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Evaluation of morphometric and molecular variations among some Egyptian brine shrimps comparatively with other *Artemia* species

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ABSTRACT

Up to date, the true phylogenetic relationships among the *Artemia* species are under debate. In this study, some morphometric and molecular (Inter-simple sequence repeats, ISSR) variations were analyzed to evaluate the biodiversity among some Egyptian *Artemia* species comparatively with other brine shrimps (*A. parthenogenetica, A. salina and A. franciscana*). The highest and lowest Wilks' lambda values were calculated for the Length of furca and the abdominal length. The cluster analysis based on the *Artemia* morphological variations was an agreement with the re-constructed dendrogram based on ISSR markers. The ISSR variations were comparatively analyzed with the *Artemia* species Cytochrome oxidase subunit I gene (COI) sequence variations. Based on the COI consensus sequences, the distance value between *A. salina* and *A. parthenogenetica* was higher than the distance value between *A. salina* and *A. franciscana.* The ISSR could be an effective method in *Artemia* molecular characterization and evolutionary studies. The results could be helpful in the conservation of the evaluated *Artemia* species. The combination of more informative molecular markers with the selected morphometric characters should be carried out to understand the true evolutionary variations in the *Artemia* resources.

INTRODUCTION

The *Artemia* species (The 2n ranged from 42 to 44) are widely distributed crustacean organisms that can inhabit hypersaline lakes and lagoons (Kong *et al.*, 2019). Also, the osmotolerance of *Artemia* was extensively explained in many biological investigations (Gajardo and Beardmore, 2012; Kong *et al.*, 2019).

The economic values of the *Artemia* in research laboratories and aquaculture as natural feeding stuff were extensively explained (Gajardo and Beardmore, 2012; Jamali *et al*., 2018).

Up to date, the true phylogenetic relationships among *Artemia* species especially the Egyptian *Artemia* resources are under debate (Saad *et al*., 2014; Eimanifar *et al.*, 2015).

There are different laboratory techniques were applied for evaluating the biological differences among and within *Artemia* resources such as biometry,

biochemical and molecular methods (Mahdhi *et al.*, 2012; Saad *et al*., 2014; Saad and Elsebaie,2017).

Artemia is including a set morphologically similar species that mainly characterized by the criteria of reproductive isolation (Badaracco *et al*., 1995).

The body length character in *Artemia* (such as in *A. sinica*) was investigated and characterized by low heritability values (Kong *et al*., 2019). Therefore the morphological characterization might be not sufficient for inference the true evolutionary variations.

Regarding the molecular characterization, the accuracy in the *Artemia* species characterization was associated with the molecular identification system efficiency (Saad and Elsebaie 2017). Some molecular characterization systems such as 16S ribosomal RNA, COI (Tizol-Correa *et al*., 2009; Maccari *et al.*, 2013), AFLP (De-Vos *et al*., 2013) and RAPD (Saad *et al.*, 2014), were applied for estimating the genetic variability levels among and within many *Artemia* species and/or populations. Eimanifar *et al*. (2015) used COI barcoding system for exploring the population structures among some *A. franciscana* populations collected from different geographical localities of Great Salt Lake (the largest hypersaline lake in North USA).

Recently, Asem *et al.* (2019) used four molecular markers (Na+/K+ ATPase, COI, 16s rRNA and ITS1) to investigate the effects of environmental changes on *Artemia* genetic variability in the Urmia Lake.

Concerning the *Artemia* biological resources in Egypt, genetic diversity and speciation have not been evaluated in detail. In addition, no management strategies were applied for the utilization of *Artemia* genetic resources (Saad *et al*., 2014). The advantages of different molecular methods in detecting the molecular genetic variations among different animal taxa were discussed and confirmed (Tizol-Correa *et al*., 2009; Saad *et al*., 2013; Eimanifar *et al*., 2015; Saad *et al.*, 2019).

The present study aimed to evaluate the evolutionary variations among some Egyptian brine shrimps comparatively with other *Artemia* species using some morphometric and molecular variations.

MATERIALS AND METHODS

Artemia samples

The *Artemia* samples were collected from three distantly Egyptian locations. These samples were *A. parthenogenetica* (MX from Alex. Salt marshes), *A. salina* (FA from El Fayoum Salt marshes) and *A. salina* (MN from Wadi El Natrun Valley). Also, two *Artemia* resources (*A. franciscana* from the Great Salt Lake, USA and *A. parthenogenetica* from China) were obtained from conservation of aquatic biological resources research group (DSR, King Abdulaziz University, KSA). The morphological characterization was carried out according to Clark and Bowen (1976).

Morphometric analysis

The morphometric parameters among the evaluated *Artemia* species were measured as described by Triantaphyllidis *et al.* (1997). A total of 12 morphometric parameters were scored for estimating the morphological variations among the evaluated *Artemia* species. The morphological parameters were the abdominal length (al), abdominal width (aw), distance between compound eyes (de), eye diameter (ed), length of furca (lf), length of 1st antenna (la), total length (tl), head width (hw), ratio abdominal length/total length (B/A), ratio length of furca/total length (G/A), ratio

head width/total length (I/A) and the length of 1st antenna/ total length (H/A). Also, the (λ) Wilks' lambda is calculated. The (λ) was given by the equation ($1- \eta^2$), where η^2 is the ratio of the between-groups sum of squares to the total sum of squares. This parameter has represented the proportion of the total variance attributable to differentiate among the groups.

Analysis of molecular variations among the evaluated *Artemia* species DNA extraction and PCR amplification

The DNA samples were extracted from 15 Adults *Artemia* individuals from each evaluated *Artemia* species. DNA extraction and purification were carried out according to Badaracco *et al*. (1995) with some modifications (Saad *et al*., 2014).

Analysis of ISSR markers

Eleven ISSR primers were used to investigate the molecular variability among all the applied *Artemia* samples. The ISSR primer codes and sequences are presented in Table (1).

The PCR reactions were prepared as described by Saad *et al.* (2013). The PCR program consisted of one cycle for 3 min. at 94^{o}C, 35 cycles for (30 sec. at 94^{o}C, 45 sec. at 44^{o}C and 1min. at 72^{o}C) and one cycle for 15 min. at 72^{o}C. The amplification products were separated on 1.5 % agarose gels.

Analysis of *Artemia* mitochondrial Cytochrome oxidase subunit I gene (COI) sequences

A total of 30 *Artemia* COI gene sequence accessions were alignment and analyzed. Theses accessions were DQ119645, DQ119646 (Hou *et al*., 2006), DQ401269 (Tizol *et al*., 2009), DQ426856 (Munoz *et al*., 2008), DQ426832, DQ426834, DQ426841, DQ426846, DQ426848, DQ426849, DQ426854, DQ426855, DQ426856 & DQ426858 (Munoz *et al.*, 2008), from DQ426824 to DQ426826, from GU591380 to GU591384 (Munoz *et al.,* 2010), from KC193638, KC193640 (Maccari *et al*., 2013), X69067.1 and NC_001620 (Perez *et al*., 1994), MK393285, MK393283, MK393289 and GU944723 (unpublished data obtained from NCBI).

Table 1: The ISSR primer codes, sequences, pattern evaluation, total number of generated bands and averages of band frequencies.

Code	Sequence	pattern	TB	BF±SD
IT1	CACACACACACACAGT	good	10	0.53±0.24
IT2	CACACACACACACACACAAC	good	7	0.88±0.31
IT3	GAG GAG GAG GAG AG	good	12	0.75±0.31
17898A	CAC ACA CAC ACA AC	good	16	0.74±0.25
SAS1	GTG GTG GTG GTGGC	good	12	0.81±0.20
SAS3	CAG GAG GAGGAGG	good	8	0.75±0.28
HB12	CACCACCACGC	good	15	0.69±0.28
HB13	GAG GAG GAG GC	good	11	0.85±0.26
HB15	GTG GTG GTG GC	good	7	1±0
PT1	*GTGTGTGTGTGTGTGTC*	*NC*	-	-
HB8	GAGAGAGAGAGAGG	NC	-	-

TB= Total bands, BF=average of band frequencies, SD= Standard deviation and NC= Unclear.

Data analysis

The morphometric variations and dendrogram construction were analyzed using the PAleontological Statistics, (PAST) Version 3.22 (Qyvind *et al.,* 2001).

The ISSR profiles were scored and analyzed. The distance values were calculated using the Popgene. The results were introduced separately to two programs: Dendro UPGMA (a dendrogram construction utility) and PAST (Yeh and

Boyle, 1997; Qyvind *et al*., 2001) for constructing the phylogenetic relationships among the evaluated *Artemia* species.

The COI sequences (617bp) analysis involved 30 nucleotide sequences. The COI sequences were aligned and the phylogenetic tree was re-constructed among the evaluated *Artemia* species using MEGA V6 (Tamura *et al.*, 2013) based on the Maximum Likelihood method (MLM). Analysis of SNPs (single nucleotide polymorphisms) was carried out using DNAsp. (Ver.5.10.01).

RESULTS AND DISCUSSION

Morphological variations

The average values of the 12 morphometric characters (measured on the adult *Artemia* female samples) were calculated and analyzed. The interspecific variation values shared to the separation of the evaluated *Artemia* species were the total length (tl), abdominal length (al), the distance between compound eyes (de) and length of 1st antenna (la). The lowest of these values were calculated for *A. salina* (FA).

The Ratio of the abdominal length/total length (B/A) values were succeeded in the discrimination among the evaluated *Artemia* samples comparatively with the other calculated *Artemia* body ratios (G/A, I/A and H/A) as presented in Table (2).

The Wilks' lambda (λ) was calculated to explore the differences between group means for a particular combination of dependent variables (Figure1). The highest (λ) value was calculated for (lf) character. On the other hand, the lowest (λ) value (means more the variable) was calculated for (al).

Table 2: The ratio abdominal length/ total length, ratio length of furca / total length, ratio head width /total length and ratio length of 1st antenna/ total length within evaluated *Artemia* species.

	B/A	**G/A**	**I/A**	**H/A**
A. salina (FA)	0.3813	0.048	0.0938	0.0520
A.parthenogenetica (MX)	0.5394	0.028	0.0552	0.0740
A. salina (MN)	0.3374	0.036	0.0626	0.0495
A.parthenogenetica (SC)	0.5375	0.028	0.0799	0.1066
A. franciscana (SA)	0.45207	0.033	0.1066	0.1514

B/A= Ratio abdominal length/total length, G/A=Ratio Length of furca/total length, I/A=Ratio Head width / total length and H/A= Length of 1st antenna/ total length.

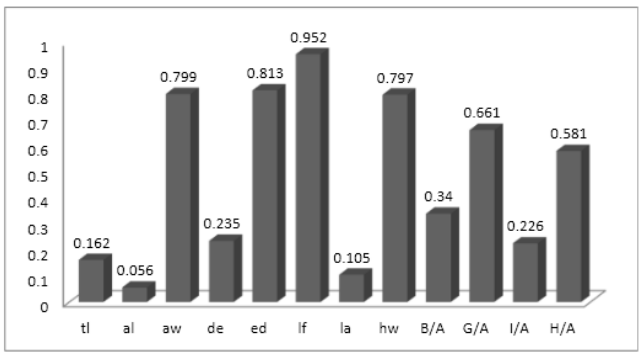

Fig.1: The Wilks' lambda (λ=1- η2) values of the selected morphometric female *Artemia* characters. tl= Total length, al= Abdominal length, aw= Abdominal width, de= Distance between compound eyes, ed= Eye diameter, If= Length of furca, la= Length of 1st antenna, hw=Head width, B/A= Ratio abdominal length/ total length, (G/A)= Ratio Length of furca/total length, (I/A)= Ratio Head width/total length and H/A= Length of 1st antenna/ total length.

Analysis of ISSR markers

Out of the 11 ISSR primers used, only two ISSR primers generated unclear banding patterns (PT1 and HB8). To evaluate the genetic variations and reconstructing the phylogenetic relations among the *Artemia* samples, the ISSR bands generated by the best ISSR primers (nine primers) were scored and analyzed. The total number of generated ISSR bands were detected and scored (98 bands). The number of generated ISSR bands were ranged from 7 (Primers IT2 and HB15) to 16 (primer 17898A). Also, the band frequency values were calculated for each selected ISSR primer in the evaluated *Artemia* species. The averages and standard deviations of these values were presented in Table (1). It was ranged from 0.53 (primer IT1) to 1 (primer HB15).

The phylogenetic relationships among the evaluated *Artemia* species

The phylogenetic relationships among the evaluated *Artemia* samples were constructed based on morphological (Figure 2a) and ISSR (Figure 2b) variations.

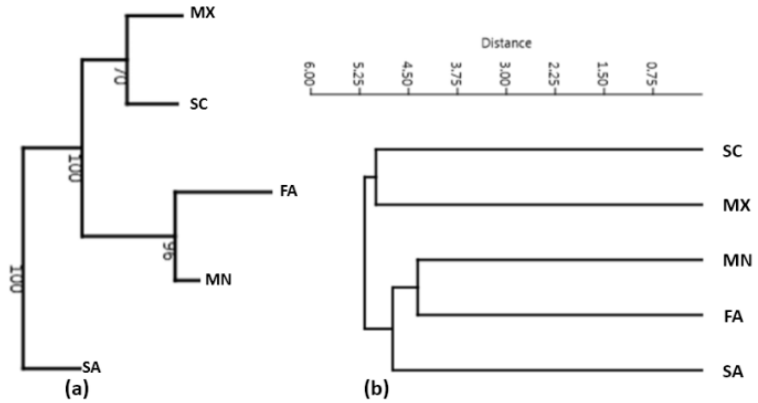

Fig. 2: The constructed dendrogram (UPGMA methods) among the evaluated *Artemia* species based on morphological (a) and ISSR (b) variations. MX= *A.parthenogenetica*, MN= *A. salina*, FA= *A. salina*, SA= *A. franciscana*, SC= *A. parthenogenetica*.

The relationships among evaluated *Artemia* species based on morphometric variations were similar that revealed from the molecular variations.

Concerning the molecular divergences, the lowest divergence value (distance = 0.193) was observed between the two Egyptian *A. salina* biotypes (FA and MN). The genetic distance value between MX and SC *Artemia* species was lower than the distance between MX and all the other evaluated *Artemia* species. The similarity values among evaluated *Artemia* species were detected and presented in Table (3).

Table 3: The genetic identity (Above diagonal) and genetic distance (Below diagonal) among the evaluated *Artemia* species based on ISSR markers.

	MN	**FA**	**SA**	**SC**	**MX**
MN		0.824	0.749	0.785	0.770
FA	0.193		0.797	0.770	0.774
SA	0.288	0.225		0.788	0.775
SC	0.241	0.260	0.237		0.801
MX	0.261	0.255	0.254	0.221	

MX= *A.parthenogenetica*, MN= *A. salina*, FA= *A. salina*,
SA= *A. franciscana*, SC= *A. parthenogenetica*.

Inference of the evolutionary variations among the three *Artemia* species based on COI gene sequence variations

A total of 30 *COI* DNA fragment sequences (617bp) in the three *Artemia* species (*A. parthenogenetica, A. salina and A. franciscana*) were analyzed.

The average values of GC (0.440), GC_2 (0.374) and GC_3 (0.509) were calculated. The number of haplotypes (h=24), single nucleotide polymorphism (SNPs= 186), estimates of haplotype diversity (hd=0.977), nucleotide diversity (Pi=0.1326), theta from polymorphic sites (Ɵ= 0.0924), average number of nucleotide differences (k=81.814), conservation threshold (CT=0.8) and sequence conservation value (Sc=0.699) were calculated in all evaluated fragment sequences (Table 4).

The previous parameters were applied for estimating the genetic variations within each evaluated *Artemia* species. All DNA polymorphism were affected by the SNP value within each *Artemia* species. The highest single nucleotide polymorphism (SNPs=30) were calculated in *A. salina.*

These results were reflected also by the calculated genetic distance and haplotype diversity values (ranged between 0.8 to1) within each *Artemia* species. The GC (0.415 to 0.471), GC_2 (0.314 to 0.370) and GC_3 (0.491 to 0.540) values were varied among the estimated COI sequences.

The estimated value of the shape parameter for the discrete Gamma Distribution is 0.3747. A discrete Gamma distribution was used to model evolutionary rate differences among sites. Mean evolutionary rates in these categories were 0.01, 0.09, 0.35, 1.01, 3.55 substitutions per site. The nucleotide frequencies were A = 22.05%, T = 33.98%, C = 24.24%, and G = 19.72%.

The phylogenetic relationships among the three *Artemia* species based on COI gene nucleotide variations were presented in Figure (3). The evolutionary history was inferred by using the Maximum Likelihood method.

Fig. 3: Phylogenetic relationships among the *Artemia* species based on COI gene nucleotide variations (accessions were obtained from the NCBI). The evolutionary history was inferred by using the Maximum Likelihood method.

Based on the consensus sequences, the distance value between *A.salina* and *A.parthenogenetica* is higher than the distance value between *A.salina* and *A.franciscana*. The evaluated COI sequence sites are beneficial in discriminating closely related *Artemia* species. The Variable COI consensus sequence sites among the three *Artemia* species are detected and presented in Figure (4).

The true evolutionary variations in the genus *Artemia* is still unclear and may be uncertain (Badaracco *et al*., 1995; Saad *et al.*, 2014).

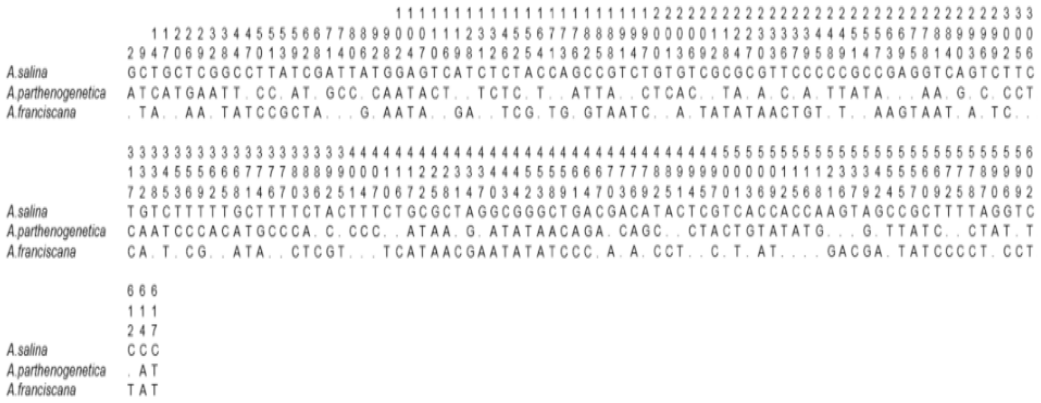

Fig. 4: The Variable COI consensus sequence sites among the three *Artemia* species.

Table 4: DNA polymorphism, sequence conservation and genetic distance values in eachevaluated *Artemia* species .

	A. salina	*A. franciscana*	*A. parthenogenetica*	**Total**
SNP	30	16	22	186
GC	0.471	0.415	0.433	0.440
GC_2	0.437	0.314	0.370	0.374
GC_3	0.540	0.491	0.496	0.509
Pi	0.0182	0.0068	0.0106	0.1326
Ɵ	0.0177	0.0093	0.0129	0.0924
K	11.267	4.200	6.578	81.814
H	10	5	9	24
Hd	1	0.8	0.978	0.977
SC	0.951	0.974	0.964	0.699
CT	0.8	0.8	0.8	0.8
Dist.	0.019	0.007	0.011	0.16

SNP=Single nucleotide polymorphism, Pi=nucleotide diversity, Ɵ =Theta from site, K= Average number of nucleotide differences, hd= Haplotype diversity= (hd), SC= Sequence conservation, CT= Conservation threshold and D= Distance value within each evaluated *Artemia* species

Some investigations recommended the analysis of different morphometric characters for exploring the evolutionary variations among different *Artemia* species such as in *A. sinica* and *A. franciscana* (Camargo *et al*., 2003; Kong *et al.*, 2019). Other studies used some biochemical analysis for differentiating the *Artemia* resources (Ruiz *et al.*, 2008).

In recent years attempts were made to correlate some *Artemia* species and/or populations living in different ecological locations around the world via molecular techniques (Eimanifar *et al.*, 2015; Dunga *et al.*, 2019). Generally, the evaluation of *Artemia* biodiversity through viable, simple and proper techniques such as ISSR is required as a basic step for good management through conservation of *Artemia* resources.

In the present study, the combination between the molecular and the morphological characterization was applied to infer the evolutionary variations in the evaluated *Artemia* resources. The optimal discriminant variables for all the evaluated *Artemia* species were the total length, abdominal length, the distance between compound eyes and length of 1st antenna. However, only abdominal length and antenna length were recommended for discriminating among some Colombian *A. franciscana* populations. Measuring the left setae and antenna length were suitable for characterizing the *Artemia* males (Camargo *et al.,* 2003).

The Wilks' lambda (as statistical parameters in multivariate analysis of variance) was applied in the present study for exploring the differences among the means of the estimated morphometric measurements. The highest (λ) value was calculated for the (lf) character. On the other hand, the lowest (λ) value (means more the variable) was calculated for (al). Application of these statistical parameters in similar data feature on some *Artemia* species (*A. franciscana*, *A. tunisiana* and *A. urmiana*) was recommended for *Artemia* species discrimination. These parameters could be informative in the classic method of *Artemia* morphological identification such as in Triantaphyllidis *et al*. (1997). Some morphological character variations might be affected by environmental conditions. Some investigations observed that the genetic variations among *Artemia* species might be correlated to geographical ranges and habitat heterogeneities of the investigated locations (Eimanifar *et al.,* 2015).

The recommended *Artemia* morphometric characters provided suitable and informative classification methods when combined with the developed molecular markers. The divergence levels that detected in the present study between *A. franciscana* and the other evaluated *Artemia* species (*A.salina and A.parthenogenetica*) were confirmed also in some previous studies using other molecular techniques such as Random Amplified Polymorphic DNA (Badaracco *et al.,* 1995).

Most of the selected ISSR primers succeeded in generating informative markers for discrimination among all investigated *Artemia* species. This finding was supported by the calculated ISSR band frequencies. All the selected primers were recommended for exploring the speciation in the *Artemia* biological resources accept three primers, HB15 (because of the mean of BF equal1), PT1 and HB8 (due to smearing in banding pattern).

The detected distance values between the two *A.salina* samples (MN and F) supporting that ISSR technique was useful for evolutionary investigation of populations belonging to the same species. The same conclusion could be revealed from the detected molecular variations between the two *A.parthenogenetica* samples (MX and SC).

For estimating the efficiency of ISSR markers the results were compared with the COI gene sequence variations among the three *Artemia* species. Detection of mitochondrial gene sequences (such as 16s rRNA and COI as a DNA barcoding system) were recommended for its successful application in estimating the genetic variations among different marine organisms (Pondella *et al.,* 2003, Ulises *et al.,* 2018; Saad *et al.,* 2019). The results showed similar evolutionary variations among *Artemia* species. Based on the consensus sequences, the distance value between *A. salina* and *A. parthenogenetica* was higher than the distance value between *A. salina* and *A. franciscana*.

The detected COI sequence polymorphism within and among the evaluated *Artemia* species were affected by the numbers of SNPs (Saad and Elsebaie, 2017). The analysis of SNPs revealed from the detected molecular markers would allow for

accurate *Artemia* species identification and exploring evolutionary variations. This observation was confirmed in other biological investigations in different biological taxa including different aquatic animal species (Saad and Elsebaie, 2017; Wenne, 2018).

On the other hand, the low level of haplotype diversity (hd=0.8) in the *A. franciscana* is due to low detected SNPs in the analyzed gene region.

The efficiency of ISSR technique in evaluating the evolutionary variations among and within different aquatic and terrestrial animal taxa was extensively confirmed (Saad *et al.*, 2013; Eimanifar *et al.*, 2015).

CONCLUSION

The calculation of (λ) as a statistical parameter was recommended for *Artemia* species discrimination based on morphological variations. The lowest (λ) value (means more the variable) was calculated for the abdominal length character. The ISSR has proven its ability to detect speciation in the *Artemia* resources. The benefits of using the ISSR system for evaluating the *Artemia* evolution and speciation are that the markers were easier to amplify and detected. The results will be helpful for the conservation of the evaluated *Artemia* species. More molecular markers such as COI, 16s rRNA and simple sequence repeats should be developed and combined with the morphological variations to understand the true evolutionary variations in *Artemia* biological resources.

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