



# DNA Barcoding Based on 16S Mitochondrial DNA (mtDNA) Molecular Marker of Mangrove Clams from the Selected Sites of Davao Region, Philippines

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## Authors' contributions

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** The taxonomic identification of mangrove clams was confirmed using DNA barcoding based on 16S Mitochondrial DNA (mtDNA) genome.

**Study design:** DNA was extracted from adductor muscle of mangrove clams sample. Purified DNA were sequenced and DNA barcode was generated.

**Place and duration of the study:** The Mangrove Clams samples used in the study were collected from Malita, Davao Occidental; Santa Cruz, Davao Del Sur; Mati, Davao Oriental, Philippines from January 2021 to June 2021.

**Methodology:** DNA was extracted from Adductor muscle of mangrove clams using the Promega GoTaq PCR kit. The extracted DNA were then purified and viewed using the Gel Electrophoresis. The Purified DNA samples were then sequenced and various softwares were then utilized such as

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sequenced assembly and alignment namely Basic Local Search Tool (BLAST) and Barcode Of Life Database(BOLD) and phylogenetic tree was generated.

**Results:** There are two species morphologically identified as *Anodontia philippiana* (Reeve, 1850) and *Austriella corrugata* (Deshayes, 1843) from Davao region. BLAST search established the two species to be closely related to *Anodontia* (Cavatidens) *omissa* (Iredale, 1930) and *Phacoides pectinatus* (Gmelin, 1791), also a member of the Lucinidae family.

**Conclusion:** In this study, the taxonomic identification of the two Mangrove Clams species were further elucidated using the 16S Mitochondrial DNA marker.

**Keywords:** *Imbaw*; DNA barcoding; 16S mtDNA; Lucinidae Family; Bivalves.

## 1. INTRODUCTION

The archipelagic features of Philippine islands suited the survival of abundant aquatic commodities. Remarkably, it provided niches to about ten(10%)percent (22,000) of the mollusk species worldwide. Mollusk constituted of the production of the inland fisheries [1]. Bivalves and gastropods are the most abundant mollusk in mangrove forest and constitute a significant trophic component of organic based foodwebs [2].

*Anodontia* sp. locally known as Imbao belongs to the family Lucinidae, they are distributed abundantly in the Indo-West pacific region, and are well known to be a delicious seafood delicacy in the Philippines. Imbao is considered as one of the major commodities in the region due to its flavor, size and demand as locally favourite shellfish in the region [3]. In Davao Region, there are two (2) locally known species namely “Imbaw laki” and “Imbaw Baye”, they are usually harvested by local fisherman by excavating in the sandy-muddy substrate. The two species were identified as *Anodontia edentula* (Linnaeus, 1758) which was renamed later to *Anodontia philippiana* (Reeve, 1850) [4] and *Austriella corrugata* (Deshayes,1843) [4] respectively (Taylor and Williams, 2008) [5].

There had been a decline in biodiversity and distinct increase in the number of endangered species observed for marine mollusk due to climatic change, marine environment deterioration and unprecedented anthropogenic activities [6]. Thus, there is a need for proper and accurate species identification of existing species for economic and conservation purposes. Despite the identity of these selected species were already established, most of the identification system were based on morphology and may not be accurate due to the existence of cryptic species. In Davao region, there had been

scarce studies on the molecular identification of the said high valued species.

DNA barcoding represents a tool for identification based on the highly established molecular marker [7]. It is currently utilized to identify invasive species and in improving biosecurity. In the past, morphology was used as the sole identifier of species population. However, it was found to be limited and would require highly trained taxonomist [8]. Moreover, there are cases when morphological characters are missing which can mislead in the identification of species. Complex morphological approaches of species in the phylum Mollusca hinder its appropriate conservation and management [6].

The molecular identification of species allows authentication of aquatic products. DNA barcoding can be useful for species identification and more reliable to assign species when traditional taxonomy is ambiguous [9]. Most DNA-based utilized a specific conserved gene region which has moderate variation. Mitochondrial DNA (mtDNA) is maternally inherited and therefore the succeeding generation would only have the maternal DNA. Due to this, mtDNA sequences can be used to differentiate species. Hebert et al. (2002) proposed the use of mtDNA sequences gene cytochrome oxidase 81 subunit I (COI) as a global identification system for animals. Previous studies have proven that mtDNA barcodes are highly effective in identification of Coleoidea: Cephalopoda [9], Canadian marine Mollusk, Deep-sea clams: Vesicomidae [10], Certhiidae: Gastropod [11] and marine mollusk *Corallina officinalis* [12].

## 1.2 Objectives of the Study

The current study is the first account in Davao region to barcode the two locally known species of Mangrove Clams using the 16S mtDNA gene

sequences as the molecular marker. It specifically aimed:

1. Provide a molecular barcode of the locally collected Mangrove Clams using the 16S Mitochondrial DNA genome;
2. Analyze the phylogenetic relationships between bivalve species using different software packages;
3. Compare DNA sequences to sequences available in Genbank using BLAST and Bold search.

## 2. MATERIALS AND METHODS

This is a descriptive study which includes methods on the collection and preservation of samples, extraction of DNA, PCR amplification, gel electrophoresis, gene sequencing and analysis of DNA sequenced using softwares and programs. The sample collection was conducted in the three provinces in Davao region namely Sta. Cruz, Davao del Sur (6° 49.16' N, 125° 23.27' E) and Malita, Davao Occidental (6° 49.16' N, 125° 35.23' E) and Mati, Davao Oriental (6.9522° N, 126.2173° E). The sample preparation was done at SPAMAST-Malita, Davao Occidental while the DNA extraction was conducted at Coral Reef Resiliency and Ecology Studies Laboratory at the University of the Philippines, Mindanao, Mintal Davao city, Philippines.

### 2.1 Sample Collection and Morphological Identification

A total of 30 samples were collected from the three provinces of Davao region but only 13 samples have successful PCR products. The muscle tissues from the Adductor Muscle of bivalves were preserved and subsequently stored in 95% alcohol. Shells were used for morphological species identification.

### 2.2 DNA Extraction and PCR Amplification

DNA extraction was performed using Promega GoTaq PCR kit. Extracted DNA was subjected to PCR amplification using CO1 primers: 16S SAR (5'-CGCCTGTTTATCAAAAACAT-3') and 16S SBR (5'-CCGGTCTGAACTCAGATCACGT-3'). Amplification was performed with a master mix based from Williams and Ozawa (2006). PCR was carried out using the protocol of Williams and Ozawa (2016) [13].

### 2.3 Gel Electrophoresis

Successfully purified PCR products were subjected to gel electrophoresis to check the presence of DNA. The size and quality of PCR product were assessed in 1.5% agarose gel and stained with ethidium bromide.

### 2.4 DNA Sequencing

Purified DNA products were sent to Macrogen, South Korea for DNA sequencing.

### 2.5 Sequence Assembly and Alignment

All sequences were assembled in Geneious R11 and aligned using MUSCLE in MEGA7.

### 2.6 BLAST and BOLD Identification

Each sequence was queried in BLAST for comparison of DNA sequences available in Genbank. All identified species under BLAST search was checked on IUCN red list of threatened species to identify endangered species.

### 2.7 Phylogenetic Analysis

For analysis of the base composition and visualization of the relationships among bivalve species included in the study, the software package MEGA7 was used. Phylogenetic analysis using the maximum likelihood tree model was conducted. Pairwise distances were also calculated.

## 3. RESULTS AND DISCUSSION

The Mangrove Clam species belongs to a shell family (Order: Veneroida; Family: Lucinidae) that accommodate symbiotic bacteria. It is a fact that brackish water pond sediments contain copious of sulfides, particularly, where the cultured animals are nourished with protein-rich diets [14]. As a constituent of family Lucinidae, some of species of Mangrove Clams have oval moderately expanded shell enveloped with closely packed greenish brown periostracum. On the inner side internal side of the shell, the anterior Adductor Muscle scar is isolated from the pallial line quarters of its length, hinge teeth are absent and shell ligament is lengthy and wide. The mantle is sturdy with folded edges and display fusion below the inhalant aperture. Foot is tubular, terminating in a muscular tip. The

gills comprise of two demi branch sheathing the visceral mass with eminent globular gonad.

*Austriella corrugata* (Fig. 1) can be identified by its ovate shell with more regular commarginal lamellae and eminent anterior and posterior sulci. The extended, wide ligament is at the rim of the hinge region of the shell and is conspicuous externally when the valves are closed meanwhile the ovate shell of *Anodontia philippiana* (Fig. 1) could be ascertained from *A. corrugata* by their fine growth lines. The long, broad ligament is relocated towards the interior and will not be visible externally when the valve joined together.

The collected samples of Mangrove Clams were identified morphologically using the taxonomic

guides and publish researches on the Lucinidae family [3], Taylor and William (2008) [13], Rochmady, R [15], Argente, F., (2018) [16]. The collected samples from fishing village, Malita, Davao Occidental were morphologically identified as *Anodontia philippiana*, meanwhile the two (2) samples from Santa Cruz, Davao del Sur were identified as *A. philippiana* while three (3) samples were identified as *Austriella corrugata*. Moreover, the collected four (4) samples from Mati, Davao Oriental were identified as *A. philippiana* and one (1) sample was identified as *A. corrugata* (Table 1). For molecular characterization, obtained DNA sequences were compared to available sequences in Genbank and BOLD system. Species with closest match was generated by BLAST was then compared with the morphological identification.



**Fig. 1. Left and Right valves of *Anodontia philippiana* (Top) and *Austriella corrugata* (Bottom). Note the prominent anterior and posterior sulci and regular growth lines of *A. corrugata*. The *A. philippiana*, on the other hand, has fine growth lines. Both valves are covered by greenish brown periostracum**

**Table 1. Morphological identification of the collected Mangrove Clams in the selected sites of Davao Region, Philippines**

Sampling sites	Sample code	Morphological Identification
Malita, Davao Occidental	FVF1	<i>Anodontia philippiana</i>
	FVF2	<i>Anodontia philippiana</i>
	FVF3	<i>Anodontia philippiana</i>
Santa Cruz, Davao Del Sur	SCM2	<i>Austriella corrugata</i>
	SCM3	<i>Austriella corrugata</i>
	SCM4	<i>Austriella corrugata</i>
	SCF1	<i>Anodontia philippiana</i>
Mati, Davao Oriental	SCF5	<i>Anodontia philippiana</i>
	MTO1	<i>Anodontia philippiana</i>
	MTO2	<i>Anodontia philippiana</i>
	MTO3	<i>Anodontia philippiana</i>
	MTO4	<i>Anodontia philippiana</i>
	MTO5	<i>Austriella corrugata</i>

The 16S rRNA sequences of the Mangrove Clams were run to Basic Local Alignment Search Tool (BLAST) to compare DNA sequences to sequence databases and calculate statistical significance, based on the Nucleotide Blast the FVF1, FVF2, FVF3, SCF1, SCF5, MTO1, MTO2, MTO3, MTO4 samples closely similar to a species belonging to Lucinidae family, *Anodontia omissa* with 79-100 % sequence similarities meanwhile the samples SCM2, SCM3, SCM4, MTO5 closely matched to another species from Lucinidae family, *Phacoides pectinatus* with 95% sequence similarities. So far based from the Nucleotide databases, there had been no reported or uploaded 16S rRNA sequences for *A. philippiana* and *A. corrugata*, the lower percentage of similarity for DNA sequences can be due to the limited nucleotides databases for the sequenced species reported in this study (Table 2). Moreover, no similar sequence was generated from Barcode of Life Data system (BOLD). This can be due to lack of deposited sequence in the database.

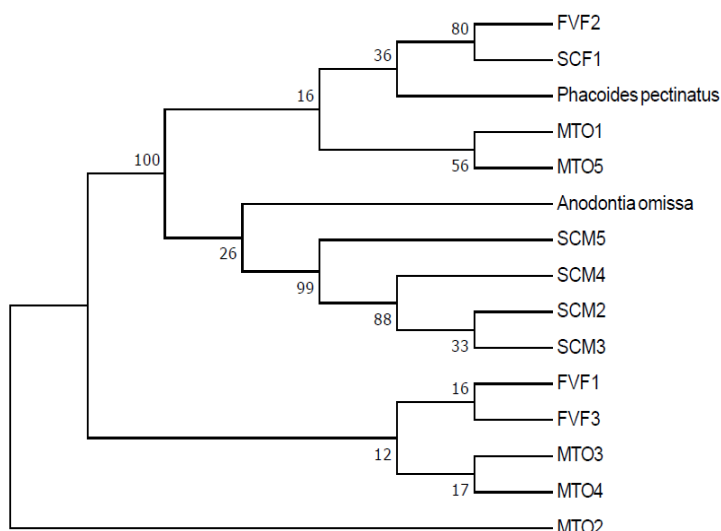
Neighbor-joining tree and bootstrapping were utilized to support the identity of successfully identified species using the BLAST search. The DNA sequence with highest possible identity percentage in Genbank(BLAST) was chosen for each specimen to identify matches for species identification. The 16S mtDNA sequence of specimens morphologically *A. philippiana* and *A. corrugata* (Fig. 2) shows they belong

to three (3) different clades under family Lucinidae.

The phylogenetic tree shows the three (3) major clades with the corresponding bootstrap value, the first clade shows FVF2, SCF1, MTO1, MTO5 and the reference sample *Phacoides pectinatus* with FVF2 and SCF1 with a bootstrap value of 80 which means it is well supported taxa and may be considered as closely related species meanwhile MTO1 and MTO5 has 56 bootstrap value which is a weakly supported taxon. The second clade constitutes the SCM2, SCM3, SCM4, SCM5 and the reference sample *Anodontia omissa*, the species in the clade has a bootstrap value of 88 and 99 which indicates a well-supported taxa. Moreover, the third clade composed the FVF1, FVF3, MTO3, MTO4 with a bootstrap value of 100 while less than 50 bootstrap value were observed at the species level. Meanwhile, the MTO2 is considered an outgroup which means it is highly genetically diverged and does not belong to any to clade. Bootstrap values represent the similarity of the sequence divergence or the degree of similarity of the DNA sequence. A bootstrap values range of 70-100 % indicated a well-supported to strongly supported clades in a 1,000 replicates phylogenetic analysis. Hesterberg et. al. (2003) affirmed that the percentage of 1000 bootstrap replications with values above 80% at the branching demonstrate very good results because these values strongly corroborated that samples which were in one branch were correct as of the species level identification [17].

**Table 2. Basic Local Alignment Search Tool (BLAST) results of the species 16S rRNA barcodes**

Species code	Blast Result	Similarity Percentage
FVF1	<i>Anodontia omissa</i>	96%
FVF2	<i>Anodontia omissa</i>	95%
FVF3	<i>Anodontia omissa</i>	96%
SCM2	<i>Phacoides pectinatus</i>	95%
SCM3	<i>Phacoides pectinatus</i>	95%
SCM4	<i>Phacoides pectinatus</i>	95%
SCF1	<i>Anodontia omissa</i>	96%
MTO1	<i>Anodontia omissa</i>	100%
MTO2	<i>Anodontia omissa</i>	96%
MTO3	<i>Anodontia omissa</i>	96%
MTO4	<i>Anodontia omissa</i>	96%
MTO5	<i>Phacoides pectinatus</i>	95%



**Fig. 2. Molecular Phylogenetic analysis by Maximum Likelihood method of Mangrove Clams showing the evolutionary distances in substitution per site at 0.50 scale**

The comparison of nucleotide sequences (Fig. 3) between the collected specimens from Davao region showed that the average nucleotide variations were between 0.600 to 0.700 towards the other Lucinidae family namely the *A. omissa* and *P. pectinatus*. The nucleotide variations within the group of the specimens range from 0 to 0.700. The lesser value of variations, the closer species are directly related or most likely belong to the same species. The greater variations might indicate the process of speciation in response to the changing environment of the species. The greater value of variations might indicate a greater evolutionary divergence or totally a different species. A genetic distance value of less than 3% indicating intraspecies species relationship [18]. The proximity of the genetic distance, the taxonomic level is at the minute level, namely the species and the close relationship between the species. Conversely, the higher the value of genetic

distance, and the greater the difference in nucleotide bases, the more distant the relationship [19]. The higher the value of genetic distance (p-distance) between a population or individual, the more isolated they are to one another. Genetic distance suggested the potential influence of geographical isolation on a population [20]. Moreover, low genetic distance is indicative of gene flow between populations. The smaller the genetic distance between individuals in a population, the more homogeneous the population of a certain species. The loss of genetic diversity will result in the detriment of growth and development, fertility, and being susceptible to various diseases, which are vital processes in life, production and reproduction. Several studies have exhibited that the genetic distance of fish and invertebrate animals is closely related to geographical conditions [21].

FVF1																						
FVF2	0.746																					
FVF3	0.000	0.746																				
SCM2	0.730	0.720	0.730																			
SCM3	0.730	0.720	0.730	0.000																		
SCM4	0.730	0.720	0.730	0.000	0.000																	
SCM5	0.735	0.720	0.735	0.011	0.011	0.011																
SCF1	0.746	0.598	0.746	0.704	0.704	0.704	0.698															
MTO1	0.704	0.725	0.704	0.735	0.735	0.735	0.735	0.757														
MTO2	0.000	0.746	0.000	0.730	0.730	0.730	0.735	0.746	0.704													
MTO3	0.000	0.746	0.000	0.730	0.730	0.730	0.735	0.746	0.704	0.000												
MTO4	0.000	0.746	0.000	0.730	0.730	0.730	0.735	0.746	0.704	0.000	0.000											
MTO5	0.735	0.688	0.735	0.746	0.746	0.746	0.746	0.709	0.672	0.735	0.735	0.735										
<i>Anodontia omissa</i>	0.651	0.720	0.651	0.656	0.656	0.656	0.656	0.730	0.693	0.651	0.651	0.651	0.677									
<i>Phacoides pectinatus</i>	0.714	0.720	0.714	0.741	0.741	0.741	0.735	0.683	0.751	0.714	0.714	0.714	0.762									

**Fig. 3. Evolutionary divergence between Mangrove Clams based on 16S rRNA gene sequence**

The molecular data further strengthened the observed morphological characteristics of Mangrove Clams in Davao region belonging to the family Lucinidae and might consist of two defined species namely *A. philippiana* and *A. corrugata*. 16S mtDNA gene sequences appear to be able to demarcate interspecific difference from the two recognized species of Mangrove Clams. When compared with sequence from other Bivalves, this resulted in clustering of the two species into different branches of family Lucinidae. *A. philippiana* clustered with other *Anodontia* species and appeared to be closely related to *A. omissa* from United states of America [22]. The high bootstrap value suggests that the two specimens although varied geographically maybe closely related species. The significant nucleotide variation between the collected specimens from Davao region may implies that the population in the region may be highly heterogeneous.

Moreover, *A. corrugata* was found to be cluster at a different branch of family Lucinidae, the groups of Lucinids living in shallow water and deep-sea vents. The 16S mtDNA gene sequences of the collected specimens from Davao region was found to be highly similar with *P. pectinatus* from Southern Australia [23].

In this study the morphological identification and molecular data of the two species (*A. philippiana* and *A. corrugata*) were studied and generated.

#### 4. CONCLUSION

DNA barcoding is an indispensable tool for identification of Bivalves species. It can be used for species identification, food safety, conservation management and animal breeding. It also allows the elucidation of putative new species and discriminate properly the molecular identity of the species. In this study, the 16S mtDNA of the morphologically identified *A. philippiana* and *A. corrugata* were sequenced and generated for future reference for further molecular characterization of other bivalve species in Davao region. Moreover, a study of the genetic diversity of Mangrove Clams in Davao region would highly be desirable to fully understand the mechanisms behind the observed genetic variability between the two species in shared habitat.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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