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The Complete Mitochondrial Genome of the Fivespot Flounder, *Pseudorhombus pentophthalmus* (Pleuronectiformes: Paralichthyidae), from Korea and Its Phylogenetic Analysis

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Abstract: The mitogenome is an important tool for researching the evolution of metazoan animals. However, until now, only few mitochondrial genes of *Pseudorhombus pentophthalmus* have been reported. Here, we report the complete mitogenome of *P. pentophthalmus*, assembled using the Illumina platform. The circular mitogenome of *P. pentophthalmus* is 16,684 bp in length, has a bias A+T content of 52.78%, encodes 37 genes (13 protein-coding genes, 22 tRNA genes, 2 rRNA genes), and has a control region. The overall nucleotide composition was A: 26.56%, T: 26.22%, G: 17.97%, and C: 29.25%. The phylogenetic tree based on the complete mitogenome *P. pentophthalmus* was shown to be monophyletic with the other *Pseudorhombus* species and was shown to be on the same branch as *P. dupliciocellatus*. This research might be useful for future studies on population genetics and evolution analysis.

Keywords: *Pseudorhombus pentophthalmus*; fivespot flounder; Paralichthyidae; mitogenome; phylogenetic analysis

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1. Introduction

The fivespot flounder, *Pseudorhombus pentophthalmus* (Gunther, 1862), is a benthic flatfish belonging to the family Paralichthyidae and the order Pleuronectiformes, which is distributed in tropical and temperate seas across the western Pacific region as well as abundantly around the eastern coast of Korea [1]. *P. pentophthalmus* is often commercially consumed as food by local people in southern Korea, and they are an economically valuable species due to their abundance in southern Korean marine habitats [2]. On the other hand, there is a high demand for flatfish in Asia. According to the United Nations Food and Agriculture Organization (FAO), Japan and the Republic of Korea caught 10,665 tons of flatfish in 2016 [3]. The official aquaculture figures show that production has increased from 1572 t in 1985 to 43,929 t in 2016, despite the fact that fishing of this species has decreased [3]. This shows that the farming of *P. pentophthalmus* may be profitable for the Korean economy and that flatfish aquaculture is a very important industry internationally. It is crucial to define the ecological and genetic features of *P. pentophthalmus* in order to understand its culture and to provide genetic tools to create fishery and aquaculture management

strategies. Furthermore, genetic data on flatfish species are also important elements in studies on the evolution of novel body plans.

The characteristics of species with an economic value have been determined using nuclear and mitochondrial genetic markers. About 20 years ago, understanding the DNA sequences of an interesting species was frequently necessary for genetic characterization. With no prior information on the DNA sequences of a specific species, next-generation sequencing (NGS) has made it possible to create innovative genetic studies today. Its significance has been acknowledged for more than ten years [4–6]. The vertebrate mitogenome is a double-stranded circular DNA structure that is generally 15 to 18 kb long. It has a number of features, including maternal inheritance, stable genetic elements, a rapid rate of evolution, a low rate of recombination, and highly conserved coding areas [7,8]. Mitogenomes have been widely used in a variety of species as molecular markers for evolutionary phylogenetics and population genetics [9]. To date, a few mitochondrial gene sequences of *P. pentophthalmus* were identified and are available in the GenBank database [10,11]. Therefore, the complete mitogenome of *P. pentophthalmus* was sequenced in this study in order to determine the mitogenome of this species and to reconstruct a phylogenetic tree of Paralichthyidae. The complete mitogenome sequence may assist greatly in future research in the development of molecular tools for fish-origin detection.

2. Materials and Methods

2.1. Sample and DNA Extraction

P. pentophthalmus was captured from the coast of Jeju Island in South Korea (33°23′06.31″ N 126°56′04.70″ E) and deposited at the Department of Marine Biology, Pukyong National University, Busan, Republic of Korea (Jin-Koo Kim, taengko@pknu.ac.kr) under the voucher number PKU-50007 (Figure 1). Total genomic DNA was extracted from muscle tissues using the DNeasy Blood and Tissue Kit (Qiagen, Germany) in accordance with the manufacturer's protocol, and a NanoDrop spectrophotometer was used to measure the genomic DNA's quality and amount (Thermo Fisher Scientific D1000, Waltham, MA, USA). As-prepared genomic DNA was preserved at −4 °C for further analysis.

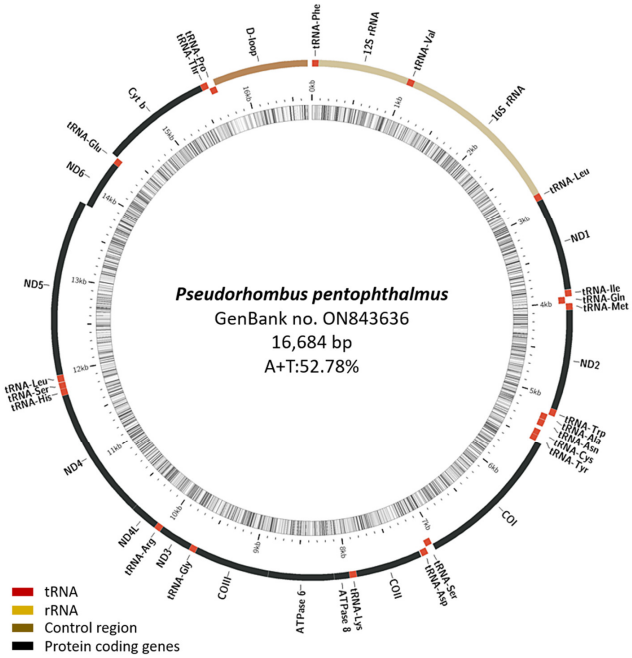

Figure 1. The complete mitochondrial genome of fivespot flounder, *Pseudorhombus pentophthalmus*, GenBank accession number ON843636, drawn by the MitoFish/MitoAnnotator online server (http://mitofish.aori.u-tokyo.ac.jp/annotation/input/, accessed on 3 February 2023). Genes outside the circle are transcribed in a clockwise direction, and those inside are transcribed in a counterclockwise direction.

2.2. Illumina Sequencing, and Mitogenome Assembly and Annotation

The DNA library was generated using the TrueSeq Nano DNA Kit and sequenced on the Illumina platform with 150 bp paired-end reads (Illumina, HiSeq 2500, San Diego, CA, USA) at Macrogen (Daejeon, South Korea). The obtained reads were cleaned with cutadapt 1.9 [12], and the low-quality reads (Q < 20) were removed. The overall quality of the produced sequencing reads was verified with FastQC v0.11.5 (Babraham Institute, Bioinformatics) [13]. The cleaned sequences were used for de novo assembly using SPAdes v3.13.0 [14]. The contig, protein-coding genes, ribosomal and transfer RNA genes, and directions were confirmed by the MitoFish (http://mitofish.aori.u-tokyo.ac.jp/, accessed on 3 February 2023) [15] and MITOS (http://mitos.bioinf.uni-leipzig.de/index.py, accessed on 3 February 2023) online web-servers [16]. The MitoAnnotator internet server (http://mitofish.aori.u-tokyo.ac.jp/annotation/input/, accessed on 3 February 2023) was used to create the mitochondrial genome map [15].

The filtered Illumina reads and assembled mitogenome were submitted to the GenBank database of NCBI at (https://www.ncbi.nlm.nih.gov/nuccore/ON843636, accessed on 3 February 2023) using the BankIt submission tool under accession no. ON843636. The associated BioProject, BioSample, and SRA numbers are PRJNA856092, SAMN29515092, and SRR19995053, respectively.

2.3. Sequence Analysis

The proportions of mitogenome nucleotides and relative use of synonymous codons (RSCU) were determined and estimated using MEGA11 v11.0.8 [17]. The asymmetric base composition of mitogenome was determined by using the formula: AT-skew = [A − T]/[A + T] and GC-skew = [G − C]/[G + C] [18]. The tRNA secondary structure was predicted and confirmed using ARWEN [19].

2.4. Phylogenetic Analysis

The phylogenetic relationships of *P. pentophthalmus* (ON843636) with other species of the family Paralichthyidae were studied using complete mitogenome sequences. One species, *Paraplagusia bilineata* (JQ379001) of Cynoglossidae, was chosen as the outgroup. All available complete mitochondrial sequences of 10 species used in this study were obtained from GenBank (Table 1). First, multiple sequence alignments were performed using ClustalW [20], and then a phylogenetic tree was constructed based on the maximum-likelihood (ML) approach [21]. ML analysis was conducted using default parameters (Tamaru-Nei model, 1000 bootstraps replications) in MEGA11 v11.0.8 [17].

Table 1. Species composition and the comparison of the whole sequence of mitogenome with the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 3 February 2023).

Species Studied	GenBank (BlastN)				Reference
	Species Identified	GenBank no.	Length (bp)	Similarity (%)	
Pseudorhombus pentophthalmus (Accession no.: ON843636; Length: 16,684 bp)	*Pseudorhombus dupliciocellatus*	KJ433562	16,621	83.06	[22]
	Paralichthys californicus	MT859134	16,858	79.30	[23]
	Paralichthys adspersus	MW288827	17,060	78.80	[24]
	Pseudorhombus levisquamis	OK509079	16,604	78.74	NA
	Paralichthys lethostigma	KT896534	16,843	78.71	[25]
	Pseudorhombus cinnamoneus	JQ639069	16,599	78.58	[26]
	Paralichthys olivaceus	AB028664	17,090	78.54	[27]
	Paralichthys dentatus	KU053334	17,033	78.18	[28]
	Paraplagusia bilineata	JQ349001	16,985	75.15	NA

3. Results and Discussion

3.1. Mitogenome Organization of Pseudorhombus Pentophthalmus

This study explored the circular mitogenome of *P. pentophthalmus* (16,684 bp, GenBank accession number ON843636) (Figure 1). The complete mitogenome of *P. pentophthalmus* is longer than that of other known *Pseudorhombus* species (Table 1). Mitogenomes of closely related species usually have small differences in length because of changes in tandem repeats in the control region and the lengths of intergenic regions or gene overlaps [23,29]. It has an overall nucleotide composition (Table S1) of A, T, G, and C of 26.56%, 26.22%, 17.97%, and 29.25%, respectively, with a slight bias A+T composition (52.78%), and it contains 13 protein-coding genes (PCGs), 2 ribosomal RNA genes (rRNA), 22 transfer RNA (tRNA), and a control region (D-loop). Similar mitogenome properties of *P. pentophthalmus* were also observed in other Paralichthyidae fishes [22–28]. All genes are encoded on the H-strand except *ND6* and eight tRNAs (*tRNA-Gln*, *tRNA-Ala*, *tRNA-Asn*, *tRNA-Cys*, *tRNA-Tyr*, *tRNA-Ser*, *tRNA-Glu*, *tRNA-Pro*), which are encoded on the L-strand (Table 2). The A+T bias composition and orientation of genes (ND6 and 8-tRNAs) were similar to those of the mitogenomes of other vertebrates [23,24].

Table 2. Gene order of the complete mitogenome of the *Pseudorhombus pentophthalmus*.

Gene	Strand	Start	End	Size (bp)	Start Codon	Stop Codon	Intergenic Nucleotides *
tRNA-Phe	H	1	69	69	-	-	-
12SrRNA	H	70	1011	942	-	-	-
tRNA-Val	H	1012	1084	73	-	-	-
16SrRNA	H	1085	2811	1727	-	-	-
tRNA-Leu	H	2812	2885	74	-	-	-
ND1	H	2886	3860	975	ATA	TAG	3
tRNA-Ile	H	3864	3934	71	-	-	−1
tRNA-Gln	L	3934	4004	71	-	-	−1
tRNA-Met	H	4004	4072	69	-	-	-
ND2	H	4073	5117	1045	GTG	T–	-
tRNA-Trp	H	5118	5190	73	-	-	1
tRNA-Ala	L	5192	5260	69	-	-	1
tRNA-Asn	L	5262	5334	73	-	-	37
tRNA-Cys	L	5372	5436	65	-	-	-
tRNA-Tyr	L	5437	5503	67	-	-	1
COX1	H	5505	7049	1545	GTG	TAG	3
tRNA-Ser	L	7053	7123	71	-	-	9
tRNA-Asp	H	7133	7203	71	-	-	7
COX2	H	7211	7901	691	ATG	T–	-
tRNA-Lys	H	7902	7974	73	-	-	1
ATP8	H	7976	8143	168	ATG	TAA	−10
ATP6	H	8134	8816	683	ATG	TA-	-
COX3	H	8817	9601	785	ATG	TA-	-
tRNA-Gly	H	9602	9673	72	-	-	-

Table 2. *Cont.*

Gene	Strand	Start	End	Size (bp)	Start Codon	Stop Codon	Intergenic Nucleotides *
ND3	H	9674	10022	349	GTG	T–	-
tRNA-Arg	H	10023	10091	69	-	-	-
ND4L	H	10092	10388	297	ATG	TAA	−7
ND4	H	10382	11762	1381	ATG	T–	-
tRNA-His	H	11763	11832	70	-	-	-
tRNA-Ser	H	11833	11899	67	-	-	4
tRNA-Leu	H	11904	11976	73	-	-	2
ND5	H	11979	13817	1839	ATG	TAA	−4
ND6	L	13814	14335	522	ATG	TAG	-
tRNA-Glu	L	14336	14404	69	-	-	2
CytB	H	14407	15546	1140	ATG	TAA	1
tRNA-Thr	H	15548	15619	72	-	-	1
tRNA-Pro	L	15621	15691	71	-	-	-
D-loop	H	15692	16684	993	-	-	-

Notes: * The numbers of nucleotides between the given and previous gene, with negative values indicating an overlap; TA-/T– indicated incomplete stop codon; H and L indicated that the genes are transcribed on the heavy and light strand, respectively.

3.2. Protein-Coding Genes (PCGs)

In *P. pentophthalmus*, all 13 PCGs made up 68.45% of the total mitogenome, which had a length of 11,420 bp. Among all PCGs, *ATP8* is the shortest (168 bp), whereas *ND5* is the longest (1839 bp). The start codons of nine PCGs (*COX2*, *ATP8*, *ATP6*, *COX3*, *ND4L*, *ND4*, *ND5*, *ND6*, and *CytB*) were ATG, while those of *ND2*, *COX1*, and *ND3* were GTG, and *ND1* started with ATA. The stop codons of *ND1*, *COX1*, and *ND6* were TAG, whereas those of *ATP8*, *ND4L*, *ND5*, and *CytB* were TAA. In contrast, *ND2*, *COX2*, *ATP6*, *COX3*, *ND3*, and *ND4* had incomplete TAA stop codons. These incomplete termination codons may be completed to TAA by RNA processing by the addition of a poly-A tail [29,30].

The total number of amino-acid triplets expressed by the 13 PCGs was 3570, ignoring the stop codon (Table S2). According to the analysis on the RSCU, leucine was used most frequently. GCC (Ala, total of 148 times) and CTT and CTC (Leu, total of 145 times and total of 140 times, respectively) were found as regularly utilized codons more than 140 times. However, the triplet codons of serine, AGA and AGG, were not utilized by PCGs. Previous research has demonstrated that similar codon usage patterns exist among members of the family Paralichthyidae [23,24].

3.3. Ribosomal RNA and Transfer RNA Genes

In *P. pentophthalmus*, the rRNA genes were 2669 bp in length (15.99% of the complete mitogenome), and they were made up of two distinct rRNAs: a small rRNA (*12S rRNA*, 942 bp) and a large ribosomal RNA (*16S rRNA*, 1727 bp) (Table 2). Both rRNAs were encoded on the H-strand, and the *12S rRNA* and *16S rRNA* genes were located between the *tRNA-Phe* and *tRNA-Leu* and separated by the *tRNA-Val*. The above-mentioned characteristics were consistent with the typical Paralichthyidae fish mitogenome [23,24]. Similar to other vertebrates, the *P. pentophthalmus* mitogenome had 22 tRNAs. Individual tRNAs varied in length from 65 to 74 base pairs, and the sum of the lengths of all tRNAs was 1552 bp (9.30% of the complete mitogenome). All tRNAs fold into typical cloverleaf secondary structures, except the tRNA-Ser-2, which lacked the dihydrouridine (DHU) arm (Figure 2). In addition, twelve tRNAs (Ala, Arg, Asp, Cys, Glu, His, Leu-1, Met, Pro, Ser-2, Thr, Tyr)

showed mismatched base pairs in the amino acid acceptor (AA) arm, and eight tRNAs (Ala, Glu, Met, Pro, Ser-1, Ser-2, Thr, Tyr) did so in the variable (v) arm. Overall, the secondary structure of tRNAs in *P. pentophthalmus* was similar to that of vertebrate mitogenomes, with typical Watson–Crick pairings [7].

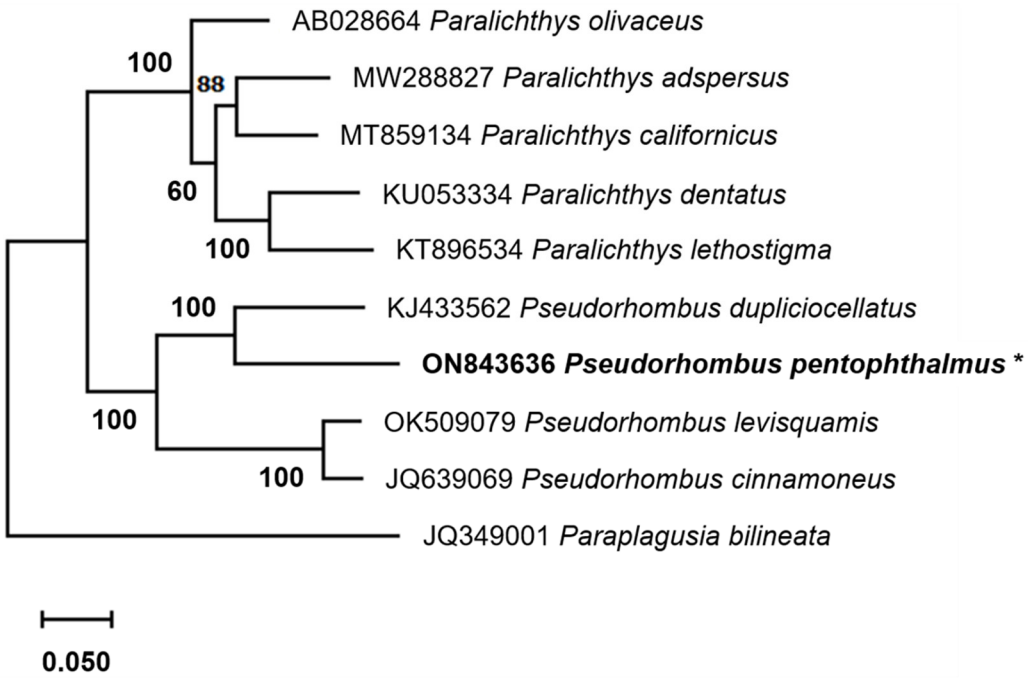

Figure 2. Maximum-likelihood phylogenetic-tree reconstruction of *Pseudorhombus pentophthalmus* (GenBank accession no. ON843636; indicated by an asterisk) in Paralichthyidae using complete mitogenome data. The GenBank accession numbers of all mitogenomes used for phylogenetic analysis are followed by species names. The number above the branches represents the maximum-probability bootstrap values.

3.4. Phylogenetic Relationships

The phylogenetic tree was constructed using complete mitogenomes of the species within the family Paralichthyidae (Figure 2). The results show that the *P. pentophthalmus* (ON843636) is placed in a sister clade with *P. dupliciocellatus* (KJ433562) and is monophyletic with *P. levisquamis* (OK509079) and *P. cinnamoneus* (JQ639069), with a high supporting bootstrap value, suggesting a close relationship with other species in the Paralichthyidae family. The species of *Paralichthys* and *Pseudorhombus* are monophyletic, and *Paraplagusia bilineata* (JQ379001) is an outgroup member. At the genus level, *Paralichthys* species and *Pseudorhombus* species were placed separately in the same clades, consistent with a mitochondrial study using PCG and a complete mitochondrial genome sequence [23,24]. To better understand the phylogenetic relationship among Pleuronectiformes species, mitogenome studies within the order must be enhanced.

4. Conclusions

The fivespot flounder, *P. pentophthalmus*, has a mitogenome that is 16,684 bp long and comprises 13 PCGs, 22 tRNAs, 2 rRNAs, and the control region, according to our results. PCGs, tRNAs, rRNAs, and the control region were distributed and oriented in *P. pentophthalmus* in a manner that was very comparable to that seen in the mitogenomes of other Paralichthyidae species. Based on phylogenetic analysis, *P. pentophthalmus* was monophyletic with other *Pseudorhombus* species and on the same branch as *P. dupliciocellatus*. A comprehensive study needs to be conducted for species status confirmation. This study describes the complete mitogenome of *P. pentophthalmus* and its phylogenetic relationship

within the family Paralichthyidae. To better understand the phylogenetic relationship among Pleuronectiformes species, it will be important to expand the mitochondrial genome analysis within the order.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8030150/s1, Table S1: General metrics of nucleotide composition of *P. pentophthalmus*.

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Institutional Review Board Statement: The sample used for this study was a dead body of fish and as per the animal experimental ethics in the Republic of Korea (Standard operating guideline; IACUC—Institutional Animal Care and Use Committee, Book no. 11-1543061-000457-01, effective from Dec. 2020), it does not need any approval from an Ethics Committee.

Data Availability Statement: The genome sequence data that support the findings of this study are openly available in GenBank of NCBI at (https://www.ncbi.nlm.nih.gov/, accessed on 3 February 2023) under accession no. ON843636. The associated BioProject, BioSample, and SRA numbers are PRJNA856092, SAMN29515092, and SRR19995053, respectively.

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Conflicts of Interest: The authors declare no conflict of interest.

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