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Morphological and molecular clues for recording the first appearance of *Artemia franciscana* (Kellogg, 1906) in Egypt

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Abstract

Artemia franciscana is a native species to the New World, and became an exotic species to most parts of the world. The Egyptian hypersaline, continental Qaroun Lake (Fayoum Governorate, Middle of Egypt) is subjected to a gradually increasing salinity rates that approximate or exceed these of seawater. *Artemia* populations there are known to be parthenogenetic. Yet, these populations started to exhibit abnormal morphologies. Therefore, Qaroun Lake samples of *Artemia* were subjected to several morphological, biometric, and molecular phylogenetic analyses for accurate species identification and phylogeographic origin approximation. These analyses revealed the existence of the alien sexual species of brine shrimp *A. franciscana* in Qaroun Lake. The characteristics of the subspherical frontal knob with several spines on the top, ovisac lateral triangular lobe on both sides and its projection together with the biometrics confirmed this

species morphotype. DNA barcoding and other molecular analyses based on PCR-based amplification and sequencing of the barcode region of the cytochrome oxidase subunit I gene (COI) exhibited that all the collected samples belong to five haplotypes. Egyptian *A. franciscana* COI sequences phylogeny and pairwise distances analysis exhibited closer proximity to Latin American strains than to the Northern American ones. *A. franciscana* presence may be ascribed to the migratory birds present in Qaroun Lake protectorate, since no marine aquaculture activity in Qaroun Lake is known. Therefore, and for the best of our knowledge, this is the first record of the invasive *A. franciscana* in Egypt.

Keywords: Ecology, Genetics, Molecular biology, Systematics, Zoology

1. Introduction

The brine shrimp, *Artemia* (*Branchiopoda*, *Anostraca*) is a keystone species in hypersaline food webs, a major resource for feeding marine aquaculture larvae, and a widely-applied animal model for ecotoxicology, developmental biology, ecology and evolutionary biology (Lenormand et al., 2018). Its use in carnivorous larviculture is exceedingly a common protocol due the easiness of hatching of its nauplii and being the least labour-intensive live feed, besides being a plausible food source for species whose larvae are relatively large at hatching (Conceição et al., 2010). A single species, *A. franciscana* (Kellogg, 1906), that is native in North, Central and South America, is of great economic importance due to its use in aquaculture as live feed for larvae. Since 1950, *A. franciscana* have been exported to many countries worldwide for the use in fish hatcheries. Up to 90 % of global trade of brine shrimp cysts come from the population of this species in the Great Salt Lake (GSL) in the USA (Ruebhart et al., 2008a). For more than 30 years since the beginning of commercial marine aquaculture activity, GSL was under a massive, selective pressure of fishing floating cysts especially, which was found to threaten the genetic structure and diversity of strains of this species (Sura and Belovsky, 2016). *A. franciscana* was recorded in many countries other than the USA as invasive species, chiefly Australia, Brazil, China, Morocco, Iran, France, Tunisia, India and Kenya (Ruebhart et al., 2008a,b; Camara, 2001; Zheng et al., 2004; Hajirostamloo and Pourrabbi, 2011; Scalone and Rabet, 2013; Ben Naceur et al., 2013; Krishnakumar and Munuswamy, 2014; Ogello et al., 2014). Non-indigenous species may cause economic gains in some parts, however, can cause great damage to others (Pimentel et al., 2000; Vijayan and Syad Rao, 2009). Aided by its enormous euryhaline, eurythermal, high fecundity, and high levels of phenotype plasticity, *A. franciscana* could invade many areas in South America, Asia, Africa, and Europe, eliminating most of the autochthonous species (Amat et al., 2007). This species appearance in the Mediterranean is dated to 1980s (Hontoria et al., 1987). It poses

serious threat to the genetic diversity of local species like the Mediterranean *A. salina* (Muñoz et al., 2008). *A. franciscana* was intentionally introduced to India in the early 1980s as live food in aquaculture. This anthropogenic interference led to a gradual loss of native parthenogenetic *Artemia* to be replaced by the invasive American one, even at areas where climatic conditions are suitable (Vikas et al., 2012). Even its introduction in waters that devoid native species of *Artemia* can alter food webs, nutrient cycles, algal biomass, and primary production; i.e. disturbs the entire ecosystem balance (Ruebhart et al., 2008b).

Artemia species are categorized in two differentially reproducing groups; that are the bisexual and the parthenogenetic (Browne and Bowen, 1991). The bisexual *Artemia* species were recorded in USA (*A. franciscana*), Iran (*A. urmiana*), Italy and North Africa (*A. salina*) and China (*A. sinica*) (Hontoria; Amat, 1992; Van Stappen et al., 2001; Triantaphyllidis et al., 1997; Naihong et al., 2000). Despite being an essential resource for aquaculture and a major biomarker species for nomadic birds' movement and salinity changes, morphological and genetic data about Egyptian *Artemia* species (Arthropoda: Anostraca) are very scarcely available. Few species of *Artemia* were discovered to be native, mainly *A. salina* and *A. parthenogenetica* (El-Bermawi et al., 2004). Both species are known to co-exist in the Mediterranean and several areas of the old world (Vikas et al., 2012).

For morphological identification of *Artemia* species, furca, penis, ovisac and frontal knobs were the most body parts that are applied for species systematics (Mura et al., 2006; Vetriselvan and Munuswamy, 2011; Scalone and Rabet, 2013). Other taxonomists focus on the biometrics of body, abdominal, furcal and antennal lengths, abdominal, ovisacs, head and eyes widths; number of setae of the furca (Amat et al., 2004; Agh et al., 2009; Krishnakumar and Munuswamy, 2014). For DNA barcoding, and since its identification by Hebert et al. (2003), its utilization in aquaculture and fisheries-related activities is continuously increasing. Use of short DNA sequences, i.e. barcodes, could successfully characterize species and populations of great importance both as economic and hazardous, such as populations of Japanese oyster *Crassostrea gigas* (Semeraro et al., 2016), salmon louse *Lepeophtheirus salmonis* (Boulding et al., 2009), blend of silver catfish *Rhamdia* species (Scaranto et al., 2018). In Egypt, the application of such taxonomic tool is still in an early stage. For example, it was used for characterization of different shrimp species in the Mediterranean and Red Seas (Sharawy et al., 2017; Abbas et al., 2018), as well as identification of commercial alteration in fisheries-related products (Galal-Khallaf et al., 2014). Genetic and morphological characterization of aquatic resources in Egypt can provide tools of great importance for aquaculture development.

Qaroun Lake is one of oldest lakes in Egypt, known as Lake Moeris at the ancient Egypt time and located at Al-Faiyum province. The Lake is a hypersaline depression, with salinity (salt budget) ranges between 29.2 and 35.4 ‰ at winter, 27.1

and 36.2 ‰ at Spring, 29.3 and 38.0 ‰ at Summer and 29.7 and 35.4 ‰ at Autumn (Abd Ellah, 2009). It used to be fed with the Nile but its opening was closed and the only water it receives is from agricultural drainage, besides high degree of water evaporation that led to increased salinity (El-Bermawi et al., 2004). It previously gained its importance in Egypt from providing the highest fisheries yield of any Egyptian inland lake, constituting a large portion of the local economy and fish and shellfish supplies to regional markets throughout Egypt (Hussein et al., 2012). The major fish species present there include the Redbelly Tilapia, *Tilapia zillii*, soles, *Solea* spp. and mullets (*Mugil* spp. and *Liza* spp.). Penaeid shrimps were introduced successfully into the lake in 1970s (Abdel-Malek et al., 1990). The species of *Artemia* known in Qaroun Lake was *A. parthenogenetica*, occupying the west side where Sodium Chloride and Sulphate are produced for commercial uses (El-Bermawi et al., 2004). However, and starting from March 2013, and in some of our field trips to identify an unknown cymothoid parasite that emerged there, we noted that the species identified as *A. parthenogenetica* in Qaroun Lake exhibits unusual morphological characteristics. Thus, this study had two main aims. First, to assess the species of *Artemia* that is currently present in the most active fish landing location in Qaroun Lake from both morphological and molecular viewpoints. This was expected to provide a clear image about whether the species present in the Lake is suffering morphological alterations due to the increasing salinity of the Lake, or it is a completely new species that was deliberately or intentionally introduced to the Lake. Presence of a new species can have unexpected impacts on the lake's food web, especially since this lake is already suffering a number of invasive species that could change its ecological balance massively (for example, see El-Shabrawy and Dumont, 2016; Ali and Aboyadak, 2018). Different stages and varying ecological conditions control main aspects in the life of *Artemia* as growth and grazing rates (Sura et al., 2017). Hence, identification of the clear identity of Lake Qaroun *Artemia* is crucial for Lake Ecosystem maintenance, controlling, and risk assessment. The second aim of this study was to apply genetic phylogeography whenever possible to detect the possible origin of the new lake inhabitant *Artemia*, which can be an accurate approach to predict its coming impacts and behaviour in relation to physiological capabilities it exhibits in its natural range, in the same way as usually done for other invasive species.

2. Materials and methods

2.1. Study area and samples collection

Qaroun Lake is a sabkha present at Al-fayoum province, Egypt. It exhibits a bottom of sand and gravels (Fig. 1). In March 2016, *Artemia* specimens were collected from Qaroun Lake near Shakshouk Village (coordinates, 29.465229 N, 30.707076 E, water salinity: 37‰). These samples were immediately preserved in 70 and 100 % ethyl

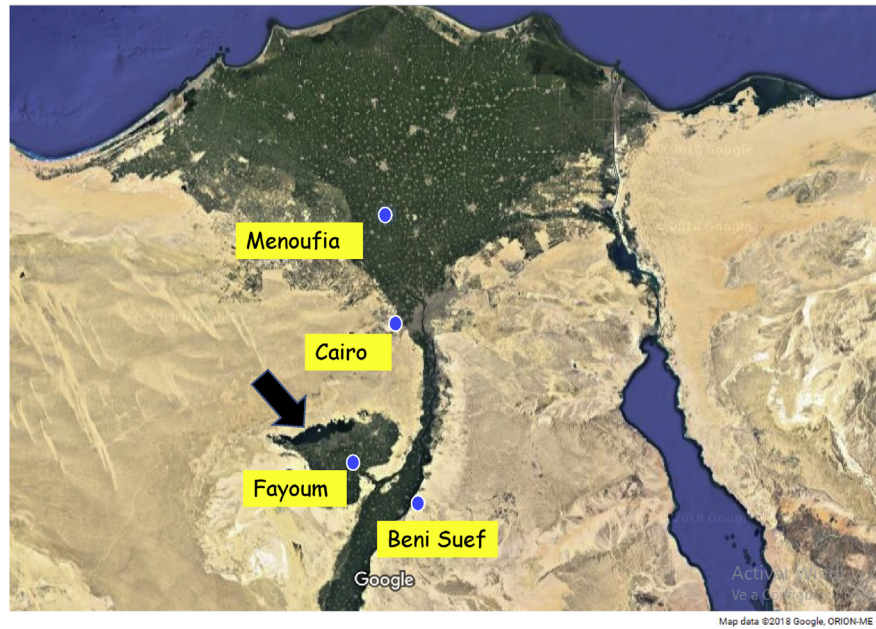


Fig. 1. The location of Qaroun Lake (black arrow) in Fayoum governorate, Egypt. Image credits: Map Data ©2018 Google, ORION-ME.

alcohol in tightly closed Eppendorf tubes. All individuals were morphologically identified. 70 % ethyl alcohol preserved samples were photographed with digital camera attached to research microscope. After morphological identification, the samples were subjected to genetic analyses, as it will be mentioned later in details.

2.2. Samples preservation and preparation of the whole mount

Artemia samples ($n = 30$), preserved in 70% ethyl alcohol and flattened by placing between two slides, were hydrated in a descending grade of ethyl alcohol then stained with Allum- Borax carmine. Dehydration was done using ascending series of ethyl alcohol, then, the samples were cleared in clove oil and finally mounted in DPX (El- Banhawy and El- Gansory, 1989).

2.3. Biometric parameters

Samples were examined morphologically. The biometric parameters of males and females were recorded as follows: total body, thorax and abdomen lengths, compound eye and head diameters, 1st antennae and 2nd antennae last segment lengths, thoracic widths, abdomen widths, ovisac diameters, and furcal lengths. These biometrics were measured using a calibrated ocular micrometer under a light microscope to the nearest millimeter (mm).

2.4. Morphometrics' statistical analysis

Data were analysed using Statgraphics (v5 software) to detect significant differences between male and female biometrics. Data were expressed as mean \pm SE. The statistical analysis was carried by unpaired student *t*-test setting the probability level when $P \leq 0.05$. When Student *t*-test is not applied; otherwise, Mann-Whitney test was applied to compare medians. Simple regression was used to detect the correlation between abdomen width and ovisac diameter in females and 2nd antennal length and thorax width in males.

2.5. DNA barcoding and genetic analyses

2.5.1. DNA extraction

DNA was extracted from entire bodies using 5 % Chelex[®] 100 sodium form resin (Sigma-Aldrich, Madrid, Spain) in TE buffer (pH 8) according to the protocol described by [Wolff and Gemmel \(2008\)](#). 2.4 U of Proteinase K (ThermoFisher) was added to each tube. Samples were incubated at 55 °C with shaking at 30 min intervals overnight. Samples were then boiled in a 100 °C water bath for 20 minutes and finally they were stored at 4 °C until COI gene amplification.

2.5.2. PCR amplification for COI gene

The barcode region located at the 5' area of the COI gene was amplified by PCR using the conditions and primers pair *tgLCOI490* and *tgHCO2198* of [Geller et al. \(2013\)](#). Final PCR volume in all cases was adjusted to 50 μ L. 200 ng μ L⁻¹ of bovine serum albumin (BSA) were used as PCR additive. The PCR was performed using DreamTaq[™] Green PCR Master Mix 2X (ThermoFisher) according to the manufacturer's instructions. 3 μ L of each PCR amplicon were electrophoresed in a 2 % agarose gel, stained with 0.5 μ g mL⁻¹ of Ethidium bromide, and visualized using an UV-transilluminator (Biometra, Gernany). The PCRs with positive results were sent to MACROGEN Inc. (Seoul, South Korea) for standard Sanger sequencing of the genetic barcodes.

2.5.3. Sequences, DNA barcodes, and phylogenetic analyses

The resulting COI sequences were first checked for correcting any possible errors in base recalls. Then, these sequences were compared to references present in GenBank database using BLAST, and with BOLD using IDS tools. They were aligned using CLUSTALW integrated with the program MEGA 6 ([Tamura et al., 2013](#)) in order to be uploaded to DNasp 5.0 Software ([Rozas et al., 2003](#)) for haplotypes' determination. Other COI sequences belonging to *A. franciscana* from all over the world were retrieved from GenBank database, together with other species' COI sequences from

A. parthenogenetica, *A. salina*, *A. tibetiana*, *A. persimilis*, and *A. urmiana*. All sequences were aligned with those obtained from our Qaroun Lake samples using CLUSTALW integrated to MEGA 6 software. To avoid false positives, species identifications were determined with similarity percentages (i.e., the percentage of similar and different nucleotides in the same site between Qaroun Lake samples and reference samples) >99% (Côté et al., 2013). The aligned sequences were either used for calculation of pairwise distances or saved as fasta format. Then, the fasta file was exported to JModel-Test software V. 2.1.10 for finding the best fit nucleotide substitution model to construct the maximum likelihood phylogenetic tree. Later on, the fasta file was uploaded to Beauti software V. 1.8.3. to estimate the tree topologies, applying 10,000,000 Markov chains. This number of Markov chains was used once and the program ran again twice with 50,000,000 and 100,000,000 chains. Then, the resulting three .xml files were separately uploaded to BEAST software V. 1.8.3. The .log files resulted from the three trials were uploaded to the program Tracer v1.6; to evaluate the quality of the results and the only accepted results were that above 200 Effective Sample Size (ESS). LogCombiner software V. 1.8.3 was used to combine the results of the three trees. The result was uploaded to Tree Annotator software V. 1.8.3 in order to summarize the information that was retrieved from tree samples created using BEAST. Finally, FigTree software V. 1.4.2 was applied to obtain the final consensus tree.

2.5.4. Calculation of pairwise distances and Principal Coordinate Analysis (PCoA)

Pairwise genetic distances were calculated among all haplotypes of *A. franciscana* found in the current study and those retrieved from the GenBank database were calculated using MEGA 6 software, after selecting the best substitution model using the same software. The resulting file was exported as excel file and transformed to the format adequate for carrying out the principle coordinate analysis (PCoA) using GenAlex software 6.501 (Peakall and Smouse, 2012) in order to infer the genetic relationships for the geographic area where those haplotypes are present.

3. Results

3.1. Sexual dimorphism of *Artemia*

In the present study, the collected samples of *Artemia* were bisexual, composed of males with clasps and females with ovisacs, indicating the absence of parthenogenetic *Artemia* populations. The ratios males to females were 1:1, with 10.3 % of females without ovisacs and 89.7 % with ovisacs.

3.2. Remarks of species *franciscana*

Measurements showed significant difference between males and female in the total body length. Males were shorter than females (8.6 ± 0.2 and 9.4 ± 0.3 mm, respectively, Mann-Whitney, $P = 0.04$, Table 1).

For the head, the eyes were semicircular in shape and consisted of several ommatidia and attached to the head by eye stalk. There were pigmentations on the eye stalk. There was no significant differences between right and left eyes diameter of males (1.4 ± 0.1 and 1.5 ± 0.1 mm) and females (1.1 ± 0.1 and 1.2 ± 0.1 mm, t -test, $P = 0.3$, Table 1), respectively. However, there was significant difference in eye diameters between males and females (t -test, $P \leq 0.03$). Which indicated sexual dimorphism based on eye size (Fig. 2a and b). The head diameter (distance between eye stalks) of males and females were 2.5 ± 0.2 and 2.5 ± 0.4 mm, respectively, with no significance difference between them (t -test, $P = 0.1$).

The first antennae were dramatically shorter than the second antenna with at least three terminal spines. The length of the 1st antennae was 2.9 ± 0.4 and 2.7 ± 0.2 mm for males and females, respectively (Fig. 2c and d). There was significant difference between males and females 1st antennae length (t -test, $P = 0.003$, Table 1), where males 1st antennae were longer than females'. That referred to sexual dimorphism based on 1st antennal length.

The second antennae were long and flat at the distal part. The distal part is an acute equilateral triangle shape and their lengths ranged between 5 to 6.4 mm. However, the proximal part has several curves, three at each side. The morphology of the frontal knobs was subspherical with several very short spines on the top. There were pigmentations distributed on 2nd antennae including the knobs (Fig. 3a, b, c and d). A positive significant correlation found between 2nd antennal length of males and their thorax width (Fig. 6a, $r = 0.9$ and $P = 0.0001$). The mouth located in the ventral surface of the body. It located below, parallel position to the naupilar eye, middle position to the maxillary glands and above the neck organs (Fig. 2a).

Table 1. Biometric parameters of males and females *A. franciscana* of Qaroun Lake.

Biometric parameters (mm)	Total length	Left eye diameter	Right eye diameter	Head diameter	1 st antennae	2 nd antennae (last seg.)
Males	$8.6 \pm 0.3^*$	$1.4 \pm 0.1^*$	$1.5 \pm 0.1^*$	2.5 ± 0.2	$2.9 \pm 0.4^*$	5.8 ± 0.3
Females	9.3 ± 0.7	1.1 ± 0.1	1.2 ± 0.1	2.5 ± 0.4	2.7 ± 0.2	NA
	Thorax length	Thorax width	Abdomen length	Abdomen width	Ovisac diameter	Furcal length
Males	$3.3 \pm 0.1^*$	2.2 ± 0.1	$3.8 \pm 0.1^*$	1.8 ± 0.2	NA	1.1 ± 0.1
Females	3.7 ± 0.2	2.4 ± 0.3	4.2 ± 0.2	1.9 ± 0.2	5.8 ± 0.6	1.0 ± 0.1

Note. $n = 30$ individuals, values are means \pm SE, * represent significant difference between males and females biometrics (when $P \leq 0.05$, t -test/Mann Whitney). NA, not applicable.

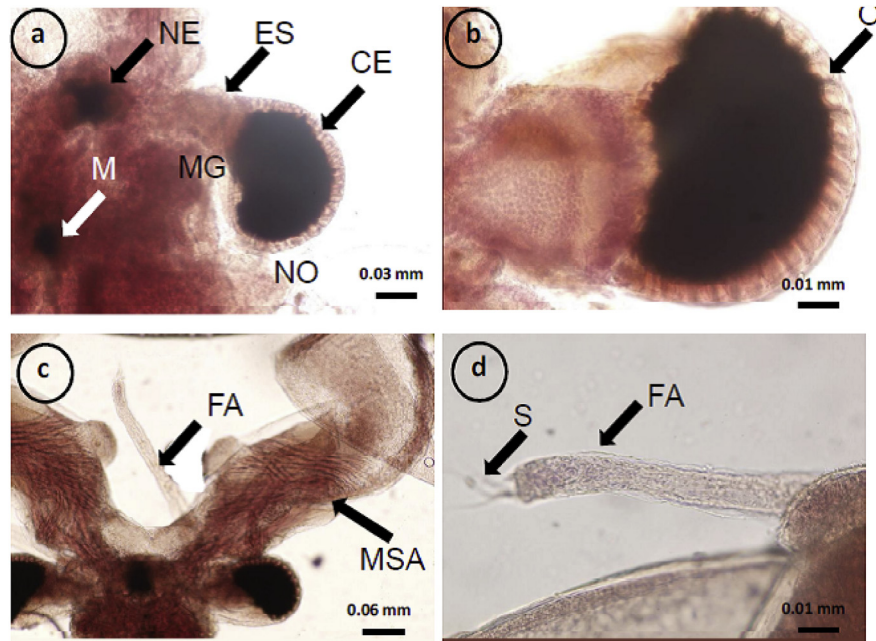


Fig. 2. The head and its appendages of male *A. franciscana* a) the head, b) the compound eye, c) the 2nd antennae and d) the 1st antennae. NE, naupilar eye, ES, eye stalk, CE, compound eye, M, mouth, O, ommatidia, s, spines, FA, first antenna, MSA, male second antenna, MG, maxillary gland; NO, neck organ.

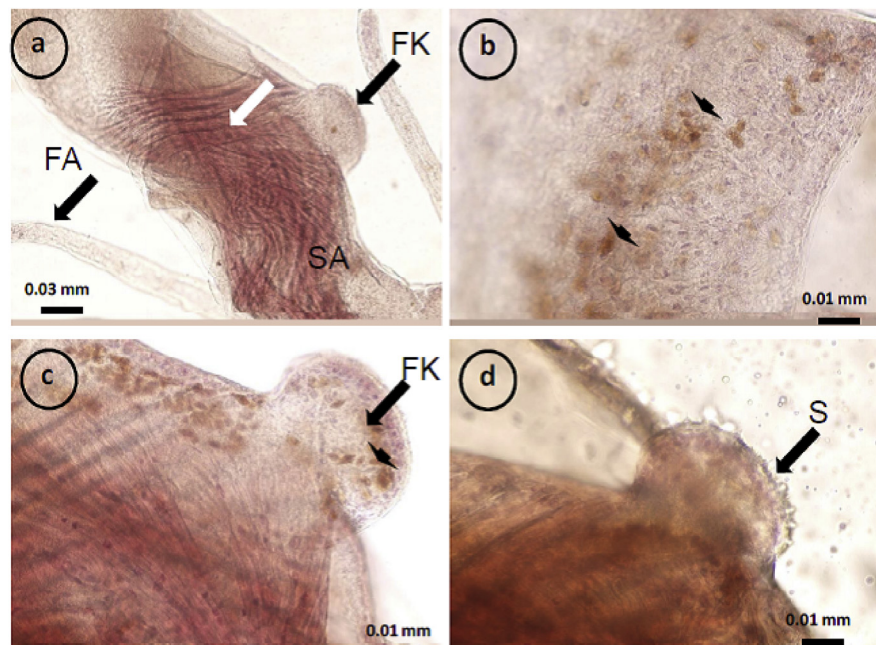


Fig. 3. The 2nd antenna of male *A. franciscana* a) the 1st antennae and 2nd antenna, b) the pigmentation on the 2nd antenna, c) the pigmentation on the subspherical frontal knob and d) spines on the frontal knob. SA, second antenna, FK, frontal knob, white arrow, striations, S, spines, arrow heads, pigmentations.

For the thorax, thoracic lengths of males and females were 3.3 ± 0.1 and 3.7 ± 0.2 mm, respectively. Females exhibited significantly longer thoraxes than males (Mann-Whitney, $P = 0.04$). However, there was no significant difference between males and females thorax width (3.2 ± 0.1 and 3.4 ± 0.3 mm, t -test, $P = 0.4$, Table 1). Thoracic appendages are 11 pairs on both sides of the thorax segments. They divided into three parts, oval exopodite, triangle telopodite and semicircular endopodite. All of them were surrounded by long spines (Fig. 4a, b, c and d).

For the abdomen, the abdominal segments were 6 in number, followed by a telson which was divided terminally into two furcae (right and left). Furcal setae were distributed on the furca. The 1st abdominal segment bore a pair of male genital organs in males and an ovisac in females (Fig. 5a, b, c and d). The ovisacs were with its dilated end toward the thorax and its narrow end toward the abdomen (Fig. 5d). ovisacs were laterally curved with pointed lateral triangular lobes of both sides and a right sided projection was found on its ventral side. Male penis has a pointed distal end internally. The abdomen length of males were 3.8 ± 0.1 mm and females were 4.2 ± 0.2 mm. Males' abdomens were significantly shorter than females' ($P = 0.04$). Meanwhile, males and females abdominal widths did not differ significantly (1.8 ± 0.2 and 1.9 ± 0.2 mm, t -test, $P = 0.7$, Table 1). The ovisacs diameter of females ranged between 4.9 to 6.9 mm. There was a strong, positive and significant correlation between the abdominal width and ovisacs diameter of female samples (Fig. 6b,

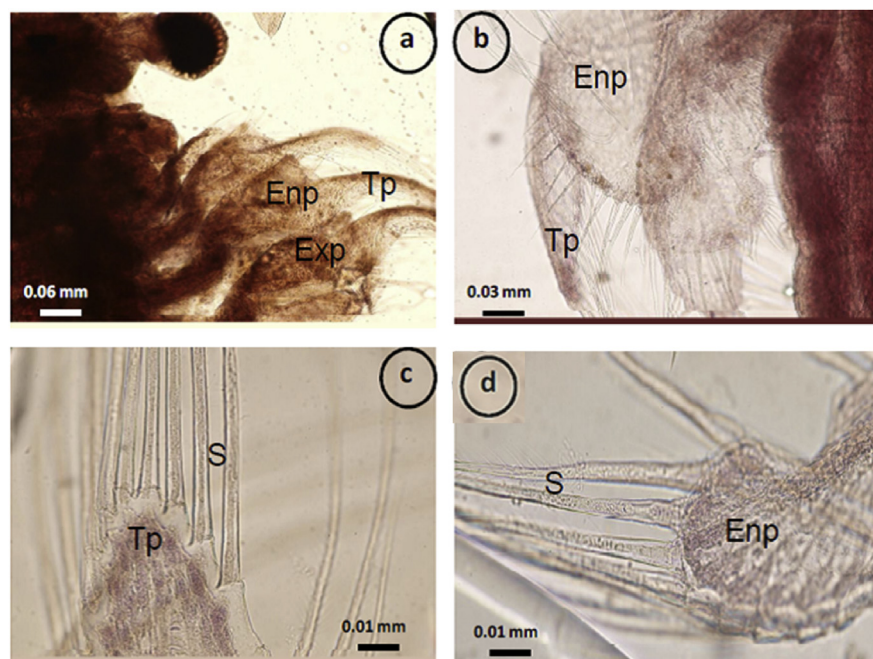


Fig. 4. The thorax and its appendages of male *A. franciscana* a) thoracic appendages, b) enlarged thoracic appendages, c) enlarged telopodite with spines and d) enlarged endopodite with spines. Tp, telopodite; Enp, endopodite; Exp endopodite, s, spines.

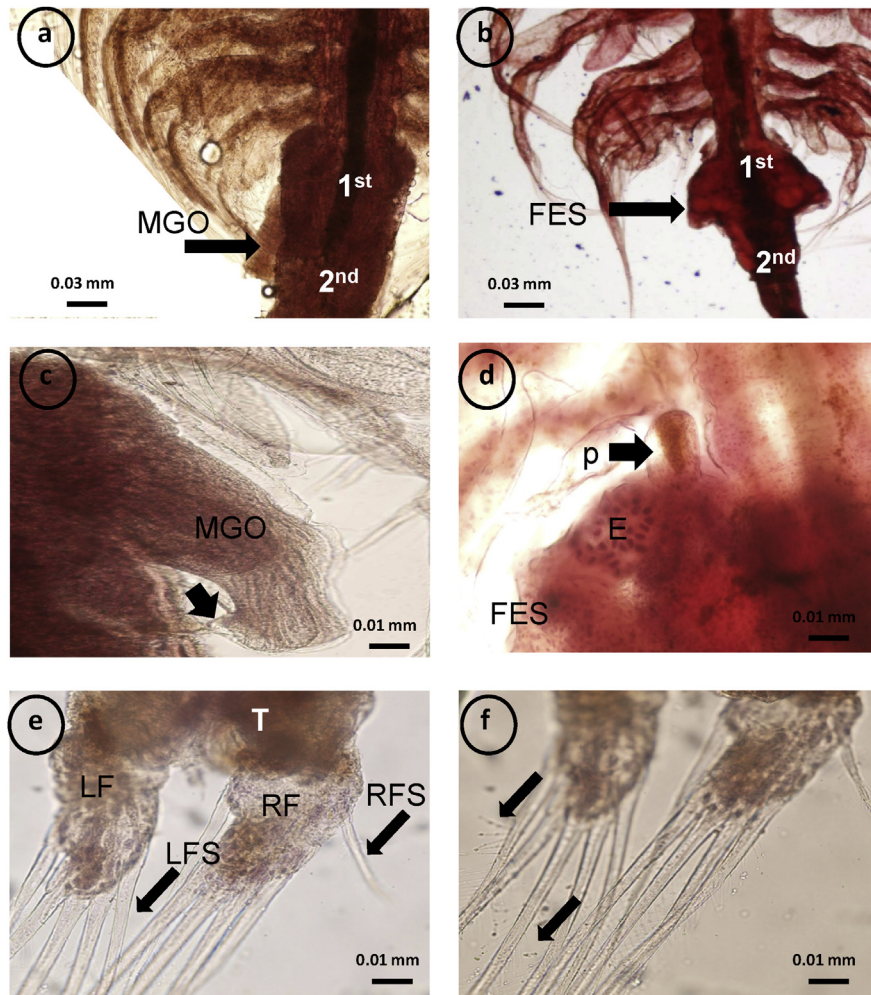


Fig. 5. The abdomen and its appendages of male and female *A. franciscana*. a) abdomen with male claspers, b) abdomen of female bearing ovisac with pointed ends (arrow), c) enlarged male claspers with pointed end (arrow), d) enlarged female ovisac with projection, e) telson and f) caudal furca. MGO, male genital organ; FES, female ovisac; E, eggs; p, projection from the ovisac; T, telson; lf, left furca; rf, right furca; lfs, left furcal setae; rfs, right furcal setae (arrows), spines-like hairs on furcal setae (arrow).

$r = 0.8$ and $P = 0.01$). The caudal rami/furcae were short. Each caudal furca bore 6 long setae. Each setae had several spiny hairs (Fig. 5e and f). The length of caudal furcae were 1.1 ± 0.1 and 1.0 ± 0.1 mm for males and females, respectively, with no significant difference (t -test, $P = 0.4$, Table 1).

3.3. DNA barcoding and phylogenetic analyses

PCR amplification for Folmer region of the COI gene of the sampled Qaroun Lake could be successfully carried out. They were deposited in the GenBank/BOLD/EMBL/DBJ databases with accession numbers MF817948–MF817977. BLAST

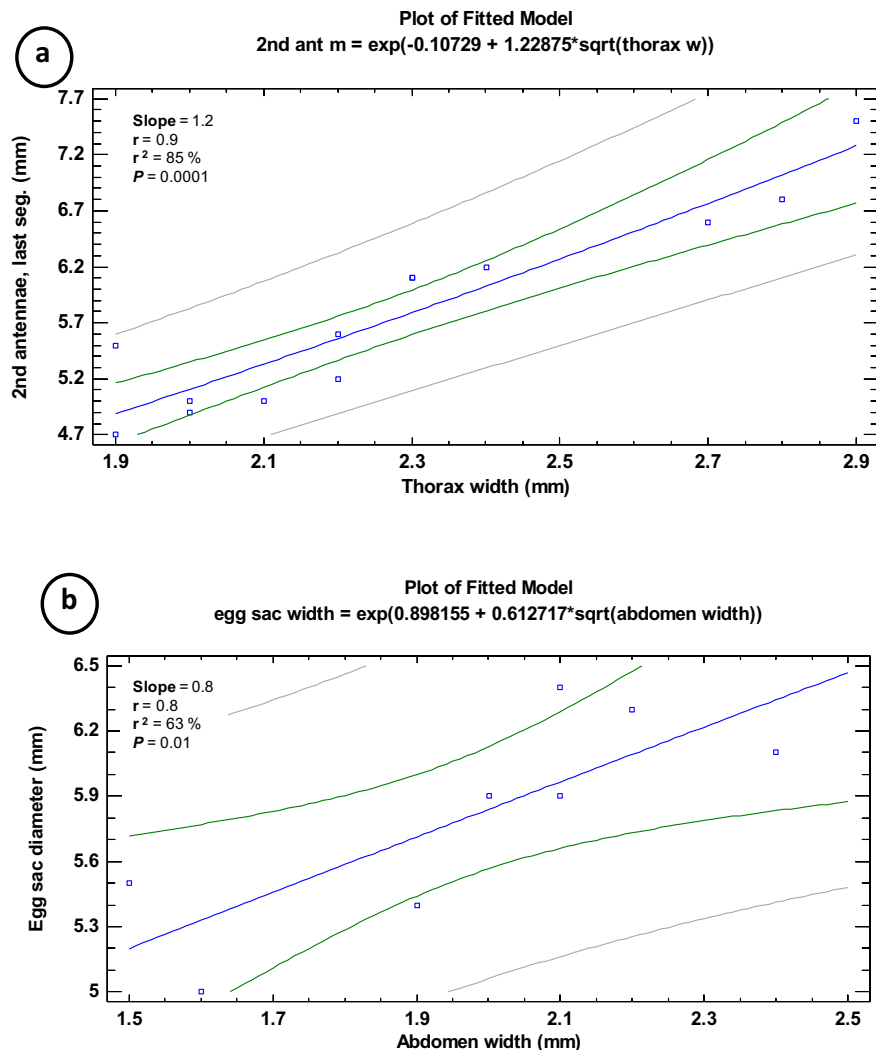


Fig. 6. Relationships of a) 2nd antennal length and thorax width in males and b) ovisacs diameters and abdomen width in females *A. Franciscana* of Qaroun Lake.

and BOLD comparisons resulted in 99–100 % sequence identity with *A. franciscana* present in different areas on the New and Old Worlds. The identity was much lower with other species of *Artemia* that are known to be native or co-existing; for example 85 % with *A. parthenogenetica* (accession number KU183969.1), 82 % with *A. salina* (acc. no. EU543470.1), 85 % with *A. sinica* (acc. no. EU543470.1), 84 % with *A. tibetiana* (acc. no. EU543470.1); 85 % with *A. sinica* (acc. no. EU543470.1). The analyzed thirty samples could be allocated to five haplotypes. Median joining network and maximum likelihood tree (Figs. 7 and 8), with the latter being constructed basing on Tamura 3-parameter substitution model with a Gamma value of 0.28, coincided in the close proximity between the Egyptian haplotypes with the Latin American ones of *A. Franciscana* in its native range, but much more divergent from these present in Canada and USA where the commercial strains of the

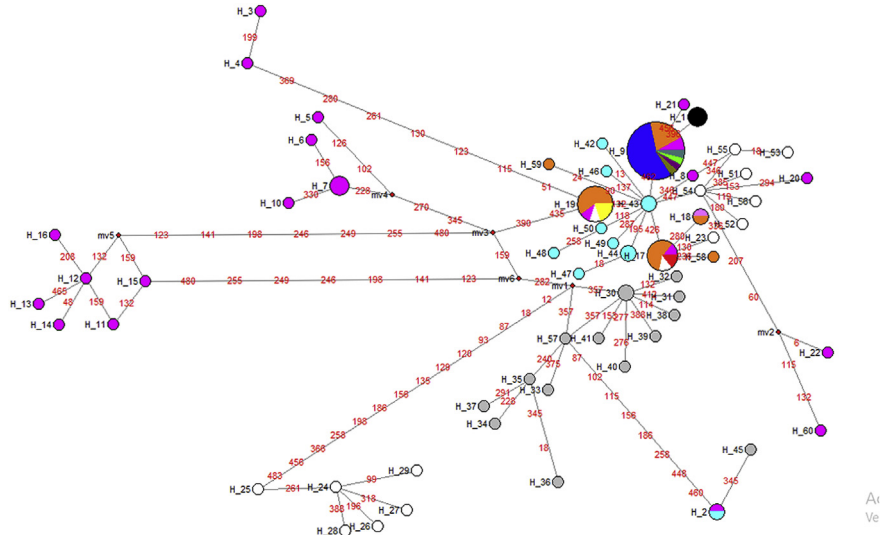


Fig. 7. Median-joining haplotype network. Circles' diameters are proportionate to the number of samples in a given country. Color key, USA: White, Canada: Grey, Mexico: Pink, Egypt: Blue sea, Colombia: Light Blue, India: Green, VietNam: Violet, Portugal: Greyish blue, Iran: Yellow, Iraq: Red, China: Orange, Cuba: Black, Sri Lanka: Greyish yellow.

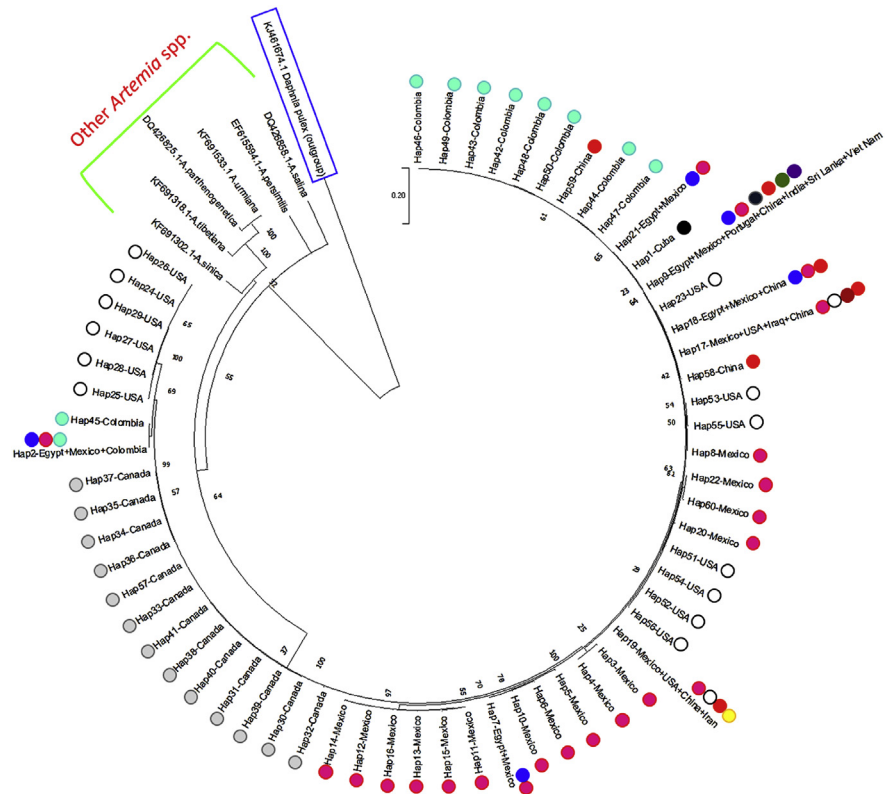


Fig. 8. COI-based maximum likelihood phylogenetic tree for world *A. franciscana* haplotypes (Hap, black colour) in different geographical locations and in comparison to other *Artemia* species of the old world. Coloured circles refer to the origin of each sequence. Colours' key is the same as of Fig. 7.

organism are usually fished and sold. Out of its native range, the same Egyptian haplotypes were also differentially found in Mexico, Columbia, China, Sri-Lanka, Viet Nam, and Portugal (Figs. 7 and 8).

3.4. PCoA

PCoA resulted in a clear degree of proximity among most haplotypes present in the Latin America and USA together, against another group of haplotypes that are uniquely present in Canada. Most Egyptian haplotypes detected herein fallen more in the Latin American-USA group of haplotypes (Fig. 9).

4. Discussion

Lake Qaroun in Egypt represents one of the unique inland marine lakes in the entire world. Its continuous salinity increase since 1901 until now permitted the survival of many transferred marine species there including the soles *Solea vulgaris* and *S. aegyptiaca*, marine prawns, and the cockle *Cerastoderma glaucum* (Fathi and Flower, 2005; El-Shabrawy et al., 2015; Kandeel et al., 2017). However, in the last year, the fisheries of bony fishes in the lake were severely reduced due to the presence of massive infestation with tongue-eating, tissue-damaging isopod parasites (Ali and Aboyadak, 2018). Herein, we could detect the presence of a new population of *A. franciscana* in Lake Qaroun in Egypt. This, in light of the increasing salinity and nutrients accumulated in the Lake (see Kandeel et al., 2017) and the decrease in the annual fishery yields in Lake Qaroun from 4,518 tonnes at 2014 to 1,124 tonnes at 2015, falling even further to 873 tonnes at 2016 (Gafred, 2017); can represent a

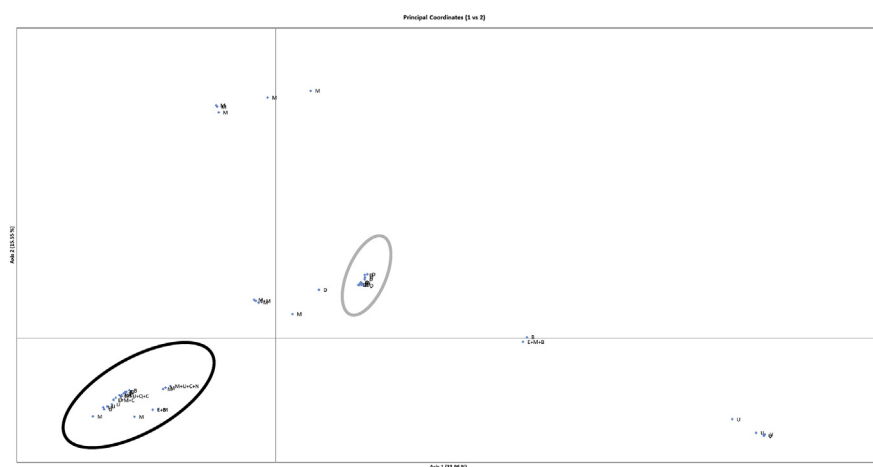


Fig. 9. Principal Coordinate Analysis (PCoA) run on GenAlEx for 60 COI haplotypes of *A. franciscana* found in our study and retrieved from the GenBank database. Grey oval refers to the haplotypes in Canada, Black oval encompasses most South American, USA and Egyptian haplotypes. Letters: E: Egypt, M: Mexico, U: USA, D: Canada, C: China, Q: Iraq, N: Iran, A: Cuba, B: Colombia.

new, unexpected, space for the growth and flourishing of *A. franciscana* as a direct result of removing its direct predators from the Lake's food web.

Morphological parameters have been widely applied in classification of species for decades. The modern methodologies for identification based on DNA barcoding provided more accurate taxonomic data. Both of these approaches successfully elucidated the first record of *A. franciscana* as a wild population in Egypt. This record was in Qaroun Lake, which can be considered then the first Egyptian reservoir recorded to harbor this non-native, economically-important *Artemia* species. Our phylogenetic analyses placed 80 % of the identified samples in the allochthonous clade of *A. franciscana* combining all haplotypes present out of its native American homelands. Application of COI gene hypervariable barcode region sequencing was shown as an efficient marker for *Artemia* species and populations discrimination. In general, it was suggested as a powerful system to reconstruct the phylogenetic relationships among different crustaceans (Saad and El-Sadek, 2017). It could reveal the phylogenetic relationships among Asian *Artemia* species, being native or invasive (Eimanifar et al., 2015). *Artemia* different species composition in five major salt lakes in the Tibetan Plateau was elucidated successfully using COI-based barcoding (Wang et al., 2008). Genomic richness of *A. franciscana* in the Great Salt Lake (USA) could be identified using COI sequences analysis (Eimanifar et al., 2015). Eastern spreads of this American species to the Mediterranean, prior to Egypt, could be confirmed through hypervariable barcoding region of COI (Ben Naceur et al., 2010; Horváth et al., 2018). COI sequences exhibited a great degree of divergence among diploid and tetraploid individuals of *A. parthenogenetica* (Perez et al., 1994). A great regional endemism in the extremely halophilic Mediterranean *A. salina* could be detected applying COI gene sequencing (MuñOz et al., 2008).

In almost all cases where *A. franciscana* was registered as an invasive, it was able to almost completely dominate over the native species of *Artemia*. The entire Northwest Mediterranean countries, Portugal; Spain; and France, are now dominated by this species (Ruebhart et al., 2008a,b). In Brazil, it were only three years (1977–1890) between the first inoculation of few grams of *A. franciscana* cysts for use in aquaculture, and harvesting tonnes of cysts of the same species (Persoone and Sorgeloos, 1980). In general, it appears that the invasive filters that control their propagation are the least. It is well known that invasion retains the species that have advantage over the residents in trophic interactions, and the residents may rapidly evolve traits to better tolerate the invaders (David et al., 2017). In the epoch when we found *A. franciscana* population, Lake Qaroun fishes were under heavy infestation with isopod parasites and organic contamination that led to massive reduction of predatory fishes (Ali and Aboyadak, 2018). This might have provided a transient metacommunity alteration in the lake with extinction-colonization dynamics, making some zooplanktonic communities in much better state to evolve and may be to dominate. As *A. franciscana* is known to exhibit higher reproductive rates and exploit food

more rapidly and efficiently than native species of *Artemia*, as many invasive species do (Browne and Halanych, 1989; Morrison and Hay, 2011). Yet, invasion by *A. franciscana* doesn't always implicate a complete disappearance of native populations, such as the case of Aigues-Mortes saltern in South France (Lenormand et al., 2018) where this species was intentionally introduced in the 1970s, as the usual case of invasion limited by competition which leads to the coexistence (David et al., 2017). Moreover, cestode parasites change the colour of native *Artemia* species to intense red and their phototaxis (parasite-induced positive phototaxis); increased time of surfacing behaviour, and the normal photophobic behaviour of adults to favour luminated microhabitats, active movement to light stimuli and increased time of swimming near water surface- but these responses were not apparent in the invasive *A. franciscana* (Georgiev et al., 2007; Dunn, 2009).

Being more tolerant to environmental fluctuations and parasites, and capable of exploiting alimentary resources in a superior way than native residents, native species of *Artemia* are expected to face extreme survival challenge upon *A. franciscana* invasion. *Artemia* species are known as intermediates hosts for cestode parasites of water birds. A demographic release of the invasive species could ensue directly due to the high infection level of native species with cestode parasites the coevolved with *A. franciscana*, in comparison to the rate of infection of *A. franciscana* itself, thus propagation of the latter one could be best aided by the presence of the co-evolved parasite (Redón et al., 2009). *A. franciscana* was susceptible to only 6 of 10 cestode parasites impacting native Mediterranean species of *Artemia*.

In the present investigation, *Artemia* collected from Qaroun Lake have the same characteristics as the invasive *A. franciscana* collected from different parts around the world. From these characters, subspherical frontal knob, penis of males and ovisacs of females were an identification key for *A. franciscana* morphology as mentioned by Mura and Brecciaroli (2004) and Mura et al. (2006). In the current work, females were significantly taller than males, thorax and abdomen lengths, however, males had bigger eyes and 1st antennae length than females. For the biometrics comparison with *A. franciscana* from nearby locations like Tunisia, the work of Ben Naceur et al. (2013) was a good example, analysing native Tunisian and American samples of *Artemia* spp. They recorded that females exhibit longer body sizes, bigger abdominal lengths and widths, shorter furcal and 1st antennae lengths than males, but no difference in the head diameters between the two sexes and males' eyes were bigger than females'. However, the mean values of *A. franciscana* biometrics in the previous researches were slightly larger than ours. This can be a result of location specificity and environmental conditions as mentioned by Amat et al. (2004) where *A. franciscana* collected from warmer areas (Krishnakumar and Munuswamy, 2014) in the world were shorter than the same species in colder areas.

Phylogenetic analysis exhibited similar pattern to that found using the same COI and/or ITS genes in different species of *Artemia* of the old and new world by Eimanifar et al. (2015), Baxevanis et al. (2006), and others. Most species of *Artemia* of the old world, beside *A. persimilis*, grouped together in a single clade that was older than the one encompassing all *A. franciscana* world haplotypes. *A. salina* and *A. persimilis* were closer to the outgroup of the tree than other *Artemia* species, being the first of the most ancestral species of old world *Artemia* and the latter, land-locked species identified as the First New World species that separated from the common ancestor of *Artemia* species at the time of separation of Africa from South America (Baxevanis et al., 2006). The close phylogenetic relations among *A. urmiana*, *A. tibitiana*, and *A. sinica* is well-identified (for examples, Eimanifar et al., 2015). *A. parthenogenetica* grouped together in the single clade encompassing all Asian *Artemia* species, which agrees with the assumption of parthenogenetic *Artemia* from Asia (Muñoz et al., 2010).

All conditions in the location in Lake Qaroun where *A. franciscana* was collected seem to be optimal for this species, including the temperature, salinity and pH (Sorgeloos et al., 1986). Moreover, *A. franciscana* is known as a strong competitor to the native *Artemia* species, in a similar way to what happened in India where this species appeared previously and successfully abolished the native, parthenogenetic ones (Vikas et al., 2012). Local populations of *Artemia* may be more susceptible to different stresses than the introduced ones. The advantage of the sexual reproduction of *A. franciscana* contributes much to its success as a competitor over the asexual species (Wilson, 1992). In addition, *A. franciscana* tolerate a wide range of temperatures through active expression of different stress proteins (e.g. hsp70, artemin and p26) (Tanguay et al., 2004). Moreover, *A. franciscana* exhibited lesser susceptibility to parasites than *A. salina* and *A. parthenogenetica*. Many of these infections cause changes in behavior, energetic costs, and appearance of infected individuals, making them more susceptible for predation by birds as the end host of these pathogens (Georgiev et al., 2007; Sánchez et al., 2012). Conclusively, the successive criteria of the invasive species over the native ones are, 1) fast growth rates, 2) early maturity, 3) increased fecundity allowing quick population recovery to withstand hostile environmental conditions and 4) short life spans (McMahon, 2002).

Since the first appearance of *A. franciscana* out of the Americas was in Portugal in 1981, several pathways have been postulated for its transfer. Its common use in aquaculture as live food for larvae is one of these ways (Mura et al., 2006). This pathway was directly implicated in the spread of *A. franciscana* in India, Australia and China (Van Stappen et al., 2007; Ruebhart et al., 2008b; Vikas et al., 2012). Another pathway of transfer was through migratory water fowl. This pathway may be the most acceptable for introduction of the species in Qaroun Lake. Since, firstly, no *Artemia* –based aquaculture activity is known in the Lake or the nearby regions in general, and secondly, that the lake area was officially considered a

protectorate by the Egyptian Prime Minister Decision 943/1989, due to the wide variety of migratory birds' passing/sheltering/or breeding there. The role of migratory water birds to transfer *A. franciscana* via long distances has been confirmed by several researches (see Green et al., 2005). In addition, *A. franciscana* cysts were extracted from the excreta of shore birds (Green et al., 2005; Amat et al., 2005). The long distance dispersal (LDD) of aquatic invertebrates through geographically isolated areas is well known since Darwin work (2004). Many cases were recorded for invertebrates spreading through migratory water birds, including the transfer of bivalve molluscs and their eggs, resting eggs (ephippia) of cladocerans, statoblasts of bryozoans, all could be found attached to legs or feathers of the migratory birds, as reviewed by Green et al. (2005). The transportation of animal propagules through birds' digestive system after resisting the digestive process, known as endozoochory, achieves the maximum LDD and the longer the length of guts of a migratory bird, the farther the propagule can be transmitted (1000–3000 km: Charalambidou and Santamaría, 2002; Green and Figuerola, 2005). *Artemia* cysts are resistant enough for gastric digestion, with elevated possibility of endozoochory if the bird exhibited herbivorous habits (Charalambidou et al., 2005). Transfer through migration of birds is responsible for significant component of gene flow of some organisms, as in the case of bryozoans, *Daphnia*, and *Artemia* (Okamura and Freeland, 2002; Figuerola et al., 2005; Coughlan et al., 2017). In this context, 20 % of genetic variations of *A. franciscana* in America could be directly linked to the Pacific, Atlantic, and Central American birds' migrations (Muñoz et al., 2013). Most Egyptian haplotypes were reported in Mexico, belonging to a phylogenetic clade falling within the Pacific route of bird migrations. However, we cannot definitely confirm a direct origination of our Egyptian samples from North America, since no American birds are known as nomadic species in Qaroun Lake protectorate and the nearby wetlands. Our phylogenetic analysis exhibited that the haplotypes of *A. franciscana* detected in Qaroun Lake in Egypt not only present in Mexico but also in Columbia, China, India, Sri Lanka, and Portugal. A prominent bird migration route from Eurasia is known to pass through Qaroun Lake protectorate. Many water birds migrate from Asia, including China, and reach Egypt from their main lands for wintering. Birdlife database (<http://datazone.birdlife.org/home>) counts about 100 water birds species that move between the Siberian part of Russia and/or China and Egypt. The following species are found or come in contact during their lives and native grounds in salt marshes, according to the IUCN red List of Threatened Species and del Hoyo et al. (1996, 2014): the Red-breasted Merganser *Mergus serrator*, the *Tadorna tadorna* (usually present in salty lakes), the Marbled Duck *Marmaronetta angustirostris*, the Garganey *Spatula querquedula*, the Great Crested Grebe *Podiceps cristatus*, the Black-necked Grebe *Podiceps nigricollis*, the Dalmatian Pelican *Pelecanus crispus*, the Eurasian Oystercatcher *Haematopus ostralegus*, the Little Ringed Plover *Charadrius dubius*, the Greater Sandplover *Charadrius leschenaultia*, the Caspian Plover *Charadrius asiaticus*, the White-tailed Plover *Vanellus leucurus*, the

Eurasian Curlew *Numenius arquata*, the Black-tailed Godwit *Limosa limosa*, the Broad-billed Sandpiper *Calidris falcinellus*, the Ruff *Calidris pugnax*, the Broad-billed Sandpiper *Calidris falcinellus*, the Curlew Sandpiper *Calidris ferruginea*, and the Temminck's Stint *Calidris temminckii*. Future work can be carried out by analyzing these birds' excreta in the protectorate area for assessment of *A. franciscana* propagules presence.

However, the existence of few haplotypes of *A. franciscana* in the Lake may raise some debate about the bird-based transfer, since this transfer is usually for many cysts. In all cases, an accidental, unintentional transfer happened and led to *A. franciscana* appearance in Qaroun Lake in Egypt where this species could be recorded. Some future studies can be carried out in order to assess the state of other *Artemia* populations and characterize the real standing diversity of *Artemia* spp. present in the Lake. This theme that has not been revisited since the work of [El-Bermawi et al. \(2004\)](#), but now it gains special importance in light of the massive appearance of invasive invertebrate species ([El-Shabrawy and Dumont, 2016](#)).

In conclusions, a new *Artemia* population from Qaroun Lake was found to be the invasive, bisexual *A. franciscana*. From the morphological and molecular observations, no variations were recorded with respect to the different body parts of *A. franciscana* biometric characters. The presence of this species in that area may be secondary to recent birds' migration. Future works can be of especial importance for both detecting whether there are possible role of birds' migrations on the gene flow of this newly-introduced species in Qaroun Lake in Egypt, and assessing the future possibilities of expanding the use of Lake Qaroun as a reservoir for *A. franciscana* native production in Egypt for national aquaculture activities. The damage to the economic finfish fishery of Lake Qaroun due to different infestations and heavy load of biological invasions can be overcome by raising and/or conservation of the other economic, non-fish species less susceptible to these threats, including the new inhabitant of the lake, *A. franciscana*, and the marine prawns.

Declarations

Author contribution statement

Khaled Mohammed-Geba: Performed the experiments; Wrote the paper.

Asmaa Galal-Khallaf, Sherin Sheir: Performed the experiments; Analyzed and interpreted the data.

Azza Mohamed: Conceived and designed the experiments.

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