Original Research Article

DNA barcoding based on 16s mitochondrial DNA (mtDNA) gene sequence of ~~Mangrove~~ mangrove clams (*Anodontia* sp.) collected from the selected sites of Davao region, Philippines

Abstract

Aims: The taxonomic identification of ~~Mangrove~~ mangrove clams was confirmed using DNA barcoding based on 16s mitochondrial DNA (mtDNA) gene sequence.

Study design: The adductor muscle was processed and DNA were extracted for DNA barcoding.

Place and duration of the study: The mangrove samples used in the study were collected from Malita, Davao O~~o~~ccidental~~,~~; Santa Cruz, Davao Del Sur~~,~~; Baganga, Davao Del Norte and Mati, Davao O~~o~~riental, Philippines from January 2021 to June 2021.

Methodology: The DNA of collected samples of mangrove clams were extracted from adductor muscle using the polymerase chain reaction. 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 namely sequenced assembly and alignment and BLAST and BOLD identification and Phylogenetic tree were generated.

Results: There are two species morphologically identified from Davao region namely *A. edentula* and *A. corrugata*. The BLAST search established the two species to be closely related to *A. omissa* and *P. pectinatus*, a member of the Lucinidae family.

Conclusion: In this study, the morphological identification of the two mangrove clams were confirmed by the 16s mitochondrial DNA barcode.

Keywords: Imbaw**,** DNA barcoding**,** 16s mtDNA**,** Lucinidae family**,** Bivalves.

Introduction

The archipelagic features of Philippine islands suited the survival of abundant aquatic commodities. Remarkably, it provided niches to about 10 % (22,000) of the mollusk species

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worldwide. Mollusk constituted of the production of the inland fisheries[1]. Bivalves and gastropods are among the most abundant mollusk in mangrove forest and represent an important 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) 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) and *Austriella corrugata* (Deshayes, 1843) respectively (Taylor and Williams, 2008)[4].

There had been a decline in biodiversity and distinct increase in the number of endangered species observed for marine mollusk due to climatic change, coastal environment deterioration and anthropogenic activities[5]. Thus, there is a need for proper and accurate species identification of still 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 no studies on the molecular identification of the locally known species of ~~Mangrove~~ mangrove clams.

DNA barcoding represents a tool for identification based on the highly established molecular marker[6]. It is currently being used to identify invasive species and improving biosecurity. In the past, morphology was used as the sole identifier of species population. However, it was to be unproductive[7]. There are cases when morphological characters are missing which can mislead in the identification of species. Complex morphological

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approaches of species in the phylum Mollusca hinder its appropriate conservation and management[5].

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[8]. 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[8], Canadian marine Mollusk, Deep-sea clams: Vesicomyidae[12], Certhiidae: Gastropod[13] and marine mollusk *Corallina officinalis*[14].

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~~ mangrove clams using the 16s mitochondrial DNA;~~.~~

2. Analyze the phylogenetic relationships between bivalve species using different software packages;~~.~~

3. Compare DNA sequences to sequences available in in Genbank using BLAST and Bold search.

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 samples using softwares and programs. The sample collection was conducted in the three provinces in Davao region namely Baganga, Davao Oriental (7^0 34.12' N, 126^0 33.36' E), Sta. Cruz, Davao del Sur (6^0 49.16' N, 125^0 23.27' E) and Malita, Davao Occidental (6^0 49.16' N, 125^0 35.23' E) and Mati, Davao Oriental (6.9522° N, 126.2173° E). The sample preparation was conducted at SPAMAST-Malita, Davao Occidental while the DNA extraction was conducted at Philippine Genome Center Mindanao at the University of the Philippines, Mindanao, Mintal Davao Ceity, Philippines.

Sample collection and Morphological Identification.

A total of 30 samples were collected from the three provinces of Davao rRegion. The muscle tissues from the adductor muscle of bivalves were preserved and subsequently stored in 95% alcohol. Basing on vernacular names, 15 samples were selected for DNA extraction. Shells were used for morphological species level identification.

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)[15].

Gel Electrophoresis.

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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.

***DNA sequencing*.**

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

Sequence Assembly and Alignment.

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

BLAST and BOLD Identification.

Each sequence was queried in BLAST for comparison of DNA sequences available in Genbank. Along with BLAST, BOLD was used to minimize the risk of using contaminated sequences. All identified species under BLAST search was checked on IUCN red list of threatened species to identify endangered species.

Phylogenetic Analysis

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

Results and Discussion

The ~~Mangrove~~ 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[16]. As a constituent of family Lucinidae, some of species of

m~~M~~angrove 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 **(Figure 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 edentula* (**Figure 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.

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

The collected samples of ~~Mangrove~~ mangrove clams were identified morphologically using the taxonomic guides and publish researches on the Lucinidae family[3], Taylor and William (2008)[15], Rochmady, R[17], Argente, F., (2018)[18]. The collected samples from fishing village, Malita, Davao Occidental were morphologically identified as *Anodontia edentula*, meanwhile the two (2) samples from Santa Cruz, Davao del Sur were identified as *A. edentula* while three (3) samples were identified as *Austriella corrugata.* Moreover, the collected four (4) samples from Mati, Davao Oriental were identified as *A. edentula* and one (1) sample was identified as *A. corrugata* (Table 1).

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SAMPLING SITES	SAMPLE CODE	Morphological Identification
Fishing Village, Malita Davao Occidental	FVF1	*Anodontia edentula*
	FVF2	*Anodontia edentula*
	FVF3	*Anodontia edentula*
Santa Cruz, Davao Del Sur	SCM2	*Austriella corrugata*
	SCM3	*Austriella corrugata*
	SCM4	*Austriella corrugata*
	SCF1	*Anodontia edentula*
	SCF5	*Anodontia edentula*
Mati, Davao Oriental	MTO1	*Anodontia edentula*
	MTO2	*Anodontia edentula*
	MTO3	*Anodontia edentula*
	MTO4	*Anodontia edentula*
	MTO5	*Austriella corrugata*

Table 1: Morphological identification of the collected mangrove clams in the selected sites of Davao region

Comment [M6]: In the materials and methods section you said that 30 samples were collected, but 13 samples were analyzed. Did you analyze the rest of the samples? If not, you can explain why you didn't.

The 16s rDNA sequences of the ~~Mangrove~~ mangrove clams were run to Basic Llocal Alignment Ssearch Ttool (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~~ 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 loaded 16s rDNA sequences for

A. edentula 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).

SPECIES CODE	BLAST RESULT	SIMILARITY PERCENTAGE
FVF1	*Anodontia omissa*	96 %
FVF2	*Anodontia omissa*	95%
FVF3	*Anodontia omissa*	96%
SCM2	*Phacoides pectinatus*	95%
SCM3	*Phacoides pectinatus*	95%
SCM4	*Phacoides pectinatus*	95%
SCF1	*Anodontia omissa*	96%
MTO1	*Anodontia omissa*	100%
MTO2	*Anodontia omissa*	96%
MTO3	*Anodontia omissa*	96%
MTO4	*Anodontia omissa*	96%
MTO5	*Phacoides pectinatus*	95%

Table 2: Basic Local Alignment Search Tool (BLAST) results of the species 16s rDNA barcodes

The 16s mtDNA sequence of specimens morphologically *A. edentula* and *A. corrugata* (**Figure 2**) shows they belong to three (3) different clades under family Lucinidae. *A. edentula* specimen from Davao region was in a branch clustered with other *Anodontia* species from United States of America, the *Anodontia omissa*. The morphologically identified *A. corrugata* specimens from Davao region was in a branch with another Lucinidae family, *Phacoides pectinatus* from Australia. Moreover, FVF1, FVF3, MTO3 and MTO4 (**Figure 2**) which were morphologically identified as *A. edentula* were in separate clades mostly for another Lucinidae family.

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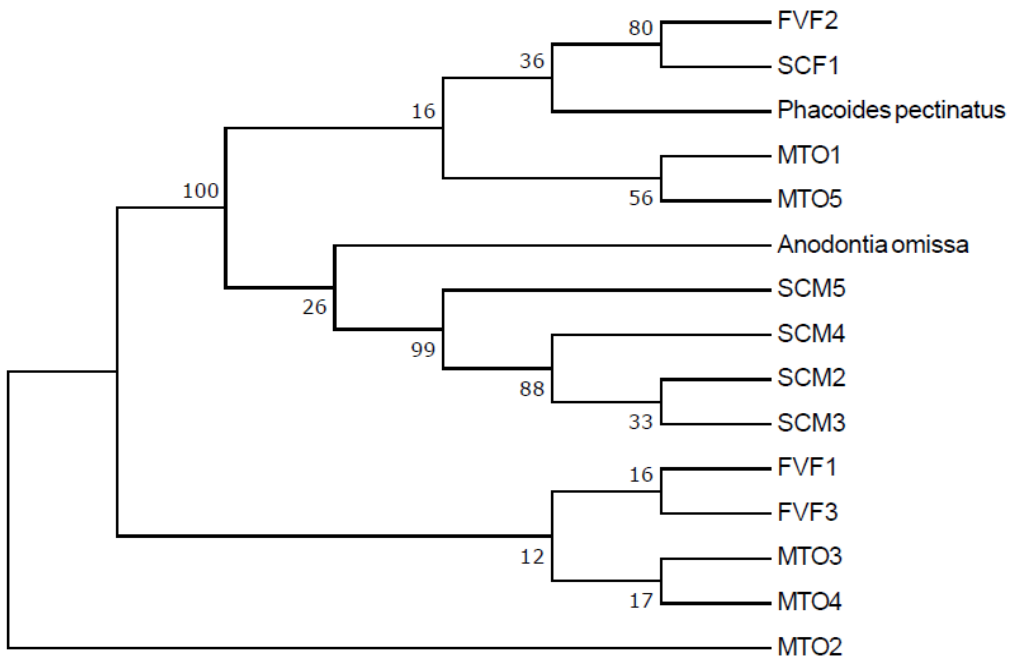

Figure 2: Molecular Phylogenetic analysis by Maximum Likelihood method of mangrove clams showing the evolutionary distances in substitution per site at 0.50 scale.

The comparison of nucleotide sequences **(Figure 3)** between the collected specimens from Davao ~~Region~~ region showed that the average nucleo~~u~~tide variations were between 0.600 to 0.700 towards the other Lucinidae family namely the *A. omissa* and *P. pectinatus*. The nucleo~~u~~tide variations within the group of the specimens range from 0 to 0.700. The lesser ~~the~~ value of variations, the closer ~~the~~ species are directly related or most likely belong to the same species. The greater ~~the~~ variations might indicate the process of speciation in response to the changing environment of the species. The greater ~~the~~ value of variations might indicate a greater evolutionary divergence or totally a different species.

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	
Anodontia omissa	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
Phacoides pectinatus	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

Figure 3: Estimate of Evolutionary divergence between ~~Mangrove~~ mangrove clams based on 16s rDNA sequences.

The molecular data substantiated the observed morphological characteristics that mangrove clams in Davao region belong to the family Lucinidae and might consist of two defined species namely *A. edentula* 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. edentula* clustered with other Anodontia species and appeared to be closely related to *A. omissa* from USA. The high bootstrap value suggests that the two specimens although varied geographically maybe similar species. The significant nucleo~~u~~tide 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.

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

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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. edentula* and *A. corrugata* were sequenced and generated for future reference for further molecular characterization of Bivalve species in Davao region.

Consent

Informed consent is not applicable on the nature of the study.

Ethical approval

Ethics clearance is not applicable on the nature of the study.

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