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Molecular identification of new global isolate of *Brachionus plicatilis* named HH2

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Abstract

The target of this work was extracted DNA from cultured *Brachionus plicatilis* which then subjected to PCR amplification and after that sequenced .The PCR reactions were performed with only one isolate of *Brachionus plicatilis* having identical sequences of 18S rDNA and recorded as one global isolate in the (NCBI of USA, ENA of Europe and DDBJ of Japan).

Key words: 18s rDNA, PCR ammplification, *Brachionus plicatilis,* Iraq-Basrah.

Introduction

The phylum Rotifera is a relatively small group of microscopic aquatic or semi-aquatic invertebrates, encompassing about 2,000 species of unsegmented, bilaterally symmetrical pseudocoelomatest they increase in marine fishes larvae rearing around the world has been due partly to the 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* rotifers are widely used in aquaculture systems as the first living food to the larvae of fishes, and considered as the main food sources of the marine finfish industry (Lubzens *et al*., 2001). Because of their high economical value, extensive research had been carried out on the ecophysiology of *Brachionus* strains, types, or species (King, 1972; Gallardo *et al*., 2000). These studies focused on the understanding of *Brachionus* population dynamics both at laboratory and feild, resulting in improved culture efficiency in hatcheries (Snell and Serra, 1998; Yoshinaga *et al.*, 2001; Dhert *et al*., 2001; Sarma *et al.*, 2001). Methods for identification are essential to a better understanding of *Brachionus* rotifers both in research and aquaculture. As cryptic species cannot be distinguished using morphological data, molecular markers can be used instead. 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), mainly for phylogenetic purposes, these sequences can

be used for the genetic identification of the various *Brachionus* species and/or biotypes . 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, within the *Brachionus plicatilis* species complex (Papakostas *et al*., 2006a; Suatoni *et al*., 2006). In addition, it had been shown that the freshwater rotifer also comprises a species complex (Gilbert & Walsh, 2005). 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* rotifers, methods of genetic identification need to be incorporated in the rotifer culturing industry to uncover possible species interactions which were not yet described. Different *Brachionus* species or biotypes may have different optima with respect to culture conditions. (Ortells *et al*., 2003).

Experimental Methodology

Samples collection

The samples have been collected from Shatt Al-Arab River by conical net of 1meter length and 40 cm diameter with mesh size of 50 µm as a routine monthly sampling were carried out between September 2013 and March 2014 from eight selected stations. The conical net was thrown into the water and pulled to a distance of 3 meters by tied rope and then the collected amount of water were poured in a plastic bottles . (Hammadi , 2010).

The Purification of samples

The samples were purified immediately after reached the lab. for purity after phynotypic diagnosis by using an disecting microscope according to (Battish 1992, Fernando, 2002, De Smet 2007, Sharma 2007, Segers and De Smet, 2008 , Fontaneto 2010 , Petersen 2010, and Hammadi et al.,2012) to isolate the rotifers, two sieve [90 and 43micron] were used , the first one used to obscure the large organisms while the small organisms including rotifers collected in glass beaker. The second one (43 micron) were used to collect the rotifers which assemble on the top sieve by washing the sieve with sterilized water , then anatomical microscope used to exam the rotifers and collect the *Brachionus plicatilis* only according to the key and put in tank (Ghazi, 2005).

***Brachionus* Culture**

After the rotifer were isolated and purified, 50 individual / ml were taken and placed in a tank of 5 liter capacity, where the laboratory conditions were appropriate for the reclamation process which include (salinity 7.1- 8.73 g / L, dissolved oxygen 6.5- 7.3 mg / L, pH 6.5- 7.5 and the water temperature (21- 22ºC) the process of feeding started by dissolving 250g of yeast in sterilized water using a barrier lumbar. The animal manure 5.012g using oven 60 ºC for 24hours , then covered with gauze topic in the tank and fed for seven days at the rate of once per day.

Laboratory conditions

Configured laboratory in the Marine Science Center-Marine Biology Department in the range 21-22ºC. which thermally the condition area is 4 meters and is equipped with four plastic tanks of the same dimensions (40 cm length, 30 cm width and 20 cm height).which secured from the oxygen needed by a ventilator electric-type (RS electrical 5010) a Chinese made. In addition to artificial light source (Florescence) tank surrounding culture from all sides needed to secure the object from the light.

Nutrition

Three types of food, including animal manure (5.012 gm) ; Baker´s yeast (250 mg \ 50 individuals) and a mixture of animal manure and Baker´s yeast Saccharomyces cerevisiae (255.012 gm) were used and the ratio was adjusted daily depending on the increasing numerical of *Brachionus plicatilis.*

DNA Extraction

According to (Genaid Kit Serial No JM23411) for alcohol embedded sample.

DNA Concentration

The concentration of DNA was calculated by nanodrop spectrophotometer (OPTIZN-Japan).

Identification of *Brachionus plicatilis* by using specific 18S rDNA amplification

The 18S rDNA gene was amplified using primers corresponding to conserved regions as 200bp (winnepenninckx et al. ,1995), were designed on the basis of 18S rDNA published sequence data (Genbank: U29235) see tables (1-3).

Table 1. Oligonucleotide primer sequences used for PCR amplification of 18S rDNA gene

Primer	**Sequence**	**No**
Forword primer	18Sr RNA(5-AGATTAAGCCATGCATGCGTAAG-3	23
Reverse primer	18S rRNA(5-TGATCCTTCTGCAGGTTCACCTAC-3	24

Table 2. Reagents of PCR amplification (50 µl) for 18S rDNA

No	**Reagent**	**Volum**
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. Touch down PCR Condition for Specific 18S rDNA gene

Steps	**Temperature**	**Time**
Step 1	95 ºC	2 min
Step 2	95º C	30 sec
Step 3	61.3 ºC decrease 0.5 ºC percycle	30 sec
Step 4	72 ºC	20.0 sec
Step 5	Repeate steps 2-4	14 more time
Step 6	95 ºC	30 sec
Step 7	54.3 ºC	30 sec
Step 8	72 ºC	20 sec
Step 9	Repeate steps 6-8	19 more time
Step 10	72 ºC	5 min

Experimental Results and Discussion

Molecular identification of *Brachionus plicatilis* using 18S ribosomal DNA

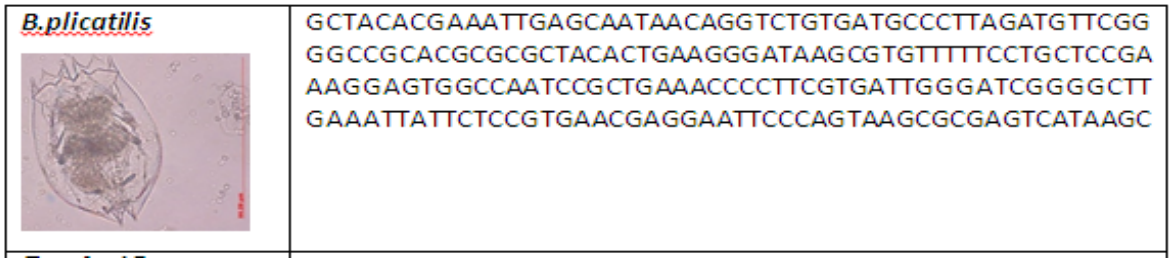
The extracted DNA from each isolate (n=10) was subjected to PCR for amplifying 18S rDNA. The individual band of the gene was characterized by 200 bp due to comparison with the standard molecular DNA Ladder (100bp). Agarose gel (2%gm and 60V,2MA) electrophoresis patterns show PCR amplified products of gene 18S rDNA. Lane M: 1kb DNA ladder, lanes 1-9: Gene 18S rDNA bands of *Brachionus plicatilis*.

Sequencing for 18S rDNA and identification of *Brachionus plicatilis*.

The results of 18S rDNA nucleotides sequencing for each isolate are presented in Table (4) .

Identification of new global isolate of *Brachionus plicatilis*.

There is one *Brachionus plicatilis* isolate (No. 2) which is different from their reference strain in one position of nucleotide sequence. So we were recorded this isolate as a new global strain and this isolate was published by The National Center for Biotechnology Information (NCBI) , The European Nucleotide Archive (ENA) and DNA Data Bank of Japan (DDBJ) .The databases of this strain was recorded in the GenBank for DNA sequences. This isolate (No.2) *Brachionus plicatilis* named isolate HH2 with ID number which is (GenBank: KM191797.1) was closely related (99%) to *Brachionus plicatilis* isolate isolate A759 -B3 but with a Gene or Point mutation type Transversion (C instead G) at the position 112 changing the amino acid Alanine to Proline. (Figure 2).



Figure(1): Global new record of *Brachionus plicatilis* sequence

***Brachionus plicatilis* isolate A759-B3 18S ribosomal RNA gene**

GCTACACGAAATTGAGCAATAACAGGTCTGTGATGCCCTTAGATGTTCGGGGCCG
CACGCGCGCTACACTGAAGGGATAAGCGTGTTTTTCCTGCTCCGAAAGGAGTGGG
CAATCCGCTGAAACCCCTTCGTGATTGGGATCGGGGCTTGAAATTATTCTCCGTG
AACGAGGAATTCCCAGTAAGCGCGAGTCATAAGC

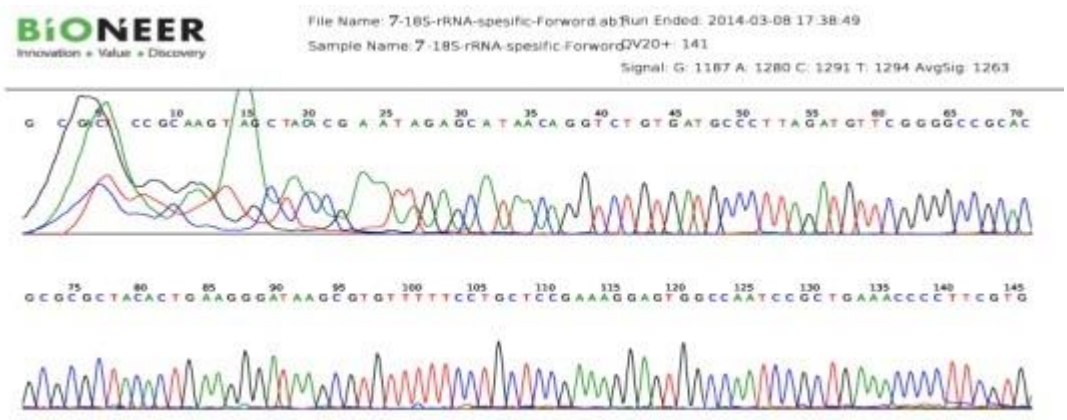


Figure (2): Sequence of *Brachionus plicatilis* 18S ribosomal DNA gene

***Brachionus plicatilis* 18S ribosomal RNA gene**

GCTACACGAAATTGAGCAATAACAGGTCTGTGATGCCCTTAGATGTTCGGGGCCG
CACGCGCGCTACACTGAAGGGATAAGCGTGTTTTTCCTGCTCCGAAAGGAGTGGC
CAATCCGCTGAAACCCCTTCGTGATTGGGATCGGGGCTTGAAATTATTCTCCGTG
AACGAGGAATTCCCAGTAAGCGCGAGTCATAAGC

Comparison of 18S rDNA nucleotide sequences (200bp) for the isolate ***Brachionus plicatilis*** (with peaks) from present study and reference isolate A759-B3. A Point mutation type Transversion (C in red color instead G) at the position 112bp changing the amino acid Alanine to Proline.

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التشخيص الجزيئي لعزلة مسجلة عالمياً للدولابي المستزرع *Brachionus plicatilis*

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المستخلص

الهدف من البحث كان معتمداً على استخلاص الحامض النووي منقوص DNA الاوكسجين من الدولابي المستزرع و تعريضه الى التضخيم الجيني بواسطة التفاعل متعدد السلسلة PCR ومن ثم عمل تحليل جيني له من خلال جهاز التحليل الجيني للمحتوى الوراثي للدولابي DNA Sequencing Analyzer. اظهرت النتائج وجود عزلة وحدة تعود للدولابي المستزرع حيث تتطابق بنسبة ٩٩% مع العزلات العالمية وتم تسجيلها كعزلة جديدة في المركز الدولي لبنك المعلومات الوراثية الامريكي والارشيف الوراثي الاوربي وبنك البيانات الوراثية في اليابان