

## DNA Barcoding Analysis and Phylogenetic Relationship of the Blood Cockle, *Tegillarca granosa* in Malaysia using the *Cytochrome Oxidase Subunit 1* (CO1) Gene

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**Abstract:** Blood cockles are the most economically important brackish water invertebrates found in Malaysia. However, our knowledge of blood cockle phylogeny and systematics is rudimentary, especially for the species *Tegillarca granosa*. It is unclear, for instance, whether the cockles occurring on the west coast of Peninsular Malaysia constitute a single species, or multiple, phylogenetically distinct species. We performed the DNA molecular phylogenetic analysis of *T. granosa* to distinguish it from other related species found in other parts of the world. An approximately 585-nucleotide fragment of the mitochondrial DNA (cytochrome oxidase I, COI) was sequenced for 180 individual cockles, representing 9 populations: three from the north, four from the central part and two from the southern part of Peninsular Malaysia. Phylogenetic analyses of the resulting dataset yielded tree topologies that not only showed the relationship between *T. granosa* and its closest relatives but its position in the evolutionary tree. Two mitochondrial clades were evident, each containing an individual genus. Using the mutation rate of the COI gene, the divergence time between *T. granosa* and its closest related species was estimated to be 320 thousand years ago. This study provides a phylogenetic framework for this ecologically prominent and commercially important cockle species.

**Keywords:** *Tegillarca granosa*, CO1, bivalve, mudflat, intertidal species

**Abstrak:** Kerang merupakan invertebrata air payau yang utama dalam ekonomi Malaysia. Walau bagaimanapun, pengetahuan kita tentang filogeni dan sistematik kerang sangat rendah, terutama untuk spesies *Tegillarca granosa*. Tidak jelas, misalnya, apakah kerang yang wujud di pantai barat semenanjung Malaysia merupakan satu spesies tunggal, atau beberapa spesies yang boleh diasingkan secara filogenetik. Kami melakukan analisis filogenetik molekul DNA *T. granosa* untuk membezakannya dengan spesies lain yang berkaitan yang terdapat di bahagian lain di dunia. Sebanyak

585-nukleotida fragmen DNA mitokondria (sitokrom oksidase I, COI) diujukkan untuk 180 sampel, mewakili 9 populasi: tiga dari utara, empat dari bahagian tengah dan dua dari bahagian selatan Semenanjung Malaysia. Analisis filogenetik dari set data yang terkumpul menghasilkan pokok topologi yang tidak hanya menunjukkan hubungan antara *T. granosa* dan spesies terdekatnya tetapi kedudukannya dalam pokok evolusi. Dua kumpulan mitokondria terbukti, masing-masing mengandungi genus individu. Dengan menggunakan kadar mutasi gen COI, masa perbezaan antara *T. granosa* dan spesies yang berkaitan dengannya dianggarkan 320 ribu tahun yang lalu. Kajian ini menyediakan kerangka filogenetik untuk spesies kerang yang sangat penting dari sudut ekonomi dan ekologi.

## Introduction

The blood cockle, *Tegillarca granosa*, an arcid bivalve mollusc, is an important marine commodity in Malaysia. Blood cockles are benthic organisms with a sessile adult stage and a pelagic larval stage (Pathansali, 1963). Their distributions depend heavily on the salinity of the water and are found most commonly under brackish sand and fine, soft, mud bottoms (Pathansali and Soong, 1958; Pathansali, 1963). In 1984, about 65,000 tonnes of cockles were produced from cultured stocks, making it the most important aquaculture industry in Malaysia (Ng, 1986). However, according to the annual statistics released by the Department of Fisheries Malaysia (quote reference here), cockle production from 1990 to 2017 was fluctuating. The highest production of cockle occurred in 1995 with a total yield 100,275.76 tonnes and the lowest production was in 2016 recorded at 9,596.76 tonnes only.

While many molecular markers have been discovered for a wide range of bivalves globally, there are still species of economic significance that have received little attention, such as *T. granosa*. Like most invertebrates, the larval dispersal patterns of the cockles are poorly understood (Banks et al., 2006). Origins of naturally recruited spat of this cockle species are unknown, as is the impact of translocations and natural population structure. Thus, the cytochrome oxidase I (COI) gene was investigated in this phylogenetic study using primers designed for bivalves of the same genus. The application of molecular methods to questions of blood cockle phylogeny and systematics has provided valuable new insights into blood cockle evolutionary relationships and has allowed independent testing of pre-existing hypotheses.

A phylogenetic framework for the blood cockles is needed in order for cockles to survive the effects of water pollution common on the west coast of Peninsular Malaysia, introduction of exotic species and negative effects of aquaculture. It is also needed to replace organism classification based on morphological characteristics, which

are known to be inaccurate and unreliable (Doadrio and Dominguez, 2004). Proper classification and identification of *T. granosa* will help ensure effective management of the species in aquaculture and in the wild. Our primary goal in this study was to find out the position of the *T. granosa* in the evolutionary tree utilizing approximately 585 bp of the mitochondrial COI gene.

## Materials and Methods

A total of 180 samples from 9 populations were collected for this study in the period of 2017-2019 along the west coast of Peninsular Malaysia; Kedah (Tanjung Dawai), Pulau Pinang (Juru and Bukit Tambun), Perak (Kerian and Kuala Terong), Selangor (Sungai Besar and Kuala Selangor), and Johor (Parit Besar and Parit Raja), (Figure 1). Table 1 shows the coordinates of the sites at which the cockles were collected.

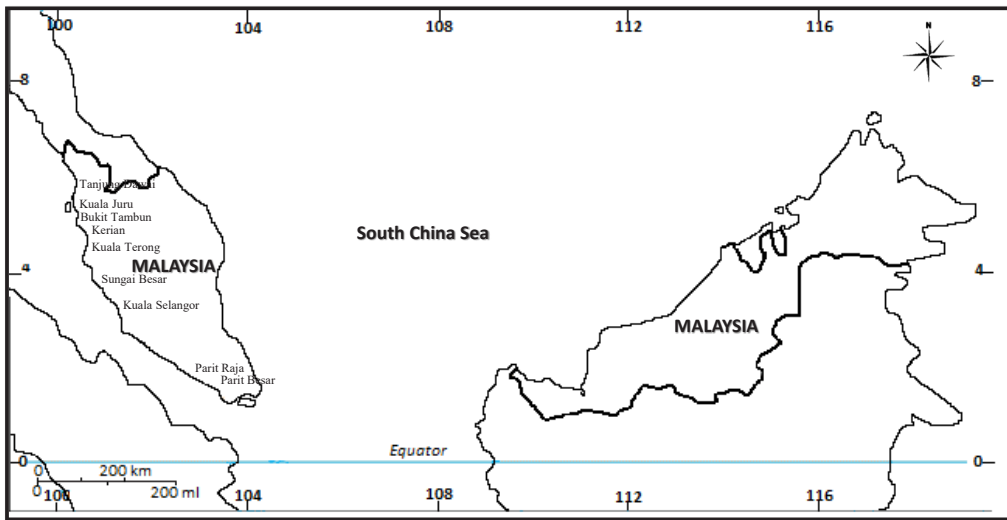


Figure 1: *Tegillarca granosa* sampling areas in Peninsular Malaysia

Table 1: Coordinates of sites at which cockles were obtained

Sampling Site	Coordinates
Tanjung Dawai (Kedah)	5°40'43.28"N, 100°20'23.24"E
Kuala Juru (Pulau Pinang)	5°19'59.94"N, 100°23'55.34"E
Bukit Tambun (Pulau Pinang)	5°16'44.02"N, 100°24'27.00"E
Kerian (Perak)	4°57'56.10"N, 100°23'49.14"E
Kuala Terong (Perak)	4°38'45.49"N, 100°33'54.45"E
Sungai Besar (Selangor)	3°49'20.94"N, 100°47'07.93"E
Kuala Selangor (Selangor)	3°19'37.02"N, 101°13'25.85"E
Parit Raja (Johor)	1°47'57.48"N, 102°52'37.83"E
Parit Besar (Johor)	1°43'23.99"N, 103°00'49.09"E

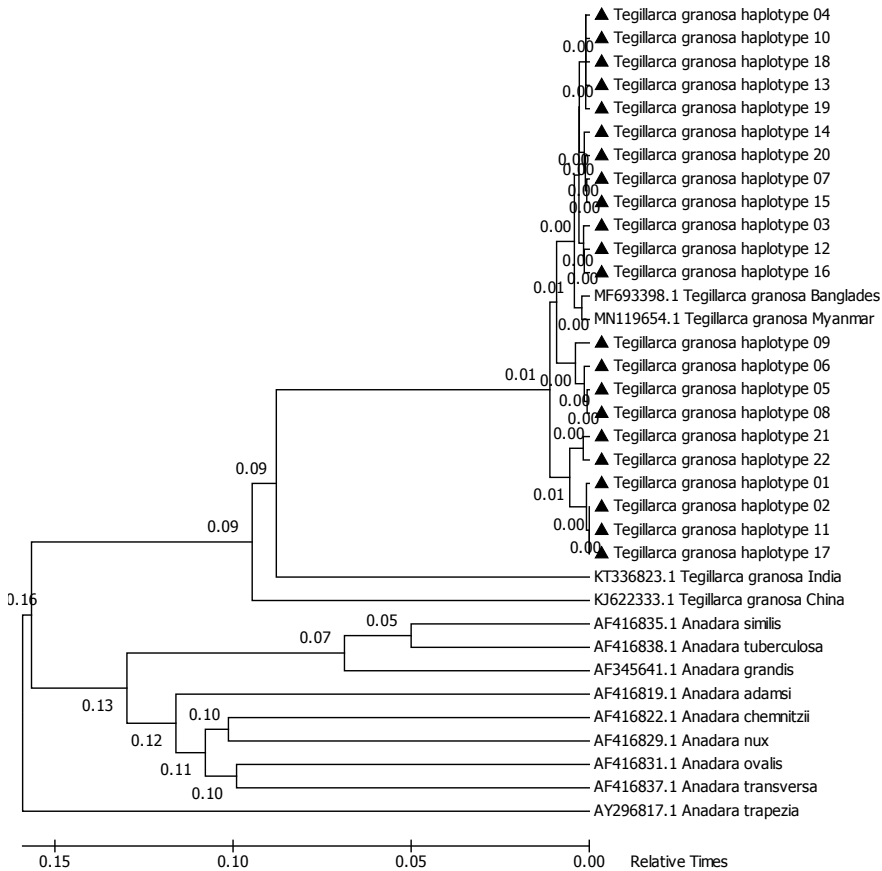
DNA was extracted from 25 mg gills using Dneasy Blood and Tissue kit (Qiagen, Germany). DNA amplification was conducted in a 25- $\mu$ L reaction mixture containing 12.5  $\mu$ L MyTaq mix (Bioline), 1.0  $\mu$ L of each forward (5'-CWA-ATC-AYA-AAG-ATA-TTG-GAA-C-3') (Colgan *et al.*, 2003) and reverse (5'-AAT-ATA-WAC-TTC-WGG-GTG-ACC-3') primer and 2.0  $\mu$ L template DNA. The amplifications were performed in Kyratec thermocycler (Australia), which was programmed for 35 cycles: 10 s at 94 °C, 10 s at 43 °C and 15 s at 72 °C. The purified PCR products were directly sequenced using an automated DNA sequencer (Apical Scientific Sdn Bhd).

All aligned sequences (MEGA 6.0) were collapsed to unique haplotypes or sequences using the DnaSP5.10 software (Julio Rozas *et al.*, 2010). The partial COI gene of *T. granosa* was then “blasted” with the COI gene of other species from the same genus using *blastn* from GenBank.

All sequences (*T. granosa* from Malaysia and other countries, and other bivalves from the same family obtained from GenBank) were aligned and a dendrogram was built to depict the phylogeographic relationship using a distance tree of pairwise comparisons provided by NCBI.

## Results

A 585-bp segment of the COI gene of *T. granosa* from Malaysia was sequenced. Collapsing the 180 sequences yielded 22 haplotypes. The *T. granosa* sequences had a maximum identity of 98-99% with each other. The sequences also had 77% maximum identity with the COI gene of *Anadara grandis* (603 bp) from Colombia, 75% with *A. tuberculosa* (603 bp) from Costa Rica, 75% with *A. similis* (603 bp) from Ecuador, 75% with *A. adamsi* and *A. nux* (both 586 bp) from Panama, 75% with *A. trapezia* (580 bp) from Queensland, 75% with *A. ovalis* (586 bp) from Florida, 73% with *A. chemnitzii* (586 bp) from Costa Rica, and 73% with *A. transversa* (570 bp) from Florida over the average query coverage of 86% (Figure 3). *Tegillarca granosa* is the only cockle among the other mentioned species found in Asia, whereas the majority are found in Northeast Australia, North and South America and the Caribbean.



**Figure 2:** Phylogenetic tree of the cockle species analysed as deduced from their COI gene sequences using the neighbour-joining phylogenetic-inference method. Divergence times for all branching points in the topology were calculated with the RelTime method using the branch lengths contained in the inferred tree.

## Discussions

The presence of conserved regions in the mitochondrial genome is often utilized, particularly in phylogenetic studies ranging from parasites (Gibson *et al.*, 2005) to fish (Abbasi *et al.*, 2007). According to Remigio and Hebert (2003), shellfish identification using the conserved regions of a sequence is remarkable especially with the COI gene because it enables the recovery of shallow divergences and more robust estimates of shellfish relationships. The results of their study also suggested that COI sequences are well suited to give an indication of shifts in rates of molecular diversity as well as evidence of molecular evolution. The COI sequences have been used to characterize and infer phylogenies across vertebrates and invertebrates. However, in order to assess individuals that are closely related to each other, microsatellites should be employed.

The studies of Canapa *et al.* (1999) on bivalves and Yoon and Kim (2005) on gastropods also supported the utilization of mtDNA, which includes both conserved and variable regions for phylogenetic investigations.

In order to estimate the time of divergence of *T. granosa* from the other closely re-lated species, the COI sequence of *T. granosa* was aligned with sequences of related species deposited in GenBank. This analysis revealed that it had a moderate percentage of intrageneric similarity with other species found in Central America, Caribbean and Australia ( $\approx 77\%$ ). Thus, evolutionary separation of *T. granosa* from its Australian as well as North and South American and Caribbean relatives could be assumed to have occurred at about the same time. However, when the sequence of *A. grandis* from Colombia was “blasted” with the sequences of the other species, it was found to be more related to the other South American species such as *A. tuberculosa* and *A. similis* (88-100%) than to *T. granosa* from Peninsular Malaysia and *A. trapezia* from Australia. Cunningham (2008) stated in his study done on *Littorina littorea* in North America that new alleles only arose 20 kilo years ago (kya). Based on this piece of information by Cunningham (2008), the South American species are estimated to have diverged from each other less than 140 kya. On the other hand, since *T. granosa* diverged by 23% from its South American relatives, it can be estimated that the Malaysian species evolved from the rest, including the Australian species about 320 kya.

A study conducted by Benson *et al.* (2001) on the green mussel (*Perna viridis*) found in Tampa Bay, Florida, also had a similarity of 78.2-99.5% to the *P. viridis* found in its native country, Trinidad. They discussed that the larvae of the species were probably entrained in ship ballast water. The conducive environments, which include the right temperature and the right substrates, contributed to their successful reproduction in Tampa Bay. Continued studies carried out by Fajans *et al.* (2004) three years after the first study had been done showed that the species had moved southward establishing new populations in Florida and Georgia. This finding shows how a closely related species can be found across large geographical distances, thrive and, to some extent, evolve over time to adapt to their surroundings.

The results of this study are beneficial in terms of identifying and classifying the cockles. Many times, the identification of specimens based on just the species' morphological attributes is inaccurate, leading to wrong conclusions in breeding and harvesting the cockles. Wang *et al.* (2008) used the fragments of the COI gene to classify the oysters in China using samples found in the Yangtze River. This study was able to distinguish the small oysters found in intertidal rocks as *Crassostrea gigas*, not *C. piculata* as widely assumed. They state that the small irregular shell characteristics are reflections of the stressful intertidal environment they inhabit and not reliable characters

for classification. Therefore, their study highlighted the need for reclassifying oysters of China with molecular data to facilitate sampling activities.

### Conclusion

This study serves as a basis for future studies possibly involving the conservation and management of the species. Phylogenies are powerful in drawing inferences from the structure of the tree, which can then be used to build hypotheses and models of important events in history. The relationships established by phylogenetic trees describe the species' evolutionary history and, hence, the historical relationships among lineages or organisms or their parts, such as genes.

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