTATTCTTAA AAGACGAGAAGACCCCATAAAACTTAATTTTTTCATTTCTTT TTATCGTTTAAAAATTTAAATGGGGACTTTTGAGTACTTTAA TAACCTTTTGGACCTATATTTTTATTTTCTGAGAAGCTACTT TGGGGATAACAGGGTAAAATATTTAGAGAGTTCATATCGAT AAATATGATTACTACCTCGATGTTGGATTA

B. manjavacas 	AATGGCCGCAGTACCCTGACTGTGCTAAGGTAGCGTGATTC TTGTTTTCTTAATTAGAAATCTGTTCAAAGAATGAAATGGA GAGAATCTATTGTTTCTATAGCAAGTGAATTTAAAATGTTA GTGAAAATTCTGACAAGTCCTTAAAAGACGAGAAGACCCCA TAAAACTTAATTTGATCTCTTTCTTAAGAGTTTAAAATTTAA ATGGGGACTTTAGAGTATAATAATAACTTAAAGACCTATAT TTATATTATTAGTGAAGCTACTTTGGGGATAACAGGGTAAA ATATTTAGAGAGTTCATATCGATAA
B. forficula 	GGCCGCAGTACTCTGACTGTGCTAAGGTAGCGTGATTCTTG TTTTCTTAATTAGAAATCTGTTCAAAGAATGATACGTAGAA AATCTTTTGTTTTCATATTTAATGAACTTAAATTGTTAGTGA AAATTCTGACGTTTTCTTAAAAGACGAGAAGACCCCATAAA ACTTTATTTTGCTGAGTTTACAGGATTCTTTTAAATTTAAAT GGGGACTTTAGAGTAATTAAATAACTTAATGACCTATTTTT ATATTTTTAGAGAAGCTACTTTGGGGATAACAGGGTAAAAT ATTTAGAGAGTTCTTATCGATAAGTATGTTTACTACCTCGAT GTGGATTA
B. caudatus 	TTAAATGGCCGCAGTACCCTGACTGTGCTAAGGTAGCGTGA TTCTTGTTTTCTTAATTAGAAATCTGTTCAAAGAATGATATG TAGAAAATCTATTGTTTTTGTATAAAATGAAGTTAAATTGTG AGTGAAAATTCTCATTTATTTATAAAAGACGAGAAGACCCC ATAAAACTTAATTTGATTATTCTTTTTAGACTGTTAAAATTT AGATGGGAACTTTAGAGTATAATAATAACTTATATGACCTA TATTTTTATTTTTTGAGAAGCTACTTTGGGGATAACAGGGT AAAATATTTAGAGAGTTCTTATCGATAAATGTGCTTACTACC TCGATGTTGGATT
B. ibericus 	GGTAGCGTGATTCTTGTTTTCTTAATTAGAAATCTGTTCAAA GAATGATATGGAGAGATTCTCTTGTTTTCGTATTTAGTGAA CTTAAAATGTTAGTGAAAATTCTGACTTACTCTTAAAAGAC GAGAAGACCCCATAAAGCTTAATTTGATCTATTTATTTATAG TTTAAAATTTAAATGGGGACTTTAGAGTATTCTAATAACTTA AAGACCTATTTTTATATTAATTGAAAAGTTACTTTGGGGATA ACAGGGTAAAGTATTTAGAGAGTCCTTATCGATAAACACAA CTACTACCTCGATGTTGGAT
B. urceus	CGGTAGCGTGATTCTTGTTTTCTTAATTAGAAATCTGTTCAA

	AGAATGAAATGAAGAGAATCTATTGTTTCTATACAAAATGA ATTTAAATTGTTAGTGAAAATTCTGACTAGAACTTAAAAGA CGAGAAGACCCCATAAAACTTAATTTGATCTCTTTTTCAAGT GTTTAAAATTTAAATGGGGACTTTAGAGTATAACAATAACT CAAAGACCTATATTTATATTATTAGTGAAGCTACTTTGGGG ATAACAGGGTAAAATATTTAGAGAGTTCTTATCGATAAATA TATCTACTACCTCGAT

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تسجيل جديد لست انواع من جنس *Brachionus* المعزول في جنوب العراق

حلا فاضل حسن[1]*iD و مالك حسن علي[1]iD و عدنان عيسى البدران[2] iD

[1] قسم الاحياء البحرية، مركز علوم البحار ، جامعة البصرة

[2]قسم علوم الحياة ، كلية العلوم، جامعة البصرة

*[1]e-mail: hala.hassan@uobasrah.edu.iq

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المستخلص

في هذه الدراسة تم تسجيل ستة أنواع من جنس *Brachionus* على انها انواع جديدة تسجل لأول مرة في جنوب العراق. تم نشر هذه الانواع في بنك S rRNA18 اعتماداً التشخيص الجزيئي المتضمن استخلاص وتضخيم و تحليل تتابعات الجين. بالتتابع (NCBI,ENA and DDBJ) المعلومات الجينية الامريكي والاوربي والياباني.

الكلمات المفتاحية: *Brachionus*، الروتيفيرا، تحليل تتابعات الجين.

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