Molecular identification of new global isolate record of *Brachionus calyciflorus* named HH1

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Abstract

This work was involved DNA extraction from cultured *Brachionus calyciflorus* subjected to PCR .The PCR reaction was performed with *Brachionus calyciflorus* which having identical sequences of 18S rDNA and recorded as a global isolates of *Brachionus calyciflorus* in the (NCBI of USA, ENA of Europe and DDBJ of Japan) with Genbank ID: KM191796.1.

Key words: 18s rDNA, Touch down PCR, *Brachionus calyciflorus,* HH1.

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. In addition, it had been shown that the freshwater rotifer *Brachionus calyciflorus* 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 were collected from Shatt Al-Arab River by conical net of one meter 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 phenotypic 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 species only according to the key and put in tank (Ghazi, 2005).

Culture of *Brachionus calyciflorus*

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- 22^{o}C) 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 ^{o}C 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 21-22^{o}C. 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*

DNA Extraction from *Brachionus calyciflorus*

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

Experimental Results and Discussion

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

The whole genomic extracted DNA from each isolate (n=7) was subjected to PCR for amplifying 18S rDNA (Figures 1). 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* sp.

DNA Concentration of *Brachionus calyciflorus*

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

Identification of *Brachionus calyciflorus* by 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/ng) 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 amplification program

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

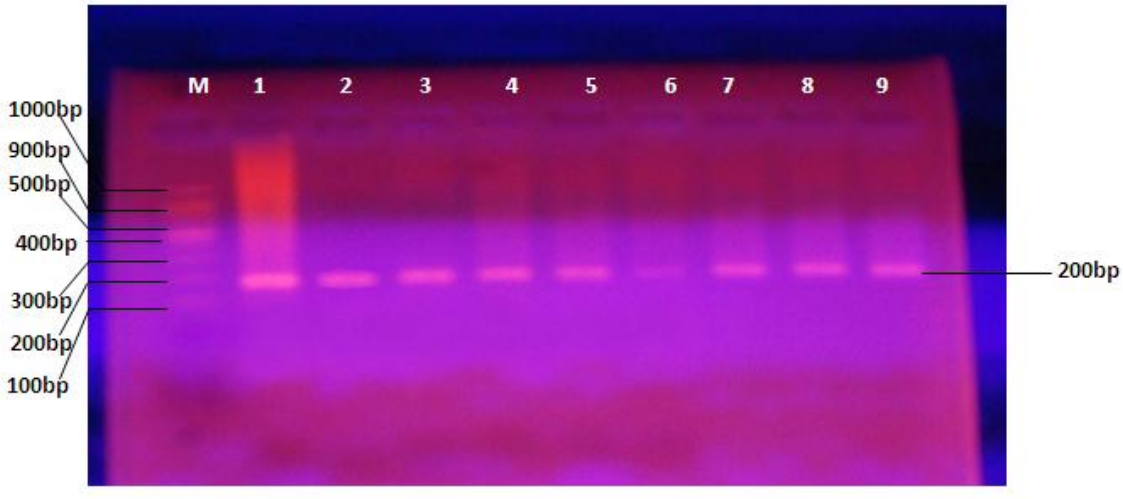


Figure 1. PCR amplification results show 1-9 Gene 18S rDNA bands of *Brachionus* sp. And M: 1kb DNA ladder.

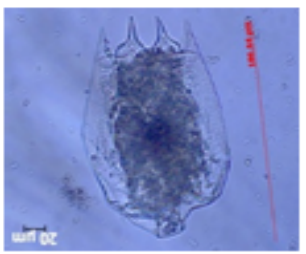
Sequencing for 18S rDNA and identification of *Brachionus calyciflorus* species.

All the 18S rDNA nucleotides sequencing of the isolate presented in Table (4) .

Identification of new global isolates

The sequence of *Brachionus calyciflorus* isolate (No. 2) which is different from the reference strains sequencing in several positions of nucleotide sequences. So when we were recorded the isolate as a new global strain in gene bank and the 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 the strain was recorded in the GenBank for DNA sequences entitled HH1 with ID: (GenBank: KM191796.1) and was closely related (before publication, 99%) with *Brachionus calyciflorus* isolate 20120630-14 but with Gene or Point mutation type Transition (A instead G) at the position 112 changing the amino acid Valine to Isoleucine. (Figure 3).
Comparison of 18S rDNA nucleotide sequences (200bp) for the isolate *Brachionus calyciflorus* (with peaks) from present study and reference isolate 20120630-14. A Point mutation type Transition (A instead G) at the position 112bp changing the amino acid Valine to Isoleucine.

Table 4. Global new record of *Brachionus calyciflores* species sequences.

B.calyciflorus 	AGCTACACGAAATTGAGCAATAACAGGTCTGTGATGCCCTTAGATGTTCG GGGCCGCACGCGCGCTACACTGAAGGGATAAGCGTGTTTTTCCTGCTCCG AAAGGAGTGGATAATCCGCTGAAACCCCTTCGTGATTGGGATCGGGGCTT GAAATTATTCTCCGTGAACGAGGAATTCCCAGTAAGCGCGAGTCATAAGC

Brachionus calyciflorus isolate 20120630-14 18S ribosomal DNA gene

AGCTACACGAAATTGAGCAATAACAGGTCTGTGATGCCCTTAGATGTTCGGGGCCGCACGCGCGCTACACTGAAGG
GATAAGCGTGTTTTTC

CTGCTCCGAAAGGAGTGGGTAATCCGCTGAAACCCCTTCGTGATTGGGATCGGGGCTTGAAATTATTCTCCGTGAA
CGAGGAATTCCCAGTA

AGCGCGAGTCATAAGC

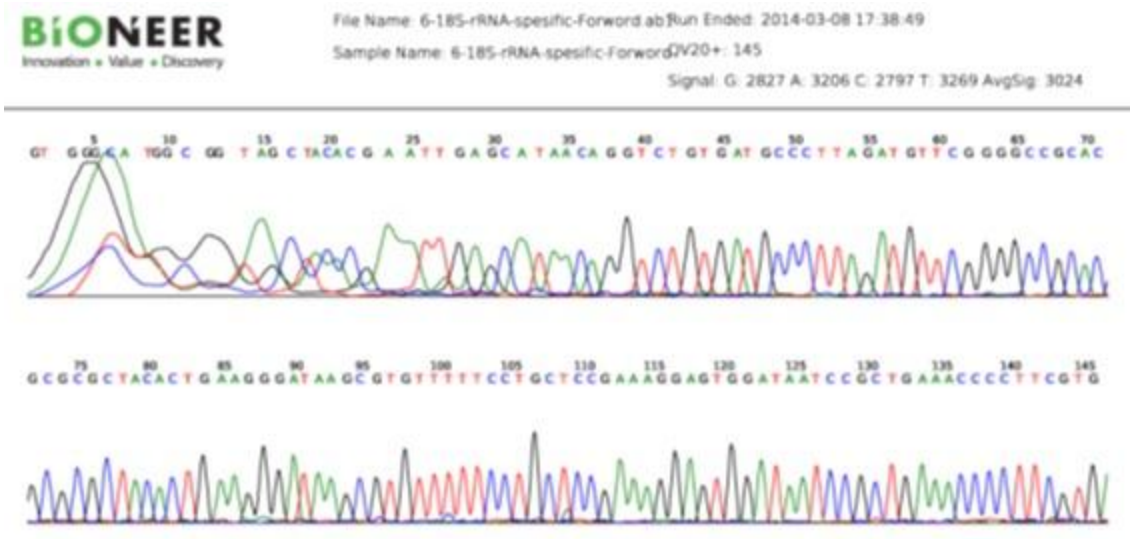


Figure (2):Sequence of *Brachionus calyciflorus* 18S ribosomal DNA gene

***Brachionus calyciflorus* 18S ribosomal RNA gene**

AGCTACACGAAATTGAGCAATAACAGGTCTGTGATGCCCTTAGATGTTCGGGGCCGCACGCGCGCTACACTGAAGG
GATAAGCGTGTTTTTCCTGCTCCGAAAGGAGTGGAT

AATCCGCTGAAACCCCTTCGTGATTGGGATCGGGGCTTGAAATTATTCTCCGTGAACGAGGAATTCCCAGTAAGCG
CGAGTCATAAGC

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التشخيص الجزيئي لعزلة *Brachionus calyciflorus* والمسمى *HH1* جديدة مسجلة عالمياً تعود لجنس الدولابي المستزرع

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الخلاصة

اشتمل العمل على أستزراع الدولابي *Brachionus calyciflorus* وأظهرت نتائج تحديد التتابعات للقواعد النتروجينية للجين 18S rDNA والمضخم بتقنية التفاعل متعدد السلسلة بحجم 388 زوج قاعدة في كل عزلة وتم تسجيل العزلة عالميا في كل من (NCBI) المركز الدولي الأمريكي للمعلومات الوراثية و(ENA) الأرشيف الدولي الأوربي للمعلومات الوراثية وكذلك في (DDBJ) بنك المعلومات الوراثية الياباني حسب الرقم الدولي Genbank ID: KM191796.1.

لكلمات المفتاحية: 18s rDNA, Touch down PCR, *Brachionus calyciflorus,* HH1