

Article

The Complete Mitochondrial Genome of *Hyotissa hyotis* (Bivalvia: Gryphaeidae) Reveals a Unique Gene Order within Ostreoidea

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Abstract: The mitochondrial (mt) genome is an important tool when studying the evolution of metazoan animals. The oyster family Gryphaeidae, together with Ostreidae, is one of the two extant taxa of superfamily Ostreoidea. Up until now, the available mitochondrial genomes of oysters were all limited to family Ostreidae. In the present study, the first complete mtDNA of family Gryphaeidae represented by *Hyotissa hyotis* was sequenced and compared with other available ostreoid mtDNA. The mtDNA of *H. hyotis* is 22,185 bp in length, encoding 13 protein-coding-genes (PCGs), two ribosomal RNA (rRNA) and 23 transfer RNA (tRNA) genes. Within all the intergenic regions that range from 2 to 1528 bp, two large non-coding regions were identified. The first large non-coding region, located between *Cox1* and *trnA*, contains 1,528 nucleotides, while the second one is 1,191 bp in length and positioned between *Cytb* and *Nad2*. The nucleotide composition of the whole mtDNA is A + T biased, accounting for 59.2%, with a negative AT skew value of -0.20 and a positive GC skew value of 0.33. In contrast to the mtDNA of Ostreidae, neither the split of *rrnL* nor *rrnS* was detected in that of *H. hyotis*. The duplication of *trnW* of *H. hyotis* was also discovered for the first time within Ostreoidea. The gene order of *H. hyotis* is quite different from those of ostreids, indicating extensive rearrangements within superfamily Ostreoidea. The reconstructed phylogeny supported *H. hyotis* as sister to Ostreidae, with the latter clade formed by *Ostrea* + (*Saccostrea* + *Crassostrea*). This study could provide important information for further understanding the mitochondrial evolution of oysters.

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1. Introduction

The mitochondrial (mt) genome within multicellular animals was characteristic of a closed-circular molecule that contains 37 genes, including 13 protein coding genes (PCGs), two ribosomal RNA (rRNA) genes, and the 22 transfer RNA (tRNA) genes [1]. With the increase of mtDNA data collected in the last two decades, considerable diversity in the organization of animal mtDNA has been detected especially in nonbilaterian groups, including phyla Cnidaria, Ctenophora, Placozoa, and Porifera [2]. Within these groups, the differences that challenged conventional defined mtDNA characteristics mainly lie in the numbers of both linear and circular chromosomes, the presence of additional PCGs (e.g., *Atp9* and *TatC*), large variation in the number of tRNAs (0 to 25), changes in genetic codes, presence of introns, tRNA and mRNA editing, translational frameshifting, and variable rates of sequence evolution [2–5]. The size of most bilaterian mtDNA ranges from 15 to 20 kb [1]. Even though some larger mtDNA have been reported, the differences in

mitogenome size were attributed to variations in length of certain portions (usually non-coding regions) instead of changes in gene content [6,7]. The sequences and structures of animal mtDNA have been widely used for species delimitation [8,9], classification [10], and phylogenetic analyses [11–13] due to its advantages including high abundance, lack of recombination, a higher rate of evolution and maternal inheritance [14]. However, the following studies have indicated that molluscan mitochondrial genomes broke the rules [15] in terms of the discovery of doubly uniparental inheritance (DUI) and homologous recombination within some bivalve taxa [16–19]. In addition, mitochondrial heteroplasmy that has no correlation with DUI has also been reported in Mytilidae [20].

Compared with other metazoans, mollusks in general, and bivalves in particular, are more prone to gene rearrangements [10,21]. Some bivalves also show variations in the number of mitochondrial tRNA genes [22] and genome size, ranging from 14,622 bp (*Lanternula elliptica* [23]) to 56,170 bp (*Scapharca kagoshimensis* [6]) according to our current knowledge. Even within some closely related taxa, considerable differences in size of mtDNA can still be discovered [6,24]. A previous study indicated that size variation was mostly caused by the different lengths of the non-coding regions [25].

Oysters are marine bivalves distributed worldwide, and many of them are important fishery and aquaculture species [26]. Belonging to superfamily Ostreoidea, the extant oysters were divided into two families, Gryphaeidae and Ostreidae [27]. Since the shell morphology of oysters was proved to be highly plastic, molecular data have been applied for the revision of oyster classifications and understanding of their evolution [28]. With the development of sequencing technologies, more and more complete mtDNA of oysters are accessible (Table 1), which provides new insights into oyster phylogenetics [25,26,29–31]. Previous comparative mitogenomic analyses of oysters were limited to family Ostreidae, and suggested several shared characteristics, such as a split of the *rrnL* gene and the duplication of *trnM*. Within Ostreidae, although the duplications and rearrangements of tRNA genes are frequently observed, the order of PCGs is relatively conserved within the same genus in *Crassostrea*, *Ostrea* and *Saccostrea*. While the PCG rearrangements between different genera could be explained as the result of transversion and/or translocation of the plesiomorphic gene order belonging to *Ostrea* [26]. In addition to family Ostreidae, there is still no information with respect to the mtDNA of other ostreoid species, especially within Gryphaeidae.

Table 1. List of mtDNA used in the present study.

New Mitochondrial Genome				
Family	Species	Length (bp)	Sampling Time	Accession No.
Gryphaeidae	<i>Hyotissa hyotis</i>	22,185	May, 2022	OP151093
GenBank Mitochondrial Genome				
Family	Species	Length (bp)	Accession No.	Reference
Ostreidae	<i>Crassostrea hongkongensis</i>	18,617	MZ337404	Liu et al. (2022) [32]
Ostreidae	<i>Crassostrea bilineata</i>	22,420	MT985154	Arshad et al. (2020)
Ostreidae	<i>Crassostrea belcheri</i>	21,020	MH051332	Gastineau et al. (2018) [33]
Ostreidae	<i>Crassostrea gasar</i>	17,685	KR856227	Cavaleiro et al. (2016) [34]
Ostreidae	<i>Crassostrea nippona</i>	20,030	HM015198	Yu and Li (2012) [35]
Ostreidae	<i>Crassostrea iredalei</i>	22,446	FJ841967	Wu et al. (2010) [36]
Ostreidae	<i>Crassostrea ariakensis</i>	18,414	EU672835	Ren et al. (2010) [25]
Ostreidae	<i>Crassostrea sikamea</i>	18,243	EU672833	Ren et al. (2010) [25]
Ostreidae	<i>Crassostrea angulata</i>	18,225	EU672832	Ren et al. (2010) [25]
Ostreidae	<i>Crassostrea gigas</i>	18,225	EU672831	Ren et al. (2010) [25]
Ostreidae	<i>Crassostrea virginica</i>	17,244	AY905542	Milbury and Gaffney (2005) [37]
Ostreidae	<i>Ostrea denselamellosa</i>	16,277	HM015199	Yu and Li (2011) [38]

Ostreidae	<i>Ostrea edulis</i>	16,320	JF274008	Danic-Tchaleu et al. (2011) [29]
Ostreidae	<i>Ostrea lurida</i>	16,344	KC768038	Xiao et al. (2015) [39]
Ostreidae	<i>Saccostrea mordax</i>	16,532	FJ841968	Wu et al. (2009)
Ostreidae	<i>Saccostrea cucullata</i>	16,396	KP967577	Volatiana et al. (2016) [30]
Ostreidae	<i>Saccostrea kegaki</i>	16,260	KX065089	Hsiao (2016)
Margaritidae	<i>Pinctada margaritifera</i>	15,680	HM467838	Wu et al. (2010)
Margaritidae	<i>Pinctada maxima</i>	16,994	GQ452847	Wu et al. (2012) [40]

As the representative of family Gryphaeidae, *Hyotissa hyotis* (Linnaeus, 1758) is distributed in the tropical regions of the Indo-Pacific Ocean, and it is an important fishery resource in coastal countries like China. Although *H. hyotis* has also been found in the Florida Keys of the United States, it was proven to be a recent introduction from the Indo-Pacific [41]. In the present study, the complete mtDNA of *Hyotissa hyotis* was sequenced and compared with those of other ostreoids available on GenBank (Table 1), with the following aims: 1) to characterize the mitogenomic features of *H. hyotis*; 2) to explore its gene order; and 3) to determine its phylogenetic position within Ostreoidae.

2. Materials and Methods

2.1. Samples and DNA Extraction

The specimen of *H. hyotis* was collected in a local market of Ximaozhou Island (18°14'22" N; 109°22'42" E). The soft tissue of the specimen was deposited in 95% alcohol in the Laboratory of Economic Shellfish Genetic Breeding and Culture Technology (LESGBCT), Hainan University.

Genomic DNA was extracted from small pieces of adductor muscle using TIANamp Marine Animals DNA Kit (Tiangen, Beijing, China) following the instructions. Only one specimen of *H. hyotis* was used for DNA extraction. The genomic DNA was visualized on 1% agarose gel for quality inspection.

2.2. Illumina Sequencing and Mitogenome Assembly

Genomic DNA of *H. hyotis* was sent to Novogene Company (Beijing, China) for library construction and next-generation sequencing. The DNA library was generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer's instructions. One library with insert size of approximately 300 bp was prepared and then sequenced on the Illumina NovaSeq 6000 platform with 150 bp paired-end reads. Finally, 37,526,636 clean reads of each direction were generated for the library. Adapters and low-quality reads were filtered using Trimmomatic [42]. The generated clean data were imported in Geneious Prime 2021.0.1 [43] for mitogenome assembly, with the strategy following Irwin et al. [44]. The filtered Illumina reads and assembled mitogenome were submitted to Sequence Read Archive (SRA) and GenBank, with the accession numbers SRR21135036 (under Bioproject PRJNA871102) and OP151093, respectively.

2.3. Mitogenome Annotation and Sequence Analysis

The mitogenome of *H. hyotis* was annotated using MITOS Webserver [45]. The PCGs were also determined by ORF Finder (<http://www.ncbi.nlm.nih.gov/orffinder>) with the invertebrate mitochondrial genetic code, and their boundaries were modified by comparing them with those of other ostreoids. The identification of *Atp8* was done by the comparison of the possible open reading frames predicted by ORF Finder with the *Atp8* genes of *Ostrea lurida* (KC768038) and *Crassostrea gasar* (KR856227) that were available on GenBank. The secondary structure of tRNA and rRNA genes were predicted by MITOS Webserver. The tRNAs were also verified using ARWEN [46].

The nucleotide composition of the mitochondrial genome, PCGs, rRNA, tRNA genes and major non-coding region were calculated using MEGA X [47]. The base skew values for a given strand were calculated according to the following formulas: AT skew = $(A - T)/(A + T)$ and GC skew = $(G - C)/(G + C)$ [48], where A, T, G, and C are the occurrences of the four nucleotides. Codon usage of PCGs was also conducted by MEGA X. The mitochondrial genome map was generated using CGView [49].

2.4. Gene Order Comparisons

The mtDNA of *H. hyotis*, along with those of other ostreoids available on GenBank (Table 1), were used for comparative gene order analysis. Mitochondrial gene orders of all mtDNA within Ostreoidea were conducted manually. The rearrangements were analyzed directly by comparing only the 12 PCG rearrangement steps between the common gene order of each genus and visualized using Microsoft Visio 2016. *Atp8* gene was not included in PCG rearrangements following Guo et al. (2018) [26].

2.5. Phylogenetic Analysis

In order to clarify the phylogenetic position of *H. hyotis* within Ostreoidea, a total of 18 ostreoid species (including 17 previously published mitogenomes) were used in the present study (Table 1). *Pinctata maxima* and *P. margaritifera* were selected as outgroups according to the topology by Sun et al. (2021) [7]. Phylogenetic trees were reconstructed using maximum likelihood (ML) and Bayesian inference (BI) analyses based on the nucleotide sequences of 12 PCGs. The *Atp8* gene was not included since it is missing in most oyster mitogenomes, and due to its high substitution rate, it is not appropriate for phylogenetic construction. Maximum likelihood analyses were performed using IQtree 1.6.10 [50], allowing partitions to have different evolutionary rates (-spp option) and with 10,000 ultrafast bootstrap pseudoreplications (-bb option). Bayesian inference analyses were carried out by MrBayes 3.2.6 [51], running four simultaneous Monte Carlo Markov chains (MCMC) for 10,000,000 generations, sampling every 1000 generations. Two independent Bayesian inference runs were performed to increase the chance of adequate mixing of the Markov chains and to increase the chance of detecting failure to converge, as determined using Tracer v1.6. The initial 25% of generations were discarded as burn-in according to the excellent effective sample size (ESS) values (>200) of all parameters as measured by Tracer v1.6.

The best partition schemes and best-fit substitution models for BI analyses were conducted using PartitionFinder 2 [52], under the Bayesian Information Criterion (BIC). For the nucleotide sequences of 12 PCGs dataset, the partitions tested were: all genes combined; all genes separated (except *Nad4-Nad4L*); and genes grouped by subunits (*Atp*, *Cytb*, *Cox* and *Nad*). Additionally, these three partition schemes were tested considering separately the three codon positions. The best partition schemes for ML analyses were also determined by PartitionFinder 2, while the best-fit substitution models for ML analyses were calculated with ModelFinder [53] as implemented in IQ-TREE 1.6.12.

3. Results and Discussion

3.1. Genome Organization of *Hyotissa hyotis*

The assembled mtDNA includes 343,733 short reads, which account for 0.45% of total data. The mean coverage reaches 2,302. The complete mtDNA of *H. hyotis* is 22,185 bp in length, containing 13 protein coding genes (PCGs), two rRNA and 23 tRNA genes (Figure 1, Table 2). This size is longer than most ostreoid mtDNA, but still falling within the size range of Ostreoidea, from 16,277 bp for *O. denselamellosa* [31] to 22,446 bp for *C. iredalei* [36]. All 38 genes are encoded on the major strand, like all marine bivalves [15]. Compared with the typical metazoan mtDNA, a duplication of *trnW* has been detected. The non-coding regions are distributed throughout the mtDNA, ranging from 2 to 1,528 bp (Table 2), accounting for 28.9% of the whole mtDNA. Among these intergenic regions, two large

non-coding regions (LNR) were identified. The first one (LNR1), which contains 1,528 nucleotides, is located between *Cox1* and *trnA*, while the second one (LNR2) is 1,191 bp in length and positioned between *Cytb* and *Nad2*. The AT skew value of LNR1 (64.8%) is significantly higher than that of LNR2 (51.7%), as well as the remainder of the mtDNA (Table 3). The LNR with higher AT content values is typically used for the determination of Ostreidae mitochondrial control region and considered to contain the initiation of replication and transcription [29]. The presence of two LNRs is not common in the reported Ostreidae mtDNA, and has only been found in that of *C. iredalei*, within which the two regions were presumed to originate from the duplications of *Nad2*, followed by separate mutations and functional gene loss [36].

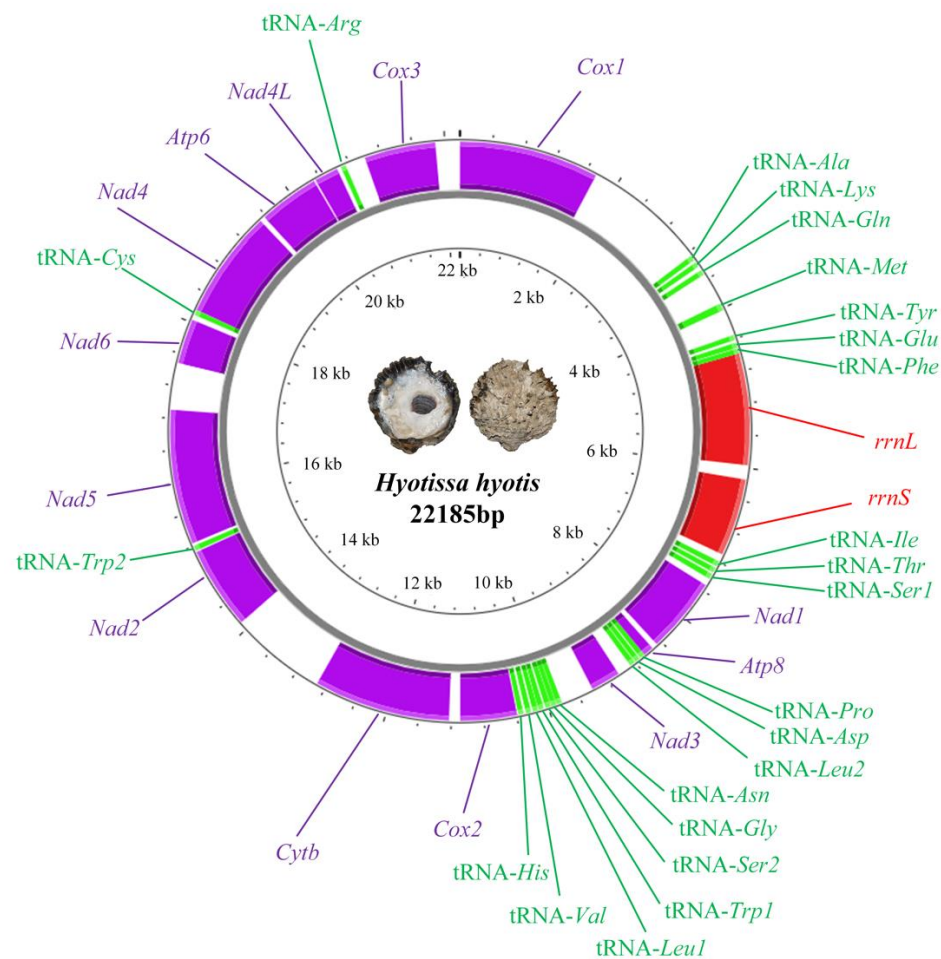


Figure 1. Mitochondrial genome map of *Hyotissa hyotis*.

Table 2. Gene annotations of the mtDNA of *Hyotissa hyotis*.

Gene	Strand	Location	Size (bp)	Start Codon	Stop Codon	Intergenic Nucleotides
<i>Cox1</i>	H	1–1725	1725	ATG	TAG	1528
tRNA- <i>Ala</i>	H	3254–3319	66			31
tRNA- <i>Gln</i>	H	3351–3413	63			51
tRNA- <i>Lys</i>	H	3465–3531	67			403
tRNA- <i>Met</i>	H	3935–4025	91			325
tRNA- <i>Tyr</i>	H	4351–4414	64			36
tRNA- <i>Glu</i>	H	4451–4516	66			8
tRNA- <i>Phe</i>	H	4525–4587	63			0

<i>rrnL</i>	H	4588–5966	1379			175
<i>rrnS</i>	H	6142–7099	958			93
tRNA- <i>Ile</i>	H	7193–7260	68			22
tRNA- <i>Thr</i>	H	7283–7345	63			20
tRNA- <i>Ser1</i>	H	7366–7435	70			90
<i>Nad1</i>	H	7526–8460	935	ATG	TA-	71
<i>Atp8</i>	H	8532–8654	123	ATG	missing	1
tRNA- <i>Pro</i>	H	8656–8720	65			13
tRNA- <i>Asp</i>	H	8734–8797	64			10
tRNA- <i>Leu2</i>	H	8808–8873	66			177
<i>Nad3</i>	H	9051–9417	367	TTT	T--	408
tRNA- <i>Asn</i>	H	9826–9892	67			4
tRNA- <i>Gly</i>	H	9897–9961	65			3
tRNA- <i>Ser2</i>	H	9965–10034	70			21
tRNA- <i>Trp1</i>	H	10056–10122	67			7
tRNA- <i>Leu1</i>	H	10130–10194	65			20
tRNA- <i>Val</i>	H	10215–10281	67			29
tRNA- <i>His</i>	H	10311–10375	65			15
<i>Cox2</i>	H	10391–11089	699	TTG	TAA	138
<i>Cytb</i>	H	11228–12910	1683	ATG	TAA	1191
<i>Nad2</i>	H	14102–15121	1020	ATT	TAG	13
tRNA- <i>Trp2</i>	H	15135–15201	67			35
<i>Nad5</i>	H	15237–16901	1667	AAG	TAA	578
<i>Nad6</i>	H	17480–18034	555	ATG	TAA	64
tRNA- <i>Cys</i>	H	18099–18162	64			2
<i>Nad4</i>	H	18165–19508	1344	GTG	TAA	66
<i>Atp6</i>	H	19575–20324	750	ATG	TAA	20
<i>Nad4L</i>	H	20345–20638	294	TAT	TAA	52
tRNA- <i>Arg</i>	H	20691–20753	63			252
<i>Cox3</i>	H	21006–21884	879	ATA	TAA	301

Table 3. List of AT content, AT skew, and GC skew of *Hyotissa hyotis*.

Feature	(A+T)%	AT skew	GC skew
Whole genome	59.2	−0.20	0.33
PCGs	59.4	−0.29	0.34
PCGs1	57.3	−0.09	0.38
PCGs2	59.9	−0.34	0.08
PCGs3	61.0	−0.42	0.57
<i>Atp6</i>	59.8	−0.23	0.40
<i>Atp8</i>	60.1	−0.30	0.14
<i>Cox1</i>	58.6	−0.28	0.29
<i>Cox2</i>	59.0	−0.25	0.37
<i>Cox3</i>	59.3	−0.31	0.30
<i>Cytb</i>	60.5	−0.20	0.27
<i>Nad1</i>	59.5	−0.36	0.36
<i>Nad2</i>	59.1	−0.30	0.39
<i>Nad3</i>	58.4	−0.43	0.50
<i>Nad4</i>	61.6	−0.33	0.35
<i>Nad4L</i>	58.5	−0.33	0.43
<i>Nad5</i>	59.1	−0.29	0.39
<i>Nad6</i>	57.7	−0.24	0.39

tRNAs	57.9	-0.12	0.25
<i>rrnS</i>	53.7	0.11	0.11
<i>rrnL</i>	58.8	-0.02	0.20
LNR1	64.8	-0.08	0.16
LNR2	51.7	-0.21	0.52

Note: LNR is the abbreviation of large non-coding region.

The molecule has an overall AT content of 59.2% (Table 3), which is lower than those of Ostreidae species (60.7% in *O. denselamellosa* to 65.3% in *C. hongkongensis* [29]). The nucleotide composition is strongly skewed away from C in favor of G (with the GC skew value of 0.33) and from A in favor of T (with the AT skew value of -0.20) (Table 3). Although the negative AT skew and positive GC skew has been detected in all Ostreoida species, the absolute values of the two parameters in *H. hyotis* are relatively higher than those of other ostreoids [36].

3.2. Protein Coding Genes (PCGs)

A total of 13 PCGs were annotated in the mtDNA of *H. hyotis* (Figure 1, Table 2). The detection of *Atp8* is quite difficult since it is a short gene that is under low selective pressure and exhibits great variability at both amino acid and nucleotide levels [54]. Previous studies indicated that *Atp8* was missing in all oyster mtDNA [25,29,36,55], however, following studies suggested the identification of this short gene in some oyster species [54]. In this study, a potential *Atp8* which is located between *Nad1* and *trnP* was detected based on the slight similarity between *Atp8* sequences here and those in the mtDNA of *Osrea lurida* (KC768038) and *C. gasar* (KR856227). The *Atp8* of *H. hyotis* encodes 41 amino acids, similar to those of other oysters [54]. It is worth noting that the stop codon of *Atp8* is missing. This may be caused by the *trnP* that breaks the transcript and makes the gene similar in length to other *Atp8* genes in this superfamily.

The AT content, AT and GC skew values of the PCGs as well as of the three positions of PCGs, show the same tendency of asymmetry as the mitochondrial genome (Table 3). The 13 PCGs are 12,039 bp in length, accounting for 54.3% of whole mtDNA.

Among the 13 PCGs, seven genes start with the conventional initiation codons ATG (*Cox1*, *Nad1*, *Cytb*, *Nad6*, *Atp6* and *Atp8*) and ATA (*Cox3*), while the remaining genes employ alternative start codons, including TTT (*Nad3*), TTG (*Cox2*), ATT (*Nad2*), AAG (*Nad5*), GTG (*Nad4*) and TAT (*Nad4L*) (Table 2). Eight genes stop with TAA (*Atp6*, *Cox2*, *Cox3*, *Nad4*, *Nad4L*, *Nad5* and *Nad6*) and two use TAG (*Cox1* and *Nad2*). Finally, *Nad1* and *Nad3* exhibit the incomplete stop codons TA and T, respectively. The truncated stop codons are common in the metazoan mtDNA [35,56], and might be modified to the TAA termini via post-transcriptional polyadenylation [57]. The relative synonymous codon usage (RSCU) values are shown in Table 4. The mtDNA encodes 3961 amino acids (excluding all stop codons), among which leucine is the most frequently chosen one (Table 4) as indicated in other mollusk taxa [58]. Considerable synonymous codon usage bias was observed in the present study (Figure 2), and these preferred codons mostly ended in T, resulting in a significant negative AT skew value at the third codon position (Table 3). Previous studies have indicated that the codon usage pattern might vary even within the same family [59].

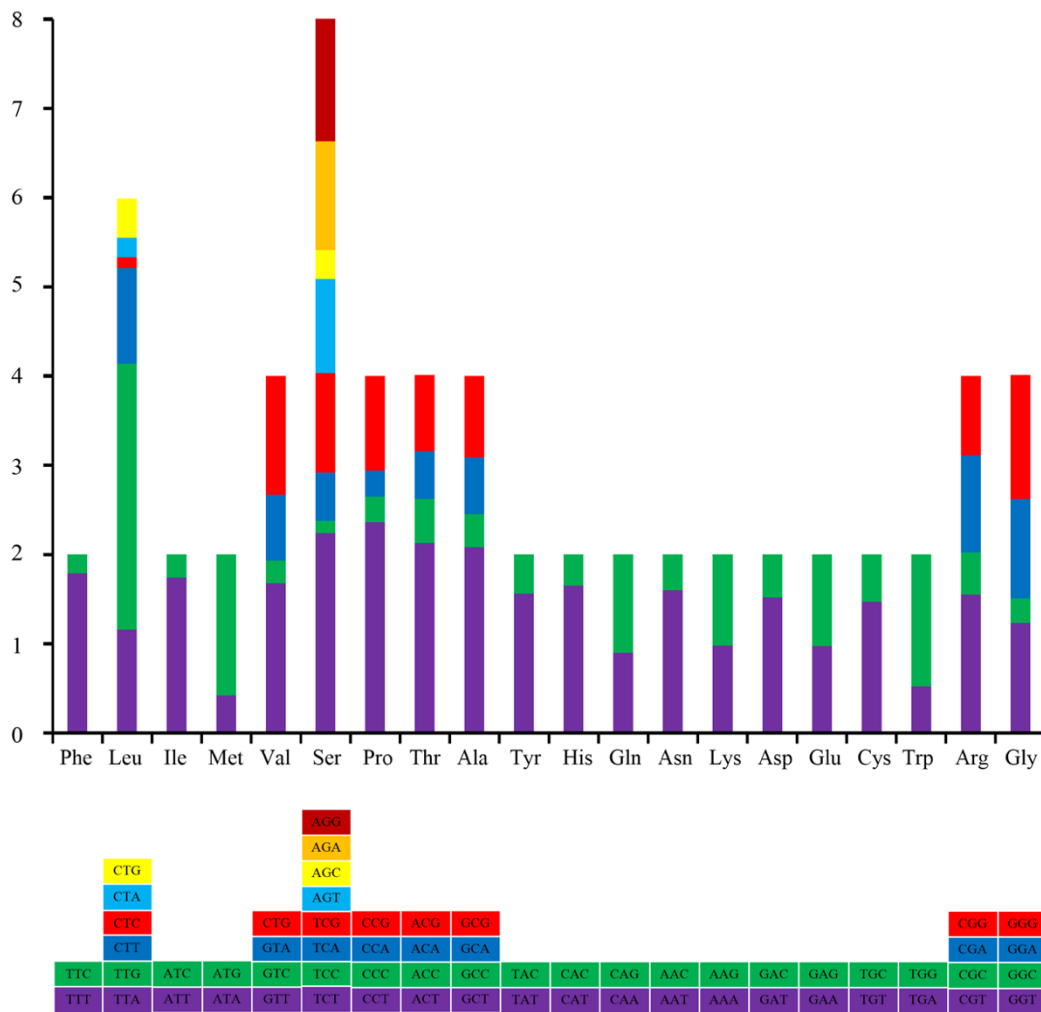


Figure 2. Relative synonymous codon usage (RSCU) of mitochondrial genome for *Hyotissa hyotis*.

Table 4. Codon and relative synonymous codon usage (RSCU) of 12 protein-coding genes (PCGs) in the mtDNA of *Hyotissa hyotis*.

Amino Acid	Codon	Count (RSCU)	Amino	Codon	Count (RSCU)
Phe	TTT	289.0(1.78)	Ala	GCT	134.0(2.08)
	TTC	35.0(0.22)		GCC	24.0(0.37)
Leu	TTA	91.0(1.16)	Gly	GCA	41.0(0.64)
	TTG	232.0(2.96)		GCG	59.0(0.91)
	CTT	84.0(1.07)		GGT	101.0(1.22)
	CTC	9.0(0.11)		GGC	24.0(0.29)
	CTA	18.0(0.23)		GGA	91.0(1.10)
Ile	CTG	36.0(0.46)	Arg	GGG	115.0(1.39)
	ATT	193.0(1.74)		CGT	40.0(1.52)
ATC	29.0(0.26)	CGC		12.0(0.46)	
Met	ATA	47.0(0.44)	Tyr	CGA	28.0(1.07)
	ATG	169.0(1.56)		CGG	25.0(0.95)
Val	GTT	155.0(1.68)	His	TAT	135.0(1.56)
	GTC	25.0(0.27)		TAC	38.0(0.44)
	GTA	68.0(0.74)	Gln	CAT	70.0(1.65)
	GTG	122.0(1.32)		CAC	15.0(0.35)
Ser	TCT	99.0(2.21)	Cys	CAA	22.0(0.90)
	TCC	6.0(0.13)		CAG	27.0(1.10)

	TCA	25.0(0.56)	Asn	AAT	81.0(1.59)
	TCG	49.0(1.09)		AAC	21.0(0.41)
	AGT	47.0(1.05)	Lys	AAA	81.0(0.98)
	AGC	15.0(0.34)		AAG	84.0(1.02)
	AGA	54.0(1.21)	Asp	GAT	67.0(1.52)
	AGG	63.0(1.41)		GAC	21.0(0.48)
Pro	CCT	82.0(2.33)	Glu	GAA	65.0(0.97)
	CCC	11.0(0.31)		GAG	69.0(1.03)
	CCA	11.0(0.31)	Cys	TGT	77.0(1.47)
	CCG	37.0(1.05)		TGC	28.0(0.53)
Thr	ACT	83.0(2.11)	Trp	TGA	39.0(0.52)
	ACC	19.0(0.48)		TGG	110.0(1.48)
	ACA	22.0(0.56)			
	ACG	33.0(0.84)			

It is also worth mentioning that the boundaries of some PCGs predicted by ORF Finder are quite long. For example, the predicted length of *Nad3* in *H. hyotis* mtDNA reached 1000 bp, far beyond the normal length 351 bp within the superfamily. On the other hand, if the indicated boundary of *Nad1* was accepted, it would overlap the *Atp8* gene. Therefore, some genes were truncated to similar length according to the corresponding PCGs in this superfamily. This operation also resulted in the appearance of incomplete stop codons mentioned above. These codons are explained to be completed via post-transcriptional polyadenylation, however, no stable secondary structures that could break translation were detected after these genes. The NGS RNAseq reads are needed to finally determine the accurate boundaries of these genes in the future.

3.3. Ribosomal RNA and Transfer RNA Genes

The split of rRNAs has been considered as a common feature of Ostreidae. Firstly, the split of *rrnL* was found in all ostreid mtDNA [25,29,36]. Furthermore, Asian species within *Crassostrea* also contained a duplicated *rrnS* gene [25,36]. In this study, however, neither *rrnL* nor *rrnS* of *H. hyotis* is separated. The *rrnS* and *rrnL* genes of *H. hyotis* are 958 and 1,379 bp, with AT content of 53.7% and 58.8% respectively. The *rrnL* shows a weakly negative AT skew and strongly positive GC skew compared with the *rrnS* that shows both positive AT and GC skew values (Table 3).

The typical metazoan mtDNA encodes 22 tRNAs, including two copies of *trnL* and two of *trnS* [1]. Nevertheless, some bivalve mtDNA have been found to show exceptions which were related to the duplication of certain tRNAs. A duplicated *trnM* has been detected in all Ostreidae species, as well as in other bivalve taxa, such as some species within Veneridae [60]. In addition to *trnM*, most Asian oysters of *Crassostrea* also possess two more duplications of *trnK* and *trnQ* [29]. A total of 23 tRNAs was found within the mtDNA of *H. hyotis* (Figure 1), but the duplication of *trnW* has never been reported within Ostreidae previously. The AT and GC skew values of all tRNAs are -0.12 and 0.25 (Table 3), respectively, showing the same tendency of asymmetry as the whole mtDNA. The lengths of the tRNAs range from 63 to 91 bp (Table 2). The *trnM* which was predicted by different software is significantly longer than other tRNAs of *H. hyotis* (Figure 3). This strange secondary structure has never been found in oyster mtDNA, but reported in the *trnM* of the bivalve family Arcidae [61]. All tRNA genes could be folded into typical clover-leaf secondary structures except for the *trnS*-AGU due to the absence of dihydrouracil (DHU) arm (Figure 3), which was common in metazoan mtDNA [62].

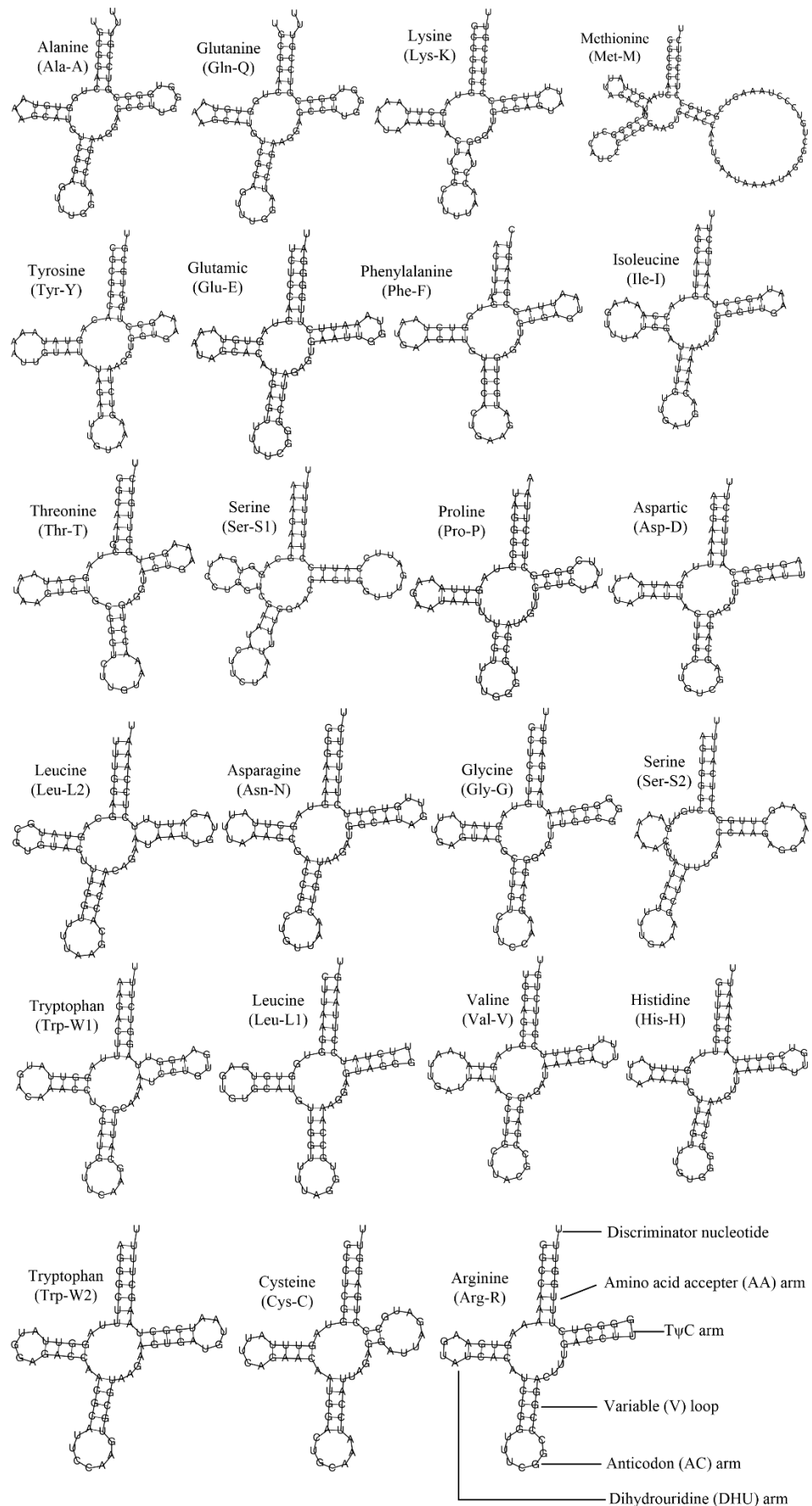


Figure 3. Inferred secondary structures of 23 transfer RNAs from *Hyotissa hyotis*.

3.4. Gene Rearrangement

Within Bivalvia, gene rearrangements are most common for tRNAs. Even within *Crassostrea*, variable gene orders of tRNAs could be detected [25]. The change of tRNA gene orders might be explained by the ‘duplication-random loss model’ [63], since duplicated tRNAs could often be found within ostreid mtDNA in addition to the 22 essential tRNAs. The tRNA gene order of *H. hyotis* shows extensive rearrangements within Ostreoidea, with little gene blocks shared with ostreids (Figure 4).

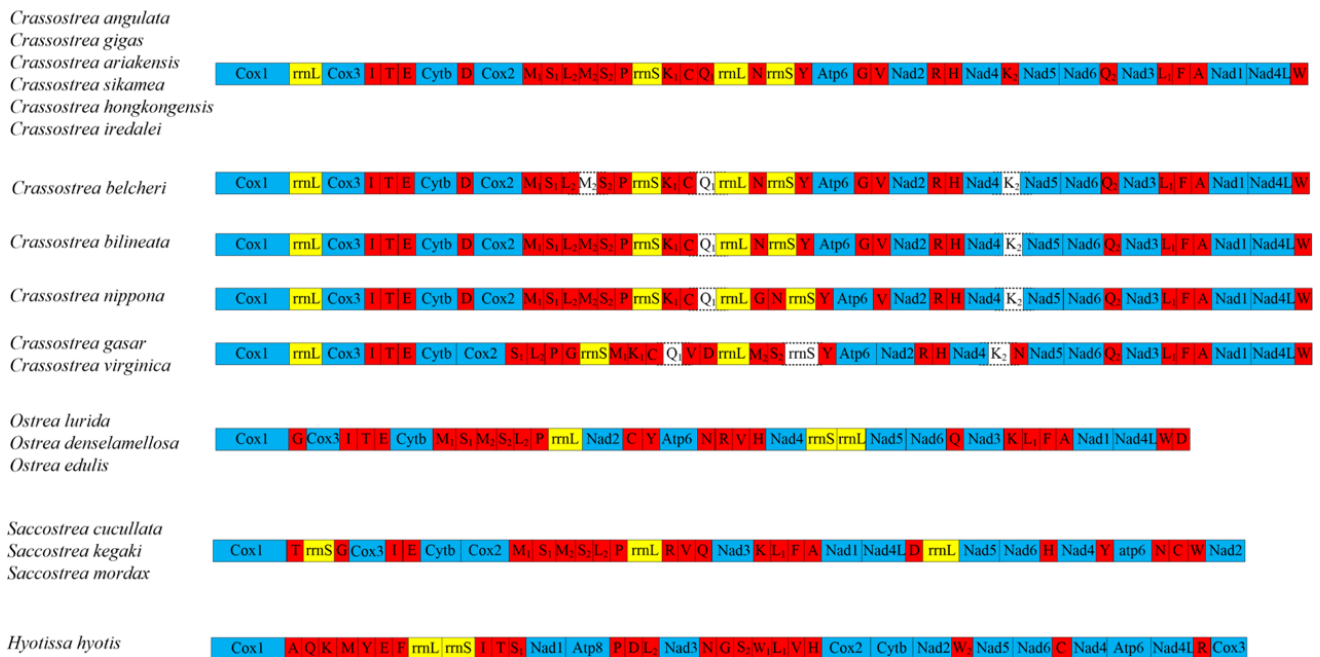


Figure 4. Linearized mitochondrial gene orders of the represented oyster mitogenomes available on GenBank (Accession numbers are provided in Table 1). tRNA genes are marked by the single-letter codes of the encoded amino acids. tRNAs in the dashed box means the missing genes compared with the congeneric species.

Compared with tRNAs, PCG orders are more conserved at genus level within Ostreidae. The PCG rearrangements among *Crassostrea*, *Ostrea*, and *Saccostrea* was explained by assuming the PCG order in *Ostrea* as the ancestral characteristic of Ostreidae, and those of *Crassostrea* and *Saccostrea* resulted from one transversion and one transversion plus one translocation, respectively [26]. However, the PCG order of *H. hyotis* shows little shared gene blocks with the pleisomorphic gene order of *Ostrea* (Figure 5). Instead, one identical gene block (*Nad5-Nad6-Nad4-Atp6*) as well as one inverted gene block (*Nad1-Nad3-Cox2-Cytb*) was detected between *H. hyotis* and *Saccostrea* (Figure 5). To understand how the gene order of *H. hyotis* evolved within superfamily Ostreoidea, more data with respect to the ostreid mtDNA together with a robust phylogenetic framework are still needed.

Bivalves are sometimes notoriously tricky to amplify PCR products from especially typical barcoding genes often used in species identification. The potential reasons may lie in, such as the gene order rearranging and differing start codons as mentioned above. The first mtDNA of family Gryphaeidae could also help provide those working with oysters a tool for better designing relevant primers.

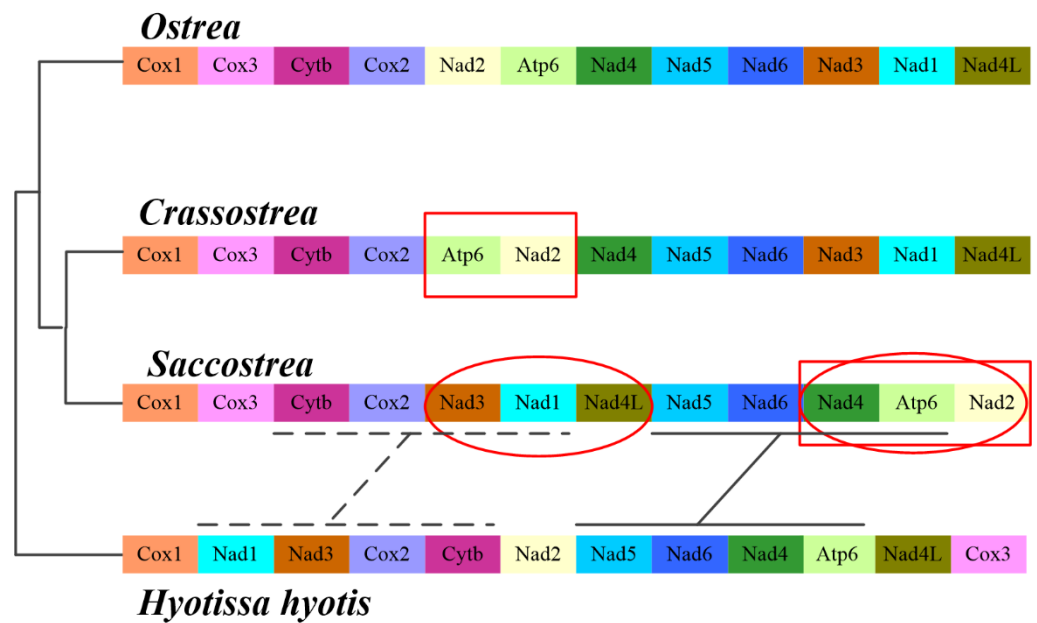


Figure 5. Linearized PCG order of Ostreidae (including *Crassostrea*, *Saccostrea* and *Ostrea*) and Gryphaeidae (represented by *Hyotissa hyotis* only) based on the phylogenetic tree (see Figure 6 for detailed relationships). By assuming the PCG order in *Ostrea* as pleisomorphic of Ostreidae, the transversions and translocations (happened in *Crassostrea* and *Saccostrea*) are indicated by red rectangular and oval boxes, respectively. The identical gene block (*Nad5-Nad6-Nad4-Atp6*) and the inverted gene block (*Nad1-Nad3-Cox2-Cytb*) between *H. hyotis* and *Saccostrea* are marked by solid and dotted underlines, respectively.

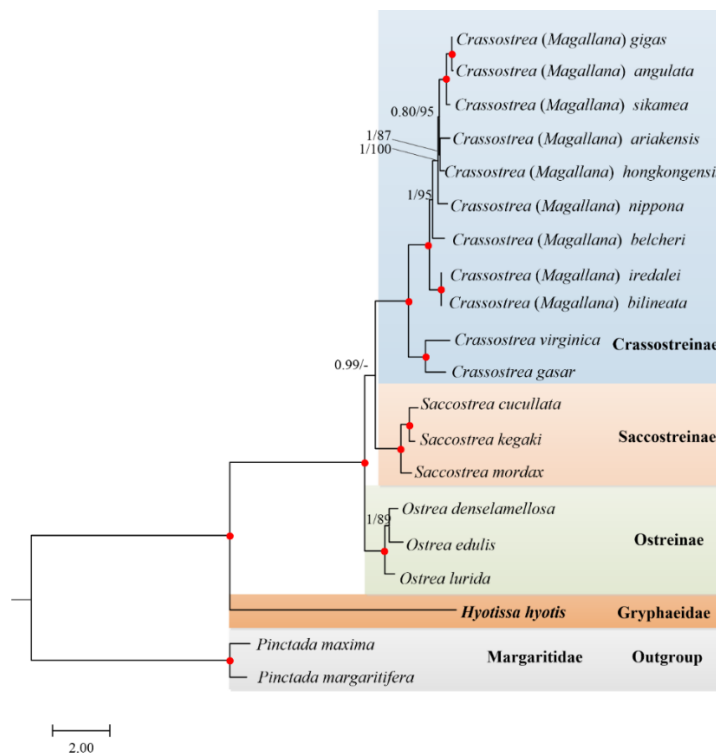


Figure 6. Phylogenetic relationships of Ostreidae based on the concatenated nucleotide sequences of 12 mitochondrial protein-coding genes. The reconstructed Bayesian inference (BI) phylogram using *Pinctada margaritifera* and *P. maxima* as outgroup is shown. The first number at each node is Bayesian posterior probability (PP), and the second number is bootstrap proportion (BP) of maximum likelihood (ML) analyses. Nodal with maximum statistical supports (PP = 1; BP = 100) is marked with a solid red circle. BP values under 80 are marked as dash line.

3.5. Phylogenetic Relationships

The identification of *H. hyotis* was based on morphological and molecular data. The *rrnL* fragment in this study is 100% matched to another individual also distributed in the South China Sea (GenBank Accession No. MT332228). However, the genetic distance of *Cox1* fragment between specimens from Ximaozhou Island (this study) and Madagascar (GQ166583)/Singapore (OM946454) reaches 18%, which indicates that the *H. hyotis* on GenBank is comprised of at least two different species. In the future, studies including more population genetic data of *Hyotissa* are necessary to distinguish the existence of cryptic diversity within *H. hyotis* or incorrect species identification. It is also necessary to include more complete mtDNA of different species belonging to Gryphaeidae, in order to determine whether the unusual mitogenomic characteristics of *H. hyotis* mentioned above is species-specific or common within the whole family.

Phylogenetic relationships of Ostreoidae were reconstructed based on the concatenated nucleotide sequences of the 12 PCGs (11,118 positions in length) using probabilistic methods (Figure 6). According to the BIC, the best partition scheme was the one combining genes by subunits but analyzing each codon position separately (Table S1). Both ML ($-\ln L = 134,336.36$) and BI ($-\ln L = 130,276.19$ for run 1; $-\ln L = 130,276.78$ for run 2) analyses arrived at identical topologies (Figure 6).

Within Ostreoidae, *H. hyotis* was recovered as sister to Ostreidae. This result is consistent with traditional classification, which was based on shell morphology and anatomic characteristics, suggesting that living oysters should be divided into two families Ostreidae and Gryphaeidae [64]. Since *H. hyotis* was the only representative of Gryphaeidae, it is still necessary to include more taxa of the same family (e.g., *Neopycnodonte* and *Pycnodonte*) to test the monophyly of Gryphaeidae in the future.

The phylogeny within Ostreidae was represented as Ostreinae + (Saccostreinae + Crassostreinae). In the present study, the three ostreid subfamilies were represented by three single genera (*Ostrea*, *Saccostrea*, and *Crassostrea* respectively). The close affinity of *Saccostrea* and *Crassostrea* here (strongly supported by BI and poorly resolved by ML, Figure 6) has been supported by morphological characteristics as they were previously classified within the same subfamily Crassostreinae [65]. However, previous molecular phylogenies mostly based on short gene fragments revealed a paraphyletic relationship between *Crassostrea* and *Saccostrea*, which did not form a monophyletic group, and supported the establishment of Saccostreinae for *Saccostrea* [66–68]. Even though the present phylogeny within Ostreidae (Figure 6) is inconsistent with previous results, the inter-relationships between *Ostrea*, *Saccostrea* and *Crassostrea* still remains unresolved, since different datasets or methods could result in different topologies. For example, the COI- and 16S-based NJ trees [67], as well as the ML tree of four combined sequences (16S, COI, ITS2 and 28S fragments [68]), revealed a closer relationship between *Crassostrea* and *Ostrea*, whereas *Saccostrea* and *Ostrea* were recovered more related in the BI tree of the combined dataset [68]. It is necessary to develop phylogenomic resources, such as the anchored hybrid enrichment probe set that is capable of capturing hundreds of molecular markers from diverse taxa [69], to resolve the controversial phylogeny within Ostreidae.

Within *Crassostrea*, the Asia-Pacific and Atlantic species formed two monophyletic lineages as previously suggested [26,68,70]. These two geographical clades could be recognized by several other molecular landmarks, such as different number of duplicated mitochondrial tRNA genes and mt gene rearrangements [25], diverse ITS2 rRNA secondary structures [68], as well as various size and shape of the rDNA-bearing chromosome [71]. Based on such molecular and biogeographical evidence, these two lineages were proposed to be assigned to different genera, and a new genus *Magallana* was proposed to include the Asian species of *Crassostrea* [68,70]. This proposal, however, has been opposed since genetic evidence is not supposed to be considered as a sole justification to define genus, without being supported by morphological and anatomical data [26,72]. In this study, we insist that *Crassostrea* is the valid genus name for both Asia-Pacific and Atlantic oysters and treat *Magallana* as a subgenus under *Crassostrea*.

4. Conclusions

The complete mitogenome of *H. hyotis* is 22,185 bp in length, including 13 PCGs, two rRNA and 23 tRNA genes as well as several non-coding regions. The AT content, AT skew and GC skew values are slightly different from those of ostreids. The gene contents, including the single *rrnL* and *rrnS* and a duplicated *trnW*, and gene orders of *H. hyotis* exhibited extensive differences with ostreid mtDNA. The phylogenetic analysis revealed that *H. hyotis* was sister to Ostreidae, which was grouped by *Ostrea* + (*Saccostrea* + *Crassostrea*) and needed further determined by phylogenomic data. In addition, this study indicates considerable *Cox1* genetic divergence of *H. hyotis* between the sequence here and the previously published ones, suggesting either the existence of cryptic species or the possibility of incorrect species identification. However, this needs to be further determined with the inclusion of more population genetic data of *Hyotissa*. To finally understand the gene order evolution of *H. hyotis*, this study calls for a comprehensive mitogenomic information of superfamily Ostreoidea, together with a robust phylogenetic framework.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/fishes7060317/s1>, Table S1: Best fit partitions and substitution models.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by The Committee and Laboratory Animal Department of Hainan University.

Data Availability Statement: The mitochondrial genome was deposited at NCBI, with accession number OP151093. The filtered Illumina reads were submitted to NCBI Sequence Read Archive (SRA) and are available online at <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA871102> (accessed on 19 August 2022).

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