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Microsatellite DNA Marker Analysis in Brown Mussels, *Perna perna* (Linnaeus, 1758) from the Coastal Waters of Oman

[1]S.M. Al-Barwani, [2]D. Aziz, [2]S.M.N. Amin and [2]A. Arshad
[1]Sultan Qaboos University, P.O. Box 50, Muscat 123, Sultanate of Oman
[2]Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

Corresponding Author: S.M. Al-Barwani, Sultan Qaboos University, P.O. Box 50, Muscat 123, Sultanate of Oman, Malaysia

ABSTRACT

Thirteen highly polymorphic microsatellite primer pairs developed for *Perna viridis* from the gene bank were tested on 3 populations of *Perna perna* samples with the aim to adapt a fast, reliable method for preliminary screening and to genetically characterize the wild populations of *P. perna* in the Oman waters. The samples were collected from three different locations viz Ras Al-Had, Ras Madrakah and Mirbat. All primers showed high level of polymorphism for all populations. The mean observed heterozygosity was lower than the expected heterozygosity across the three populations which means there was a probability of inbreeding occurred in the populations. Both the chi-square (χ^2) and likelihood (G^2) ratio tests detected significant differences ($p<0.05$), which showed deviations from Hardy-Weinberg equilibrium. Cluster analysis revealed a close genetic relationship of *P. perna* between all populations and they were clustered according to their geographical origins into two major groups which include one cluster that grouped the Mirbat and the Ras Madrakah populations together while the other cluster showed the Ras Al Had domain. The highest genetic distance (1.2666) was observed between the Mirbat and the Ras Al Had populations while the lowest genetic distance (0.4746) was recorded between the Mirbat and Ras Madrakah populations. This study demonstrated that microsatellite markers with thirteen *P. viridis* primer pairs tested can be applied to genetically characterize the brown mussel populations in Oman waters.

Key words: Genetic variability, genetic distance, brown mussels, population genetics, molecular markers, genetic differences

INTRODUCTION

Perna perna is a mussel species that is easily recognized by its brown colour and belongs to the family Mytilidae (Gosling, 2003). It is commonly known as the Brown mussel or the Mexilhao mussel and it is an economically important species which is harvested as a food source but is also known to harbour toxins and can cause damage to marine submerged structures. It is native to the waters of Africa, Europe and South America and has also been found along the Arabian Sea. *P. perna* has been found colonising jetties, navigation buoys, wrecks, petroleum platforms and other artificial hard substrata, as well as natural rocky shores in the Gulf of Mexico (Hicks and Jr. Tunnell, 1995). It could also be mistakenly identified as the greenish-brown species *P. viridis*, as their shell shape and colour is capable to change and adjust depending on environmental conditions.

Microsatellite is one of the most powerful genetic marker with the potential to provide researcher with new insights into the behaviour, ecology and genetic structure of a species (O'Connell and Wright, 1997). Microsatellite is commonly known as Simple Sequence Repeats (SSRs), Short Tandem Repeats (STRs) and also Variable Number Tandem Repeats (VNTRs). Microsatellite itself is a repetitive DNA consisting of numerous short base sequences randomly interspersed in eukaryotes genome. It is a tandemly repeated DNA sequence of 1-6 bases (Zane *et al.*, 2002) with high frequency found in the nuclear genomes of most taxa. Microsatellite DNA is a valuable marker for both assessing and monitoring genetic structure and genetic changes resulting from a restocking program due to high levels of polymorphism (Yang *et al.*, 2008). They are widely used in the study of genetic variations (Kumagai *et al.*, 2004; Watanabe *et al.*, 2004), species identifications (Du *et al.*, 2011) and population characterizations (Al-Atiyat *et al.*, 2012).

Although, research on DNA microsatellite as genetic markers for mussels has been extensive, more studies are needed to be done to develop a complete understanding and microsatellite marker of all *Perna* species in the world. This is the first work that had been carried out to gain the information on the genetic structure and diversity of natural populations of *P. perna* from the Oman waters. The objective of this study is to apply DNA microsatellite as genetic marker for population characterization of *P. perna*.

MATERIALS AND METHODS

Sampling sites: A total of 150 wild samples of the brown mussels, *P. perna* weighing from 100-200 g were collected from three different locations (Ras Al-Had, Ras Madrakah and Mirbat) in Oman (Fig. 1). The samples were preserved in absolute ethanol to maintain the freshness prior to sampling and were packed and sent to the Genetics laboratory of the Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia. The methodology consisted of four main steps which were the extraction of DNA, PCR amplification, electrophoresis of PCR products and data analysis. Thirty microsatellite primer pairs from Biobasic Canada, developed for the green mussels, *P. viridis* were used for the cross amplification in this study.

DNA extraction: Extraction of DNA of the prawns was carried out using the protocol of the Promega 7 Wizard Genomic DNA Purification Kit. The DNA was extracted from the muscle tissue (20 g wet weight). DNA quantification was run using a 0.8% agarose gel and the concentration of the DNA was determined.

PCR amplification and gel electrophoresis: Standard PCR amplification was performed in the Eppendorf mastercycler gradient and electrophoresis of the PCR product was run at 78 Volt for 90 min using the 4% metaphor agarose gel, stained using a non-carcinogenic stainer GelRed and finally visualized under the UV light.

Data analysis: The software POPGENE version 1.32 was used to analyse the populations. The extent of inbreeding was further studied with GENEPOP software (Raymond and Rousset, 1995) by estimation of the F_{IS} values and their significance level within each of the populations and the pair-wise F_{ST} values were also computed using GENEPOP software and a cluster analysis was constructed using the NTSYS version 2.1 software to visualize the relationship among the populations of the *P. perna*.

Fig. 1: Sampling locations of the brown mussels in coastal waters of Oman

RESULTS

Out of the 30 cross amplified *P. viridis* primer pairs tested, only thirteen showed successful amplification on the target loci (Fig. 2). Nine primers showed a small degree of successful loci amplification. The remaining eight primers did not show any successful product amplification. The total number of alleles per locus ranged from 3 to 36 with the allele size ranging from 100-275 bp. The 13 selected microsatellite primer pairs showed high allele frequency at 125 bp while the lowest value of allele frequency was 0.0167. The mean observed number of alleles for all loci is 3.846 (Table 1). The mean of the expected heterozygosity (Table 2) is higher than the mean of the observed heterozygosity. The observed heterozygosity of all the loci ranged from 0.040-1.000 with a mean of 0.516 while the mean of the expected heterozygosity is higher with a value of 0.545.

The F data showed significant genetic differentiation among all the populations. In the results, on average, the genetic differentiation (F_{ST}) among breeds was moderate (59.1%) (Table 3). The FIT

Table 1: Summary of genic variation statistics for all loci

Locus	na	ne	I
BP2-35-2	2	1.041	0.099
BP2-49-1	4	2.324	1.019
BP2-49-2	3	1.807	0.736
BP9-7-1	5	4.439	1.550
BP9-13-2	5	3.387	1.327
BP9-16-2	5	2.383	1.073
BP9-19-2	5	2.602	1.125
BP9-27-1	3	1.293	0.461
BP10-5-2	4	2.004	0.797
BP10-16-1	4	2.440	1.047
BP10-17-2	6	1.987	1.067
BP14-7-1	2	2.000	0.693
LR1-58-1	2	1.999	0.693
Mean	3.846	2.285	0.899
SD	1.345	0.87	0.377

na: Observed number of alleles, ne: Effective number of alleles (Kimura and Crow, 1964), I: Shannon's Information index (Lewontin, 1972)

Table 2: Summary of heterozygosity statistics for all loci

Locus	Observed heterozygosity	Expected heterozygosity*	Expected heterozygosity**	Average heterozygosity
BP2-35-2	0.040	0.040	0.040	0.026
BP2-49-1	0.329	0.572	0.5698	0.355
BP2-49-2	0.216	0.449	0.447	0.226
BP9-7-1	0.579	0.778	0.775	0.322
BP9-13-2	0.122	0.707	0.705	0.308
BP9-16-2	0.493	0.583	0.58	0.313
BP9-19-2	0.692	0.621	0.616	0.414
BP9-27-1	0.25	0.228	0.226	0.122
BP10-5-2	0.529	0.508	0.501	0.324
BP10-16-1	0.862	0.593	0.59	0.344
BP10-17-2	0.615	0.500	0.497	0.328
BP14-7-1	1.000	1.000	0.500	0.167
LR1-58-1	0.98	0.505	0.500	0.167
Mean	0.516	0.545	0.504	0.263
SD	0.315	0.236	0.192	0.112

*Levene (1949), **Nei (1973)

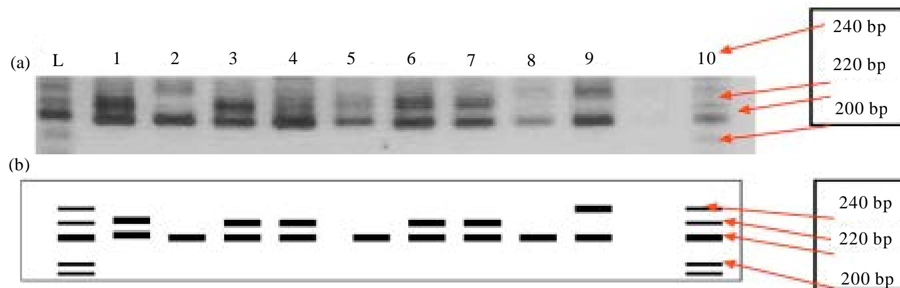

Fig. 2(a-b): Microsatellite banding profile of mussel samples from Mirbat using primer pair BP2-35-2. A diagrammatic representation of the microsatellite bands in a. Lane L: 20 bp extended ladder. Lane 1-10: Individuals of Mirbat

Table 3: F-statistics and gene flow for all loci

Locus	F_{IS}	F_{IT}	F_{ST}	Nm
BP2-35-2	-0.043	0.953	0.955	0.012
BP2-49-1	0.068	0.432	0.39	0.391
BP2-49-2	0.019	0.491	0.481	0.27
BP9-7-1	-0.8	0.251	0.584	0.178
BP9-13-2	0.608	0.832	0.571	0.188
BP9-16-2	-0.596	0.148	0.466	0.287
BP9-19-2	0.047	0.533	0.51	0.241
BP9-27-1	-0.2	0.773	0.811	0.058
BP10-5-2	-0.561	0.43	0.635	0.144
BP10-16-1	-0.657	0.304	0.58	0.181
BP10-17-2	-0.284	0.46	0.58	0.181
BP14-7-1	-1	0.647	0.824	0.054
LR1-58-1	-0.961	0.654	0.824	0.054
Mean	-0.27	0.481	0.591	0.173

Nm: Gene flow estimated from F_{ST}: 0.25 $(1-F_{ST})/F_{ST}$

Table 4: Genetic distance based on data generated utilizing thirteen microsatellite loci using Nei (1973)'s analysis

Populations ID	Mirbat	Ras Madrakah	Ras Al-Had
Mirbat	-	-	-
Ras Madrakah	0.4746	-	-
Ras Al-Had	1.2666	0.8869	-

-: No computerized value because the value differences are the same as compared or the populatious are from the same ID thus no comparison can be made

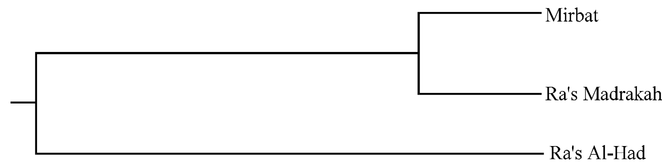

Fig. 3: UPGMA dendrogram showing the clustering pattern of *P. perna* populations in the coastal waters of Oman

value was 0.481 while the F_{ST} value was 0.591. Chi-square (χ^2) and likelihood ratio (G^2) tests showed significant deviation ($p<0.05$) from Hardy-Weinberg equilibrium for all the loci.

Considerable genetic distances (Table 4) were observed between all possible pairs of the *P. perna* from the three populations. The genetic distance values ranged from 0.4746 to 1.2666 with the highest genetic distance was observed between the Mirbat and Ras Al Had populations. The least genetic distance was observed between the Mirbat and Ras Madrakah with a value of 0.4746. The UPGMA dendrogram (Fig. 3) constructed based on the genetic distances generated from thirteen microsatellite loci showed two major clusters. One cluster grouped the Mirbat and the Ras Madrakah populations together while the other cluster is the Ras Al Had by itself.

DISCUSSION

Thirty primers were tested in this study, however only 13 primers were selected due to their successful loci amplification. Nine primers showed a small degree of successful amplification and

this was not enough to conduct a population analysis because of high degree of null allele present in all the populations studied. The mean of the expected heterozygosity can roughly reflect the variation of genetic structure of the mussel populations thus generally, all the populations are showing considerable degree of genetic diversity. The mean of the expected heterozygosity is higher than the mean of the observed heterozygosity which indicates that there is a deficit in the heterozygosities in the populations. Inbreeding may perhaps have contributed to the deficiency in heterozygosity, although it is a highly plausible point since the *P. perna* stocks were obtained from the wild, which were supposed to be out-bred unless there was a form of bottlenecking in the populations. A more probable reason for the deficiency in heterozygosity is the mutation at the flanking regions of the primer binding sites. Such mutation causes the sequences of the flanking regions to change and the primers are unable to bind to the target locus, resulting in failure of amplification (O'Connell and Wright, 1997).

Significant deviation ($p<0.05$) from the Hardy Weinberg Equilibrium (HWE) could be attributed to several factors for instance high in mutational rate in the loci, the non-random mating of the populations and population size limitation as well as overlapping between generation and migration which will cause homozygosity of the population (Lowe *et al.*, 2004). A relative high value and extremely significant ($p<0.001$), indicated that there was a differentiation among the populations. Raymond and Rousset (1995) in their study of testing heterozygote excess and deficiency also showed that a deviation from HWE could have resulted from either selection, population mixing or non-random mating. Furthermore, scoring errors could have contributed to the deviation in HWE and the effects of inbreeding might also contributed to the deficiency in heterozygosity. The FIT value was 0.481 while the FST value was 0.591. This shows that, it was clear that about 59.1% of the total genetic variation corresponded to differences of populations and the remaining 40.9% was the result of differences among individuals. The overall F_{IT} and F_{IS} values were highly significant ($p<0.05$) throughout the populations, suggesting inbreeding within population might have occurred in the *P. perna*.

All the populations are found to be clustered according to their geographical origins with the closest clustering was in accordance to their genetic distance are the Mirbat and the Ras Madrakah populations. The genetic structure among populations of *P. perna* within each region that can be seen from the dendrogram implies that mixing of individuals might have occurred and the from the close values of the genetic distances, it highly indicates that the two populations were of a closely related ancestor. Probable reason for the slight inbreeding detected in this study would be that there are no specific barrier in the Oman waters to prevent migrations and genetic flow thus there is still some form of interaction via migration and mating between the populations despite the geographical distance. Another probable reason for the inbreeding between the populations that has occurred is it could be because of rafting of juvenile or adult mussels on drifting macroalgae, wood or other material which is a plausible alternative dispersal mechanism. *Perna* spp. pediveliger are known to settle on seaweed, wood and plastic and later attach to a range of surfaces, most commonly rocks and woods (Jeffs *et al.*, 1999). Helmuth *et al.* (1994) found the *Gaimardia trapesina* bivalve up with the shell length of up to 38 mm on drifting macroalgae between South America and South Georgia, so it is possible that drifting macroalgae and certainly wood or plastic material, would have sufficient buoyancy to support *Perna* individuals after a year's growth. Rafting on drifting macroalgae or pumice has also been implicated as a long-distance dispersal mechanism for several other marine invertebrates such as the oyster *Ostrea chilensis* (Foighil *et al.*, 1999) and gastropods *Diloma* sp. (Donald *et al.*, 2005).

The reason Ras Al-Had population was individually grouped could be because of the unique and prominent environment generated during the southwest monsoon tend to affect the population during the fall intermonsoon particularly on the formation of cyclonic and anticyclonic eddies associated with the Ras Al Had Jet (Kindle and Arnone, 2001). Flagg and Kim (1998) noted the continued presence of the Ras Al Had Jet, which appeared to maintain its intensity during the intermonsoon period due to the intensification of the southward flow along the northeast coast of Oman thus does not cause further intermingling between the populations to occur while the transition from the fall intermonsoon to the north-east or winter monsoon occurs in November. In the northern Arabian Sea, the primary circulation response to the onset of the northeasterly winds is the reversal of the Oman coastal current to southeastward flow thereby yielding a continuous southward current that extends along the northeast coast of Oman and continues southward along the coast until it is entrained into offshore directed squirts and jets south. Anticyclonic features that were once directly connected to the coastal circulation during the South west and fall monsoon periods now evolve into separated eddies that exhibit a tendency to propagate southward along the coast and may occasionally directly impact the coastal circulation.

CONCLUSION

All the microsatellite loci in this study exhibited high or medium polymorphic and the mean effective number of the alleles was quite high, which could provide enough effective and reliable information for the assessment of genetic diversity of the brown mussels populations in Oman. However, the individuals from the populations may have undergone a bottleneck effects which cause some of the populations of the *P. perna* to have more homozygote individuals. This study information can be applied for future genetic improvement of selective breeding and in the design of suitable management guidelines for these genetic materials.

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