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Original Paper

Morphometric and molecular sequencing evidence for the identification of *Artemia franciscana* (Kellogg, 1906) in Southern India

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Abstract

A native of North America, the brine shrimp *Artemia franciscana* is now a very invasive foreign species that has already been imported to most of the world. Before the introduction of *A. franciscana*, Asia was home to a wide range of bisexual *Artemia* species, which is no longer the current case. The collected *Artemia* samples from salt pans in south India were subjected to exact species identification and determination of phylogeographic origin through morphometric and molecular phylogenetic analysis. The *Artemia* species was validated with the observation of spiny subspherical frontal knob and distinctive lateral triangular ovisac lobes with projections on its body. The phylogenetic analysis of these *Artemia* species from two sampling localities was carried out after nucleic acid sequencing. After DNA barcoding of the cytochrome oxidase *COI* gene, the samples were identified as belonging to *A. franciscana*.

Key words: *Artemia*, cytochrome c oxidase I gene, DNA sequencing, morphometric analysis, solar saltpan.

Introduction

The brine shrimp *Artemia* (Branchiopoda, Anostraca) is a halophilic zooplankton distributed in hypersaline habitats such as salt lakes and lagoons worldwide, and is one of the major fodders in aquaculture. *Artemia* is a sustaining species in hypersaline food webs, and a widely-applied animal model for ecotoxicology, developmental biology, evolutionary biology and ecology (Lenormand et al. 2018). In the salt industry, *Artemia* has also been employed to raise the quality of sodium chloride produced in solar salt pans (Ruebhart et al. 2008). With more than 530 *Artemia* sites now known, Asia has a rich biodiversity of *Artemia* (Naganawa, Mura 2017). Among all the *Artemia* species, *Artemia franciscana* is of great economic importance due to its usage as live feed for larvae in aquaculture industry and is a native of North, Central and South America. Due to its strong ability for reproduction, superior physiological immune system, and quicker filter feeding rate than native species, *A. franciscana* is an invasive species in salt water habitats (Sanchez et al. 2016).

Artemia are characterized into two distinct reproducing groups – the bisexual and parthenogenetic types (Browne, Bowen 1991). The bisexual *Artemia* were recorded in China (*Artemia sinica*), Iran (*Artemia urmiana*), Italy and North Africa (*Artemia salina*), USA (*A. franciscana*), and Egypt (*A. franciscana*) (Naihong et al. 2000; Van Stappen et al. 2001; Sherin et al. 2018). In India, *A. franciscana* was purposefully introduced in the early 1980s as live food for aquaculture and has currently become an invasive species as in many other countries (Krishnakumar, Munuswamy 2014). This anthropogenic interruption has led to loss of native parthenogenetic *Artemia* due to gradual substitution by the highly adaptive exotic species (Vikas et al., 2012).

Morphological and molecular characterization can be tools of great importance in aquaculture industry. Morphological identification of *Artemia* is done mainly by features of penis, ovisac, length and width of abdomen, furca, antenna, eyes, head, and number of setae over the frontal knobs (Vetriselvan, Munuswamy 2011; Scalone, Rabet 2013; Krishnakumar, Munuswamy 2014). Traditional morpho-biometric based taxonomy has some limitations, such as mimetic polymorphism which depends on need of taxonomists' expertise. There is little doubt that there is a need of complementary evidence at DNA molecular sequencing levels. The standard molecular sequencing identification system was introduced during the 1990s with DNA sequencing by polymerase chain reaction. DNA barcoding was successfully used for detection of aquaculture-based product modifications and also to identify different shrimp species from the Mediterranean and Red Sea (Asmaa Galal-Khallaf et al. 2014; El-Sharawy et al. 2017; Ahmed et al. 2021).

Genomic changes in *Artemia* populations after their

introduction to a non-indigenous hypersaline habitat are among the chief developmental traits in their successful establishment (Lee 2002). *A. franciscana* have well documented genetic variations in invasive populations relative to the native American populations (Munoz et al. 2014). Various polyphyletic obligatory parthenogenetic populations, including diploid, triploid, tetraploid, and pentaploid types, and numerous bisexual species constitute the genus (Asem et al. 2016). In this context, the study was aimed to apply both morphometric and phylogenetic analysis to detect the possible origin of the new solar salt pan inhabitant *Artemia*. This can be a precise approach to predict its behaviour and impact in relation to physiological parameters, in comparison to other invasive species.

Materials and methods

Study area

Two different saltpans (Fig. 1; Samithoppu, station 1 and Puthalam, station 2) from the southern peninsular region of India were selected as study areas for the present morphometric and molecular characterization of Artemia. Station 1 is situated northwest of Cape Comorin coast around 13km with GPS coordinates of 8°10'28.0200"N and 77°25'55.7724"E. Station 2 is situated northwest of Cape Comorin coast around 16 km with GPS coordinates of 8° 6' 21.9816" N and 77° 28' 0.7968" E. Both station 1 and 2 are surrounded by an estuary on one side and agriculture land on the other sides. The distance between the two study sites was about 5 km and distance from the Cape Comorin coast was about 12 km.

Sample collection and preparation

Artemia samples (20 males and 20 females per station) were collected in sterile bags separately in morning between 6 AM to 8 AM on a fortnight basis from both study site. The *Artemia* samples were collected separately from the reservoir, condenser and crystallizer units of the saltpan. The collected samples were packed, labelled and brought to the laboratory for biometric and molecular analysis.

Artemia samples were treated with 70% ethyl alcohol and were then compressed between two glass slides. Hydration with decreasing concentrations of ethyl alcohol was done and allum-borax carmine was used to stain the samples. Final dehydration using increasing concentrations of ethyl alcohol was done before observation. The processed samples were treated with clove oil before mounting in dibutylphthalate polystyrene xylene (El-Banhawy, El-Gansory 1989).

Morphometric parameters

Artemia collected from solar salt pans (stations 1 and 2) were treated with 2% Lugol solution to measure morphological and biometric characteristics – total length, eye diameter, head diameter, antennae length, thorax length, thorax width, length of abdomen, width of abdomen, ovisac diameter, and furcal length under a compound microscope using micrometer (Basbug, Demirkalp 1997).

Molecular analysis

DNA was extracted using the ammonium pyrrolidin dithiocarbamate method (Manaffar et al. 2010) with 2 h incubation at 55 °C. Ammonium pyrrolidin dithiocarbamate complex (200 µL) was transferred to 2 mL vial containing five fresh *Artemia* animals and 15 µL of protease K solution (20 mg mL^{-1}) was added. The tube was mixed well to increase the protease activity. The vial was shaken well by vortex every 10 min. Then the tube was chilled in an ice box and centrifuged at 2500 rpm 15 min. The supernatant was transferred carefully to a fresh tube. The DNA was precipitated in 100% ethyl alcohol in a 1:2 ratio at –20 °C for 1 h. The pellet was dissolved in 25 µL of

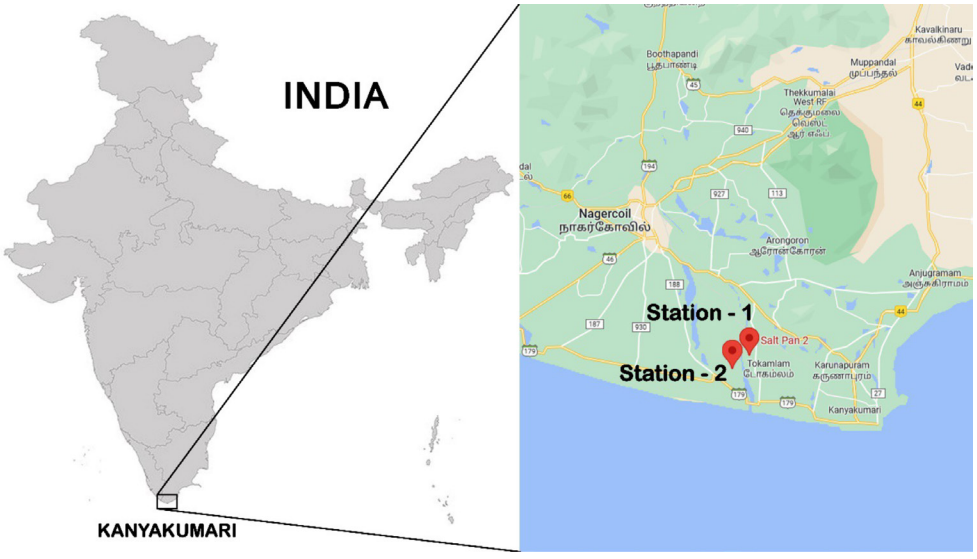

Fig. 1. Map showing the two sampling stations from the study area.

TE buffer with pH value 8.

To determine the concentration of extracted DNA, absorbance of UV light was measured using spectrophotometer at wavelength 260 nm.

The extracted DNA was evaluated by PCR amplification with the forward primer HCO2198 (5'TAAACTTCAGGGTGACCAAAAAATCA3') and with the reverse primer LCO1490 (5'GGTCAACAAATCATAAAGATATTGG3') for cytochrome c oxidase I gene (Folmer et al. 1994). The following PCR steps were used with minor modifications from Folmer et al. (1994): 5 min at 95 °C for initial denaturation followed by 30 steps of 30 s denaturation at 95 °C, annealing at 45 °C for 30 s and finally 1 min at 70 °C for annealing. The PCR product was sequenced using an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kit with AmpliTaq® DNA polymerase (FS enzyme) in an ABI 3730xl sequencer (Applied Biosystems).

The obtained sequence was subjected to local alignment analysis using the NCBI BLAST similarity search tool. The phylogeny analysis of the query and its closely related sequences was performed using PhyML 3.0 after multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The results were cured using G-blocks 0.91b after alignment (Talavera, Castresana 2007). Finally, Tree Dyn 198.3 was used for tree rendering (Dereeper et al. 2008).

Statistical analysis

Morphometric data were analysed using Microsoft Excel for identification of male and female animal differences and differences between sampling stations. All data were expressed as mean ± standard error. The statistical analysis was performed using the unpaired *t*-test with the probability of $P \leq 0.01$.

Results

Artemia species can be divided into two groups (bisexual and parthenogenetic) based on their reproduction. Phenotypic characteristics such as furca, penis, ovisac, and frontal knobs are the most commonly used body parts for morphological identification of *Artemia* species. Male and female total body length were found to differ significantly in morphometric measurements. Table 1 show morphometric measures of *Artemia* taken at stations 1 and 2 in the saltpans of Swamithopu and Puthalam. In both study regions, males were smaller than females. Male and female lengths were 8.28 ± 1.40 and 9.1 ± 1.48 mm in station 1 and 8.62 ± 0.26 and 9.49 ± 0.22 mm in station 2, respectively.

The head was joined to the eyes. On both sites, the male eye was slightly bigger than the female eye. Male eyes measured 1.46 ± 0.21 mm in diameter in station 1, whereas female eyes measured 1.2 ± 0.35 mm. Male eyes in station 2 had a diameter of 1.4 ± 0.18 mm, whereas female eyes had a diameter of 1.19 ± 0.14 mm, which illustrates sexual dimorphism in Artemia depending on eye size. However, there was no appreciable variation in the left and right eye diameter for males (1.46 ± 0.21 and 1.63 ± 0.61 mm) and females (1.2 ± 0.35 and 1.39 ± 0.78 mm) in stations 1 and 2, or for females (1.19 ± 0.14 and 1.25 ± 0.11 mm). The head diameter is indicated by the space between the eye stalks. The male and female head diameter was 2.42 ± 0.28 and 2.64 ± 0.31 mm in station 1. The head diameter of males and females in station 2 was 2.48 ± 0.23 and 2.62 ± 0.3 mm, respectively.

The first antenna was significantly shorter than the second antenna in both sites. The length of the first antenna was 2.78 ± 0.23 and 2.88 ± 0.75 mm for males and females in station 1 and 2.76 ± 0.22 and 2.67 ± 0.2 mm in station 2, respectively. There was significant difference between males and females in first antennae length in both study areas, but

Table 1. Morphometric parameters of males and female *A. franciscana* of Swamithopu saltpan (station 1) and Puthalam saltpan (station 2). In total, 20 males and 20 females per station were measured. Data was statistically analyzed by unpaired *t*-test with Welch's correction, $^{**}P \leq 0.01$ significant difference between males and females morphometrics within respective stations, NA, not applicable

Parameter	Station 1 (Swamithopu saltpan)		Station 2 (Puthalam saltpan)	
	Male	Female	Male	Female
Total length	8.28 ± 1.4	9.1 ± 1.48	8.62 ± 0.26**	9.49 ± 0.22
Left eye diameter	1.46 ± 0.21	1.2 ± 0.35	1.40 ± 0.18	1.19 ± 0.14
Right eye diameter	1.63 ± 0.61	1.39 ± 0.78	1.49 ± 0.24	1.25 ± 0.11
Head diameter	2.42 ± 0.28	2.64 ± 0.31	2.48 ± 0.23	2.62 ± 0.3
1st antennae	2.78 ± 0.23	2.88 ± 0.75	2.76 ± 0.22	2.67 ± 0.2
2nd antennae (last leg)	5.32 ± 1.15	NA	5.61 ± 0.24	NA
Thorax length	3.27 ± 0.11**	3.68 ± 0.17	3.28 ± 0.17**	3.67 ± 0.2
Thorax width	2.17 ± 0.10	2.48 ± 0.24	2.24 ± 0.15	2.48 ± 0.24
Abdomen length	3.79 ± 0.11**	4.38 ± 0.17	3.73 ± 0.16**	4.42 ± 0.18
Abdomen width	1.74 ± 0.15	1.86 ± 0.14	1.70 ± 0.18	1.84 ± 0.17
Ovisac diameter	NA	5.54 ± 0.24	NA	5.55 ± 0.25
Furcal length	1.14 ± 0.11	1.10 ± 0.08	1.18 ± 0.15	1.18 ± 0.12

first antenna of males was slightly longer than for females, which suggests sexual dimorphism of *Artemia* based on first antenna length. The second antenna was quite long and flat at the distal part. The length ranged between 5.2 to 6.4 mm. The length of second antenna of males was 5.32 ± 1.15 mm in station 1 and 5.61 ± 0.24 mm in station 2, respectively. A positive important correlation was found between second antenna length of males and width of thorax. The mouth was located in the ventral surface of the *Artemia*.

Female *Artemia* displayed a significantly longer thorax than males. Thoracic length of males and females was 3.27 ± 0.11 mm and 3.68 ± 0.17 mm in station 1 and 3.28 ± 0.17 mm and 3.67 ± 0.2 mm in station 2 respectively. There was no significant difference between thorax width in males and females of both study sites. Thoracic width of males and females was 2.17 ± 0.1 and 2.48 ± 0.24 mm in station 1 and 2.24 ± 0.15 and 2.48 ± 0.24 mm in station 2 respectively.

Abdomen length of males and females was 3.79 ± 0.11 and 4.38 ± 0.17 mm in station 1 and 3.73 ± 0.16 and 4.42 ± 0.18 mm in station 2, respectively. Abdomen width of males and females was 1.74 ± 0.15 and 1.86 ± 0.14 mm in station 1 and 1.7 ± 0.18 and 1.84 ± 0.17 mm in station 2, respectively. The male abdomen were slightly shorter than for females, but there was no significant differences between abdomen width of males and females in both study sites. The ovisac diameter of females in station 1 was 5.54 ± 0.24 mm and in station 2 – 5.55 ± 0.25 mm. There was no significant difference of ovisac diameter in both study area. Furcal length of males and females was 1.14 ± 0.11 and 1.10 ± 0.08 mm in station 1 and 1.18 ± 0.15 and 1.18 ± 0.12 mm in station 2, respectively, with no significant differences. Significant differences ($P \leq 0.01$) were seen for thorax length and abdomen length between male and female individuals in both the stations. No significant changes were seen between individuals of the two sampling stations.

The sequences of *A. franciscana* samples were deposited in the GenBank databases with the accession numbers MZ674398 and MZ674401. Fig. 2 shows dendrogram constructed based on the COI sequence phylogenetic tree of the collected *A. franciscana* samples.

Discussion

The brine shrimp *Artemia* is a well-known hypersaline crustacean with over 600 species, found in geographically isolated places such natural inland salt lakes, coastal lagoons, and man-made salterns (Van Stappen 2002). In a similar previous study, it was reported that the abundance of the *Artemia* population in the crystallizer pond of the salt pan environment varied significantly with temperature, pH, salinity, dissolved oxygen, and water depth (Biju et al. 2020). *Artemia* is a taxon that is difficult to describe due to lack of obvious traits. Some phenotypic features are considered as beneficial in identifying *Artemia* species in general. The collected *Artemia* samples were bisexual, composed of males with claspers and females with ovisacs, demonstrating the absence of parthenogenetic *Artemia* populations in the present study site. The *Artemia* populations from these stations are considerably smaller compared to the *Artemia* KKT1 studied at Tamaraikulam within Kanyakumari District (Marian et al. 2004). The lack of a gonopod basal spine and the male antenna's conical frontal knob set *A. salina* apart from other bisexual species. The only species with orthostichous spines on their gonopods are those from Chinam – *A. sinica* and *A. tibetana* (Zheng, Sun 2008).

Morphological traits have been utilized often in the

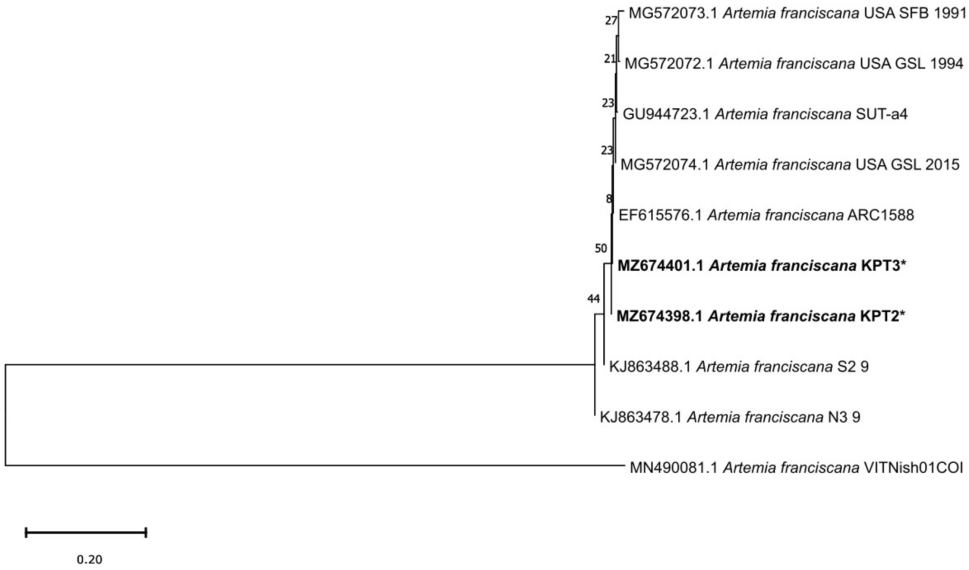

Fig. 2. The dendrogram constructed based on *COI* sequence showing phylogenetic tree of *Artemia franciscana*. Samples from the present study are shown in bold and marked with an asterisk.

classification of species for many years. More detailed taxonomic information was obtained using modern DNA barcoding-based identification techniques. These two approaches both worked well in identifying the initial wild population of *A. franciscana*. It has been shown that barcode sequencing of the hypervariable *COI* gene region can be used to distinguish between different *Artemia* species and populations. It was suggested as a potent method for retracing the evolutionary links among all crustaceans. It may reveal the evolutionary connections between native and invading Asian *Artemia* species. (Saad, El-Sadek 2017).

Phenotypical characteristics have been frequently used in the classification of Artemia species. Modern technologies are now being utilized to identify species based on genotypic criteria (DNA barcoding), which has resulted in a more precise taxonomic position. As a natural population, *A. franciscana* can be found all over the world (Sherin et al. 2018). Using the mitochondrial *COI* sequence marker, the occurrence of invasive *A. franciscana* from Iran and Iraq was documented (Eimanifar et al. 2014). The phylogenetic analysis of our sequences revealed that both of the sequences were closely related to each other and the closest related sequence in a BLAST search belonged to the Chinese strain *A. franciscana* voucher ARC1588.

The temperature, salinity, and pH in the area of study of the solar slat pan where franciscana was obtained appear to be ideal for this species. Additionally, *A. franciscana* fiercely competes with native *Artemia* species; when it first arrived, it successfully displaced the native parthenogenetic *Artemia* species (Vikas et al. 2012). Local *Artemia* populations may be more vulnerable to various pressures than introduced Artemia populations. The benefit of *A. franciscana* sexual reproduction greatly increases its competitive advantage over asexual species. Furthermore, *A. franciscana* can withstand a greater temperature range due to the active expression of many stress proteins like p26, hsp70, and artemin (Tanguay et al. 2004). Two factors distinguish invading species from native ones: (1) rapid growth rates and (2) short life spans. Early maturity and higher fertility allow populations to quickly recover and tolerate adverse environmental conditions (McMahon 2002). *A. franciscana* was designated as an invasive species, as it was able to entirely overrun the native *Artemia* species. The filters that stop them from spreading seem to be the least intrusive. It is well known that invasion retains species that have a trophic advantage over natives, and local species may quickly develop adaptations to better tolerate the invaders (David et al. 2017). As with many invasive species, *A. franciscana* has a faster reproduction rate and exploits food more quickly and efficiently than native *Artemia* species (Morrison, Hay 2011). In the present study, *Artemia* collected from Samithoppu and Puthalam had the same characteristics as the invasive species *A. franciscana* collected from different zones across the world. Female ovisac morphological features were reported to be a significant identification key for *A. franciscana* (Mura et al. 2006). However, in this study, females were slightly taller than males in terms of abdomen lengths and thorax, while males had longer first antennae and larger eyes than females. The introduction of *A. franciscana* has affected the local parthenogenetic *A. salina* population reported previously (Marian et al. 2014). This is problematic in the sense that "In any case the need to conserve local *Artemia* biodiversity should be of paramount importance when designing future inoculation activities, as *Artemia* resources stand as a natural patrimony supporting the sustainable expansion of aquaculture" (Van Stappen et al. 2020). The loss of the previously reported larger, native *A. salina* can have genetic implications such as loss of information on genes related to local adaptation, which is considered vital in *Artemia* genome research, and consecutively effective aquaculture technology research and development.

Conclusions

The morphometric and molecular observations of *A. franciscana* biometric characters showed no variations with respect to the different body parts. A new *Artemia* population from Samithoppu and Puthalam salt pan was found to be the invasive, bisexual *A. franciscana*. The newly recorded *Artemia* species from the Samithoppu and Puthalam salt pans in South India caused loss of the previously observed native *A. salina*.

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