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The Complete Mitochondrial Genome of the Chemosymbiotic Lucinid Bivalve *Pillucina pisidium* (Dunker, 1860) Occurring in Seagrass *Zostera marina* Bed in a Lagoon in Jeju Island, Korea

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Abstract: Commonly found in tropic and subtropic seagrass beds, lucinid clams host sulfur-oxidizing bacteria within their gills. These symbionts are crucial in converting phytotoxic sulfide in the sediment into less harmful sulfate, thus enhancing the environment for seagrasses and associated biota. We recently uncovered small clams within a *Zostera marina* seagrass bed situated in a lagoon on Jeju Island, off the south coast of Korea. These bivalves, with shell lengths of up to 7 mm, exhibited distinct features, including thick and hypertrophied gills, inflated and ovoid shells with a shell height/shell length ratio of 0.99, and the absence of a sulcus on the external shell surface. These characteristics align closely with those of *Pillucina pisidium*, a lucinid clam species originally reported in Japan. Analysis of the cytochrome b gene partial sequences of the clams from Jeju Island revealed a 100% match with *P. pisidium* reported in Japan, confirming their identity. Moreover, we successfully assembled the complete mitochondrial genome of *P. pisidium* for the first time, revealing a circular genome spanning 21,059 bp. Additionally, we constructed a phylogenetic tree using 13 protein-coding genes (PCGs) extracted from the mitochondrial genome of *P. pisidium*. Notably, *P. pisidium* formed a distinct clade within the subclass Autobranchia alongside other lucinid clams in the phylogenetic tree. However, within the family Lucinidae, synteny analysis of the 13 PCGs revealed diverse gene arrangement patterns, indicating considerable divergence. This divergence underscores the need for an extensive examination of Lucinidae mitochondrial genomes to elucidate the phylogenetic ties more precisely within the family, highlighting *P. pisidium*'s distinct evolutionary path within the family Lucinidae.

Keywords: Lucinidae; chemosymbiosis; morphology; phylogeny; genome

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1. Introduction

Chemosymbiosis constitutes a mutualistic relationship between marine invertebrates and chemolithoautotrophic bacteria, wherein the bacteria oxidize sulfide derived from the environment to produce organic carbons, subsequently transferring nutrients to their host organisms [1]. This phenomenon gained recognition with the initial discovery of mutualistic chemosymbiotic bacteria in the giant tube worm *Riftia pachyptila*, thriving in deep-sea hydrothermal vents approximately 2500 m deep near the Galapagos Islands. Subsequent research has revealed similar symbiotic associations between marine invertebrates and chemolithoautotrophic bacteria across diverse marine habitats [1–3]. Notably, this chemosynthetic symbiosis is particularly prevalent among marine bivalves, with numerous species in families such as Mytilidae, Lucinidae, Thyasiridae, and Solemyidae harboring symbiotic bacteria within their gill tissues [3–6]. These chemosynthetic marine bivalves inhabit a wide array of marine environments, ranging from deep-sea hydrothermal vents

and cold seeps to hypoxic intertidal zones and seagrass beds. In these habitats, chemical substrates like sulfide and methane are continuously supplied to the symbionts, serving as fuels for the production of organic matter [7–12].

The Lucinidae family represents one of the most diverse groups of chemosynthetic bivalves found across a range of marine habitats, spanning from deep-sea hydrothermal vents to seagrass beds and mangrove forests within tropical and subtropical estuaries and lagoons [6,13,14]. Predominantly, lucinid clams are obligate chemosymbionts, relying on symbiotic bacterial production for sustenance. These clams harbor sulfur-oxidizing gammaproteobacteria within specialized bacteriocytes located in their gill filaments, where the symbiotic bacteria oxidize sulfide and synthesize organic matter that is then transferred back to the host [15–18]. In seagrass beds, lucinid clams often play critical roles in enhancing the subsurface sediment environment by converting toxic sulfide accumulated in the sediments into less harmful sulfate [12]. Furthermore, according to König et al. (2016) [19] and Petersen et al. (2016) [20], the chemosymbionts within lucinid clams, such as *Lucinoma lucinalis* and *Codakia orbicularis* in seagrass meadows, also facilitate the fixation of inorganic nitrogen into organic nitrogen, providing a readily available nutrient source for marine plants and animals.

Common in seagrass meadows, the lucinid clam *Pillucina pisidium* has a broad geographical distribution range from the southern Pacific region, encompassing Madagascar and eastern Australia, to the northwestern Pacific, including Japan, Korea, and Vladivostok, Russia [21–25]. Through analysis of fatty acid profiles and the sulfur-stable isotope, Zhukova et al. (1992) [26] and Kharlamenko et al. (2001) [27] have affirmed that *P. pisidium* in seagrass beds in Vladivostok, Russia, harbor chemolithoautotrophic bacteria, potentially pivotal in neutralizing phytotoxins accumulated in the seagrass bed sediments. Furthermore, various studies have documented the presence of *P. pisidium* in intertidal areas along the south coast and Jeju Island in South Korea [28–30]. Nevertheless, the molecular identification and phylogenetic affiliation of *P. pisidium* in Korean waters remain unresolved. Moreover, recent studies on the phylogeny of the Lucinidae family have primarily used nuclear genes (12S and 16S rRNA) and mitochondrial cytochrome b (COB) to construct phylogenetic trees [23,31]. These studies have reported that the genus *Pillucina* does not form a monophyletic group, which contrasts with its morphological classification [23,31]. This indicates that a more comprehensive approach, utilizing multiple genes, is necessary for a clearer and more accurate interpretation of the phylogeny within the Lucinidae family.

Mitochondria trace their origins back to a common ancestor approximately two billion years ago and have become ubiquitous among eukaryotic organisms [32]. Due to the accessibility of mitochondrial genomic DNA, with its abundance of copies and conservation across diverse taxa, mitochondrial gene sequences (mtDNA) have found extensive application in fields like molecular evolution, phylogenetics, and population genetics [33,34]. Notably, cytochrome c oxidase subunit I (COX1), among the most conserved protein-coding genes (PCGs) within the mitochondrial genome, has been extensively employed for efficient species identification and phylogenetic analyses [34–36]. Recent research has demonstrated that analyzing the entire mitochondrial genome yields more informative data than focusing solely on the COX1 gene [33,35]. Consequently, this study marks the first to document the mitochondrial genome of the chemosymbiotic Lucinidae bivalve *P. pisidium*, integrating morphological and ecological insights. Moreover, we conducted a phylogenetic analysis employing 13 PCGs within the family Lucinidae.

2. Materials and Methods

Sampling Effort. In July 2022, small clams were harvested from sediment approximately 10 cm deep near the rhizomes and roots of *Zostera marina*, forming a meadow in the Ojo-ri lagoon (33.47° N, 126.92° E) along the eastern coast of Jeju Island (see Figure 1). A steel core with a 25 cm diameter was employed to extract sediment samples to collect the clams. Subsequently, the sediment containing clams underwent sieving through a 1 mm mesh screen. At the laboratory, the shell length (SL, maximum distance on the

anterior-posterior axis) and shell height (SH, maximum distance on the umbo-ventral axis) of the lucinid clams were measured using vernier calipers. Subsequently, the entire soft tissue was separated from the shell, and the wet tissue weight was determined using an electronic balance.

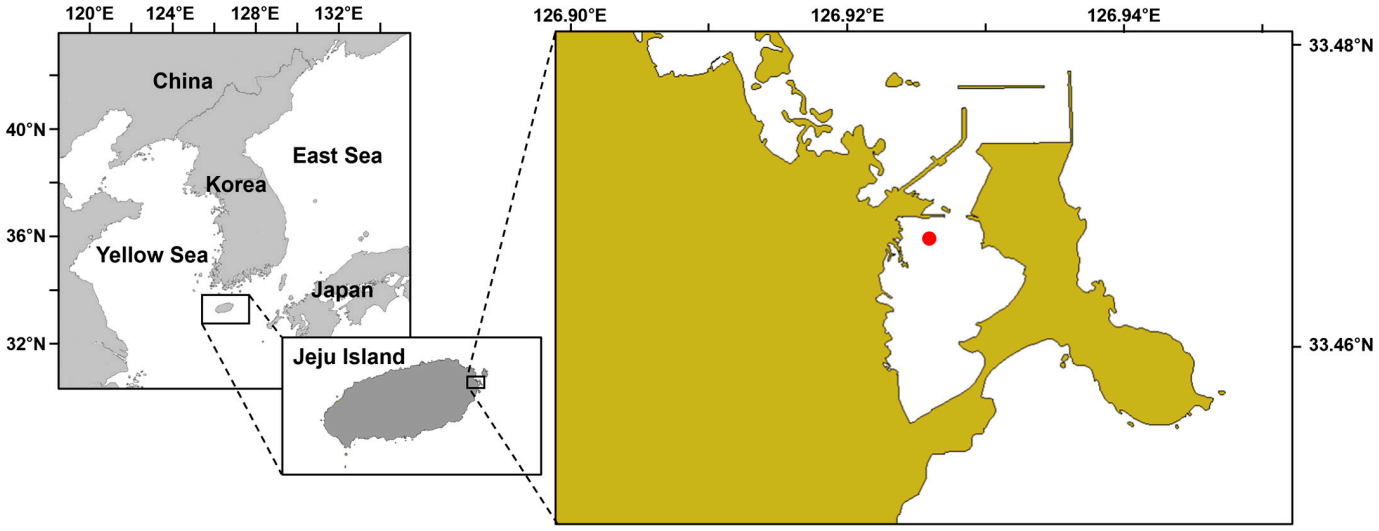

Figure 1. Map showing the study area Ojo-ri lagoon on the east coast of Jeju Island, Korea. The dot indicates the seagrass *Zostera marina* bed where the lucinid clams were collected.

DNA Isolation, Next-Generation Sequencing and Mitochondrial Genome Assembly. Genomic DNA extraction was conducted from the foot tissue, considered an organ free of symbiont, for mitochondrial genomic analysis. Approximately 20 mg of foot tissue was excised, and DNA extraction followed the manufacturer's protocol using the DNeasy Blood and Tissue Kit (QIAGEN, San Diego, CA, USA). Subsequently, a 151 bp paired-end DNA library (with an insert size of 550 bp) was prepared using a TruSeq DNA Nano kit (Illumina, San Diego, CA, USA). The sequencing procedure was carried out on a NovaSeq 6000 platform (Illumina), facilitated by DNAlink Inc. (Seoul, Republic of Korea). The raw sequencing data of the closely related species, *P. pacifica*, was obtained from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (accession number: SRR131632239, Osvatic et al., 2021) [36] to compare the DNA sequence obtained in this study.

Raw sequence data underwent quality filtering using Trim Galore! (ver. 0.6.10) with the specified parameters: `--quality 20, --length 120, and --max_n 0`, as documented at (https://github.com/FelixKrueger/TrimGalore, assessed on 8 January 2024) [37]. Subsequently, de novo assemblies of the mitochondrial genomes for both *P. pisidium* and *P. pacifica* were generated using the metaSpades mode (k-mer length: 21, 33, and 55) of MitoZ (ver. 3.6) [38–42]. The assembled circular forms of the two circular mitochondrial genomes were annotated utilizing MITOS2 (ver. 2.1.6) [43], with visualization accomplished using Circos (ver. 0.69-8) [44].

Phylogenetic Analysis. We conducted a phylogenetic analysis utilizing 13 protein-coding genes (PCGs) extracted from 26 species, all of which have complete mitochondrial genome sequences available in the NCBI database. This investigation aimed to elucidate the phylogenetic relationships within the subclass Autobranchia, encompassing chemosymbiotic lucinid clams (as outlined in Table 1). Four species from the genus *Solemya* (subclass Protobranchia) were included as outgroup representatives. Multiple sequence alignments (MSA) of the 13 PCGs were performed using MAFFT (ver. 7.520) with default parameters [45]. Subsequently, we employed PartitionFinder2 (ver. 2.1.1), utilizing Akaike's Information Criterion (AICc), along with the '`--raxml`' option, to identify the optimal partitioning schemes and models of molecular evolution [46,47]. The best fit evolutionary

models for each gene are as follows: ATP6, COX1, COX2, COX3, CYTB, ND1, ND2, ND3, ND4, ND4L, ND5, ND6 (GTR + I + G), and ATP8 (GTR + G).

Table 1. GenBank accession list of the mitochondrial genomes of the 26 clams used in the analysis.

Subclass	Infraclass	Order	Family	Species Name	Accession Number
Autobranchia	Heteroconchia	Adapedonta	Pharidae	*Sinohyriopsis schlegelii*	AP018551.1
				Sinonovacula constricta	EU880278.1
			Solenidae	*Solen grandis*	HQ703012.1
		Lucinida	Lucinidae	*Loripes lacteus*	EF043341.1
				Lucinella divaricata	EF043342.1
				Pillucina pacifica	BK067723
				Pillucina pisidium	NC_071184.1
			Thyasiridae	*Conchocele bisecta*	LC126312.1
		Myida	Dreissenidae	*Dreissena polymorpha*	MT483676.1
			Myidae	*Mya arenaria*	MW727516.1
		Venerida	Veneridae	*Ruditapes decussatus*	KP089983.1
			Vesicomyidae	*Calyptogena magnifica*	KR862368.1
				Calyptogena pacifica	MT947386.1
				Calyptogena rectimargo	MT947387.1
	Pteriomorphia	Arcida	Arcidae	*Tegillarca granosa*	KJ607173.1
		Mytilida	Mytilidae	*Bathymodiolus brooksi*	MT916743.1
				Gigantidas platifrons	AP014561.1
				Mytilus galloprovincialis	DQ399833.1
		Ostreida	Ostreidae	*Crassostrea gigas*	AF177226.1
		Pectinida	Pectinidae	*Argopecten irradians*	EU023915.1
				Mizuhopecten yessoensis	AB271769.1
				Pecten maximus	KP900975.1
Protobranchia	-	Solemyida	Solemyidae	*Solemya elarraichensis*	KY244079.1
				Solemya pervernicosa	KY244080.1
				Solemya velesiana	NC_034906.1
				Solemya velum	JQ728447.1

Phylogenetic analyses were conducted through the integration of maximum likelihood (ML) and Bayesian inference (BI) methods. The ML tree was reconstructed using RAxML-ng (ver. 1.2.0) with 1000 bootstrap replicates [48]. For the BI tree, MrBayes (ver. 3.2.7) was employed [49]. Two independent Markov chain Monte Carlo (MCMC) runs were executed, each comprising 1×10^6 generations with sampling every 1000 generations. Additionally, 25% of the initial data were discarded as burn-in data. The ML and BI tree were visualized using FigTree (ver. 1.4.4) (http://tree.bio.ed.ac.uk/software/figtree, accessed on 22 February 2024).

3. Results and Discussion

Morphological and Molecular Identification. Morphologically, the overall shell shape of Lucinidae clams found in Jeju Island closely resembles the type specimens reported by Dunker (1861) [50] in Japan, identified initially as *Lucina pisidium*. The SL of the small white clams collected from the *Z. marina* beds in Jeju Island measures 7.0 mm, and the mean SH to SL ratio was 0.99, suggesting a rounded and expanded form (see Figure 2A,B). Furthermore, a single cardinal tooth on the left and two cardinal teeth on the right valve align with the shell features typical of the genus *Pillucina* (Figure 2C). The distinctive triangular ligament presents on both the left and right valves, along with the

absence of an anterior lateral tooth on the right valve, further differentiate this species, clearly distinguishing it from other species within the genus *Pillucina* (Figure 2C).

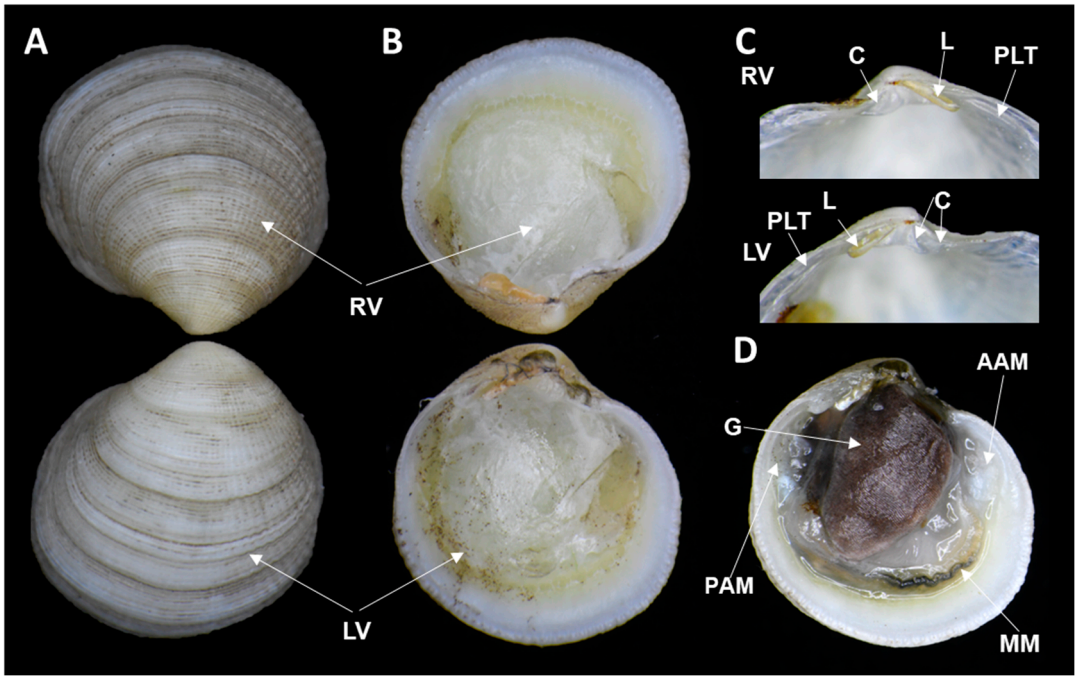

Figure 2. *Pillucina pisidium*. (**A**), External view of the shell valves. (**B**), Internal view of the shell valves. (**C**), Shell hinge, and teeth. (**D**), Gross anatomy of the highly developed gill after removal of the right valve and most of its corresponding mantle lobe. SL = 7.55 mm. Abbreviations: LV: left valve; RV: right valve; L: ligament; C: cardinal tooth; PLT: posterior lateral tooth; G: gill; AAM: anterior adductor muscle; PAM: posterior adductor muscle; MM: mantle margin.

The clams exhibited a single pair of gills covering the mantle (Figure 2D). The gills were dark brown in color, thick, and occupied 24% of the total shell inner space and 42% of the mantle area, indicating a wide surface area (Figure 2D). Additionally, the weight of the gills accounted for approximately 17% of the total tissue weight. Thick, dark brown gills are a typical characteristic of Lucinidae clams and serve as an effective habitat for symbiotic bacteria. In Lucinidae gills, the presence of a lateral zone densely populated with bacteriocyte-containing symbionts contributes to their thick and dark appearance [51,52]. The high gill-to-somatic tissue weight ratio in Lucinidae bivalves indicates their reliance on organic matter synthesized by gill symbionts as a major energy source. Several studies have reported that the chemosynthetic *P. pisidium* in seagrass beds exhibits a high gill weight ratio, a unique characteristic of lucinid clams [22,26,27]. The gill-to-total tissue weight ratio of the Lucinidae clam *Lucinoma aequizonata*, collected from 550 m depth at the sill of the Santa Barbara Basin USA, reaches up to 35% [52] and 32.5% in *Loripes lacteus*, inhabiting a seagrass bed in Corsica, France [53].

The similarity between the Lucinidae clam from Jeju Island and comparable nucleotide sequences in the National Center for Biotechnology Information (NCBI) was compared. The 1143 bp COB gene, extracted from the analyzed mitochondrial genome, showed 100% similarity with the 355 bp partial sequence (KF741678.1) of *P. pisidium* in Japan reported by Taylor et al. (2014) [23]. Based on the shell morphology and the COB gene sequences, we concluded that the lucinid clam occurring in the *Z. marina* seagrass bed on Jeju Island is *P. pisidium*.

Mitochondrial Genome Characterization. We generated a total of 213,214,754 paired-end raw sequence reads from *P. pisidium*. A quality filtering process that retained bases with a Q-score above 20 and forward-reverse reads of at least 120 in length yielded

210,719,044 reads (98.8%). Subsequently, the mitochondrial genome was constructed using filtered reads.

We successfully generated the complete mitochondrial genome of the Chemosymbiotic Lucinid Clam, *P. pisidium* from the lagoon in Jeju Island for the first time. This circular genome spanned 21,059 bp and contained a complete set of 37 genes, including 13 PCGs (i.e., COX1, COX2, COX3, COB, ND1, ND2, ND3, ND4, ND4L, ND5, ND6, ATP6, and ATP8), two rRNAs (12S rRNA, and 16S rRNA), and 22 tRNAs (Table 2 and Figure 3). The nucleotide composition of the mitochondrial genome was as follows: A: 23.78%; T: 39.68%; G: 24.73%; and C: 11.81%, with an A + T content of 63.46% and G + C content of 36.54%. The average length of the intergenic regions between coding sequences was 175 bp. The *P. pisidium* genome is longer than the average mitochondrial genome length of 17,693 bp in other three Lucinidae species, due to its extended intergenic regions (Table 1). The mitochondrial genome of *P. pisidium* has been registered in the NCBI as a reference sequence (accession number: NC_071184.1).

Table 2. Annotation of the *Pillucina pisidium* mitochondrial genome.

Gene	Position	Length (bp)	Initiation Codon	Stop Codon	Intergenic Nucleotide (bp)
Cytochrome c oxidase subunit I (COX1)	1–1536	1536	ATG	TAA	2112
tRNA-Phe (trnF)	2973–3039	67			1436
tRNA-Lys (trnK)	3109–3173	65			69
tRNA-His (trnH)	3185–3244	60			14
Cytochrome c oxidase subunit III (COX3)	3257–4054	798	ATG	TAG	12
tRNA-Met (trnM)	4105–4173	69			50
NADH dehydrogenase subunit 4L (ND4L)	4239–4517	279	ATG	TAA	65
Cytochrome c oxidase subunit II (COX2)	5243–6118	876	ATA	TAA	725
tRNA-Ser (trnS2)	6145–6209	65			26
NADH dehydrogenase subunit 6 (NAD6)	6210–6704	495	ATT	TAG	0
Cytochrome b (COB)	6709–7851	1143	ATG	TAA	4
ATP synthase F0 subunit 6 (ATP6)	7870–8574	705	ATT	TAA	18
tRNA-Leu (trnL1)	8594–8660	67			19
tRNA-Glu (trnE)	8916–8980	65			255
12S ribosomal RNA (rrnS)	8994–9808	815			13
NADH dehydrogenase subunit 4 (NAD4)	9854–11,194	1341	ATT	TAA	45
tRNA-Asn (trnN)	11,230–11,301	72			35
tRNA-Asp (trnD)	11,343–11,411	69			41
tRNA-Pro (trnP)	11,426–11,493	68			14
tRNA-Trp (trnW)	11,509–11,582	74			15
tRNA-Gly (trnG)	11,589–11,656	68			6
tRNA-Arg (trnR)	11,667–11,735	69			10
tRNA-Thr (trnT)	11,738–11,802	65			202
tRNA-Val (trnV)	11,819–11,885	67			16
tRNA-Leu (trnL2)	11,903–11,971	69			17
NADH dehydrogenase subunit 3 (NAD3)	11,972–12,319	348	ATT	TAG	0
tRNA-Ile (trnI)	12,369–12,435	67			49
16S ribosomal RNA	12,435–13,596	1162			−1
tRNA-Tyr (trnY)	13,597–13,676	80			0
RNA-Ala (trnA)	13,820–13,883	64			143
tRNA-Gln (trnQ)	13,887–13,952	66			3

Table 2. *Cont.*

Gene	Position	Length (bp)	Initiation Codon	Stop Codon	Intergenic Nucleotide (bp)
ATP synthase F0 subunit 8 (ATP8)	13,975–14,088	114	ATT	TAA	22
NADH dehydrogenase subunit 5 (NAD5)	14,161–15,891	1731	ATA	TAA	72
NADH dehydrogenase subunit 1 (NAD1)	16,389–17,342	954	ATG	TAA	497
tRNA-Cys (trnC)	17,402–17,464	63			59
tRNA-Ser (trnS1)	17,830–17,897	68			366
NADH dehydrogenase subunit 2 (NAD 2)	17,949–18,947	999	ATA	TAA	51

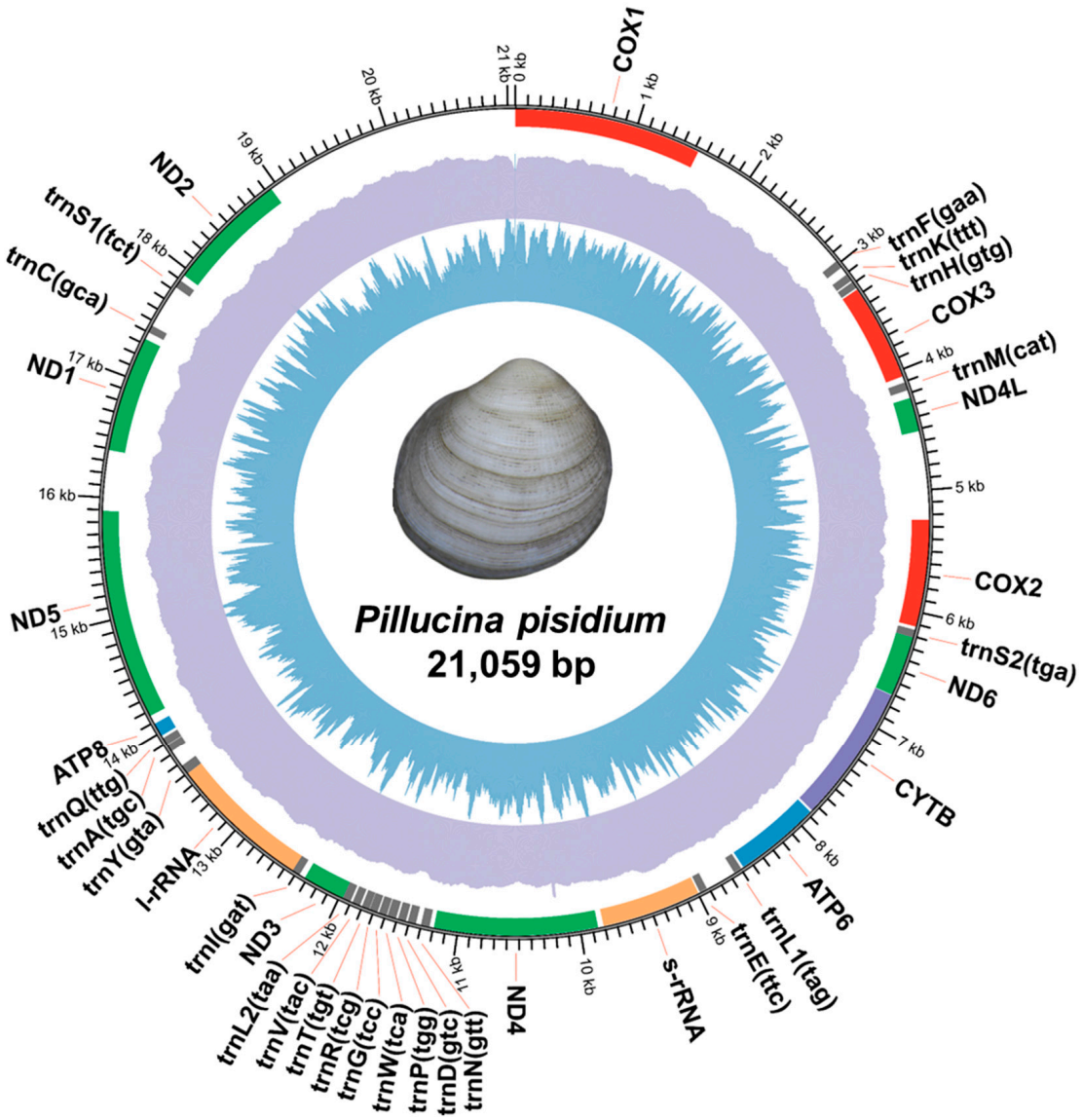

Figure 3. The mitochondrial genome map of *Pillucina pisidium* inhabiting the *Zostera marina* bed of Jeju Island. The innermost blue bar plot represents GC content. The middle purple circle shows sequencing depth. The outermost circle depicts gene order: red for COX genes, blue for ATP synthase, purple for COB, and green for ND genes. The two rRNAs are in orange, and the 22 tRNAs are in gray.

In this study, we also assembled the mitochondrial genome of *P. pacifica* distributed in the northern Atlantic coast of Australia after downloading the WGS Illumina sequence from the NCBI Sequence Read Archive (SRA) database (SRR131632239) reported by Osvatic et al. (2021) to compare the mitochondrial genomes of closely related species. The raw data downloaded for *P. pacifica* consisted of a total of 5,341,670 paired-end reads. After quality filtering, we obtained 2,297,300 clean reads (43%). The *P. pacifica* complete mitochondrial genome was circular with a total length of 16,819 bp. The mitochondrial genome contained a complete set of 37 genes, including 13 PCGs (COX1, COX3, ND4L, ND2, COX2, ND6, COB, ATP6, ND4, ND3, ND1, ATP8, and ND5), two rRNAs (12S rRNA and 16S rRNA), and 22 tRNAs (Table 3). The nucleotide composition of the mitochondrial genome was as follows: A: 25.85%, T: 42.25%, G: 22.38%, and C: 9.52%, with A + T and G + C contents of 68.10% and 31.90%, respectively. The average length of the intergenic regions between coding sequences was 53.8 bp (Table 3). The mitochondrial genome of *P. pacifica* was registered in the NCBI's GenBank database (accession number: BK067723).

Table 3. Annotation of the *Pillucina pacifica* mitochondrial genome.

Gene	Position	Length (bp)	Initiation Codon	Stop Codon	Intergenic Nucleotide (bp)
Cytochrome c oxidase subunit I (COX1)	1–1557	1557	TTG	TAA	4
tRNA-Thr (trnT)	1610–1679	70			52
tRNA-Pro (trnP)	1681–1747	67			1
tRNA-Phe (trnF)	1770–1836	67			22
tRNA-Lys (trnK)	1851–1914	64			14
tRNA-His (trnH)	1921–1985	65			6
Cytochrome c oxidase subunit III (COX3)	1996–2793	798	ATG	TAA	10
tRNA-Met (trnM)	2814–2883	70			20
NADH dehydrogenase subunit 4L (ND4L)	2947–3234	288	ATG	TAA	63
tRNA-Ser (trnS1)	3248–3316	69			13
NADH dehydrogenase subunit 2 (NAD2)	3369–4370	1002	ATT	TAG	52
Cytochrome c oxidase subunit II (COX2)	4377–5312	936	TTG	TAG	6
tRNA-Val (trnV)	5331–5395	65			18
tRNA-Ser (trnS2)	5410–5472	63			14
NADH dehydrogenase subunit 6 (NAD6)	5480–5972	493	ATT	TAA	7
Cytochrome b (COB)	5973–7106	1134	ATG	TAG	0
ATP synthase F0 subunit 6 (ATP6)	7136–7781	646	ATA	TAA	29
tRNA-Glu (trnE)	8780–8845	66			998
tRNA-Trp (trnW)	8889–8954	66			43
NADH dehydrogenase subunit 4 (NAD4)	8973–10,331	1359	ATT	TAA	18
tRNA-Tyr (trnY)	10,521–10,584	64			189
tRNA-Leu (trnL1)	10,595–10,661	67			10
12S ribosomal RNA (rrnS)	10,682–11,511	830			20
tRNA-Arg (trnR)	11,578–11,648	71			66
tRNA-Leu (trnL2)	11,766–11,833	68			117
NADH dehydrogenase subunit 3 (NAD3)	11,833–12,180	348	ATA	TAG	-1
tRNA-Ile (trnI)	12,191–12,258	68			10

Table 3. *Cont.*

Gene	Position	Length (bp)	Initiation Codon	Stop Codon	Intergenic Nucleotide (bp)
16S ribosomal RNA (rrnL)	12,289–13,455	1167			30
NADH dehydrogenase subunit 1 (NAD1)	13,483–14,442	960	ATA	TAA	27
tRNA-Asn (trnN)	14,449–14,513	65			6
tRNA-Gly (trnG)	14,517–14,583	67			3
tRNA-Gln (trnQ)	14,616–14,694	79			32
ATP synthase F0 subunit 8 (ATP8)	14,702–14,815	114	ATT	TAG	7
tRNA-Ala (trnA)	14,862–14,928	67			46
tRNA-Cys (trnC)	14,961–15,023	63			32
NADH dehydrogenase subunit 5 (NAD5)	15,025–16,740	1716	TTG	TAG	1
tRNA-Asp (trnD)	16,748–16,814	67			7

Phylogeny and Synteny. A phylogenetic tree was constructed using the nucleotide sequences of the 13 PCGs to investigate the phylogenetic relationships of *P. pisidium*. An additional phylogenetic tree was reconstructed using 25 mitochondrial genomes from bivalve species, including our assembled mitochondrial genome of *P. pacifica*, along with existing raw data sequences (Table 1). Upon examination, the subclass Autobranchia and its outgroup, the subclass Protobranchia, were delineated (Figure 4). Within Autobranchia, the infraclasses Pteriomorphia and Heterochonchia formed sister groups. However, despite being a member of Heterochonchia, *Sinohyriopsis schlegelii* was grouped as an outgroup to the rest of the Autobranchia. Additionally, each family formed appropriate clusters with high reliability (bootstrap value > 90; Bayesian posterior probability > 0.9) (Figure 4). These results were consistent with the topology observed in phylogenetic trees constructed using various marker genes from different clam species [54,55].

P. pisidium was grouped with three other species in the Lucinidae family. However, it did not form a sister group with *P. pacifica* despite belonging to the same genus. Instead, it formed a sister group with *L. lacteus* and *Lucinella divaricate* with intermediate supporting value (bootstrap value = 63; Bayesian posterior probability = 0.97), with *P. pacifica* as the outgroup (Figure 4). The similarity between *P. pisidium* and *P. pacifica* was analyzed using the Basic Local Alignment Search Tool for nucleotides (BLASTn) (ver. 2.13.0+) for the 13 PCGs. Despite belonging to the same genus, the two species exhibited low average identity (74.28%), suggesting significant divergence. This divergence likely contributes to the inability of the genus *Pillucina* to form a monophyletic cluster. Therefore, it is imperative to conduct further studies that incorporate more species to meticulously examine the phylogenetic relationships within the family Lucinidae, ensuring a thorough understanding of their evolutionary history.

Gene arrangement of 13 PCGs among the 26 clam species displayed a wide variety of patterns (right panel of Figure 4). Only certain species with relatively close phylogenetic relationships shared gene orders at the family level (Mytilidae, Pectinidae, Lucinidae, and Vesicomyidae) and consistent gene orders were observed at the genus level (*Solemya* and *Calyptogena*). However, *P. pisidium* and *P. pacifica* did not exhibit matching synteny among the 13 PCGs, despite belonging to the same genus. However, *P. pisidium*, which forms a sister group with two other species (*L. lacteus* and *L. divaricata*), had a distinct gene order within the family Lucinidae. Conversely, *P. pacifica*, an outgroup within the family Lucinidae, shared a gene arrangement with *L. lacteus* and *L. divaricata*.

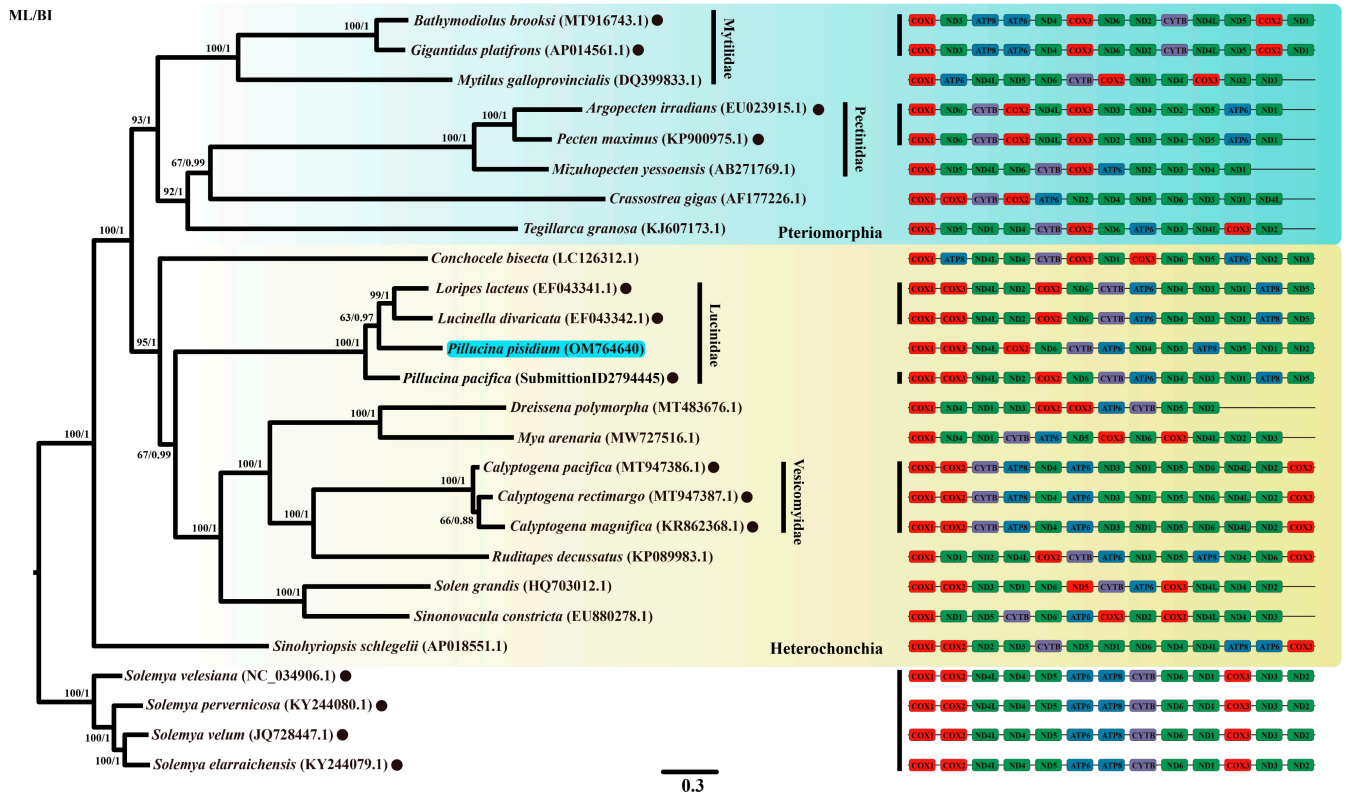

Figure 4. The maximum likelihood (ML) phylogenetic tree of the subclass Autobranchia. The tree was reconstructed based on 13 protein-coding genes (PCGs). Four species in the genus *Solemya* were used as the outgroup. Bootstrap supports a value of >60% for ML (upper), and posterior probabilities of > 80% for Bayesian inference (BI) (lower) are indicated around each node. The background colors represent different infraclasses. The scale bar represents the relative substitution rates per site. Horizontal lines next to species names indicate members of the same family. The synteny of 13 mitochondrial PCGs was placed to the right of the tree. The horizontal bars to the left of the synteny and the dots next to the species names indicate the same order of 13 PCGs arrangement.

4. Conclusions

We analyzed molecular biology data of the chemosymbiotic clam *P. pisidium*, which is distributed in *Z. marina* beds in a lagoon on the eastern coast of Jeju Island. The shell morphology of *P. pisidium* matched the type material and the thick and hypertrophied gills reflected the characteristics of chemosynthetic clams. Including *P. pisidium*, phylogenetic analyses utilizing all 13 PCGs from the mitochondrial genomes of Autobranchia reported to date revealed phylogenetic relationships similar to those found in previous studies. Additionally, synteny analysis of the 13 PCGs revealed some similarities within the same genus/family; however, diverse gene arrangement patterns were observed. Specifically, *P. pisidium* belonged to the Lucinidae family but did not form a sister group with *P. pacifica*, even though they belong to the same genus. Moreover, *P. pisidium* exhibited distinctive features, i.e., the length of the entire mitochondrial genome and the arrangement of the PCGs, within the Lucinidae family. Therefore, a comprehensive investigation of the mitochondrial genomes of Lucinidae species is necessary to clarify phylogenetic relationships within this group.

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Data Availability Statement: The raw sequencing data of *Pillucinia pisidinum*, is available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (accession number: SRR28961031). The mitochondrial sequence of *P. pisidinum* is available from the NCBI RefSeq of NCBI under accession no. NC_071184. The mitochondrial sequence of *Pillucinia pacifica* is available from the NCBI GenBank accession No. BK067723.

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