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Assessment of Genetic Relatedness of Clams (*Egeria radiata*) Using Inter Simple Sequence Repeat (ISSR) Markers

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AJH and EVI designed the study and wrote the protocol of the study. Authors UUE and EEE wrote the first draft of the manuscript, statistical analysis and managed the analyses of the study. Authors UUE and AH managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Forty-four (44) samples of clams were obtained from three locations between Cross River and Akwa Ibom States. Genomic DNA was extracted from tissues of the clams using cetyl trimethylammonium bromide (CTAB). Following DNA extraction from the tissues, ultraviolet radiation spectroscopy was used to get the concentration of the DNA isolated from the tissue. From the study, the mean gene diversity obtained was 0.8950 while polymorphic information content was 0.8860. A dendogram of the 44 samples using Weighted Neighbour – Joining (WNJ) procedure clustered them into four major groups. Group I had 78% bootstrap value, group II had 85% bootstrap value, group III had 74% bootstrap value, while group IV had 80% bootstrap. The results of genetic study of the three populations of *Egeria radiata* showed that they were diverse. Similarly,

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the principal component analysis (PCA) indicated that the four(4) clusters had samples across all the populations of clams studied, showing that there was an active migration of the clams among the three populations leading to a high genetic diversity of the clams. The implication of the results taking together showed that further genotyping should be carried out using other DNA markers that could add further understanding to the genetic diversity of clams.

Keywords: Egeria radiata; genetic diversity; ISSR; population.

1. INTRODUCTION

Aquaculture is the fastest growing food-producing sector [1] in the world. With the continuous increase in the human population, the demand for food has been a significant concern especially in Africa and other developing world. In Nigeria, the human population is estimated to be over 180 million [2]. This has placed the country in a pitiable state of food insecurity and hunger. According to FAOUN [3], malnutrition contributes 54% of mortality in children younger than five years old. A higher percentage of this mortality may be attributed to protein malnutrition, and this is more frequent in Africa. The contribution of aquaculture in ensuring food security and economic development in Nigeria is yet to be fully explored and exploited. Aquatic foods such as clams (*Egeria radiata*) are an important source of animal protein that could be harnessed and incorporated into aquaculture schemes and exploited in the food security agenda of the federal government.

Fresh water clams (*Egeria radiata*) are among the organisms found along the coastal lagoons and mangrove swamps of West Africa and inhabit the bottom of some rivers ranging from the Gulf of Guinea to Congo in sub-regions [4,5]. They are also dominant in Volta River in Ghana, riverine areas of Nigeria including Cross River and Akwa Ibom States and Sanga River in Cameroon where they serve as an affordable source of animal protein as well as providing employment opportunities to several communities therein [6,7]. *E. radiata* is highly recommended in dietary regimes as a result of high protein content, low calorie, low fat and cholesterol profile, low amount of saturated fat, a significant amount of omega-3-fatty acid, vitamins A and B12 and important minerals needed for healthy human growth [8,9]. The medicinal value of the species was earlier remarked [10]. The shells are used as a source of calcium in poultry feed formulation [11].

Despite the myriad of the benefits inherent in *E. radiata,* their potentials are yet to fully exploited. This is because of the general lack of research interest that can open up a deeper understanding of the genetic nature of this important species. To overcome this drawback, genetic diversity study of *E. radiata* becomes sacrosanct. Genetic diversity quantifies the magnitude of genetic variability within a population [12] and provides information required for species conservation [13]. Genetic variability studies which assess the variation and relationships existing within and between populations have recently become an integral part in agricultural programmes as a tool for selection of breeding stock and characterization of species for possible conservation [14]. It is valuable for management of species, selection in breeding programmes and estimation of the genetic contribution to stocks [15,16,17].

Several methods of assessing genetic relatedness of species exist. The morphometric methods utilise information gained from the phenotypic expressions to assess genetic diversity. The morphometric method is however very subjective to environmental influences [18,19,20]. Protein profiling has also been used to assess the genetic diversity of species [21,22], although this method serves as a preliminary investigative tool. Interestingly, molecular markers have been more reliable in the assessment of genetic variation among species and populations since they utilise information directly from the DNA. Molecular markers together with statistical tools have revolutionized population studies adapted towards exploring the genetic diversity within and between populations [17]. Among the various molecular markers, ISSR has good reproducibility, low technical constraint and affordability which are advantageous in population studies [23,24]. With ISSR, the knowledge of the sequence information for genomic DNA is not required to be known [25]. ISSR marker techniques involve the use of single primer composing of microsatellite sequence together with polymerase chain reaction and amplification of genetic material. Previous authors have used ISSR markers to characterize animal genetic

resources including clams [26,27,28,29,30,31, 32]. The current research employed ISSR marker to assess the genetic relatedness of freshwater clams (*Egeria radiata*) from different populations.

2. MATERIALS AND METHODS

2.1 Study Location and Sample Collection

The research was conducted at the Biotechnology Laboratory, Ebonyi State University, Abakiliki, Nigeria. A total of 44 samples of *Egeria radiata,* were collected from three different locations; 15 from Calabar River (CR) in Cross River State, 15 from Mbak Itam (MI) and 14 from Itu Head Bridge (IT) in Akwa Ibom State (Plate 1). The samples were sustained in freshwater from the points of collection and transferred to the laboratory for analysis.

2.2 DNA Extraction

The shells were rinsed out of water and placed in a clean container. The valves were open to obtain the flesh (Plate 2) which were properly washed and placed in Eppendorf tubes. DNA was isolated from the tissue samples of all the clams using the CTAB method according to Rolfs et al. [33]. The tiny pieces of the clam samples in the Eppendorf tubes were removed and placed each in a different sterile

Sample from MI **Sample from IT** **Sample from CR**

Plate 1. Samples of *Egeria radiata* obtained from three locations for the study

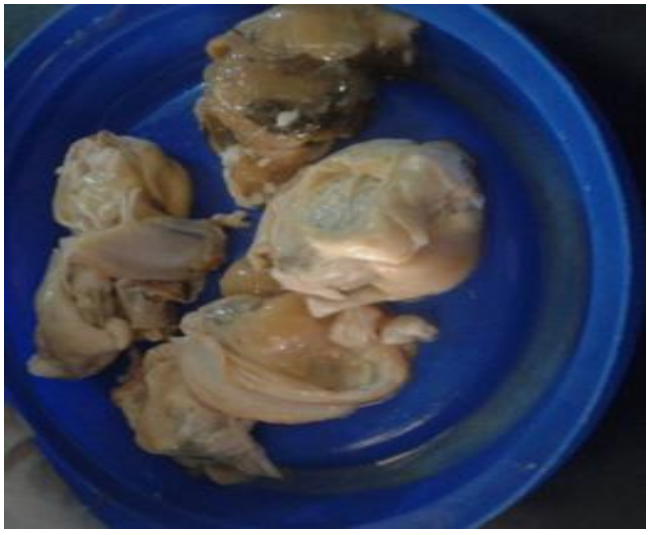

Plate 2. Flesh of *Egeria radiata* for DNA extraction

mortar and ground using liquid nitrogen in order to degrade the cell completely. 20 µl of proteinase K (10 mg/ml) was added to each tube. The tubes were briefly homogenized and incubated in water bath at 60°C for 10 minutes until the tissue slices were completely digested. Equal volume of phenol, chloroform and iso-amyl alcohol at the ratio of 25: 24: 1 was added at room temperature. The tubes were vortexed and centrifuged at 12000 rpm for 10 minutes, after which 450 µl of the supernatants were removed into new 1.5 ml sterile tubes. 400 µl of cold Isopropanol was added, mixed and incubated for 30 minutes to 1 hour – overnight at -20°C. The tubes were centrifuged at 12000 rpm for 10 minutes to sediment the DNA. The supernatants were gently decanted and the pellets were undisturbed. 500 µl of 70% ethanol was added to the pellets and then centrifuged at 12000 rpm for 5 minutes to wash the pellet. The ethanol was decanted and air-dried at room temperature. The pellets were suspended in 100µl to 200 µl TE buffer. RNase was added to the extracted mix to remove the RNAs in the nucleic acid, leaving only the genomic DNAs. DNA concentration was determined spectro-photometrically and isolated DNA was diluted to 30 – 50ng/µl in ddH_20 and stored at -20°C until required.

2.3 ISSR – Polymerase Chain Reaction (PCR) Amplification

A total of 13 primers (UBC and EB) were screened and five (5) primers which produced clearly reproducible bands were selected for ISSR analysis (Table 1). Polymerase chain reaction was performed according to the method of Islam et al. [34]. DNA amplification was carried out using Acculpower PCR premix bead (Bioneer Co., USA) containing 2.50 µl each of 10 x Buffer, 1.25 µl of $MgCl_2$, 2.0 µl dNTPs (2.5 mm), 1.0 of ISSR primer, 2.0 (100ng/µl) DNA taq and 16.05 µl of ultra pure water. The PCR cycling profile used for the reaction consisted of an initial step at 94°C for 2 minutes and 72°C for 1 minutes, 10 minutes final extension at 72°C maintained at 4°C as its soaking temperature. The PCR products were viewed under UV-light after being electro-phoresed on 1.5% of agarose gel stained with 10g/ml ethidium bromide.

2.4 Statistical Analysis

Data matrix of ISSR profile for fragments of similar molecular weight from each amplicon was severed as 1 (presence of alleles) and 0 (absence of alleles). The data obtained from the scoring of the ISSR amplicons were used for construction of phylogenetic tree using molecular Evolution Genetic Analysis (MEGA.6) [35]. The dissimilarity index was obtained using Jaccard's option [36]. The data were further subject to NTSYSpc software version 2.02. Molecular data generated were further subjected to power marker software version 3.25 [37] to review genetic parameters of the clams such as genetic diversity, allele frequency and polymorphic information content (PIC).

3. RESULTS

3.1 Genetic Diversity of Clams

The Inter Simple Sequence Repeat (ISSR) banding patterns of the 44 samples of clams are shown in Plates 3, 4 and 5.

3.2 Genetic Parameters of ISSR Primers Used

From Table 2, it was observed that the major allele frequency ranged from 0.1364 to 0.3182 with mean of 0.2083, while the gene diversity ranged from 0.8213 to 0.9556 with mean of 0.8950. The highest major allele frequency of 0.3182 was obtained with UBC 811 and UBC 901, respectively, while the least was recorded from EB891 (0.1364). The highest gene diversity was obtained from EB-891 (0.9556) with allele number of 33 followed by EB-827 (0.9184) with 21 allele number, while the least was from UBC-901 (0.8213) with 12 allele number. The mean polymorphic information content (PIC) was 0.8860 with the highest recorded from EB-891 (0.9129).

3.3 Principal Component Analysis (PCA)

The principal component analysis based on data generated from ISSR marker clustered the samples into 4 different coordinates labeled as cluster I-IV (Fig. 1). Cluster I consisted of more samples from Itu population (IT-3, IT-4, IT-7, IT-8, and IT-9), Calabar River population (CR-3, CR-4, CR-9 and CR-12) with only MI-13 from Mbak Itam population. Cluster II had more samples from Mbak Itam population (MI-4, MI-5, MI-7, MI-10, MI-11 and CR-5), only CR-6 from Calabar River population and IT-2 and IT-6 from Itu population. Cluster III had more samples from Itu population (IT-1, IT-10, IT-11, IT-12, IT-13 and IT-14) with MI-6, MI-12 and MI-14 from Mbak Itam population and CR-7, CR-13 and CR-14 from Calabar River population. Cluster IV had more samples from Calabar River Population (CR-1, CR-2, CR-8, CR-10 and CR-15), MI-2,

MI-3 and MI-9 from Mbak Itam population with only IT-5 from Itu population.

3.4 Phylogenetic Analysis

The 44 samples of clams were clustered into four (4) major groups (Fig. 2). Group I consisted of clams from the three populations. Group II consisted of clams from Calabar River and Mbak Itam, respectively. Group III consisted of clams from Itu and Calabar River, while Group IV was made up of clams from Itu and Mbak Itam populations.

4. DISCUSSION

In the era where insecurity is a significant challenge especially in Nigeria, many people are displaced from their homes. Most of these people are gathered in camps and in such camps, the primary challenge is not just lack of food, but having the right proportion of food for consumption. Many protein sources have been sorted to meet the need of these unfortunate individuals as well as the general public and animal protein remains indispensable in this quest due to its high protein content and safety. Clams have been reported as a rich source of animal protein with essential elements that support healthy human growth [8,9]. The assessment of genetic diversity of clams in the quest for genetic improvement, conservation and contribution to food security cannot be overemphasized.

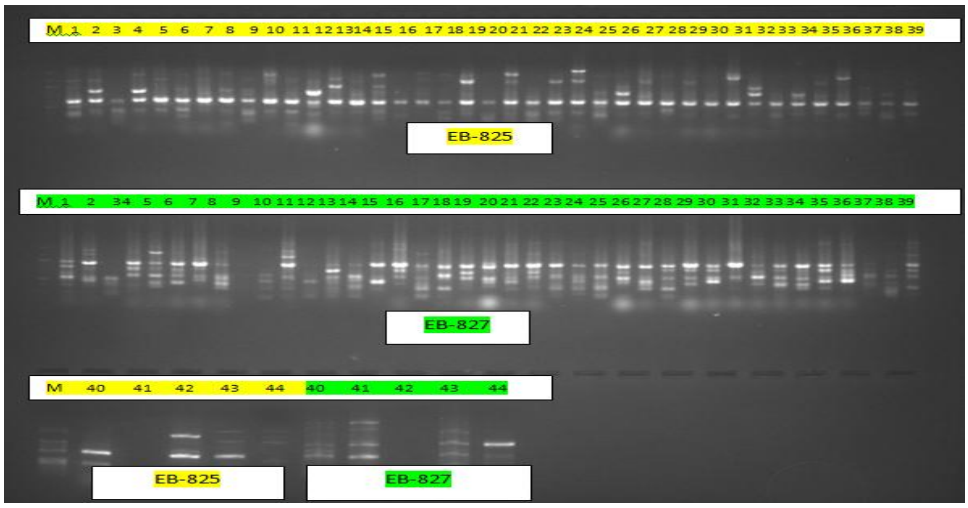

Plate 3. ISSR profile of *E. radiata* for primer EB – 825 and EB – 827

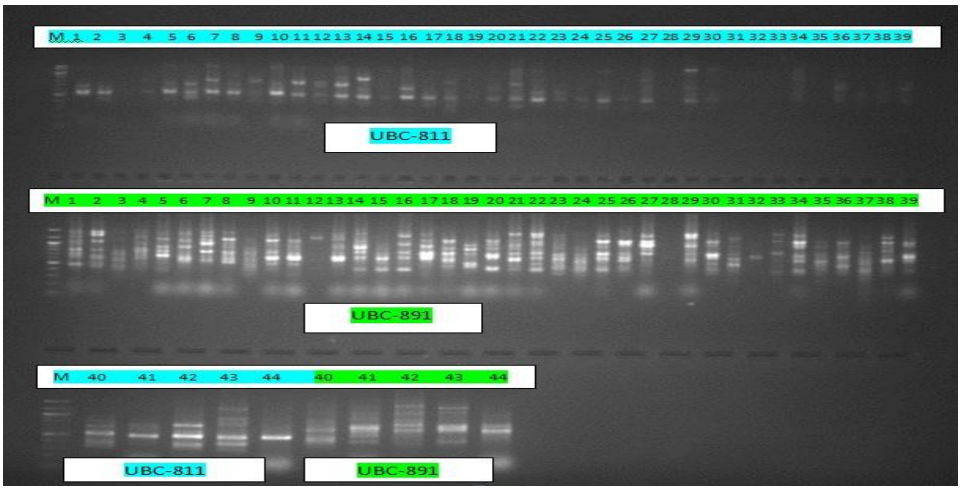

Plate 4. ISSR profile of *E. radiata* for primer UBC– 811 and UBC – 891

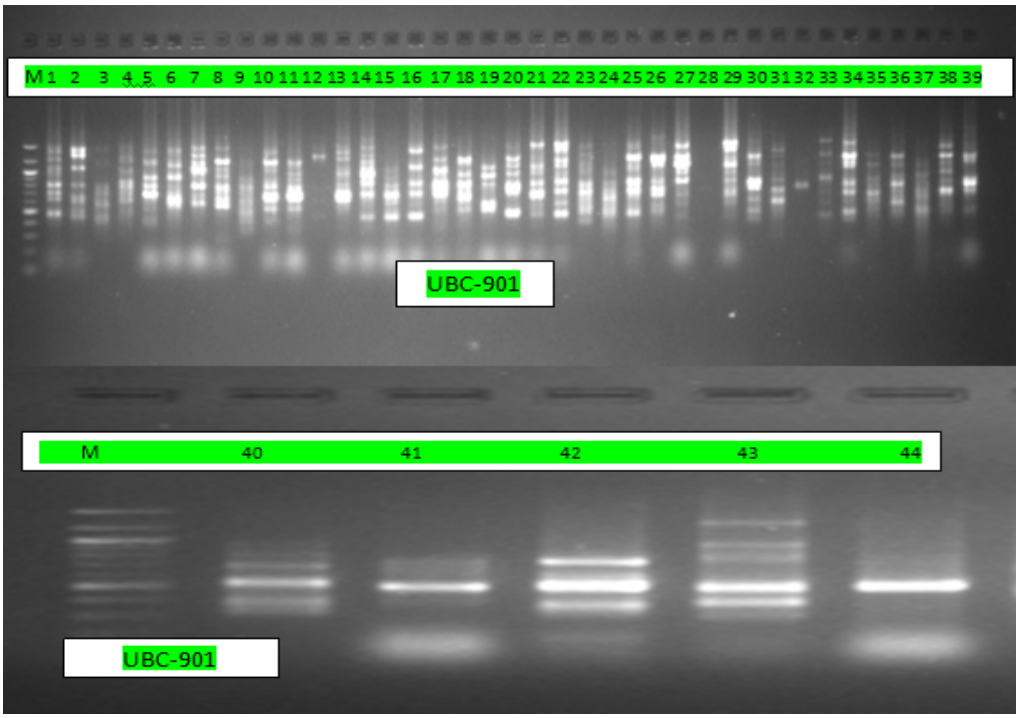

Plate 5. ISSR profile of *E. radiata* for primer UBC-901

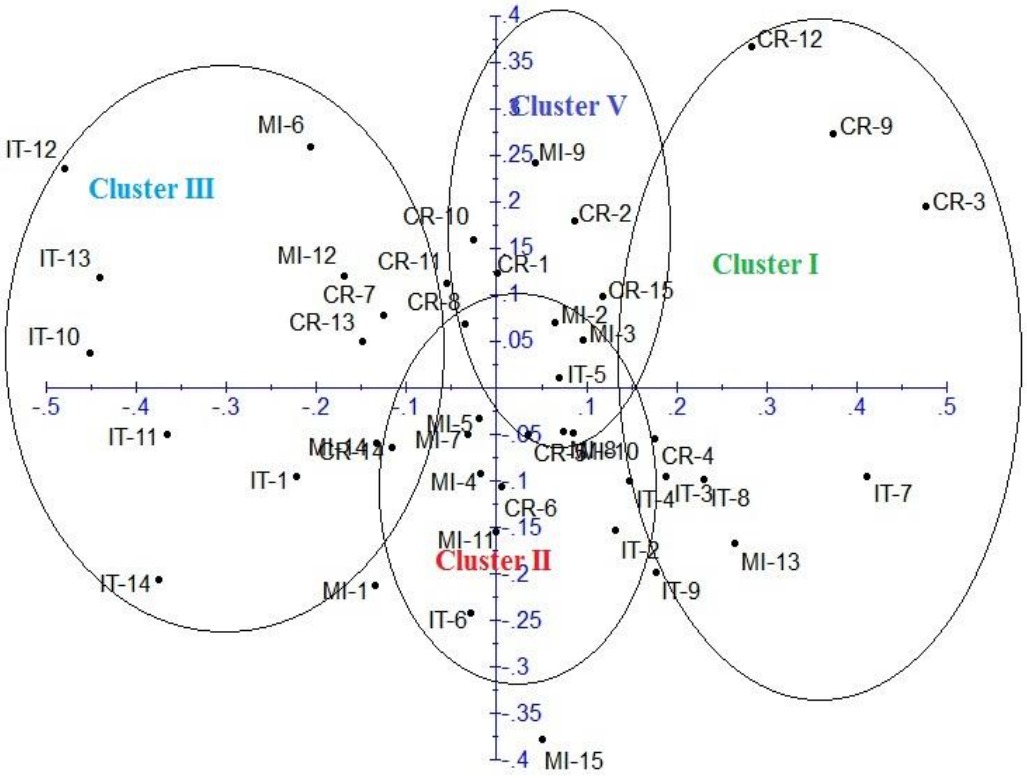

Fig. 1. Principal component analysis of *E. radiata* samples

Table 1. List of primer sequences used for the study

S/N	Primer name	Primer sequence
1	UBC-901	CACACACACACACACA
2	EB-825	ACACACACACACACACT
3	EB-827	ACACACACACACACACG
4	UBC-811	GAGAGAGAGAGAGAGAC
5	EB-891	ACG TGTGTGTGTGTGTG

Table 2. Genetic parameters and percentage polymorphism of the ISSR markers used

Marker	Major allele frequency	Number of observation	Allele number	Gene diversity	PIC
EB - 825	0.2955	44.0000	19.0000	0.8688	0.8597
EB - 827	0.1591	44.0000	21.0000	0.9184	0.9129
UBC - 811	0.3182	44.0000	14.0000	0.8285	0.8108
EB - 891	0.1364	44.0000	33.0000	0.9556	0.9540
UBC - 901	0.3182	44.0000	12.0000	0.8213	0.8018
Mean	0.2083	44.0000	23.0000	0.8950	0.8860

PIC=Polymorphic information content

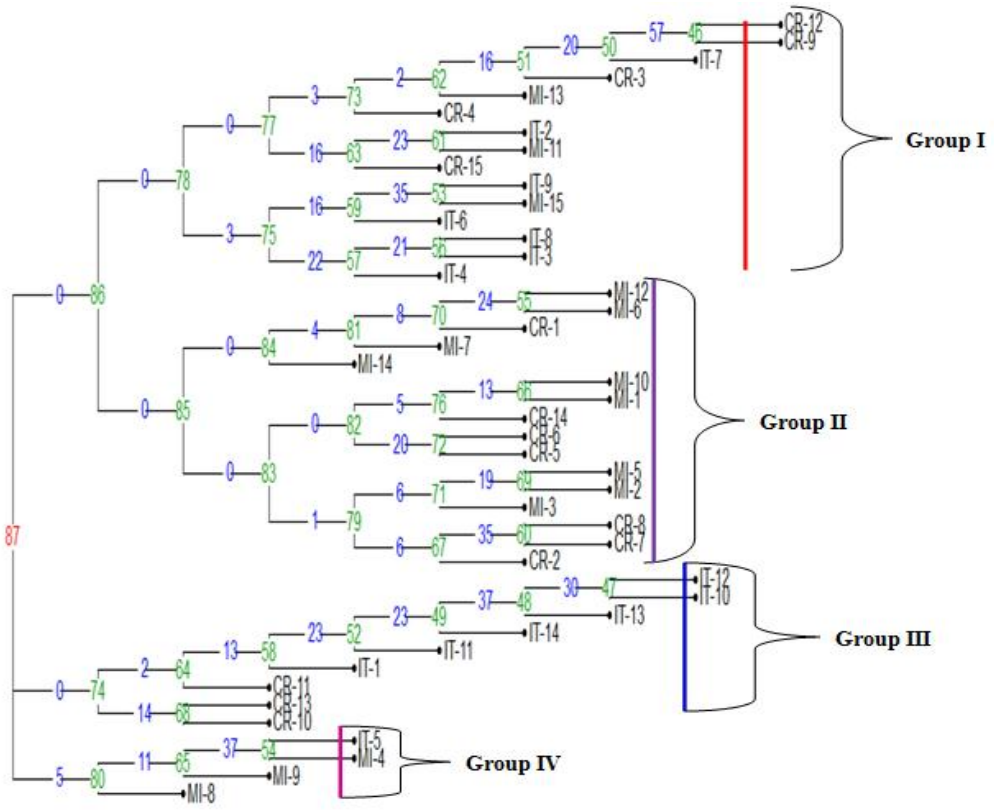

Fig. 2. Phylogenetic tree of the 44 samples of *E. radiata*

Results obtained on the genetic diversity of *E. radiata* used in this study revealed high genetic diversity from the ISSR markers used. Similar results were previously obtained by Nuryanto and Kochzius [38], Kochzius and Nuryanto [39], DeBoer and Barber [40], Grulois et al. [41], Mohamed et al. [42] in clams. With this, it is possible to utilise clams from the populations used in this study in selective breeding for possible genetic improvement of the species.

Polymorphic information content (PIC) is a major genetic parameter in assessing genetic diversity [43]. The mean PIC of 0.8860 obtained in this study is a further indication of the genetic variation that is inherent among the clam samples from the different populations. This was lower than the earlier report of Etukudo et al. [43], Sastry and Thomas [44] in *B. aeruginosa* and giant African land snails, respectively.

The results obtained from the genetic diversity parameters corroborates the phylogenetic tree constructed for the 44 samples of *E. radiata.* There were four clusters with the 44 samples spread across all the clusters. This could suggest that though there are genetic variations among the samples, they may share a common ancestral lineage irrespective of the population where they are found [45]. Apart from a common ancestral line, inbreeding activities within a population also increase the relatedness of species which may bring about similar clustering pattern in phylogenetic constructions. As was revealed in both the phylogenetic tree and principal component analysis (PCA), the 44 samples from the three populations interacted in the different groups/clusters suggesting active migration between the different populations. According to Sastry and Thomas [45], migration brings about the introduction of new genes into a population, thus increasing genetic diversity. This migration may be by natural processes such as tides and changes in water current which could lead to introduction of new genetic material to an existing gene pool with resultant genetic divergence as seen in the genetic diversity parameters of *E. radiata* assessed in this study.

5. CONCLUSION

This study revealed genetic relatedness among *E. radiata* as samples from the different populations were grouped in similar clusters. Also, the gene diversity as revealed by the ISSR markers used were high, suggesting heterogeneity within *E. radiata* samples used in this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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