ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Occurrence of morphotypes in the invader species, *Artemia franciscana* Kellogg, 1906 (Crustacea: Anostraca) from Covelong salt works, South India

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Manuscript Info

Manuscript History:

Received: 18 September 2014
Final Accepted: 29 October 2014
Published Online: November 2014

Key words:

Artemia, Morphometry, Morphotypes, Principal Components Analysis, Kelambakkam

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Abstract

The present paper documents the occurrence of morphotypes in the natural population of *Artemia franciscana*, colonized in Covelong salterns, Kelambakkam (South India). Morphotyping was done based on the variations in the structure and morphology of graspers and its basel width in male and brood pouch morphology in female. These observations resulted in four types in male and three in female population. To further confirm, the morphometric measurements of specific characters were made with male and female morphotypes. Principal Components Analysis revealed that male morphotypes were distributed into four different groups and the female morphotypes almost clustered in to one group. The discriminant analysis performed, based on the morphological variations, registered as much as 98% and 67% with male and female morphotypes respectively. These morphological variations based on statistical analysis, further confirm the occurrence of morphotypes in male and female populations. However, further genomic studies on these morphotypes would provide more light on these aspects.

Introduction

The brine shrimp, *Artemia* (Branchiopoda, Anostraca) is an organism of great economical importance in aquaculture industry [Bengtson et al., 1991; Sorgeloos et al., 2001]. The brine shrimp has wide distribution in salt pans, inland salt lakes, and hypersaline water bodies throughout the world (Van Stappen, 2002), except Antarctica (Van Stappen, 2002; Gajardo, 2002). It is being assumed that this species is diverged about five to six million years ago from an ancestral form living in the Mediterranean area (Abatzopoulos et al., 2002; Munoz et al., 2008). The distribution of *Artemia* is not continuous; the populations are found throughout the tropical, subtropical and temperate climatic zones (Van Stappen, 1996). The geographical isolation of *Artemia* populations has led to numerous geographical strains that have adapted to conditions that fluctuate widely with regard to temperature, salinity and ionic composition of the biotope (Bowen et al., 1988).

Nowadays the genus *Artemia* is considered as a complex of taxon with sexual and asexual species and superspecies (Browne and Bowen, 1991), which are very similar in their morphological characters. Speciation of *Artemia* is still a highly puzzled topic and is evident from the existence of many species concepts and speciation mode. Based on morphology, six bisexual species and one parthenogenetic population are recorded and are grouped as the New World and the Old World species (Pilla and Beardmore, 1994; Abatzopoulos et al., 2002).

In India, parthenogenetic strain was reported in many salt pans (Kulkarni, 1953; Baid, 1958; Royan, 1979; John et al., 2004). But due to the introduction of the exotic (non-native) strain for culture aspects, the parthenogenetic strain, native

to Indian subcontinent, could not be traced (Sivagnanam et al., 2011) and the invader *A. franciscana* is colonised in most of the saltpans in India and is proved genetically (Vikas et al., 2012; Sivagnanam et al., 2013; Krishnakumar et al., 2014). However, there exists no published report on the morphology and biometry of this invader *A. franciscana* colonized in temperate regions, like India. The present study aimed to characterize *A. franciscana* morphologically, from Covelong saltern, Kelambakkam and to investigate the morphological variations in this tiny crustacean that belongs to the homogenous population. The scope of this study is to present data for further characterization of these *Artemia* populations both for taxonomic and culture perspectives.

Materials and Methods

Artemia Sampling

Samples of the invader species, *A. franciscana* were collected from a population, colonized in Covelong salt works, Kelambakkam during 2010-2011. Adult individuals were collected using 1mm scoop nets and brought to the wet laboratory, located at the salt pan and were subjected to morphological and biometric analysis.

Morphology and Biometric analysis

The collected adult *Artemia* samples were subjected to morphological and biometric analysis separately. The morphological measurements used in the present study included the structure and basel width of graspers in males and brood pouch in female and the biometric measurements represented in figure 1.

Collected adult brine shrimps were anesthetized with few drops of chloroform (2 drops mL^{-1}) in habitat seawater. The fixed samples were observed under light microscope equipped with calibrated ocular micrometer (ERMA). As many as 25 animals of each male and female morphotypes (totally 175) were used for biometric analysis. The variables used for the biometric measurements are given in the table 1. Among the variables measured, TL and AL were measured using standard graduated scale (1 mm) and the remaining variables using an ocular micrometer calibrated and attached to a compound microscope.

Statistical analysis

Morphological variables of different morphotypes were subjected to One-way ANOVA, Principal Components Analysis (PCA) and Discriminant Function Analysis (DA). In addition, DA used to appoint percent of corrected classification among stations. This was achieved by 2 methods: Calculated percent of overall correct classification as well as percent of correct classification between each pair stations. All statistical analyse were done using SPSS software, Version 11.5.

Results

Morphotypes

Results on morphometric analysis indicated considerable morphological differences on graspers morphology and diameter of basel width in males and brood pouch morphology females. Such variations were prominent and consistent two generations observed and hence they are denoted as 'morphotypes' as reported by Fu et al. (1991) in rotifers.

Description of male morphotypes

In males, the II antennae had modified into hooked claspers, by which they hold the females during copulation. During morphometric analysis, four different morphotypes were encountered and designated as 'M_1, M_2, M_3 and M_4'. In M_1, the antennae were curved and pointed at the tip region and the basel width was 'valley' shaped structure (Fig. 2a). The M_2 had blunt end antennae and 'Pot' shaped basel width (Fig. 2b). The antennae in M_3 showed spine like appearance and with sharp tip. The basel width showed 'U' shape (Fig. 2c). In M_4, the antennae resembled that of M_1 males but not pointed at the tip and the basel width was 'V' shaped (Fig. 2d).

Measurements of II antennae showed a maximum total length of 9.32 ± 1.11 mm in M_3. The basel width between the two antennae and frontal knob diameter in M_1 measured 0.22 ± 0.07 and 0.15 ± 0.07 mm respectively. These structures were smaller in size compared to other three morphotypes (Table 2). Type M_2 male had maximum basel width of 0.49 ± 0.01 mm followed by M_3 (0.42 ± 0.01 mm) and M_4 (0.34 ± 0.02 mm). On the other hand, the frontal knob diameter of M_2, M_3 and M_4 measured 0.16 ± 0.01 mm and no significant difference was observed among the male morphotypes studied ($P>0.05$).

Morphometric variables observed in the male types differed significantly ($P<0.05$) for BW (F value – 269.920), LA (F value – 7.518), FKD (F value – 5.012) and ED (F value – 4.177). Thus, these variations contribute the separation of the various morphotypes in male *Artemia* samples. These characters were formed to be of high diagnostic value for the characterization of morphotypes.

Discriminant analysis based on the morphotypes in the homogenous population as a separation criterion resulted in 98% separation. However, morphotypes M_2, M_3 and M_4 exhibited 100% separation among the four morphotypes studied

(Table 3). Figure 3 depicts the plot of the discriminant analysis based on the first and second (out of 3) roots that were produced. The percentage of corrected classified individuals for the four morphotypes ranged from 92.0 to 100.0%. Discriminant analysis resulted in 3 canonical discriminant functions. The first two were statistically highly significant ($P < 0.001$) obtaining a cumulative separation percentage of 98.0% (Table 4).

Description of female morphotypes

The differentiated female morphotypes were designated as 'F_1, F_2 and F_3 females'. The morphology of the brood pouch of F_1 was laterally triangular and pointed with vestigial spines (Fig. 4a). In F_2, the brood pouch was convex in shape and no lateral spines were observed (Fig. 4b). The brood pouch in F_3 was umbrella shaped and found without lateral spines (Fig. 4c).

Four morphotypes and the mean (cm) values of morphometric variables are given in table 5. Among the three morphotypes encountered, F_3 female was larger and measured around 10.28 ± 1.06 mm in total length, where as F_2 and F_1 measured 9.62 ± 1.27 and 9.46 ± 1.04 mm respectively. But F_2 females showed similarities with that of F_3 in total length. In F_1, F_2 and F_3 the brood pouch measured 1.32 ± 0.20, 1.39 ± 0.32 and 1.33 ± 0.17 mm respectively and no significant difference was observed (Table 5), but they differed in TL, LF and DF.

The statistical analysis indicated significant differences for DF (F value – 6.157), LF (F value – 5.291) and TL (F value – 3.732). These are the most contributing characters for morphotypes in female populations. Discriminant analysis based on the morphotypes in a homogenous population as a separation criterion resulted in 67% variation (Table 6). The plot of the discriminant analysis based on the first and second (out of 3) roots that were produced (Fig. 5). The percentage of corrected classified individuals for the three morphotypes ranged from 2 to 92%. Discriminant analysis resulted in 3 canonical discriminant functions (Table 7).

TABLES

Table 1: Variables used for morphometric analysis in *Artemia*

TL	Total length
AL	Abdominal length
LF	Length of furca
LA	Length of antenna
ED	Eye diameter
SFL	Number of setae on furca left
SFR	Number of setae on furca right
DF	Distance between furca
LAL	Length of last abdominal segment
BW	Basel width of clasper
FKD	Frontal knob diameter

Table 2: Measurement (in mm) of variables of male morphotypes

	MALE MORPHOTYPES					
Variables	M_1	M_2	M_3	M_4	F value	P value
TL[#]	8.72 ± 1.02[a]	8.72 ± 0.97[a]	9.32 ± 1.11[a]	8.70 ± 1.02[a]	2.167	0.097
AL[#]	3.76 ± 0.72[a]	3.68 ± 0.68[a]	4.04 ± 1.01[a]	3.66 ± 0.84[a]	1.139	0.337
LF	0.16 ± 0.03[a]	0.16 ± 0.05[a]	0.18 ± 0.06[a]	0.16 ± 0.06[a]	1.315	0.274
LA	0.94 ± 0.25[b]	0.89 ± 0.19[b]	1.15 ± 0.22[a]	0.94 ± 0.18[b]	7.518	0.000
ED	0.32 ± 0.05[b]	0.33 ± 0.06[ab]	0.37 ± 0.05[a]	0.32 ± 0.05[b]	4.177	0.008
SFL	11.08 ± 2.12[a]	9.76 ± 2.26[a]	10.84 ± 3.33[a]	9.68 ± 3.97[a]	1.438	0.237
SFR	10.48 ± 1.98[a]	10.12 ± 2.54[a]	10.68 ± 3.12[a]	9.60 ± 4.00[a]	0.622	0.602
DF	0.14 ± 0.03[b]	0.15 ± 0.02[ab]	0.17 ± 0.05[a]	0.15 ± 0.03[ab]	1.891	0.136
LAL	1.06 ± 0.12[a]	1.02 ± 0.16[a]	1.08 ± 0.13[a]	1.03 ± 0.16[a]	0.697	0.556
FKD	0.15 ± 0.07[c]	0.16 ± 0.01[bc]	0.16 ± 0.01[ab]	0.16 ± 0.01[a]	5.012	0.003
BW	0.22 ± 0.07[d]	0.49 ± 0.01[a]	0.42 ± 0.02[b]	0.34 ± 0.02[c]	269.920	0.000

Mean ±/SD of 25 replicates; means within a column followed by the same letter are not significantly different (P>0.05)
[#] values in cm

Table 3: Percentage of complete original male samples grouped classification by discriminate function analysis (classification results [1,2])

			Predicted Group Membership				
			M_1	M_2	M_3	M_4	Total
Original	Count	M_1	23	0	0	2	25
		M_2	0	25	0	0	25
		M_3	0	1	25	0	25
		M_4	0	0	0	25	25
	%	M_1	92.0	0.0	0.0	8.0	100.0
		M_2	0.0	100.0	0.0	0.0	100.0
		M_3	0.0	0.0	100.0	0.0	100.0
		M_4	0.0	0.0	0.0	100.0	100.0

[1]98.0% of original grouped cases correctly classified.
[2]92.0% of cross-validated grouped cases correctly classified

Table 4: Discriminant analysis of morphometric variables for male morphotypes. Classification functions produced by discriminant analysis for each types, Standardized coefficients for canonical variables, Eigenvalues and Cumulative percentages are presented.

Standardized coefficients for canonical variables			
	Root 1	Root 2	Root 3
TL	0.083	0.594	-0.120
AL	0.218	-0.837	0.285
LF	0.031	0.230	0.000
LA	-0.273	1.403	-0.152
ED	0.042	-0.157	-0.419
SFL	-0.403	1.422	-0.612
SFR	0.353	-1.348	-0.032
DF	0.012	0.143	0.131
LSL	-0.079	-0.076	0.167
FD	0.032	0.346	0.984
BW	1.014	0.005	-0.037
Eigenvalue[a]	9.013	0.655	0.196
Cumulative %	91.4	98.0	100.0

[a]First 3 canonical discriminant functions were used in the analysis.

Table 5: Measurement (in mm) of variables of female morphotypes

	FEMALE MORPHOTYPES				
Variables	F_1	F_2	F_3	F value	P value
TL[#]	9.46 ± 1.04[b]	9.62 ± 1.27[b]	10.28 ± 1.06[a]	3.732	0.029
AL[#]	4.08 ± 0.76[a]	4.30 ± 0.85[a]	4.28 ± 0.68[a]	0.648	0.526
WB	1.32 ± 0.20[a]	1.39 ± 0.32[a]	1.33 ± 0.17[a]	0.577	0.564
LF	0.17 ± 0.06[ab]	0.19 ± 0.05[a]	0.14 ± 0.03[c]	5.291	0.007
LA	0.59 ± 0.15[ab]	0.63 ± 0.11[a]	0.64 ± 0.14[a]	1.167	0.317
ED	0.31 ± 0.13[a]	0.29 ± 0.06[a]	0.30 ± 0.11[a]	0.224	0.800
SFL	9.42 ± 3.05[a]	10.08 ± 2.25[a]	9.28 ± 2.92[a]	0.599	0.552
SFR	9.13 ± 2.42[a]	9.56 ± 2.04[a]	9.04 ± 2.24[a]	0.384	0.682
DF	0.18 ± 0.07[a]	0.18 ± 0.04[a]	0.14 ± 0.02[b]	6.157	0.003
LAL	1.22 ± 0.14[a]	1.14 ± 0.52[a]	1.29 ± 0.24[a]	1.139	0.326

Mean ± SD of 25 replicates; means within a column followed by the letter are not significantly different (P>0.05)
[#] values in cm

Table 6: Percentage of complete original female samples grouped classification by discriminate function analysis (classification results)[a,b]

			Predicted Group Membership			
			F_1	F_2	F_3	Total
Original	Count	F_1	19	4	2	25
		F_2	9	12	4	25
		F_3	2	0	23	25
	%	F_1	36.0	48.0	16.0	100.0
		F_2	8.0	0.0	92.0	100.0
		F_3	19	4	2	25

[a] 67.0% of original grouped cases correctly classified.
[b] 55.5% of cross-validated grouped cases correctly classified

Table 7: Discriminant analysis of morphometric variables for female morphotypes. Classification functions produced by discriminant analysis for each type, Standardized coefficients for canonical variables, Eigenvalues and Cumulative percentages are presented.

Standardized coefficients for canonical variables		
	Root 1	Root 2
TL	-0.847	-0.603
AL	0.436	0.603
WB	-0.061	0.120
LF	0.721	0.347
LA	-0.106	0.714
ED	0.248	-0.100
SFL	0.007	0.182
SFR	-0.131	-0.249
DF	0.751	-0.603
LAL	-0.029	-0.211
TL	0.657	0.073
Eigenvalue[a]	90.0	100.0

Cumulative %	-0.847	-0.603

[a]First 2 canonical discriminant functions were used in the analysis.

FIGURE

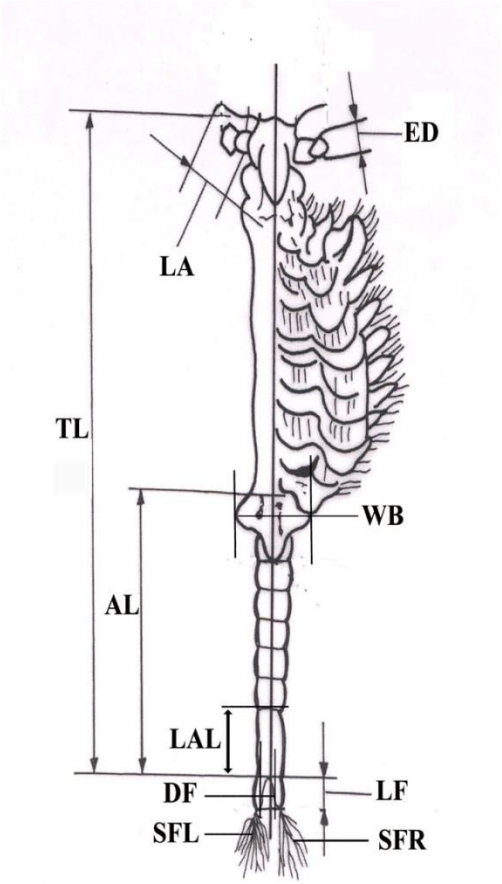

Figure 1: Morphometric measurements (Gajardo et al., 1998)

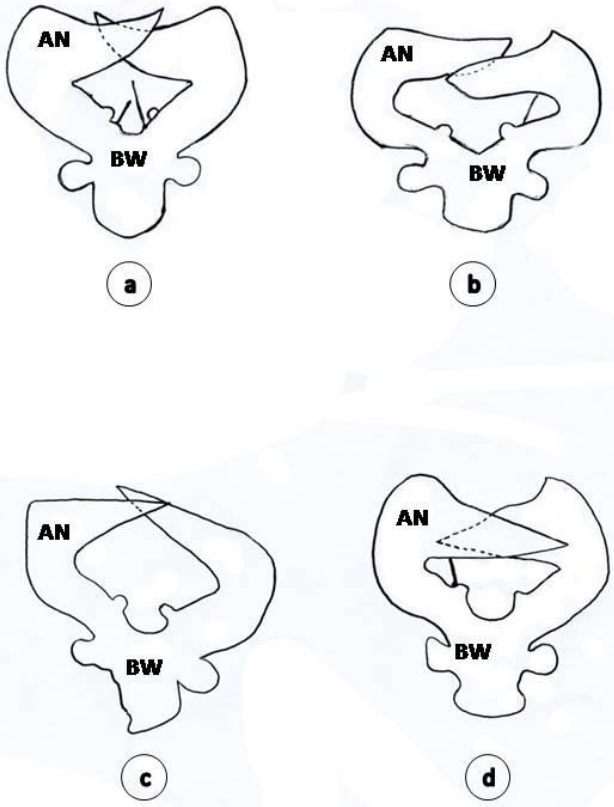

Figure 2: Morphotypes in males (AN: Anetnnae; BW: Basel width)

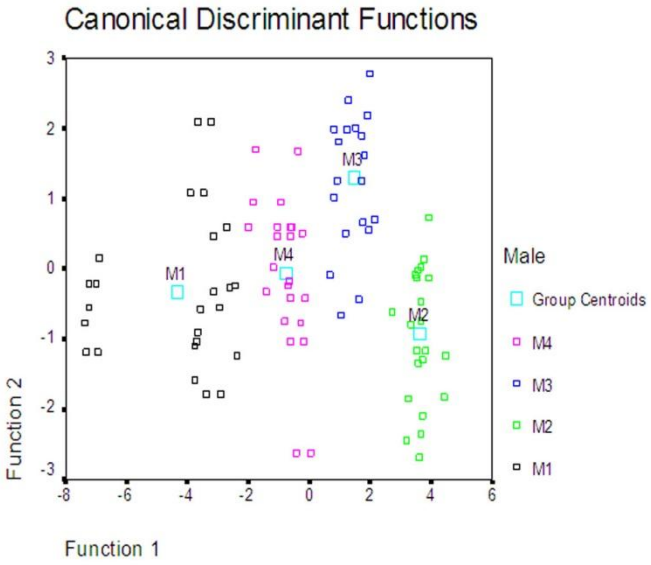

Figure 3: Scatterplot resulting from the discriminant analysis (canonical scores) based on morphometrics and using the male morphotypes in a homogenous population as a separation criterion. Borderlines represent 95% confidence level.

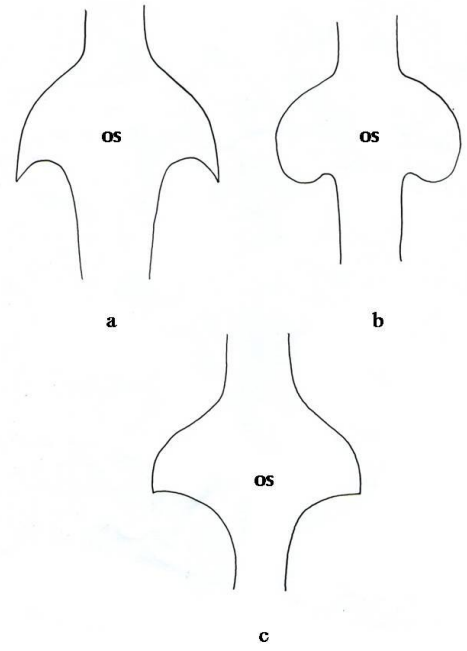

Figure 4: Morphotypes in female (OS: Ovi sac)

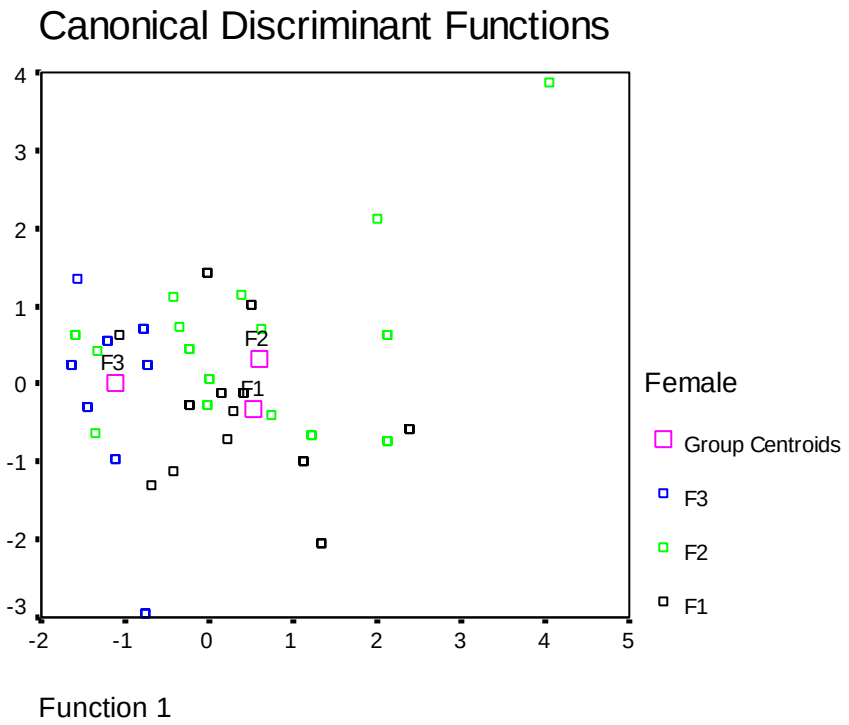

Figure 5: Scatterplot resulting from the discriminant analysis (canonical scores) based on morphometrics and using the male morphotypes in a homogenous population as a separation criterion. Borderlines represent 92% confidence level.

Discussion

In *Artemia*, morphological characters have been used as a basis to describe populations and species, though controversy exists on ways of selecting and using the best traits, as well as on their degree of genetic and environmental determination (Gajardo et al., 1998; Baxevanis et al., 2005). Since morphological traits may change, depending on environmental variations, particularly salinity and/or other culture conditions, the simplest and perhaps most effective procedure to study the relationships between species is to gather qualitative and quantitative data for different populations under carefully standardized laboratory conditions (Naceur et al., 2012). The present study offered an opportunity to expand the knowledge on conditions that promote or constrain morphological differences in the homogenous population of *Artemia franciscana*, colonized in Kelambakkam saltpan, India.

The complex structures like frontal knob spines and the basel width of the II antennae of *Artemia* showed taxonomic importance than simple structures. The morphotypes in male and female adult *Artemia franciscana* of Kelambakkam were significantly different from each other in some but not all of the biometric and morphological characters studied and these morphological differences could be used to identify the populations and to construct taxonomic keys (Mayer, 2002). Among male morphotypes, the general structure of the II antennae and the basel width of the antennae can be used to discriminate between the four morphotypes encountered. The result of Torrentera and Dodson (1995), discriminated the three Caribbean populations based on the brood pouch morphology in *Artemia* females and suggested considerable intrageneric variability of brood pouch morphology. Similarly, the present study also discriminate the presence of three and four morphotypes in female and male *Artemia* populations respectively in intrapopulation variability. Females have laterally triangular, umbrella shaped and round brood pouch with and without lateral lobes and spines. Considering morphometric variables among the different male morphotypes, length of antennae, eye diameter, frontal knob diameter and basel width in male morphotypes and total length, length of furca and distance between furca in female differed significantly among the morphotypes.

In male morphotypes, the two canonical axes explain most (98%) of the variation. Loading suggests that frontal knob size and basel width of the claspers are characteristics with respect to males. This is an important contribution of our study, because the characteristics like clasper and the basel width of the claspers have not been described in detail in previous studies. On the other hand, the canonical axes explain no much variation between female morphotypes. Amat

(1980) described width and length of the brood pouch as differential characters between Spanish versus SFB strains. Although, no significant variations were observed in the brood pouch diameter, they can be considered as distinguishable characters in *Artemia*.

Knowledge of cyst and nauplii size is important if these populations are to be considered as food sources for the early larval stages of fish and shrimp. Among various morphotypes observed in the Kelambakkam populations, no significant difference was observed with reference to the diameter of hydrated cysts and I-instar nauplliar length. The intrapopulation variation in cyst diameter, if any, may be caused due to physical and chemical conditions on ovipositing females at the collection site (Agh et al., 2009).

Polyploidy is also a common phenomenon among parthenogenetic populations (Abatzopoulos et al., 1986; Abreu-Grobois, 1987). Amat (1980) and Hontoria and Amat (1992) studying parthenogenetic populations from the Western Mediterranean basin demonstrated that ploidy level affects the morphology of *Artemia*. There are many ecologically and reproductively isolated bisexual populations of *Artemia* in the New World which have been grouped together in the *A. franciscana* superspecies; but according to Torrentera and Dodson (1995) and Camargo et al., (2002), it is possible that speciation had occurred in the Caribbean area as indicated by morphological and genetic traits in some of the populations.

Amat (1980) distinguished two knob morphologies and differentiated New World populations and Old World populations. Torrentera and Dodson (1995) also suggested that knob morphology must be studied in a quantitative analysis. In our study, we focussed on the diameter of the frontal knob and differentiated four morphotypes of a homogenous population. Amat (1980) and Hontoria and Amat (1992a) described differences among populations using furcal shape, number of furcal setae, antenna, and head size. However, within a population, high variability in appendage characters, morphological changes in furcal lobes and setae between field and culture organisms are reported.

Our finding agrees with Hontoria and Amat (1992b) who have suggested that these two populations may be incipient different species within the *A. franciscana* complex. Similarly, the molecular analysis revealed that the population exists in Kelambakkam saltpan is the complex of *A. franciscana* (Sivagnanam, 2005; Vikas et al., 2012). The morphological difference may be a result of geographical and ecological isolation. These morphological differences may be due to isolation resulting from adaptations to different kinds of temporary ponds and possibly genetic.

Abreu-Grobois (1987) indicated that strong salinity of the medium may influence the morphological changes in *Artemia*. In the present study, there exists high variability in appendage characters within a population, with considerable changes in adult brine shrimp morphology, cultured at similar environmental conditions. Thus, present observations prove the existence of various morphotypes in the salterns of Kelambakkam. However, further molecular studies are highly warranted to augment the new species/ populations with the variable characters of the brine shrimp.

Acknowledgements

Financial assistance from the Ministry of Earth Sciences (MoES/11-MRDF/1/8/P/07), Government of India is gratefully acknowledged. Thanks are due to M/s. SKMS saltpan, Kelambakkam and Salt Commissioner, Government of India for extending the salt pan facility for brine shrimp culture project.

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