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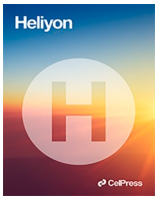

Research article

Morphology and morphometry of morphotypes in the population of *Artemia franciscana* (Kellogg, 1906) from salterns of the southeastern coast of India

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ABSTRACT

We document the morphology, morphometric variations among the morphotypes of the brine shrimp, *Artemia franciscana*. From the samples collected at four different locations in South India, Tamil Nadu *viz.* Kelambakkam, Vedaranyam, Tuticorin and Nagarcoil we identified six morphotypes: M1, M3, M4 in males and F1, F2, F3 in females. The Scanning electron micrographs of male morphotypes show distinct variation in the basal width, shape and number of cuticular cones on the second antennae. Similarly, the female morphotypes show various shape and sizes of the ovisac with or without spines. However, the cyst surface topography is smooth without any specific variation/ornamentation in all three female morphotypes. Multivariate analysis of eighteen morphological traits measured in males and fifteen in females to elucidate the intraspecific variations among morphotypes indicate significant dissimilarity between males and females. Furthermore, relative length measurements showed distinct morphometric variation of traits between the morphotypes encountered at different sampling sites.

1. Introduction

Globally, the brine shrimp *Artemia franciscana* (Kellogg, 1906; Article 4; ICZN, 2000) is known as universal live feed and contribute about 85 % of the feeds in marine larviculture [1,2]. To meet the demand of local aqua farmers at hatchery levels various several countries import *Artemia* cysts from the USA. This paved the way for culture of *A. franciscana* as live feed to improve their aquaculture productions and also its introduction into aquafarm saline habitats as a non-native species [3–5]. Although maximum profit (25 % in total yield) was gained, this practice has reducing the use of native populations in aquaculture [6]. Non-native species contribute up to 17 % of the world aquaculture production: for example, 50–60 % of aquaculture production from Brazil and the Philippines depends on non-native species as live feed [7]. However, uncontrolled cultivation and unplanned introduction of non-native species have led to habitat losses of native species [8,9].

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In India, *A. franciscana* was introduced as a live feed in several aqua farms [[4,10,11]]. Earlier studies have reported the existence of parthenogentic *Artemia* in the saline ecosystems of Vedaranyam, Tuticorin, Nagarcoil and Kelambakkam, South Coast of India [12, 13]. These parthenogenetic (*A. parthenogenetica*) populations, however, existed up to 1990 and now the scenario is very different. They are displaced by the non-native *A. franciscana*. This non-native species is bisexual, highly euryhaline and eurythermal when compared to parthenogenetic and other species of the genus. Above characteristics probably enable *A. franciscana* to dominate and colonize several salterns on the Indian subcontinent outcompeting with the native congeneric species *A. parthenogenetica* [[4,13,14],]. Similar invasion of *A. franciscana* has been reported from Portugal around the 1980s [15], France [16], Morocco, Italy and Spain [17,18]. Rapid growth of aquaculture is attributed to the invasion *A. franciscana* around the world's hypersaline habitats and the extinction of native populations like *A. parthenogenetica* and *A. salina* (Linnaeus, 1758) [18]. A combination of habitat loss and the establishment of *A. franciscana* have resulted in the loss of 55–74 % of native *Artemia* population habitats from Portugal, France and Spain [18]. Invasiveness of *A. franciscana* has been the central topic of several studies, while its speciation is highly puzzling and complicated due to the morphological variations among populations, which again could be related to its increased survival and fitness in extreme hypersaline environments [19–21]. Thus, the taxonomic status of *A. franciscana* has long been controversial because of existence of considerable morphological variability within the species [18–22].

It is well known that morphological traits have been the basis for describing the *A. franciscana* [23–26]. Hontoria & Amat [23,24] have reported morphometry as a useful tool for differentiation and identification of species when they are morphologically identical. Also, morphometric differences between individuals in the same species might be correlated to their environment and/or genotypic variation [27,28]. The presence of *A. franciscana* in Indian salterns have been confirmed through morphological and molecular characterization both at genotype and phenotype level [29–31]. Different morphotypes of this species have been reported by Ref. [32] from Covelong saltern, Kelambakkam, Tamil Nadu, India based on morphological variations of second antennae and the clasper structure in males and the shape of ovisacs in females. Earlier reports on this population were described as different morphotypes, namely M1, M2, M3, M4 in males and F1, F2, F3 in females carrying specific morphological traits (shapes of the ovisac and structure of second antennea) of the population documented in Kelambakkam saltern, Tamilnadu [32]. However, the morphotype of M2 reported by Krishnakumar & Munuswamy [32] was not encountered throughout the sampling periods in all salterns of southern India. The more clear and precise morphological traits were provided through valid photomicrographs and scanning electron microscopic observations. At present, morphological characterization is the most widely used approach to distinguish *Artemia* species [23,24]. Earlier studies on the morphometrics and morphology of *Artemia* spp. have shown striking differences of morphological traits among the populations encountered from different geographical sites [23,24,33], especially from the coastal sites of India, inland China, Europe and Africa [33]. The occurrence of the non native population of *A. franciscana* from the saltern of Kelambakkam (Tamil Nadu, India) and also other salterns in southern India has been genetically proven as *A. franciscana* [4,32]. But studies on the presence of

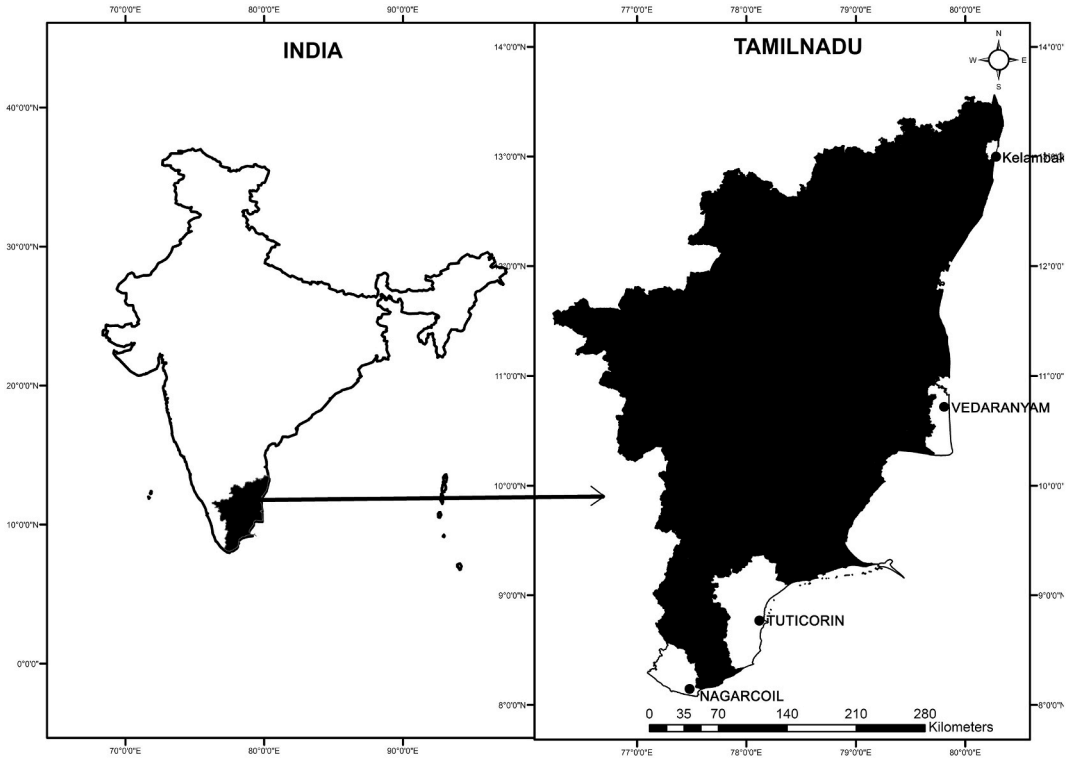

Fig. 1. Map showing the sampling locations of southeastern part of India.

morphotypes and their occurrence in homogenous populations of *A. franciscana* are scare and our earlier study reported the occurrence of morphotypes from the Kelambakkam saltern by both wild and laboratory cultures with molecular markers such as p26 and 16S rRNA, confirming that all morphotypes belong to the single isolated population of *A. franciscana*. However, the present study hypothesizes that all the salterns had similar effects of morphotype distribution with varying environmental conditions in southeastern India to document the presence of similar morphological variation in the encountered morphotypes within *A. franciscana*. Therefore, an attempt was made to document the specific variation among different morphotypes using light and scanning electron microscopy. These observations are supported by the morphometric analysis of designated traits of the morphotypes.

2. Materials and methods

2.1. Sample collection

Cysts and biomass of the brine shrimp were collected in the morning hours during December 2014–August 2018 (on a seasonal basis *viz* summer (April–June); premonsoon (July–September); monsoon (October–December) and postmonsoon (January–March)) from Kelambakkam (KBM) (12° 45′ 26.62″ N; 80° 12′ 43.80″S), Vedaranyam (VDM) (10° 20′ 108″ N; 079° 50′ 191″ E), Tuticorin (TUT) (08° 58′ 343″N; 078° 12′ 132″E), and Nagarcoil (NGC) (08° 58′ 227 62″ N; 78° 11′ 326 E″) Tamil Nadu, India (Fig. 1). Cysts were collected using a 100 μm mesh net and stored in 1 L screw capped polypropylene bottles along with saline habitat water (100–250 psμ). Biomass was collected using a 500 μm mesh and preserved in 4 % neutral buffered formalin (NBF) for morphological analysis.

Cyst samples (1 gm) from each location was processed and subjected to hatching and animals were cultured in the laboratory under controlled environmental conditions as described by Lavens et al. (1986). The field collected adult biomass and the cultured brine shrimp from four sites were segregated into different morphotypes based on the shape and structure of the basal width of the second antennae for males and the shape of the ovisac for females under a stereo zoom microscope (Leica MZ6), Germany [5,32].

2.2. Scanning electron microscopy (SEM)

Individuals from different morphotypes isolated from the samples were similar to earlier descriptions of the structure of the second antennae in males and shapes of the ovisac in females [26,32]. They were fixed in a fresh solution of 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 90 min [34]. Then the samples were dehydrated through a graded alcohol series and subjected to critical point drying (CPD) by CPD apparatus of Polaron E3000 CPD. These dried samples were carefully mounted on adhesive tape adhered on metal stubs and sputter gold coated for visualization by a Scanning electron microscope (Hitachi S–3000 N, Tokyo, Japan). The scanning electron micrographs of basal width and cuticular cones in males and ovisacs in females were taken by accelerating the voltage of 15 kV [34–36].

2.3. Morphometric analysis

The following morphological traits were measured for male and female morphotypes (Table 1 and Fig. 2): total length (TL), abdominal length (AL), head width (HW), distance between compound eyes (DE), eye diameter (left) (EDL), eye diameter (right) (EDR), abdominal width (AW), distance between 3rd and 8th abdominal segment (DAS), length of telson (LTE), length of furca (LF), length of IInd antenna (right) (LAR), length of IInd antenna (left) (LAL), ovisac width (OW), frontal knob diameter (left) (FKDL), frontal

Table 1
Morphological traits used to distinguish the morphotypes of Artemia franciscana.

Morphological traits	Abbreviations
TL	Total length
AL	Abdominal length
HW	Head width
DE	Distance between compound eyes
EDL	Eye diameter (left)
EDR	Eye diameter (right)
AW	Abdominal width
DAS	Distance between 3rd to 8th abdominal segment
LTE	Length of telson
LF	Length of furca
LAR	Length of antenna (right)
LAL	Length of antenna (left)
LO	Length of Ovisac
SFR	Number of setae per furca (right branch)
SFL	Number of setae per furca (left branch)
OW	Width of ovisac
FKDL	Frontal knob diameter left
FKDR	Frontal knob diameter right
BW	Basal width
DF	Distance between furcae

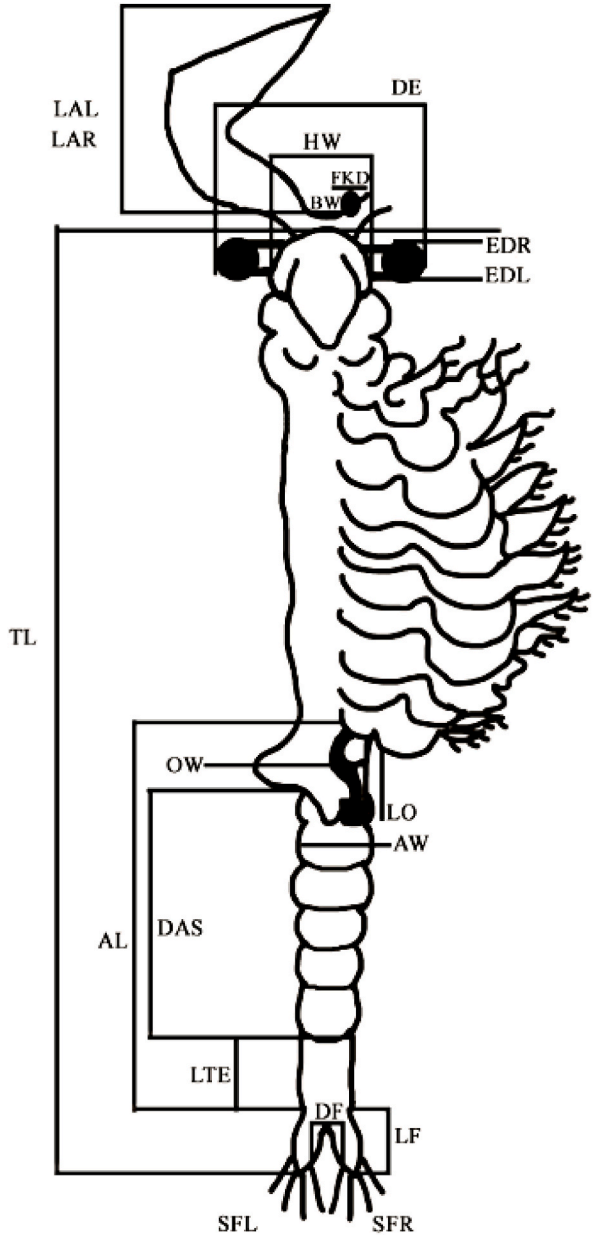

Fig. 2. Morphological traits used for analysis of morphotypes of *A. franciscana*.

knob diameter (right) (FKDR), basal width (BW), length of ovisac (LO), distances between furcae (DF), number of setae per furca (right branch) (SFR) and number of setae per furca (left branch) (SFL) [25,37]. For morphometric analysis of 20 individuals per morphotype (10 reared and 10 from biomass) from each site were measured with the help of a bright field microscope calibrated with an ocular micrometer. Photomicrographs of these structures were taken at different magnifications.

The data on measurements of traits were subjected to Multivariate Discriminant Analysis (MDA) to study the homogeneity and variation of male and female morphotypes from various salterns through analysis of variance (ANOVA) that was conducted to determine significant variances among the morphotypes encountered in this study. A pair-wise comparison (Tukey method; $P < 0.05$) was used to compare the significant variances among populations applying the statistical package SPSS for Windows, version 21.0.

3. Results

3.1. Gross morphology of morphotypes

The three male morphotypes M1, M3 and M4 were differentiated based on the basal width, shape and structure of second antennae. The M1 morphotype presents curved and pointed second antennae with pot shaped basal width (Fig. 3a). M3 has a straight and pointed second antenna with valley shaped basal width (Fig. 3b), whereas the morphotype M4 has U- shaped basal width with blunt second antennae (Fig. 3c). In contrast, throughout sampling periods from the different sampling sites the M2 type which was described earlier [32] was not encountered.

In females also three morphotypes F1, F2 and F3 were differentiated based on the shape and structure of the ovisac. The ovisac of F1 morphotype is triangular shape with the presence of spines at the bilateral ends (Fig. 3d) whereas F2 hshows an umbrella shaped ovisac with blut bilateral ends (Fig. 3e). The F3 morphotype has convex ovisacs with bulging apical areas without any spines at the bilateral ends (Fig. 3f).

3.2. Scanning electron microscopy of morphotypes

The scanning electron micrographs of both male and female morphotypes showed variations in their structure and shape as observed under light microscopy but with more details. In the male morphotypes, basal part of the M1 second antennae is with prominent three cuticular cones of which one is larger and other two are smaller (Fig. 4a & 5a). There are four cones on the basal part of M3 of second antenna with spines at their base (Fig. 4b & 5b). In the morphotype M4, the basal part of second antennae is with two cuticular cones comparatively smaller than other types (Fig. 4c & 5c). Female morphotypes showed variations in their shape and structure of ovisac as observed under the light microscopy (Fig. 6a–f). However, no significant difference was noted in surface topography of cysts of F1, F2 and F3 morphotypes. All the cysts showed smooth surface topography without any specific

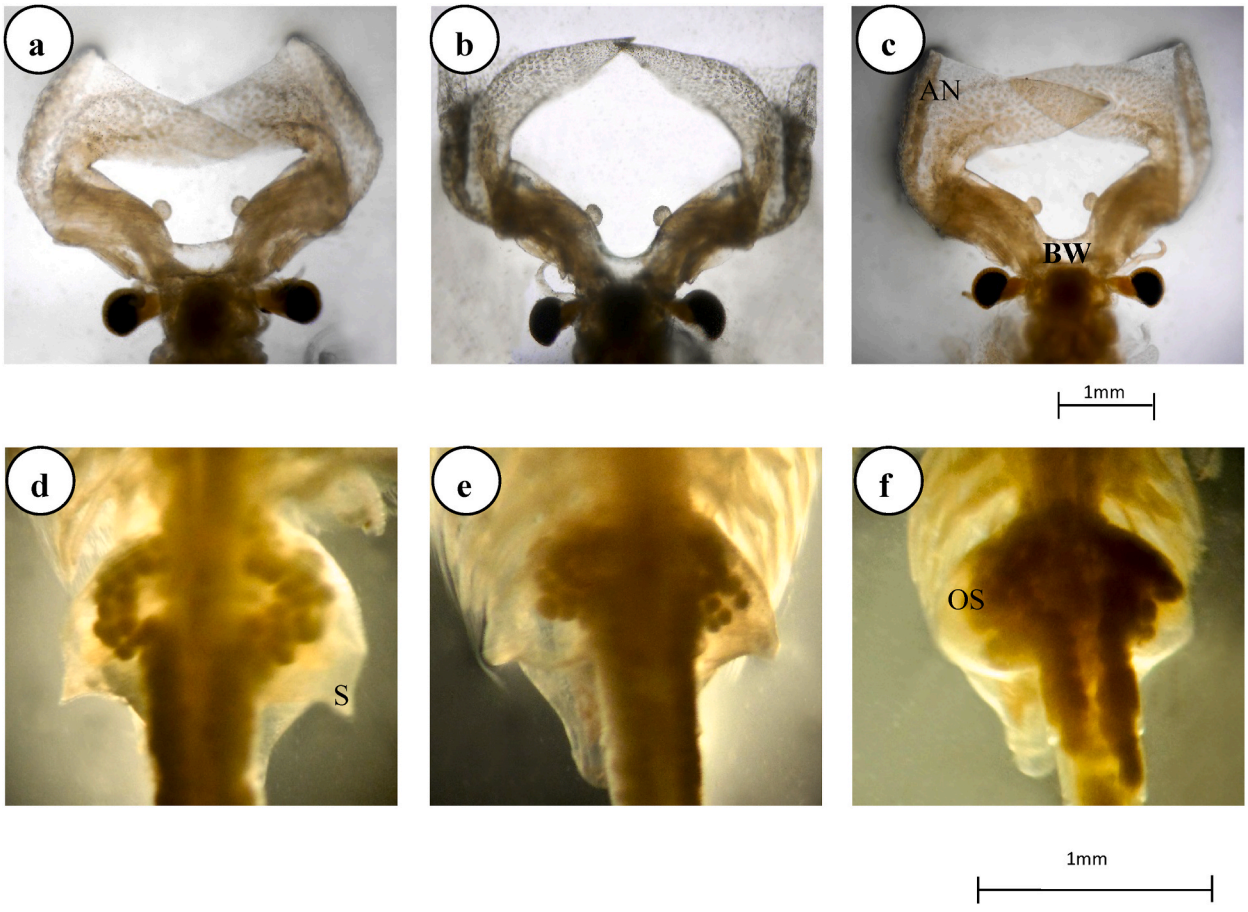

Fig. 3. Photomicrographs of male [(a) M1 (b) M2 (c) M3] and female [(d) F1 (e) F2 (f) F3] morphotypes showing variations in the structure of the second antennae and shape of ovisac. Note the variations in the shape and structure of the second antenna (AN), basal width (BW) shape and structure of ovisac (OS) and spines (S) (for details refer to text).

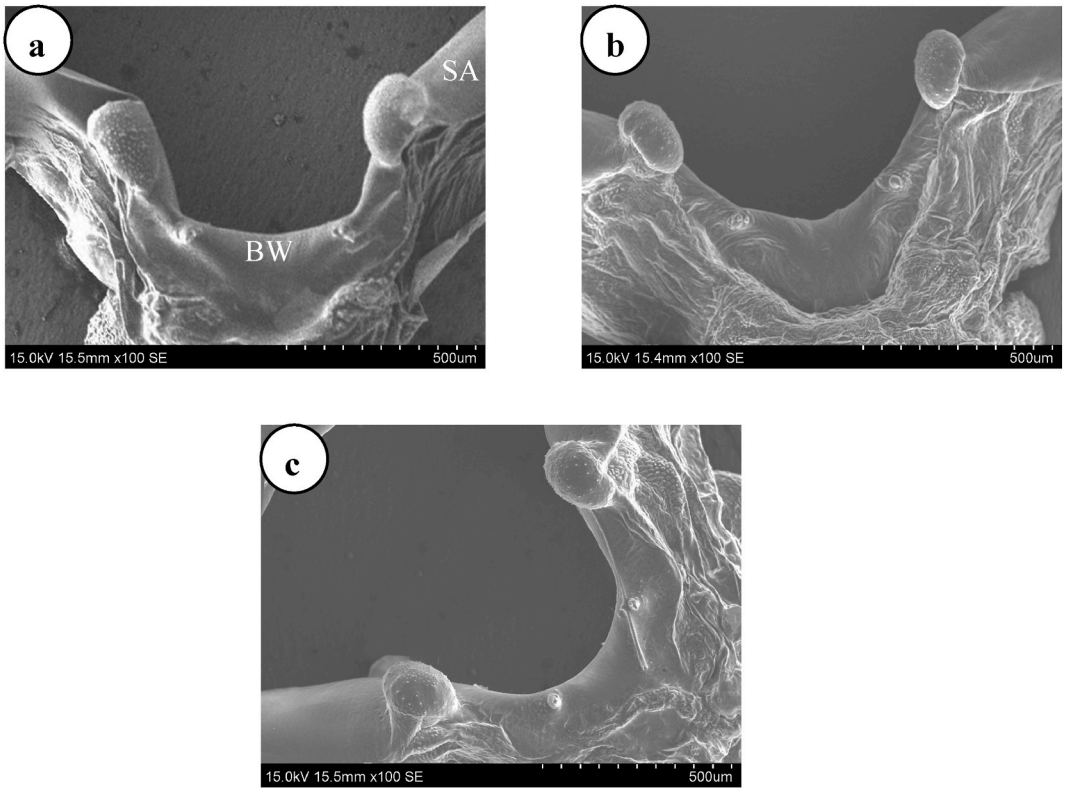

Fig. 4. Scanning electron micrographs of basal width of second antennae (SA) in male morphotypes (a) M1 (b) M3 and (c) M4, note smooth surface topography and variation in the basal width (BW) of male morphotypes.

ornamentation (Fig. 7a–c).

3.3. Morphometry of morphotypes

The average body length of within M1 was 9.04 ± 0.64 mm in male and 9.62 ± 1.13 mm with F1 in female; while the minimum average length of 6.02 ± 0.59 mm was observed in M3 and 6.75 ± 0.58 mm in F3. The average length of the second antennae was recorded in M4 (mean = 4.21 ± 0.50 mm) and M3 (2.63 ± 0.65 mm) (Supplementary Table 2). Morphotype F1 had an average size of the ovisac 2.12 ± 0.70 mm length and 2.13 ± 0.71 mm width while F3 had an average ovisac size of 1.38 ± 0.35 mm length and 1.14 ± 0.40 mm width (Supplementary Table 3). The statistical analysis of variance (ANOVA) showed that significant differences in morphometric traits of both male and female morphotypes ($P < 0.05$): in male, DE ($P < 0.000$), LTE ($P < 0.004$), LAR ($P < 0.001$), LAL ($P < 0.001$) and in female DE ($P < 0.001$), EDL ($P < 0.005$), EDR ($P < 0.005$), LF ($P < 0.006$), LO ($P < 0.008$).

Morphometric data of traits subjected to multivariate analysis revealed significant differences in morphometric traits between morphotypes. Discriminant analysis of both male and female morphometric data showed 11 discriminating traits. Six of these functions corresponded to TL, AL, HW, LA, OW and LO gave variance percentages of 57.5 % for males and 51.30 % females. These were characterized through the values of Wilks' Lambda (0.010–0.673 in males and 0.005 to 0.626 in females) and Chi-square (1031.542–88.667 in males and 1176.606 to 105.584 in females). The smallest values of Wilks' Lambda and the higher values of the Chi-square test showed the presence of significant morphometric variations among the studied morphotypes (Supplementary Tables 4 and 5). The percentage of variance in the morphometric traits were evident and presented a clear differentiation between morphotypes of *A. franciscana*.

The morphological trait values were subjected to Discriminant function analysis through canonical discriminant functions to authenticate the morphometric variations among the male and female morphotypes, explaining 81.10 %–18.90 % and 94.00 %–6.00 % of the first and second functions with their specific morphological traits within populations, respectively. Morphological traits used as a separation factor for the scatter plot analysis showed that male morphotypes (M1, M3 and M4) were grouped separately according to their specific morphometric traits (Fig. 8a). In the case of females (F1, F2 and F3) mophotypes were grouped separately with minimum overlapping (Fig. 8b). These results indicated subtle variation in morphological and morphometric traits among the six morphotypes of *A. franciscana*. Interestingly, a maximum of variations was found between morphotypes of males compared to females.

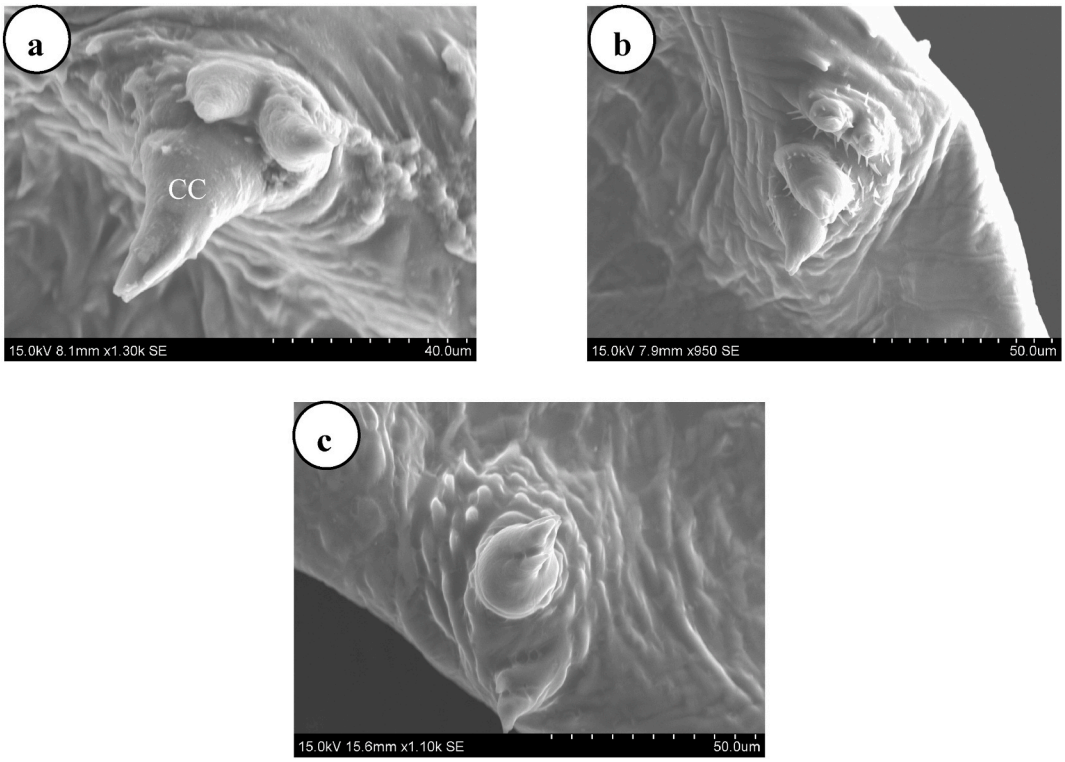

Fig. 5. Scanning electron micrographs of cuticulor cones (CC) of second antennae in male morphotypes (a) M1 (b) M3 and (c) M4, note smooth surface topography and varying numbers of cuticulor cones in each morphotype.

4. Discussion

Earlier studies on the *Artemia* population from various hypersaline ecosystems reported that distinct morphological variations were persistent among *Artemia* species exposed to different environmental conditions [25,38,39]. These different morphological variations are elucidated by different morphometric traits and are useful for *Artemia* taxonomy to authenticate distinct morphological variations. Hence, morphometric traits and the obtained data from different morphotypes are subjected to multivariate discriminant analysis of six different morphotypes of *A. franciscana*. A previous studies by Amat [40], reported that morphological changes were found in 22 different *Artemia* populations from the Mediterranean region. Persistent variations between males and females, elucidated that during geological timescales *Artemia* populations consistently adapted to changing climate conditions for their survival and this separated these populations into several sibling species. However, it is challenging to discriminate single isolated populations from subtropical ecosystems of Indian salterns due to ambient climate and seasonal variations. Interestingly, such climate conditions correlate with the morphotypes of isolated populations of *A. franciscana* from the different sampling sites of the southeast coast of India. Hence, the present observation on these population clearly demonstrated that the morphotypes of the same populations were separated by their specific morphological and morphometric characteristics indicating their intraspecific variability. However, previous studies reported that all salt pans in southern India were occupied by the invader brine shrimp *Artemia franciscana*. Similarly, native populations could not be traced in southern Indian salterns, and all the salterns were occupied by *Artemia franciscana* instead of *Artemia parthenogenetica* [4,13,41]. Hence, based on evidence of the present study, different morphotypes of *Artemia franciscana* were reported based on secondary sexual characters such as ovisac shapes in females and the structure of the second antennae in the males. Secondary sexual characters were the main discriminating factor among *Artemia* populations to confirm their sibling species with distinct morphological traits. This finding was strongly supported by earlier findings of Naceur et al. [42,43], where morphological variations of Tunisian *Artemia* populations were analyzed using secondary sexual characters such as ovisac morphology in females and the frontal knob morphology and basal part of the penis in males. Similarly, the morphology of secondary sexual characters varied from one population to the other giving rise to several siblings from the main mother population [38]. The present study showed that morphometric data of secondary sexual characters such as ovisac and second antennae showed more striking discrimination among morphotypes.

Furthermore, a discriminant function analysis of male and female morphotypes indicated that all types were separated by their specific morphometric characteristics. Results indicate that morphotypes vary from one another based on morphometric traits. Our results are greatly supported by previous observations [25], reporting interpopulation relationships in *Artemia franciscana* populations (Colombian Caribbean coast) based on morphometric characters. Male morphometric characters showed more separation without overlaps compared to female morphotypes. Because all male morphotypes were correctly positioned in the different populations

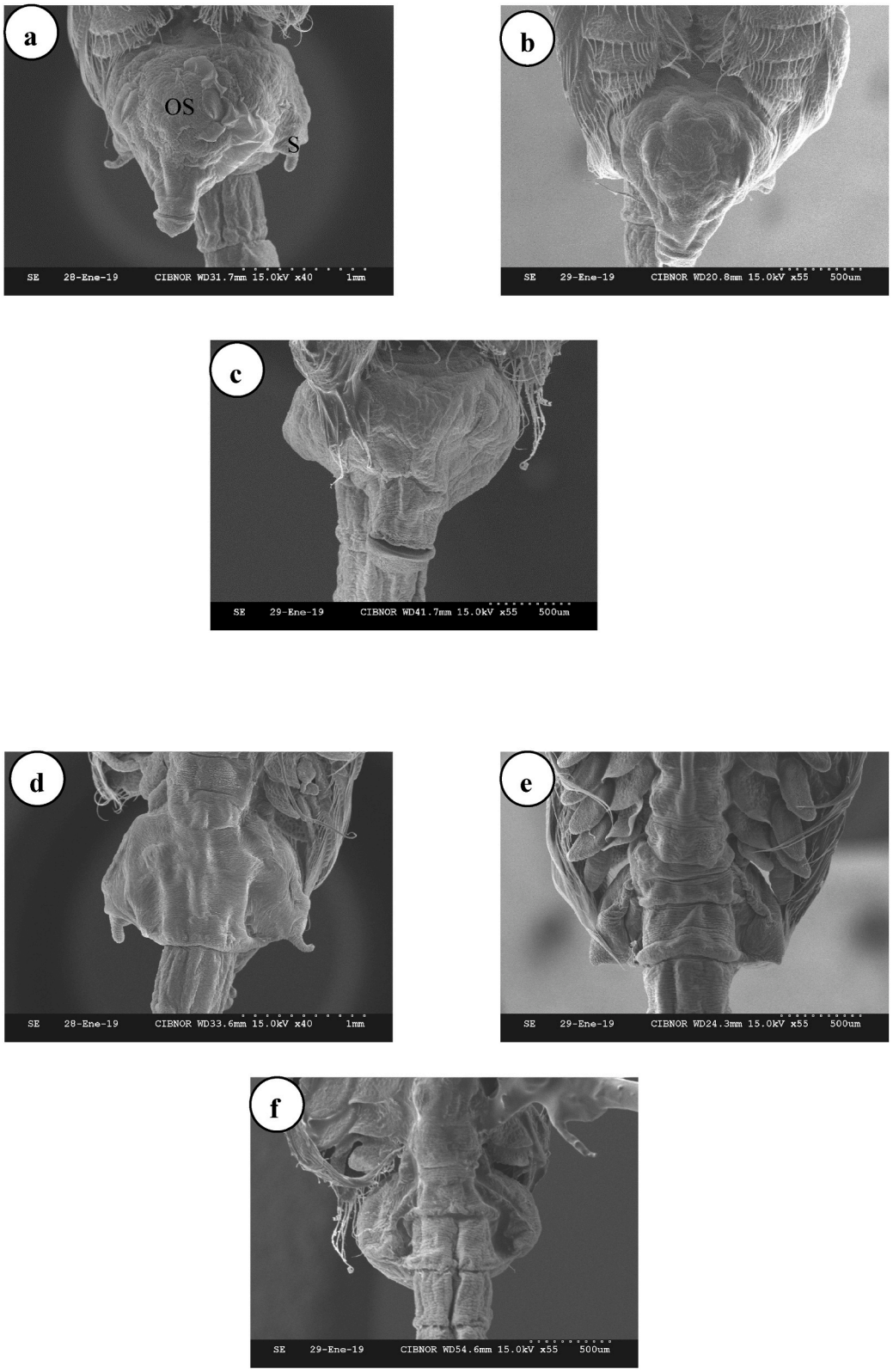

Fig. 6. Scanning electron micrographs of ovisac (OS) of female morphotypes (a) F1 (b) F2 (c) F3 (Ventral view); (d) F1 (e) F2 (f) F3 (Dorsal view) Note smooth surface topography of ovisac and presence of a pair of spines (S) in F1 on either side.

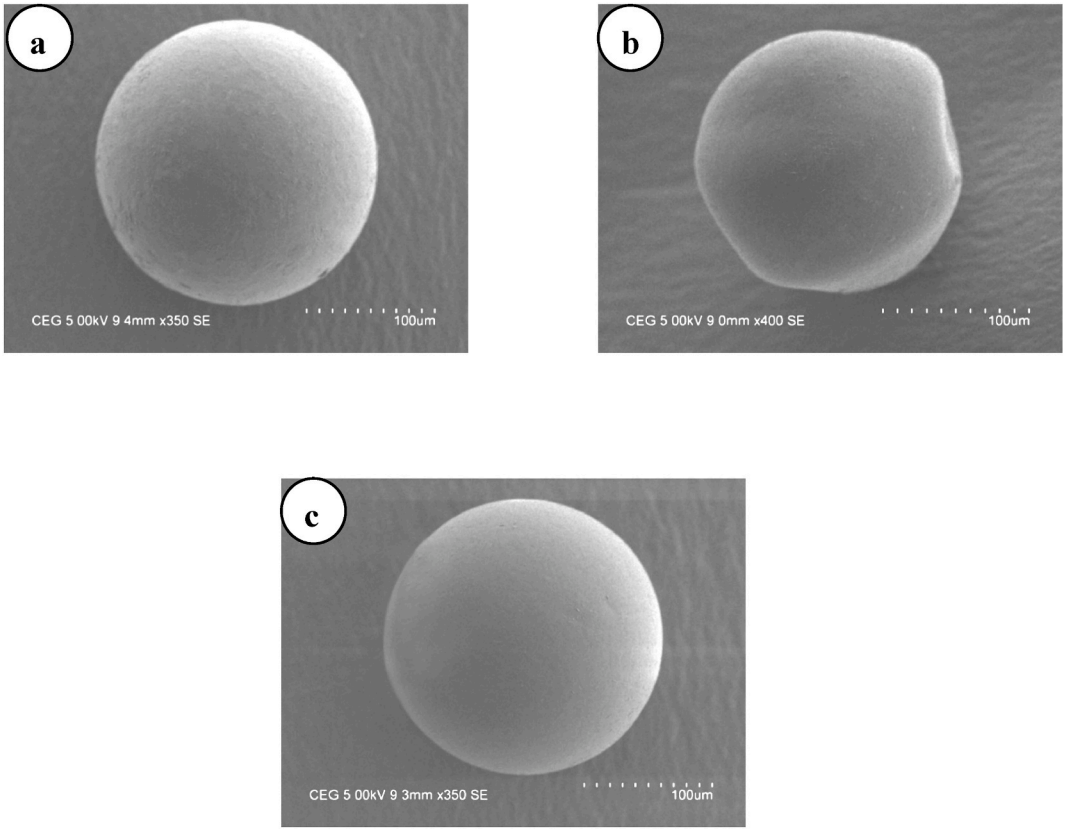

Fig. 7. Scanning electron micrographs of cyst of three female morphotypes, F1 (b) F2 (c) F3. Note the smooth surface topography of the cysts without any specific ornamentation.

studied. Earlier studies reported that the variation in these characters observed among male and female *Artemia* from different populations allowed to classify different *Artemia* populations. However, as mentioned above, it is difficult to distinguish among populations of *Artemia franciscana* [44–46]. To clarify this issue, brine shrimps were cultured in the laboratory, and the same types were observed and compared with those collected from the different sampling sites. This could help in the discrimination of morphotypes from the wild type which were raised from cysts collected from the sampling site [47].

With reference to morphometric data, it was reported that the most discriminant variables, such as cyst biometry, the diameter of the eyes, the distance between the eyes, the length of the first and second antennae, and the width of the ovisac, were used to discriminate between *Artemia* population [23,24]. The present study indicated that these particular characters showed a more striking discrimination among the morphotypes, in particular the second antennae in males and the width of the ovisac in females. Even the antennae of males were significantly different from each other. This was supported by the observation of Triantaphyllidis et al. [44] and Amat [40], that the first antenna was found to be the best tool to discriminate between parthenogenetic and bisexual individuals. However, the furcal morphology and its variation are the most important characters for the identification of different morphotypes. Similarly, the present study found variations in the shape of the furca - similar to how Amat et al. [48] found variations among populations in southern Spain and the rest of the spanish bisexual population using the size of the furca and the number of its setae. The present study emphasizes this parameter because of its usefulness in distinguishing the six different morphotypes.

The present work clearly indicates the taxonomic separation of six different morphotypes of *Artemia franciscana*. The occurrence of morphotypes does not seem to be influenced by prevailing environmental factors during different seasons. Thus, the clear separation of morphotypes on the basis of morphological and morphometric characteristics emphasizes a high degree of intra-population variability.

Scanning electron microscopic observations showed subtle variations among female morphotypes and the presence of lateral spines on the ovisac. Asem and Sun [36] reported different phenotypes in seven parthenogenetic populations of *Artemia franciscana* with reference to pattern of ovisac shape and structure. They used different shapes of ovisac such as rounded/ellipsoid, pear-shaped, triangular and rounded/heart-shaped in parthenogenetic populations. In the present study, the three *A. franciscana* female morphotypes were observed with triangular, umbrella and convex shaped ovisac.

Tyson & Sullivan [49] characterized frontal knob structure in male *A. franciscana* and proposed its role during mating. Later Tyson et al. [34] reported different types of cuticular cones on the knob. In general, the frontal knob cuticular cones are useful for the mating process especially for riding behaviour of the males as shown by Mura et al. [50,51]. In our study, we found significant differences in the cuticular cones at the basal part of the second antennae of male morphotypes in *A. franciscana*. The three *A. franciscana*

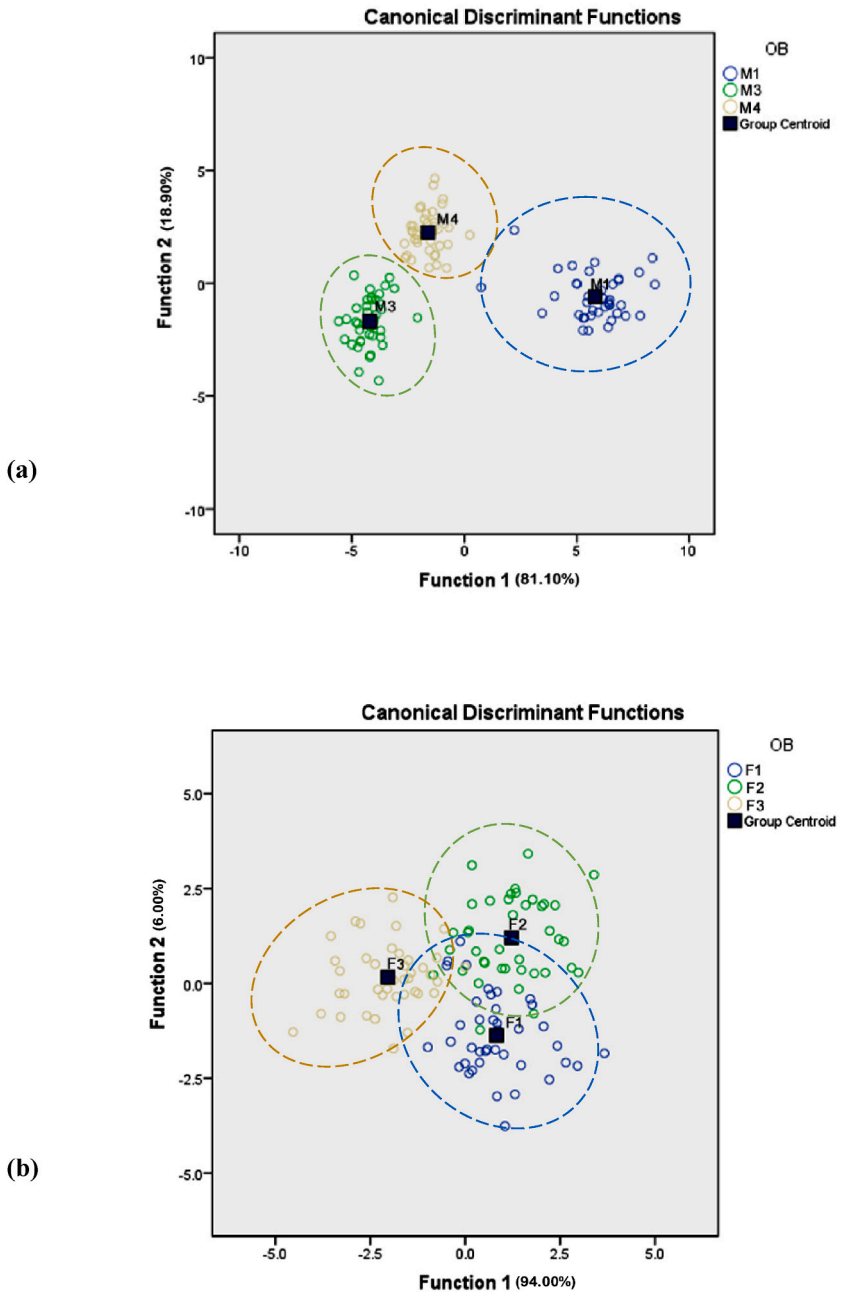

Fig. 8. Scatterplot resulting from the discriminant analysis (canonical score) for male and female morphotypes as separating factor from different sampling sites. Border lines represent 95 % confidence level: (a) Male (b) Female.

morphotypes with different shaped second antennae and numbers of cuticular cones probably serve as morphotype specific mating preference by the males.

Measurements of morphological traits are found to be key to distinguish *Artemia* species [52,53]. In this study, morphometric analysis of traits of *A. franciscana* showed more significant variations among the male and female morphotypes. Discriminant function analysis of male and female traits indicated specific morphometric variation of traits among the morphotypes. Camargo et al. [25] also reported interpopulation relationships among *A. franciscana* populations (Colombian Caribbean coast) based on morphometric traits similar to our study. Male and female traits showed distinctive morphometric variation of traits between the morphotypes compared with each other. Different traits such as diameter of eyes, distance between the eyes, and length of the first and second antennae and width of ovisac were generally used to differentiate *Artemia* species [23,24]. We also found differences in these parameters among the morphotypes, in particular the second antennae in male and width of ovisac in female, even the antennae of males was significantly

different from each other [40,44].

Our study confirmed the presence of six morphotypes of *A. franciscana* in the salterns of southeastern India with animals cultured under controlled laboratory conditions. Additional studies using molecular analysis will provide a more comprehensive interpretation of the taxonomy and adaptive strategies of the different morphotypes encountered in *A. franciscana*. On the other hand, studies are required to test whether these morphotypes offer more valuable contributions to the ecosystem than the native species and their suitability in aquaculture.

Ethical statements

1)The manuscript is not currently being considered for publication in another journal.
2)All authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

CRediT authorship contribution statement

Subramani Thirunavukkarasu: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gopal Murugan:** Writing – review & editing, Visualization, Validation, Investigation, Formal analysis, Data curation. **Jiang-Shiou Hwang:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis. **Natesan Munuswamy:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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Appendix A. Supplementary data

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