Songklanakarin J. Sci. Technol.
30 (2), 141-146, Mar. - Apr. 2008

Songklanakarin Journal of Science and Technology
http://www.sjst.psu.ac.th

Original Article

Phylogeography of Ivory shell (*Babylonia areolata*) in the Gulf of Thailand revealed by COI gene structure and differentiation of shell color by ITS1 DNA

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Received 15 October 2007; Accepted 3 March 2008

Abstract

Babylonia areolata is an aquatic mollusk in the genus of the gastropod family Buccinidae. Genetic variations were observed in the 542 base pairs of the sequences from its cytochrome oxidase subunit I (COI) gene of mtDNA. COI alignments showed that there were two monophyletic clades on the phylogenetic tree, referred as *B. areolata*. These results point towards the possibility of *B. areolata* being a single species with strong phylogeographic subdivisions. About 520 base pairs of the internal transcribed spacer 1 (ITS1) sequence were able to distinguish between the shell colors (brown, cream and white) of *B. areolata*.

Keywords: *Babylonia areolata*, cytochrome oxidase subunit I, phylogeographic subdivision, internal transcribed spacer 1

1. Introduction

Babylonia areolata, ivory shell is a gastropod family Buccinidae. Mollusks live in almost every habitat, including deep-sea vents, coral reefs, estuaries, rocky and sandy shorelines, freshwater lakes, rivers and almost everywhere on land from deserts to rainforests (Lydeard and Lindberg, 2003). This species distributes from Ceylon and the Nicobar Islands through the Gulf of Thailand, along the Vietnamese and Chinese coasts to Taiwan (Altena and Gittenberger, 1981) and inhabits sandy or muddy bottoms in shallow water. Economically, mollusks have Figureured prominently in fisheries and mariculture, being used as food (e.g., clams, scallops, abalone, calamari and conch) and commercial pearl production (Landman *et al*., 2001) *Babylonia areolata* is going to be a target species of commercial fisheries in Thailand. At present, catches are decreasing and the size of mollusk obtained from the Thai coast is small, therefore aquaculture has increasingly attempted to supply the market and the enhancement of stock. There are three different shell colors (brown, cream and white) of *Babylonia areolata* found in the Gulf of Thailand. Different shell colors have different values (Figure 1). The brown shell has the highest price while the white shell has the lowest price. There is a big export demand for brown shells to countries such as China, Taiwan, Hong Kong, and Japan.

In Thailand, surprisingly, only the brown shells are only found in Phetchaburi and Rayong (the upper of the Gulf of Thailand) while all three colors are found in Songkhla and Pattani (the lower of the Gulf of Thailand). It is known, that *Babylonia areolata* from the upper and lower Gulf are the same species, so the question is what is the genetic information that make one only brown and the other mixed colors, respectively.

In this study, we have used the differences in shell

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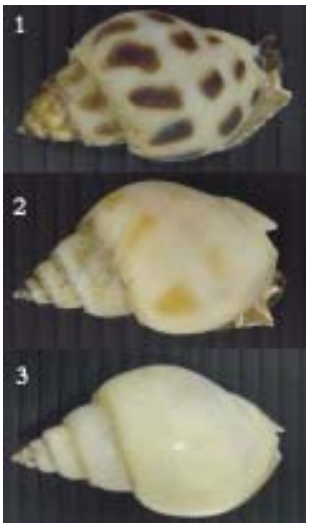

Figure 1. The phenotypic differences in the shell color of *Babylonia areolata*, 1) brown shell, 2) cream shell, 3) white shell.

color as a basis to analyse the genetic information obtained from mitochondrial (COI) and nuclear (ITS) DNA regions. In particular, we have focused on the two questions : (i) Can we find any ambiguity in the genetic data that might indicate that these two populations with different phenotypes are not the same species (*Babylonia areolata*)? (ii) Do any of the observed variations in the DNA of COI and ITS genes bears any relationship to the differences in shell color?

2. Material and Methods

Fifteen ivory shells were collected from each of four locations in the Gulf of Thailand; Phetchaburi, Rayong, Songkhla and Pattani (Figure 2). Samples covered the three colors; brown, cream and white. Total genomic DNA was extracted from each individuals foot muscle. The tissue was chopped into small pieces and homogenised in an appropriate volume of the extraction solution (10 mM Tris-HCl, pH 7.5, 100 mM EDTA, 1% SDS and 1 mg/ml proteinase K). The homogenate was incubated at 55°C, for 3 h. DNA was

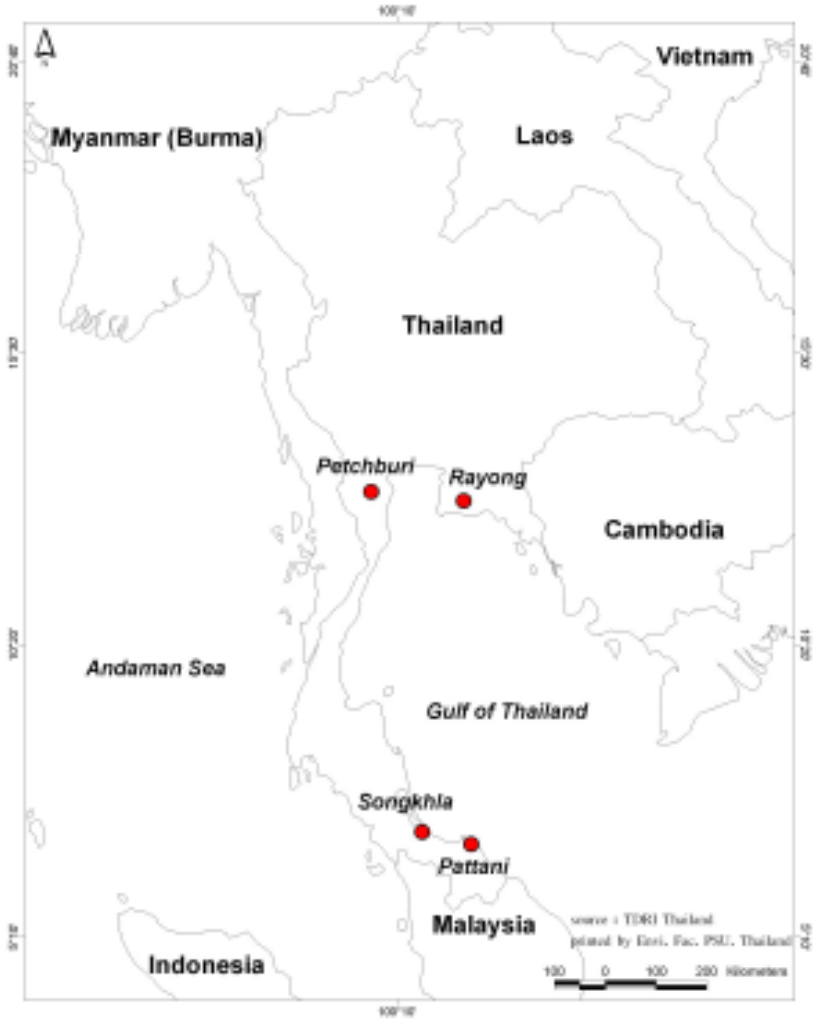

Figure 2. The four locations in the Gulf of Thailand where samples were collected, Phetchaburi, Rayong, Songkhla and Pattani.

extracted twice using phenol/chloroform/isoamyl alcohol (25:24:1) and recovered by ethanol precipitation. DNA concentrations were spectro-photometrically determined.

2.1 Polymerase Chain Reaction (PCR) of COI and ITS

PCR was carried out in 25 ml reaction mixtures, containing 200 mM each of dNTPs, 1.5 mM $MgCl_2$, 1 unit of *Taq DNA Polymerase*, 0.25 mM of the COI primer pair: (5'-TTA GTT GGT ACT GCT CTT AGA TTG CTA AT-3') and (5'-ATC GTA ATA GCT CCA GCT AAT ACT GG-3'): or the ITS1 primer pair: (5'-TGA ACC TGC CGG AGG ATC AT-3') and (5'- GTY CTT CMT CGA CSC ACG AG-3') and 50 ng of the total genomic DNA using a 2400 Perkin Elmer™ thermal cycler. PCR conditions were predenaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C - 50°C for 30 s and extension at 72°C for 60, s and the final extension at 72°C for 5 min.

For DNA sequencing, the amplification product was electrophoretically fractionated through a 1.8 % agarose gel. The PCR fragment was recovered from the gel, directly cloned into pGEM-TEasy™ (Promega) and transformed into *E. coli* Top 10 F' (Invitrogen). The nucleotide sequence of each clone was examined by a BigDye™ Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase (PE Applied Biosystems) using an Applied Biosystems 377 sequencer (Perkin-Elmer).

2.2 Data analysis

The COI nucleotide sequences from *Babylonia areolata* from 4 locations in Thailand were deposited in GenBank (Accession no. EF490190-EF490205) and those retrieved from the GenBank including *B. formosae* (DQ 314764), *B. japonica* (AF373888), *B. areolata* (DQ314763), *B. formosae forhabei* (AY819773), and *B. spirata* (AY 819774), were aligned by using Clustal W (Thompson *et al.*, 1994). The ITS sequences of 5 samples with different shell colors were deposited in GenBank (Accession no.EF490206-EF490210). The sequence divergence between pairs of sequences was estimated using Kimura's (1980) two-parameter model. Data were bootstrapped 1000 times. A consensus neighbor-joining tree based on divergence between pairs of sequences was then constructed using Neighbor-Joining (Saitou and Nei, 1987) in PHYLIP (Felsenstein, 1993).

3. Results

3.1 COI sequence analysis

A 542 bp fragment of COI-DNA was amplified. The sequences of this 542 bp fragment from each sample were compared. The percentage of AT (62%) and GC (38%) of this part of the COI gene was consistent with the mtDNA

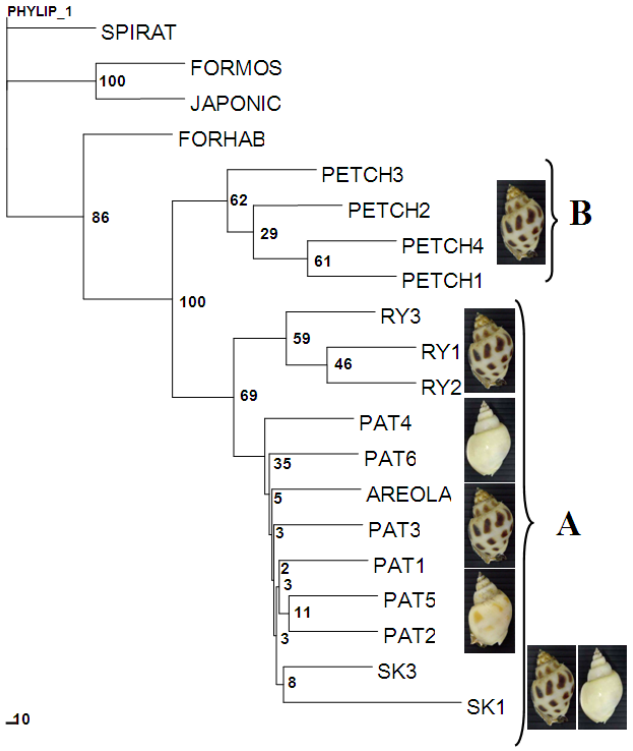

Figure 3. A neighbor-joining tree constructed from the percentage of sequence divergence between pairs of COI sequences. The percent bootstrapping values (1000 replicates) between branching groups are indicated. Data from GenBank included *B. formosae* (DQ314764), *B. japonica* (AF373888), *B. areolata* (DQ314763), *B. formosae forhabei* (AY819773), and *B. spirata* (AY819774).

sequences of *B. areolata* in the GenBank database. There were no insertions or deletions. The base positions can be used to distinguish our samples from Phetchaburi, Rayong, Songkhla, and Pattani. Samples from Phetchburi have base G at position 270 and 513, while from Rayong the base at these positions is A. Samples from Songkhla and Pattani cannot be differentiated by the bases at these two positions as they are always G at 270 and A at 513.(Table 1) The COI sequences divergence amongst the *B. areolata* shells was high compared with other species, *B. formosae forhabei* (14.22 %), *B. formosae* and *B. japonica* (11.21%), and *B. spirata* (18.33%), but there was low divergence in our samples in clade A (0-0.74%) and clade B (0.84%). These results show that all phenotypic variations (brown, cream, and white shell) were all the same species, *B. areolata*.

3.2 The genetic distance and phylogenetic analysis by COI sequence

The phylogenetic tree shows that there are two clades, A and B identified as *B. areolata* (Figure 3). Clade A consists of samples from Songkhla, Pattani and Rayong and also includes the GenBank *B. areolata*. Clade B consists of

Table 1. The relationships among colors, gene structure of COI and geography in three locations from the Gulf of Thailand

Sample number	Sample name	Sex	Colorson	COIBase at 270	COIbase at 513
1	SK1	F	Brown	G	A
2	SK2	M	White	G	A
3	Petch1	M	Brown	G	G
4	Petch2	F	Brown	G	G
5	Petch3	M	Brown	G	G
6	Petch4	F	Brown	G	G
7	RY1	M	Brown	A	A
8	RY2	F	Brown	A	A
9	RY3	M	Brown	A	A
10	Pat1	F	White	G	A
11	Pat2	M	Brown	G	A
12	Pat3	M	Cream	G	A
13	Pat4	F	Brown	G	A
14	Pat5	F	Cream	G	A
15	Pat6	M	White	G	A

SK = Songkhla, Petch = Petchburi, Pat = Pattani, RY = Rayong

samples from Phetchaburi. The distance between *B. areolata* (clade A) and clade B has a divergence of about 0.84%. The distance between *B. areolata* and *B. formosae forhabei* is 14.22%, between *B. areolata* and *B. formosae* and *B. japonica* is 11.21%, between *B. areolata* and *B. spirata* is 18.33%. Our samples from Clade A and B indicate that *B. areolata* is a paraphyletic group.

3.3 Genetic variation within clades

There is low COI variation within clade A and B of *B. areolata*. The divergence within clade A and B are 0.00-0.74 and 0.37-1.67%, respectively. This variation does not show strong geographical identity within clade A as samples from Pattani and Songkhla are closely aligned in clade A together with those from Rayong. On the other hand, the variation within clade B shows strong geographic identity. All samples in clade B come from Phetchaburi.

3.4 ITS1 sequence analysis

Primer ITS1 used in this work was designed by Wanna *et al.* (2004) to amplify the ITS1 region of the nuclear rDNA coding site. We consistently amplified a fragment of approximately 520 bp in five samples (from Pattani and Songkhla) and for the three colors. When compared to the GenBank database, they are similar to the gastropod mollusk *Littorina littorea*. Our ITS sequences were GC-rich (60%) and this corresponds to the gastropods (Schilthuizen *et al.* 1995) and snails (Gonzalez *et al.* 1990) (59-61%).

The length variation of *B. areolata* ITS1 sequences was 515-520 bp which is similar to the range for the *Bulinus* snail (450-516 bp) (Stothard *et al.*, 1996). Nevertheless, DeJong *et al.* (2001), studying American and African *Biomphalaria* mollusks, mentioned that the ITS1 regions over such a diverse group had extreme length variation ranging from 548-778 bp. The ITS1 sequence length of the white shell was 515 bp, the cream shell was 520 bp and the brown shell was 515 and 516 bp.

The sequence analysis found that four bases changed at position 8, 14, 137, and 452 while six positions (69, 70, 71, 72, 73, and 460) are inserted (Table 2). The base changes are related to the different shell colors. For example, the white shell (Pat6 and Song2) has bases C and G at positions 8 and 14, respectively. The brown shell (Song1 and Pat2) has base A at position 8 and base C or G at position 14. The cream shell (Song3) has base A and C at position 8 and 14, respectively, and has an insertion from position 69 to 73. We found the repeated sequence GTTTC at the 5' end, with three copies (54-58, 59-63 and 64-68) found in the white and brown shell, but four copies (54-58, 59-63, 64-68 and 69-73) in the cream shell.

4. Discussion

4.1 Phylogeography followed by COI sequences

Clades A are identified within *B. areolata*. Clade A consists of specimens from Songkhla, Pattani and Rayong. Samples from Rayong are cluster separated from Songkhla and Pattani in low bootstrap values. Songkhla and Pattani are in the same cluster and this follows from their genetic distance. There is low genetic variation within Songkhla and Pattani and a possible reason for this might be that genetic exchange is occurring among their populations.

Clade B consists of specimens only from Petchburi

Table 2. The relationships among shell colors, gene structure of ITS1 and locations from the Gulf of Thailand

Sample name	Sex	Samplesize	Shell Colors	base at position				Insertion position
				8	14	137	452	
SK1	F	3	Brown	A	C/G	A	T	T (460)
SK2	M	3	White	C	G	A	T	-
SK3	M	3	Cream	A	C	A	T	G(69), T(70), T(71), T(72), C(73)
Pat2	M	3	Brown	A	C/G	A	C	-
Pat6	M	3	White	C	G	G	T	-

SK = Songkhla and Pat = Pattani

with a much higher genetic distance than clade A. The pattern of genetic differentiation in our samples (in clade A and B) is like a stepping stone gene flow, as genetic differentiation between populations increases with increased geographic distance (Hellberg *et al.*, 2002).

The patterns of COI from *B. areolata* in the four regions of the Gulf of Thailand may be because most or all haplotypes are related closely, yet are localized geographically. The implication is that contemporary gene flow has been low enough in relation to population size to have permitted lineage sorting and random drift to promote genetic divergence among population that nonetheless were in historical contact recently, long-term biogeographic barriers (Avise, 2000).

The samples from Petchburi and Rayong have only the spotted brown shell. We could not obtain other colors from these two regions while three colors were found in the Pattani and Songkhla. This may be why there are phylogenetic gaps between some branches in a gene tree, with the principal lineages being co-distributed over a wide area. This pattern could arise in a species with a large evolutionary propensity and high gene flow. Then, some anciently separated lineages might by chance have been retained, whereas many intermediate genotypes were lost over time by gradual lineage sorting (Avise, 2000).

Because of the higher value of the spotted brown shell we tried to use genetic markers to distinguish between the three colors and provide benefits for aquaculture. From the COI sequence, we could not distinguish between the three different shell colors. The COI data can be used as a link between the deeper branches of the tree to its shallow, species-level branches. The possibility from this result is that there is only a single species with strong phylogeographic subdivision.

4.2 Differentiating shell color by ITS1 sequences

The ITS1 sequences can be used to identify the shell color of the samples. The bais position to be in would be an ability to differentiate all three shell colors but there were no more than six variant positions found in any individual and this is similar to that found in the *Haliotis* ITS sequences (Coleman and Vacquier, 2002). The genetic variation between white and cream shell is about 1%, between white and brown is about 1%, between brown and cream is about 0.1%. Our ITS sequences have been shown to be phylogenetically informative at the species level. This, however, is only a preliminary study of nuclear markers and more samples will allow for more confidence about this marker.

The aquaculture of this species is developing; it requires more molecular markers for genetic resource management and breeding programs such as the use of microsatellite markers leading to production of a good quality product for the market in the future.

Acknowledgements

This work was supported by a grant from the National Science and Technology Development Agency, Thailand, and the Faculty of Art and Science, Prince of Songkla University, Surat Thani campus, Thailand, and a TRF Master Research Grant to Koravee Tongchuai. We thank Dr. Brian Hodgson for valuable comments.

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