CHAPTER IV

RESULTS

4.1 Distribution and shell color variation of *B*abylonia *areolata*

Samples of *Babylonia areolata* were planed to collects from the Gulf of Thailand and Andaman Sea, but the samples were obtained only from the Gulf of Thailand because in this investigation, there is no *B. areolata* in Andaman Sea. The amounts of collected samples were between 40-65 individuals per location, excepted for Pattani site only 18 samples were obtained (Table 4.1).

Table 4.1 The sampling sites and number of samples.

No.	Sampling site	Location	NO. of samples
1	Trad	11°46′ 38.79″N 102°52′ 38.05″E	40
2	Chanthaburi	12°34′ 19.72″N 101°53′ 38.48″E	50
3	Rayong	12°36′ 47.65″N 101°25′ 23.87″E	50
4	Chonburi	-	-
5	Samut Songkhram	-	-
6	Phetchaburi	12°48′ 48.78″N 99°59′ 44.70″E	40
7	Prachuap Khiri khan	11°11′ 54.91″N 99°31′ 31.65″E	45
8	Surat Thani	-	-
9	Nakhon Si thammarat	8°58′ 58.51″N 99°54′ 54.29″E	65
10	Songkhla	6°58′ 27.67″N 100°46′ 42.91″E	65
11	Satun	-	-
12	Krabi	-	-
13	Ranong	-	-
14	Pattani	6°53′ 56.27″N 101°22′ 26.45″E	18

Babylonia areolata samples collected in this study were different in shell color patterns, which could be divided into 5 categories, brown, orange, white, rust and dark brown stripe (Figure 4.1). In the upper part of the Gulf of Thailand, most of spotted babylon were brown and only a little amount of rust samples were found in Trad. In the lower part of the Gulf of Thailand, all five shell color patterns were found and dark brown stripe samples were found only in Prachuap Khiri khan (Table 4.2).

(a) (b) (c) (d) (e)

Figure 4.1 Various shell color patterns of *Babylonia areolata.* (a) brown (b) orange (c) white (d) rust and (e) dark brown stripe.

Table 4.2 The number and location of samples collections.

populations	Brown	Orange	White	Rust	Dark brown stripe	Total
Trad	32	-	-	8	-	40
Chanthaburi	50	-	-	-	-	50
Rayong	50	-	-	-	-	50
Phetchaburi	40	-	-	-	-	50
Prachuap Khiri khan	18	-	-	-	10	40
Nakhorn Si Thammarat	38	8	19	-	-	65
Songkhla	32	18	7	10	-	65
Pattani	9	5	3	1	-	18

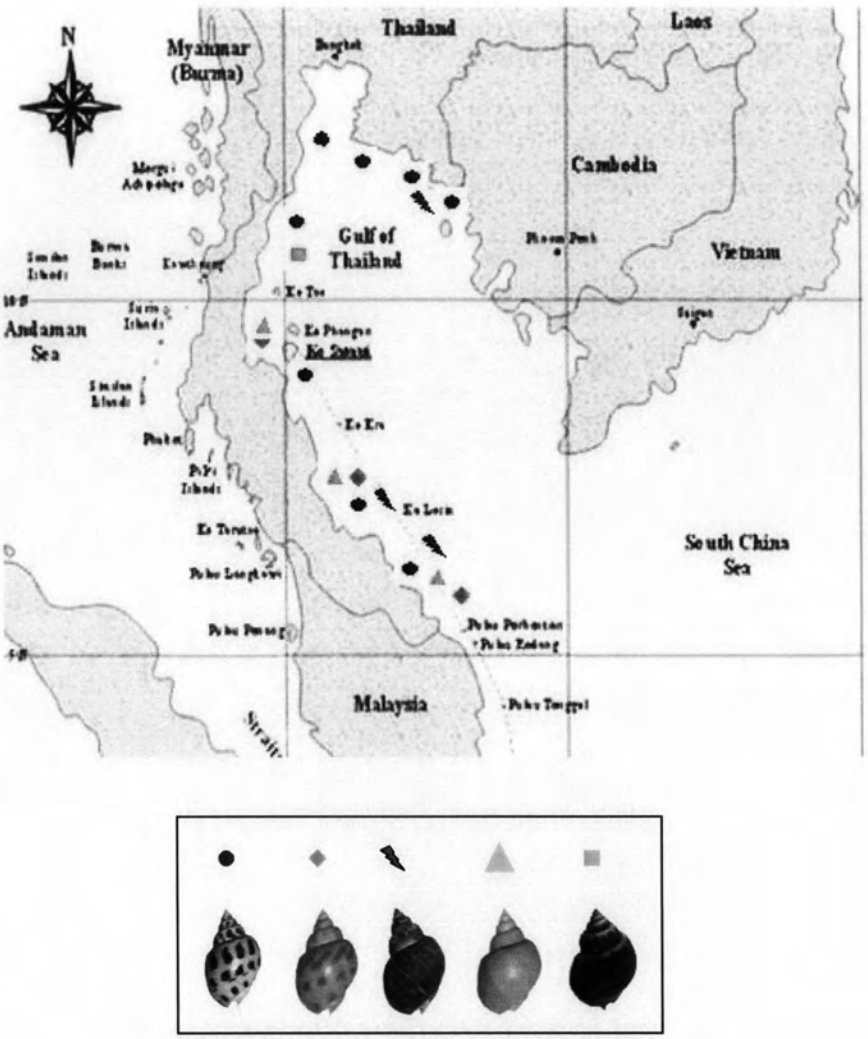

Figure 4.2 Distribution map indicating approximate sampling locations of *Babylonia* within the Gulf of Thailand.

4.2. DNA extraction

DNA was extracted from foot tissue *B. areolata* which keep in 95 % ethanol using CTAB extraction method (as described in chapter III). Good quality and quantity of genomic DNA was obtained, approximately 20–50 ng (Figure 4.3).

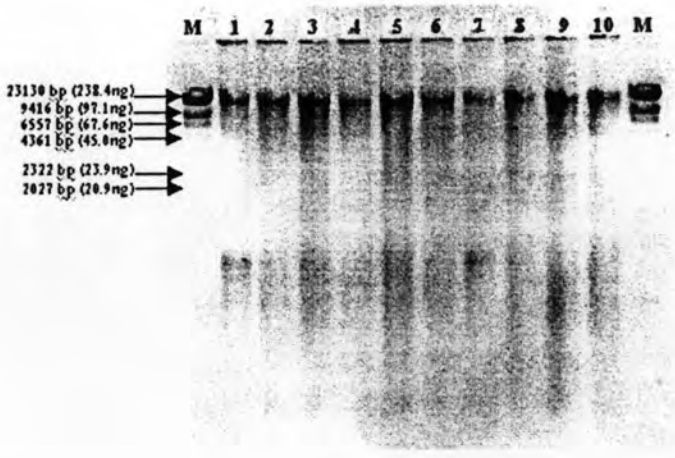

Figure 4.3 Extracted genomic DNA on 0.8% agarose gel stained with EtBr, Lane M: λ Hind III as DNA marker, lane1-10: samples from Songkhla.

4.3 ISSR–PCR

Forty-eight ISSR primers were screened on five randomly selected individuals using PCR condition as described in chapter III (section 3.3.2). Eight primers were selected as a potential primer to use in this study. After, the adjustment of magnesium concentrations and annealing temperature there were 4 primers producing clear and reproducible fragments. Then they were examined for the repeatability of bands by repeating the ISSR process. It proved that pattern of ISSR was highly reproducible. Using those primers to screen 48 of *B. areolata* samples with different shell color patterns, the primers produced a total of 21 reproducible bands ranging from 200 to 1100 bp in size. The 21 loci were surveyed, 14 were polymorphic, and the percentage of polymorphic loci was 66.67%. The highest number of polymorphic bands was produced by primer 814 (Figure 4.4). However each of the 48 individuals has a unique ISSR genotype, indicating extensive genetic variation in this study. The parameters of PCR reaction for each of primers were showed in Table 4.3.

Table 4.3 Primers sequences used in the ISSR amplification, concentration of $MgCl_2$, annealing temperature (Tm.), number of bands, number of polymorphic bands and size range of fragments.

Primers	Sequence (5'-3')	$MgCl_2$ (mM)	Tm. (^oC)	No. of bands	No. of Polymorphic bands	Size range of fragments (bp)
UBC841	$(GA)_8YC$	2.0	38	5	3	570-1000
UBC845	$(CT)_8RG$	2.0	51	5	3	270-1000
814	$(CT)_8A$	2.0	52	5	5	400-1100
T8707	$(GAG)_4RC$	2.0	45	6	3	200-800

*Y= C/T; R= A/G

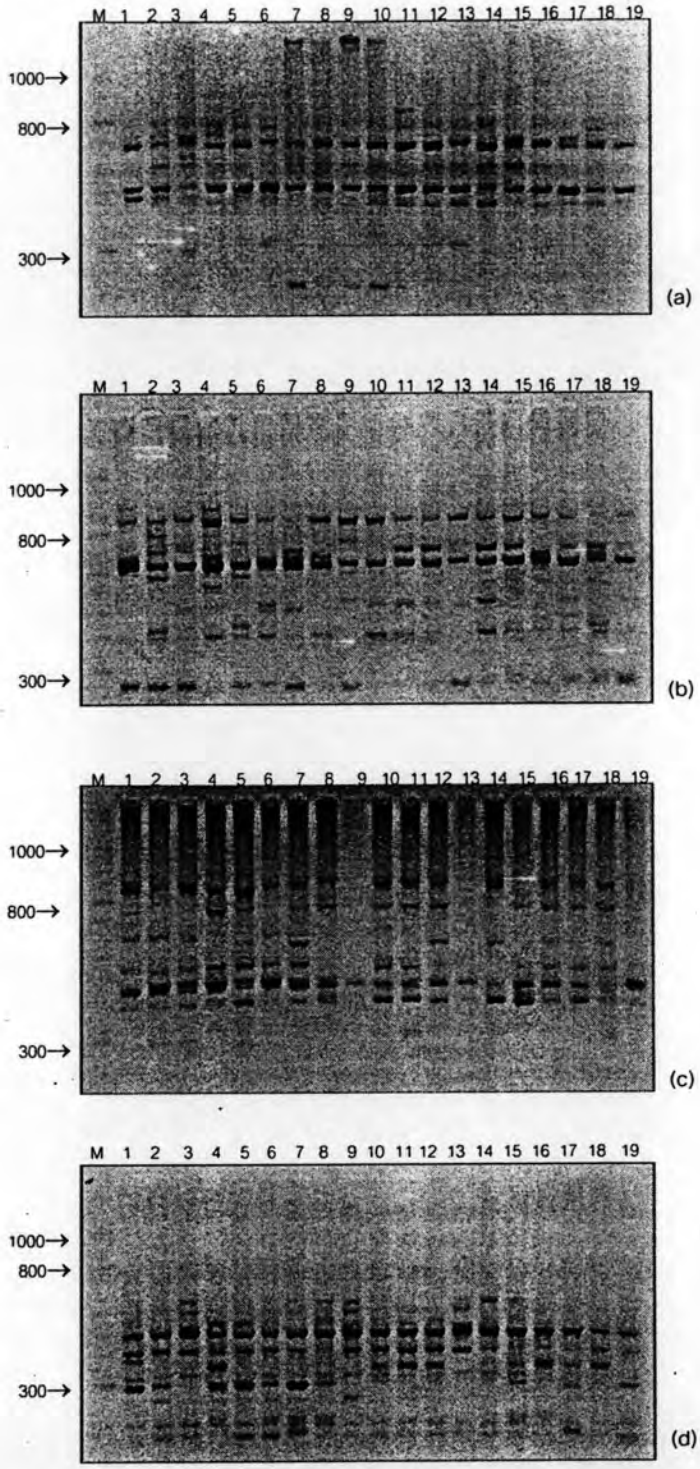

Figure 4.4 ISSR profiles of 19 samples using primer (a) UBC841 (b) UBC845, (c) 814 and (d) T8707.

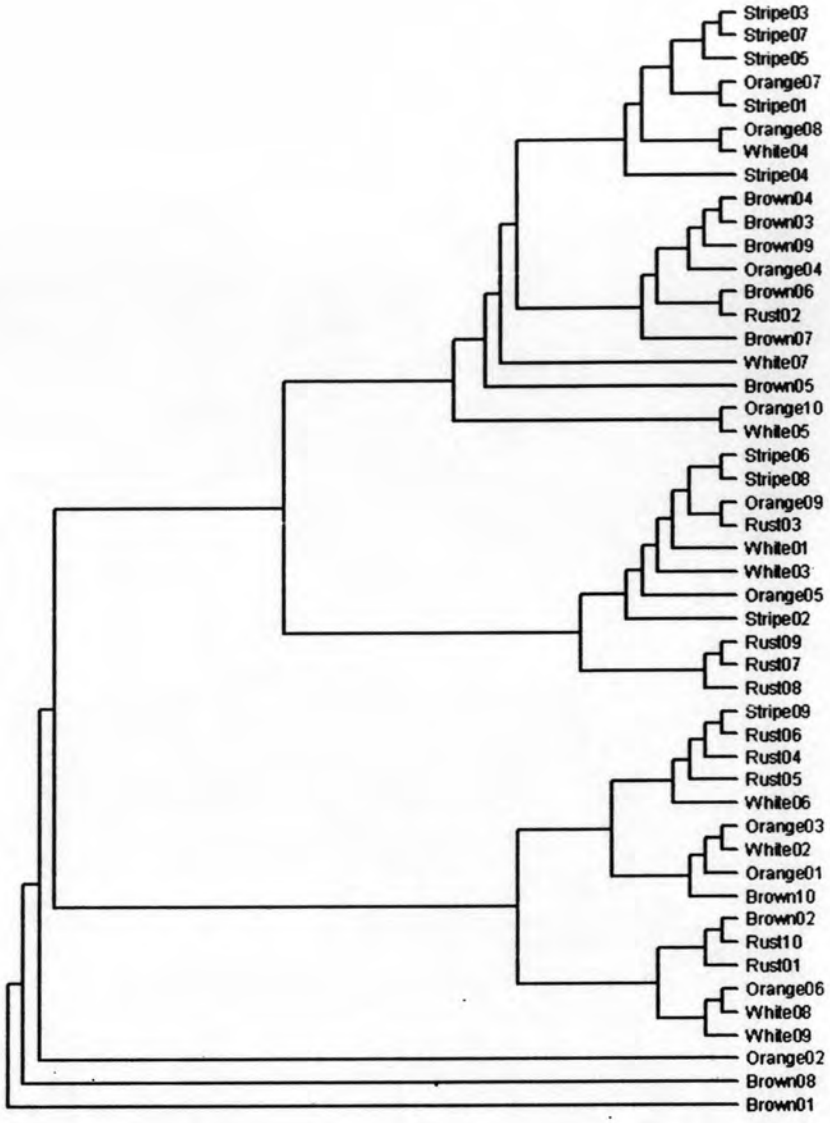

Figure 4.5 The dendrogram of ISSR profile showing relationships within *Babylonia* each color based on Penny-Branch.

The Penny-Branch and bound produced 40 pasimonious trees. All of then showed no clear structure on shell color pattern of *B. areolata*. Thus, one dendrogram was selected to show in this study (Figure 4.5).

4.4 DNA sequencing

16S rRNA primers produced PCR product about 550 bp in size and the COI primers product about 700 bp (Figure 4.6). The sequences of 16S rRNA gene (435 bp) and COI gene (460 bp) were and used to search for similarity using Blasts

(http://www.ncbi.nlm.nih.gov/). The result showed that the sequences were similar to 16S rRNA gene of GenBank accession number DQ314761.1 *Babylonia areolata* percentage similarity 99%, and cytochrome oxidase I gene accession number AJ623008.1 *Echinolittorina hawaiiensis* mitochondrial partial COI gene percentage similarity 84%, which were gastropods. This is to confirm that the sequences were each gene of *B. areolata*. Among five shell color patterns snails, the 16S rRNA of 5 specimens and COI genes 15 specimens were examined (Table 4.4).

Table 4.4 Selection of specimens used in DNA sequencing

Sample	16s rRNA gene	COI gene		
Brown	Chanthaburi20	Chanthaburi20	Prachuabkhirikhan25	Songkhla42
Orange	Nakornsithammarat56	Pattani4	Nakornsithammarat 56	Songkhla58
Rust	Trad29	Songkhla40	Trad29	Songkhla35
White	Nakornsithammarat 40	Pattani8	Nakornsithammarat 40	Songkhla10
Stripe	Prachuabkhirikhan2	Prachuabkhirikhan2	Prachuabkhirikhan5	Prachuabkhirikhan10

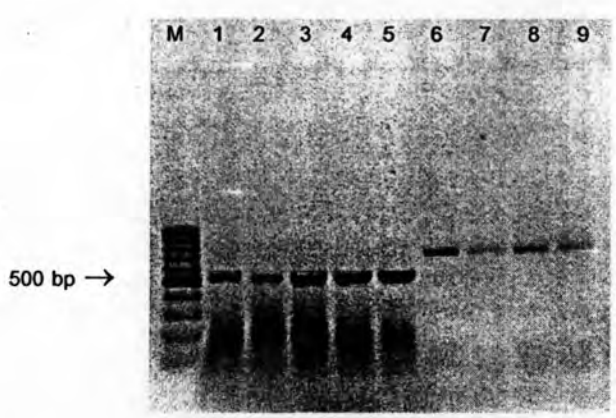

Figure 4.6 The PCR products of 16s rRNA (lane 1-5) and COI genes (lane 6-9) were assessed by 1.0% agarose gel and compared to 100 bp DNA ladder.

4.5 Genetic variation of 16srRNA and COI sequence

Percentages of nitrogenous base composition (A, C, G and T) were presented %GC content of 16S rRNA 35.67%, COI 36.07% and % AT contents for both gene approximately 65% (Table 4.5). This characteristic is common in mtDNA genome of plant and animals.

Table 4.5 Percentages of nitrogenous base composition of sequences of *B. areolata*.

primer	Samples	A	C	G	T
16s rRNA	Brown1	35.63	14.48	21.15	28.74
	Orange1	35.63	14.48	21.15	28.74
	White1	35.40	14.71	21.15	28.74
	Rust1	35.63	14.48	21.15	28.74
	Stripe1	35.63	14.48	21.15	28.74
	Means	**35.58**	**14.52**	**21.15**	**28.74**
COI	Brown1	25.75	16.81	19.36	38.09
	Brown2	25.95	16.81	19.15	38.09
	Brown3	25.75	16.81	19.36	38.09
	Orange1	25.96	16.81	19.15	38.09
	Orange2	25.96	16.81	19.15	38.09
	Orange3	25.96	16.81	19.15	38.09
	White1	25.96	16.81	19.15	38.09
	White2	25.96	17.02	19.15	37.87
	White3	25.96	16.81	19.15	38.09
	Rust1	25.96	17.02	19.15	37.87
	Rust2	25.96	16.81	19.15	38.09
	Rust3	25.96	16.81	19.15	38.09
	Stripe1	25.53	16.81	19.57	38.09
	Stripe2	25.75	16.81	19.36	38.09
	Stripe3	25.75	16.81	19.36	38.09
	Means	**25.87**	**16.84**	**19.23**	**38.06**

Table 4.6 Percentages differences of 16S rRNA sequences among five shell color patterns of snails.

color pattern	Brown	Orange	White	Rust	Stripe	*B.spirata*
1.Brown	-					
2.Orange	0.00	-				
3.White	0.05	0.05	-			
4.Rust	0.00	0.00	0.05	-		
5.Stripe	0.00	0.00	0.05	0.00	-	
6.*B.spirata*	6.33	6.33	6.85	6.33	6.33	-

The percent differences of 16s rRNA sequences among five shell color patterns are very low (0.00-0.05%) compared with percent differences of those with *B. spirata*. 6.33 – 6.85% (Table 4.6). Higher percent differences were found among COI sequences of the examined snails ranging from 0.00-0.64%. The highest differences (0.64%) were found between the stripe1 sample from Prachuab Khiri Khan and the sample white2 from Nakorn Si Thammarat and between the stripe1 sample from Prachuab Khiri Khan and the sample rust1 from Songkhla (Table 4.7). However the difference of COI sequence among those samples were low compared with the percentage differences of those with *B. spirata* (12.72- 13.56%). Therefore, it is likely that spotted babylon with difference shell color pattern is the same species.

Table 4.7 Percentage differences of cytochrome oxidase I sequences among five shell color patterns of snails.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1.Brown2	---															
2.Stripe2	0	---														
3.Stripe3	0	0	---													
4.Brown1	0	0.21	0	---												
5.Stripe1	0.21	0.21	0.21	0.21	---											
6.Rust2	0.21	0.21	0.21	0.21	0.43	---										
7.Rust3 .	0.21	0.21	0.21	0.21	0.43	0	---									
8.White3	0.21	0.21	0.21	0.21	0.43	0	0	---								
9.Orange1	0.21	0.21	0.21	0.21	0.43	0	0	0	---							
10.White1	0.21	0.21	0.21	0.21	0.43	0	0	0	0	---						
11.Orange3	0.21	0.21	0.21	0.21	0.43	0	0	0	0	0	---					
12.Brown3	0.21	0.21	0.21	0.21	0.43	0	0	0	0	0	0	---				
13.Orange2	0.21	0.21	0.21	0.21	0.43	0	0	0	0	0	0	0	---			
14.White2	0.43	0.43	0.43	0.43	0.64	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	---		
15.Rust1	0.43	0.43	0.43	0.43	0.64	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.43	---	
16.*B.spirata*	13.28	13.28	13.3	13.28	13.56	13.00	13.00	13.00	13.00	13.00	13.00	13.00	13.00	13.27	12.72	---

4.6 Cross breeding

The preliminary cross breeding experiment was carried out during June 2007 to January 2008. The result showed that there was no spawning of eggs occurred throughout the experimental period for all breeding treatments. Only one spawning took place for female of white pattern and male of brown spotted pattern within five days of the experiment but it may due to the former breeding. In addition, the egg capsules obtained was small about 1 cm in length and they were all unfertilized eggs. Therefore the experiment had to test by adding amount of male and female broodstocks from 1:1 to 2:6 after 2 months of observation.