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Genetic Diversity of Freshwater Bivalves Using Inter Simple Sequence Repeat Markers

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ABSTRACT

Freshwater mussels are highly impacted by many human activities. As a result, it is considered one of the most threatened ecosystems worldwide. Studying genetic diversity is crucial for any further research on conservation efforts. In the present study, inter simple sequence repeat (ISSR) marker analysis was employed to study genetic diversity between 8 randomly selected specimens of bivalve. A total of 53 bands were produced from the 10 primers. Of these 37 bands were polymorphic, resulting in 69.8% polymorphism. Some primers showed more polymorphic bands than others. For example, primer ISSR M17 produced no polymorphic bands. While primer ISSR M1 produced 3 polymorphic bands (100% polymorphism). Cluster analysis, based on a similarity matrix, obtained showed that the 8 bivalve specimens can be clustered into two groups, one group containing specimens 2 and 6, and the other group representing specimens 4, 5, 1, 3, 7, and 8. These data may suggest that the bivalves in Abohomos, Behera Governorate, Egypt, may have more than one species. The implication of these data on the conservation of the bivalve is explained. The conclusion is that the bivalve population has enough genetic diversity. These populations are adapted to the environment they live in and to the climatic changes.

INTRODUCTION

Little is known about the biology of freshwater bivalves in Egypt, except the information provided on the anatomy of one species through which the ultrastructural of the digestive tubules of the freshwater mussel *Caelatura parreyssi* from the Nile Riverwas subjected to study (**Soliman, 2001; Awad, 2018**).

The freshwater bivalves are eaten in other parts of the world, such as Asian countries, where they are used as a supplemental protein source (**Ibrahim *et al.*, 1999**). Most mussels exhibit excellent ecological adaptability, and the diverse characteristics of their shell shapes create confusion in nomenclature and classification. Recently, molecular techniques have been employed to conduct phylogenetic analyses of 70 species within the Unionidae family, complementing their morphological and behavioral

properties **(Lopes-Lima *et al.*, 2017)**. Consequently, researchers phylogenetically divided the Unionidae into 6 subfamilies and 18 tribes, including the newly described tribes Chamberlainiini, Cristariini, and Lanceolariini. This study led to the proposal of a new classification system for Unionidae **(Lopes-Lima *et al.*, 2017)**.

The application of molecular technology in the study of freshwater mussels has revolutionized our understanding of their evolutionary relationships. The pioneering work of **Graf and Cummings (2006, 2007)** presented the first molecular phylogeny of freshwater mussels, reshaping our knowledge of their evolutionary history. Subsequent research by **Pfeiffer and Graf (2013, 2015)** further refined our understanding of the evolution and classification of freshwater mussels. Recent attention has focused on freshwater mussels, particularly in understanding species-level diversity and distribution, using advanced molecular approaches **(Zieritz *et al*., 2018)**.

In our study, we aimed to provide insights into the biodiversity of freshwater bivalves in Abohomos, Behera Governorate. A field survey conducted in May 2017 involved the identification of materials at least to the genus level based on shell morphology. To explore the genetic diversity among the eight specimens, we employed the ISSR technique. The genetic data generated were utilized to assess the variation among individuals and explore the possibility of these individuals forming a species flock.

MATERIALS AND METHODS

Bivalve collection

A total of 8 samples were randomly collected from a freshwater lake in Abohomos, Behera Governorate.

Inter simple sequence repeat (ISSR)

The current molecular biology work as well as data analysis were done at the Alexbiotechnology Molecular Biology Service Lab (Sidi Gaber, Alexandria, Egypt). The DNA was extracted from bivalve's soft tissue using a standard phenol-chloroform extraction (**Taggart *et al.*, 1992; Aranishi & Okimoto, 2006**). DNA Quantification and purity measurement was done using Nano Drop ND-200 Spectrophotometer (AOSHENG, China) and was used as well to quantify the DNA present in all samples. The quality or purity of the elution in terms of the presence of humic acids (indicated by the absorbance ratio at 260nm/ 230nm) and protein contaminants (indicated by the absorbance ratio at 260nm/ 280nm) was also assessed using the ND-200 Spectrophotometer. The primers used in this study were synthesized by Jena Bioscience Company (Germany). The sequence of these primers and the annealing temperature for each primer is presented in Table (1).

PCR amplification conditions

PCR Reactions were performed in 200µL micro-centrifuge tubes containing template DNA, primers, heat stable Taq DNA polymerase (FastGene, Taq ReadyMix PCR, Nippon Genetics, Germany) and PCR-grade water (Jena Bioscience). PCR reactions were performed using a thermocycler (TC-3000, Techne). The PCR reaction volume was 25ul containing 50ng of genomic DNA, 20mM Tris-HCl (pH 8.4), 50mM

KCl, 250µM each of dNTP, 3mM $MgCl_2$, 0.6µM primer, 1U of Taq polymerase. PCR-ISSR cycles conditions were as follows: 1 cycle of denaturation at 94^{o}C for 4min, 40 cycles of denaturation at 94^{o}C for 1min, annealing at 49 -50^{o}C for 1.30min, extension at 72^{o}C for 3min and a final extension at 72^{o}C for 5min a final hold at 20^{o}C, as mentioned by **Obeed *et al.* (2008)**.

The PCR products were analyzed by electrophoresis on 2% agarose-gel. The DNA size marker used was the 100 bp DNA Ladder (MWD100, Nippon Genetics, Germany). The PCR products were electrophoresed at 100V/ 30min using DNA gel electrophoresis (Mupid–One, JAPAN) containing 1× TBE buffer (0.045M Tris-borate, 0.001M EDTA) and 0.5µg/ ml ethidium bromide for 6.5h at 90V, and finally examined using gel documentation system (Nippon Genetics, Europe) **(Obeed *et al.*, 2008)**.

ISSR data analysis

Only the reproducible and consistent bands were recorded manually for further analysis. Amplified products were scored for band presence (1) or absence (0), and a binary qualitative data matrix was constructed. Fragments which could not be un-ambiguously recognized were not scored for analysis. Analysis of molecular variance (AMOVA) was performed to analyze genetic distance among samples using PAST program. A cluster dendrogram based on similarity matrix obtained with unweighted pair group method using arithmetic average (UPGMA) was constructed based on the Nei's genetic distances for determining the genetic relationship among populations **(Nei, 1978)**.

RESULTS

The results shown here are based on DNA profiles using ISSR marker analysis of 10 primers (Table 1). The band area and the number of monotypic and polymorphic bands produced by each ISSR primer are also shown in Table (1). A total of 53 bands were generated from the 10 primers, of which 37 bands were polymorphic. Some primers showed more polymorphic bands than others. For example, the primer ISSRM17 did not produce a polymorphic band. On the other hand, primer ISSR M1 produced three polymorphic bands (100% polymorphism).

The cluster analysis of the eight samples based on the UPGMA is shown in Fig. (1). The phylogenetic tree analysis revealed that the specimens 2 and 6 were grouped in one cluster, while the specimens 1, 5, 4, 8, 7, 8 and 3 were grouped in another cluster. On the other hand, both specimens 4 and 5 were closely related to each other. Similarly specimens (3, 7) and specimens (2, 6) were closely related specimens.

Table 1. ISSR primer sequence, number of monomorphic band, number of polymorphic band and annealing temperature for each primer

Primer	*Primer sequence*	*Number of bands*	*Polymorphic Band*	*Polymorphism %*	*Annealing temperature*
ISSR M1	***5- AGC AGC AGC AGC AGC AGC -3***	***3***	***3***	***100***	***50° C***
ISSR M2	***5- ACC ACC ACC ACC ACC ACC -3***	***0***	***0***	***0***	***50° C***
ISSR M3	***5- AGC AGC AGC AGC AGC AGC -3***	***6***	***3***	***50***	***50° C***
ISSR M8	***5- ACA CAC ACA CAC ACA CAC -3***	***9***	***7***	***78***	***50° C***
ISSR M9	***5- ACA CAC ACA CAC ACA CCG -3***	***0***	***1***	***100***	***49° C***
ISSR M12	***5- GAC ACG ACA CGA CAC GAC AC- 3***	***9***	***9***	***0***	***50° C***
ISSR M17	***5- CAG CAC ACA CAC ACA CAC -3***	***5***	***5***	***0***	***50° C***
ISSR F2	***5- AGA GAG AGA GAG AGA GCG -3***	***6***	***2***	***33.3***	***50° C***
ISSR F4	***5- AGA GAG AGA GAG AGA GTG -3***	***8***	***2***	***25***	***50° C***
ISSR F9	***5- GAA GAA GAA GAA GAA -3***	***7***	***5***	***71***	***50° C***
Total		***53***	***37***	***69.8***	

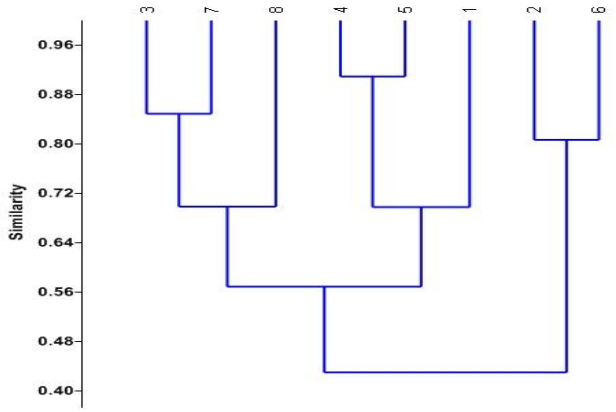

Fig. 1. The phylogenetic tree (similarity index) constructed on the basis of ISSR profile of the eight specimens

Table (2) shows the similarity and distance indicators for the 8 samples. It shows the highest similarity and distance index with a genetic distance of 0.90927 between samples (4, 5), and the lowest similarity and distance index with samples (2, 3) and samples (2, 7) at a distance of 0.11066. On the other hand, the groups of samples (2, 3) and samples (2, 7) had the same similarity index at 0.11066. Furthermore, the similarity index between samples (3, 1) and samples (3, 6) had the same similarity index of 0.47247.

Table 2. Similarity indices of the eight specimens of bivalves under study

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	*1*							
2	*0.51814*	*1*						
3	*0.47247*	*0.11066*	*1*					
4	*0.66243*	*0.30159*	*0.75984*	*1*				
5	*0.73352*	*0.33773*	*0.66139*	*0.90927*	*1*			
6	*0.64228*	*0.80672*	*0.47247*	*0.66243*	*0.57608*	*1*		
7	*0.33835*	*0.11066*	*0.84914*	*0.59755*	*0.4843*	*0.33835*	*1*	
8	*0.49048*	*0.48731*	*0.6988*	*0.62476*	*0.69305*	*0.60406*	*0.6988*	*1.00*

The freshwater environment is becoming increasingly polluted throughout the biosphere. The pollutants bring about damage to different organs in the organism. Naturally, there is protective mechanism of the body to resist and combat the toxic effect of the pollutant. Prolonged exposure to organisms produces cellular alteration in variety of body tissues.

Choosing an effective method to assess genetic variability in a group of individuals is of great interest to many researchers studying population genetics. In recent years, different molecular markers based on PCR amplification have been developed and have rapidly become essential tools in this field. Some of these markers are microsatellite-based, such as the inter-simple sequence repeat (ISSR) markers. ISSR markers are generated from nucleotide sequences located between two microsatellite priming sites inversely oriented on opposite DNA strands and near enough to be amplified by PCR. This technique relies on the high polymorphism and wide distribution of microsatellites to detect low differentiation levels. Prior knowledge of the sequence is not needed, and numerous polymorphic bands are generated. Like other dominant markers, such as RAPDs, the ISSR amplification products are scored as present or absent without distinguishing between heterozygous and dominant homozygous. On the other hand, ISSR markers have more stringent primer annealing conditions than RAPDs, which leads to a higher reproducibility. Those features, along with the ease and cost, have brought attention to these markers.

The Nile River is the longest river in the world, running through ten African countries; it is a source of life to millions of people. The Nile starts in Burundi and flows into the Mediterranean Sea in the north part of Egypt. With the progression of development in agriculture, industry, and urbanization, the associated side effects have increased. These come in the form of various pollutants, including agrochemicals, heavy

metals, and human waste products. In addition, pollution caused by inadequate drainage systems in rural villages, and irrigation wastewater filled with fertilizers and pesticides, flows into the river channel. In general, different analytical methods were constructed to monitor the water quality status in freshwater ecosystems .

The current results demonstrate the DNA profiles obtained through ISSR analysis using 10 primers (ISSR M1, ISSR M2, ISSR M3, ISSR M8, ISSR M9, ISSR M12, ISSR M17, ISSR F2, ISSR F4, and ISSR F9), as indicated in Table (1). The range of monomorphic and polymorphic bands produced by these 10 ISSR primers is presented in Table (3). These results indicate that there were 12 polymorphic bands produced by the ten primers. The primers ISSR M8, ISSR F2 , ISSR F4 and ISSR F9 recorded the highest number of polymorphic bands (2), the primers ISSR M1, ISSR M3, ISSR M9, ISSR M12 and ISSR M17 showed (1) polymorphic band, while the primer ISSR M2 showed no polymorphic bands. An example of the PCR product generated by ISSR primers, specifically ISSR M1, is illustrated in Fig. (2).

Table 2. Number of amplified and polymorphism bands in the studied bivalves produced by ten ISSR primers

Polymorphism %	Total bands	Polymorphic band	Monomorphic band	Primer
100	1	1	0	ISSR M1
0	0	0	0	ISSR M2
100	1	1	0	ISSR M3
50	2	1	1	ISSR M8
100	1	1	0	ISSR M9
100	1	1	0	ISSRM12
100	1	1	0	ISSR 17
100	2	2	0	ISSR F2
100	2	2	0	ISSR F4
100	2	2	0	ISSR F9
		12	1	Total

The similarity and distance indices of the eight genotypes are shown in Table (3). Data presented in Table (3) show that the cluster of genotypes (4) and (5) recorded the highest similarity and distance indices at a distance of 0.90927, while genotypes (2, 3) and genotypes(2, 7) recorded the lowest similarity and distance indices at a distance of 0.11066. On the other hand, the cluster of genotypes (2, 3) and genotypes (2, 7) had the same similarity and distance indices at a distance of 0.11066. Moreover, the cluster of genotypes (3, 1) and genotypes (3, 6) had the same similarity and distance indices at a distance of 0.47247.

Table 3. Similarity and distance indices bands in the eight genotypes of bivalves under test

8	7	6	5	4	3	2	1	0
0.49048	0.33835	0.64228	0.73352	0.66243	0.47247	0.51814	1	1
0.48731	0.11066	0.80672	0.33773	0.30159	0.11066	1	0.51814	2
0.6988	0.84914	0.47247	0.66139	0.75984	1	0.11066	0.47247	3
0.62476	0.59755	0.66243	0.90927	1	0.75984	0.30159	0.66243	4
0.69305	0.4843	0.57608	1	0.90927	0.66139	0.33773	0.73352	5
0.60406	0.33835	1	0.57608	0.66243	0.47247	0.80672	0.64228	6
0.6988	1	0.33835	0.4843	0.59755	0.84914	0.11066	0.33835	7
1	0.6988	0.60406	0.69305	0.62476	0.6988	0.48731	0.49048	8

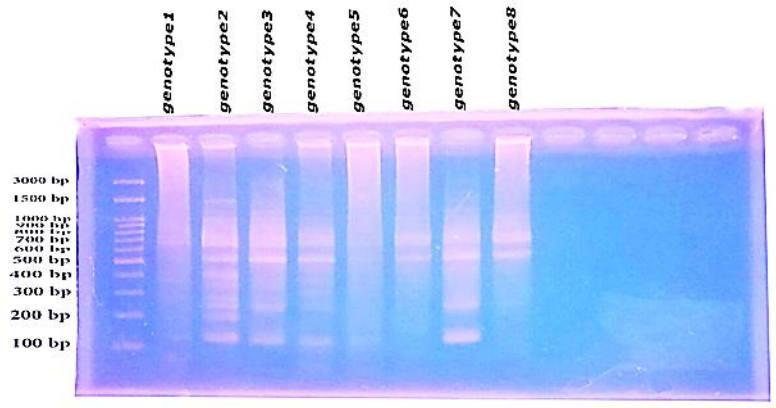

Fig. 2. An example of the PCR product of ISSR primers. The primers used in this ISSR M1

DISCUSSION

Since information regarding contaminated regions in tropical areas is limited, studies on pollution monitoring in freshwater lake environments have been conducted. These studies have utilized various indicator species, including water sediments as well as the shells of bivalves and gastropods. These indicators have been instrumental in describing the behavior of heavy metals and monitoring their contamination levels within these compartments **(Ayodele & Abubakar, 2000)**.

Intersimple repeat (ISSR) consists of semi-arbitrary markers amplified by the polymerase chain reaction (PCR) using primers complementary to the target microsatellite. ISSR does not require genomic sequence information. In addition, it produces polymorphic, highly polymorphic patterns and produces dominant markers. Furthermore, as a genotyping technique, ISSR-PCR is fast, inexpensive, based on region variation between microsatellites, and is technically simpler than many other marker systems. This method provides reproducible results and produces abundant polymorphisms in many systems **(Wolfe *et al.*, 1998; Abdel-Mawgood, 2012)**.

Genetic variation is an important tool for assessing the biological potential of an organism. Populations with high genetic variation can cope well with environmental changes, such as water fluctuations, temperature fluctuations, and epidemics **(Alam *et al.*, 2010)**. On the other hand, reducing genetic diversity may increase susceptibility to environmental changes and ultimately lead to species extinction **(Evans *et al.*, 2010**). This reduction in genetic diversity can affect growth and reproduction (**Dixon *et al.*, 2008)**. Therefore, maintaining a high level of genetic diversity is very important for species conservation **(Barroso *et al.*, 2008)**. Individuals need to possess the ability to adapt to environmental changes and thrive. Therefore, genetic monitoring is ideal for use in reproductive programs aimed at genetic conservation (storage). Molecular markers are a viable and useful tool for studying, while monitoring is ideal for use in a reproduction program with the aim of genetic conservation (stocking). Molecular markers are a viable and useful tool for studying and monitoring the genetic status of both natural populations **(Alam & Islam, 2005)**. In this study, the similarity between specimens 2 and 3 is 0.11, which means that the genetic distance between some specimens is high, and the genetic distance between these two specimens is 0.89. Similarly, the genetic distance between samples 6 and 7 is 0.67. This suggests that rivers may be inhabited by multiple species of shellfish and need to be categorized. However, certain individuals exhibit remarkable similarities and can be classified as one species. For instance, consider items 3 and 7, as well as items 4 and 5, found on different pages.

The first study of the Egyptian freshwater mussels involved two populations collected from the Nile in Giza Governorate, Egypt **(Mandahl-Barth, 1988)**. He classified them as a species belonging to the Unionidae family. However, others separated them as two different species **(Ibrahim *et al.*, 1999)**. In addition, a randomly amplified DNA polymerase polymorphism chain reaction marker (RAPD-PCR) was used to determine the genetic distance (D) between the two Unionidae species **(Sleem & Ali, 2009**). They concluded that the high genetic distance between the two specimens (0.64) suggests that they are two different species for a very similar group of individuals. In addition, copies 2 and 6 form the third branch. In general, the genetic diversity of freshwater mussels seems to be relatively high. This could be because they are made up of multiple species. For example, the RAPD technique was used on populations collected from three water bodies in India, and the genetic diversity value reached 0.99, indicating that there is high genetic diversity between the population and individuals of the same species **(Alam *et al.*, 2010)**. Several other studies support the discovery of the presence of high genetic diversity in freshwater mussels. Using 19 microsatellite markers and 64 specimens, one study considered the freshwater mussels (Unionidae) in the Yangtze River basin of China to

be one of the most diverse communities on earth **(Liu *et al.*, 2017)**. Another study concluded that global estimates of freshwater mussel diversity are 840 species in 161 genera. Regional diversity is as follows: Nearctica: 302 species, Neotropica: 172 species, Afrotropica: 85 species, Palearctica: 45 species, Indotropica: 219 species, and Australasia: 33 species. The largest family is Unionidae with 674 species **(Graf & Cummings, 2007)**.

Genetic diversity plays a crucial role in enabling species to withstand environmental changes. High genetic diversity within aquatic populations significantly enhances the potential for evolutionary adaptations to habitat alterations, pathogen infections, and other selective pressures **(Freeland *et al.*, 2011; MacDonald *et al.*, 2011; Liu & Yao, 2013)**. The results presented in this study indicate that the samples collected in Avohomos, Behera province, exhibit moderate genetic diversity. These samples possess slightly higher levels of genetic variation, suggesting a relatively greater capacity to adapt to their environment. However, it is important to note that other studies have indicated freshwater mussels as one of the most endangered animal groups globally **(Aldridge *et al.*, 2007; Vaughn, 2010)**. Similarly, it has been suggested that freshwater mussels are a vulnerable group and may face global decline (**Gallardo, 2018**). Later studies predicted a major distribution contraction of the endemic freshwater fauna and fragmentation of the remaining suitable habitat. This research has led to future expeditions to monitor the conservation status of freshwater biodiversity. A summary was conducted on the national conservation status of mussels in East and Southeast Asian countries (**Zieritz *et al.*, 2018**). The major threats to the demographics of these countries are pollution, deforestation, interaction with alien species, changes in land use, dam construction and mining, climate change, sediment accumulation, overfishing and alien species predation. It was revealed that the major threats to the population diversity in these countries were pollution, deforestation, interaction with nonnative species, land-use change, dam construction and mining, climate change, sediment accumulation, overharvesting, nonnative predators, hydrological alterations, and urbanization (**Zieritz *et al.*, 2018**).

CONCLUSION

In conclusion, the bivalve population have enough genetic diversity. These populations are adapted to the environment they live in and to the climatic changes.

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