New geographic site records for Artemia in Iran

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Abstract

The last distribution map of different Artemia populations in Iran dates back to 2006, which reported the identification of Artemia from 17 different geographical areas. Iran climate changes and Artemia compatibility to new environments, reinforce the importance of the study of its new habitats. In this study, in addition to introducing three new habitats for Artemia, scientific information on the species and population characteristics of Artemia found from these new habitats was presented. Moreover, cyst samples were recorded and stored in the cyst bank of National Artemia Research Center, Urmia, Iran. Cyst samples collected from three new areas of Iran including Qazvin, Behshahr and Khoy were transferred to the laboratory for general biometric study of cysts, molecular studies and species differentiation by examining the nucleotide diversity of the COI-mtDNA region and the marker of the Na/K ATPase pump. The results showed that the identified Artemia were all bisexual and in terms of species, the Artemia found in Behshahr region was *Artemia sinica* and those found in Qazvin-Abyek and Khoy-Qotur were *A. urmiana*. The mean diameter (mean ± standard deviation) of the Artemia cyst in Behshahr region was significantly different from that of the other two regions (p<0.05). The largest cyst diameter was related to *A. sinica* from Behshahr region (280.53±19.64 µm) and the smallest cyst diameter was related to Artemia in Khoy-Qotur region (255.15±5.51 µm). For the first time, the isolation and identification of *A. sinica* in Behshahr and *A. urmiana* in Qazvin and Khoy were reported in Iran. The reason for the presence of Artemia in these areas seems to be the presence of nearby fish farms, which may have used Artemia as a live food for larvae, from which Artemia cysts spread out and became a permanent habitat of the reported areas in recent years.

Keywords: Artemia, Distribution, Population, Genetics, Iran

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Introduction

Artemia is a zooplankton crustacean found in high-salinity environments such as salt lakes, coastal salt ponds, and solar salt ponds worldwide (Vanhaecke *et al*., 1987; Abatzopoulos *et al*., 2009; Berindean *et al*., 2020), that has a special important place as a live feed in aquaculture especially for aquatic larviculture (Sorgeloos *et al*., 1986; Berindean *et al*., 2020; Madkour et al., 2023). Artemia was first described by Schosser in 1755 (Sorgeloos, 1980; Asem, 2008) and since then, many habitats have been reported for Artemia and its populations have been identified in all parts of the world except North Pole (Van Stappen, 2001). In 1915, Abonyi published a list of 80 Artemia sites in 21 countries. Similar lists were published by Artom in 1922 with 18 locations, Stella in 1933 with 28 locations, and Barigozzi in 1946 with 29 locations. In 1980, Persoone and Sorgeloos published a list of 250 places spread across more than 48 countries. Vanhaecke *et al*. (1987) published new studies covering 350 natural sites in Artemia. Subsequent discoveries of Artemia continued, and useful information from a list of about more than 500 Artemia sites was reported by Van Stappen (2001) (Asem, 2008; Abatzopoulos *et al*., 2009).

In addition to special lists covering the world, several studies have devoted their studies on Artemia sites to the study of specific geographical boundaries, generally of a particular country or region, such as China (Xin *et al*., 1994), Spain (Amat *et al*., 1995), Iran (Abatzopoulos *et al*., 2006), Mexico and Chile (Castro *et al*., 2006) and Africa (Kaiser *et al*., 2006). In recent decades, a new population of Artemia sp. which has features to be named as a species has also been identified from an unknown location in Kazakhstan (Pilla, 1992; Pilla and Beardmore, 1994). The latest details on Iranian population of artemia are described by Abatzopoulos *et al*. (2006). Due to the importance of Artemia in the world, it is necessary to study its new habitats and update the distribution list of Artemia. In this study, the species and biometric characteristics of Artemia cysts found in these habitats were investigated.

Material and methods

This study was conducted in order to establish a cyst bank of different populations of Artemia during 2019-2020. Based on local reports received and in coordination with the Fisheries Management and the General Department of Environmental Protection located in different provinces of Iran, new Artemia habitats were identified, then, samples were collected and transferred to the Artemia Biotechnology Laboratory in National Artemia Research Center, Urmia, Iran for further analysis. The study locations are described in Table 1.

Biometric examination of the cyst

Samples of cysts were taken in conical containers with a capacity of 50 ml by Dietrich and Kalle solution. For this purpose, besides the above solution, Lugel solution was added to each container at a rate of 1% and gently aerated in the dark for 3 hours (Vanhaecke *et al*., 1980).

Table 1: Geographical locations of Artemia new habitats.

Location	Latitude & Longitude
Behshahr, Mazandaran Province	36.751859 – 53.544863
Khoy, West Azerbaijan Province	38.520496 – 44920308
Qazvin, Qazvin Province	36.011637 – 50.300251

The cysts of each conical vessel were then collected using a 100-micron mesh. Approximately 1000 complete cysts were biometrically investigated using binocular microscope equipped with a 2000 Mutic loop motor, connected to a biometric monitor on which MLC-150C software was installed and the mean diameter of cysts (Mean±SE) was calculated. Then, the same number of cysts were decapsulated according to the method of Bruggman *et al*. (1980) using sodium hypochlorite containing 5% active ingredient and their chorion thickness was calculated according to the following formula:

$$ch=\frac{(ND-D)}{2}$$

Ch: Chorion Thickness; ND: Non - Dencapsulated cysts; D: Decapsulated cysts

Molecular investigations

DNA of cysts collected from each habitat was extracted individually by Chelex method. The COI region of the mtDNA genomic region was used for molecular studies and species segregation and Na/K ATPase was used for determining bisexuality and parthenogenesis (Mousavi, 2010; Manaffar *et al*., 2011a). For this purpose, DNA of Artemia cysts was extracted from each cyst sample using Chelex method, then a spectrophotometer was used to confirm the success of DNA extraction. A 280 bp fragment of the nuclear Na/K ATPase gene was amplified to investigate the parthenogenesis or bisexuality of these Artemia (Manaffar *et al*., 2011a). The PCR product was enzymatically cut with Tru1I enzyme to identity confirmation according to the protocol. PCR product was evaluated in all experiments using 2% agarose gel electrophoresis and GeneFlash gel imaging device. The primers and PCR program used in these two molecular methods are summarized in Table 2.

Table 2: Primers and PCR program related to amplification of COI-mtDNA and Na/K ATPase regions in this study

Parameters	Primers	Program
COI-mtDNA	GGT-CAA-CAA-ATC-ATA-AAG-ATA-TTG-G TAA-ACT-TCA-GGG-TGA-CCA-AAA-AAT-C-A	94 ºC for 3 min 94 ºC for 1 second 50 ºC for 1 second 72 ºC for 1.5 min 72 ºC for 3 min 22 cycles
Na/K ATPase	CAG-CCA-AAC-GTA-TGG-CTT-C' GAA-TTC-AGC-ACG-ACT-GCA-AA-G	94 ºC for 2 min 94 ºC for 25 second 56 ºC for 45 second 72 ºC for 25 min 72 ºC for 3 min 22 cycles

Data analysis

Statistical analysis of the data was performed using SPSS software. First, the normality of the data was assessed using the Kolomogorav-Smirnov test and then the homogeneity of variances was performed using the Leven test. Duncan's test was used to compare the means. In all analysis, the allowable error of 5% was considered. The obtained data were analyzed on the NCBI/BLAST website, Sequence Alignment and Chromas program.

Results

Khoy study station

The location was 4 km far from the Khoy city, West Azerbaijan, Iran with an altitude of 1909 meters above sea level and with an area called Doz Daghi located on the road of Amir Beyg village, which contains 2 reservoirs (Fig. 1). Based on reports recieved, the pond has existed from 17 years ago and it was temporarily fed by spring water and rain in the seasons of the year and its depth was between 10 to 30 cm. Currently, some parts of the pond have been filled with soil due to road construction operations. Its water salinity was 16 ppt and its acidity was pH=7. In this station due to the arrival of fresh water and also possibly to fight the malaria-carrying mosquito, Gambusia fish was released, a live specimen of which was observed during study period. The origin of Artemia in this station is not known, but it was probably transferred to it from Urmia Lake by migratory birds.

Figure 1: Khoy city sampling station.

Qazvin study station

This sampling place was located in Qazvin province in Basharyan, Abyek city, from 20 km of Amorian village-Aladaghloo village (Fig. 2). These drains were drains in agricultural lands and pistachio orchards and its soil contains salt. Its water was originated through drainage which have been constructed to reduce water salinity. The population of the area was 50 people and the distance from the main road was 7 km. The land was privately owned and there has previously been limited trout, carps and sturgeon fish farming in the area. However, no farmed fish were observed during the sampling period. The above farm has a water well with a depth of 18 meters that had a salinity of 32 ppt. The drainage had a width of 3 m and a length of 800 m which fed with a water source of around drainage (agriculture). According to the information obtained, the available Artemia has probably been transferred to these drains since the activity of the fish culturing farm in this area.

Figure 2: Qazvin city sampling station.

Behshahr study station

The samples were collected from aquaculture earth ponds (50 hectares) of Sadaf Mahi Company located in the north of Behshahr city, Mazandaran province (longitude 53º, 32′, 21″ and latitude 36º, 46′, 29″) (Fig. 3). Around 45 hectares of the ponds were related to the culture of *Litopenaeus vannamei* (30 hectares) and sturgeon fish (15 hectares) and Artemia has also been cultured in 3-5 hectares since its first introducing into these ponds in 1999. The most important factors for selecting these ponds for Artemia culture were the appropriate climate and physicochemical conditions of the stored water (salinity, temperature and pH) and the presence of phytoplankton and micronutrients. There was enough fresh water to watering all the existing ponds through the nearby traditional water supply canal (salinity 0), but the saline water supply has been provided by digging canals six meters deep (salinity 50) next to this farm (Table 3).

The results of molecular studies are shown in Figures 4 and 5. The results of biometric experiments of cysts obtained from study stations are presented in Table 4.

In this study, Artemia cyst of Qazvin station with a mean diameter of about 280 microns was the largest cyst and the other two samples obtained from Khoy and Behshahr had diameter of 255 and 254 microns, respectively.

Figure 3: Behshahr city sampling station.

Table 3: Results obtained from Blast of obtained nucleotide sequences and related comparison in the NCBI database.

Station / location	Species	Percentage of overlap	Percentage of homogeneity
Behshahr	*A. sinica*	96	76.17
Khoy	*A. urmiana*	97	98.60
Qazvin	*A. urmiana*	95	96.50

Table 4: Comparison of the mean (± Standard deviation) of cyst diameter of the identified Artemia population.

Sampling location	Identified species	Average cyst diameter	Registration date in Cyst Bank
Behshahr - Sadaf Mahi Co.	*A. sinica*	254.61 ± 12.36	23/10/2018
Khoy - Razi Border Road	*A. urmiana*	255.15 ± 5.51	20/11/2019
Qazvin - Abyek	*A. urmiana*	280.53 ± 19.64	7/2/2020

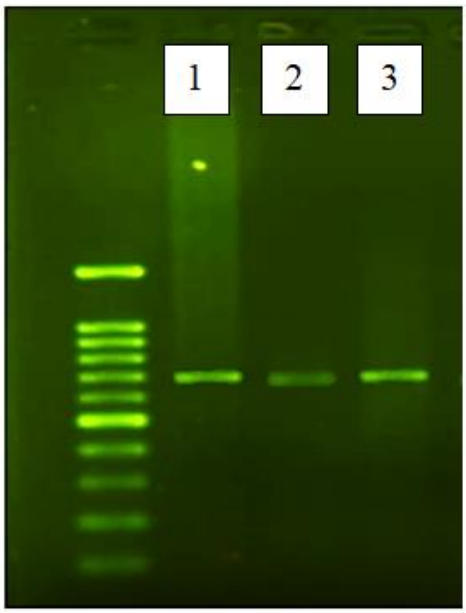

Figure 4: Gel electrophoresis related to COI sequence in the studied populations. The firs column) 100 bp DNA ladder, 1) Behshahr, 2) Qazvin, 3) Khoy.

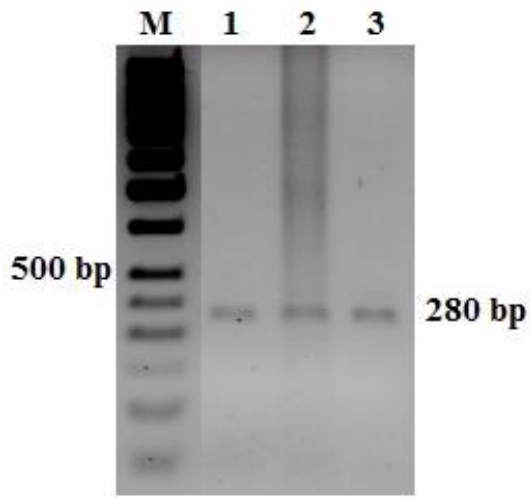

Figure 5: Gel Electrophoresis related to the 280 bp region of Na/K Atpase gene in the studied Artemia. The presence of a single band in the study indicates that bisexuality of Artemia. M) 100 bp DNA ladder, 1) Behshahr, 2) Qazvin, 3) Khoy.

Discussion

The most important habitat of Artemia in Iran is the Lake Urmia, where the presence of Artemia has been reported by Gunther for the first time in 1900 (Gunther,1990; Asem, 2008), and then in 1976, it was named by Clark and Bowen under a separate species called *Artemia urmiana* (Clark and Bowen, 1976). In addition to the bisexual Artemia, which lives in the Lake Urmia, there is also a type of parthenogenetic Artemia in the ponds around the Lake Urmia, which was first reported by Agh and Noori (1997).

Also, the existence of Artemia in the Shorabil Lake, Ardabil, Iran has been reported by Ahmadi (1987). In addition to the above cases, the existence of Artemia in several other parts of Iran has been reported including: the Namak Lake and Qom Sultan Basin and the surrounding water catchments in Qom Province, the Incheh and Shur Reservoirs near Aqqala, Golestan Province (Makhdomi, 1992), the Jazmourian Lake in Kerman Province, Vermal Reservoir in Sistan and Baluchestan Province (Piri and Tehrani, 1997), the Maharloo, the Bakhtegan and the Tashk lakes in Fars Province, the Nogha reservoir in Rafsanjan, Kerman Province, the Gavkhuni Wetland in Yazd Province, the Miqan lake in Markazi Province, the Gonabad Kal Shoor in Gonabad, Khorasan Province, the Khoramabad Kal Shoor, Khoramabad Provice, the Hashtgerd Kal Shoor in Tehran, Tehran Province, the ponds around Lake Urmia, Urmia and the catchments of Masjed Soleiman in Khuzestan. The presence of Artemia in some of the mentioned areas has not been carefully studied, furthermore, the presence of Artemia in other regions of Iran has not been ruled out and needs further investigation (Abatzopoulos, *et al*., 2006).

The results of the study showed that the identified Artemia were all bisexual and in terms of species, the artemia found in Behshahr region was *Artemia sinica* and the species found in Qazvin-Abyek and Khoy-Qotur was *A. urmiana*. So, the isolation and identification of *A. sinica* in Behshahr and *A. urmiana* in Qazvin and Khoy were reported in Iran for the first time in this study. In the present study, except Behshahr station, the other two stations were diagnosed with endemic bisexual artemia (*A. urmiana*). Numerous studies have been conducted on the genetic diversity of Artemia, in order to study the genetic differences between Artemia species of the Lake Urmia and the parthenogenetic populations around this lake and also to study the phylogenic relationships between some Artemia populations of Iran (Qom, Arak, Golestan and Shiraz cities) and a number of bisexual and parthenogenetic foreign species using PCR-RAPD molecular index (Manaffar *et al*., 2011a; 2011b). In studies conducted by Eimanifar *et al*. (2013), it was reported that the classification pattern led to the separation of specimens into two separate clusters when studying *A. urmiana* and samples around the lake Urmia and four separate clusters when studying Iranian and foreign artemia species.

Although salt water Artemia are all of the same genus, there is a genetically farther relationship between bisexual and parthenogenic Artemia populations of the Lake Urmia (Manaffar *et al*., 2011a), which in the present study, their identification was confirmed using a Na/K pump marker.

In a study by Joaquin *et al*. (2008), using the cytochrome oxidase (COl) mitochondrial gene sequence on the biodiversity and phylogeny of bisexual Artemia, *A. salina* and *A. franciscana*, it was concluded that the reason that *A. salina* spread well in the Mediterranean region may be due to scattering factors such as bird migration, human management as well as intra-population potential of this species. In contrast, the genus *A. franciscana* has a very low distribution in this region (Hami Tabari *et al*., 2010).

Numerous morphological studies have been performed on the Artemia sources of the Lake Urmia, other parts of Iran and foreign sources. However, limited studies have been performed on Artemia of the lake Urmia and other Artemia of Iran based on the study of physical characteristics. In our study, morphological comparison of the mean diameter index (±standard deviation) of Artemia cyst population in Behshahr region with the other two regions showed a significant difference ($p<0.05$). The largest cyst diameter was related to Artemia from Behshahr region (*A. sinica*: 280.53±19.64 µm) and the smallest cyst diameter was related to bisexual artemia in Khoy-Qotur region (255.15±5.51 µm).

Evaluation studies of genetic diversity and population structure of the Lake Urmia Artemia have been conducted by Eimanifar *et al*. (2006) and Baxevanis *et al*., (2005, 2006), however, none of the conducted studies cover the information gaps related to the artemia gene bank of the Urmia Lake and other parts of Iran

and gene and cyst products of each region is not available yet.

Conclusion

The results showed that the identified Artemia were all bisexual and in terms of species, the artemia found in Behshahr region was *Artemia sinica* and the species found in Qazvin-Abyek and Khoy-Qotur was *A. urmiana*. For the first time, the isolation and identification of *A .sinica* in Behshahr and *A. urmiana* in Qazvin and Khoy were reported in Iran. The reason for the presence of Artemia in these areas seems to be the close near several fish farms, which may have used Artemia as a live feed for larvae, from which Artemia cysts spread out and became a permanent habitat of the reported areas in recent years. Due to the climate change occurring in inland water resources of Iran, it is suggested that the devices related to environmental protection support the Cyst Bank of different populations of Artemia established and launched in National Artemia Research Center under the support of Iranian Fisheries Science Research Institute and to take effective and intelligent steps to register and maintain Artemia populations and develop the Artemia gene bank to protect Iran's animal genetic resources.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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