MOLECULAR AND HISTOLOGICAL IDENTIFICATION OF COMMERCIALLY IMPORTANT BAMBOO CLAM, *CULTELLUS SP*. IN KYAUKPYU, RAKHINE STATE

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Abstract

The razor or bamboo clam *Cultellus* is widely distributed in the intertidal zones and estuarine waters along the coast of western Pacific Ocean and is extensively cultured. In the present study, commercially important bamboo clams, *Cultellus* sp., were collected from the deep sea of Kyaukphyu, Rakhine coastal area from October 2020 through September 2021 to investigate the histological features of the bivalve species and the sex ratio of the population. The relation between shell length (mm), wet tissue weight (g), dry tissue weight (g) and sex of the clams was examined. Histological analysis of the gonad was conducted to reveal the gonad development of the clam. Except the undifferentiated stage, four different gonad development stages (developing, ripe, spawning and spent) were found in both male and female clams. In addition, the taxonomic position of the bamboo clam was studied by morphological characters and mitochondrial DNA sequences. Regarding sexual differentiation, higher numbers of male bamboo-clams were recorded than female bamboo clams. DNA sequence of a 1395 bp was obtained from the collected species and the species was identified as *Cultellus maximus*.

Keywords reproductive biology, histological, *Cultellus* sp., bamboo clam

Introduction

The razor clam, *Cultellus* sp., is a near shore marine bivalve known locally in Myanmar as bamboo clam. This razor clam species occurs in Asian pacific coastal waters and lives buried in the relatively flat sandy bottom between depths of 2.5 and 10 m (Nguyen, 2016). There are several known species of allied genus *Solen* Lin-naeus, 1758, but to date, *Cultellus* which is often misidentified as *Sinonovacula* sp. (Nguyen, 2016). *Cultellus* sp. is among the most important commercial bivalves to be considered for aquaculture recently (Nguyen, 1996) due to its potential economic value but its natural production continues to decline (Vu, 2021). However, little information is known about the genetic population structure and reproductive biology of this species within Kyaukphyu, precluding an informed and sustainable harvest program.

Bamboo clam trading has been started in Kyaukphyu area but knowledge of that species is limited. Differentiating between bivalve species based on their morphology is a challenging task due to the wide range of forms and sizes observed in their shells. The unique characteristics of their habitats greatly influence the tremendous variation in shell shapes, even within the same species, and this makes it very difficult to identify the species of any wild individual (Comesana *et. al*., 2001). In order to overcome the confusion stemming from the morphological characteristics of bivalves, recent years have witnessed the utilization of molecular biological techniques. These methods aim to identify individuals by examining a variety of genetic markers. By employing such techniques, scientists strive to enhance accuracy and precision in species identification. In this study, the suitability of mitochondrial DNA sequencing for the identification of clam was evaluated which is a fundamental requirement to extend the research work on that clam.

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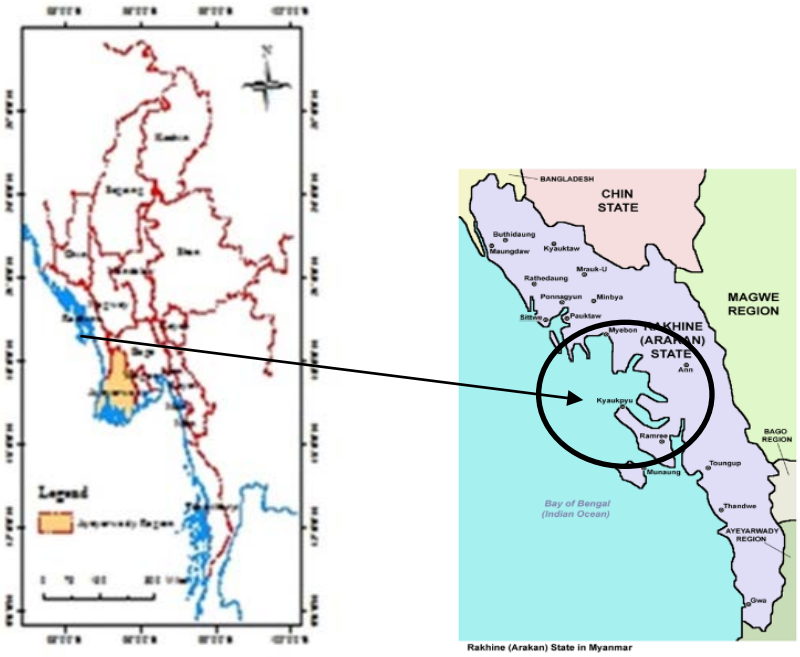
Understanding the reproductive cycle and spawning period of this species will provide information needed for the planning of a better conservation strategy for *Cultellus sp.* and recruitment period of this population. In Myanmar, the reproductive biology of this species is not well understood, and characterization of its gonad development is necessary step that would contribute to a better understanding of its reproductive biology. The objective of the present study was to investigate the DNA sequencing identification and reproductive biology of the bamboo clam *Cultellus* sp. in Kyaukphyu, Rakhine State, Myanmar.

Materials and Methods

Sampling site and the study period

The bamboo clam samples were collected from the intertidal zone of in Kyaukphyu Township of Rakhine State, Myanmar. This study area is located on the north western corner of Yanbye Island on Combermere Bay. It is situated at Latitude 19° 12’ 58” North and Longitude 93° 43’ 56” East (Fig. 1). The study was conducted from October 2020 to September 2021.

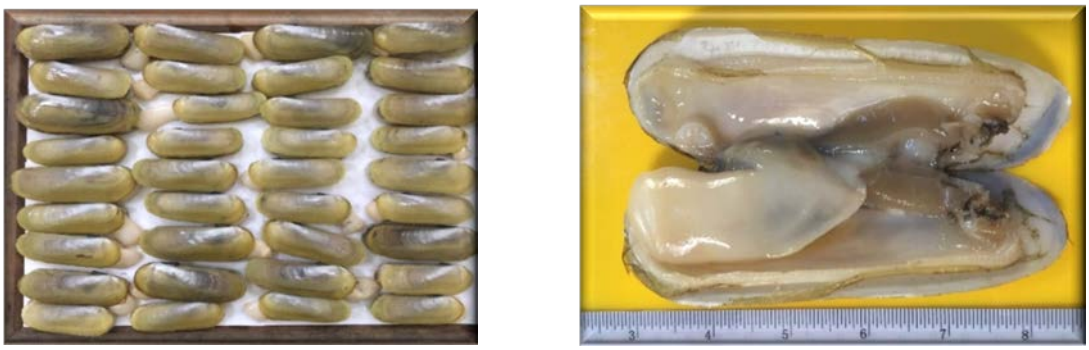


Myanmar Kyaukphyu Township, Rakhine State

Figure 1 Map showing location of Kyaukphyu Township of Rakhine State, Myanmar

Sample collection

Samples were collected during the low tide period. Low tide is the most favorable time for collecting bamboo clams that inhabits the intertidal zone. All samples were transported to the Laboratory of Aquatic Bioscience, University of Yangon immediately after harvest.



(A) (B)

Figure 2 (A) *Cultellus* spp. (B) Internal shell of the studied bamboo clam

Morphological analysis

A total of 100 clams were collected during the study period. In the laboratory, each clam was measured along its shell length to the nearest millimeter with vernier caliper. Immediately, the clams were opened and wet tissue weight was determined after removing excessive water using an absorbent tissue paper. Sexes were examined under the light microscope to determine the gender. Clams were identified by morphometric characters according to Morton (1984) (Fig. 3).

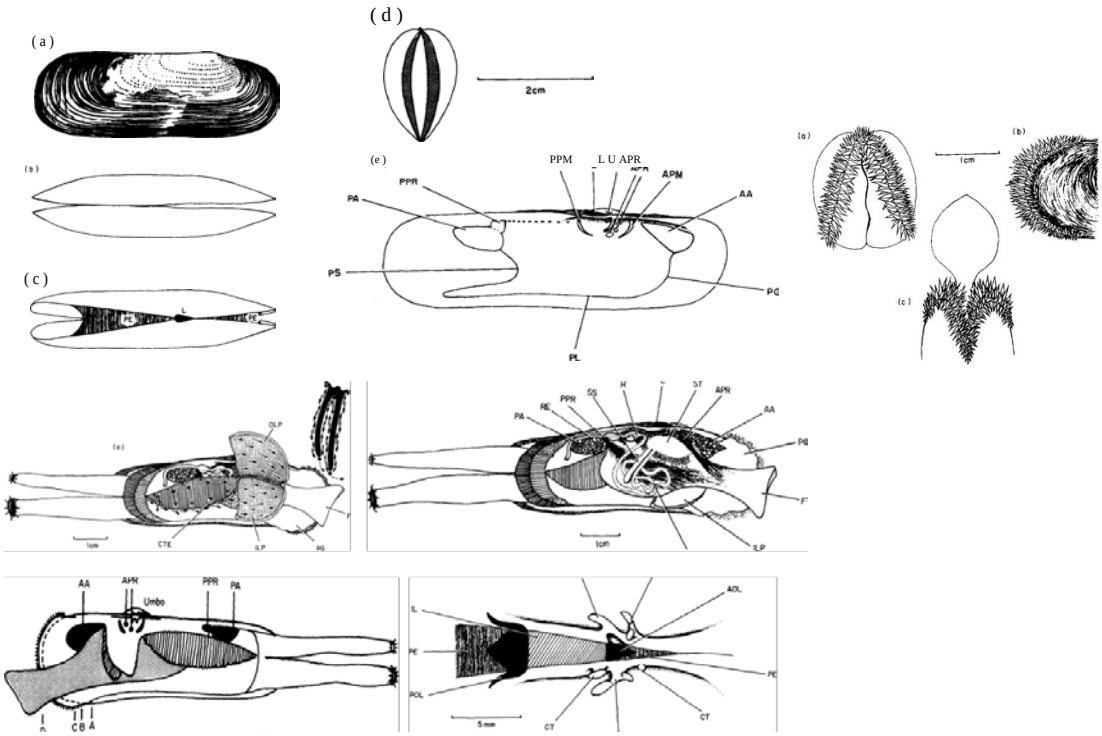


Figure 3 External and internal characters (a) The right; (b) ventral; (c) dorsal and (d) posterior aspects. (e) An internal view of the left shell valve; PPM-Posterior pedal protractor muscle or scar), PPR- Posterior pedal retractor muscle, PPM- Pallial retractor muscles, PS- Pallial sinus, PA- Posterior adductor muscle or scar, L-Ligament., U-Umbo, APR-Anterior pedal retractor muscle or scar, APM-Anterior pedal protractor muscle or scar, AA- Anterior adductor muscle or scar, (f) Pedal gape (i) anterior; (ii) lateral and (iii) dorsal aspects, OLP-Outer labial palp, CTE- Ctenidium, ILP-Inner labial palp. , F- Foot, ES-Exhalant siphon, IS-Inhalant siphon, RE-Rectum, SS-Style sac, H-Heart, ST-Stomach, CT-Cardinal tooth (Morton, 1984).

Histological preparation

A small piece of gonad (3 mm) under the mid region of the mantle lobe was removed, fixed individual pieces with Davidson's solution for 48 h. Samples were then dehydrated in a graded ethanol series, cleared in xylene and blocked-in paraffin wax. All sections, 5 μm in thickness, were cut using a rotary microtome and stained with Harris hematoxylin and counterstained with eosin. The histologically prepared slides were checked under a compound microscope.

Reproductive development analysis

The gonad development of bamboo clam was categorized into 5 stages and assigned stages 1 to 5 for undifferentiated, developing, ripe, spawning and spent, respectively, based on the microscopic appearance of the gonad tissue in histological sections [modified after Heffernan *et al*. (1989)]. Gonad development stages proposed for *Cultellus* sp. are described in Table (1).

Table 1 Gonad development stages proposed for *Cultellus* sp.

Sexual phase	Sex	Description
(1) Undifferentiated		Gonadal area replaced with connective tissue, but no trace of gonadal material
(2) Developing	Male	Spermatogonia and spermatocyte abundant, few spermatids and sperm present
	Female	Mostly oogonia, and attached oocytes and occasional free oocytes present
(3) Ripe	Male	Mostly spermatide, few spermatocyte present
	Female	Free oocytes with distinct nucleus and nucleoi present
(4) Spawning	Male	Follicles partly empty, few sperm present
	Female	Follicles partly empty, few free oocytes present
(5) Spent	Male	Follicles contracted, few in number, occasionally residual sperm present, connective tissue abundant
	Female	Follicles contracted, few in number, occasionally residual oocytes present, connective tissue abundant

DNA extraction

Only ten clams were used for DNA extraction and gene sequencing. The clams were fixed in 70% ethanol before the preparation of DNA extraction (Fig 4 A). PetNAD nucleic acids co-prep kit was used to extract DNA from the gill tissue of the clam (Fig. 4 B). DNA extraction was conducted according to the instructions in PetNAD nucleic acids co-prep kit.

(A) (B)

Figure 4 (A) Fixation of *Cultellus* spp. (B) PetNAD Nucleic Acid Co-prep Kit

PCR amplification and gene sequencing

In order to amplify mitochondrial cytochrome gene, universal primers for bivalve COI region, 28S- LCO1490F (5'- ggtcaacaaatcataaagatattgg -3') and 28S- HCO2198R (5'- taaacttcagggtgaccaaaaaatca -3') were designed (Table 2). For PCR, 5 µL of the extracted DNA in 75.5 µL of water was mixed with 3.0 µL of 25 µM of each primer, 0.5 µL of HS Taq DNA polymerase, 10 µL l of 10 × PCR buffer and 8.0 µL of dNTPs (WizPure, Seongnam, South Korea). The PCR conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72°C for 1 min, and a final extension at 72 °C for 7 min. The PCR products were separated and visualized by electrophoresis on 1.5 % agarose gel containing SYBR Safe DNA gel stain ((WizPure, Seongnam, South Korea) and the DNA was extracted and purified from the distinct bands (around 680 bp) and using FastGene® Gel/PCR Extraction Kit (Nippon Genetics Europe GmbH, Bunkyo, Tokyo). Purified DNA products were quantified and used for direct DNA sequencing by the PCR primers with an ABI gene sequencer. Genes sequence of the clam was compared with that of other bivalve species using the BLAST search located in the National Center for Biotechnology Information (NCBI) (http//www.ncbi.nih.gov).

Table 2 Primer details for the PCR detection of the studied clam

Primers	Direction	Sequences	Tm
LCO1490	Forward	5’-ggtcaacaaatcataaagatattgg-3’	51°C
HCO2198	Reverse	5’- taaacttcagggtgaccaaaaaatca-3’	44°C

Tm = melting temperature

Results

Morphological description

The clam had two thin, equal-sized valves that were elliptical in shape and marked with conspicuous concentric rings. The valves were joined dorsally by a dark brown ligament which was external and triangular. Opposite the hinge was a distinct heart shaped lunule. The external shell was generally pale yellow-colored or off-white. The internal shell was white or pale yellow margin. Three cardinal teeth were found in left valve, two in right valve. No lateral teeth found in this calm. Right anterior and posterior cardinal teeth were smooth while remaining cardinal tooth was usually bifid. Inner margin was smooth and located at the posterior third.

Posterior pedal retractor muscle and PRM- pallial retractor muscles was present. Pallial sinus was internally deep. Anterior and posterior adductor muscle or scar was generally equal in size. Umbo was posteriorly elongate and Pedal gape was present. Anterior pedal protractor muscle or scar was present where anterior pedal retractor muscle or scar was smaller in size. Periostracum was thickened in this species. Pedal gape, (i) anterior; (ii) lateral and (iii) dorsal aspects, was wide. OLP-outer labial palp, was unridged and CTE- ctenidium was large.

Inner labial palp was ridged where foot was elongate. The body of the clam was surrounded by a mantle. A pair of siphons fused at the bases. Exhalant siphon and inhalant siphon were separate. The siphons were generally creamy white, but the margins were colored in a range of yellows or browns. Rectum and style sac were present in this clam. Stomach was small. According to morphological characters, the clam was tentatively identified as belonging to the families Pharidae or Solenidae or Tellinidae.

DNA sequencing

DNA sequences of a 1395 bp fragment were obtained from the clam (Fig. 5). The BLAST search of database located in the NCBI revealed that the clam DNA sequence was highly homologous to the genus *Cultellus.* Sequence identities are 99.85 % to *Cultellus maximus* (MN119650), 89.74 % to *C. attenuatus* (OQ434723), 88.17 % to *Novaculina myanmarensis* (MW549330) and 87.58 % to *N. gangetica* (MF958991). Identities with the genus *Novaculina* are much lower than to the genus *Cultellus*; 88.17% to *N. myanmarensis* (MW549330) and *N. gangetica* (MF958991) and 86.77% to *Pharella acutidens* (MW311106) (Table. 3).

CCAATAAAATATGATTTTGGCGTTTTGATCTGGATTAGTTGGAACTAGGTTGAGGC……..56
TATTGATTCGTTTGGAGTTGGCTCGGCCTGGTGGTTTTATGGGGGATGAACATTTA…… 112
TATAATGTTATTGTTACTGCTCATGCTTTTGTTATGATTTTTTTTTTGGTTATGCC……..150
AATAATGGTTGGTGGGTTTGGTAATTGATTATTACCTTTAATGTTAACTTCTCCTG……200
ATATGTCTTTTCCTCGAATAAATAATTTGAGGTTTTGGTTGCTTCCTTTTGCGTTG……250
TTTTTTTTGTTATGGTCTGGGTTTGTTGGGAGGGGTATTGGTGCTGGTTG……300
AACTATTTATCCTCCTTTATCTGGAAATTTGGCCCATAGGAGTCCTTCAATGGATT…… 350
ATGGAATTTTTTCTATACATTTGGCGGGTGCTTCTTCTATTTTAGGTGCAGCTAAT…… 400
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GT TGTTTGTTTGGTCAGTAGTTATTACTGCTTTTTTATTGGTTTTGGCTATATGTTTG ……..500
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GAGGGGTGGTGGTGACCCTATTTTGTTTGTTCATTTGTTTTGATTTTTTGGTCACC……..650
TGGAAAGTTTAAGGGGGGGTGGTGCTGATTGCGAGGGGGCGCGGGGGTGG…… 700
GCCGCGCGGCTGACATACACAGCCTGGTCGCCCACGCCGACGACGGTGCA…… 750
AGGTAGAGGGTGGGGTTCCATGTAGGACGTGCCAGCCCTCATCATACTAAGTGCGC……. 80
CGCCCGGCGAGACCAGGACGGACGGCAAGAATACAACGGAAATGGAATGGAGCGGG……850
CTCAAGCATAAGCGTCCGAGCCTTCGAATCAATACGATGCGGAAGAGCCGAAACCT……900
CGCCTGGGCTTGCACTGCAGGGCAACCCGGGACGAGACGCCCCCTTCCGC……950
CCAGGGATCCAGCAGGGGGGGGGCCTGACCCTACCCCCACGGTCGTGGTAAT……1000
GAGAGGTGAGGGTCCACCACCCAGCAGCGAGCCATACCCTTGTTTGGCTG……1050
GTTGCCACAGGCCCGGGGGGGCGGGGCGCCCAGTATACCCCCCCCGGGCG……1100
CCGCGTACCAAAGAAGGGGAGGGGGGGGGGCGGACAAAAAAAAACAAACC……1150
CCCCCCTTCCTTTTATGGGGGGGGTGGCCAACTAGATAAACACTTAGAAA……1200
GGGTTATTTAAAAAACATTTTGCCAGAAAAAAGCGGCGGGGGGGGTCCA……1250
AACAAAAAACAAAAATCAAGGGGCTGTTTTTTTGGGAGAGGGGTTGGCGGC……1300
GAACCC CCTACCCCGCCCCGAAGTAGTGAGAATGAATACAAAAAAAAAAAAA ……1395

Figure 5 Partial sequences of mitochondrial cytochrome gene of the studied clam showing designed primer annealing sites

Table 3 Mitochondrial cytochrome gene partial sequence identities of the studied clam to other species in the NCBI data base

Accession	Description	%
MN119650	*Cultellus maximus* (Gmelin, 1791)	99.85 %
OQ434723	*C. attenatus* Dunker, 1862	89.74 %
MW549330	*Novaculina myanmarensis* (Lamarck, 1818)	88.17 %
MF958991	*N. gangetica* Benson, 1830	87.58 %
MW311106	*Pharella acutidens* (Broderip & Sowerby, 1853)	86.77 %

Growth monitoring and sex ratio of *Cultellus maximus* in natural population

The soft tissue weight of male was 80.4 ± 1.2 g while that of female was 110 ± 1.1g. The mean length of male and female were 10.1 ± 0.8 cm and 13.5 ± 1.5 cm, respectively. The soft tissue weight and the length were not significantly different in males and females clam (t test, P>0.05).

The numbers of females and males *Cultellus maximus* collected from Kyaukphyu were shown in (Table 4). Samples contained a relatively larger number of male clams than females throughout the study period. Significant differences in the sex ratio of male and female were not found in this study.

Table 4 The sex ratio, mean shell length, wet weight and the dry weight of studied clams

Gender	Mean Length	Mean Wet Weight	Sex Ratio
Male	10.1 ± 0.8 cm	80.4 ± 1.2 g	54
Female	13.5 ± 1.5 cm	110 ± 1.1g	46

Gonad development stages of *Cultellus maximus*

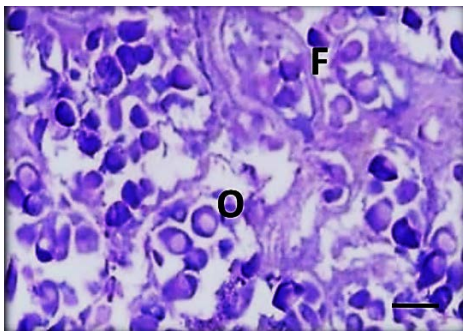
The reproductive development stages of *Cultellus maximus* males and females were examined. Surprisingly, undifferentiated stage was not found. Gonad development of female clams was reasonably synchronized with that of males.

Developing (Plate 1A and 2E): In developing stage, the follicular wall was lined with small oogonia in females or darkly stained spermatogonia in males but most of the follicles were empty.

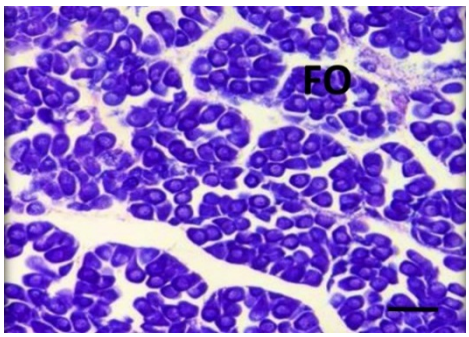
Ripe (Plate 1B and 2F): In ripe stage, oogonia attached to the follicle while oocytes in female and sperm in male escape to the lumen showing that clam was near to spawning. Follicles filled with mature oocytes with distinct nucleus in females and spermatozoa with tails in males, follicles wall was usually very thin and distended. Little or no connective tissue could be observed in this stage. Interestingly, ripe stage in male and female clams was abundantly found.

Spawning (Plate 1C and 2G): In spawning stage, some follicles were empty due to the releasing of oocytes/sperm and some collapse follicles were found. Residual and abnormal oocytes were found in female while small amount and pale color of sperm were found in male. Therefore, the most dominant reproductive stage in this population was the spawning stage followed by the ripe stage.

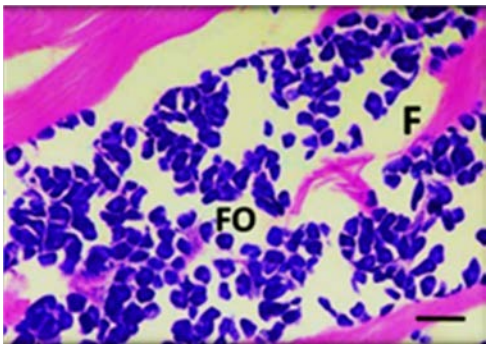
Spent (Plate 1D and 2H): In spent stage, small gametes were present near the follicle periphery during and immediately after spawning indicating that in clam's gonads did not have a resting period between gametogenic stages. Follicles containing degenerating oocytes, often elongated in shape and phagocytes were also present. Connective tissue began to reappear in this stage. The majority of *Cultellus maximus* individuals with spent gonads were seen.



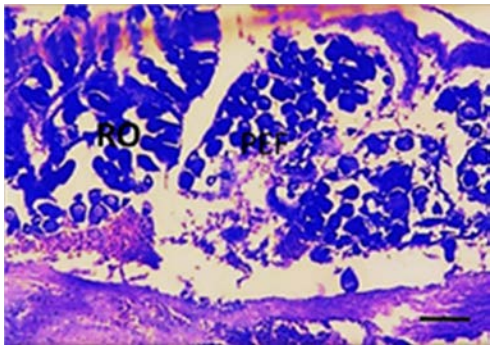
(A) Developing



(B) Ripe

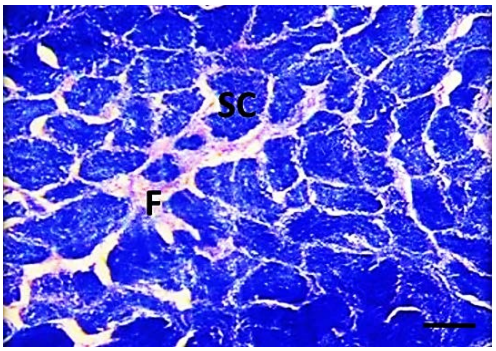


(C) Spawning

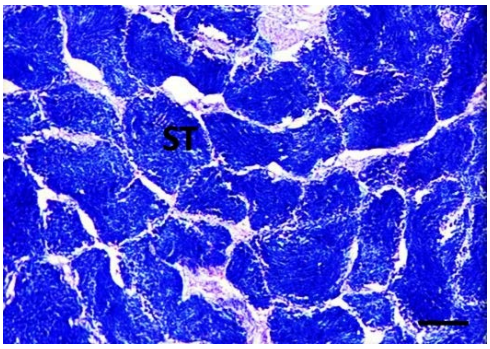


(D) Spent

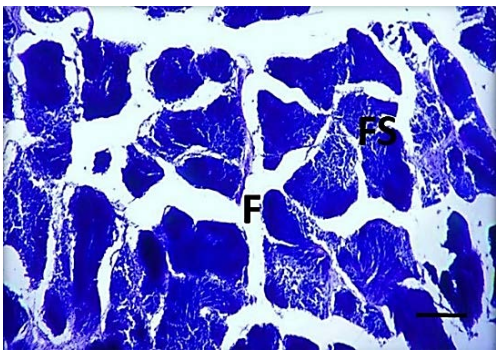
Plate 1 Gonad developmental stages observed in female (A-D) *Cultellus maximus*. All photographs are to the scale /70 µm. O= Oogonia, F = Follicle, FO = Free oocytes with distinct nucleus, PEF = partly empty follicle, RO = Residual oocytes; CT = Connective tissue



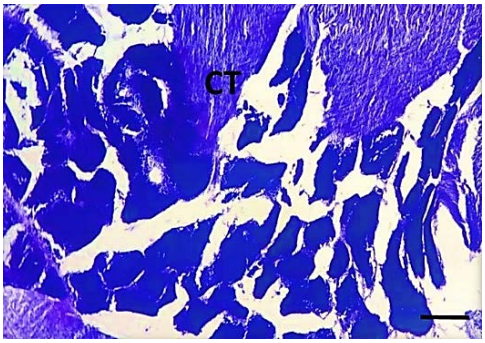
(E) Developing



(F) Ripe



(G) Spawming



(H) Spent

Plate 2 Gonad developmental stages observed in male (E-H) *Cultellus maximus*. All photographs are to the scale /70 µm. F = Follicle, PEF = partly empty follicle, SC = Spermatocysts, ST= Spermatids, FS = Few sperms in follicle, CT = Connective tissue

Discussion

In the present study, mitochondrial cytochrome gene sequencing for the identification of clam was conducted. It revealed that sequence identities of studied clam species indicated to be *Cultellus* sp. with 99.85%. The bamboo clam *Cultellus* species also known as the razor clam, belongs to the family Pharidae (Morton, 1984). Morphological characters revealed that the studied clam could belong to the family Pharidae or Solenidae or Tellinidae. The different

characters among those families were: (1) shell layers (2) hinge teeth (3) pedal gape (4) siphons (5) stomach and (6) style sac. Shell layer of *Cultellus* and *Pharella* has two layers and three in *Novaculina*. (Morton, 1984). In hinge teeth of *Cultellus*, three cardinal teeth in left valve, two in right and no lateral teeth where *Pharella* has two cardinal teeth in each valve and lateral teeth well developed and one to three cardinals in each valve and lateral teeth absent or weak in *Novaculina*. In addition, those characters were insufficient to identify the clam down to the species.

To confirm the genus, DNA analysis was conducted. Although, there was no matched sequence to the mitochondrial cytochrome gene sequence of the studied clam in the NCBI database, the most similar sequence indicated to be *Cultellus maximus* with 99.85% homology. Identities with the genus *Novaculina* are much lower than that of the genus *Cultellus*; 88.17% to *N. myanmarensis* (MW549330) and *N. gangetica* (MF958991) and 86.77% to *Pharella acutidens* (MW311106). Therefore, it is highly possible that the studied clam could not belong to genus *Novaculina*. The clam is therefore identity as *Cultellus maximus*. There are only 2 to 3 base pairs of *Cultellus maximus* and differ from those of clam reported in Korea.

The reproductive cycle with different reproductive stages and sex ratio of the *Cultellus maximus.* were revealed in the present study. They are generally divided into five stages: undifferentiated, developing, ripe, spawning and spent (Choi and Chang, 2003). Four reproductive stages were recorded in the studied clams: developing, ripe, spawning and spent. This classification was based on the histological similarity between the bivalves mentioned above. However, immature-undifferentiated stage was not observed in this species. Lai (2022) studied the reproductive development and conditioning of the clam *Cultellus sp.* in Vietnam and undifferentiated/primordial stage clams were recorded in February and March when salinity dropped less than 5 ppm. However, developing individuals increased from January to March when water temperature decreased < 20º C. Spawning individuals increased after salinity fluctuations in August and September. It is assumed that reproductive development of clam in Kyaukphyu is related to water temperature and salinity.

The reproductive cycle and gonad development of *Cultellus maximus* in Kyaukphyu were investigated. Although, there was no statistically significant difference in the shell length distribution, males were generally smaller in size than the females. The length at first maturity in this study (10.12 cm, 9.65–10.49 cm) is longer than the *Solen thachi* population in the central coast of Vietnam (i.e., 7 cm; Hoang and Tuyen 2016). Kim and Lee (2008) reported that the length at first maturity of *Sinonovacula constricta* (Lamarck, 1818) in western South Korea was 5–6.1 cm.

In *Cultellus maximus*, spawning and ripe stages were abundantly found. Clemente and Ingole (2009) studied the gametogenic development of the clam, *Cultellus sp.* at Chorao Island, Goa. They discussed that clam reached sex maturity when the shell length was over 3.4 cm. Shell length of clams collected in the present study was > 6 cm. All clams collected from Kyaukphyu area were assumed that they were in maturity stage. Various studies on the reproductive biology of bivalves have demonstrated that temperature and food are the most important exogenous factors influencing the reproductive cycle (Beer, 2000; Morriconi *et al*., 2002; Dalai and Goswami, 2001). Devassy and Goes (1989) reported an excess stock of phytoplankton available in the Mangrove areas. Since the role of food supply is vital in the development of gonad (Williams & Babcock 2004), the rapid metabolic reserves in *Cultellus sp.* coincided closely with the periods of high food abundance. Accordingly, the availability of food can be considered as a major determining factor of the seasonal gonadal cycle.

Bivalves often exhibit roughly equal numbers of males and females within a population (Gosling, 2015). However, the sex ratio imbalance observed here is indicative of the unique

geophysical location and environmental conditions such as temperature and food availability, or sampling bias (Rinyod and Rahim, 2011; Hoang and Tuyen, 2016; Trisyani et al., 2019). Individuals' sex at birth is determined by environmental conditions and genetic variation, or the combination thereof (Yusa, 2007). The temporal sex ratio variation has been observed in razor clam populations such as in a *Solen regularis* Dunker, 1862 population in Malaysia (Rinyod and Rahim 2011), in a *Solen* sp. population in Indonesia (Trisyani et al. 2019), and in a *Solen thachi* Cosel, 2002 population in the central coast of Vietnam (Hoang and Tuyen, 2016). Trisyani et al. (2019) hypothesized that the sex imbalance could result from the impact of excessive exploitation on one of the sexes of the *Solen* sp. populations in Indonesia. Bivalve species collected in the present study were from natural population. They might have undergone a greater temperature stresses and presumably had lower food availability (due to short periods of inundation during high tight). These environmental factors may have caused a higher male ratio in the bivalve species.

Conclusion

Morphology and molecular identification of bamboo clam from Kyaukphyu, Rakhine State was conducted. The BLAST search of database located in the NCBI revealed that the clam DNA sequence was highly homologous to mitochondrial cytochrome gene sequence of the species *Cultellus maximus*. The bamboo clam that had been traded in Rakhine state was confirmed as *Cultellus maximus*.

The reproductive cycle and gonad development of *Cultellus maximus* in Kyaukphyu were investigated. It was concluded that *Cultellus maximus* is a species with a continuous development of ganads throughout the year in Kyaukphyu area. The data presented here provided valuable information on the timing of spawning events for *Cultellus maximus* which had utmost importance for the development of sustainable management regimes for bivalve community.

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