CHAPTER III

MATERIALS AND METHODS

3.1 Samples

The hatchery-propagated *H. asinina* specimens were collected form Sichang Marine Science Research and Training Station (SMaRT). Chulalongkorn University (G8; N = 102). Epipodial tentacles (Figure 3.1) of fifty individuals of abalone were excised, placed in absolute ethanol and kept at the -20 °C until used. Subsequently, the foot muscle was additional collected from the remaining abalone and kept at -80 °C. In addition, wild specimens originating from Talibong Island, Trang province located in the Andaman Sea were collected (N = 25).

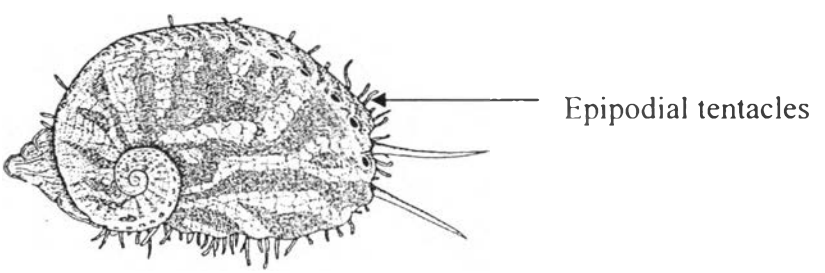

Figure 3.1 Epipodial tentacles (arrow) of abalone (Fallu, 1991)

To examine the possible correlation between genotypes of microsatellites and the body weight of the hatchery-propagated sample, the body weight of 280 individuals of the group B sample was weighed. Fifteen percent of abalone exhibiting from the top (BL, N = 40, $\overline{X}$ = 12.2 ± 1.451 g), the middle (BM, N = 40, $\overline{X}$ = 7.40 ± 0.372 g) and the bottom (BS, N = 40, $\overline{X}$ = 3.90 ± 0.580 g) according to the body weight were selected and used for association analysis between microsatellite genotypes and the body weight of these abalone. The foot muscle of each abalone was dissected out and kept at -80 °C.

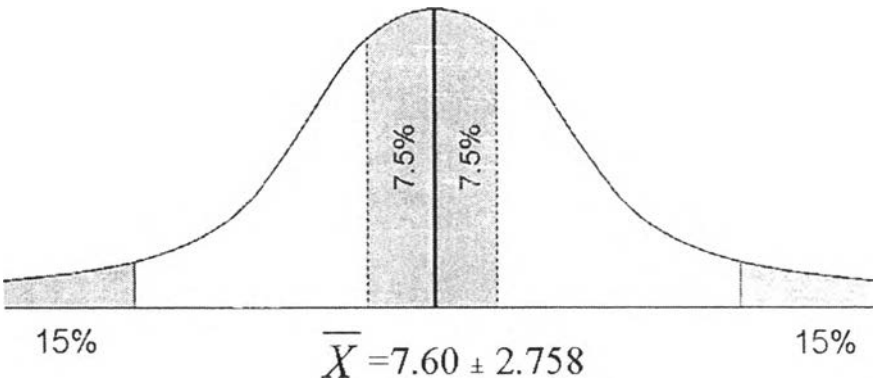

Figure 3.2 Diagram showing the sampling collection scheme of the group B sample used for association analysis between microsatellite genotype and the body weight of abalone.

Table 3.1 Date of culture, collection date and age at the collection date of samples used in this thesis

Sample	Date of culture	Collection date	Age at the collection date
G8	Nov 2006	22-23 Mar 2007	4 months (N = 49)
		and 24 Jun 2007	and 8 months (N = 53)
B	Sep 2006	30 Aug 2007	11 months

3.2 DNA extraction

Genomic DNA was extracted from each abalone using a phenol chloroform–proteinase K method (Klinbunga et al., 1999). Approximately 50-100 mg of foot of *H. asinina* was sniped into tiny pieces in a 1.5 ml microcentrifuge tube containing 600 µl of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl; pH8.0) and briefly homogenized with a micropestle. SDS 10% and RNase A (10 mg/ml) solutions were added to a final concentration of 1.0% (w/v) and 100 µl/ml, respectively. The resulting mixture was then incubated at 37°C for 1 hour. At the end

of the incubation period, a proteinase K solution (10 mg/ml) was added to the final concentration of 200 µg/ml and further incubated at 55°C for 3-4 hours. An equal volume of buffer-equilibrated phenol was added and gently mixed for 10 min. The solution was centrifuged at 12,000 rpm for 10 min at room temperature. The upper aqueous phase was transferred to a newly sterile microcentrifuge tube. This extraction process was then repeated once with phenol:chlorofrom:isoamyl alcohol (P:C:I = 25:24:1) and twice with chlorofrom:isoamyl alcohol (C:I = 24:1). The aqueous phase was transferred into a sterile microcentrifuge tube. DNA was precipitated by an addition of two volume of cold absolute ethanol and mixed thoroughly. The mixture was incubated at -80°C for 30 minutes. The precipitated DNA was recovered by centrifuged at 12,000 rpm for 10 min at room temperature and washed twice with 400 µl of 70% cold ethanol for overnight. After centrifugation, the supernatant was removed. The DNA pellet was air-dried and resuspended in 15-30 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). The DNA solution was incubated at -80°C for 1-2 hours and kept at 4 °C until further use.

3.3 Measuring DNA concentrations using electrophoresis and spectrophotometer

3.3.1 Estimation of DNA and RNA concentration by spectrophotometry

The concentration of extracted DNA or RNA was estimated by measuring the optical density at 260 nanometre (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml double stranded DNA and 33 µg/ml oligonucleotide (Sambrook and Russell, 2001). Therefore, the concentration of DNA/RNA samples were estimated by multiplying an OD_{260} value with a dilution factor 50 and 33 for DNA and oligonucleotides, respectively.

The purity of DNA samples can be guided by a ratio of OD_{260} /OD_{280}. For the extracted DNA, the ratio much lower than 1.8 indicated contamination of residual proteins or organic solvents whereas the ratio greater than this value indicate contamination of RNA in the DNA solution (Kirby, 1992).

3.3.2 Estimation of the amount of DNA by mini-gel electrophoresis

The amount of high molecular weight DNA can be roughly estimated on the basis of the direct relationship between the amount of DNA and the level of fluorescence after ethidium bromide staining using agarose gel electrophoresis. Genomic DNA was run in a 0.8 - 1.0% agarose gel prepared in 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.0 mM EDTA, pH 8.3) at 4 V/cm. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml). DNA concentration was estimated from the intensity of the fluorescent band by comparing with that of undigested λDNA.

3.4 Polymerase Chain Reaction (PCR) using homospecific microsatellite primers

Four microsatellites (*CUHas2, CUHas8, Haμ2J* and *Haμ13*) was amplified and detected by silver staining whereas the 5′ terminus of the forward primer of *CUHas3* was fluorescently labeled by TAMRA (Table 3.2). The PCR product of that locus was detected by a fluorescent scanner (PhosphorFX, BioRad).

Table 3.2 Nucleotide sequence and melting temperature of microsatellite primers for *H. asinina*.

Primer	Repeat unit (bp)	Sequence	Tm (°C)
CUHas3	$(GT)_{24}(GA)_{18}$	F: 5′–TCCAGACTGCACGTTATTATTCC-3′	55
		R: 5′–GCACCCTGTCTCCCTTGAAC-3′	
CUHas2	$(AT)_{7}(GT)_{37}$	F: 5′–ATGGAAGTCAACAATAGACAGG-3′	55
		R: 5′–CCCAGATCAGTTCCACAATAC-3′	
CUHas8	$(AGTG)_{16}$	F: 5′–GTATTACTTGACTTTGAGCC-3′	53
		R: 5′–TGTATGTCCTATCACAGCAT-3′	
Haμ2J	$(CA)_{10}T(CA)_{3}$	F: 5′–TGAGGTGTGTAGTTTGGAAGGA-3′	55
		R: 5′–CTCCCCTGGAAAATGCACATA-3′	
Haμ13	$(CA)_{5}AG(CA)_{11}$	F: 5′–CTAATTGCAGTTATGGGGTTTTGG-3′	60
		R: 5′–GCGGAGGAAGTACTATAACCAG-3′	

PCR was carried out in either 12.5 (*CUHas2* and *CUHas8*) or 15 µl (*CUHas3*, *Haµ2J* and *Haµ13*) containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 200 µM of dNTP, 2.0 mM $MgCl_2$, 0.4 - 0.45 µM of each primer, 0.3 - 1.0 unit of DyNAzyme^{TM} DNA polymerase and 50 ng of DNA template. Amplification was performed as described by Table 3.3. The PCR product was electrophoretically analyzed as soon as possible after amplification.

Table 3.3 PCR condition used for amplification of genomic DNA of *H. asinina*

Locus	**dNTP (µM)**	**$MgCl_2$ (mM)**	**Primer (µM)**	***Taq* (unit)**	**PCR condition**
CUHas2	200	2.0	0.4	0.3	-94°C, 3 min followed by -94°C, 45 s, 55°C, 90s and 72°C, 60s for 35 cycles and -72°C, 10 min
CUHas3	200	2.0	0.4	1.0	-94°C, 3 min followed by -94°C, 20 s, 60°C with a reduction of 1°C for each cycle for 25s, 72°C, 10s for 6 cycles and -94°C, 25 s, 55°C, 25 s and 72°C, 5 s for 25 cycles
CUHas8	200	2.0	0.45	0.75	-94°C, 3 min followed by -94°C, 45s, 53°C, 1 min and 72°C, 45s for 32 cycles and -72°C, 10 min
Haµ2J	200	2.0	0.4	1.0	-same condition as *CUHas3*
Haµ13	200	2.0	0.4	1.0	-94°C, 3 min, 1 cycle followed by -94°C, 20s, 65°C with a reduction of 1°C for each cycle for 25s, 72°C, 10s for 6 cycles and -94°C, 25s, 60°C, 25s and 72°C, 5s for 25 cycles

3.5 Agarose gel elctrophoresis

An appropriate amount of agarose was weighed out and mixed with an desired volume of 1x TBE buffer (89 mM Tris-HCl, 89 mm boric acid and 2 mM EDTA, pH 8.3) The gel slurry was boiled in a microwave oven to complete solubilization and allowed to lower than 60 °C before poured into the gel mold. A comb was inserted. The agarose gel was left to solidify. When needed, enough amount of 1x TBE buffer covering the gel for approximately 0.5 cm. the comb was removed.

The PCR product was mixed with the one-fourth volume of the 10x loading dye (0.25% bromophenol blue and 25% ficoll in water) and loaded into the well. A 100 bp DNA ladder was used as the standard DNA marker. Electrophoresis was carried out at 5 - 6 volts/cm until bromophenol blue move to approximately one-half of gel. The electrophoresed gel was stained with an ethidium bromide solution (0.5 µg/ml) for 5 min and destained in the water to remove unbound ethidium bromide from the gel. DNA fragments were visualized by a gel documentation (BioRad).

3.6 Polyacrylamide gel electrophoresis

3.6.1 Preparation of glass plates

The long glass plate was thoroughly wiped with 1 ml of 95% commercial grade ethanol with a piece of the tissue paper in one direction. This process was then repeated twice. Afterwards, the long glass plate was coated with 1 ml of freshly prepared Bind silane (10 µl of Bind silane, Amersham Biosciences, 980 µl of 95% ethanol and 10 µl of 5% glacial acetic acid) and left for approximately 10 - 15minutes. Excess binding solution was removed with a piece of the tissue paper. The long glass plate was further cleaned with 95% ethanol for 3 times.

The short glass plate was treated as described above with the exception that the binding solution was replaced by the Repel silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone). The cleaned glass plates were assembled with a pair of 1.5 mm spacers.

3.6.2 Preparation of denaturing polyacrylamide gel electrophoresis

Desired concentrations (4.5 - 6.0%) of denaturing polyacrylamide gels were prepared by combining 40 ml of the acrylamide solution (19:1 acrylamide: bisacrylamide with 7 M urea in 1x TBE buffer) with 300 µl of freshly prepared 10 % ammonium persulphate and 30 µl of TEMED. The acrylamide solution was gently swirled and degassed for 15 minutes. The assembled plate sandwich was hold at a 45 degree angle on the bottom corner. The acrylamide solution was then gently injected into one side of the assembled plates. The filled plate sandwich was left in the horizontal position. The flat edge of the shark-tooth comb was then inserted. The gel was left at room temperature for 1 hour. After that, the polymerized gel was covered by the water-soaked tissue papers and left at room temperature for 4 hours (or overnight) for complete polymerization. When required, the spring clips and the sealing tape were carefully removed. The top of the gel was rinsed with deionized H_2O.

3.6.3 Electrophoresis

The gel sandwich was placed in the vertical sequencing apparatus with the short glass plate inward. The gel sandwich was securely clamped with the integral gel clamps along the sides of the sequencing apparatus. The upper and lower buffer chambers were filled with approximately 300 ml of 1x TBE. The shark-tooth comb was reinserted into the gel until the teeth just touched the surface of the gel. Three microlitres of the polyacrylamide gel loading dye (98 % formamide, 200 µl EDTA, 0.25 % bromophenol blue and 0.25 % xylene cyanol) was loaded into each well. The gel was prerun at 35 W for 20 minutes.

Six microlitres of the amplification products were mixed with 3 µl of the loading buffer and heated at 95°C for 5 minutes before snapped cooled on ice for 3 minutes. The sample was carefully loaded into the well. Electrophoresis was carried out at 35 W for approximately 2.5 - 3 hours (XC moved to the end of the gels). Microsatellite bands were visualized by silver staining for *CUHas2, CUHas8, Haµ2J* and *Haµ13* and fluorescent detection for *CUHas3*.

3.6.4 Silver staining

The gel plates were carefully separated using a plastic wedge. The long glass plate with the attached gel was placed in a plastic tray containing 3 litres of the fix/stop solution (10% glacial acetic acid) and agitated well for 30 minutes. The gel was briefly soaked with shaking 3 times for 2 minutes with deionized water. The gel was lifted out from the tray between each wash and allowed the washed water draining out of the gel for 5 seconds. The gel was transferred to 0.1% silver nitrate solution (3 litres) and incubated with agitation at room temperature for 30 minutes. The gel was soaked in 3 litres of deionized water with shaking (10 forward and 10 backward agitation) and immediately placed in the tray containing 3 litres of the chilled developing solution. This step is crucial and the time taken to soak the gel in the water and transfer it to chilled developing solution (Sodium carbonate) should be no longer than 5 - 10 seconds. The gel was well agitated until the first bands are visible (usually 1.5 - 2 minutes). The gel was then transferred to another tray containing 3 litres of chilled developer and shaken until bands from every lane were observed (usually 2 – 3 minutes). One litre of the fix/stop solution was directly added to the developing solution and continued shaking for 3 minutes. The stained gel was soaked in deionized water twice for 3 minutes each. The gel was placed in the plastic bag and air-dried.

3.7 Scoring of microsatellite alleles

Sizes of microsatellite allele were determined by scoring of that of the PCR product that separated in a denaturing polyacrylamide gel. In general, a microsatellite allele from an electrophoretically pattern was not a singer band, but a ladder of bands called stutter bands. Therefore scoring of a particular band was carried out by making an assumption that an actual band of given allele was the most intense band located at the biggest in size compared to the neighbor group of stuttered bands.

Since microsatellite is a co-dominant marker providing 1 or 2 alleles per individual, the genotype of the individual *H. asinina* for each locus could be divided into homozygotic or heterozygotic states. For microsatellites detected by silver

staining (*CUHas2, CUHas8, Haμ2J* and *Haμ13*), both alleles of a particular locus were visualized and only one allele was scored.

3.8 Data analysis

3.8.1 Genetic variation and heterozygosity

The number of alleles per locus was directly estimated from obtained data. The frequency of a particular allele in a sample at a given locus was calculated as

$$P = (2N_{AA} + N_{Aa})/2N$$

where P is the frequency of the A allele. N is a total number of investigated individuals within a sample. N_{AA} and N_{Aa} are numbers of homozygotes and heterozygotes for such a locus.

Heterozygosity can be calculated as observed (direct-counted, h_{obs}) and expected heterozygosity. The former is a proportion of heterozygous individuals and overall investigated specimens in a sample or species. The latter (h_{exp}) is computed from allele frequencies of a locus using the formulae;

$$h_{exp} = 1 - \Sigma p_i^2$$

Assuming that investigated samples conform Hardy-Weinberg equilibrium.

Practically, those parameters were estimated using GENEPOP Ver. 3.2 (Raymond and Rousset, 1995).

3.8.2 Discrimination capacity (DC)

The discrimination capacity was introduced to eliminate the possible bias on using the number of genotype as the diversity index. The DC can be calculated as

DC = numbers of genotype/number of investigated individuals.

3.8.3 Hardy-Weinberg equilibrium

Once allele and genotype frequencies have been estimated, association of two alleles that an individual receives at a locus should be considered. Without

significantly disturbing forces (e.g. selection, mutation or migration) which would change allele frequencies over time and mating is actually occurred at random in a large population. Pairs of allele are not associated.

In this study, the null hypothesis (H_0) that observed genotypes frequencies of an investigated sample at a given locus conform Hardy-Weinberg equilibrium and an alternative hypothesis (H_1) for heterozygote deficiency were tested using permutation version of the exact test based on a Markov chain following the algorithm of Guo and Thomsom (1992) routine in GENEPOP.

3.8.4 Parameters to indicate the ability to carry out individuality and parentage analysis of each microsatellite locus was calculated

3.8.4.1 Matching probability (MP)

$$MP = \sum_{i=a}^{n} G_i^2$$

where G = the frequency of sample with genotypes i.

For multiple loci:

$$MP = (MP1).(MP2).(MP3)\ldots$$

3.8.4.2 Power of discrimination (PD)

$$PD = 1-\sum_{i=a}^{n} G_i^2$$

where G = the frequency of sample with genotypes i.

For multiple loci:

$$PD = 1-[(1-PD1).(1-PD2).(1-PD3)\ldots.]$$

3.8.4.3 Power of exclusion (PE)

$$PE = h^2 (1-2*h*H^2)$$

where G = the frequency of sample with genotypes i.

h = the observed frequency of heterozygotes

H = the observed frequency of homozygotes

For multiple loci:

$$PE = 1-[(1-PE1).(1-PE2).(1-PE3)....]$$

3.8.5 The effective population size and inbreeding coefficient

Initially, the numbers of dams and sires contributed in the previous generation of the present stock were estimated using PARENTAGE version 1.0 (Emery et al., 2001).

The effective number of population size (N_e) was then calculated using the formulae (Gail, 1987);

$$Ne = 4N_fN_m/(N_f + N_m)$$

where N_f is the number of breeding females and N_m is the number of breeding males.

Inbreeding coefficient of the present stock was calculated using the equation.

$$F=1/2Ne$$

where *Ne* is the effective popultaion size assuming no inbreeding in the previous generation.

3.8.6 Genetic heterogeneity between subgroups of the hatchery-propagated *H. asinina*

Genetic heterogeneity between subgroups of *H. asinina* was tested based on the null hypothesis that the allelic distribution of paired samples at a given locus is not statistically significant difference using the exact test described by Raymond and Rousset (1995) implemented in GENEPOP. Results are expressed as the probability of homogeneity between compared samples.

3.8.7 Association analysis of microsatellite genotypes and the body weight in the hatchery sample (B) of *H. asinina*

Association between genotypes (fix effect) and the body weight (variable effect) was initially tested at each locus using simple linear regression analysis. Results from the *Hau13* locus were further analyzed. Significant differences between the body weight of the B sample having different genotypes were analyzed using ANOVA followed by Duncan's new multiple range test. Significant comparisons were considered when the P value was < 0.05.