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First Report on the Occurrence of the Comb Pen Shell, *Atrina pectinata* (Linnaeus, 1767) (Bivalvia: Pinnidae) in Ulleungdo Island in the East Sea: Ecology and Molecular Identification of the Species using COI Gene Sequence

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Abstract – Pen shell is one of the largest marine bivalves inhabiting shallow subtidal soft bottoms in the west Pacific and Indian Oceans. In Korea, the comb pen shell *Atrina pectinata* fisheries has been established on the south and west coasts. Recently, a pen shell population has been discovered from a subtidal sand flat (25–30 m depth) in Ulleungdo Island located in the East Sea of Korea, suggesting a potential shellfish resource in this area. In the present study, we first surveyed the population density and size of the unique pen shell using SCUBA, and identified the pen shell to species level using mitochondrial cytochrome oxidase I gene (COI) sequence. An underwater survey carried out from July to September 2013 revealed that populations of pen shell patched on subtidal sand flat at a depth of 20–25 m. Grain size analysis indicated that sand particles accounted for 99% of the 600 × 700 m sand flat. The underwater survey also indicated that density of the pen shell ranged between 6–19 ind/m^2, with a mean of 11 ind/m^2. Shell height (i.e. longest axis of the shell) of the pen shell on the sand flat varied between 17.2 cm to 28.8 cm, with a mean of 25.1 cm, and the age was estimated to range between 1.5–7.5 yrs, with a mean of 5 yr. COI DNA sequence obtained from the pen shell in this study showed 98.9–99.2% similarity to *Atrina pectinata* (Linnaeus 1767) reported from Japan. In the cluster analysis, the COI DNA sequence of the pen shells from Ulleungdo Island was grouped with *A. pectinata* reported from Japan and China, indicating that the pen shell discovered in this study was *A. pectinata*, commonly distributed on the west and south coasts of Korea.

Key words – *Atrina pectinata*, Pinnidae, Ulleungdo Island, East Sea, COI sequence, underwater sand flat

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1. Introduction

Pen shell is one of the biggest suspension-feeding marine bivalves occurring in shallow subtidal soft bottoms in the Pacific, Indian and Atlantic Oceans. According to Schultz and Huber (2013), a total of 55 species in the Pinnidae have been identified from temperate to tropical regions. In Korean waters, 5 species of pen shell have been reported, while 14 and 9 species of pen shells have been identified in Japan and in China (NFRDI 1997; Okutani 2000; Min 2004; Zhongyan 2004). In small bays on the west and south coast of Korea, *Atrina pectinata* (Linnaeus 1767) is widely distributed on subtidal sandy-mud substrate at depths of 20–50 m (Kim and Hur 1998; Hong et al. 2002; Min 2004; An et al. 2012). Due to the high market value of the adductor muscle, *A. pectinata* culture and/or fishery has been popular in the bays of Gamakman, Yeojaman, Deungyangman and Jinhaeman on the south coast and Cheonsuman Bay, Boryeong and Gunsan on the west coast (NFRDI 1997; Lee et al. 2015). According to the Korea Ministry of Oceans and Fisheries statistics, annual landings of *A. pectinata* have been declining for the past decade, possibly due to their habitat destruction and overfishing (Ryu et al. 2001).

Ulleungdo Island is a volcanic island located 120 km east of the Korean east coast with characteristic high wave activities and rocky shoreline extending to the subtidal as a steep cliff.

Relatively few studies have investigated the subtidal benthic community of Ulleungdo Island, despite its unique geographic locality. A recent underwater survey carried out on the south coast of Ulleungdo Island revealed that the shallow subtidal zone has high species diversity both in benthic flora and fauna (Ulleung-Gun 2013). The underwater survey also identified unique patches of pen shell populations on a subtidal sand flat at a depth of 20–25 m, which was not previously reported from Ulleungdo Island. Choe et al. (1994) first reported marine mollusks in Ulleungdo Island based on an expedition from June 1989 to August 1992, reporting 33 species of marine bivalves in this area. It is noticeable that pen shell was not listed in the molluscan species report, possibly due to the difficulties involved in the sampling.

From July to September 2013, we surveyed benthic organisms on the subtidal sand flat off the south coast of Ulleungdo Island using SCUBA. During the survey, we found numerous patches of adult pen shells with the sea grass *Zostera caulescens* on sand flat. In the present study, we report for the first time the genetic identification of the pen shell in Ulleungdo Island using a molecular marker.

2. Materials and Methods

Sampling effort

During the underwater survey of benthic organisms in Ulleungdo Island in 2013, patches of pen shell colonies were identified from sand flat at a depth of 20–30 m off the south coast (Fig. 1). To estimate the population density, a series of SCUBA divings were made from July 2013 to September 2013 and 1 × 1 m plastic quadrat was applied to 10 randomly selected sites on the sand flat. The number of pen shell in the quadrat was counted by the SCUBA divers. To measure the size, the pen shells were removed from the habitat and the longest axis (i.e. shell height) of the shell was measured to mm *in situ* (Fig. 2A). All the pen shells used in the size estimation were then re-planted to the substrate where they were removed by the SCUBA divers, after the size measurements were completed (Fig. 2B). In July, 10 adult pen shells were collected and transported to the laboratory for the molecular identification of the species. To obtain total DNA of the pen shell, the adductor muscles were excised and stored in absolute ethanol.

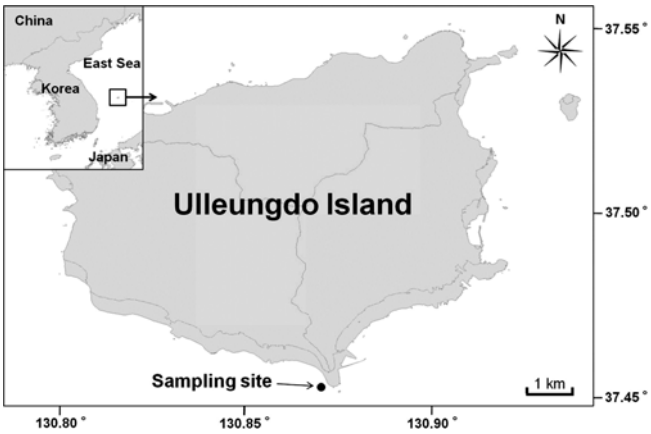

Fig. 1. Ulleungdo Island, showing the sampling site of *A. pectinata*

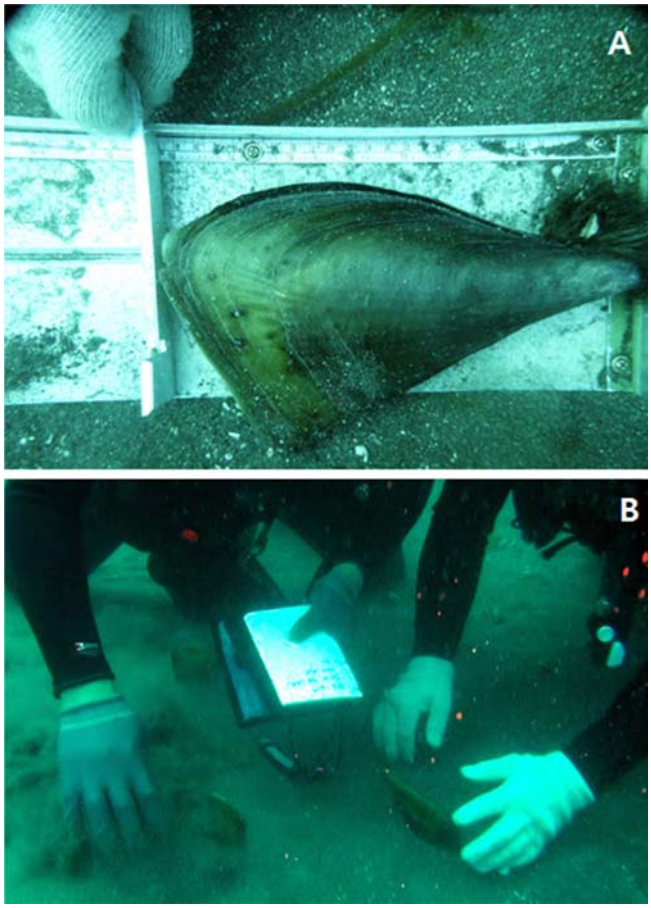

Fig. 2. Size measurement of the pen shell in situ (A) and re-plantation of the pen shell after the size measurement (B)

Grain-size analysis

Surface sediment where the pen shell population was located was collected by SCUBA to understand the depositional environment of the pen shell habit. For the analysis, approximately 80 g of sediment was treated with hydrogen peroxide (35%) to remove carbonate and organic matters and dried at 100°C.

Size distribution of the grains in the sediment was determined using standard sieving (< 4Φ) and pipetting techniques (> 4Φ, Folk 1968). Statistical parameters of the grain size distribution including mean, sorting, skewness and kurtosis were calculated using the graphic method of Folk and Ward (1957).

DNA extraction, PCR amplification, sequencing and sequence analysis

To identify species of the pen shell in a molecular way, the mitochondrial cytochrome oxidase I gene (COI) universal primers (LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') designed by Folmer et al. (1994) were applied in this study. The COI gene has been widely used in animal DNA barcoding technology to identify different species of animals (Folmer et al. 1994). For the analysis, the total DNA was extracted from 25 mg of the ethanol preserved adductor muscle using DNeasy Tissue Kit (Qiagen, Germany) and approximately 700 bp of the partial mitochondrial COI gene was amplified in PCR. The PCR mixture contained 50 ng of extracted DNA, 0.5 μm of each primer, 200 μM of dNTPs, 10 × Ex Taq polymerase buffer and 2.5 unit of Ex Taq polymerase (Takara, Japan) in 50 μL volume. The DNA samples were pre-denatured at 94°C for 5 min and the reaction was processed 30 times following 94°C for 30s, 50°C for 30s, 72°C for 50s and a final extension at 72°C for 5 min. Four microliter of each PCR product was visualized on 1.2% agarose gel after ethidium bromide staining. The PCR products were finally purified and cloned into pGEM-T easy vector (Promega, USA). The cloned plasmid DNAs were sequenced using PRISM Big Dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

Resulting COI sequences from the 10 pen shells were used to search for similar nucleotide sequences registered in the National Center for Biotechnology Information (NCBI) GenBank database using BLAST program. The COI sequences were aligned to available sequences of *A. pectinata* and other species belonging to the Genus *Atrina* and *Pinna* using CLUSTAL-W algorithm (Thompson et al. 1994). Finally, phylogenetic affinity of the Ulleungdo Island pen shell population was compared with other pen shell species using the neighbor-joining method in MEGA 5.0 (Tamura et al. 2011) with 1000 replicates.

3. Results and Discussions

Sediment particle size analysis indicated that most of the surface sediment of the sand flat consists of sand, accounting for 99% of the total sediment particles (Fig. 3). The sand grain size ranged from 0.063 to 2 mm and was categorized as slightly gravel sand ((g)S) having the mean grain size 2.75 phi (0.168 mm). Sediment type is one of the most important environmental factors governing pen shell distribution, influencing pen shell larval settlement and growth.

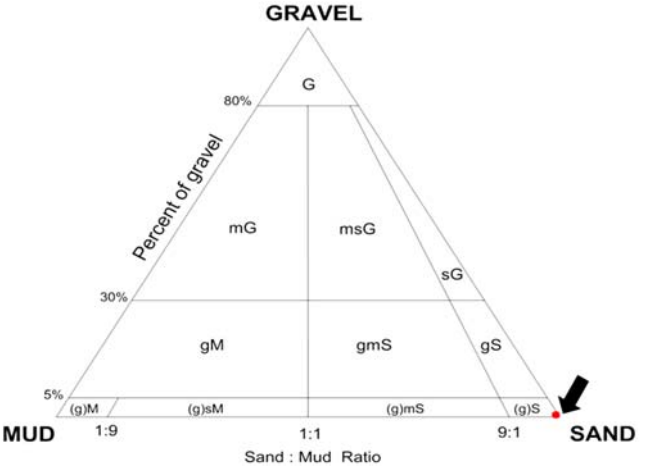

Fig. 3. Ternary diagrams showing the sediment composition. The arrow and red dot indicate sediment type of the sand flat discovered in the present study. G, gravel; M, mud; S, sand; mG, muddy gravel; msG, muddy sandy gravel; sG, sandy gravel; gM, gravelly mud; gmS, gravelly muddy sand; gS, gravelly sand; (g)M, slightly gravelly mud; (g)sM, slightly gravelly sand mud; (g)mS, slightly gravelly mud sand; (g)S, slightly gravelly sand

Hong et al. (2002) first investigated sediment types of the pen shell *A. pectinata* fishing grounds located off the west coast in Chungnam Province. The survey reported that bottom types of the 5 licensed pen shell fishing grounds in Chungnam Province were mostly sandy to sandy-mud, where the sand contents varied between 50–80% of the bottom substrate. In the survey, higher density of the pen shell occurred at the subtidal sand flat where the sand proportion in the sediment accounted for 80%. In contrast, no pen shell distribution was confirmed on the subtidal mud flat in the survey, suggesting that muddy substrate is not a suitable habitat for pen shell, possibly due to the deleterious effects of fine re-suspended particles on feeding and respiratory activities of the pen shell. According to Kondo et al. (2003), *A. pectinata* mortality in Ariake Bay in Kyushu Japan is linked to very high turbidity caused by re-suspension of fine sediment particles in the muddy tidal flat environment. Such negative impacts of fine sediment particles on survival and growth of pen shell were experimentally demonstrated by Yurimoto et al. (2008).

Fig. 4. The giant seagrass *Zostrea caulescens* observed at depth 20–25 m off the south coast of Ulleungdo Island

Accordingly, it is believed that the subtidal sand flat discovered in this study provides a suitable habitat for pen shell.

At shallow part of the sand flat, a forest of giant sea grass with a shoot length of 1.5–2.0 m was observed (Fig. 4). The giant seagrass was identified to be *Zostrea caulescens*, endemic to the Northeast Pacific and distributed in shallow subtidal sand flat on the east and south coast of Korea (Lee et al. 2002, 2005; Kim et al. 2015). *Z. caulescens* is considered to be the tallest seagrass in the world and plays an important ecological role in shallow subtidal sand flat ecosystems, providing shelters and nursery grounds for fish and invertebrate animals, as well as stabilizing the sand flat substrate (Nakaoka et al. 2003; Kim et al. 2015). This giant seagrass is not common in Korea and Japan and is listed as an endangered plant species in Japan (Nakaoka et al. 2003). On the east and south coast, *Z. caulescens* is present mostly at a depth between 8–10 m, while this giant seagrass occurs at a depth between 20–25 m in Ulleungdo Island. The deeper distribution of *Z. caulenscens* in Ulleungdo Island is possibly linked to the unusually high transparency of seawater along the island (SR Park, personnel communication).

As summarized in Table 1, we visited the subtidal sand flat in July, August and September 2013 and surveyed the pen shell populations scattered on the sand flat. The mean number of pen shell in the 1 × 1 m quadrat (Fig. 5A) varied between 9.5 ind/m^2 (September) and 12.8 ind/m^2 (July), with its monthly range between 6–12 ind/m^2 (September) and 7–19 ind/m^2 (July). As shown in Fig. 5B, the live pen shells exhibited inhalant and exhalent holes at the posterior-ventral part of the shell, exposed to the water column for respiration and feeding. The mean shell height (i.e. the longest axis of the shell, Fig. 5C) of the pen shell recorded *in situ* varied between 24.5 cm (September) and 26.4 cm (July). The largest pen shell recorded in this study was 28.8 cm (July), while the smallest was 21.7 cm (August). It was noticeable that no pen shell size under 21 cm in shell height was observed in this survey, although a pen shell of 17.2 cm in shell height was collected in the preliminary survey carried out in the sand flat in April 2013 (Fig. 5C).

A few studies have reported on the morphological features of *A. pectinata*. According to Min (2004), Okutani (2000) and Zhongyan (2004), *A. pectinata* described in Korea, Japan and China is characterized as a shell with a comparatively thin and brownish to dark-brown color, having approximately 10 radial ribs without scales on the right valve. As shown in Fig. 5C, the right valves of the pen shell from the subtidal sand flat exhibit the characteristics of *A. pectinata*, dark brown color with no scales and remarkable radial ribs

The age of marine bivalves is often inferred from their exoskeleton, by counting the external growth marks or so-

Table 1. Total number of pen shell counted from the 10 1 × 1 m quadrats during the sampling in July, August and September 2013. Mean shell height (i.e. the longest axis of the shell) and density of *A. pectinata* from each sampling period

Sampling Period (2013)	Number of Quadrat	Pen Shell Density (Ind/m^2)			Shell Height (cm)	
		Average	Range	Total	Average	Range
July	10	12.8	7-19	128	26.4	23.4–28.8
August	10	10.3	6-18	103	24.6	21.7–27.1
September	10	9.5	6-12	95	24.5	23.0–26.7

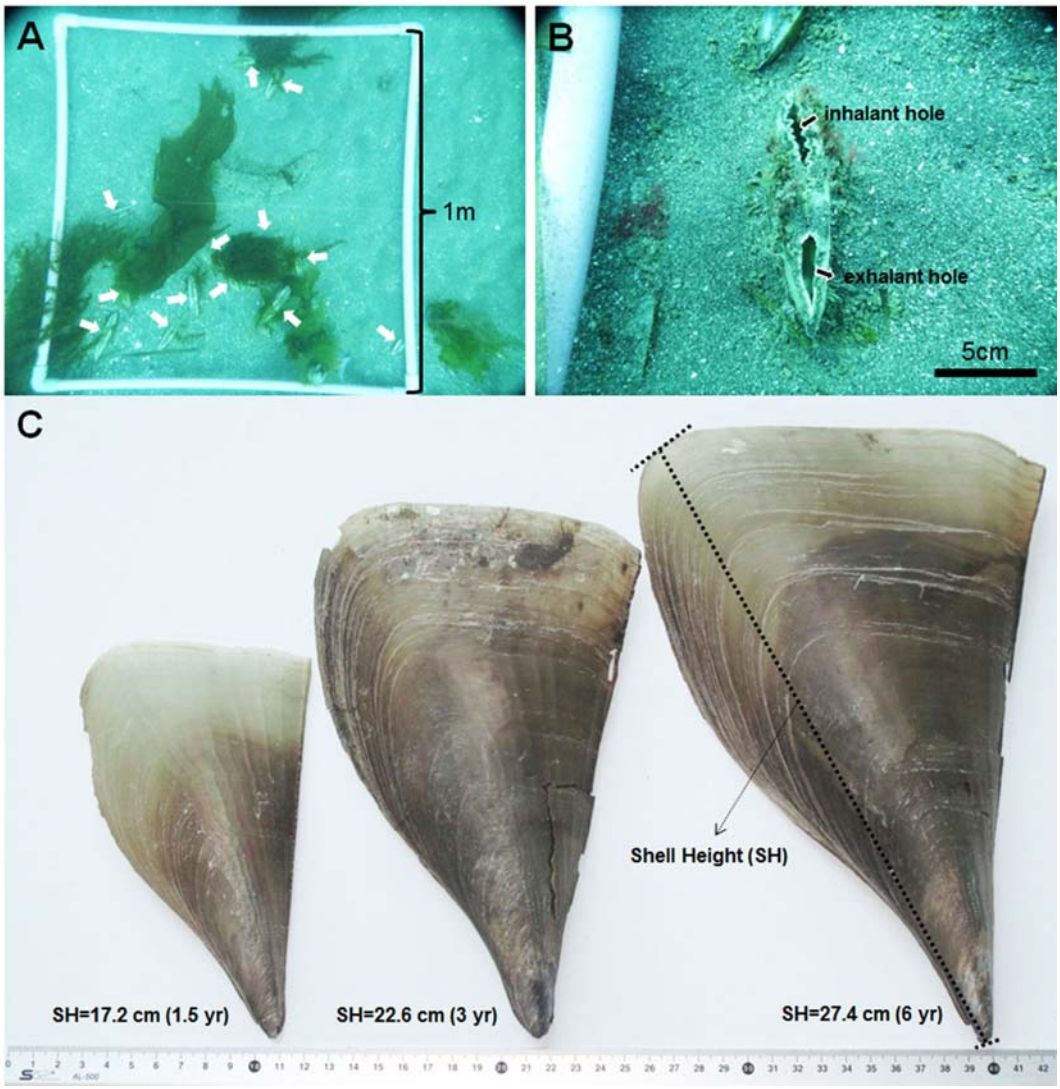

Fig. 5. (A) Underwater photography showing *A. pectinata* in 1 × 1 m quadrat; arrows indicate individual pen shells. (B) Close-up photograph of *A. pectinata* exhibiting the mantle tissues. (C) Different size of pen shell collected from the sampling site and the estimated ages

called growth rings (Seed 1980; Vakily 1992). In particular, the exterior surface of the pen shell exhibits the clear mark of a growth band, which has been utilized in determining age. Based on the annual growth band formed on the exterior surface of the shell, Silina (2012) successfully estimated the age of the flag pen shell *A. vexillum* in the Gulf of Thailand. Qiu et al. (1996) also investigated the shell growth pattern of *A. pectinata* distributed on the northern coast of Shandong peninsula, China. Qie et al. (1996) reported that 2 different types of growth bands could be identified from the shell surface, the winter and summer rings, suggesting that two growth bands are formed annually in the study area. In contrast, Ryu et al. (2001) reported that the growth band is formed only once in a year in *A. pectinata* distributed on the west coast of Korea. Accordingly, the age of *A. pectinata* on the west could be estimated using the von Bertalanffy's growth equation; shell height of 14 cm as a 1 yr old and 29 cm as an 8 yr old.

In the present study, we were unable to examine the growth banding pattern of pen shells discovered in Ulleungdo Island, since the pen shells used in the analysis were re-planted to the original habitat after recording the shell height *in situ*. According to Ryu et al. (2001), the age of the pen shell distributed on the sand flat in Ulleungdo Island could range between 1.5 yr (17.2 cm in shell height) and 7.5 yr (28.8 cm in shell height) , although more studies need to be carried out to verify the relationship between age and shell height.

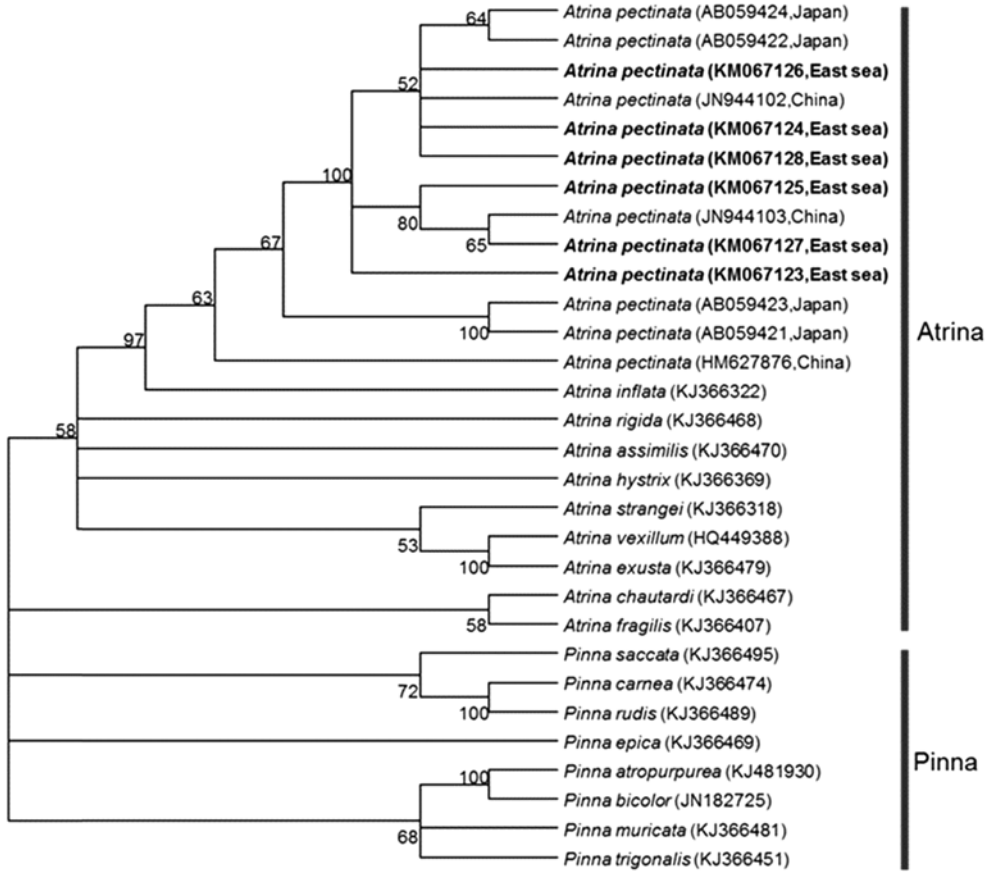

Fig. 6. Phylogenetic analysis of cytochrome oxidase I (COI) sequences of *A. pectinata* from Ulleungdo Island and other related species in the Genus *Atrina* and *Pinna* using the neighbor joining method based on Kimura 2 parameter model. The boostrap values were calculated with 1000 replicates and values > 50% are shown at the nodes. The determined COI sequences of *A. pectinata* in this study is indicated in bold

The mitochondrial COI gene was successfully isolated from the adductor muscles of the 10 pen shells collected in July and amplified using PCR. All the pen shells used in the analysis yielded 703 bp of nucleotides, which encode 203 amino acids. Subsequently, the COI gene nucleotide sequence was registered in GenBank under accession number KM067123-067128. In the BLAST search, the COI sequences of the pen shell from Ulleungdo Island were 98.9–99.2%, similar to the COI sequences of *A. pectinata* (Accession number AB059422) distributed in Ariake Bay in Japan. In the cluster analysis, the COI gene sequence of the Ulleungdo Island pen shell was aligned within the Genus *Atrina*, and the sequence also formed a sub-clade with *A. pectinata* sequences reported from Japan and China (Accession numbers AB059422, AB059424, JN944102-3, Fig. 6). The phylogenetic analysis indicated that the pen shell distributed on the subtidal sand flat in Ulleungdo Island is *A. pectinata*. The cluster analysis also demonstrated that the sequences of *A. pectinata* population in Ulleungdo Island are different from those previously reported for other species in the Genus *Atrina*. Accordingly, it is confirmed that the pen shells distributed in Ulleungdo Island is *A. pectinata*.

In summary, the present study first identified species of pen shell distributed on a subtidal sand flat off the south coast of Ulleungdo Island using PCR with COI gene marker. The pen shell populations were composed mostly of 3–6 yrs old adults with a shell height of 22–27 cm and the population density varied between 6–19 ind/m^2. The COI gene sequence analysis revealed that the pen shell discovered in Ulleungdo Island was *A. pectinata*, which is widely distributed on the south and west coast of Korea.

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