Genetics of Aquatic Organisms 6(2), GA501
https://doi.org/10.4194/GA501

RESEARCH PAPER

Analysis of Genetic Diversity in Wild and Domesticated Stocks of the Tropical Abalone *Haliotis asinina* by Microsatellite Polymorphism

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How to cite

Praipue, P., Klinbunga,S., Prasertlux, S., Janpoom, S., Rongmung, P., Ratdee, O., Ittarat, W., Boonphimpapha, P., Jarayabhand, P., Khamnamtong B. (2022). Analysis of Genetic Diversity in Wild and Domesticated Stocks of the Tropical Abalone *Haliotis asinina* by Microsatellite Polymorphism. *Genetics of Aquatic Organisms, 6(2), GA501*. https://doi.org/10.4194/GA501

Article History
Received 22 March 2022
Accepted 21 May 2022
First Online 02 June 2022

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Keywords
Genetic diversity
Population differentiation
Abalone
Domesticated stocks
Microsatellites

Abstract

The basic information on genetic diversity of wild and hatchery-propagated stocks of tropical abalone, *Haliotis asinina* is important for the construction of a breeding scheme leading to the sustainable culturing activity of this species. In this study, 2,876 expressed sequence tags (ESTs) were *in silico* analyzed and 178 EST sequences contained microsatellite motifs. Four loci (*DW455*, *DW503*, *PHe177*, and *PT102*) of type I and two loci (*Haμ9* and *Haμ10*) of type II microsatellites were applied for genetic diversity studies. The mean number of alleles per locus and observed heterogeneity in wild populations were 4.167 and 0.483, and 4.833 and 0.528 for CAME (east coast) and TRGW (west coast) while those of hatchery-propagated samples were 3.000 and 0.708, 4.500 and 0.479, 5.167 and 0.524, and 6.333 and 0.527 for PHIH (2nd generation, G2; Philippines), SAMH (G1), SMaRT-TRGH (G8) and SMaRT-SICH (G8), respectively. The numbers of alleles, observed heterozygosity and effective population sizes suggested no severe reduction of genetic diversity in our breeding program of *H. asinina*. However, reduced N_e was observed in the cultured stocks from the Philippines (PHIH). F_{ST}-statistics and the exact test between pairs of samples revealed significant genetic differences between all pairwise comparisons of samples in this study.

Introduction

Farming of abalone has been carried out commercially in various parts of the world (Shepherd et al., 1992; Gordon, 2000). The estimated world's supply (including fisheries landings, legal catch and aquaculture production) in 2017 were 174,162 metric tons (Cook, 2019). Over 15 species of abalone dominated by temperate species (Geiger, 1998 and 2000; Jarayabhand & Paphavasit, 1996; Gordon, 2000) are farmed and main contributes are China (139,697 mt) and South Korea (16,042 mt) (Cook, 2019).

The tropical abalone, *Haliotis asinina* is distributed in the Indo-West Pacific region (Jeffrey et al., 2007). It is a year-round spawner. The spawning cycle is highly predictable in hatchery conditions. The market price is about $25-30/kg. Therefore, *H. asinina* is the target species for the culture industry in Thailand (Jarayabhand

& Paphavasit, 1996; Selvamani et al., 2001; Klinbunga et al., 2004; Tang et al., 2005). The breeding program of *H. asinina* has been carried out at Sichang Marine Science Research and Training Station (SMaRT), Chulalongkorn University since the last decade. Founders of the established stocks were originated from various geographic locations of both the Gulf of Thailand (east) and the Andaman Sea (west).

To minimize the reduction of genetic diversity of our domesticated stocks, mating between different abalone lines has been performed. Male and female used for mass spawning each time was about 80 individuals for each sex. The genetic diversity levels of domesticated stocks should be maximized by efficient breeding plans and regularly monitored by appropriate genetic markers to reveal possible changes in variability caused by genetic drift, inbreeding, or selection (Allendorf & Ryman, 1987).

Previously, genetic diversity of wild *H. asinina* in Thailand were examined by six microsatellite loci (*CUHas1*, *CUHas2*, *CUHas3*, *CUHas4*, *CUHas5* and *CUHas8*). Analysis of genetic heterogeneity, including allele frequency comparison and pairwise F_{ST} analysis, indicated population differentiation between natural *H. asinina* from the Gulf of Thailand and the Andaman Sea ($P<0.0001$; Tang et al., 2004; 2005).

In this study, EST-derived (type I) microsatellites were developed. The developed markers along with previously published non-coding (type II) microsatellites were tested against wild and hatchery-propagated stocks of *H. asinina*. The basic information is useful for improving the efficiency of breeding and domestication of *H. asinina*.

Materials and Methods

Experimental Animals

Wild specimens of *H. asinina* were collected from Koh Kong, Cambodia (CAME, *N* = 20) and Talibong Island, Trang province (TRGW located in the south of Thailand, *N* = 25). Hatchery populations were also collected including the first generation (G1) progeny established from founders originating from Samet Island, Rayong province (SAMH, *N* = 16) and the second generation (G2) progeny cultured at the Southeast Asian Fisheries Development Center-Aquaculture Department (SEAFDEC/AQD), Iloilo, Phillipines (PHIH, *N* = 20). Moreover, the eight generation (G8) sample was bred from abalone broodstock maintained at Sichang Marine Research and Training Station (SMaRT), Chonburi province by mass spawning. Founders of this population were originated from various geographic locations in Thailand with no record. The propagated stocks were divided to 2 groups and cultured separately at Sichang Island (SMaRT-SICH, *N* = 181) and at a hatchery in Trang province (SMaRT-TRGH, *N* = 48) (Table 1 and Figure 1). Dissected foot tissue or epipodial tentacles were sampled and kept at -20 °C or in absolute ethanol until needed.

DNA Extraction

Total DNA was extracted from the epipodial tentacles or foot muscles of each abalone using a phenol-chloroform-proteinase K method (Klinbunga et al., 2001). The concentration of extracted DNA was spectrophotometrically measured. DNA was kept at 4 °C until required.

Identification of ESTs Containing Microsatellites and Genotyping

Six hundred forty-nine EST sequences from testis, ovary, and hemocyte cDNA libraries of *H. asinina* (Amparyup et al., 2004) and additional 2227 ESTs from other *Haliotis* species deposited in GenBank (http://ncbi.nlm.nih.gov) were *in silico* analyzed for the existence of simple repeats using the Tandem Repeat Finder 2.2 (Benson, 1999). Sequences that contained the number of repeats greater than 10 for mononucleotide, 6 for dinucleotide, 4 for trinucleotide, and 3 for tetranucleotide, pentanucleotide, and hexanucleotide repeats were regarded as ESTs containing microsatellites (Stalling et al., 1991; Tassanakajon et al., 1998).

Primer Design and Genotyping

Twelve primer pairs that flank microsatellites in ESTs were designed using Primer Premier 5.0 (Table 2). The amplification reaction was performed against

Table 1. Sample collection sites, geographic coordinates and sample sizes of *H. asinina* specimens

Sampling site	Geographic coordinates	Abbreviation	Sample size (*N*)
Wild specimens			
Talibong Island, Trang (Andaman Sea)	7°15′0′′N, 90°22′60′′E	TRGW	25
Koh Kong, Cambodia (Gulf of Thailand)	11°36′55.12′′N, 102°59′1.68′′E	CAME	20
Hatchery-propagated stocks			
First generation (G1), Samet Island, Rayong (Gulf of Thailand)	12°34′2.39′′N, 101°27′10.19′′E	SAMH	16
Second generation (G2), the Philippines	10°30′35′′N, 122°29′59′′E	PHIH	20
Eighth generation (G8), Trang hatchery	13°8′58.76′′N, 100°48′29.54′′E	SMaRT-TRGH	48
Eighth generation (G8), Sichang Marine Research and Training Station, Chonburi	13°8′58.76′′N, 100°48′29.54′′E	SMaRT-SICH	181
Total			310

Table 2. Names of loci, characteristics of repeats primer sequences, and annealing temperature (Ta) of type I and type II microsatellite loci used in this study

Locus	Gene homologue	Repeat motif	Primer Sequence (5' - 3')	Ta (°C)
Type I microsatellites				
PHe4	*B-cell translocation gene 2*	$(CAG)_4(CAA)_4$	F: ACAAGGAAAGCAGTCTCAGC R: TCTTACACGAATACCCAATC	50
PHe24	Unknown	$(CCGA)_8$	F: TATTCCGCTTCATAGATTGT R: AGAGCCTATTCTGCCACG	50
PHe156	Unknown	$(TG)_4(GTGTGC)_6$	F: ACCCCACGAAACAAGCAAT R: ATTGGCTTCCCCTTTTACAT	49
PHe177	Unknown	$(TA)_7$	F: AACACATTCACTGATTTAGG R: ATTATTATCTGACCGAGCA	50
PHe221	*Thrombospondin 1*	$(TAC)_5$	F: CTGTTGGCGGTTGTCCTT R: TATCTGGTGTAGCCGTATCC	50
PO97	Unknown	$(TAG)_{10}$	F: CAGGGGCTTGGTGGATGGAG R: CAAACAGTGAACAGAGGGGG	58
PO179	*Cysteine proteinase 1 precursor*	$(TGAG)_5$	F: CACGCATACGCTAGCAGTCA R: GGGAAGCAGGGGTCTTTGTT	53
PT101	*Ribosomal protein L41*	$(CAG)_7$	F: CTTGGTGTTGTGGCGTGTG R: TCTACTGACTATCTCATTCTCTGG	50
PT102	*Ribosomal protein L41*	$(ATG)_{10}$	F: TGCTTGTGAAGGACAGTATC R: ACCAACACGCACTGACAT	50
DW404	Unknown	$(GAT)_8$	F: TCCCTATCGGTGTGGTCG R: GCTCTCCTGAGGAATGCGT	57
DW455	Unknown	$(TG)_{13}(GC)_7$	F: CGGACAAAGATACTTGCTCA R: ATCAATGCTGGTCAAACGGA	59
DW503	Unknown	$(ATG)_{12}$	F: AGTCCCAGGTGTAAGAGCAT R: TGAACAGAGGGGTTGTCGTG	57
Type II microsatellites				
Haμ9	-	$(CA)_8CG(CA)_4AA(CA)_3$	F: CCACCCCCATCCCACCCCT R: ATTGCATCACCTACGAAAACAAA	57
Haμ10	-	$(CA)_{10}AA(CA)_2(GA)_6CA(GA)_6$	F: CTAATTGCAGTTATGGGCGTTC R: AAGGCTTTCGGAGGACGTGTC	63

Figure 1. Map of Thailand indicating sampling sites of wild and hatchery-propagated samples of *H. asinina* used in this study. Dots represent sample locations from which *H. asinina* was collected. Wild abalone were collected from Trang (TRGW) and Koh Kong (Cambodia, CAME). Hatchery stocks were collected from Samei Island (SAMH). G8 abalone bred at Sichang Marine Research and Traning Station (SMaRT) were cultivated at Sichang Island (SMaRT-SICH) and at a hatchery in Trang province (SMaRT-TRGH). The Philippines sample (PHIH) is not on this map.

genomic DNA of 20 individuals of natural and cultivated abalone in a 15 µl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each primer, 1 unit of DyNazyme^{TM} II DNA Polymerase (Finnzymes) and 50 ng of DNA template. PCR was carried out by predenaturation at 94 °C for 3 min, followed by 10 cycles of 94 °C for 45 s, 10 °C above the annealing temperature of each primer pair for 1 min, with a reduction of 2 °C in every other cycle, and extension at 72 °C for 45 s and 30 cycles of 94 °C for 45 s, at the annealing temperature for 1 min and 72 °C for 45 s. The final extension was carried out at 72 °C for 10 min. The PCR products were electrophoretically analyzed through 1.5 - 2.0% agarose gels. The successfully amplified products were further analyzed by 6% denaturing polyacrylamide gels (Sambrook & Russell, 2001). Electrophoresis was carried out at 35 W for approximately 2.5 - 3 h and bands were visualized by silver staining.

In addition, six loci of type II microsatellites; *Haµ1M*, *Haµ2K*, *Haµ3C*, *Haµ3E*, *Haµ9* and *Haµ10* (Selvamani et al., 2000); were also screened against hatchery-propagated and natural abalone specimens (*N* = 20). Loci showing successful amplification results were selected to examine genetic diversity of abalone samples. Subsequently, the reverse primer of *Haµ9* and *Haµ10* was 5´-terminally labeled with HEX and FITC, respectively. The PCR products of those loci were detected by a fluorescent scanner (Molecular Image Phosphor^{FX}, BioRad).

Data Analysis

Observed and expected heterozygosity were calculated (Nei, 1987). Hardy-Weinberg expectations and genotypic disequilibrium were analyzed using the exact test (Guo & Thompson, 1992) using Genepop (Raymond & Rousset, 1995; Rousset, 2005). The number of alleles per locus, allelic richness (A_R) for each locus and inbreeding coefficient (F_{IS}) were calculated and statistically tested using FSTAT version 2.9.3.2 (Goudet, 2002). Geographic heterogeneity in allele and genotype distribution frequencies among compared samples was carried out using the exact test (Guo & Thompson, 1992) using Genepop (Raymond & Rousset 1995; Rousset, 2005). F_{ST} between pairs of samples (Weir & Cockerham, 1984) was determined if it was significantly different from zero using FSTAT version (Goudet, 2002). The significance levels for multiple comparisons were adjusted using a Bonferroni correction (Rice, 1989).

Results

Identification of ESTs Containing Microsatellites

In total, 178 microsatellite-containing motifs were found from data mining. Twelve EST sequences were selected and a primer pair that flank microsatellites regions of each EST was designed. These type I microsatellites included 9 loci from local cDNA libraries and 3 loci (*DW404*, *DW455*, and *DW503*) from GenBank (Table 2). In addition, six loci of type II microsatellites; *Haµ1M*, *Haµ2K*, *Haµ3C*, *Haµ3E*, *Haµ9* and *Haµ10* (Selvamani et al. 2000) were also screened.

All type I microsatellite were successfully amplified against genomic DNA of *H. asinina*. However, non-specific amplification products were found at *PHe156*, *PO97* and *PO179* loci. The PCR products of loci *PHe221* (approximately 700 bp) and *DW404* (approximately 1700 bp) were larger than that of the expected product (121 and 289 bp, respectively) suggesting that the amplified region contained the relatively large intron (s). Accordingly, PCR products of 7 loci of type I microsatellites (*PHe4*, *PHe24*, *PHe177*, *PT101*, *PT102*, *DW455* and *DW503*) were further tested. For type II microsatellite, *Haµ3C* generated non-specific amplification products whereas 5 loci (*Haµ1M*, *Haµ2K*, *Haµ3E*, *Haµ9* and *Haµ10*) were further tested.

Four loci of type I (*DW455*, *DW503*, *PHe177*, and *PT102*) and 2 loci of type II microsatellites (*Haµ9* and *Haµ10*) were polymorphic. They were selected for determination of genetic diversity in wild and hatchery-propagated *H. asinina*. *DW455*, *DW503*, *PHe177, PT102, Haµ9* and *Haµ10* generated 10, 7, 6, 6, 10, and 12 alleles, respectively. Sizes of allele distribution for respective loci were 191 - 271, 174 - 201, 157 - 183, 173 - 190, 106 - 137, and 134 - 181 bp (Table 3).

Genotyping and Allele Distribution Frequencies of Developed Microsatellites

At the *DW455* locus, 6 and 5 alleles were found in wild samples (CAME and TRGW) while 7 and 3 alleles were observed in G1 and G2 generations from Rayong (SAMH) and the Philippines (PHIH). The domesticated SMaRT-TRGH and SMaRT-SICH stocks had 7 and 8 alleles. A 191 bp allele was the most common allele in CAME (0.550) and SAMH (0.406) abalone from the Gulf of Thailand whereas a 267 bp alleles had the highest frequency (0.520) in the Andaman sample (TRGW). A 233 bp allele (0.500) had the greatest frequency from the Philippines. Two alleles (233 and 251 bp) were found in both coastal populations of Thai abalone at low frequencies. For hatchery samples, the 191 bp was the most common allele for both SMaRT-TRGH and SMaRT-SICH samples. A 267 bp alleles commonly found in wild abalone from the Andaman Sea was found in only 0.010 and 0.014 of respective stocks.

At the locus *DW503*, 7 alleles were found in abalone from the TRGW sample for which a 174 bp allele is the most common allele (0.540). A lower number of alleles (2 - 3) per locus was observed in SAMH, CAME and PHIH. The 174 allele was not found in these samples. The most common allele in our domesticated stocks (SMaRT-TRGH and SMaRT-SICH) was the same as that of the Gulf of Thailand samples (195 bp). A 174 bp allele commonly found in TRGW was found at extremely low frequencies (0.021 and 0.017) in domesticated stocks.

Table 3. Allele sizes (base pairs, bp), allele frequencies, number of alleles per locus (NA), allelic richness (A_R), number of specimens (*N*), observed (H_o) and expected (H_e) heterozygosity and inbreeding coefficient (F_{is}) of each microsatellite locus in wild and hatchery stocks of *H. asinine*. Effective population size (N_e) of each stock is also shown

Locus	Allele (bp)	Wild			Hatchery		
		CAME	TRGW	SAMH	PHIH	SMaRT-TRGH	SMaRT-SICH
DW455	191	0.550	-	0.406	-	0.448	0.470
	195	0.075	-	0.031	-	-	0.028
	203	0.050	-	0.031	0.200	0.146	0.028
	209	0.150	-	0.094	0.300	0.198	0.185
	217	0.075	-	0.344	-	0.021	0.072
	233	0.100	0.040	0.063	0.500	0.135	0.044
	251	-	0.120	0.031	-	0.042	0.160
	255	-	0.180	-	-	-	-
	267	-	0.520	-	-	0.010	0.014
	271	-	0.140	-	-	-	-
NA		6	5	7	3	7	8
A_R		5.788	4.874	7.000	3.000	5.695	6.328
N		20	25	16	20	48	181
H_o		0.700	0.440	0.875	1.000	0.875	0.691
H_e		0.668	0.675	0.724	0.636	0.726	0.690
F_{is}		0.014	0.307	0.217	0.600	0.208	0.046
DW503	174	-	0.540	-	-	0.021	0.017
	177	-	0.060	-	-	-	-
	180	-	0.100	0.031	0.200	0.094	0.177
	183	-	0.140	-	0.175	-	-
	189	-	0.020	-	-	-	-
	195	0.700	0.120	0.938	-	0.802	0.762
	201	0.300	0.020	0.031	0.625	0.083	0.044
NA		2	7	3	3	4	4
A_R		2.000	6.236	3.000	3.000	3.511	3.547
N		20	25	16	20	48	181
H_o		0.400	0.800	0.125	0.550	0.333	0.440
H_e		0.431	0.674	0.123	0.553	0.344	0.402
F_{is}		0.073	-0.222	-0.017	0.005	0.072	-0.078
PHe177	157	-	0.160	-	0.200	0.042	0.086
	163	0.075	-	-	0.525	0.083	0.122
	167	-	0.260	-	0.275	0.094	0.008
	171	-	0.580	-	-	-	0.025
	181	0.925	-	0.969	-	0.719	0.693
	183	-	-	0.031	-	0.063	0.066
NA		2	3	2	3	5	6
A_R		1.994	3.000	2.000	3.000	4.661	4.634
N		20	25	16	20	48	181
H_o		0.150	0.360	0.063	0.750	0.458	0.463
H_e		0.142	0.582	0.063	0.624	0.467	0.575
F_{is}		-0.056	0.423*	0.000	-0.208	-0.057	0.058
PT102	173	-	0.460	-	-	0.021	0.025
	177	-	0.120	0.031	0.200	0.156	0.188
	183	-	0.080	-	0.175	-	-
	186	0.725	0.100	0.938	-	0.750	0.738
	188	0.275	0.220	0.031	-	0.073	0.050
	190	-	0.020	-	0.625	-	-
NA		2	6	3	3	4	4
A_R		2.000	5.613	3.000	3.000	3.613	3.302
N		20	25	16	20	48	181
H_o		0.350	0.409	0.125	0.550	0.396	0.476
H_e		0.409	0.520	0.123	0.553	0.412	0.416
F_{is}		0.147	0.293	-0.017	0.005	0.114	-0.107
Haμ9	106	-	-	-	0.200	-	-
	110	-	-	-	0.200	-	-
	122	0.325	0.600	-	0.300	0.031	0.088
	124	0.250	-	0.219	-	0.490	0.268
	126	0.325	0.080	0.156	0.300	0.417	0.271
	128	0.050	-	0.375	-	0.042	0.207
	130	0.050	-	0.250	-	0.021	0.160

Table 3 (cont.)

Locus	Allele (bp)	Wild CAME	Hatchery TRGW	Locus	Allele (bp)	Wild CAME	Hatchery TRGW
	132	-	0.260	-	-	-	0.003
	137	-	0.060	-	-	-	0.003
NA		5	4	4	4	5	7
A_R		4.928	3.973	4.000	4.000	3.975	5.283
N		20	25	16	20	48	181
H_o		0.450	0.680	1.000	1.000	0.458	0.564
H_e		0.740	0.574	0.748	0.759	0.590	0.755
F_{is}		0.398	-0.215	0.352	-0.329	0.250	0.231*
Haμ10	134	-	0.220	-	-	-	-
	140	-	0.280	-	-	-	-
	142	-	0.260	-	-	-	-
	144	-	0.240	-	-	-	0.008
	154	0.050	-	0.063	-	-	0.036
	158	0.150	-	0.125	0.350	0.042	0.047
	161	0.100	-	0.031	0.650	-	0.130
	163	0.025	-	0.281	-	0.271	0.320
	167	0.275	-	0.156	-	0.021	0.221
	173	0.100	-	0.219	-	0.375	0.066
	177	0.275	-	0.031	-	0.115	0.077
	181	0.025	-	0.094	-	0.177	0.094
NA		8	4	8	2	6	9
A_R		8.000	4.000	7.563	2.000	5.353	7.632
N		20	25	16	20	48	181
H_o		0.850	0.480	0.688	0.400	0.625	0.526
H_e		0.823	0.763	0.845	0.467	0.747	0.777
F_{is}		-0.034	0.426*	0.191	0.146	0.163	0.323*
Mean NA		4.167	4.833	4.500	3.000	5.167	6.333
Mean H_o		0.483	0.528	0.479	0.708	0.524	0.527
Mean H_e		0.534	0.631	0.438	0.599	0.548	0.602
Overall F_{is}		0.108	0.176*	-0.099	0.189	0.050	0.119*
N_e		64.0	14.1	13.8	1.5	20.9	25.7

*Significance at *P*-value < 0.0083 following Bonferroni's adjustment. The most common allele of each locus for each group of sample is boldfaced. H_o and H_e are illustrated in boldface if significant deviation from HWE is observed.

Limited numbers of alleles of *PHe177* were observed in CAME (2 alleles), TRGW (3 alleles), SAMH (2 alleles) and the Philippines (3 alleles) but a greater number of alleles was observed in SMaRT-TRGH (5 alleles) and SMaRT-SICH (6 alleles). The most common alleles (180 bp) in SAMH, CAME and domesticated stocks (SMaRT-TRGH and SMaRT-SICH) was 0.969, 0.925, 0.719 and 0.693, respectively. The TRGW and PHIH samples possessed the non-overlapped common alleles of 171 (0.580) and 163 (0.525) bp.

A greater number of alleles in TRGW (6 alleles) than the Gulf of Thailand (2 - 3 alleles) samples were observed at the *PT102* locus. The most common allele in the Gulf of Thailand (SAMH and CAME, 186 bp with the frequencies of 0.938 and 0.725), the Andaman Sea (TRGW, 173 bp with the frequency of 0.460) and the Philippines (PHIH, 190 bp with the frequency of 0.625) was different. A 186 bp allele was also common in domesticated stocks (SMaRT-TRGH and SMaRT-SICH with the frequencies of 0.750 and 0.738).

Comparable polymorphic levels were observed when the same sample set was screened with type II microsatellites. At the locus *Haμ9*, 5 and 7 alleles were found in SMaRT-TRGH and SMaRT-SICH whereas only 4 - 5 alleles were observed in other groups of samples. The most common allele in TRGW (122 bp, with the frequency of 0.600) was found in lower frequencies in other samples (0.031 – 0.325).

Haμ10 showed non-overlapping allele distribution between abalone originating from the east (CAME and SAMH) and the west (TRGW) coasts. More numbers of alleles were found in the Gulf of Thailand (6 - 9 alleles) than the Andaman Sea (4 alleles) samples. Only a few individuals (allele frequency = 0.008) of the domesticated SMaRT-SICH stock exhibited the Andaman Sea allele (143 bp) while no Andaman Sea allele was observed in SMaRT-TRGH.

Genetic Diversity and Effective Population Size in Domesticated and Wild Stocks of *H. asinina*

The average number of alleles per locus and average observed heterogeneity in wild populations were 4.167 and 0.483 for CAME (east coast) and 4.833 and 0.528 for TRGW (west coast). Relatively high genetic diversity was found in the hatchery stocks which were 3.000 and 0.708, 4.500 and 0.479, 5.167 and 0.524, and 6.333 and 0.527 for PHIH (G2, Philippines), SAMH (G1), SMaRT-TRGH (G8) and SMaRT-SICH (G8), respectively (Table 3).

Effective population sizes were estimated. The highest N_e was found in wild CAME sample (N_e = 64.0) while a lower N_e was found in wild TRGW sample (N_e = 14.1). For hatchery-propagated samples, the lowest N_e was found in PHIH (N_e = 1.5) and greater N_e values were found in SAMH, SMaRT-TRGH and SMaRT-SICH (N_e = 13.8, 20.9 and 25.7, respectively) (Table 3).

Genetic Differences Between Wild Populations and Between Domesticated Stocks of *H. asinina*

F_{ST}-statistics and the exact test between pairs of samples at each locus clearly revealed significant differences between wild abalone from the east (CAME) and west (TRGW) coasts and between different groups of domesticated abalone from the Philippines and Thailand (Table 4). Genetic heterogeneity between the Gulf of Thailand (CAME and SAMH) samples were observed at *DW503*, *PT102*, *Haμ9* and *Haμ10* loci (P<0.05). SMaRT-TRGH showed a genetically difference from SAMH at 3 loci (*DW455, Haμ9* and *Haμ10*) and from CAME at 6 loci (*DW455*, *DW503*, *PHe177, PT102*, *Haμ9* and *Haμ10*).

SMaRT-SICH showed a genetically difference from SAMH at only 1 locus (*DW455*) and from CAME at 4 loci (*DW455*, *DW503*, *PT102* and *Haμ10*). Both domesticated stocks (SMaRT-TRGH and SMaRT-SICH) were genetically different from TRGW (west) and PHIH at all loci. Interestingly, the domesticated SMaRT-TRGH and SMaRT-SICH bred from the same groups of brooders but cultured at different locations exhibited genetic heterogeneity at 4 loci (*DW455, Phe177*, *Haμ9* and *Haμ10*).

Discussion

Loss of genetic variation in small populations owing to genetic drift and inbreeding is commonly observed in captive stocks (Sbordoni et al., 1986; Allendorf & Ryman, 1987; Goyard et al., 2003; Evans et al., 2004; Dixon et al., 2008). Inbreeding depression in hatchery-propagated populations affect growth, viability and reproductive performance of stocks with limited genetic diversity (Gjedrem, 2005). Microsatellites have been used for identification of population structure, parentage assignment, construction of genetic linkage map and monitoring changes in genetic diversity in domesticated stocks of various aquaculture species (Selvamani et al., 2000 and 2001; Li et al., 2004; Pan et al., 2004; Hara & Sekino, 2007; Staelens et al., 2008).

Although type I microsatellites developed from ESTs may less polymorphic than type II (non-coding) microsatellites, they are more conserved making them more transferable across species for comparative analysis (Liu & Cordes, 2004). In the Pacific abalone (*Haliotis discus*), ten type I microsatellites were developed from 1476 EST sequences deposited in GenBank. The number of alleles ranged from 2 to 13 and the values of H_o and H_e varied from 0.122 to 0.861 and 0.245 to 0.931, respectively. Six of ten loci conformed to the Hardy-Weinberg equilibrium (Zhan et al., 2008).

In the present study, 4 EST-derived microsatellites were successfully developed. The number of alleles per locus of type I microsatellites developed in this study (*DW455*, *DW503*, *PHe177*, and *PT102*) varied from 6 to 10 which is comparable those of EST-derived microsatellite in *H. discus* (Zhan et al., 2008). However, higher numbers of alleles per locus were observed in type II microsatellites of several abalone species; for instance, 3 - 26 alleles in *H. asinina* (Tang et al., 2004 and 2005), 2 - 32 alleles in *H. midae* (Evans et al., 2004); 6 - 45 alleles in *H. discus hannai* (Li et al., 2004); and 10 - 24 alleles in *H. fulgens* (Gutierrez-Gonzalez & Perez-Enriquez, 2005).

Our domesticated stocks at SMaRT were initially established from abalone covering both coastal sides of Thai waters (Gulf of Thailand and Andaman Sea). Therefore, genotyping of the maintained stocks is carried out and specimens originating from Cambodia (CAME) and Samet Island (SAMH) located in the east coast and Talibong Island (TRGW) located in the west coast were included to identify whether the domesticated stocks were biasedly contributed from founders originating from different coastal regions of Thai waters.

In the present study, specimens were genotyped by both type I (*DW455*, *DW503*, *PHe177*, and *PT102*) and type II microsatellites (*Haμ9* and *Haμ10*). The allele

Table 4. Genetic heterogeneity analysis based on the exact test of allelic (above diagonal) and genotype (above diagonal in brackets) distribution frequencies and F_{ST} values (below diagonal) between pairs of *H. asinina* samples.

	TRGW	CAME	SAMH	PHIH	SMaRT-TRGH	SMaRT-SICH
TRGW	-	*P*<0.0001 (*P*<0.0001)	*P*<0.0001 (*P*<0.0001)	*P*<0.0001 (*P*<0.0001)	*P*<0.0001 (*P*<0.0001)	*P*<0.0001 (*P*<0.0001)
CAME	0.410*	-	*P*<0.0001 (*P*<0.0001)	*P*<0.0001 (*P*<0.0001)	*P*<0.0001 (*P*<0.0001)	*P*<0.0001 (*P*<0.0001)
SAMH	0.096*	0.346*	-	*P*<0.0001 (*P*<0.0001)	*P*<0.0001 (*P*<0.0001)	*P*<0.0001 (*P*=0.0001)
PHIH	0.461*	0.316*	0.369*	-	*P*<0.0001 (*P*<0.0001)	*P*<0.0001 (*P*<0.0001)
SMaRT-TRGH	0.080*	0.365*	0.077*	0.384*	-	*P*<0.0001 (*P*<0.0001)
SMaRT-SICH	0.040*	0.332*	0.057*	0.348*	0.041*	-

*P<0.0001

distribution frequencies of microsatellites indicated that the present generation of SMaRT-TRGH and SMaRT-SICH stocks exhibited greater levels of the "Gulf of Thailand alleles" than the "Andaman alleles" at all loci. More importantly, *Haμ10* showed non-overlapping alleles between wild abalone originating from the east and the west coasts and only 3 individuals (0.83%) of the domesticated SMaRT-SICH stock exhibited the Andaman allele (144 bp). Similarly, only the Gulf of Thailand alleles were observed in the SMaRT-TRGH sample. This clearly indicated that founders originating from the Gulf of Thailand have been more genetically contributed to the present stocks than those from the Andaman Sea. The information opens the possibility that *H. asinina* from the east coast should have better survival rates and be more adaptable to the cultivated conditions than those from the west coast.

Different contribution of founders from different coastal regions of abalone in Thai waters should be further verified by additional molecular markers exhibiting fixed genotypes (or alleles) in either coastal sample to confirm this circumstance. The information will partially resolve problems from mass mortality previously occurred in our propagated stocks leading to the sustainability of abalone breeding programs.

Previously, genetic diversity of our hatchery-propagated stocks (6^{th} generation, group A; N = 102 and group B; N = 120 maintained at SMaRT) and a wild sample originating from Talibong Island (N = 25) was examined using 5 loci of type II microsatellites. The number alleles of *CUHas2, CUHas3, CUHas8* and *Haμ13* in the hatchery-propagated abalone was greater than that of the Talibong sample. In contrast, those of the *Haμ2J* locus in the wild sample (6 alleles) were greater than in the hatchery samples (4 alleles). Observed heterozygosity in the respective hatchery samples was 0.69 ± 0.308 and 0.56 ± 0.241 which is slightly greater than that of the Talibong sample (0.52 ± 0.180; Rungpituckmana, 2007). Results from that and our studies suggested no obvious reduction of genetic diversity in domesticated *H. asinina* after propagated for several succeeding generations by mass spawning.

The reduction in genetic diversity (i.e., number of alleles per locus) relative to wild populations was reported in hatchery-propagated *H. discus hannai*. Three hatchery strains and two wild populations of *H. discus hannai* were investigated with six microsatellite loci. The hatchery strains possessed less genetic variation as revealed in lower number of alleles and lower expected heterozygosity (2 – 14 alleles per locus and 0.238 – 0.876, respectively) than natural populations (5 – 45 alleles and 0.525 – 0.973, respectively). This circumstance may be caused by suboptimal contribution of parents in the previous generation increasing the effect of genetic drift (Li et al., 2004).

Disregarding the PHIH sample, the number of alleles per locus and expected heterozygosity of abalone in this study were 2 – 8 and 0.063 – 0.845 in the cultured stocks and were 2 - 8 alleles and 0.142 – 0.823 in wild stocks, respectively. Practically, eighty pairs of abalone were used for the generation of subsequent generations of *H. asinina* stocks for our breeding programs (SMaRT-TRGH and SMaRT-SICH). This reflects moderate number of estimated N_e in these stocks. Therefore, severe reduction of genetic diversity in domesticated *H. asinina* is not observed at present.

Previous studies based on PCR-RFLP of *16S* and *18S rDNA* did not reveal genetic heterogeneity between wild *H. asinina* in Thailand (Klinbunga et al., 2003). In contrast, there are obviously significant differences between pairs of samples in the present study. Result from this study strongly supported the previous reports on the existence of intraspecific population differentiation of wild *H. asinina* in Thai waters based on microsatellite (Tang et al., 2004; 2005) where wild samples from the east coast and the west coast were genetically differentiated ($P<0.05$). The Philippines sample is recognized as a different stock. Moreover, the present study provided the further insights on differentiation between CAME and SAMH samples originated from the same coastal region.

The F_{ST}-statistics and exact test also indicated significant genetic heterogeneity between the hatchery-propagated stocks. Numbers of alleles and levels of observed heterozygosity in SMaRT-TRGH and SMaRT-SICH after domesticated for 8 generations and wild abalone were comparable. Since these stocks were originally bred from the same groups of founders but separated maintained at SMaRT (SMaRT-SICH) and at a farm in Trang province (SMaRT-TRGH), significant genetic differentiation reflects the occurrence of genetic drift in these propagated stocks. As a result, a larger number of brooders should be used for breeding of *H. asinina* in each generation.

In conclusions, levels of genetic diversity in wild and the present generation of domesticated stocks of *H. asinina* were examined and provided the useful information to elevate management efficiency of breeding program of this economically important species. Appropriate breeding plans have been implemented to reduce the inbreeding coefficient of the hatchery stock.

Ethical Statement

All authors declare that the present study was conducted in an ethical, professional and responsible manner following the regulation for animal care and use for scientific research of the National Center for Genetic Engineering and Biotechnology (BIOTEC) Animal Welfare Committee.

Funding Information

This research received no funding.

Author Contribution

P.P, S.P., P.R. and P.B. performed the experiments. S.J., O.R. and W.I. analyzed the data. S.K. and P.J. conceptualization, provided critical comments and final approval of the manuscript. B.K. supervised the findings and reviewed the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

We thank the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand for providing research facilities.

References

Allendorf, F. W. & Ryman, N. (1987). Genetic management of hatchery stocks. In: Ryman N & Utter F (Eds) Population genetics and fishery management. Washington Sea Grant Program, University of Washington Press. Seattle, WA.

Amparyup, P. (2004). Development of genetic markers for increasing production efficiency of the tropical abalone, *Haliotis asinina* in Thailand. [Unpublished doctoral dissertation], Chulalongkorn University.

Amparyup, P, Klinbunga, S., Preechaphol, R., Tassanakajon, A., Hirono, I., Aoki, T. & Jarayabhand, P. (2004). Expressed sequence tag analysis of ovaries and testes from tropical abalone (*Haliotis asinina*). Marine Biotechnology 6, S1-S6.

Benson, G. (1999). Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Research 27(2), 573-580. https://doi.org/10.1093/nar/27.2.573

Cook, P. A. 2019. Worldwide Abalone Production Statistics," Journal of Shellfish Research, 38(2), 401-404. https://doi.org/10.2983/035.038.0222

Dixon, T. J., Coman, G. J., Arnold, S. J., Sellars, M. J., Lyons, R. E., Dierens, L., Preston, N. P. & Li, Y. (2008). Shifts in genetic diversity during domestication of Black Tiger shrimp, *Penaeus monodon*, monitored using two multiplexed microsatellite systems. Aquaculture, 283(1), 1-6. https://doi.org/10.1016/j.aquaculture.2008.07.009

Evans, B., Bartletta, B.J., Sweijde, N., Cooke, P. & Elliotta, N. G. (2004). Loss of genetic variation at microsatellite loci in hatchery produced abalone in Australia (*Haliotis rubra*) and South Africa (*Haliotis midae*). Aquaculture, 233(1-4), 109-127. https://doi.org/10.1016/j.aquaculture.2003.09.037

Geiger, D. L. (1998). Recent Genera and Species of the Family Haliotidae Rafinesque, 1815 (Gastropoda: *Vetigastropoda*). Nautilus 111(3), 85-116.

Geiger, D. L. (2000). Distribution and biogeography of the recent Haliotidae (Gastropoda: *Vetigastropoda*) worldwide. Bolletino Malacologico 35: 57–120.

Gjedrem, T. 2005. Selection and Breeding Programs in Aquaculture. Springer, Dordrecht

Gordon, H. R. (2000). World abalone supply, markets and pricing: historical, current and future prospective. In: Abstracts of the 4th International Abalone Symposium, University of Cape Town, Cape Town, South Africa, 6-11th February 2000.

Goyard, E., Arnaud, S., Vonau, V., Bishoff, V., Mouchel, O., Pham, D., Wyban, J., Boudry, P. & AQUACOP (2003) Residual genetic variability in domesticated populations of the Pacific blue shrimp (*Litopenaeus stylirostris*) of New Caledonia, French Polynesia and Hawaii and some management recommendations. Aquatic Living Resources, 16(6), 501-508. https://doi.org/10.1016/j.aquliv.2003.07.001

Guo, S. W. & Thompson, E. A. (1992). Performing the extract test of Hardy-Weinberg proportion for multiple alleles. Biometrics, 48(2), 361-372.

Gutierrez-Gonzalez, J. L. & Perez-Enriquez, R. (2005). A genetic evaluation of stock enhancement of blue abalone *Haliotis fulgens* in Baja California, Mexico. Aquaculture, 247(1), 233-242. https://doi.org/10.1016/j.aquaculture.2005.02.021

Hara, M. & Sekino, M. (2007). Genetic differences between hatchery stocks and natural populations in Pacific abalone (*Haliotis discus*) estimated using microsatellite DNA markers. Marine Biotechnology, 9(1), 74-81. https://doi.org/10.1007/s10126-006-6060-2

Jarayabhand, P. & Paphavasit, N. (1996). A review of the culture of tropical abalone with special reference to Thailand. Aquaculture, 140(1-2), 159-168. https://doi.org/10.1016/0044-8486(95)01194-3

Jeffrey, B., Hale, P., Degnan, B. M. & Degnan, S. M. (2007). Pleistocene isolation and recent gene flow in *Haliotis asinina,* an Indo-Pacific vetigastropod with limited dispersal capacity. Mol. Ecol. 16(2), 289-304. https://doi.org/10.1111/j.1365-294X.2006.03141.x

Klinbunga, S., Siludjai, D., Wuthijinda, W., Tassanakajon, A., Jarayabhand, A. & Menasveta, P. (2001). Genetic heterogeneity of the giant tiger shrimp (*Penaeus monodon*) in Thailand revealed by RAPD and mtDNA-RFLP analyses. Marine Biotechnology, 3(5), 428-438. https://doi.org/10.1007/s10126-001-0055-9

Klinbunga, S., Pripue, P., Khamnamtong, N., Tassanakajon, A., Jarayabhan, P., Hirono, I., Aoki, T., Menaseveta, P. (2003). Genetic diversity and molecular markers of the tropical abalone (*Haliotis asinina*) in Thailand. Marine Biotechnology, 5(5), 505–517. https://doi.org/10.1007/s10126-002-0108-8

Klinbunga, S., Amparyup, P., Leelatanawit, R., Tassanakajon, A., Hirono, I., Aoki, T., Jarayabhand, A. & Menasveta, P. (2004). Species-identification of the tropical abalone (*Haliotis asinina*, *Haliotis ovina* and *Haliotis varia*) in Thailand using RAPD and SCAR markers. Journal Biochemistry and Molecular Biology, 37(2), 213-222. https://doi.org/10.5483/bmbrep.2004.37.2.213

Li, Q., Park, C., Endo, T. & Kijima, A. (2004). Loss of genetic variation at microsatellite loci in hatchery strains of the Pacific abalone (*Haliotis discus hannai*). Aquaculture, 235(1), 207-222. https://doi.org/10.1016/j.aquaculture.2003.12.018

Liu, Z. F. & Cordes, J. F. (2004). DNA marker technologies and their applications in aquaculture genetics. Aquaculture, 238(1-4), 1-37. https://doi.org/10.1016/j.aquaculture.2004.05.027

Nei, M. (1987). Molecular evolutionary genetics, Columbia University Press, New York.

Pan, Y. W., Chou, H. H., You, E. M. & Yu, H. T. (2004). Isolation and characterization of 23 polymorphic microsatellite markers for diversity and stock analysis in tiger shrimp (*Penaeus monodon*). Molecular Ecology Notes, 4(3), 345–347. https://doi.org/10.1111/j.1471-8286.2004.00692.x

Raymond, M. & Rousset, F. (1995). GENEPOP (Version 1.2): a population genetics solfware for exact tests and ecumenicism. Journal of Heredity, 86, 248-249. https://doi.org/10.1093/OXFORDJOURNALS.JHERED.A111573

Rungpituckmana, T. (2007). Genetic diversity and microsatellite patterns in hatchery-produced Thai abalone *Haliotis asinina*. [Unpublished master dissertation], Chulalongkorn University.

Sambrook, J., & Russell, D. W. (2001). Molecular Cloning: A Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press, New York.

Sbordoni, V., De Matthaeis, E., Cobolli-Sbordoni, M., La Rosa, G. & Mattoccia, M. (1986). Bottleneck effects and the depression of genetic variability in hatchery stocks of *Penaeus japonicus* (Crustacea, Decapoda). Aquaculture, 57, 239–251.

Selvamani, M. J. P., Degnan, S. M., Degnan, B.M. (2001). Microsatellite genotyping of individual abalone larvae: parentage assignment in aquaculture. Marine Biotechnology, 3(5), 478-485. https://doi.org/10.1007/s1012601-0062-x

Selvamani, M. J. P., Degnan, S. M., Paetkau, D., Degnan, B. M. (2000). Highly polymorphic microsatellite loci in the Heron Reef population of the tropical abalone *Haliotis asinina*. Molecular Ecology, 9(8), 1184-1186. https://doi.org/10.1046/j.1365-294x.2000.00954-9.x

Shepherd, S. A., Lowe, D. & Partington, D. (1992). Studies of southern Australian abalone (genus *Haliotis*) XIII: larval dispersal and recruitment. Journal Experimental Marine Biology and Ecology 164:247-260.

Staelens, J., Rombaut, D., Vercauteren, I., Argue, B., Benzie, J. & Vuylsteke, M. (2008). High-Density Linkage Maps and Sex-Linked Markers for the Black Tiger Shrimp (*Penaeus monodon*). Genetics, 179(2), 917-925. https://doi.org/10.1534/genetics.107.080150

Stalling, R. L., Ford, A. F., Nelson, D., Torney, D. C., Hildebrand, C. E. & Moyzis, R.K. (1991). Evolution and distribution of (GT)n repetitive sequences in mammalian genomes. Genomics, 10(3), 807-815. https://doi.org/10.1016/0888-7543(91)90467-S

Tang, S., Popongviwat, A., Klinbunga, S., Tassanakajon, A., Jarayabhand, P. & Menasveta, P. (2005). Genetic heterogeneity of the tropical abalone (*Haliotis asinina*) revealed by RAPD and microsatellite analyses. Journal Biochemistry and Molecular Biology, 38(2), 182-190. https://doi.org/10.5483/bmbrep.2005.38.2.182.

Tang, S., Tassanakajon, A., Klinbunga, S., Jarayabhand, P. & Menasveta, P. (2004). Population structure of tropical abalone (*Haliotis asinina*) in coastal waters of Thailand determined using microsatellite markers. Marine Biotechnology, 6(6), 604-611. https://doi.org/10.1007/s10126-004-2300-5

Tassanakajon, A., Pongsomboon, S., Jarayabhand, P., Klinbunga, S. & Boonsaeng, V. (1998). Genetic structure in wild populations of the black tiger shrimp (*Penaeus monodon*) using randomly amplified polymorphic DNA analysis. Journal of Marine Biotechnology, 6(4), 249-254.

Weir, B. S. & Cockerham, C. C. (1984). Estimation *F*-statistics for the analysis of population structure. Evolution, 38(6), 1358-1370. https://doi.org/10.1111/j.1558-5646.1984.tb05657.x

Zhan, A., Bao, Z., Wang, M., Chang, D., Yuan, J., Wand, X., Hu, X., Liang, C. & Hu, J. (2008). Development and characterization of microsatellite markers for the Pacific abalone (*Haliotis discus*) via EST database mining. Journal of Ocean University of China (Oceanic and Coastal Sea Research), 7(2), 219-222. https://doi.org/DOI:10.1007/S11802-008-0219-6