Reconstruction of phylogenetic relations among some *Artemia* species

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Abstract: The application of DNA-based genetic analysis in brine shrimp (*Artemia)* research, stock development and management in Egypt is still not fully maximized. RAPD (Random amplified polymorphic DNA) was used to detect the general molecular polymorphism among some *Artemia* species collected from distantly different Egyptian locations (EMISAL at El-Fayoum, Netroun valley and EL Max Co., Alex.). The main objective of this study is to select the suitable method for reconstruction of phylogenetic relationships among the estimated *Artemia* species. RAPD markers were powerful tools to estimate the genetic diversity and detecting genetic polymorphism among the applied *Artemia* species. The number of RAPD-DNA markers (generated by ten RAPD primers) was 132 in all performed PCRs. Out of the 132 markers, 107 were polymorphic. Some of the tested RAPD primers generated species specific DNA markers. The similarity values between each estimated *Artemia* species pair was relatively low.

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1. Introduction

The brine shrimp *Artemia* are extremely euryhaline (able to adapt to a wide range of salinities), with standing salinities from 3 ppt to 300 ppt. *Artemia* survive temperatures ranging from 15 to 55 ^{o}C (Treece, 2000 and Kaiser *et al*., 2006). Therefore, studying the *Artemia* biodiversity has been a continuous since the second half of the previous century. The genus *Artemia* (Crustacea, Anostraca) are of interest to both biologists (studying their evolution and developmental biology) and aquaculturists. They used *Artemia* as live food in fish and shrimp larvae culture (Abatzopoulos *et al*., 2002 and Dhont and Sorgeloos, 2002).

Scientific knowledge about *Artemia* distribution, ecology, characterization and aquaculture applications in Egypt are not fully maximized. So, molecular characterization as an important step for good management of aquatic biological resources (Saad *et al*., 2013) should be conducted to estimate the biodiversity (based on genetic markers) among Egyptian *Artemia* species and probable *Artemia* sub species.

Generally, the genetic markers will provide the information needed for management of aquatic species such as Fish (Rashed *et al*., 2008, Rashed *et al*., 2009, Saad *et al*. 2011, Saad *et al*., 2012), shrimp (Saad *et al.*, 2013) and *Artemia* (El- Gamal 2010). The advantage of RAPD to generate molecular characterization is the production of molecular markers without any previous genomic information on the target species.

Analysis methods such as Dice and simple match coefficients are commonly employed in the analyses of similarity and/or dissimilarity values among individuals in the absence of knowledge of ancestry of all individuals of species and sub species such as in Tilapia species (Rashed *et al*, 2011).

The main objective of this study is to select the suitable analysis method for reconstruction of phylogenetic relationships among some Egyptian *Artemia* species. Determining true genetic dissimilarity between individuals is a decisive point for clustering and analyzing diversity within and among aquatic species such as *Artemia* species, because different dissimilarity indices may yield conflicting outcomes.

2.Material And Methods

Brine shrimp samples belong genus *Artemia* were collected from three distantly different Egyptian locations (EMISAL at El Fayoum, Netroun valley and EL Max Co., Alex.) for DNA extraction, purification and molecular analysis. *Artemia* samples were classified according to morphological characterization. These *Artemia* species were *Artemia salina* from Fayoum (F), *A. parthenogenetica* from EL Max Co., Alex. (X) and two *Artemia* species from Netroun valley (Wa & Wb).

Ten *Artemia* samples were applied from each collected species. DNA extraction and purification were carried out according to Hillis *et al*.,(1996) with

some modifications. Ten RAPD primers were tested to study the genetic diversity among the applied *Artemia* species.

RAPD-PCR reaction mixture was carried out as the following:

RAPD-PCR reaction mixture was carried out as described by (Rashed *et al*., 2008) with some modifications. The reaction conditions involved initial denaturation of DNA for 4 minutes at 94 °C, 35 cycles of 45 sec (denaturation) at 94° C, 45 sec (annealing) at 36° C, 1min. (extension) at 72° C, and one 10 min cycle at 72° C for final extension.

The amplification products were separated on 1.5 % agarose gels according to Rashed *et al*. (2008) with some modifications. The molecular sizes of each generated RAPD band was detected. The RAPD Primers used in the study and their sequences were A2 (3'- TGC CGA GCT G-5'), A3 (3'- AGT CAG CCA C-5'), B3 (3'-CAT CCC CCT G-5'), C2(3'-GTG AGG CGT C-5'), D1 (3'-ACC GCG AAG G-5'), D2 (3'-GGA CCC AAC C-5'), D3 (3'-GTC GCC GTC A-5'), E1 (3'-CCC AAG GTC C-5'), E2 (3'-GGT GCG GGA A-5') and E3 (3'-CCA GAT GCA C-5').

Data analysis:

Data were analyzed as described by Rashed *et al*., (2011). SPSS (10, and 15) software were used to estimate the similarity percentages among the applied *Artemia* species and reconstructing the phylogenetic relationships (using Sokal & SneathI, Dice and Simple match coefficients).

3. Results

In the present study, RAPD markers were used to discriminate genetic variations among the applied *Artemia* species. In this situation, 10 different random primers (A2, A3, B3, C2, D1, D2, D3, E1, E2 and E3) were tested. The molecular sizes of these bands and there relative fronts for all the 10 used primers were estimated.

The data obtained from all primers were combined together to calculate the similarity index and to reconstruct the phylogenetic relationships among all studied *Artemia* species.

Genetic polymorphism generated by the RAPD primers:

The number of RAPD-DNA markers (generated by all the ten used primers) was 132 in all performed PCRs. Out of the 132 amplicons, 107 were polymorphic. The frequencies of RAPD bands were calculated and presented in (Table 1). The number of detected bands, specific RAPD markers and range of separated bands were varied among applied *Artemia* species.

Table (1): Total number of detected bands, polymorphic bands, monomorphic bands and range of band frequency based on relative front of bands

P.C.	TB	PB	MB	RBF	P.C.	TB	PB	MB	RBF
A2	8	6	2	0.25-1	D2	16	12	4	0.25-1
A3	15	15	0	0.25-0.75	D3	8	2	6	0.5-1
B3	18	18	0	0.25-0.75	E1	15	14	1	0.25-1
C2	18	11	7	0.25-1	E2	11	8	3	0.25-1
D1	9	9	0	0.25-0.75	E3	14	12	2	0.25-1
P.C.=Primer code, TB=Total number bands, PB=polymorphic bands, MB=monomorphic bands and RBF =range of band frequency.									

Analysis of RAPD markers for the applied *Artemia* species:

The numbers of detected bands were 87, 74, 85 and 64 in F, Wa, Wb and X respectively. The ranges of separated RAPD bands were presented in (Table 2). Some of the tested RAPD primers generated species specific DNA markers.

Reconstruction of phylogenetic relationships among applied *Artemia* species:

To assess the genetic similarity, dissimilarity (Table3) and Phylogenetic relationships among the applied *Artemia* species (Figure 1a, b, and c), three most frequently similarity equations were used(Dice, Simple matching and Sokal & Sneath). As presented in Table (3) the similarity value between each estimated *Artemia* species pair was relatively low and reflect high genetic distance values among these *Artemia* species. The lowest similarity values were detected between F and Wb *Artemia* species. These values were 0.523, 0.379 and 0.549 using Dice, Simple matching and Sokal & Sneath coefficients. The similarity values were high between (F & Wa) and (Wb & X) *Artemia* samples relatively using the three used similarity coefficients. The lowest distance values were calculated between (X & Wb) as presented in Table (3).

The phylogenetic trees were reconstructed based on RAPD data (Figure 1a, b and c).

Table (2): Total number of bands, number of specific bands and range of MW (Kb) for detected bands generated by 10 RAPD primers in each studied *Artemia* species.

Primer	*Artemia*	F	Wa	Wb	X
	TNB	3	4	8	2
A2	SB	0	0	4	0
	RMW(Kb)	0.097-0.45	0.07-0.45	0.07-0.96	0.09-0.29
	TNB	9	9	8	2
A3	SB	4	0	0	1
	RMW(Kb)	0.039-1.13	0.065-1.13	0.065-1.13	0.099-0.175
	TNB	9	5	13	10
B3	SB	3	0	1	0
	RMW(Kb)	0.098-1.24	0.198-0.495	0.108-1.24	0.177-1.24
	TNB	15	10	10	10
C2	SB	5	0	2	0
	RMW(Kb)	0.079-1.61	0.146-1.61	0.146-1.51	0.146-1.61
	TNB	4	6	3	4
D1	SB	2	0	0	2
	RMW(Kb)	0.188-0.722	0.124-0.722	0.124-0.257	0. 09-0.257
	TNB	14	9	12	10
D2	SB	3	0	0	0
	RMW(Kb)	0.88-1.475	0.188-1.475	0.187-1.475	0.188-0.991
	TNB	7	7	7	7
D3	SB	0	0	0	1
	RMW(Kb)	0.109-1.699	0.109-1.699	0.109-1.699	0.109-1.699
	TNB	8	10	8	10
E1	SB	1	1	1	0
	RMW(Kb)	0.118-1.22	1.32-0.131	0.076-0.817	0.188-1.226
	TNB	10	8	6	6
E2	SB	1	0	1	0
	RMW(Kb)	0.078-0.931	0.078-0.931	0.078-1.192	0.078-0.821
	TNB	8	6	10	4
E3	SB	2	0	4	1
	RMW(Kb)	0.049-0.601	0.069-0.601	0.069-0.866	0.221-0.756

RMW=Range of detected molecular weight, S.B= species specific RAPD bands, TNB=Total number of detected bands, *Artemia salina* from Fayoum =F, *A.* ***parthenogenetica*** from Alex. =X and two *Artemia* species from Netroun valley(Wa & *A*.sp.=Wb).

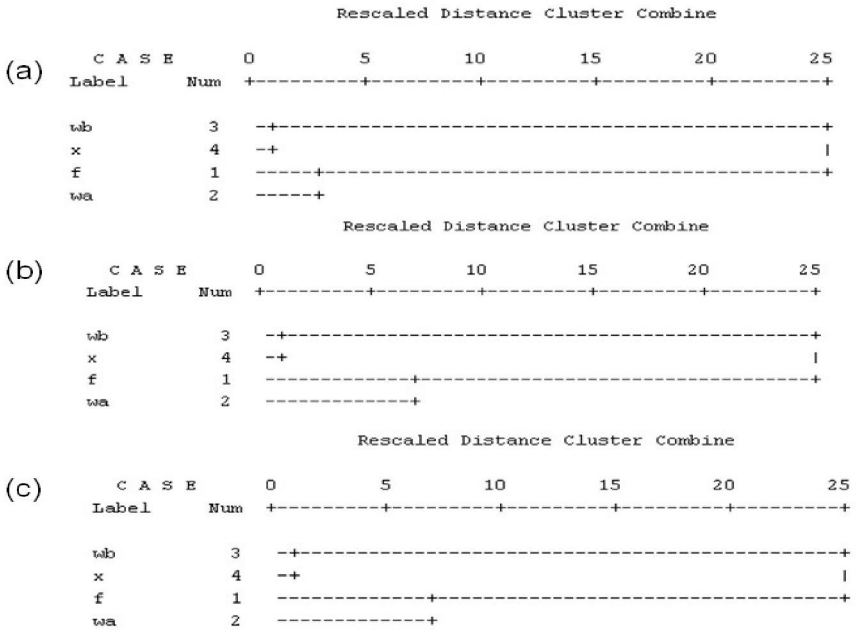

Figure (1): Reconstruction of Phylogenetic relationships among applied *Artemia* species (*Artemia salina* from Fayoum =F, *A. parthenogenetica* from EL Max Co. Alex. =X and two *Artemia* species from Netroun valley, (Wa & Wb) based on RAPD polymorphism using the three coefficients (Dice=a, Simple matching=b and Sokal & Sneath=c).

Table (3): Similarity and Dissimilarity values among the applied *Artemia* species based on RAPD markers via three similarity coefficients (Dice, Simple matching and Sokal & Sneath I).

	Dice		Simple matching		Sokal & Sneath I	
	Si	Di	Si	Di	Si	Di
F&Wa	0.683	0.317	0.614	0.386	0.761	0.239
F&Wb	0.523	0.477	0.379	0.621	0.549	0.451
F&X	0.553	0.447	0.485	0.515	0.653	0.347
Wa&Wb	0.667	0.333	0.598	0.402	0.749	0.251
Wa&X	0.619	0.381	0.598	0.402	0.749	0.251
Wb&X	0.693	0.307	0.652	0.348	0.789	0.211
F=Fayoum, Wa=Netroun valley(a), Wb=Netroun valley(b), X=EL Max, Si=similarity and Di= Dissimilarity						

4. Discussion

In the present study, *Artemia* species *(Artemia salina* from Fayoum =F, *A. **parthenogenetica*** from EL Max Co. =X and two *Artemia* species from Netroun valley or Wa & Wb) were characterized and used as models to select the suitable method for reconstruction the phylogenetic relations among them.

Up to date, knowledge about *Artemia* biodiversity and characterization around the world (especially in Egypt) are not fully maximized. So, molecular characterization using simple and suitable molecular techniques such as RAPD is an important step for good management of these aquatic biological resources. RAPD was proved to be a discriminatory and suitable method to identify the animal species like buffalo, cow, pig, goat, chicken, frogs, snakes (Rastogi *et al*., 2007), brine shrimp (El- Gamal 2010) and fishes (Saad *et al.,* 2012). So, RAPD (as a simple molecular technique) was used to generate DNA markers for characterization of the applied *Artemia* species.

Generally, the DNA variations generated by RAPD primers (Rashed *et al.,* 2011) can be detected using two viewpoints (band present or band absent and changes in the intensity of fragments at the same size). In the present study, RAPD polymorphism were analyzed based on the band present or band absent to reconstruct the phylogenetic relations among the applied *Artemia* species.

RAPD enables arbitrary amplification of genomic sites, it can generate unlimited number of markers which are inherited mainly as dominant markers (Bardakci and Skibinski, 1994 and Rashed *et al*., 2011).

In the present study, three equations (Dice equation, Simple matching and Sokal and Sneath1) were used for calculating the dissimilarity among the applied *Artemia* species.

As confirmed by Rashed *et al*., (2011), Dice equation uses only the shared present fragments so its equation estimates the similarity based on the observed fragments between any two estimated species and ignores any other fragments in all studied species. In addition, Dice ignores the shared absent fragments and gives double weight for the shared-present matched fragment between any two estimated individuals. On the other hand, Simple matching includes both shared present and absent fragments and gives equal weight for shared and un-shared fragments. Sokal and Sneath1 include both shared present and absent fragments and gives double weight to shared fragments.

Kosman and Leonard (2005) couldn't detect a universal methods to investigate the distance between individuals with DNA markers**.** They concluded that, Dice coefficient is a good measure of polymorphism with co-dominant markers and it can be applied directly to (0, 1) data representing banding profiles of individuals within a species.

In the present study, Dice coefficient is appropriate for diploids with RAPD as dominant markers. On the other hand, Kosman and Leonard (2005) found that, none of the common measures, Dice, and simple mismatch coefficient is appropriate for diploids with co-dominant markers.

In the present study, the values of similarity among the studied *Artemia* species were relatively high in Sokal and Sneath I and moderately in Simple matching and Dice (due to the use of shared present and absent fragments between each two *Artemia* species in case of Simple matching and Sokal and Sneath I equations). So, the estimated similarity between every two species was increased.

The values of Sokal & Sneath I dissimilarity are always differ from those of the Dice dissimilarity and the simple match coefficient. In addition, the values of the Dice dissimilarity may be differ (smaller or greater) than the values of the simple match coefficient. This is depending on whether the number of positions with shared bands is less or greater than the number of positions with shared the absence of bands (Rashed *et al*., 2011).

The data of the present study suggested that, RAPD analysis was suitable method to differentiate between the studied *Artemia* species. RAPD is an efficient tool in allowing multiple loci to be analyzed for each individual in a single gel run (Rashed *et al*,. 2011 and Saad *et al*., 2012).

The distance between the combined clusters of estimated *Artemia* species reflects that, the Egyptian ecology has a lot of *Artemia* species genetic resources. In addition, these resources needs for more biological

studies including molecular methods for good characterization and management.

RAPD was a suitable tool for characterizing the applied *Artemia* species. In addition, analysis of data using Dice coefficient was suitable measure of similarity and/or dissimilarity values among the studied *Artemia* species.

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