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Genetic Integrity in Wild Stock of *Babylonia spirata* (Linnaeus, 1758) and *Babylonia zeylanica* (Bruguiere, 1789) from Southeast Coast of India: An important Mariculture Gastropod Species

M. Chandra Shekar[1]*, S. Arularasan[1], Neelam M. Nathani[2], Shefali Macwan[3] and S. T. Somasundaram[1]

[1]Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai-608502, Tamilnadu, India; chandu.avi@gmail.com; drarulcas76@gmail.com; drstscas@yahoo.com
[2]Department of Biosciences, Saurashtra University, Rajkot-360005, Gujarat, India; neelam.nathani@yahoo.com
[3]Department of Animal Genetics & Breeding, Veterinary College, Anand Agricultural University, Anand-388001, India; shefali2790@gmail.com

Abstract

Background/Objectives: Determining the effect of high demand of whelk meat, overexploitation and indiscriminate fishing on the genetic diversity in two economically important whelk species viz., *Babylonia spirata* and *Babylonia zeylanica* from southeast coast of India. **Methods/Statistical analysis**: The genetic diversity and population structure of two whelk species namely, *B. spirata* (62) and *B. zeylanica* (57) involving total 119 individuals were studied using standard diversity parameters. Both species were genotyped at 12 microsatellite loci to support conservation and improvement strategies. **Findings:** The results show that levels of genetic diversity in natural populations of specific genetic group are moderate to low. All the loci under study were observed to be highly polymorphic and a total of 139 alleles for all 12 markers were identified. The two genetic groups of the whelk species presented HWE deviations for majority of the loci. The range of alleles was found to be 3.5 to 7.5 with a global mean of 5.792. The overall mean of observed and expected heterozygosity were 0.489 and 0.787 respectively. Within population, inbreeding estimate (F_{IS} = 0.381) indicated shortfall of heterozygosity in the population. Microsatellite analysis revealed less genetic diversity in both the species. The Analysis of Molecular Variance (AMOVA) showed 23% of total variation between both the species. **Applications/Improvements:** With the actual genetic diversity and the population structure of these two whelk genetic groups evaluated, it was possible to clarify their importance as well as to propose some management strategies to avoid further loss of genetic diversity in these whelk species.

Keywords: Gastropod, Hardy-Weinberg Equilibrium, Heterozygosity, Polymorphic Information Content, Shannon Information Index

1. Introduction

In India, gastropod beds are abundantly present along the south east coast and coral reef ecosystem in Andaman and Nicobar Islands. Whelks, like other important edible gastropods of Indian coasts are under extensive fishing pressure along the east and west coasts of India[1]. In many cases, this has led to collapse or permanent closure of

*Author for correspondence

the fishery[2]. The production of whelks in India increased considerably from 30,499 t in 1950 to 1,21,657 t in 2011.

Among the genus *Babylonia* the two whelk species (Figure 1) i.e., *Babylonia spirata* and *B. zeylanica* are recognized as an important component of molluscan diversity of southeast and southwest coasts of India and also the important mariculture gastropod species. The *B. spirata* is distributed in the Indian Ocean up to 150 meters and *B. zeylanica* is distributed allover in the Indian and Sri Lankan waters. These are the two species of whelks, which form higher proportion as by-catch of shrimp trawlers along the southwest coast and the southeast coast of India.

Figure 1. Typical whelks (A) *Babylonia spirata* and (B) *Babylonia Zeylanica.*

The high demand for whelk meat in China, Singapore, Thailand and Europe and the high economic value for its opercula, due to its aphrodisiac qualities which is exporting to Gulf countries under the trade name "Fish nail" has led to overexploitation which includes the major proportion of undersized whelks from southeast and southwest coasts of India. This scenario is directly affecting of renewal of the population which may result the reduction of heterogeneity in the natural stocks. In several parts of the world, due to high economic value many marine gastropods have shown serious problem of overexploitation[3–6]. Moreover, recently, due to over exploitation, some of the mollusc species have been listed under the endangered species category[7].

Moreover, literatures on population genetics of marine molluscs in their natural breeding tracts are very scanty[8]. The current exploitation rates of gastropods indicate the need to have scientific data of selected gastropods on their genetic diversity in their natural habitats for planning their conservation and management. Information on genetic diversity of a particular species under natural conditions will provide inputs for their domestication also[9–12]. DNA markers have been used to study the genetic variation in mariculture[11,13], and major livestock i.e. cattle[14], pigs[15–21] and goats[22–26]. Genetic variations between species can be well determined with the help of genetic markers and the obtained genetic distance subsequently help in determining the relationships among species. Several studies have suggested microsatellite markers as a good tool to analyze the genetic variation[27]. Population genetic studies of commercially important marine molluscs based on microsatellite markers are useful for the analysis of population structure and relationships as demonstrated by various studies[28,29]. However, the genetic diversity of natural stocks of *B. spirata* and *B. zeylanica* by covering wide numbers and different avid fishing zones of India has not been assessed yet using microsatellite markers.

Therefore, in the current study, we have examined twelve well-characterized polymorphic microsatellite loci on widely separated natural populations of *B. spirata* and *B. zeylanica* from southeast coast of India in order to understand their genetic diversity and population structure in the natural breeding tract and to establish a microsatellite profile. As knowledge about genetic diversity levels and population differentiation through microsatellite analysis in native breeding tract will be useful for formulating management strategies for sustainable exploitation. The results obtained were compared with the genetic diversity in representative commercial stocks in India and from overseas for better understanding of intra and inter-population diversity.

2. Materials and Methods

2.1 Ethical Statement

No specific permissions were required in the field studies, they were obtained from the catch as these species form a part of important commercial fishery in South-east coast of India and it did not involve endangered or protected species.

2.2 Genetic stocks

A total of 119 whelks representing 2 species from family Buccinidae were sampled from major fish landing centers of southeast coast of India. Summary of whelks sampled from different localities are described in Table 1.

Sample collection and DNA isolation

About 5g of foot tissue was collected from each individual using TNES-urea buffer as a preservative[30] and stored at 4°C until DNA isolation. The geographical information of sampling sites and number of samples from respective site are furnished in Table 1. DNA extraction from tissue was performed as described earlier[31].

Table 1. Summary of sampling locations and number of whelks sampled

S. No.	Species	Sampling sites	Latitude and longitude	Number of samples
A	*B. spirata*	Cuddalore	11°43'45.74" N 79°47'31.51" E	14
		Nagapattinam	10°45'37.94" N 79°50'57.82" E	16
		Toothukudi	8°44'24.62"N 78°10'58.50" E	18
		Kanyakumari	8°05'48.80" N 77°33'42.32" E	14
Total of A				62
B	*B. zeylanica*	Mudasal Odai	11°29'07.74" N 79°46'28.10" E	09
		Nagapattinam	10°45'37.94" N 79°50'57.82" E	11
		Rameshwaram	9°16'49.46" N 79°19'02.44" E	07
		Punnaikayal	8°38'15.20" N 78°07'13.63" E	13
		Arogyapuram	8°07'10.76" N 77°33'32.25" E	12
		Colachel	8°10'20.67" N 77°14'56.42" E	05
Total of B				57
Total of A+B				119

2.3 Microsatellite Markers

The markers were selected from the available genetic maps for marine gastropods[32] such that different chromosomes were included for proper evaluation of the level of polymorphism and reliability of allele calling. Thus, to characterize two whelk species, a total of twelve microsatellite markers were included in the current study (Table 2). Forward primers of each marker were modified for labeling purpose. Extracted DNA samples were analyzed with 12 microsatellite markers. Each 10µl PCR reaction mixture consisted of 10ng of template DNA, 1X buffer, 200µM dNTPs, 2.5mM $MgCl_2$, 1U of AmpliTaq Gold and 10pM primer. PCR amplification conditions for each reaction were as follows: Initial denaturation at 95°C for 5 min, and 30 cycles with 95°C for 30 sec, respective annealing temperatures as shown in Table 2 and 30 sec at 72°C, followed by final extension of 72°C for 5 min.

2.4 Genotyping and Statistical Analysis

Genotyping was performed on automated DNA Sequencer (ABI HITACHI 3500) and the output was analyzed to generate genotype calls per locus using Gene Mapper v. 4.0 (Applied Biosystems) by considering GS 500 (- 250) LIZ as size standard.

Genetic diversity was determined in the form of various indices including allele frequencies, effective number of alleles (N_e), Hardy-Weinberg Equilibrium (HWE), observed heterozygosity (H_o), expected heterozygosity (H_e) and Shannon Information Index (I) using the software PopGene v. 1.31[33] while the Polymorphic information content (PIC) was computed using Excel Microsatellite Toolkit 3.1 software[34]. Nei's (1972) standard genetic distance was calculated using the Dispan program[35]. FSTAT v. 2.9.3[36] was used for estimating Wrights F statistics (F_{ST}, F_{IS} and F_{IT})[37] per locus within population.

Principal Component Analysis (PCA) was computed using GenAlex v. 6.5[38] which forms a representation of populations as cloud points in the metric space. Comparison between the inertia of each marker enables comparison of their typological value. The genetic divergence between groups was determined by computing the Analysis of molecular variance (AMOVA) through F_{ST} estimations[37] using Arlequin v. 3.11 software[39], and were tested with 10^5iterations of Markov Chains and 10^5 permutations.

Population structure for estimating the most probable number (K) in the samples from the generated data was performed using a Bayesian approach with Structure v. 2.3.3[40]. K value from 2 to 5 was estimated, with five simulations, tested with 10^5 repetitions and 5×10^5iterations of Markov chain Monte Carlo Simulation (MCMC). From the data probability logarithms (P Ln (D)) obtained, the best K was estimated with an *ad hoc* statistic Delta K[41]. The visual graphics of the output were computed using Distruct[42].

3. Results

3.1 Within Species Diversity

A total of 139 alleles from 12 microsatellite loci were identified in the 119 evaluated samples of Whelks.

Table 2. Details of microsatellite markers used in present study

Locus name	Primer sequences (5'-- 3')	Repeat motif	Labeled dye	T_a (°C)	Allele size range (bp)
HNI_A3	F:CCATTGCTGAGAGACTGAAGAA R: ACATTTGCGCTTAGTTTGACTG	$(CA)_{22}$	6 FAM	58	238–268
HNI_A12	F:AGTAGGCGGCATTTCACTTC R:CACGAAACTCTGCAAAGACG	$(CA)_{37}$	ROX	58	136–216
HNI_A5	F: CTGTGCAACATCTCTCATTGTT R:ATTTTGCGCTATACCAAGAATG	$(TAA)_7$	Tamra	57	164–182
HNI_A120	F:CTAGCCCCAGTGTATGGTC R:GGTGTCAGTCCTCATTTGG	$(CA)_{21}$	HEX	57	202–282
HNI_A10	F:GAATCCATCCTATGTTTTCAAG R:AAAGAGAGAGGGGAAGAATAAG	$(CA)_{31}$	6 FAM	56	133–237
HNI_B9	F:GGGGTCTACAACACGGTG R:GATGGGAATGGATGGTTG	$(CATC)_{19}$	Tamra	56	121–161
HNI_B120	F:GCAAACACACTCACACACTTT R:CATCCAAGTAAGCAGGAAGAC	$(CTAC)_{26}$	ROX	57	240–286
HNI_A117	F:GGCAGAACGGCATTAACTATG R:CAGGGATCGACAGAGAATCAG	$(TCTG)_8$	6 FAM	57	120–138
HNI_C12	F:TGTCGAATACGATGGAGAGTG R:GGTCTGCTTTACCATTGGAAG	$(TACA)_{23}$	HEX	58	229–301
HNI_B12	F:CACGCACACGTTATACATACAC R:CTTATTCTTCCCCTCTCTCTTT	$(CA)_{51}$	Tamra	58	267–329
HNI_B104	F:ATCGAAGAAGTGGGCATATTG R:ACTGGTAAGATGGGGTTGTTG	$(CATC)_{14}$	HEX	57	153–215
HNI_C102	F:TGAGGCTTCGTGTTGAAG R:CGTCATAAATGCAAACATAGTG	$(TACA)_{21}$	6 FAM	57	109–189

T_a, annealing temperature; bp, base pair.

TNA per locus (Table 3) was 5.792, ranging from 3.500 (HNI_A5) to 7.500 (HNI_B120). The amplitude of all markers in allele sizes was observed to be exceeding the expected amplitude range, indicating the presence of new alleles in the analyzed population. However, the effective number of alleles (Table 3) varied from 3.367 (HNI_A5) to 6.297 (HNI_B120), with over all mean of 5.013±0.958. The observed heterozygosity (H_o) ranged from 0.243 (HNI_A10) to 0.693 (HNI_A117), with a mean value of 0.489±0.159, *which* was lower than expected heterozygosity (H_e) (mean value 0.787±0.046) (Table 3). The *B. spirata* and *B. zeylanica* species represented the highest values of F_{IS} Index (Table 3) and were not within the expectations of Hardy-Weinberg Equilibrium, revealing an amount of homozygous individuals beyond expected. The Shannon's Information Index (I) describing the level of diversity was sufficiently high with a mean of 1.639±0.216 (Table 3). The PIC value revealed that all the studied loci were highly polymorphic in nature with a global mean of 0.885 (Table 3).

The within-species analysis (Table 4) showed that two populations of whelks had least genetic diversity (MNA, N_a, N_e, H_o and H_e). Between two whelk species, *B. zeylanica,* had smallest level of genetic diversity (H_o = 0.457), while *B. spirata* had largest level of genetic diversity (H_o = 0.520). The chi-square (χ^2) test for HWE revealed that all the investigated loci deviated from equilibrium in *B. zeylanica* and 11 out of 12 loci in *B. spirata* (Table 4).

The species differentiation measured by Wright's F-statistics revealed the divergence between expected and observed heterozygosity as measured by the F_{IT} statistic, that had a global mean of 0.454 for all the studied markers, with values ranging between 0.228 and 0.729 (Table 3). While the within-species excess heterozygosity (F_{IS} statistic) had a global mean of 0.381, and ranged from 0.129 to 0.694. And the genetic differentiation among the

Table 3. Estimates of genetic variability indexes per locus based on 119 whelks

Locus	Mean								
	TNA	N_e	H_o	H_e	I	PIC	F_{IS}	F_{IT}	F_{ST}
HNI_A3	6.000	4.846	0.475	0.788	1.642	0.885	0.398	0.469	0.118
HNI_A12	7.000	6.182	0.375	0.827	1.843	0.907	0.546	0.589	0.095
HNI_A5	3.500	3.367	0.288	0.695	1.220	0.828	0.586	0.660	0.180
HNI_A120	4.500	4.432	0.250	0.772	1.491	0.875	0.676	0.718	0.129
HNI_A10	6.500	5.604	0.243	0.794	1.712	0.891	0.694	0.729	0.115
HNI_B9	7.000	5.886	0.639	0.829	1.852	0.908	0.230	0.302	0.093
HNI_B120	7.500	6.297	0.588	0.840	1.912	0.915	0.301	0.362	0.087
HNI_A117	6.000	4.904	0.693	0.796	1.676	0.889	0.129	0.228	0.114
HNI_C12	6.500	5.535	0.575	0.819	1.778	0.902	0.298	0.368	0.099
HNI_B12	6.000	5.244	0.592	0.804	1.700	0.894	0.263	0.344	0.109
HNI_B104	5.000	4.471	0.572	0.776	1.555	0.878	0.264	0.357	0.126
HNI_C102	4.000	3.507	0.575	0.703	1.294	0.834	0.182	0.325	0.174
Global mean	5.792± 1.269	5.013± 0.958	0.489± 0.159	0.787± 0.046	1.639± 0.216	0.885± 0.027	0.381± 0.057	0.454± 0.050	0.120± 0.009

TNA, Total number of alleles; N_e, Effective or Corrected number of alleles; H_o, Observed heterozygosity; H_e, Expected heterozygosity; I, Shannon's information index; PIC, Polymorphic Information Content; F_{IS}, Inbreeding coefficient within population; F_{IT}, Inbreeding coefficient related to the whole population; F_{ST}, genetic differentiation index.

Table 4. Estimates of genetic variability indexes per population using with 12 microsatellite loci

Genetic group	TNA/ population	Alleles/locus (MNA)	Corrected Alleles/locus (N_e)	Heterozygosity		HWE deviation	F_{IS}
				Observed (H_o)	Expected (H_e)		
B. spirata	64	5.333	4.664	0.520	0.767	11 loci	0.323*
B. Zeylanica	75	6.250	5.382	0.457	0.807	12 loci	0.434*

*(P<0.05), TNA, Total number of alleles; MNA, mean number of alleles; HWE, Hardy-Weinberg Equilibrium; F_{IS}, Inbreeding coefficient within each population

species (F_{ST} statistic) had a global mean of 0.120, ranging between 0.087 to 0.180. Overall, the results indicated that 88 % of the genetic variability was due to the differences among individuals within species and 12 % was caused by the differentiation among species.

3.2 Between Species Divergence

A large genetic distance (0.3655) between the two studied whelk species was revealed by Nei's (1972) standard genetic distance measurement. Further, an AMOVA analysis performed to analyze the variation within and between species revealed 23% variation among populations and 77% variation within populations. For all the studied loci, the observed variance components among population were highly significant (Table 5), demonstrating significant geographical structuring in whelk species.

Table 5. AMOVA analysis of *B. spirata* and *B. Zeylanica* based on microsatellite DNA variation

Source of variation	Degree of freedom	Sum of squares	Variance component	Percentage of variation
Among Pops	1	185.425	4.286	23
Within Pops	78	1091.150	13.989	77
Total	79	1276.575	18.275	100

Principal component analysis was performed using all the 139 alleles frequencies for the 12 markers. The first

two principal components explained 52.47% of the total variation, and its global principal component analysis is presented in Figure 2. The first and second axis contributed about 36.88% and 15.59% of the inertia, respectively and distinguished the two whelk populations from each other into separated clusters containing *B. spirata* and *B. zeylanica*. As a result, these two axis revealed a pattern of association that supported a partition of populations into two discrete groups.

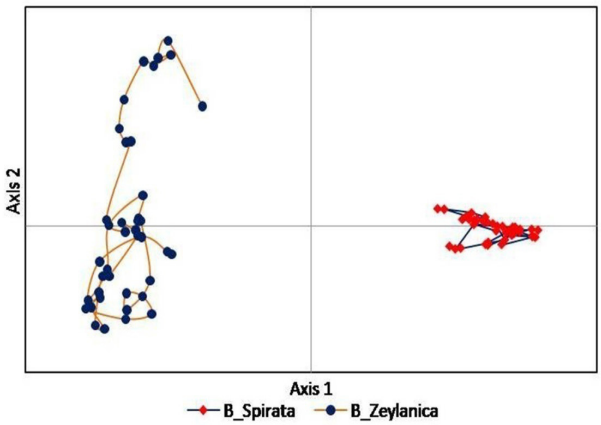

Figure 2. Global principal component analysis (First two principal components).

To supplement F_{ST} analysis, genetic structure analysis using a Bayesian approach by Structure software was performed with increasing number of inferred populations. The probability of K = 1 to 5, averaged over 10 runs for each value of K. A continued gradual increase in Pr (X/K) values was observed for increased values of K, with highest probability of K being 2. This interpretation was based on the combination of the highest –LnPr value and a low SD. The assignment of individuals to each cluster based on K = 2, from 500,000 MCMC iterations, is 99.9 per cent. The proportion of membership to each cluster for K = 2 to K = 5 is presented in Figure 3.

4. Discussion

In our study, the set of microsatellite markers (Table 2) developed for *Hexaplex nigritus*[32] were used for analyzing the genetic diversity of two whelk populations collected from different geographical locations of Southeast coast of India (Table 1) and the relationships among and within the populations.

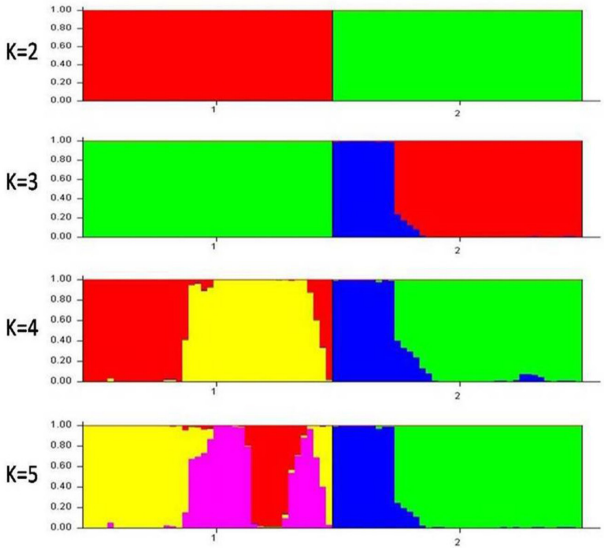

Figure 3. Distribution of the genetic structure of the two whelk species studied with the software Structure/Distruct for K = 2 to K = 5.

The *B. spirata* and *B. zeylanica* species, based on estimates of effective number alleles and observed heterozygosity, can be considered the populations with lowest genetic variability. The global mean number of alleles observed (5.792) in the study is less than the mean number reported for *B. areolata* (13.125) (Wang et al. 2011) and *H. nigritus* (19.385)[32]. However, the global mean number of effective alleles (5.013) was lower than the observed number of alleles which might be due to very low frequency of majority alleles at each locus and fewer alleles may be contributing major part of the allelic frequency. The mean H_o and H_e of *B. spirata* and *B. zeylanica* (0.520 and 0.767; 0.457 and 0.807) was lower than the mean number of H_o and H_e (0.700 and 0.854) in *B. areolata*[43]. Moreover, the present findings of observed and expected heterozygosity was also lower than the reported[32] value in *H. nigritus* populations viz., Punta Chueca (PCH) (0.608 and 0.742); El Borrascoso (EBO) (0.632 and 0.747); Isla San Jorge (ISJ) (0.679 and 0.775); San Luis Gonzaga (SLG) (0.7 and 0.762).

All of the loci possessed high PIC values (above 0.50) signifying that these markers are highly informative for characterizing whelk populations (Table 3). The deviation of most of the loci from HWE (Table 4) may be due to consequences of population size. The specimens of these two genetic groups might be sampled from wild where mating was not controlled. The selection of non-related animals in populations with these characteristics generally

is not easy, and also influences the results in relation to the HWE. Moreover, several other factors, such as non-random mating, Walhund effect; sampling errors and less proportion of male individuals, may cause deviation from HWE.

The global mean within population inbreeding estimate (F_{IS}) was 0.381. The deficiency of heterozygotes (38.1%) in both the studied genetic groups supports the random mating. The positive F_{IS} for all loci in the populations may reflect the division of general population into subpopulations due to accumulated inbreeding in smaller populations and deviations from the HWE.

The variability within genetic groups estimated by the inbreeding coefficient (F_{IS}) (Table 4) showed that the higher value was obtained for both the populations (*B. spirata*, 0.323 and *B. zeylanica,* 0.434; $P<0.05$). Due to lack of organized selection programs in the natural populations, it can be inferred that positive F_{IS} observed values are a result from the inbreeding effect, which may contribute to the observed heterozygote deficits resulting from mating between related individuals.

The F_{ST} had a global mean of 0.120, showing that 12% of the genetic variation was on account of the differences between populations. The mean F_{IT} value for all loci was 0.454, revealing difference of 45.4% between the observed and expected heterozygosity. In addition, AMOVA indicated 23% of the total genetic variation between studied whelk species, confirming moderately higher within population diversity in the investigated genetic groups. Further, the Principal Component Analysis (PCA) supported the grouping of animals and the distance between species was significant.

Results from the assignment test suggested a true genetic structure with significant differentiation among studied populations. This result from the Bayesian-based assignment test therefore supports the trend from frequency-based F_{ST} values, which showed significant differentiation among the populations. Two genetic groups displayed a pattern of strong distinctiveness, more than 98% of individual assigned to a single cluster in each case. Overall, the results from the assignment test support the hypothesis of high homogeneity within natural populations sampled. In the absence of historical data, the exact comparison with nearest genetic groups cannot be determined.

5. Conclusions

In the present study, the markers used were highly informative for characterization of diversity in whelk species as indicated by the PIC values observed. The results show that levels of genetic diversity in natural populations of both the species are moderate to low. With the actual genetic integrity of these two important mariculture gastropod species evaluated, it is possible to propose some management strategies to preserve the genetic integrity and also to avoid further loss of genetic diversity.

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