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Sympatric morphological and genetic differentiation of the pearl oyster *Pinctada radiata* (Bivalvia: Pterioida) in the northern Persian Gulf

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The pearl oyster, Pinctada radiata*, shows great variation in shell morphology throughout its distribution. This variation can be related to phenotypic plasticity, genetic variability or a combination of both. Using geometric morphometric and microsatellite DNA analyses, two morphologically distinct populations of the pearl oyster were studied in the northern Persian Gulf, i.e. from the Lavan and Hendourabi Islands. Ten landmarks were selected to define the shape of the left shell. In addition, concentration of Zn, Mg, Fe, Cu, Pb, Cd, Mn and Cr of the soft tissues were measured using atomic absorption spectrometry. Six microsatellite loci were used to assess the population genetic structure of the pearl oyster. There were morphometric differences between the populations suggesting the existence of two morphotypes. There was a significant difference between the two populations in concentrations of Fe, Mg, Zn, Cd, Mn and Cr indicating that the specimens from the Lavan Island experience a more stressful environment than those from the Hendourabi Island. Analysis of molecular variance (AMOVA) indicated that the proportion of the genetic variation attributed to differences among populations of the pearl oyster was highly significant for both* F_{ST} *and* R_{ST} *(*$F_{ST} = 0.066$*,* $R_{ST} = 0.265$*,* $P < 0.001$*). Our findings showed that stressful conditions resulting from heavy metals may have a direct influence on the separation of the populations in Lavan and Hendourabi despite the lack of a physical barrier.*

Keywords: phenotypic plasticity, genetic differentiation, *Pinctada radiata*, heavy metal

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INTRODUCTION

Many species of the pearl oyster (*Pinctada radiata*) exhibit great variation in the shell morphology throughout their distribution (Wada, 1982; Beaumont & Khamdan, 1991). This variation may be related to phenotypic plasticity (Kelly *et al.*, 2012) and/or genetic variability (Benzie & Smith-Keune, 2006). Most morphogenetic investigations on the pearl oyster have focused on allopatric process, i.e. using samples from either end of a large geographic range with dissimilar environments with different ecological characteristics (Lind *et al.*, 2007; Tlig-Zouari *et al.*, 2010) to ensure the existence of a physical barrier and lack of a gene flow between the populations (Gopurenko & Hughes, 2002; Ward *et al.*, 2006). As a result, sympatric differentiation of morphogenetic characterisics in *P. radiata* has rarely been reported, although it has been shown that morphological characteristics of *P. radiata* populations may differ without physical barriers (Rajaei *et al.*, 2014).

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The presence of the pearl oyster has a long history in the Persian Gulf (Elisabeth, 2008). There are two populations of *P. radiata* (Leach, 1814) in the northern Persian Gulf, i.e. in the Lavan and Hendourabi Islands. Despite the short distance between the two islands, these populations exhibit dissimilar morphology (Rajaei *et al.*, 2014). The Hendourabi Island population tended to be wider, while Lavan Island specimens tended to be longer, and the thickness of those from Lavan Island was greater than those of Hendourabi Island (Rajaei *et al.*, 2014). There is no physical barrier between the two islands suggesting a gene flow between the populations through westerly currents of the northern Persian Gulf (Persian Gulf Study Center, 2013). The major difference between the environmental parameters of the two islands originates in the oil industry. Lavan Island is the second largest oil-producing island in Iran with its environment having suffered from the storage and production of crude oil and ballast water. In contrast, Hendourabi Island is away from the petroleum industries suggesting that the marine biota of the island are less stressed than those from the Lavan Island.

The scope to which genetic divergence can happen in the absence of physical barriers to restrict the gene flow between populations is currently one of the most polemic topics in evolutionary biology, helping in the understanding of phenotypic plasticity, adaptive potential and speciation (Hoffman *et al.*,

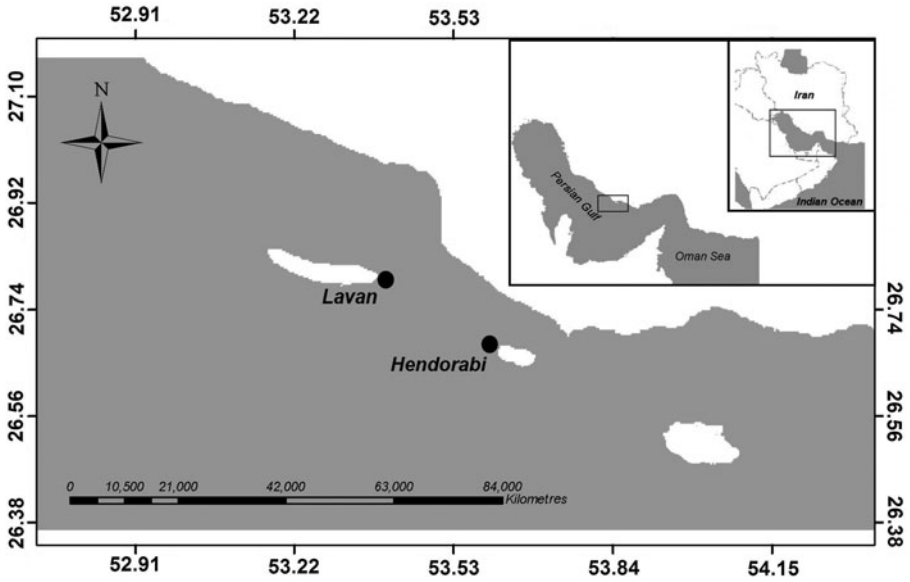

Fig. 1. Sampling points of *Pinctada radiata* populations in the northern Persian Gulf (the Hendourabi and Lavan Islands).

2010). Few studies have investigated both phenotypic plasticity and genetic variability in populations of pearl oyster with dissimilar morphological features. The present study thus aimed to investigate whether the morphological differences between the close populations of *P. radiata* in the northern Persian Gulf reflects the genetic variability and thus probable sympatric genetic differentiation in *P. radiata* populations.

MATERIALS AND METHODS

Sampling

The present study was conducted in two islands of the northern Persian Gulf, i.e. Hendourabi and Lavan (Figure 1). One hundred and nineteen specimens of *P. radiata* were collected using scuba diving at a depth of 6–8 m in coastal waters of Hendourabi and Lavan. Forty specimens were transported to the laboratory in an ice box for heavy metal analysis. Seventy-nine specimens ranging from 35.10 to 79.12 mm shell height were digitized and used for geometric analysis and 52 specimens for genetic analysis were selected.

Geometric morphometrics

The left valve of each specimen was photographed using a Sony digital camera (30 cm distance and a right angle). The images were converted to TPS files using tpsUtil (version 1.46) and 10 landmarks were placed on the pictures using tpsDig, as shown in Figure 2 (version 2.14; Rohlf, 2009, 2010). The coordinates were transformed by procrustes superimposition using Coordgen6f (Sheets, 2001). To describe the shell shape variations among the populations, a canonical variant analysis (CVA) was performed using CVAGen6. Since the CVA suggested a significant difference between the populations, the tendency of direction of the landmarks was calculated to examine the shape variation in the specimens from Lavan and Hendourabi. This analysis was carried out using the TwoGroup6 software.

Heavy metal

The frozen specimens were transported to the laboratory in plastic bags. Half a gram of soft tissue was weighed to the nearest 0.001 g using an electronic scale. The freeze-dried tissues were digested in 10 ml HNO_3 (99.99% Merck) at 150°C until appearance of a clear solution when a yellow colour was formed. The samples were filtered using an ashless filter paper. The remaining materials on the filter paper (mainly fat) were forced to pass the filter using warm distilled water and melting the materials to a final volume of 25 ml. The concentrations of metals were measured using atomic absorption spectrometry (Shaimadzu AA G70). In the present study, concentration of metals was expressed as $\mu g\ g^{-1}$ wet weight. To examine a significant difference in concentration of Zn, Mg, Fe, Cu, Pb, Cd, Mn and Cr between specimens from the two islands the Hotelling's T^2 was applied using the R package 'Hotelling' version 1.0–2.

Genetic diversity

Fifty-two specimens were randomly selected (26 from each island). The adductor muscle was sampled (≈1 g) using biopsy and preserved in 96% ethanol. The preserved tissue

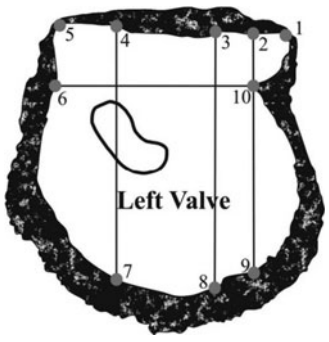

Fig. 2. The position of the 10 landmarks used to define inner surface of the left shell shape of the pearl oyster. To improve the identification of points, all landmarks were painted with large points of permanent marker.

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Table 1. Microsatellite markers used in *Pinctada radiata* population study.

Locus name	Core repeat of sequenced allele	Primers (5′–3′)	Annealing temperature	Reference
HNUPM052	$(TA)_6$	F: GATTTAAGGGAGCCGAGACC R: TAGTCGGGCGCATACTCTTC	60	Shi *et al.* (2009)
HNUPM068	$(AAT)_4$	F: GGCAAGTTCATCGCATAGTTC R: TCCACCAGAAGAGAAACCAAC	59	Shi *et al.* (2009)
HNUPM059	$(TC)_7$	F: CGAATGCGAACTACGTGAAC R: GGAGGGATGGTGCTAAAATG	57	Shi *et al.* (2009)
Pmx 16_23	$(GT)_{26}$	F: CCATACCCACCCACCATC R: TATCAAAGTCGGGAGGCA	50–60	Kuang *et al.* (2009)
Pmx + 0222	$(CT)_{17}$	F: ACA ATA AAG CGC CAT AGC R: TCA TCG TCT CAT CCT GAA C	50–60	Smith *et al.* (2003)
JCUPm 1_g8	$(CT)_8$	F: TTAGTTGTTTGGGGTTTTGAGAC R: GCAGAAGCAATTCTAAATCAATCAG	50–60	Evans *et al.* (2006)

was digested in lysis buffer (10 mg ml^{-1}) and 30 µl Proteinase K for overnight at 55°C, followed by a phenol–chloroform–isoamyl alcohol purification protocol to extract total genomic DNA (Sambrook *et al.*, 1989). The quality and quantity of the genomic DNA samples were examined using 1% agarose gel electrophoresis and NanoDrop-ND1000.

The screened microsatellites were the six polymorphic loci that had been isolated and characterized from genomic DNA (Table 1). Polymerase chain reaction (PCR) was conducted in 25 µl volumes using the conditions presented in Table 2. $MgCl_2$ concentrations varied for each marker according to the original published conditions. Thermocycler programmes began with an initial denaturation step for 7 min at 96°C followed by 35 cycles of 94°C for 1 min, annealing temperature for 1 min and 72°C for 1 min, then a final extension step at 72°C for 7 min. The annealing temperature varied for the loci. Finally, the success of amplification was examined using 1.5% agarose gel electrophoresis. The PCR products were then separated on 4% MetaPhor® Agarose (FMC, Rockland, ME, USA) gels and visualized using ethidium bromide staining.

Number of alleles, observed heterozygosity (HO), expected heterozygosity (HE) and effective number of alleles (ne) were calculated using the software Popgen 1.32 (Nei, 1973; Labate, 2000). Genalex 6 was used to identify deviations from Hardy–Weinberg equilibrium (Peakall & Smouse, 2005). Gene diversity, allelic richness (Rs) (Leberg, 2008), inbreeding coefficient (FIS) also were assessed for the two populations using FSTAT 2.9.3.2 (Goudet, 1995). For partitioning the proportion of variance among (F_{ST}) and between (F_{IS}) population genetic differences, an F-statistics was used (Weir & Cockerham, 1984). Weighted estimates of global F_{ST} and F_{IS} (AMOVA; Excoffier *et al.*, 1992) were performed using arlequin 3.1 (Excoffier & Lischer, 2010). Furthermore the Garza–Williamson test, which is a statistic for bottleneck detection using microsatellite data was performed using this program. The formula is number of alleles/R (allele range) + 1 (Garza & Williamson, 2001).

Table 2. Amount of PCR components used in this study.

	Components	Volume	Final concentration
1	ddH2O	16.5 µl	–
2	10x PCR buffer	2.5 µl	1X
3	$Mgcl_2$	1.5 µl	1.5 mM
4	dNTP	1 µl	0.2 mM each
5	Rp	1 µl	0.5 µM
6	Fp	1 µl	0.5 µM
7	DNA	1 µg	–
8	Taq DNA polymerase	0.5 µl	0.625 unit/25 µl reaction
	Total	25	

RESULTS

Geometric morphometric

The canonical variable detected a significant difference between the two populations ($P < 0.001$, Figure 3).

The shape of specimens from Hendourabi tended to be more depressed than those from Lavan. The landmark No. 10 had the least differentiation and the landmarks 5 & 6 had the most differentiation (Figure 4) indicating that the shells of the Lavan specimens were more stretched than those from Hendourabi. There was a major difference between specimens of Lavan and Hendourabi Islands in the dorsal lip (in the direction of lateral hinge teeth) with Lavan specimens having larger dorsal lips than those from Hendourabi.

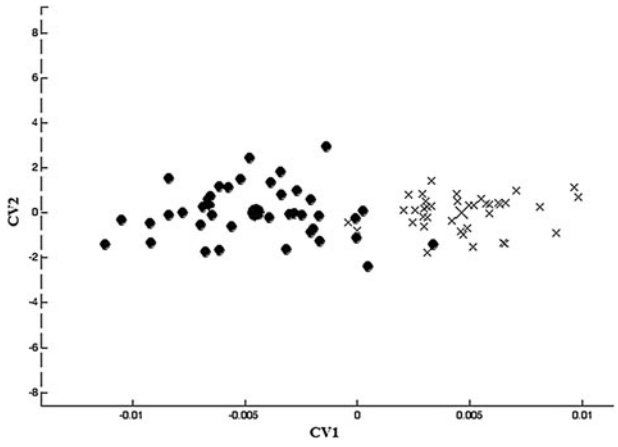

Fig. 3. Geometric morphometric. Distribution of scored frequencies generated by canonical variate analysis (CV1 and CV2 = the first and second canonical variate) from the specimens of Lavan (•) and Hendourabi (×).

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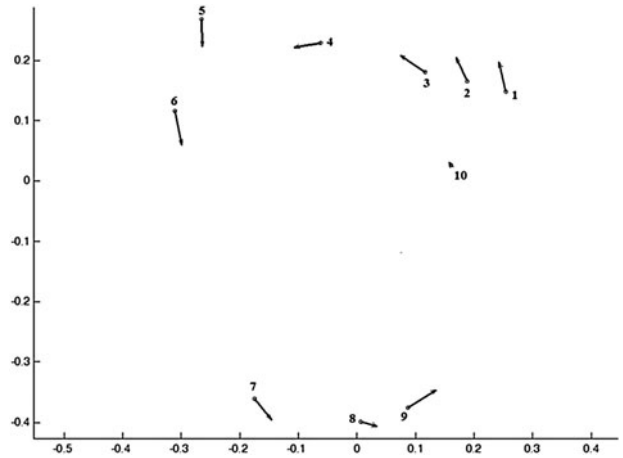

Fig. 4. Geometric morphometric. The tendency direction of landmarks from Lavan to Hendourabi generated by IMP TwoGroup6h. The numbers on the graph are the landmark as depicted in Figure 2.

Heavy metal

The average concentrations of Zn, Mg, Fe, Cu, Pb, Cd, Mn and Cr in soft tissue of the pearl oysters collected from Hendourabi and Lavan are presented in Figure 5. The highest concentration of Mn, Cr, Fe, Mg, Zn and Pb were found in the Lavan specimens while Cd and Cu were higher in the specimens from Hendourabi. Among the metals, Mg and Mn had the highest and lowest concentrations, respectively.

There was a significant difference between the specimens collected from Hendourabi and Lavan in concentrations of heavy metals (Hotelling's T^2 statistic = 30.208, df = 8, 30, $P = 8.227 \times 10^{-14}$).

Genetic diversity

The JCUPm 1_g8, Pmx + 0222 and Pmx 16_23 loci didn't show any band under different biochemical and annealing temperature condition and the HNUPM052, HNUPM059 and HNUPM068 loci are included in all analyses.

Genetic diversity varied across the samples of the two populations and loci. Levels of polymorphism among Hendourabi and Lavan in the microsatellite loci analysed in these two islands were variable, with the overall number of alleles ranging from seven alleles at the HNUPM059 locus in Hendourabi, to 13 alleles at HNUPM068 in Lavan. Expected heterozygosities were high (>0.7) in both Hendourabi and Lavan for all loci, and the observed heterozygosity for most loci was lower than expected heterozygosity. The mean observed heterozygosity (HO) differed among Hendourabi and Lavan (Hendourabi: 0.6737; Lavan: 0.8974), the locus HNUPM068 had the most difference. Estimation of effective number of alleles differs greatly between Hendourabi and Lavan (Mean Lavan: 8.77; Hendourabi: 4.3269). Allelic richness (RS) showed a distinct trend of fewer alleles per locus at Hendourabi than the Lavan, with a mean RS ranging from 8.556 in Hendourabi to 11.761 in Lavan. The mean RS of Lavan was significantly greater than that of Hendourabi (*t*-test, $P = 0.040$), which was also seen by the expected heterozygosity means (HE). The mean gene diversity of Lavan was also greater than Hendourabi (Table 3).

The pearl oyster individuals showed significant partitioning of genetic variation in Hendourabi and Lavan. The analysis of molecular variance (AMOVA) indicated that the proportion of the global genetic variation attributed to differences between the Hendourabi and Lavan populations was highly significant for both F_{ST} (based on allele frequencies) and R_{ST} (based on allele sizes) estimates ($F_{ST} = 0.06653$; $R_{ST} = 0.26537$, $P < 0.001$) (Table 4).

Except HNUPM059 in Hendourabi, all other loci had significant deviations from Hardy–Weinberg expectations of heterozygosity. The Garza–Williamson statistic for microsatellite data for all loci was lower than 0.6 which is an indicator of bottleneck (Garza & Williamson, 2001) in the populations of both Lavan and Hendourabi (Table 5).

DISCUSSION

The present study found a significant intraspecific morphological variability in *P. radiata* from two close islands of the

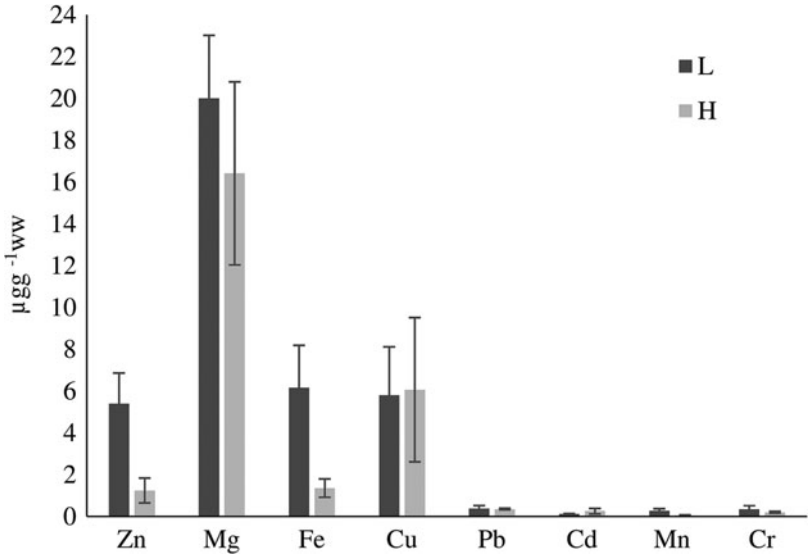

Fig. 5. The mean concentration of Zn, Mg, Fe, Cu, Pb, Cd, Mn and Cr in soft tissues of the pearl oyster collected from the Hendourabi (H) and Lavan (L) Islands.

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Table 3. Genetic diversity statistics of *Pinctada radiata* populations, showing number of alleles, observed heterozygosity (H_O); expected heterozygosity (H_E); Effective number of alleles (n_e), inbreeding coefficient (F_{IS}), allelic richness (Rs) and gene diversity.

	Hendourabi	Lavan	Mean	Total
n	**26**	**26**	**26**	**52**
Mean				
No. alleles	8.6	12	10.3	13.66666667
H_o	0.6737	0.8974	0.78555	0.7904
H_e	0.7833	0.903	0.84315	0.8836
n_e	4.3269	8.77	6.54845	8.0303
F_{IS}	0.121	0.017	0.069	0.0489
R_s	8.556	11.761	10.1585	11.989
Gene diversity	0.789	0.913	0.851	

Persian Gulf, Hendourabi and Lavan. The two islands have similar salinity, temperature and wave action (PGSC, 2013), which are the most important environmental factors influencing the morphological differentiation of the pearl oyster populations (Beaumont & Khamdan, 1991; Tlig-Zouari *et al.*, 2010). The major difference in environmental parameters of the two islands is the water and sediment characteristics, these being related to the petrochemical pollution and heavy metals concentration (Rajaei *et al.*, 2014). Our analyses of heavy metals also indicated significant differences in concentrations of the metals between the oysters collected from Hendourabi and Lavan confirming that Lavan is a more polluted and thus stressful environment than Hendourabi.

Our findings of the genetic analysis showed that the populations of *P. radiata* are genetically structured throughout Hendourabi and Lavan. The larval period of *P. radiata* lasts 16–30 days (Gervis & Sims, 1992), being long enough to let the larvae get a piggyback from the westerly currents of the Persian Gulf (PGSC, 2013) and move from Hendourabi to Lavan. Hence, the pearl oysters of Hendourabi and Lavan may have a chance to mix with each other. However, there is a poor genetic exchange between Hendourabi and Lavan populations in spite of absence of physical barriers between the two islands. The sympatric genetic differentiation of the genus *Pinctada* has been reported previously, for example, Benzie & Smith-Keune (2006) found genetic discrepancies between the populations of *P. maxima* in southern Indonesia and north-west Australia. They assigned the dominant factor shaping the population differentiation to a historical separation of the Pacific and Indian Ocean basins (Benzie & Smith-Keune, 2006). Also, Lind *et al.* (2007) found that the population of *P. maxima* is genetically structured throughout its distribution. Both of them reported F_{ST} 0.009 and 0.027, respectively, considerably lower than estimates of genetic differentiation presented here in Hendourabi and Lavan ($F_{ST} = 0.0665$, $P < 0.001$). Based on our results, which examined these populations on a small spatial scale, *P. radiata* exhibits stronger genetic population structuring than those reported previously (Benzie & Smith-Keune, 2006; Lind *et al.*, 2007). Both Lind *et al.* (2007) and Benzie & Smith-Keune (2006) assigned this differentiation to the existence of an impassable land barrier, which does not exist between Hendourabi and Lavan.

Regarding the Garza–Williamson statistic for microsatellite data (G-W stat lower than 0.6) for all loci that is an indicator of bottleneck (Garza & Williamson, 2001), the H-W disequilibrium may arise from the population demography experiencing a recent population size decrease that has caused a heterozygote deficiency.

Although the Hendourabi larvae may be able to be transferred to Lavan Island, our results suggest that they cannot tolerate the heavy metal pollution and are removed in a competition with Lavan larvae, which may be adapted to a high concentration of heavy metals. In this respect, Gifford *et al.* (2006) showed that the total growth of the oyster could be affected by high concentrations of zinc and lead. It has already been demonstrated that chemicals can support biological activities or have toxic effects on living organisms at high concentrations (Roesijadi & Robinson, 1994), influencing the biodiversity of the marine species (Andersen *et al.*, 1996). The genetic differentiation found between Hendourabi and Lavan populations can increase over time and probably result in speciation (sympatric speciation).

In conclusion, the findings of this study indicated that there are morphological and genetic differentiations in Hendourabi and Lavan pearl oysters, which are probably related to the stressful environment generated by heavy metal pollution in these islands. This seems to be a plausible explanation for the significant differences in the genetic diversity seen here between Hendourabi individuals, which generally have experienced a less polluted environment, and those from Lavan that have probably adapted to the heavy metal pollution. Since this genetic differentiation is slow due to stressful conditions in

Table 4. Analysis of molecular variance (AMOVA) describing the partitioning of genetic variation for two populations of the pearl oyster in Hendourabi and Lavan.

Source	df	SS	Est. Var.	Percentage (%)
F_{ST}				
Among populations	1	2.029	0.03038	6.65
Among individuals within populations	50	22.442	0.0225	4.93
Within individuals	52	21	0.40385	88.42
Total	103	45.471	0.45673	100
R_{ST}				
Among populations	1	7927.538	144.8871	26.54
Among individuals within populations	50	19670.423	−7.68038	−1.41
Within individuals	52	21 256	408.7692	74.87
Total	103	48853.962	545.976	100

Global $F_{ST} = 0.06653$, $P < 0.001 \pm 0.00001$.
Global $R_{ST} = 0.26537$, $P < 0.001 \pm 0.00001$.

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Table 5. Test of Hardy–Weinberg equilibrium and Garza-Williamson on two populations of the pearl oyster in Hendourabi and Lavan Islands.

Pop	Locus	DF	χ^2	Prob.	Signif.	G-W stat
Hendourabi	HNUPM052	45	97.3	0	***	0.27027
	HNUPM068	36	82.01	0	***	0.12162
	HNUPM059	28	29.597	0.383	ns	0.06957
Lavan	HNUPM052	55	115.498	0	***	0.28205
	HNUPM068	78	122.272	0.001	**	0.14286
	HNUPM059	66	89.24	0.03	*	0.14458

ns, not significant.
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Lavan, such diversity will be lost far more quickly, significant variation or differences in genetic diversity most likely arising through loss rather than gain (Amos & Harwood, 1998).

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