Journal of Applied Biology & Biotechnology Vol. 7(06), pp. 8-14, Nov-Dec, 2019
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2019.70602

Genetics characterization of fresh water clam *Spathopsis rubens* using ISSR-PCR markers

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ARTICLE INFO

Article history:
Received on: June 03, 2019
Accepted on: August 19, 2019
Available online: November 12, 2019

Key words:
Morphometric analysis, genetic variation, bivalves, ISSR markers, *Spathopsis rubens*

ABSTRACT

Spathopsis rubens is freshwater bivalves distributed in the Nile River and its main canals all over Egypt. Four populations of *S. rubens* on Al-Mahmoudia irrigation canal at Damnhour, Egypt were collected and investigated for morphometric characters and genetic diversity by using inter simple sequence repeats (ISSR). The results declared that both the length and height measurements of the collected samples from the different locations showed slight significant differences; however, no significant differences between the total weight and the width of the samples have found. High degree of correlation between temperature and total weight, height and length was reported in samples collected from the third location. Genomic DNA from the selected samples of each population was extracted and amplified using 10 ISSR primers**.** The primers (M2, M3, M8, M12, M17, F2, F4, and F9) showed 100% polymorphism. Unweighted pair group method with arithmetic mean dendrogram characterized the samples of *S. rubens* into two main definite clusters. The cluster of genotypes 2 and 3 recorded the highest similarity and distance indices at a distance of 0.60859, while genotypes 1 and 3 recorded the lowest similarity and distance indices at a distance of 0.1716.

1. INTRODUCTION

Bivalves are widespread mollusks distributed in freshwater and marine ecosystems. These mollusks are sedentary animals attached to the substratum or firmly cemented in lakes and rivers. Freshwater bivalves are able to feed on microorganisms such as diatoms, bacteria, and phytoplankton. They are also considering as a food source for many aquatic animals such as herons, fishes, and other invertebrates [1]. Freshwater bivalves are important for ecosystem services such as nutrient cycling, water filtration, and reduction of water turbidity [2]. *Spathopsis rubens* was common in the Nile and its main canals from upper to lower Egypt and inhabit all types of water bodies, swiftly and slowly running or stagnant water [3]. Threats to freshwater bivalves include pollution and climate changes, but human disturbance is the main factor affecting freshwater bivalves. These factors have resulted in a decrease in freshwater bivalve's diversity and a change in their community structure [4–6].

The utility of simple sequence repeats (SSR) or microsatellites technique had been established for a wide range of applications, such as conservation biology, systematic and molecular ecology [7]. In research, the microsatellite DNA markers have been used to detect genetic variations and to estimate population structure in organisms [8]. Having such techniques to detect the differences in the levels of genetic variety among populations of organisms, especially the aquatic ones could shed the light on the evolutionary potential for dealing with effects of infection with pathogens and changes in habitat [9–12]. Applications of inter simple sequence repeats (ISSR) markers for population studies were reported for different species of mollusks including the amethyst gem clam *Gemma gemma* [13], the giant Mediterranean limpet *Patella furruginea* [14], the giant clam *Tridacna gigas* [15], *Helix pomatia* [16], and *Cepaea vindobonensis* [17]. The present work investigated the morphometric measurement and water quality of four different populations of freshwater clam *S. rubens* along Al-Mahmoudia irrigation canal at Damanhour, Egypt. In addition, the study was extended to address the genetic differentiation and phylogenetic relationship between the collected bivalves using ISSR markers.

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2. MATERIALS AND METHODS

2.1. Study areas and samples collection

Al-Mahmoudia canal is found close to the northern edge of the western delta in Beheira Governorate, Egypt. The total length of the canal is 77 km from the Rosetta branch of the Nile down to the Mediterranean Sea at Alexandria [18]. It has economic importance of the people in Beheira and Alexandria Governorates. It has been exploited to support fisheries, agriculture, industry, public water supply, hydroelectric power, and recreation. Al-Mahmoudia canal possesses three different sources of water; two of them are freshwater sources which come from Rosetta branch via El-ATF pump stations at the head of the canal and Al-Khandaq Al-Sharqi canal, and the third one is drainage water from Zarkon drain via Edku irrigation pump station [18]. Due to the different water resources that poured in Al-Mahmoudia canal, the pollutants lead to a significant deterioration of the quality of the water in the canal [19]. *Spathopsis rubens* were collected during July 2018 from four sites on Al-Mahmoudia irrigation canal (Fig. 1). These sites are Zaweyt Ghazal (first location), Ezbet Siknidah (second location), Ezbet Qabil (third location), and Manshat Hamour (fourth location). The specimens collected from the four locations were taken to the laboratory and dead or damaged specimens were eliminated. The morphometric characteristics of the collected samples were determined by measuring the shell length (L), width (W), and height (H) to the nearest millimeter (mm) with Vernier calipers. Total wet weights (g) were also determined. The soft tissues were separated and preserved at −80°C until used.

2.2. Water quality determination

Physicochemical parameters such as dissolved oxygen, pH, temperature, and turbidity were detected in collected water samples by a water quality meter (AQUAREAD, AP-800). The data were assessed using the one-way analysis of variance Significant differences were indicated when $p < 0.05$. Tukey pairwise comparisons were used to compare the difference between group mean values.

2.3. Multivariate analysis

Canonical correspondence analysis (CCA, PAST, software V3.2) [20,21] was used as correspondence analysis of a site/species matrix where each location has given values for each one of the water quality factors (pH, dissolved oxygen, turbidity, and temperature). The ordination axes are linear combinations of the environmental variables. The ordinations given as site scores environmental variables are plotted as correlations with site scores. Scaling 2 emphasizes relationships between morphometric parameters.

2.4. DNA extraction and purification

The DNA was extracted from bivalves according to Attaran-Fariman and Javid [22] with slight modification using a standard phenol-chloroform extraction [23].

2.5. DNA Quantification and purity measurement

A NanoDrop ND-200 Spectrophotometer (AOSHENG) used to quantify the DNA in all extracts. The purity of the elution (protein contaminants) showed by the absorbance ratio at 260 nm/280 nm and humic acids showed by the absorbance ratio at 260 nm/230 nm.

2.6. Inter-simple sequence repeat (ISSR)

The template quality of extracted genomic DNA was tested by ISSR by polymerase chain reaction (PCR). The sequence of the

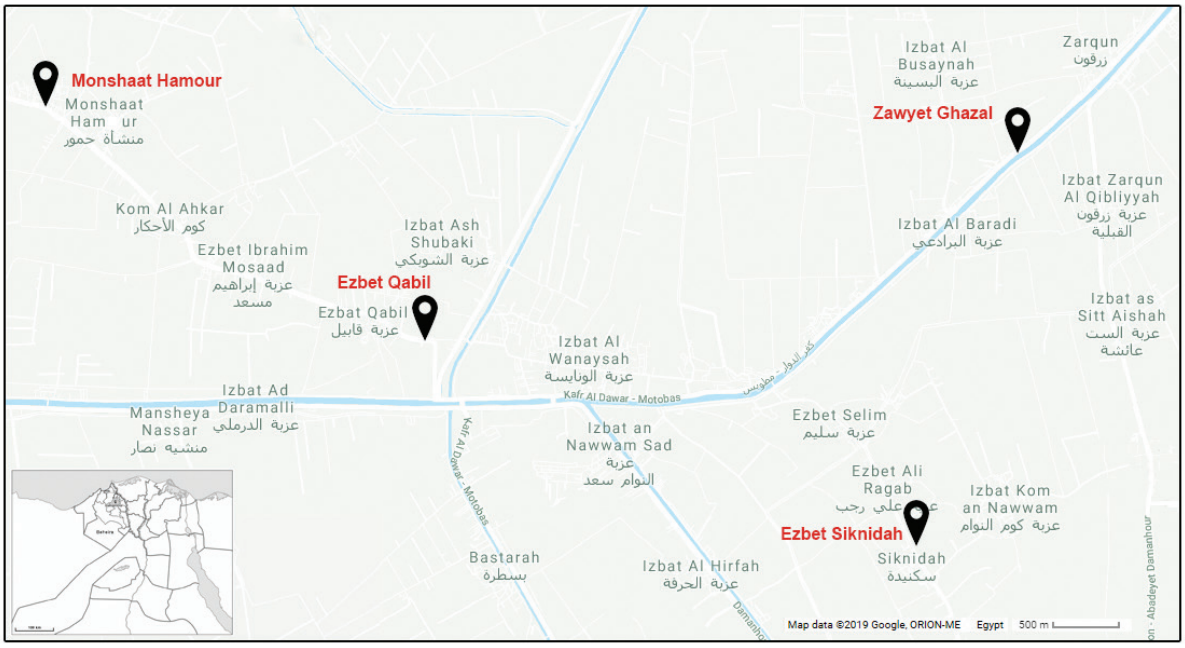

Figure 1: Map showing the collection sites (Zawyet Ghazal: location 1, Ezbet Siknidah: location 2, Ezbet Qabil: location 3, and Manshaat Hamour location 4).

primer used for ISSR that was synthesized by Jena Bioscience Company; Germany (Table 1).

2.7. PCR amplification conditions of ISSR primers

The reaction mixture for ISSR amplification assay had a total volume of 18 µl, containing 50 ng genomic DNA, 1 µl of single primer ISSR (Table 1), 9 µl *Taq* DNA polymerase (Taq polymerase Fast Gene, Ready Mix PCR Kit), and 7 µl PCR-grade water (Jena Bioscience). Each amplification reaction was performed using a single primer and repeated twice to verify band autosimilarity. PCR reactions were performed using a thermocycler (TC-3000) (TECHNE), programmed for 1 cycle of denaturation at 94°C for 4 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 49°C for 1.30 minutes, extension at 72°C for 3 minutes, and a final extension 1 cycle at 72°C for 5 minutes, a final hold at 20°C. The amplification products of each sample for each ISSR primer, along with 8 µl of a 100 bp DNA Ladder H3 RTU, were size fractionated on 2% agarose gel electrophoresis (Mupid–One, JAPAN) at 100 volts for 30 minutes. Gel documentation system was used for molecular data analysis (NIPPON GENETICS EUROPE). The sizes of DNA fragments of the amplified products were estimated by comparing the DNA bands with that of the DNA ladder

2.8. ISSR data analysis

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

Table 1: Characteristics of ISSR markers used for the analysis of *S. rubens* populations.

Primer code	Nucleotide sequence (5′-3′)
ISSR M1	5-AGC AGC AGC AGC AGC AGC-3
ISSR M2	5-ACC ACC ACC ACC ACC ACC-3
ISSR M3	5-AGC AGC AGC AGC AGC AGC-3
ISSR M8	5-ACA CAC ACA CAC ACA CAC-3
ISSR M9	5-ACA CAC ACA CAC ACA CCG-3
ISSR M12	5-GAC ACG ACA CGA CAC GAC AC-3
ISSR M17	5-CAG CAC ACA CAC ACA CAC-3
ISSR F2	5-AGA GAG AGA GAG AGA GCG-3
ISSR F4	5-AGA GAG AGA GAG AGA GTG-3
ISSR F9	5-GAA GAA GAA GAA GAA-3

3. RESULTS

The morphometric characters of *S. rubens* collected from Zaweyt Ghazal (first location), Ezbet Siknidah (second location), Ezbet Qabil (third location), and Manshat Hamour (fourth location) on Al-Mahmoudia canal are summarized in Table 2. The length and height of the collected samples from the different locations showed slight significant differences. The length of the samples ranged from 11.12 to 11.97 cm and the height ranged from 6.02 to 6.64 cm, while there are no statistical differences between the total weight and width of the samples.

The water quality parameters including temperature, dissolved oxygen, pH, and turbidity of the samples collected from the investigated locations are summarized in Table 3. The result showed that there was no variation in the water temperature among different locations, while the turbidity, dissolved oxygen, and pH of the water samples were changed according to locations. The water sample of the first location recorded the highest value of turbidity and dissolved oxygen and the lowest value of pH. Figures 2 and 3 showed that the high degree of similarity was reported between the second and fourth location and the samples collected from the first location were grouped in another cluster. High degree of correlation between temperature and total weight, as well as between height and length, was obtained in samples collected from the third location; however, there is no correlation between that parameters and pH, turbidity and dissolved oxygen. Statistical analysis also showed that the width of samples has a degree of positive correlation with pH and negative correlation with turbidity and dissolved oxygen.

ISSR analysis for 10 primers (ISSR M1, ISSR M2, ISSR M3, ISSR M8, ISSR M9, ISSR M12, ISSR M17, ISSR F2, ISSR F4, and ISSR F9) was used to detect the genetic diversity of *S. rubens* collected from four sites on Al-Mahmoudia irrigation canal. The range of bands number of monomorphic and polymorphic bands produced by different 10-ISSR primers is shown in Table 4. These results indicated that there were 49 polymorphic bands produced by the ten primers. The primers ISSR M1, ISSR M 3, ISSR M 17, and ISSR F2 showed 3-polymorphic bands, the primers ISSR M2, ISSR M8, and

Table 2: Length, height, width, and total weight of *S. rubens* collected from investigated locations.

Location	Length (cm)	Height (cm)	Width (cm)	Total weight (g)
Zawyet Ghazal (first location)	11.62 ± 0.62^{a,b}	6.31 ± 0.30^{a,b}	3.33 ± 0.34	124.30 ± 20.53
Ezbet Siknidah (second location)	11.16 ± 1.11^{b}	6.12 ± 0.57^{b}	3.56 ± 0.44	128.77 ± 43.11
Ezbet Qabil (third location)	11.97 ± 0.75^{a}	6.64 ± 0.55^{a}	3.53 ± 0.41	142.23 ± 31.23
Monshaat Hamour (fourth location)	11.12 ± 1.12^{b}	6.02 ± 0.59^{b}	3.56 ± 0.47	125.59 ± 37.50
F-value	2.85	4.08	1.12	1.00
p-value	0.046*	0.011*	0.349	0.400

*Significant at p-value ≤ 0.05.

Table 3: Environmental factors of water samples collected from investigated locations.

Location	Temperature °C	Dissolved oxygen (mg/l)	pH	Turbidity (NTU)
Zawyet Ghazal (first location)	32.67 ± 2.08	11.09 ± 1.55[a]	7.23 ± 0.05[c]	11.1 ± 1.40[a]
Ezbet Siknidah (second location)	33.00 ± 1.00	3.47 ± 1.15[c]	7.45 ± 0.03[b]	3.73 ± 1.33[b]
Ezbet Qabil (third location)	34.33 ± 1.15	7.83 ± 0.35[b]	7.52 ± 0.05[b]	8.93 ± 1.17[a]
Monshaat Hamour (fourth location)	32.00 ± 1.00	5.67 ± 1.05[b,c]	7.90 ± 0.10[a]	1.59 ± 0.57[b]
F-value	1.51	25.51	58.69	43.23
p-value	0.285	0.000***	0.000***	0.000***

***Significant at *p*-value ≤ 0.001.

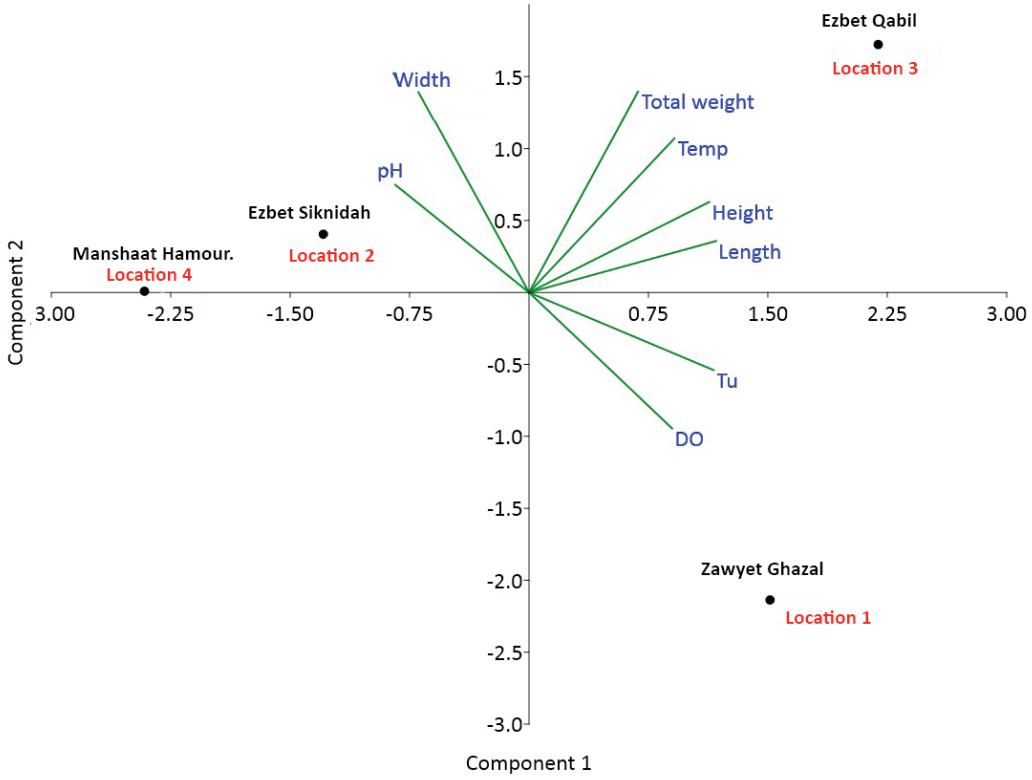

Figure 2: Biplots of the canonical correspondence analysis models for the water quality factors matrix: Locations, location (1) Zawyet Ghazal, location (2) Ezbet Siknidah, location (3) Ezbet Qabil, and location (4) Monshaat Hamour. Water quality factors are: pH, dissolved oxygen (DO), turbidity (Tu), and temperature (Temp).

ISSR F4 showed 5-polymorphic band, while the primer ISSR M9 and ISSR F9 showed 7-polymorphic bands. The primer ISSRM12 recorded the highest number of polymorphic bands (8).

Cluster dendrogram based on the similarity matrix obtained with UPGMA and the relationships between samples collected from four locations were shown in Figure 4. The phylogenetic tree analysis revealed that the samples collected from location 1 were grouped in one cluster while the samples collected from locations 2, 3, and 4 were grouped in another one. The second cluster showed that samples collected from locations 2 and 3 were closely related to each other, followed by samples collected from locations 3 and 4.

The similarity and distance indices bands in samples collected from four locations were shown in Table 5. This table shows that cluster of samples collected from locations 2 and 3 recorded the highest similarity and distance indices at a distance of 0.60859, while samples collected from locations 1 and 3 recorded the lowest similarity and distance indices at a distance of 0.1716.

4. DISCUSSION

The current study was conducted to investigate the morphometric and genetic variations of different populations of freshwater clam *S. rubens* along Al-Mahmoudia irrigation canal at Damnhour, Egypt. Environmental factors, such as temperature, dissolved oxygen, and water turbidity, significantly affect the distribution of benthic community structure [24,25]. The obtained data showed that turbidity and dissolved oxygen of water samples collected from the first location were higher than the values of water samples collected from the other locations and the samples of *S. rubens* collected from the first location were grouped in one cluster while

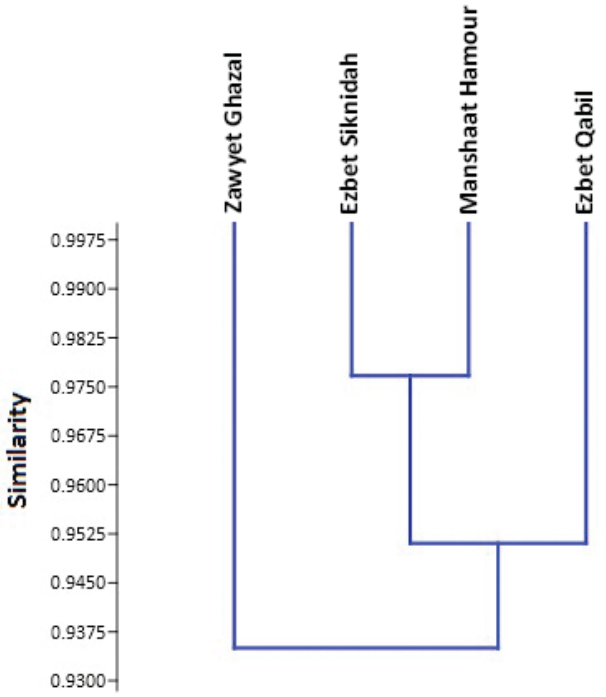

Figure 3: Dendrograms of cluster analysis based on the degree of similarity relationship between samples of *S. rubens* collected from different sites under investigation (location (1) Zawyet Ghazal, location (2) Ezbet Siknidah, location (3) Ezbet Qabil, and location (4) Monshaat Hamour.

Table 4: Number of amplified bands (monomorphic, polymorphic) and percentage of polymorphism) in *S. rubens* produced by each primer.

Primer	Monomorphic bands	Polymorphic bands	Total number of bands	Polymorphism %
ISSR M1	1	3	4	75
ISSR M2	0	5	5	100
ISSR M3	0	3	3	100
ISSR M8	0	5	5	100
ISSR M9	2	7	9	77.77
ISSR M12	0	8	8	100
ISSR M17	0	3	3	100
ISSR F2	0	3	3	100
ISSR F4	0	5	5	100
ISSR F9	0	7	7	100
Total	3	49		

the samples collected from the other locations were grouped in another one. Cao et al. [26] reported that a positive correlation was found between the dissolved oxygen and macrozoobenthos diversity and also indicated that high turbidity of water could provide rich food sources for the reproduction of bivalves.

The present study shows that a high degree of correlation between temperature and total weight, height and length, in samples collected from the third location. This agrees with the previous finding of Saucedo et al. [27] who showed that temperature is considered as a major environmental factor influencing in the

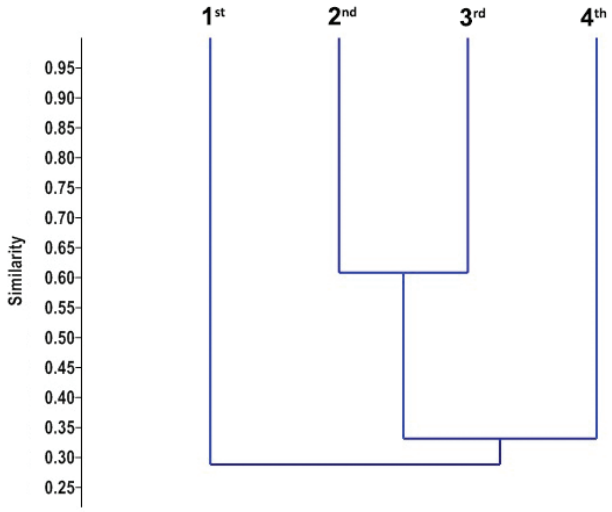

Figure 4: Dendrogram representing the genetic relationships of *S. rubens* from different sites under investigation using UPGMA cluster analysis of genetic similarity coefficients generated from studied 10 `ISSR primers.

Table 5: Similarity and distance indices bands in the 10 genotypes of *S. rubens* under test.

	Loc.1	Loc.2	Loc.3	Loc.4
Loc.1	1	0.41884	0.1716	0.27501
Loc.2	0.41884	1	0.60859	0.37522
Loc.3	0.1716	0.60859	1	0.28765
Loc.4	0.27501	0.37522	0.28765	1

physiological process of oyster and suggested that high temperature within tolerance limits associated with reducing feeding and poor growth.

Genetic variation is a proper approach used for determining the biological potential of organisms. It has shown that organisms with high levels of genetic variation were able to adapt better with changes in its surroundings environments [28]. In contrast, reduction of genetic variation could cause more sensitivity to environmental changes which in turn led to the extinction of a species [17]. Moreover, it may affect growth and reproduction [29]. Hence, the genetic variability maintenance is crucial for the conservation of a species [30]. ISSRs have become useful dominant markers for genetic investigations due to their high polymorphism, reproducibility, technical simplicity, and cost-effectiveness [31].

In the present study, the genetic structure by using ISSR analysis of *S. rubens* collected from four sites situated on Al-Mahmoudia irrigation canal, Egypt, showed that there were 49 polymorphic bands produced by the primers used in this study. The phylogenetic tree analysis revealed that the genotypes of samples collected from location 1 were grouped in one cluster while the genotypes of samples collected from locations 2, 3, and 4 grouped in another cluster. Along the coast of China, Hou *et. al.* [32] investigated seven different populations of *Mactra veneriformes* (210 individual) by ISSR fingerprinting [32]. Furthermore, Varela

et al. [33] indicated that this technique could be useful to define parentage and relatedness in mussels.

A few investigations of bivalves inhabiting similar environments, such as the surf clams *Donax serra* and *D. deltoides* [34,35], the Arctic surf clam *Mactromeris polynyma* [36], and the soft-shell clam *Mya arenaria* [37], have revealed limited genetic differences among populations separated by thousands of kilometers. Other studies revealed some differences among populations but associated with a physical barrier (*Coelomactra antiquate*) [38], a biogeographic break (*Merceneria merceneria*), or isolation by distance (*Mactra veneriformis*) [39].

It was concluded that the correlation between environmental factors and morphometric analysis and the genetic variation observed in the ISSR markers will be useful for future studies on the population structure of *S. rubens* and for aquaculture of this species.

CONFLICT OF INTEREST

All authors declare that there was no conflict of interest.

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How to cite this article:
Elkhodary GM, Ghoneim AZ. Genetics characterization of fresh water clam *Spathopsis rubens* using ISSR-PCR markers. J Appl Biol Biotech 2019;7(06):8–14. DOI: 10.7324/JABB.2019.70602