

Evaluation of within Species Diversity in Natural Stock of *Babylonia spirata* (Linnaeus, 1758), Along Tamilnadu Coast of India Using Microsatellite Markers

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Abstract: The objective of this study was to evaluate genetic structure in natural stock and to establish a microsatellite profile for *B. spirata*. A total of 12 microsatellite markers were used to investigate genetic diversity in *B. spirata* collected from four different stations in Tamilnadu coast (N=62). All studied loci were highly polymorphic and a total of 64 alleles were observed across the investigated loci. The range of alleles was found to be 3 to 9 with a mean of 5.333 ± 0.512 . The frequency distribution of microsatellite alleles in this gastropod population was 0.063 to 0.438. The expected and observed heterozygosity was 0.520 ± 0.051 and 0.767 ± 0.020 respectively. The polymorphic information content (PIC) was 0.727 ± 0.090 . The overall mean of within-population inbreeding estimate (F_{IS}) was 0.323 ± 0.063 . The Shannon's information index (I) was sufficiently high with a mean of 1.556 ± 0.092 . The bottleneck analysis revealed that population has not undergone any recent reduction. The less genetic diversity even though distinct population structure of *B. spirata* in Tamilnadu coast is noticed in microsatellite analysis. Further, this study highlights for future domestication of this species in India.

Key words: *Babylonia spirata* • Heterozygosity • PIC • Microsatellites

INTRODUCTION

Due to high economic value and excessive capture, marine gastropods show serious problems of overexploitation [1-4]. In many cases, this has led to collapse or permanent closure of the fishery. In India, molluscs have occupied a marked place in state of affair and economy of mind and aesthetic values, of religion and rites of worship and edible diet [5]. The major development in Indian marine fishing industry in recent years and considerable changes in fishing craft and gear has led to the increased use of edible gastropods resulting in the extension of the fishing zone.

Consequently, several gastropods that inhabit the deeper waters are caught in the trawl and landed as the by-catch. Among gastropod resources, *Babylonia spirata* has become economically important during last decade. A total of six species of genus, *Babylonia* are commercially important viz., *Babylonia areolata*, *B. japonica*, *B. formosae formosae*, *B. formosae habei*, *B. spirata* and *B. zeylanica*. These are very popular mainly in Southeast Asian countries. The *B. spirata* is well represented in the Indian Peninsula in Gulf of Mannar, Poompuhar, Nagapattinam, Madras and around the waters of Andaman and Nicobar Islands [6]. The *B. spirata* is fished and exported from India to China,



Fig. 1: A typical *B. spirata* from Tamilnadu coast

Singapore, Thailand and Europe. As the demand is increasing and modified gears are being used in fishing of benthic molluscs at Kollam and Tuticorin coast, these resources are regularly fished from Tamilnadu coast and as a result the number of fishing vessels targeting this gastropod species is increasing day by day. Whelk is also fished using traps at Portonovo, Malpe [7] and at Pondicherry using ring-nets [8]. The export statistics of MPEDA showed that India has exported only frozen products, since 2001, but the scenario has since changed resulting in export of live whelks as well. In 2003 about 1115 t were exported as fresh/processed.

The *B. spirata* has shell with characteristic features. The spiral Babylon is a smooth shell, ovoid, with regular spiral rows of large, rounded or squarish brown patches on a white ground. The spire is of medium height with rounded whorls while, the body whorl is inflated and the suture is channeled with sharp edge (Fig. 1). The columella is smooth, with somewhat strongly thickened callus. The aperture is large, ovate and constricted posteriorly by a single, thick ridge extending spirally inward on the columellar side.

Considering the economical prospective and utility of *B. spirata* species, it has been decided to study the genetic structure of natural population along Tamilnadu coast of India and to establish a microsatellite profile. However, microsatellite markers are short tandem repeats, exhibiting high degree of polymorphism and distributed throughout the genome. Moreover, molecular characterization of species through microsatellite analysis is one of the basic requirements for taking up conservation measures.

MATERIALS AND METHODS

Sample Collections and Preservation: A total of 62 (N) fresh sample of *B. spirata* were randomly collected from by-catch trash of four major fish landing centers (Fig. 2) of Tamil Nadu viz., Cuddalore (n=14), Nagapattinam (n=16), Tuticorin (n=18) and Kanyakumari (n=14). The samples were dissected immediately and foot tissues were preserved in TNES-Urea buffer [9] and transported to the laboratory on ice and stored at 4°C until use.

DNA Extraction and Quantification: Genomic DNA was isolated from foot tissue of whelk (*B. spirata*) by using standard phenol-chloroform method [10] with slight modifications like using of DNazol (Invitrogen) reagent instead of SDS and proteinase K. The quantity and quality of isolated DNA were confirmed. The concentrated samples were diluted to reach appropriate concentrations for the purpose of PCR amplification.

Microsatellite Analysis: A total of 12 microsatellite markers were developed by Longo *et al.* [11] for black murex, *Hexaplex nigritus*. The same set of primers was used for present investigation based on their level of polymorphism, allele size range and reliability of allele to evaluate genetic structure in *B. spirata* [12]. The forward primer of each marker was fluorescently labeled with either FAM, ROX, TAMRA or HEX dye. All microsatellite markers were first checked under single locus amplification conditions to evaluate their performance in the multiplex.

Multiplex PCR has been used for multicolor fluorescence genotyping. Based on the guidelines of Henegariu *et al.* [13] and Loffert *et al.* [14], the initial parameters of multiplex PCR were set up. The basic PCR reaction mixture (15 µl) containing 20-50 ng of template DNA; 1.5 mM MgCl₂; 5 picomoles each of forward and reverse primers; 1 unit of taq DNA polymerase and 200 mM dNTPs was prepared. Amplification was carried out with initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation (95°C for 30 sec), annealing (52°C to 58°C for 30 sec) and extension (72°C for 45 sec) using Applied Biosystems (Model #: 9902) Veriti™ 96- well thermal cycler.

Genotyping and Information Analysis: The genotyping was carried out on an automated DNA Sequencer (ABI PRISM 3130XL). The resulting data were analyzed

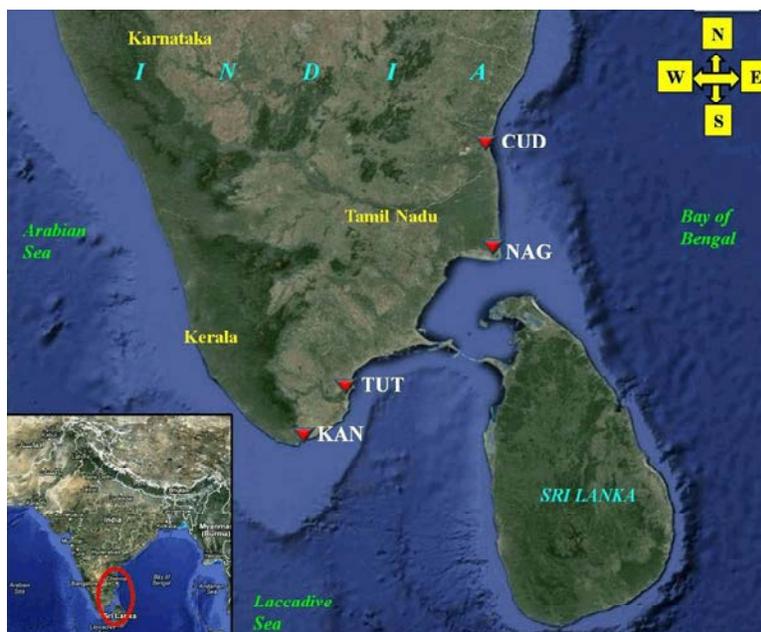


Fig. 2: Shows the sampling sites of *B. spirata* (Courtesy to Google earth map). Acronyms correspond to localities: Cuddalore (CUD); Nagapattinam (NAG); Tuticorin (TUT) and Kanyakumari (KAN)

using standard software Gene Mapper™ version 4.0 (Applied Biosystems Inc., California, USA) to generate genotype calls for each locus by using GS 500 (- 250) LIZ as size standard.

Genetic diversity was determined as allele frequencies, effective number of alleles (N_e), test of Hardy-Weingberg equilibrium (HWE), observed (H_o) and expected (H_e) heterozygosity, F-statistics and Shanon information index (I) using GenAlex v. 6.5 [15]. We checked our data for null alleles using Micro-Checker v. 2.2.1 [16]. Polymorphic information content (PIC) was calculated using Excel Microsatellite Toolkit 3.1 software [17]. The Bottleneck v. 1.2.03 [18] software was used to know whether this population exhibits a significant number of loci with excess of heterozygosity.

RESULTS

The various parameters of genetic variability per locus in whelk (*B. spirata*) population, such as allele number, effective number of allele, PIC, observed and expected heterozygosity, within- population inbreeding estimate (F_{IS}), Shanon's information index and Loci showing evidence of null alleles suggested by Micro-Checker are furnished in Table 1.

All investigated loci were polymorphic in nature. The number of observed alleles (N_a) ranged from 3

(HNI_A5 and HNI_C102) to 9 (HNI_A12), with an overall mean of 5.333 ± 0.512 and a total of 64 alleles were observed at these loci in the population. However, the effective number of alleles (N_e) ranged from 2.809 (HNI_C102) to 7.767 (HNI_A12) with a mean of 4.664 ± 0.409 . Overall allele frequency values ranged from 0.063 (at locus HNI_A12) to 0.438 (at locus HNI_C102). The PIC value ranged from 0.5682 (HNI_A5) to 0.8576 (HNI_A12) with a mean of 0.7276 ± 0.0907 . The overall means for observed (H_o) and expected (H_e) heterozygosities were 0.520 ± 0.051 and 0.767 ± 0.020 , respectively which ranged from 0.211 (HNI_A10) to 0.775 (HNI_A117) and 0.644 (HNI_A5 and HNI_C102) to 0.871 (HNI_A12) respectively.

The chi-square (χ^2) test for HWE revealed that 11 out of 12 loci deviated from equilibrium. Shannon's information index (I) which measures high diversity, was obtained with a mean of 1.556 ± 0.092 . The within population inbreeding estimate (F_{IS}) was observed to be positive at all loci with a mean of 0.323 ± 0.063 indicating significant heterozygosity deficiency in studied whelk population. Only one locus (HNI_C102) showed signs of null alleles as indicated by Micro-Checker (1000 randomizations).

Three mutation models namely, infinite allele model (IAM), two phase model (TPM), stepwise mutation model (SMM) were used for Bottleneck analysis (Table 2).

Table 1: Estimates of genetic variability per locus in whelk (*B. spirata*) population

Plex	Locus	Size range (bp)	Parameters							
			N _a	N _e	PIC	H _o	H _e	I	F _{is}	HWE
Plex 1	HNI_A3	238–268	5.000	4.087	0.7169	0.550	0.755	1.498	0.272	48.782**
	HNI_A12	136–216	9.000	7.767	0.8576	0.400	0.871	2.121	0.541	141.108**
Plex 2	HNI_A5	164–182	3.000	2.810	0.5682	0.364	0.644	1.062	0.435	14.545*
	HNI_A120	202–282	4.000	3.974	0.7013	0.243	0.748	1.383	0.675	55.029**
Plex 3	HNI_A10	133–237	4.000	3.570	0.6664	0.211	0.720	1.316	0.708	61.340**
	HNI_B9	121–161	6.000	5.527	0.7935	0.694	0.819	1.749	0.152	101.263**
Plex 4	HNI_B120	240–286	7.000	5.872	0.8084	0.650	0.830	1.860	0.217	103.773**
	HNI_A117	120–138	7.000	5.087	0.777	0.775	0.803	1.771	0.035	99.533**
Plex 5	HNI_C12	229–301	6.000	5.664	0.7987	0.575	0.823	1.763	0.302	67.598**
	HNI_B12	267–329	5.000	4.359	0.7326	0.629	0.771	1.530	0.184	49.901**
Plex 6	HNI_B104	153–215	5.000	4.436	0.7404	0.579	0.775	1.554	0.253	68.130**
	HNI_C102'	109–189	3.000	2.809	0.5695	0.575	0.644	1.064	0.107	10.363 ^{NS}
Mean overall loci			5.333±0.512	4.664±0.409	0.727±0.090	0.520±0.051	0.767±0.020	1.556±0.092	0.323±0.063	

* Significant ($P \leq 0.05$); **highly significant ($P \leq 0.01$); ^{NS} Not significant ($P > 0.05$); †Loci showing evidence of null alleles suggested by Micro-Checker; bp: Base pair; N_a: Number of alleles; N_e: Effective number of alleles; PIC: Polymorphic information content; H_o: Observed Heterozygosity; H_e: Expected Heterozygosity; I: Shannon's Information Index F_{is}: Deficit or excess of Heterozygotes, HWE: Hardy-Weinberg equilibrium.

Table 2: Bottleneck analysis in *B. spirata*

Model	Sign rank test - Number of loci with heterozygosity excess			Standardized differences test - T2 values (probability)	Wilcoxon test - Probability of heterozygosity excess
	Expected	Observed	Probability		
IAM	13.19	21	0.00020	3.842 (0.00006)	0.00000
TPM	13.14	20	0.00143	2.356 (0.00924)	0.00047
SMM	13.11	12	0.39130	0.155 (0.43842)	0.43680

IAM - Infinite allele model; TPM - Two phase model; SMM - Stepwise mutation model

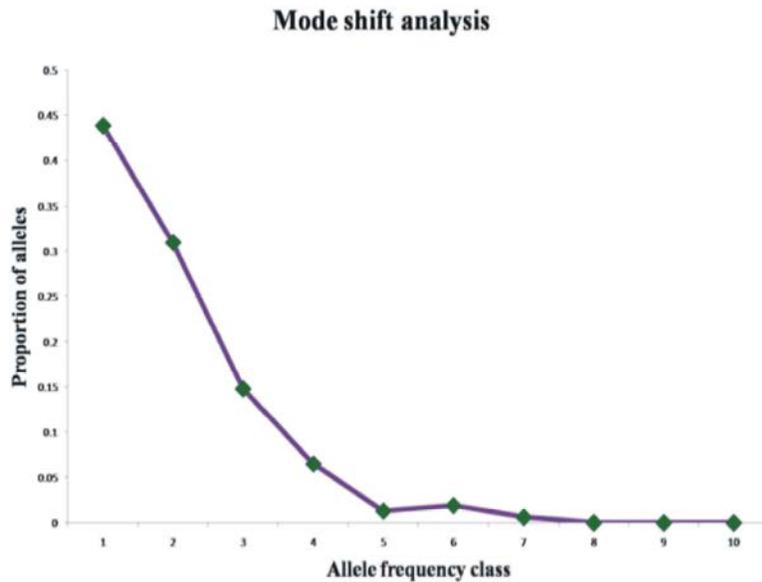


Fig. 3: Graphical representation of allele proportion and their contribution in *B. spirata*

In *B. spirata*, under Sign test, the expected number of loci with heterozygosity excess was 13.11 (SMM) which is higher than the observed number of loci 12 (SMM) with heterozygosity excess. The expected number of loci (13.19 and 13.14) with heterozygosity excess were significantly ($P < 0.05$) higher than the observed number of loci (21 and 20) with heterozygosity excess under

IAM and TPM respectively. Standard difference test (T2 statistics) in this population provided the significant gene diversity deficit under the one mutation model SMM (-0.155). Under Wilcoxon rank test, probability values of 0.00000 (IAM), 0.00047 (TPM) and 0.43680 (SMM) were found to be non-significant. The mode shift analysis [19] revealed L-shaped curve (Fig. 3) indicating no mode-shift

in the frequency distribution of alleles indicating that the studied population has not undergone any recent and/or sudden reduction in the effective population size.

DISCUSSION

Twelve polymorphic microsatellite loci developed for *Hexaplex nigritus* were used to evaluate genetic structure and genetic variation in natural stock of *B. spirata* (N=62) collected from four different stations of Tamilnadu coast of India (Fig. 2).

The mean number of alleles observed (5.333) in the study is less than the mean number reported for *B. areolata* (13.125) [20] and *H. nigritus* (19.385) [11]. However, the mean number of effective alleles (4.664) is lower than the observed number of alleles which is due to low frequency of more alleles at each locus and few alleles have contributed to allelic frequency at each locus.

All of the loci possessed high PIC values (Above 0.50) signifying that these markers are highly informative for characterization of *B. spirata* population. The observed and expected mean heterozygosity of the study species is lower (0.520 and 0.767) than the number of observed (0.700) and expected (0.854) heterozygosity in *B. areolata* [20]. Moreover, the present findings is also lower than the reported value in *H. nigritus* populations [11] 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). The mean within population inbreeding estimate (F_{is}) was 0.323. The deficiency of heterozygotes (32.3 %) in *B. spirata* supports the non-random mating of the population. The chi-square (χ^2) test for Hardy-Weinberg equilibrium revealed that 11 loci out of 12 deviated from equilibrium. The reason for deviation is most likely the genetic drift; non-amplifying alleles or the population might be divided into a series of closely related or inbred family groups. Micro-Checker analysis indicated that one locus is determined to have null alleles for studied *B. spirata* population [21].

The *B. spirata* population is non-bottlenecked as evident from the quantitative graphical method [18]. The population has not undergone any recent reduction in the effective population size and remained at mutation-drift equilibrium. In the present study, no mode-shift was detected in the frequency distribution of alleles and a normal L-shaped curve was observed.

CONCLUSION

This study represents the first attempt to genetically characterize *B. spirata* population along Tamilnadu coast of India using microsatellite markers. The PIC values observed in the present study is indicative of the markers used are highly informative for characterization of diversity in this gastropod species, *B. spirata*. The shortfall of variability in the studied population is indicative of the loss of a valuable genetic diversity. Thus, needful strategy steps should be taken to improve the genetic variation and its sustainable utilization. The population has not undergone any reduction at least in the recent past.

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REFERENCES

1. Castilla, J.C., 1996. La futura red Chjlena de parques y reservas marinas los conceptos de conservacion, preservacion y manejoen la legislacion nacional. Rev. Chil. Hits. Nat., 69: 253-270.
2. Hobday, A.J., M.J. Tegner and P.L. Haaker, 2001. Overexploitation of a broadcast spawning marine invertebrate: decline of the white abalone. Rev. Fish biol. Fish, 10: 493-514.
3. Ponce-Diaz, G., A. Vega-Velasquez, M. Ramade-Villanueva, G. Leon-Carvallo and R. Franco-Santiago, 1998. Socioeconomic characteristics of the abalone fishery along the west coast of the Baja California peninsula, Mexico. J. Shellfish. Res, 17: 853-857.
4. Tegner, M.J., 1989. The California abalone fishery: production, ecological interactions and prospects for the future. In: Marine Invertebrate Fisheries: Their Assessment and Management (Ed. C.F. Caddy). John Wiley and Sons, NY, 401-420.

5. Mukundan, C., 1968. Molluscs in Indian tradition and economy, Symposium on mollusca. Cochin, Jan., 12-16: xxix-xxxv.
6. Ayyakkannu, K., 1994. Fishery status of *Babylonia spirata* at Port Novo Southeast coast of India. Phuket. Mar. Boil. Cent. Spl. Publ., 13: 53-56.
7. Sasikumar, G., Prathibha Rohit, N. Ramachandran, D. Nagaraja and G. Sampathkumar, 2006. Emerging small-scale trap fishery for whelk (*Babylonia spirata*) in Malpe, Southern Karnataka. Mar. Fish. Infor. Serv. T&E Ser., 188: 14-17.
8. Chidambaram, L., 1997. A note on whelk (*Babylonia* spp.) fishery in Pondicherry. Mar. Fish. Infor. Serv. T&E Ser., 147: 15.
9. Asahida, T., T. Kobayashi, K. Saitoh and I. Nakayama, 1996. Tissue preservation and total DNA extraction from fish stored at ambient temperature using buffers containing of urea. Fisheries Sci., 62: 727-730.
10. Bayani, W., W. Omar, M. Shafie, B. Shafie and Z. Kasim, 2009. DNA Extraction from Different Preserved Tissue of *Cassidula aurisfelis* for PCR study. Nature and Science, 7(9): 8-14.
11. Longo, G., R. Beldade, R. Cudney-Bueno, P. Raimondi and G. Bernardi, 2011. Isolation and characterization of 13 polymorphic microsatellites for the black murex, *Hexaplex nigrinus*. Marine Genomics, 4: 69-70.
12. Linnaeus, C., 1758. Systema Naturae. Systema Naturae, 10(1): 1-823.
13. Henegariu, O., N.A. Heerema, S.R. Dlouhy, G.H. Vance and P.H. Vogt, 1997. Multiplex PCR: Critical parameters and step-by-step protocol. Biotechniques, 23: 504-511. PMID: 9298224.
14. Loffert, D., S. Karger, G. Twieling, V. Ulber and J. Kang, 1999. Optimization of multiplex PCR. Qiagen News Issue, 2: 5-8.
15. Peakall, R.O.D. and P.E. Smouse, 2006. Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes, 6(1): 288-295.
16. Van Oosterhout, C., F.H. William, P.M.W. Dere and S. Peter, 2004. Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes, 4: 535-538.
17. Park, S.D.E., 2001. Trypanotolerance I West African cattle and the population genetic effects of selection, Ph.D. thesis, University of Dublin.
18. Cornuet, J.M. and G. Luikart, 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics, 144: 2001-2014.
19. Luikart, G.L., F.W. Allendorf, J.M. Cornuet and W.B. Sherwin, 1998. Distortion of allele frequency distributions provides a test for recent population bottlenecks. J. Heredity, 89: 238-247.
20. Wang, Y., H. Lu, J. Zheng, K. Long, Y.H. Shi, Z.F. Gu and A.M. Wang, 2011. Eight polymorphic microsatellite markers for the spotted babylon, *Babylonia areolata* (Buccinidae). Genetics and Molecular Research, 10(4): 3230-3235.
21. Van Oosterhout, C., D. Weetman and W.F. Hutchinson, 2006. Estimation and adjustment of microsatellite null alleles in non-equilibrium population. Mol. Ecol. Notes, 6: 255-256.