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New Record of *Macrobrachium lar* (Fabricius, 1798) (Crustacea: Decapoda: Palaemonidae) from Al-Hammar Marsh, Southern Iraq

Abdul-Hussein H. Ghazi[1]* & Hala F. Hassan[2]

[1]Department of Natural Marine Science, Marine Science College, University of Basrah, Iraq
[2]Department of Marine Biology, Marine Science Center, University of Basrah, Iraq

*Corresponding author: abdulhussein73@yahoo.com

Abstract: A species of freshwater prawn *Macrobrachium* was newly recorded from Al-Hammar marsh, Southern Iraq. Morphological features accompanied by 18 S r DNA analyses indicated that the species is *Macrobrachum lar*. DNA sequences of specimens of this species from the marsh is deposited at the GenBank for DNA as a new global isolate and was published by The National Center for Biotechnology Information (NCBI), the European Nucleotide Archive (ENA) and DNA Data Bank of Japan (DDBJ). *M. lar* inhabit deep sections of streams and brackish water, adults live in freshwater, while juveniles can be found in brackish or saltwater, the total length of *M. lar* recorded in this study was ranged between 72 and 109 mm for males and between 61 and 93 mm for females.

Keywords: New record, *Macrobrachium lar*, Southern Iraq, DNA sequences

Introduction

Macrobrachium Bate, 1868 is a widely distributed and abundant macro-invertebrate genus in inland water. It has been recorded in several tropical and subtropical regions such as lakes, ponds, rivers, as well as in estuaries (Cook et al., 2002; Guerra et al., 2014). Species of *Macrobrachium* found in lowlands of Africa, Asia, Oceania, North, Central and South America. Although most of the species are inhabiting freshwater environments, some can tolerate higher salinities and found near sea coasts where water is rather brackish (Wowor et al., 2009; Pileggi & Mantelatto, 2010). Update, this genus includes approximately 210 species of prawns and is considered as the largest palaemonid genus, with the greatest diversity occurring in the Indo-Pacific region and the Americas (Mantelatto et al., 2008; De Grave & Fransen, 2011; Guerra et al., 2014).

The only species of *Macrobrachium* reported in Iraq is *M. nipponense* (De Haan, 1849) which was first found in Al-Hammar marsh by the end of 2002 (Salman et al., 2006). *Macrobrachium lar* is a large palaemonid freshwater prawn, commonly known as Monkey River prawn and is known throughout the Indo-West Pacific area, from East Africa to the Marquesas Islands (Holthuis,1980; Sethi et al., 2013a). According to Sarangi et al. (2001), the species has been reported as a native

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in Andaman and Nicobar islands. The information on *M. lar*, however, is relatively limited. Most investigations were oriented to study its taxonomy (Short, 2004; Sethi et al., 2013a), reproductive biology (Sethi et al., 2014), natural food and feeding habits (Sethi et al., 2013b) and morphology variation (Fadli et al., 2018). The present work adds to our knowledge more information on the environmental preference of *M. lar* as it is encountered, for the first time, in a rather brackish water habitat in Southern Iraq.

Material and Methods

The prawns were collected from Al-Hammar marsh, Al-Mashaab, Southern Iraq in October 2015. This is located within an area that lies between 30º 39´ 34.27" N; 47º 39´ 13.81" E., which is connected with the Arabian Gulf through the Shatt Al-Arab river (Figure 1). Specimens were collected at a depth between 3-4 m with a trawl net, 3 m long and 10 mm mesh size. Al-Hammar marsh is currently distinguished by being semi closed, receiving its water mainly from the Euphrates river and its branches, and it depends on the phenomenon of tide to changes its waters. The study area is characterized by the spread of a number of aquatic plants such as *Ceratophylum demersum*, *Myrophylum* sp., *Nymphoides indica*, *Typha australis*, *Potamogeton* sp. and *Phragmites australis*.

All collected samples were kept in cold box and transported to the laboratory. The work was done in the Department of Marine Biology, Marine Science Center, University of Basrah for further identification. Dissecting microscope was used for specimen's examination and depending on some keys (Riek, 1951; Short & Meek, 2000; Wowor & Choy, 2001). Water temperature, salinity and potential hydrogen ion (pH) were measured by YSI 556 MPS models 2005 which usually calibrated before every field trip.

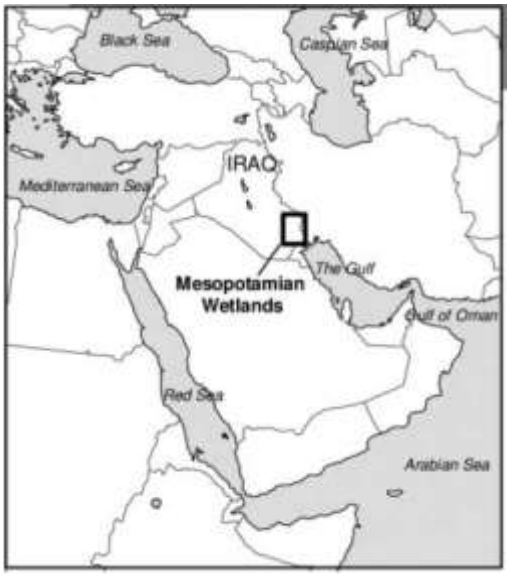

Figure 1: Geographical location of the sampling site in Al-Hammar marsh, Southern Iraq where specimens of *Macrobrachium lar* were collected.

Molecular Identification

QIAGEN (protease) or (proteinase K) 20µl was added into the 1.5 ml Eppendorf tube included 200 µl of the sample and 200 µl of the buffer AL to the solution and mixed the sample using pulse-vortex for 15 esc. and incubated at 56 °C for 10 min. Centrifugation of the mixture was done to remove drops from the inside of the lid then 200µl of ethanol (96-100%) were added to the sample and mixed again by pulse-vortex for 15 seconds. After mixing, brief, centrifugation of 1.5 Eppendorf tube were done to remove drops from the inside of the lid. The mixture was carefully applied from step 6 to the QLAamp Mini spin column (in a 2ml collection tube) without wetting the rim. Closed the cap, and centrifugation at 8,000 rpm for one minute was carried out, then the QLAamp Mini spin column was placed in a clean 2ml collection tube and discard the tube containing the filtrate. The QLAamp Mini spin column was carefully opened and 500µl buffer AW1 were added without wetting the rim. The cap was closed and centrifugation at 8000 rpm for one minute was done. The QIAamp Mini spin column was placed in a clean 2ml collection tube containing the filtrate to avoid contamination and to purify the DNA extraction, the QIAamp Mini spin column was opened and 500 µl of AW2 buffer were added without wetting the rim, the cap was closed and centrifugation at full speed 14,000 rpm for three minutes was done. The QIAamp Mini spin column was placed in a new 2ml collection tube with the filtrate (the purified DNA). Centrifugation at full speed for one minute was carried out, then the QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and discard the collection tube, the QIAamp Mini spin column was carefully opened and 200µl of AE buffer were added. Incubated at room temperature (15-25 ºC) for one minute and then centrifuged at 8000 rpm for one minute.

DNA Quantitation Procedure

The concentration of DNA was calculated by nanodrop spectrophotometer (OPTIZN). One µl of DNA sample was taken and put in the lance. The optical density was read with nanodrop spectrophotometer at wavelength 260 and 280 nm. The quality and purity were measured directly. Electrophoresis of DNA in the agarose was done according to Hassan (2015). The reagents used for this investigated were Agarose, Tris Borate EDTA buffer (TBE), Ethidium bromide, Bromo phenol blue, DNA marker (100bp) and DNA sample. The procedure for electrophoresis consisted of three steps these were:

Step 1: Preparation of agarose gel.

Twenty-five ml of 1x TBE were taken in a beaker and 0.2 g agarose was added to the buffer. The solution was heated to boiling (using hot plate) until all the gel particles were dissolved. Then the solution was allowed to cool down to 50-60 ºC.

Step 2: Casting of the horizontal agarose gel.

The gel was assembled to casting tray and the comb was positioned at one end of the tray. 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. The comb was carefully removed and the gel replaced

in the electrophoresis chamber. The chamber was filled with TBE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

Step 3: Loading and running DNA in agarose gel.

DNA (6 µL) was mixed with bromophenol blue in the ratio of 3:1 and loaded in the wells of the 0.8% agarose gel. The cathode was connected to the well side of the unit and the anode to the other side. The gel was run at 60 v and 20 mA until the bromophenol blue tracking dye migrated to the end of the gel. DNA was observed by staining the gel with Ethidium bromide and viewed under UV transilluminator.

Identification of *Macrobrachium lar* by Using Specific 18S rDNA Amplification

The 18S rDNA gene was amplified using primers corresponding to conserved regions as 866 bp (Winnepenninckx et al., 1995). Designed on the basis of 18S rDNA published sequence data (Genbank: U29235). Table 1 and 2 show the primers sequences and the volume of the mixture of reagents, respectively, while Table 3 described the Touchdown PCR condition program (Hassan, 2015).

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

Primer	Sequence	No. of nucleotides
Forward primer	18Sr RNA(5-AGATTAAGCCATGCATGCGTAAG-3	23
Reverse primer	18S r RNA(5-TGATCCTTCTGCAGGTTCACCTAC-3	24

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

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

Sequencing of 18S rDNA

The 18S rDNA fragments were sequenced at MWG Company "http://eng.mwg.com". Preparation of samples for sequencing was done according to the procedure of Barker et al. (2005).

Table 3: Touchdown PCR condition for specific 18S rDNA gene.

Steps	Temperature	Time
1	95 ºC	2 minutes
2	95 ºC	30 seconds
3	61.3 ºC decrease 0.5 ºC per cycle	30 seconds
4	72 ºC	20 seconds
5	Repeated steps 2-4	14 more time
6	95 ºC	30 seconds
7	54.3 ºC	30 seconds
8	72 ºC	20 seconds
9	Repeated steps 6-8	19 more time
10	72 ºC	5 minutes

Results

Diagnosis

In *M. lar*, major second pereopod with merus greater than or equal to carpus. Also, second pereopod with chelae completely naked or bearing only a few scattered setae not concealing surface; chela subcylindrical. The rostrum straight with 1-3 teeth on carapace; 2-4 ventral teeth; one big tooth on each finger. Carpus conical or cup-shaped; palm not swollen. Telson with apex rounded, lateral spines short. Body color deep olive brown to olive grey or blue grey, abdominal condyles light cream to orange, posterior abdomen often darker than anterior and tergum darker than pleura. Antennal flagella deep olive grey to olive brown or orange, antennule flagella orange brown to almost black. Rostrum olive grey to olive brown with dorsal teeth and plumose setae orange brown. The total length of *M. lar* recorded in this study ranged between 72 and 109 mm for males and between 61 and 93 mm for females (Figure 2).

Figure 2: *Macrobrachium lar* from Al-Hammar marsh, Southern Iraq.

Molecular Identification Study

18S Ribosomal DNA

The results of 18S rDNA nucleotides sequencing for the isolate is presented in Table 4. The extracted DNA was subjected to PCR for amplifying 18S rDNA. After measured by nanodrop, the individual band of the gene was characterized by approximately 866bp due to comparison with the standard molecular DNA Ladder (100-1200 bp) as in Figure 3 (A and B).

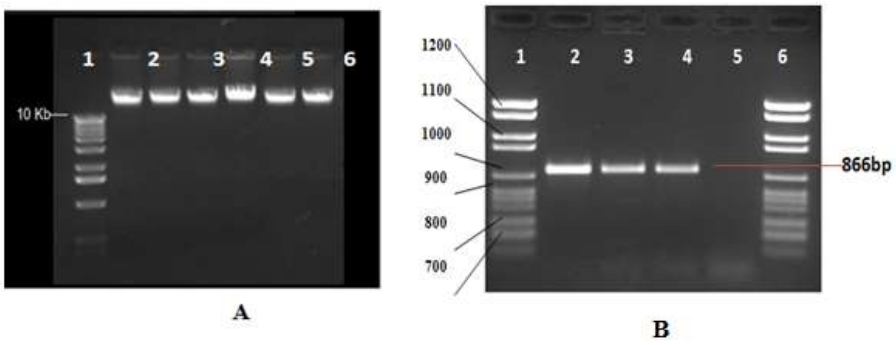

Figure 3: A- Agarose gel (0.8% and 60V 2 mA) electrophoresis patterns show DNA bands of *M. lar* from 1-6 extracted DNA from formalin embedded samples. B- Agarose gel (2% and 60V, 2mA) electrophoresis patterns show PCR bands of *M. lar*18S rRNA gene from1-6 compared with stander ladder (100-1200bp).

Table 4: Identification of global new record of *M. lar* recorded in Al-Hammar marsh, Southern Iraq by sequencing of 18S rDNA during 2015.

Macrobrachium lar 	GenBank: KX066822.1 GGTAGTGACGAAAAATAACAATGCGGGACTCTTCCGAGTC TGCGTAATTGGAATGAGCACACTTTAAATCCTTTAGCAACA ACCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAA TTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCGGTTAAA AAGCTCGTAGTTGGAGGTCAGTTGCGGACTGGGGTGGGAG GTCACCGCCCGGTGCTTCCCCTCTAGGCTCCGAACAGCTTC CGTGAGTGGAAGCCAAGCCGGCTTAGCCGGGGTGCTCTTT ACCGAGTGTCCCGCGCAACCGGCCAGTTCACCTTGAATAA ATGAGAGTGCTCAAAGCAGGCTACTCTGGAATACAATGCC CGAATGTTACCTGCATGGAATGATGGAAGATGATATCGGT TGCATTTTGTTGGTGGTGGCAGCCCGAGGTAATGATAAAG AGGGACTGTCGGGGGCATCCGTACTACGACGCGATAGGTG AAATTCAGTGACCGTCGTAGGACGAACAACAGCGAAAGCA TTTGCCAAGAATGTCTTCGTTGATCAAGAACGAACGTTAGA GGATCGAAGGCGATCAGATACCGCCCTAGTTCTAACCCTA AACGATGCTGACTAGCAATTCGCCGTCGTTATTGCCATGAC GTGGCGAGACGCCCCCGGGAAACCTCAAGTCTTTGAGTTC CGGGGGTAGTATGGTTGCAAAAGTGAAACTTAAAGGAATT GACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAAT TTGACTCAACACGGGAAACCTGACCAGGCCCGGACACCAG AAGGATTGACAGATTAAGAGCTCTTTCACGATTTGGTGGGT GGTGGTGCATGGCCGTTCTT

Discussion

The species of *M. lar* isolate (No KX066822.1) is different from their reference strain in several positions of nucleotide sequences (Table 5). So, we recorded this isolate as a new global isolate and 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 isolate were recorded in the GenBank for DNA sequences (Table 5).

Table 5: Comparative nucleotides sequence of 18S rDNA gene between the original and quarry sequence.

Original sequence GenBank: KP215302.1	Quarry sequence GenBank: KX066822.1
GGCACGCAAATTACCCAATGCCAGCTCT	GGTAGTGACGAAAAATAACAATGCGG
GGGAGGTAGTGACGAAAAATAACAATG	GACTCTTCCGAGTCTGCGTAATTGGAA
CGGGACTCTTCCGAGTCTGCGTAATTGG	TGAGCACACTTTAAATCCTTTAGCAAC
AATGAGCACACTTTAAATCCTTTAGCAA	AACCAATTGGAGGGCAAGTCTGGTGCC
CAACCAATTGGAGGGCAAGTCTGGTGCC	AGCAGCCGCGGTAATTCCAGCTCCAAT
AGCAGCCGCGGTAATTCCAGCTCCAATA	AGCGTATATTAAAGTTGTTGCGGTTAA
GCGTATATTAAAGTTGTTGCGGTTAAAA	AAAGCTCGTAGTTGGAGGTCAGTTGCG
AGCTCGTAGTTGGAGGTCAGTTGCGGAC	GACTGGG**GTG**GGAGGTCACCGCCCGG
TGGGGCGGGAGGTCACCGCCCGGTGCTT	TGCTTCCCCTCTAGGCTCCGAACAGCTT
CCCCTCTAGGCTCCGAACAGCTTCCGTG	CCGTGAGTGGAAGCCAAGCCGGCTTAG
AGTGGAAGCCAAGCCGGCTTAGCCGGG	CCGGGGTGCTCTTTACCGAGTGTCCCG
GTGCTCTTTACCGAGTGTCCCGCGCAAC	CGCAACCGGCCAGTTCACCTTGAATAA
CGGCCAGTTCACCTTGAATAAATGAGAG	ATGAGAGTGCTCAAAGCAGGCTACTCT
TGCTCAAAGCAGGCTACTCTGGAATACA	GGAATACAATGCCCGAATGTTACCTGC
ATGCCCGAATGTTACCTGCATGGAATGA	ATGGAATGATGGAAGATGA**TAT**CGGTT
TGGAAGATGATCTCGGTTGCATTTTGTTG	GCATTTTGTTGGTGGTGGCAGCCCGAG
GTGGTGGCAGCCCGAGGTAATGATAAAG	GTAATGATAAAGAGGGACTGTCGGGG
AGGGACTGTCGGGGGCATCCGTACTACG	GCATCCGTACTACGACGCG**ATA**GGTGA
ACGCGAGAGGTGAAATTCAGTGACCGTC	AATTCAGTGACCGTCGTAGGACGAACA
GTAGGACGAACAACAGCGAAAGCATTT	ACAGCGAAAGCATTTGCCAAGAATGTC
GCCAAGAATGTCTTCGTTGATCAAGAAC	TTCGTTGATCAAGAACGA**ACG**TTAGAG
GAAAGTTAGAGGATCGAAGGCGATCAG	GATCGAAGGCGATCAGATACCGCCCTA
ATACCGCCCTAGTTCTAACCCTAAACGA	GTTCTAACCCTAAACGATGCTGACTAG
TGCTGACTAGCAATTCGCCGTCGTTATTC	CAATTCGCCGTCGTTAT**TGC**CATGACG
CCATGACGTGGCGAGACGCCCCCGGGAA	TGGCGAGACGCCCCCGGGAAACCTCAA
ACCTCAAGTCTTTGAGTTCCGGGGGTAG	GTCTTTGAGTTCCGGGGGTAGTATGGT
TATGGTTGCAAAAGTGAAACTTAAAGGA	TGCAAAAGTGAAACTTAAAGGAATTGA
ATTGACGGAAGGGCACCACCAGGAGTG	CGGAAGGGCACCACCAGGAGTGGAGC
GAGCCTGCGGCTTAATTTGACTCAACAC	CTGCGGCTTAATTTGACTCAACACGGG
GGGAAACCTGACCAGGCCCGGACACCA	AAACCTGACCAGGCCCGGACACCAGA
GAAGGATTGACAGATTAAGAGCTCTTTC	AGGATTGACAGATTAAGAGCTCTTTCA
ACGATTTGGTGGGTGGTGGTGCATGGCC	CGATTTGGTGGGTGGTGGTGCATGGCC
GTTCTTAGTTGGTGGAGT	GTTCTT

The point mutations were happened in the nucleotides sequence and the amino acids translation is explained in Table 6.

Table 6: The point mutations and amino acids changes.

Original	GCG-GUG, UCU-UAU, GAG-GAU, AGU-CGU, UCC-UGC
Quarry	Ala-Val, Ser-Tyr, Glu-Asp, Ser-Arg, Ser-Cys

The family Palaemonidae represents one of the few decapod groups that has successfully colonized oceans, estuaries and rivers in the subtropics and tropics (Valencia & Campos, 2007). The genus *Macrobrachium* has a great diversity with highly variable morphology, the taxonomic identification based only on these characteristics may be difficult, so that in this study, we analyzed identification of *M. lar* by using specific 18S rDNA amplification and sequence. This genus is covering the lowlands freshwater species, although some are found near the coast in brackish water (Valencia & Campos, 2007). According to Salman et al. (2006), only one species of freshwater prawns of the genus *Macrobrachium*, *M. nipponense* has been noticed as invasive species in Al-Hammar marsh, Southern Iraq. But we expect other species belonging to the same genus entering the Iraqi waters, one of these is *M. lar* which is recorded in the present study and considered as the second species belonging to the genus *Macrobrachium* so far present in the Iraqi water.

According to Short (2004), this species had a wide range of distribution in the Indo-West Pacific, Eastern Africa to the Ryukyu Islands and Marquesas East Indies. The measurements of the present species as recorded in India and Uzbekistan are with a length range of 82-123 mm for males and 73-118 mm for females (Sethi et al., 2014). The specimens recorded during the present study had a size range between 72-109 mm for males and from 61-93 mm for females. No details are available on the apparent method of introduction of the present species to Southern Iraq, but it can be emphasized that the water of the marsh undergoes dramatic changes in water quality associated with the decline in the rates of discharge from the Tigris and Euphrates rivers (Al-Mahmood et al., 2015). The decrease of freshwater inflows allowed the extension of a saltwater front from the Arabian Gulf to the mouth of Shatt Al-Arab river and reaches the marsh (Abdullah et al., 2016**)**. *M. lar* is a strong swimmer, an adapted crawler, and its diadromous pattern as adults is restricted to freshwater environments that are connected to the sea, due to the fact that larval development must take place in seawater before individuals return to freshwater habitats as postlarvae.

The results indicate that the physical properties, particularly salinity had changed dramatically which caused serious catastrophic changes in the distribution, abundance and species composition of the flora and fauna of the region. The salinity was the foremost reason that resulted in the intrusion of the present species into the inland waters. It is well known that *M. lar* has a wide ecological tolerance for salinity, ranging from freshwater to estuarine waters depending on the life stages. The physical parameters were of critical importance for the successful invasion of any species of aquatic organism (Zheng et al., 2008). Lal et al. (2012)

suggested that *M. lar* is able to tolerate fresh or brackish-water of approximately 10‰. Also, the temperature range of 30±0.5 °C produced optimal survival and development for *M. lar* larvae. All of these factors are available in the marsh environment in Southern Iraq as a result of the processes of mixing marine and fresh water through the tidal phenomenon. *M. lar* prefers clear water and individuals are usually found or occur in well oxygenated pools below riffles or waterfalls and individuals shelter in poorly lit areas among piles of rocks, brush, burrows and fallen trees, and among tree roots.

One of the assumed reasons for entering this species are either derived from escaped aquaculture stock or perhaps deliberately introduced, as well as may have been introduced, accidentally to the Iraqi waters through ships transport activities that coming from other neighboring countries. Moreover, the species managed to live and reproduce in the local environment, especially the species has relatively fast growth rates (Seeto et al., 2012). We can conclude that the continuous changes in our local environment, global changes related to climate changes and the link between the different countries of the world contributed to the presence of this species in the waters of Al-Hammar marsh.

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