Thesis for the Degree of Master of Fisheries Science

Complete Mitogenome of Spottail Spiny Turbot, *Psettodes belcheri* (Pleuronectiformes: Psettodidae): Characterization and Phylogeny

by

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(*Psettodes belcheri* (Pleuronectiformes: Psettodidae)의 전장 미토콘드리아 유전체에 대한 분석 연구)

Advisor: Prof. Hyun-Woo Kim

by

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Abstract

Psettodes belcheri (Bennett, 1831) is known as the primitive flatfish distributed in Eastern Atlantic, starting from Western Sahara and Mauritania to Angola. In this study, we provide the complete mitogenome of *Psettodes belcheri* from Cameroon using the next-generation sequencing. The mitogenome of *Psettodes belcheri* (16,747 bp) consists of 37 genes, including 13 protein-coding genes, 2 ribosomal RNA genes, 22 transfer RNA genes, and control region. The *Psettodes belcheri* mitogenome displays an AT-biased (54.15%), which is similar to its congener, *Psettodes erumei* (53.07% and 53.61%). Most protein-coding genes (PCGs) in *Psettodes belcheri* start with an ATG start codon, with the exception of

Cytochrome Oxidase I (COI), which initiates with a GTG codon. Additionally, four PCGs use TAA as their stop codon, while seven PCGs have an incomplete stop codon. Two PCGs, NAD1 and NAD6, terminate with AGG and TAG stop codons, respectively. In the mitogenome of *Psettodes belcheri*, most transfer RNAs (tRNAs) exhibit typical cloverleaf secondary structures, except for tRNA-serine, which lacks a DHU arm. Comparative analysis of the conserved blocks within the control regions of two Psettodidae species reveals that the CSB-II block is more extensive (51 base pairs) than other blocks and contains highly variable sites. A comprehensive phylogenetic analysis, using the concatenated sequences of 13 mitochondrial protein-coding genes, classifies various Pleuronectiformes species. It highlights the Psettodidae family's basal position and demonstrates the monophyletic clustering of *Psettodes* species. The estimated divergence time between *Psettodes belcheri* and *Psettodes erumei* is approximately 20.56 million years ago, shedding light on their separation and colonization during the early Miocene. The TimeTree analysis also estimates the divergence of the two suborders, Psettodoidei and Pleuronectoidei, occurring during the late Paleocene to early Eocene, around 56.87 million years ago. The distribution patterns of *Psettodes* flatfishes are influenced by ocean currents and environmental conditions, which contribute to their ecological speciation.

Keywords: *Psettodes*, flatfish, mitogenome, phylogeny, evolution, oceanography

Introduction

1. Background of the Study

Marine waters, including brackish water and estuaries, have high biodiversity of fishes. This condition is often correlated with river flow and connectivity with the marine system (Pasquaud et al., 2015). This fertility condition also impacts a relatively high diversity of demersal fishes, including flatfishes. Flatfishes, also called flounders, soles, halibuts, turbots, plaices, and tonguefishes, belongs to the Pleuronectiformes classification with consisting of more than 800 species distributed in the global marine and estuarine, including species that inhabit deep-sea thermal vents and freshwater rivers (Munroe, 2015; Schreiber, 2013).

Flatfishes are exciting to study because of its characteristics. The unique characteristics of flatfishes appear during unique physical metamorphosis with morphological and physiological changes associated with eye migration, a 90° rotation in posture, and asymmetrical pigmentation metamorphosis at the end of the larval period (Geffen et al., 2007; Coulson & Poad, 2021). Most flatfishes species are laterally monomorphic and, as adults, display either exclusively “dextral” (both eyes are located on the right side after metamorphosis) or “sinistral” (both eyes are located on the left side) morphology

(Schreiber, 2006; Munroe, 2005; Bergstrom et al., 2007). The metamorphosis process can be used to see the evolutionary process in fish, especially the Pleuronectiformes order.

The order Pleuronectiformes consists of two suborders: the Psettoidei, comprising the family Psettodidae; and the Pleuronectoidei, containing all remaining flatfish groups. The suborder Pleuronectoidei has 13 families, which is the most species in the order Pleuronectiformes, and the suborder Psettodoidei only has one family, namely the Psettodidae (Berendzen and Dimmick, 2002; Munroe, 2015). Based on the Eschmeyer's Catalog (http://researcharchive.calacademy.org, accessed on 18 July 2023), the Psettodidae family only consists of three valid species (*Psettodes erumei*, *Psettodes bennettii*, and *Psettodes belcheri*), and all of these species belong to the same genus *Psettodes*.

The Psettodidae family is fascinating to study, especially for the phylogenetic and evolutionary of this "primitive flatfish". However, studies about the Psettodidae family somehow concluded inconsistently phylogenetic results because were limited to a single *Psettodes* species (*Psettodes erumei*) from Indo-Pacific region (Campbell et al., 2013; Bentacur and Orti, 2014; Campbell et al., 2014; Harrington et al., 2016; Shi et al., 2018; Campbell et al., 2019). This emphasizes the necessity for more extensive research incorporating additional congeners from the Atlantic Ocean to fill in the existing gap. To deepen our understanding of the evolutionary history of *Psettodes*, we choose a Spottail Spiny Turbot species, *Psettodes belcheri*, that lives in Cameroon waters as a limitation of this study.

The aims of this study are:

1) To generate the complete mitogenome of the *Psettodes belcheri* from Cameroon.
2) To characterize the genomic features (PCGs, tRNAs, rRNAs, Control Region) of *Psettodes belcheri* mitogenome
3) To trace the phylogenetic relationship of *Psettodes belcheri* with other Pleuronectiformes species, including potential evolutionary and colonization scenario of *Psettodes* species.

This research will compile the complete mitogenome of *Psettodes belcheri*, unravel its genetic characteristics, and establish its phylogenetic connections with other Pleuronectiformes fishes. Furthermore, this study also endeavors to estimate the divergence time between *Psettodes belcheri* and the congener, *Psettodes erumei,* and also explore potential evolutionary scenarios within the marine environment. The complete mitochondrial genome of these species will provide more information about its genetic diversity, phylogenetic, and evolutionary studies of *Psettodes* flatfishes.

2. Biology of Flatfishes with Special Reference to Spottail Spiny Turbot

Flatfish are well-known as asymmetrical fishes. This asymmetrical condition occurs when one eye migrates to the opposite side of the head and transitions to a lateralized swim posture (Schreiber, 2006). The side of the body that contains both eyes is referred to as the eyed side, whereas the side with no eyes is called the blind side. Flatfishes usually have a white lower side and a brown or dark color on the upper side. This upper side gave a camouflage pattern with the dark area or sand-colored flatfish habitat (Burton, 2002). During metamorphosis, flatfish are changing their behavior. This metamorphosis changes their swimming posture, which may serve to maintain binocular vision, while late-stage larvae are still pelagic (Schreiber, 2006). During the metamorphosis stage, demersal larvae consume pelagic plankton until settlement is complete (Fernandez-Diaz et al., 2001). This condition will affect the larval feeding patterns and may continue influencing feeding during metamorphosis. However, most teleost larvae exhibit ontogenic shifts in prey and learn new feeding patterns during metamorphosis (Geffen et al., 2007).

Figure1. Spottail Spiny Turbot (*Psettodes belcheri)* from Cameroon

Our species study, Spottail Spiny Turbot (*Psettodes belcheri*) (Figure 1), has a characteristic oval and flat body, but thicker than other flatfishes. This fish has a body depth of 2.7 to 3.2 times in total length. Generally in the Pleuronectiformes order, the eyes are on the same side of the head, but in the case of *Psettodes* one eye is at the dorsal midline (Friedman, 2008). This condition affects the insertion of the dorsal fin in *Psettodes*, which unlike that in other flatfish is posterior to the eye (Carpenter, 2016). Chabanaud (1937) notes that the eyes of *Psettodes* cannot be extended and do not have any skin folds around the eyes unlike Pleuronectoids fishes, which can extend the eyes and have skin folds around the eyes. In addition, *Psettodes* has distinct characteristics that are not typical of other flatfish, where *Psettodes* species may include both left- and right-sided fish, a characteristic termed asymmetry. In contrast, populations of other Pleuronectiform species have a tendency to be uniformly left or right sided (Palmer, 1996). *Psettodes belcheri* have a large mouth with a well-developed supramaxillary bone under both eyes with strong canine teeth far past the lower eye. The color of this fish is brown with spots and blotches ocular side and most often pale on the blind side. Compared to other species in the genus *Psettodes*, one special distinguishing feature for *Psettodes belcheri* is the presence of dark spots on the caudal fin (Carpenter, 2016).

Fisheries of flatfishes hold economic importance in various parts of the world. The aquaculture of flatfish, especially flounders, has gained prominence due to the high demand for these species in the global seafood market (Stieglitz et al., 2021). Moreover, some other

species of flatfish are commonly raised in captivity for commercial purposes. The majority of captured flatfishes consumed by humans are often caught through demersal trawling in marine ecosystems (Eighani, 2013), including *Psettodes* flatfishes. The Indian halibut (*Psettodes erumei*) is extensively harvested within the tropical fishing zone as designated by the United Nations Food and Agriculture Organization (FAO) (Gibson et al., 2015). This indicates that *Psettodes* flatfish also has economically important value as food.

3. Taxonomy of Spottail Spiny Turbot, *Psettodes belcheri* (Bennett, 1831)

The taxonomic order of the Spottail Spiny Turbot, *Psettodes belcheri*, is as follows (Romero, 2002):

Kingdom : Animalia

Phylum : Chordata

Superclass: Actinopterygii

Class : Teleostei

Order : Pleuronectiformes

Suborder : Psettodoidei

Family : Psettodidae

Genus : *Psettodes*

Species : *Psettodes belcheri*

Psettodes belcheri is a marine water fish under the genus *Psettodes*. This genus forms a separate clade under the suborder Psettodoidei (order Pleuronectiformes) with only one family, Psettodidae. Studies on the phylogenetic status of Psettodoid fish within the order Pleuronectiformes still need to be clearly determined.

In the last two decades, there have been several different opinions regarding the status of Psettodidae in the Pleuronectiformes order, whether monophyly or polyphyly. An anatomical study defined the monophyly of Pleuronectiformes members (including Psettodidae) based on the synapomorphies by Chapleau (1993). Those studies polarized a morphological character to produce a phylogenetic hypothesis of Pleuronectiform monophyletic based on three morphological characteristics: eyes ontogenetic migration, the origin of an anterior position of the dorsal fin (dorsal fin overlaps the cranium), and recessus orbitalis presence. But recent evidence suggests Psettodidae is excluded from Pleuronectiformas entirely because the absence of the recessus orbitalis in *Psettodes erumei*

(Campbell et al, 2013), thus concluding that the monophyletic of this order was still ambiguous based on the morphological study.

On the other hand, the molecular study of the flatfish taxonomic has been more advanced. This condition is because molecular taxonomy studies have a wide sampling of lineages possible with molecular methods with comparatively lower effort. However, differences of opinion also occur in determining the taxonomic status of Psettodidae in the order Pleuronectiformes in this molecular taxonomy study. Campbell et al. (2013) pointed out that the relationship between Psettodoid fish and the Order Pleuronectiformes does not lead to monophyletic. This inconsistent phylogenetic results regarding the placement of *Psettodes* could be due to the origin of the *Psettodes* lineage occurring very close in time to the initial diversification of Carangimorphariae or to the initial diversification of pleuronectoids (the other suborder of flatfishes). But at the same time, Betancur-R and Orti (2014) denied this conclusion and stated that Campbell et al. (2013) have misanalysed. They still believe that the relationship between Psettodoid fishes and other flatfishes is monophyletic. Responding to this, Campbell et al. (2014) wrote that a critical review of the morphological evidence supporting monophyletic flatfish indicates that this evidence warrants careful re-examination regarding Psettodoids fishes.

Recent studies of the phylogenetic placement within the order Pleuronectiformes have led to several new studies and evidence. Campbell et al. (2019) find polyphyly or paraphyly of two flatfish families, the Paralichthyidae and the Rhombosoleidae, and support the creation of two additional families, Cyclopsettidae and Oncopteridae, to resolve their non-

monophyletic status. Their findings also support the distinctiveness of Paralichthodidae and refine the placement of that lineage. Another study by Lü et al. (2021) revealed that flatfishes also have a polyphyletic origin, where the sub-order Pleuronectoidei and Psettodoidei independently evolve from their different percoid ancestors. However, Pleuronectoidei and Psettodoidei also share convergent gene alterations related to muscular development, lipid accumulation, body axis determination, and fin pattern regulation. Meanwhile, Psettodoidei also exhibited unique mutations that may contribute to their less asymmetric body plan than Pleuronectoidei. Lü et al., (2021) also explained about morphological differentiation of Psettodoidei from Pleuronectoidei, which includes: (1) lack of skin folds around the eyes; (2) posterior insertion of the dorsal fin; (3) less extensive cranial asymmetry; (4) presence of spinous rays in fins; and (5) larger mouths with specialized teeth.

4. Status and Distribution of *Psettodes* species

The International Union for Conservation of Nature (IUCN) assigns *Psettodes belcheri* to "data deficient" status in their list. This also applies to two other *Psettodes* species (*Psettodes erumei* and *Psettodes bennettii*) which are classified to "data deficient" status on the IUCN list (Figure 2A). The IUCN database notes that *Psettodes* flatfishes are distributed in the tropical ocean while *Psettodes erumei* ranges expansively across the Red Sea and the Indo-West Pacific Ocean, and the other two species (*Psettodes bennettii* and *Psettodes belcheri*) are confined to the eastern Atlantic (Figure 2B).

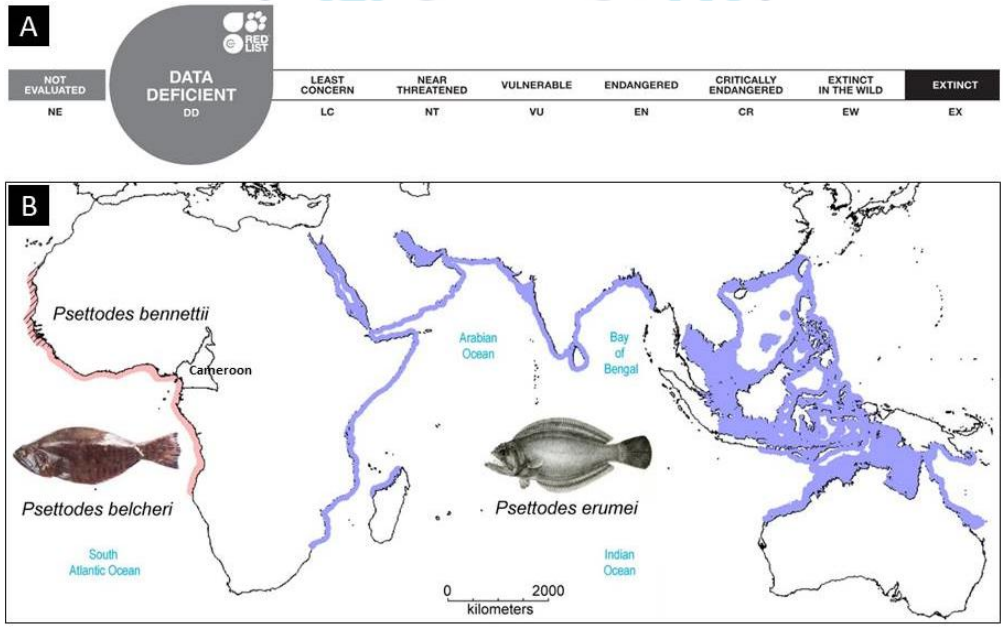

Figure 2. (A) *Psettodes belcheri* IUCN Red List status, and (B) Geographic range of *Psettodes* species (*P. erumei*, *P. bennettii*, and *P. belcheri*) based on the IUCN database.

Psettodes belcheri has a habitat distribution in the Eastern Atlantic: West African coast from Western Sahara (about 24°N) and Mauritania, to Angola (about 17°S), including Cape Verde Islands, and São Tomé and Principe. Interestingly, the ranges of *Psettodes belcheri* and *Psettodes bennettii* partially overlap, extending from the west Sahara to the Liberian coast (Eschmeyer at al., 2023; Carpenter, 2016). *Psettodes* flatfishes generally inhabit muddy, sandy and rocky bottoms in estuaries and coastal waters from the shoreline to at least 150 m deep. As a demersal fish, they do not always have close contact with the bottom of the waters. It also actively swims, looking for other fish and shrimp as their prey. *Psettodes* flatfish's bodies are almost rounded and do not have the associated bilateral asymmetry in musculature typical of other flatfishes (Munroe, 2015) and often swim in an upright orientation (Carpenter, 2016).

Materials and Methods

1. Sample Collection and Species Identification

A single live specimen of *Psettodes belcheri* was collected from the estuaries of the Kineke River (latitude 2.938611°N, longitude 9.911667°E), Kribi, Cameroon, on July 2022 (Figure 3). Based on the primary taxonomic keys, sample identification was done by morphological approach through looking at special morphological characters on the species target using a reference from Bennett (1831), and the specimen was recognized as *Psettodes belcheri*. After species identification was done, the tissue sample was aseptically dissected from the ventral thoracic region.

The fish was collected and preserved directly in 95% ethanol. The Ministry of Livestock, Fisheries, and Animal Industries (MINEPIA) in Yaounde, Cameroon provided the tissue sample, which was then stored at the Department of Marine Biology, Pukyong National University in Busan, South Korea. The Institutional Animal Care and Use Committee of the host institute granted approval (Approval Code: PKNUIACUC-2022-72, dated 16 December 2022) for the utilization of deceased fish muscle tissue in molecular investigations. To facilitate additional analysis, the tissue sample was maintained at a temperature of -20°C.

Figure 3. Location for sampling site of *Psettodes belcheri* in Estuaries of the Kineke River (2.938611N 9.911667E), Kribi, Cameroon. (Map showed by google earth: earth.google.com/web/).

2. Genomic DNA (gDNA) Extraction

A DNA extraction kit (AccuPrep® Genomic DNA Extraction Kit, Bioneer, Republic of Korea) was used to extract genomic DNA (gDNA) from the samples following the protocol provided by the manufacturer: about 100 mg of muscle tissue was taken from specimen and lysed in 1X lysis buffer (600 µl). Homogenization was then performed with a motorized Tissue lyser II (Qiagen, Hilden, Germany). Then, 40 µl of Proteinase K and 40 µl of sodium dodecyl sulfate (SDS) were added. The samples were incubated at 60°C for 12 hours or overnight for complete tissue lysis. After incubation, 600 µl of the GC buffer was added and incubated at 60°C for 10 minutes, then followed by centrifuge at 14,000 rpm for five minutes. Transfer 500 µl of supernatant, then followed add by 350 µl of isopropanol. The 650 µl of supernatant mixed with isopropanol was filtered using the column tube by centrifugation for one minute at 8,000 rpm. Washing Buffer 1 and Buffer 2 were used to wash the column twice. 30 µl of elution buffer (ET) was added to elute the gDNA. Furthermore, purity and quantity were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific D1000, WA, USA) and stored at -20°C until further analysis.

3. Sequencing by Illumina Myseq Platform

To amplify the complete mitochondrial genome of *Psettodes belcheri*, gDNA from the tissue sample extraction were sequenced commercially using the dideoxynucleotide analog method at Macrogen (https://dna.macrogen.com/, accessed on 15 August 2023) in Daejeon, Republic of Korea. The sequencing libraries were prepared following the NovaSeq platform, provided by Illumina and accessible at Macrogen (https://dna.macrogen.com/, accessed on 15 August 2023) in Daejeon, Republic of Korea. Initially, 100ng of genomic DNA was fragmented using adaptive focused acoustic technology from Covaris (Woburn, MA, USA), resulting in blunt-ended dsDNA molecules with 5'-phosphorylation. Following end-repair, DNA fragments were size selected using a bead-based method, followed by modification with the addition of a single 'A' base and ligation with TruSeq DNA UD Indexing adapters. Subsequent purification and PCR enrichment produced the final DNA library. Library quantification was performed using qPCR, following the KAPA Library Quantification kits protocol for Illumina Sequencing platforms, while quality assessment utilized Agilent Technologies 4200 TapeStation D1000 screentape. Paired-end (2 x 150 bp) sequencing was performed by Macrogen using the NovaSeq platform (Illumina, Inc., San Diego, CA, USA). Raw reads exceeding 20 million were processed with the Cutadapt tool (http://code.google.com/p/cutadapt/, accessed on 15 August 2023) to trim adapters and eliminate low-quality bases, using a Phred quality score (Q score) cutoff of 20. The

Geneious Prime version 2023.0.1 was employed for assembly, utilizing reference mapping with the mitogenome of a closely related species, employing default mapping algorithms.

4. Control Region Confirmation

After mitogenome raw data was completed by the outsource company, the partial Control Region part was confirmed by the partial Control Region amplified using the designed primer pair CF11_CYTB (5'-CCTACACACGTCCAAGCAACG-3') and CF11_12S (5'-GCTGAGCTCGTGCCTGATACC-'3) in a three-step PCR amplification. The amplification was performed in a PCR thermal cycler (TaKaRa, Japan) using the following condition:

- Initial denaturation at 94°C for 5 minutes
- 30 cycles of the following
    - Denaturation at 94°C for 30 seconds
    - Annealing at 63.9°C for 30 seconds
    - Extension at 72°C for 2 minutes
- Final extension at 72°C for 5 minutes

A total volume of 30µl PCR mixture was prepared containing the following template:

- 1µl of genomic DNA template (1/10 diluted)
- 1µl each primer (forward and reverse)

- 4µl of deoxynucleotide triphosphates (dNTPs) mixture (2.5µl Takara, Japan)
- 4µl of 10X ExTaq Buffer (TaKaRa Bio, Inc.)
- 0.4µl Ex Taq HS polymerase enzyme (TaKaRa Bio, Inc.)
- 0.9µl of dimethyl sulfoxide (DMSO 3%)
- 17.7µl sterilized deionized water

The amplified PCR products were separated in 1% agarose gel, stained with ethidium bromide (6 mg/ml), and then compared with a 100 bp DNA ladder. Bands were visualized using a digital camera intended to capture the picture of the glowing fragments from UV light. The targeted bands were cut out from the gel and purified. The process for purification was done using the AccuPrep® PCR/Gel Purification Kit (Bioneer, Republic of Korea) following the manufacturer's protocol: three volumes of the FB buffer were added in tubes containing pre-weighed gel with the fragments, then added with one (1) volume of absolute isopropanol. After the gels were completely dissolved, the mixtures were transferred to binding columns and centrifuged at 14,000 rpm for 1 min. After centrifugation, flow-through residues were poured off until all mixtures were completely filtered. Then, 500µl of W2 Buffer was added to the binding column and centrifuged at 14,000 rpm for 1 min. Again, flow-through residues were poured out and repeated the process of adding W2 buffer once. After completely washing, binding tubes containing the fragments were centrifuged at 14,000 rpm for 2 minutes to remove the washing buffer residues. Binding columns were transferred to the new 1.5 ml microcentrifuge tube. The fragments were eluted in 30µl TE buffer, incubated for 10 minutes, and centrifuged at

14,000 rpm for 2 minutes. The concentration and quality of eluted amplicons were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific D1000) and sequenced commercially using the dideoxynucleotide analog method at Macrogen, South Korea. Trimmed sequence readings were used for Control Region confirmation compared with data from outsource.

5. Sequence Assembly and Genome Annotation

The mitogenome was assembled by checking the overlying regions alignment through MEGA X (Tamura et al., 2021) and BLAST webserver (https://blast.ncbi.nlm.nih.gov, accessed on 10 July 2023). The boundary of each gene and directions were affirmed through MITOS v806 (http://mitos.bioinf.uni-leipzig.de, accessed on 10 July 2023) and MitoAnnotator (http://mitofish.aori.u-tokyo.ac.jp/annotation/input/, accessed on 10 July 2023) web servers (Iwasaki et al., 2013; Bernt et al., 2013). The calculation of intergenic spacers, which separate adjacent genes, and overlapping regions was performed manually. The result from MITOS Web Server was also used to predict the secondary structures of 22 transfer RNAs (tRNAs). The protein-coding genes (PCGs) were further confirmed through the Open Reading Frame Finder web tool (https://www.ncbi.nlm.nih.gov/orffinder/, accessed 10 July 2023) after being translated into the putative amino acids of the vertebrate mitochondrial genetic code. The generated mitogenome of *Psettodes belcheri* was submitted to the GenBank global database and had

been received the accession number (OR231239). The nucleotide composition of protein-coding genes (PCGs), ribosomal RNA (rRNA), transfer RNA (tRNA), and the control region (CR) was estimated by MEGA X (Kumar et al., 2018; Tamura et al., 2021). We estimated AT skew and GC skew using the following formulas to understand the base compositional bias of mitochondrial genomes: AT skew = (A - T)/(A + T) and GC skew = (G - C)/(G + C) (Perna and Kocher, 1995). The verification of initiation and termination codons for each PCG, as well as adherence to the vertebrate mitochondrial genetic code, was carried out using MEGA X. Additionally, the boundaries of rRNA and tRNA genes were confirmed through the use of the tRNAscan-SE Search Server 2.0 in conjunction with ARWEN 1.2 (Chan et al., 2021; Laslet and Canback, 2007). Structural domains within the control region were delineated through CLUSTAL X alignments (Thompson et al., 1997), and tandem repeats were explored utilizing the online Tandem Repeats Finder web tool (https://tandem.bu.edu/trf/trf.html, accessed on 15 August 2023) (Benson, 1999).

6. Genetic Distance, Dataset Building, Phylogenetic Analysis, and TimeTree Estimation

We calculated genetic distances using the Kimura 2-parameter (K2P) method within MEGA X. As a result of not having access to the complete mitogenome of *Psettodes bennettii*, we determined intra-species and inter-species distances by utilizing the commonly used mitochondrial COI gene. To elucidate the matrilineal phylogenetic relationships, a total of 16 mitogenomes (1 generated and 15 databases) of three from Psettodoidei suborder and twelve from Pleuronectoidei suborder were accumulated to build a dataset. The mitogenome of *Lates calcarifer* (family Centropomidae) was incorporated into the dataset as an outgroup (Appendix S1 Table). The combination of all 13 PCGs was carried out using the iTaxoTools 0.1 tool to create the dataset for phylogenetic analysis (Vences et al., 2021). To ensure a seamless dataset alignment, we intentionally left out the non-coding rRNA genes and control regions during the current phylogenetic analysis. Model selection determined the 'GTR + G + I' model as the most suitable, based on the lowest Bayesian Information Criterion (BIC) score, utilizing PartitionFinder 2 via CIPRES Science Gateway v3.3 and JModelTest v2 (Lanfear et al., 2016; Miller et al., 2015; Darriba et al., 2012). A Bayesian (BA) tree was constructed using Mr. Bayes 3.1.2, with nst = 6, employing one cold and three hot Metropolis-coupled Markov Chain Monte Carlo (MCMC) chains. The analysis spanned 10,000,000 generations, with tree sampling every 100th generation and 25% of samples discarded as burn-in (Ronquist and Huelsenbeck, 2003). The BA tree was visualized using the iTOL v4 web server (https://itol.embl.de/login.cgi, accessed on 15 August 2023) (Letunic and Bork, 2007).

Furthermore, the estimation of divergence time was conducted using the RelTime method, following the standard protocol as implemented in MEGA X (Mello, 2018). This method aimed to reduce the extensive computational time associated with Bayesian methods (Tamura et al., 2012; Mello et al., 2017). After loading the sequence data, the constructed maximum-likelihood topology (.nwk format) was utilized as a baseline tree. The TimeTree computation incorporated two calibration constraints through the calibration editor, reflecting the divergence from the sister lineage of Citharidae (55.54 MYA) and Achiridae (49.73 MYA), as established in a previous study (Campbell et al., 2014).

Results and Discussion

1. Mitogenomic Structure, Organization, and Composition

The complete mitochondrial genome of *Psettodes belcheri* was determined as closed-circular DNA molecule of 16,747 base pairs (bp) in the present study (GenBank Accession no. OR231239) (Figure 4). We were compared with two sequences of *Psettodes erumei* discovered by China (FJ606835) and Japan (AP006835). Considering the total length, the present mitogenome was longer than *Psettodes erumei* (16,683 bp; AP006835) and shorter than *Psettodes* erumei (17,315 bp; AP006835). The mitogenome of *Psettodes belcheri* constituted 13 protein-coding genes, 22 transfer RNA genes, two ribosomal RNA genes, and a single AT-rich control region. The heavy strand accommodated 28 genes (12 PCGs, two rRNAs, and 14 tRNAs), while ND6 and eight tRNAs (trnQ, trnA, trnN, trnC, trnY, trnS2, trnE, and trnP) were positioned on the light strand (Table 1, Figure 4). The mitogenome of *Psettodes belcheri* was AT biased (54.15%), with 28.09% A, 16.24% G, 29.56% C, and 26.06% T. Similar AT biasness of the nucleotide composition was also observed in other *Psettodes* species 53.61% (*Psettodes erumei*; FJ606835) to 53.07% (*Psettodes erumei*; AP006835). A similar pattern of nucleotide composition and AT biases was observed in other vertebrate mitogenomes described earlier (Kundu et al., 2023; De Alwis et al., 2023).

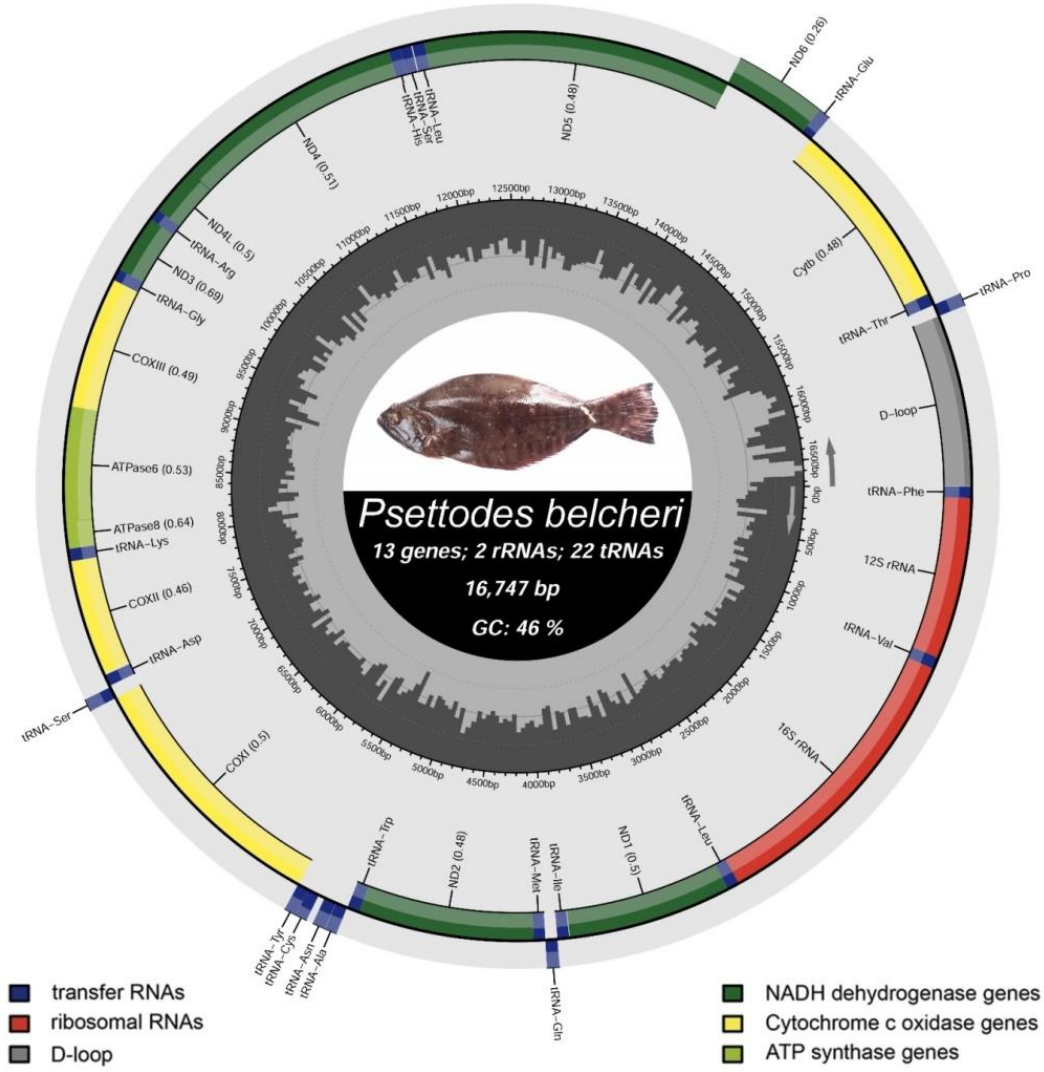

Figure 4. Circular view of the mitochondrial genome of *Psettodes belcheri*, drawn by the MitoAnnotator online server.

In the *Psettodes belcheri* mitogenome, the AT skew and GC skew were 0.037 and -0.291, respectively. The comparative analysis of other two *Psettodes* mitogenomes, *Psettodes belcheri* elucidated with the lowest AT skew (0.037) and highest in *Psettodes erumei* (AP006835; 0.077). While, GC skew was highest in generated mitogenome of *Psettodes belcheri* (-0.291) and *Psettodes erumei* (FJ606835) and *Psettodes erumei* (AP006835), -0,323, -0,328 respectively (Table 2).

A total of five overlapping regions with a total length of 14 bp were identified in the *Psettodes belcheri* mitogenome. The longest overlapping region (7 bp) was observed between ATP synthase 8 (atp8) and ATP synthase 6 (atp6) genes. Other *Psettodes* mitogenomes also had the similar overlapping patterns with the longest overlapping for *Psettodes erumei* (FJ606835) and *Psettodes erumei* (AP006835) was 10 bp between ATP synthase 8 (atp8) and ATP synthase 6 (atp6) genes. In addition, a total of 15 intergenic spacer regions with a total length of 106 bp were observed in *Psettodes belcheri*, while 15 intergenic spacer regions also found in the *Psettodes erumei* (FJ606835) and *Psettodes erumei* (AP006835) with total length 77bp and 78 bp, respectively (appendix table S2).

Table 1. List of annotated mitochondrial genes of *Psettodes belcheri*

Genes	Start	End	Strand (H/L)	Size (bp)	Intergenic Nucleotide	Anti-codon	Start Codon	Stop Codon
tRNA-Phe (F)	1	69	H	69	0	TTC	.	.
12S rRNA	70	1,030	H	961	0	.	.	.
tRNA-Val (V)	1,031	1,102	H	72	26	GTA	.	.
16S rRNA	1,129	2,830	H	1,702	0	.	.	.
tRNA-Leu (L2)	2,831	2,903	H	73	0	TTA	.	.
ND1	2,904	3,878	H	975	4	.	ATG	AGG
tRNA-Ile (I)	3,883	3,952	H	70	1	ATC	.	.
tRNA-Gln (Q)	3,954	4,024	L	71	-1	CAA	.	.
tRNA-Met (M)	4,024	4,093	H	70	0	ATG	.	.
ND2	4,094	5,138	H	1,045	0	.	ATG	T--
tRNA-Trp (W)	5,139	5,211	H	73	2	TGA	.	.
tRNA-Ala (A)	5,214	5,282	L	69	1	GCA	.	.
tRNA-Asn (N)	5,284	5,356	L	73	38	AAC	.	.
tRNA-Cys (C)	5,395	5460	L	66	0	TGC	.	.
tRNA-Tyr (Y)	5,461	5,530	L	70	1	TAC	.	.
COI	5,532	7,082	H	1,551	0	.	GTG	TAA
tRNA-Ser (S2)	7,083	7,153	L	71	8	TCA	.	.
tRNA-Asp (D)	7,162	7,230	H	69	8	GAC	.	.
COII	7,239	7,929	H	691	0	.	ATG	T--
tRNA-Lys (K)	7,930	8,004	H	75	1	AAA	.	.
ATP8	8,006	8,170	H	165	-7	.	ATG	TAA
ATP6	8,164	8,844	H	681	2	.	ATG	TA-
COIII	8,847	9,629	H	783	2	.	ATG	TA-
tRNA-Gly (G)	9,632	9,702	H	71	0	GGA	.	.
ND3	9,703	10,050	H	348	1	.	ATG	T--
tRNA-Arg (R)	10,052	10,120	H	69	0	CGA	.	.
ND4L	10,121	10,414	H	294	-4	.	ATG	TAA

ND4	10,411	11,791	H	1,381	0	.	ATG	T--
tRNA-His (H)	11,792	11,859	H	68	0	CAC	.	.
tRNA-Ser (S1)	11,860	11,927	H	68	6	AGC	.	.
tRNA-Leu (L1)	11,934	12,006	H	73	0	CTA	.	.
ND5	12,007	13,845	H	1839	-1	.	ATG	TAA
ND6	13,845	14,363	L	519	0	.	ATG	TAG
tRNA-Glu (E)	14,364	14,432	L	69	5	GAA		.
Cyt b	14,438	15,578	H	1,141	0	.	ATG	T--
tRNA-Thr (T)	15,579	15,652	H	74	-1	ACA	.	.
tRNA-Pro (P)	15,652	15,724	L	73	0	CCA	.	.
Control region	15,725	16,747	H	1,023	.	.	.	.

Tabel 2. Nucleotide composition of the mitochondrial genome in different *Psettodes* species

Species Name	Size (bp)	A%	T%	G%	C%	A+T%	AT-Skew	GC-Skew
Complete mitogenome								
P. belcheri (OR231239)	16,747	28.09	26.06	16.24	29.56	54,15	0.037	-0.291
P. erumei (FJ606835)	17,315	28.83	24.78	15.71	30.68	53.61	0.076	-0.323
P. erumei (AP006835)	16,683	28.57	24.50	15.78	31.15	53.07	0.077	-0.328
PCGs								
P. belcheri (OR231239)	11,427	25.00	27.73	16.00	31.27	52.74	-0.052	-0.323
P. erumei (FJ606835)	11,427	25.37	25.75	15.62	33.25	51.13	-0.008	-0.361
P. erumei (AP006835)	11,426	25.33	25.62	15.62	33.43	50.95	-0.006	-0.363
rRNAs								
P. belcheri (OR231239)	2,689	31.42	21.38	21.87	25.33	52.81	0.190	-0.073
P. erumei (FJ606835)	2,680	32.43	20.56	20.86	26.16	52.99	0.224	-0.113
P. erumei (AP006835)	2,680	32.43	20.45	20.90	26.23	52.87	0.227	-0.113
tRNAs								
P. belcheri (OR231239)	1,556	27.83	27.31	23.14	21.72	55.14	0.009	0.032
P. erumei (FJ606835)	1,553	27.17	26.85	23.82	22.15	54.02	0.006	0.036
P. erumei (AP006835)	1,554	27.28	26.83	23.68	22.20	54.12	0.008	0.032
CRs								
P. belcheri (OR231239)	1,015	30.25	33.40	12.51	23.84	63.65	-0.050	-0.312
P. erumei (FJ606835)	1,601	38.91	33.92	10.31	16.86	72.83	0.069	-0.241
P. erumei (AP006835)	968	42.15	36.36	7.13	14.36	78.51	0.074	-0.337

2. Protein-Coding Genes (PCGs)

Psettodes belcheri has PCGs length 11,427 bp, which counts as 68,23% of the total mitogenome. The length of PCGs was the same as the *Psettodes erumei* (FJ606835) and longer than *Psettodes erumei* (AP006835), which had an 11,426 bp. In *Psettodes belcheri* PCGs, the AT skew and GC skew were -0.052 and -0,323, respectively. Most *Psettodes belcheri* PCGS are encoded in the heavy strand (H-strand) except for the ND6 gene located in the light strand (L-strand). The shortest PCGs were initiated as ATP8 with 165 bp, while the longest PCGs were in the ND5 with 1,839 bp. The same pattern was found in the two other species (*Psettodes erumei* (FJ606835); *Psettodes erumei* (AP006835)), with the shortest PCGs being the ATP8 and the longest one being ND5 with the size 168 bp and 1,824 bp, respectively.

Based on the calculation of the total 13 PCGs in the *Psettodes belcheri*, the percentage of A+T shows the total 11 PCGs genes biased towards the Adenine (A) and Thymine (T) nucleotides and only two genes (ATP8 and ND4L) biased towards the Guanine (G) and cytosine (C) nucleotides. Six PCGs genes were found to have positive AT skews (ND2, COII, ATP8, COIII, ND5, and ND6), while seven PCGS remaining (ND1, COI, ATP6, ND3, ND4L, ND4, Cytochrome b) had negative AT skews. On the other hand, all of PGCs genes have negative GC skews.

3. Ribosomal RNA (rRNA) and transfer RNA (tRNA)

In *Psettodes belcheri*, the length of the ribosomal RNA genes was 2,689 bp (16.06% of complete mitochondrial genome) consisting of a small (12S) rRNA and a large (16S) rRNA of 961 bp and 1,702 bp in length, respectively. The 12S rRNA is located between tRNA-Phe and tRNA-Val whereas the 16S rRNA is located between the tRNA-Val and tRNA-Leu2. There is a 26 bp intergenic spaces between tRNA-Val and 16S rRNA. The rRNA of *Psettodes belcheri* is the largest compared to the other *Psettodes*, which have the same size (2,680 bp). All species biases to AT content in the ribosomal RNA genes varied from 52.81% (*Psettodes belcheri*) to 52.99% (*Psettodes erumei*, FJ606835). The AT skew in the ribosomal RNA varied from 0.190 (*Psettodes belcheri*) to 0.227 (*Psettodes erumei*, AP006835), while the GC skew ranged from -0.073 (*Psettodes belcheri*) to -0.113 (*Psettodes erumei* (FJ606835); *Psettodes erumei* (AP006835)).

Psettodes belcheri mitochondrial genome consists of 22 transfer RNA genes which are characterized by a variation in length from 66 bp (tRNA-Cys) to 75 bp (tRNA-Lys), estimating a total length of 1,556 bp and this overall length contributed 9.29% of the complete mitochondrial genome as described in Table 2, which is making the longest among all *Psettodes* species in the present dataset (Table 2). The AT richness within the transfer RNA ranges from 54.02% (*Psettodes erumei*, FJ606835) to 55.14% (*Psettodes belcheri*). The AT skew ranged from 0.006 (*Psettodes erumei*, FJ606835) to 0.009 (*Psettodes belcheri*), while the GC skew ranged from 0.032 ((*Psettodes belcheri* and

Psettodes erumei (AP006835)) to 0.036 (*Psettodes erumei*, FJ606835). This set of 22 tRNA genes is predicted to fold in the typical cloverleaf secondary structure as presented in (Figure 5). The comparative structural features of transfer RNA gene revealed 13 tRNA genes (tRNA-Phe, tRNA-Val, tRNA-Leu2, tRNA-Ile, tRNA-Gln, tRNA-Met, tRNA-Trp, tRNA-Ala, tRNA-Asn, tRNA-Cys, tRNA-Tyr, tRNA-Ser2, tRNA-Asp, tRNA-Lys, tRNA-Gly, tRNA-Arg, tRNA-His, tRNA-Ser1, tRNA-Leu1, tRNA-Glu, tRNA-Thr, and tRNA-Pro) were constituted by both conventional Watson-Crick base (A=T and G≡C) pairing and wobble base pairing (G–T), whereas the other seven tRNA gene only built with the Watson-Crick base pairs (Figure 5).

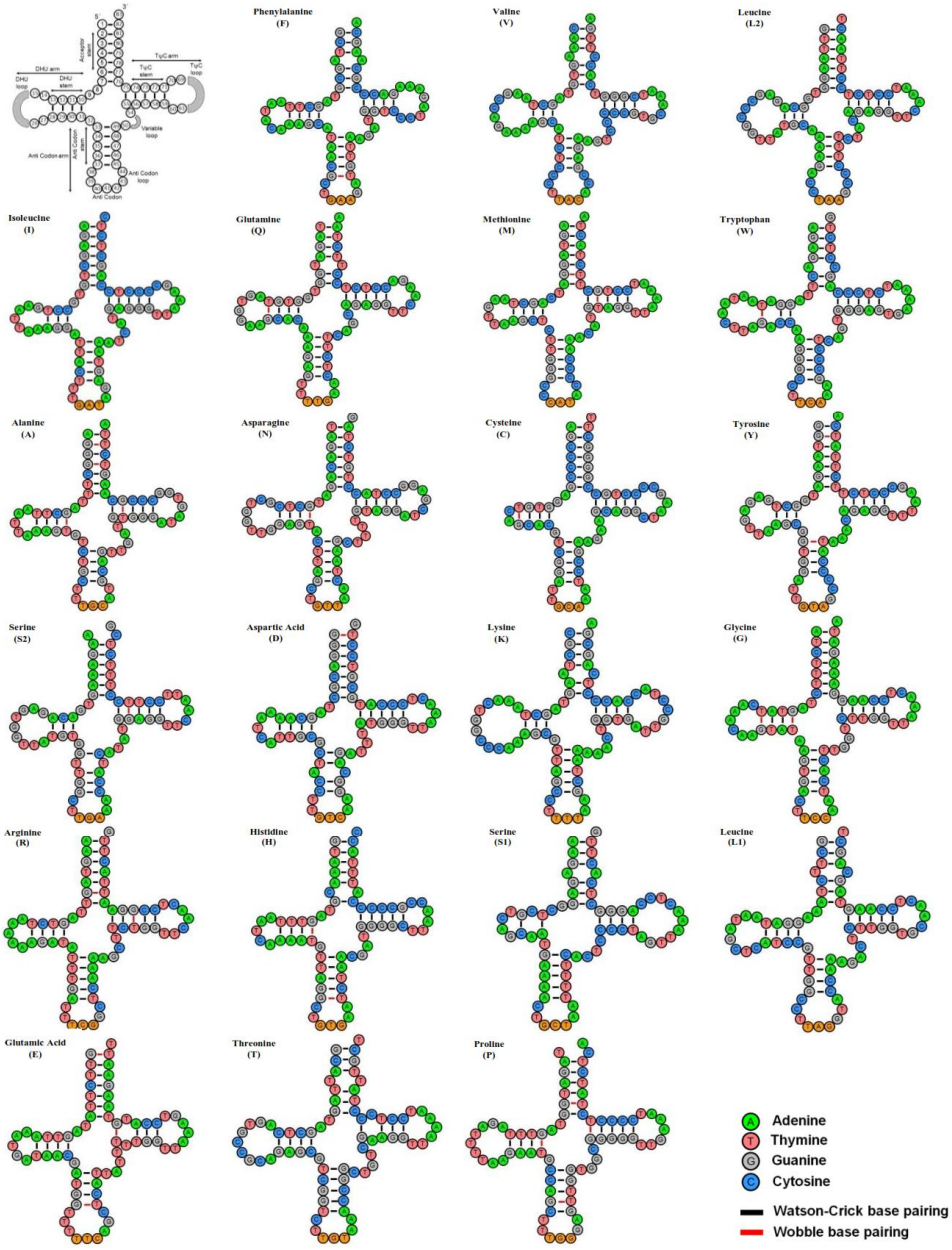

Figure 5. The secondary structure of the 22 tRNA genes in the mitogenome of *Psettodes belcheri*.

4. Features of Control Region and Gene Arrangements

The Control Region (CR) of *Psettodes belcheri* had a length of 1015 base pairs, characterized by a high AT content of 63.65% and a GC content of 36.35%. In contrast, the CR lengths of *Psettodes erumei* varied, ranging from 968 to 1601 base pairs. The AT skew ranged from -0.050 for *Psettodes belcheri* to 0.074 for *Psettodes erumei*, while the GC skew varied from -0.337 (*Psettodes erumei*, AP006835) to -0.241 (*Psettodes erumei*, FJ606835) (Table 2). Within the *Psettodes belcheri* mitogenome, more than two copies of 72 base pair tandem repeats were observed, whereas *Psettodes erumei* exhibited over eight copies (FJ606835) and more than twelve copies (AP006835) of 56 base pair repeats. Both *Psettodes belcheri* and *Psettodes erumei* mitogenomes contained four conserved blocks (CSB-D, CSB-I, CSB-II, and CSB-III), consistent with their presence in other teleost fishes (Satoh et al., 2016; Kundu et al, 2023). Among these, CSB-II was the longest at 51 base pairs, followed by CSB-D (27 bp), CSB-I (36 bp), and CSB-III (35 bp) (Figure 6A). Comparative analyses revealed significant nucleotide variability and parsimony informative nucleotides within CSB-II compared to the other three conserved domains. This AT-rich regulatory region holds promise for assessing population structures and identifying inter- and intra-specific differences among *Psettodes* species through these variable nucleotides. Like other species, these conserved domains play an essential role in mitochondrial genome replication and transcription (Kundu et al, 2023; Clayton, 1982). Notably, these primitive Pleuronectiformes fishes maintain a gene order within their

mitochondrial genomes that aligns with that of ancestral teleosts (Boore, 1999) (Figure 6B). However, Pleuronectiformes mitogenomes exhibit repeated occurrences of control region duplications and gene rearrangements (Kong et al., 2009; Shi et al., 2013; Shi et al., 2020; Gong et al., 2020; Wang et al., 2020). These mechanisms, involving genomic rearrangement through double replications, random loss, dimer-mitogenomes, and non-random loss, contribute to our understanding of the structural diversity of mitogenomes and the complexities of mitochondrial genome evolution.

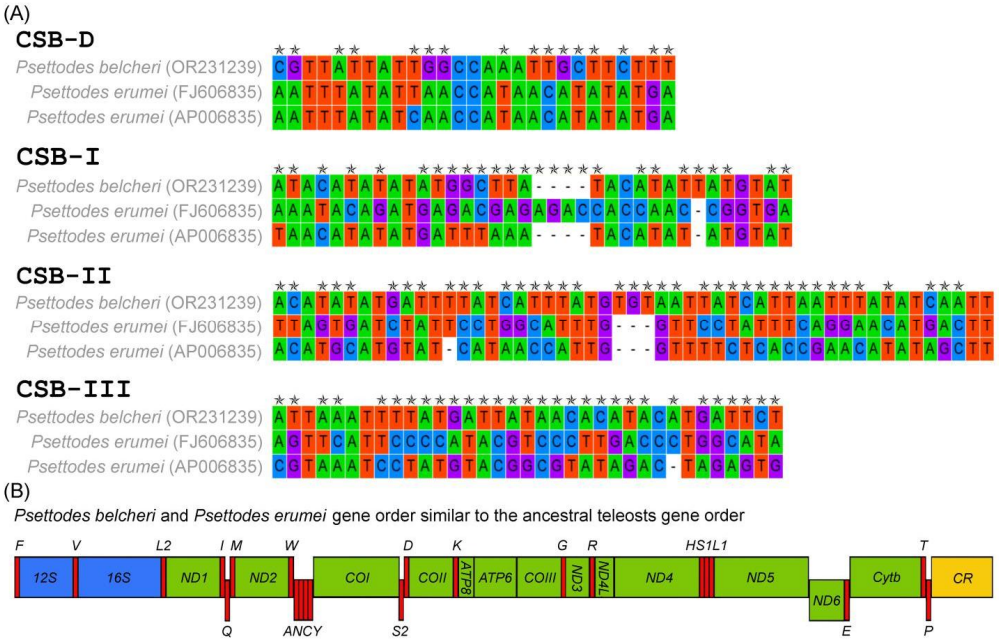

Figure 6. (A) Comparison of length and nucleotide composition of four conserved domains of two *Psettodes* species control regions. The variable nucleotides are marked in stars; (B) The gene arrangements of two *Psettodes* species mitogenomes.

5. Genetic Distances and Phylogenetic Relationship

Our species study, *Psettodes belcheri*, revealed significant genetic differences of 14.00% and 17.30% in comparison to its relatives, *Psettodes bennettii* and *Psettodes erumei*, respectively, as determined through the analysis of the COI gene (Appendix Figure S1). Notably, a noteworthy finding was the substantial genetic divergence of 14% between *Psettodes belcheri* and *Psettodes bennettii*, despite their coexistence in the Western Atlantic. This genetic distinction, significant in the context of their overlapping distributions and sympatric distribution, suggests a considerable degree of reproductive isolation between these species. The exploration of population genetic structures within *Psettodes belcheri* and *Psettodes bennettii* promises to offer valuable insights into their migration patterns within the shared habitat of the Eastern Atlantic Ocean. Utilizing a phylogenetic analysis rooted in mitogenomes, our research successfully categorized all examined Pleuronectiformes flatfishes using a concatenation of 13 PCGs, supported by a strong posterior probability (Figure 7). The resulting phylogeny derived from mitogenomes aligns closely with previous evolutionary hypotheses concerning Pleuronectiformes species (Campbell et al., 2014; Shi et al., 2018). Importantly, representative species from the suborders Psettodoidei and Pleuronectoidei formed coherent monophyletic clusters within the current topology. The Spottail Spiny Turbot, *Psettodes belcheri*, consistently clustered with its relatives, notably *Psettodes erumei*. The family Psettodidae emerged as the basal node of Pleuronectiformes, occupying a unique position as an ancestral group among other

flatfish families. Additionally, the conducted cladistic analysis demonstrated a sister relationship between *Cynoglossus gracilis* (=Cynoglossidae I) and *S. orientalis* (=Cynoglossidae II) (Figure 7). The application of mitochondrial genome-based and phylogenomic assessments has proved successful in elucidating higher teleostean phylogenies, encompassing flatfishes (Tinti et al., 2000; Saitoh et al., 2000; Miya et al., 2003). To establish the precise matrilineal evolution of *Psettodes* flatfishes within the monotypic family Psettodidae, the generation of the *Psettodes bennettii* mitogenome stands as a crucial task. Notably, a wealth of genetic data, spanning multi-locus exon-capture data, and whole-genome sequencing, has recently provided fresh insights into the phylogeny and genetic evolution of flatfishes (Chen et al, 2014; Figueras et al., 2016; Shao et al., 2017; Zhao et al., 2021; Lü et al., 2021; Atta et al., 2022). The integration of such extensive genetic information presents the potential to illuminate the evolutionary landscape of primitive *Psettodes* flatfishes in the near future.

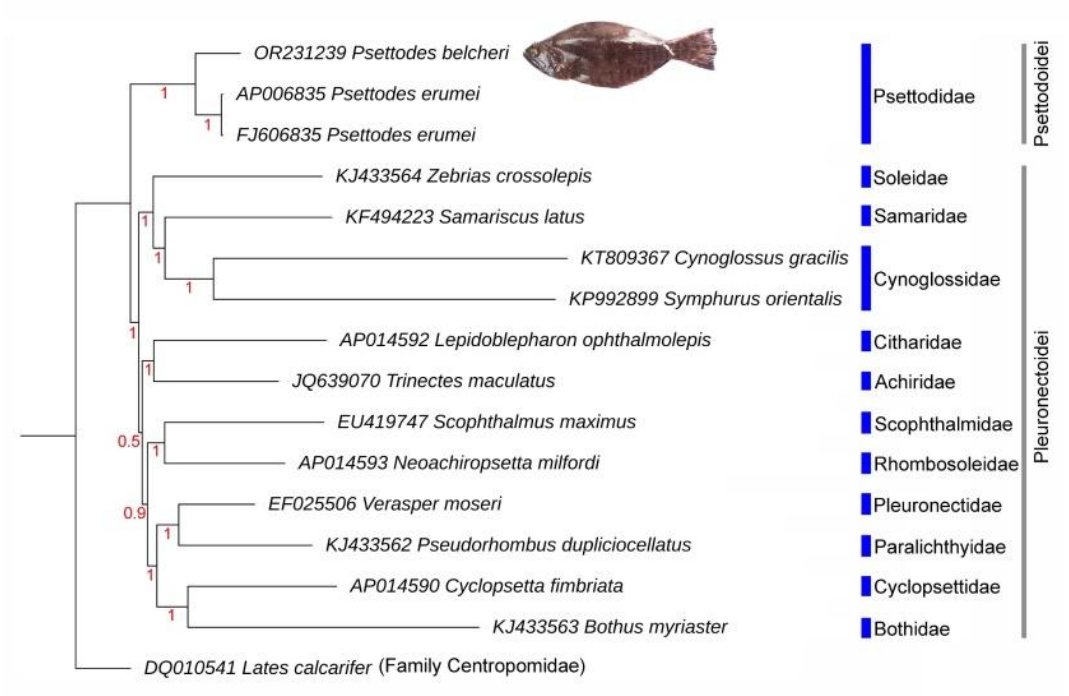

Figure 7. The Bayesian matrilineal phylogeny based on the concatenated sequences of 13 PCGs exhibits the evolutionary relationship of *Psettodes* species with other Pleuronectiformes.

6. Divergence Time and Diversification

To analyse the temporal aspects of the evolutionary history of *Psettodes belcheri* and *Psettodes erumei*, we employed the TimeTree application. According to our analysis, these two species underwent separate divergence during the early Miocene, approximately 20.56 million years ago (MYA) (Figure 8). Curiously, the family Psettodidae (suborder Psettodoidei) exhibited an early split of roughly 56.87 MYA from other flatfish families (suborder Pleuronectoidei) during the late Paleocene to early Eocene (Figure 8). On the other hand, the remaining Pleuronectoidei flatfish families diverged from each other between the Oligocene and early Eocene periods, with divergence dates ranging from 29.93 MYA to 54.98 MYA (Figure 8). The distribution pattern of *Psettodes* flatfishes, particularly within the family Psettodidae, has captured the interest of numerous ichthyologists. The year-to-year variations in the early life phenology and dispersal of flatfishes have been established as being influenced by factors such as bathymetry, alterations in water salinity, oceanic temperature, and wind conditions (Lacroix et al., 2018; Vaz et al., 2023). Therefore, the exploration of the evolution and diversification of marine fishes calls for a comprehensive discussion that encompasses genetic connectivity, divergent selection, as well as demographic and ecological influences (Reis-Santos et al., 2018).

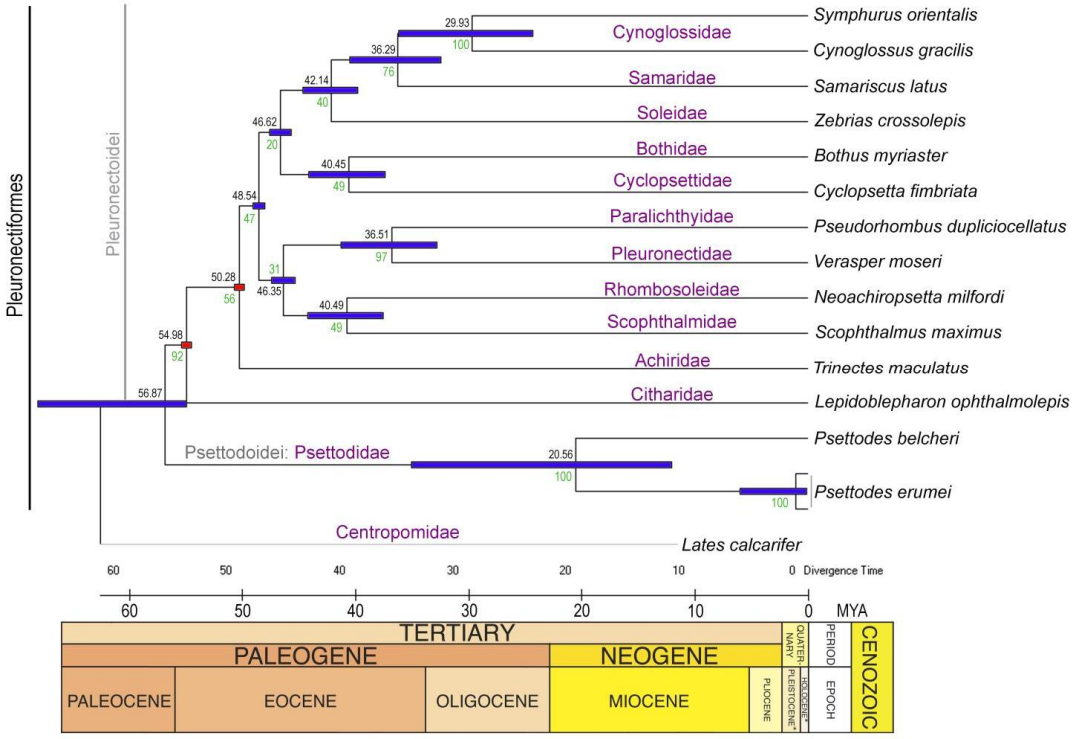

Figure 8. The maximum-likelihood-based TimeTree elucidates the approximate divergence time of the *Psettodes* species along with other Pleuronectiformes species.

To better understanding of the evolutionary patterns of *Psettodes* flatfishes subsequent to their settlement in the Eastern Atlantic and Indo-West Pacific regions through the demersal lineage, it is crucial to combine the Maximum-Likelihood time-tree computation framework with marine ecological factors. It is noteworthy that while *Psettodes belcheri* is present in both the South and North Atlantic Oceans, covering the western coast of Africa

from Angola to western Sahara, *Psettodes bennettii* is found exclusively in the North Atlantic, with a range encompassing Gambia, Guinea, Guinea-Bissau, Mauritania, Morocco, Senegal, and Western Sahara. The coexistence and differentiation of these two species could be ascribed to ecological selection within the pelagic environment. Over time, variations in hydrographic and climatic conditions in the North and South Atlantic Oceans have been substantial due to the Coriolis effect caused by the Earth's rotation (Figure 9A). In the North Atlantic, the movement of oceanic currents leads to distinct oceanic gyres, including the warm Gulf Stream current flowing northward and the cold Canary current flowing southward (O'Brien et al., 2017). The interplay of these currents, in conjunction with the North Equatorial current, contributes to the development of the North Atlantic gyre (Figure 9B). Conversely, the southern Atlantic Ocean is characterized by counterclockwise current circulation, dominated by the Anticyclonic Subtropical Gyre and bordered by several major surface ocean currents such as the Antarctic circumpolar current, the Benguela current, the South Equatorial current, the Brazil current, and the Malvinas current (Figure 9C).

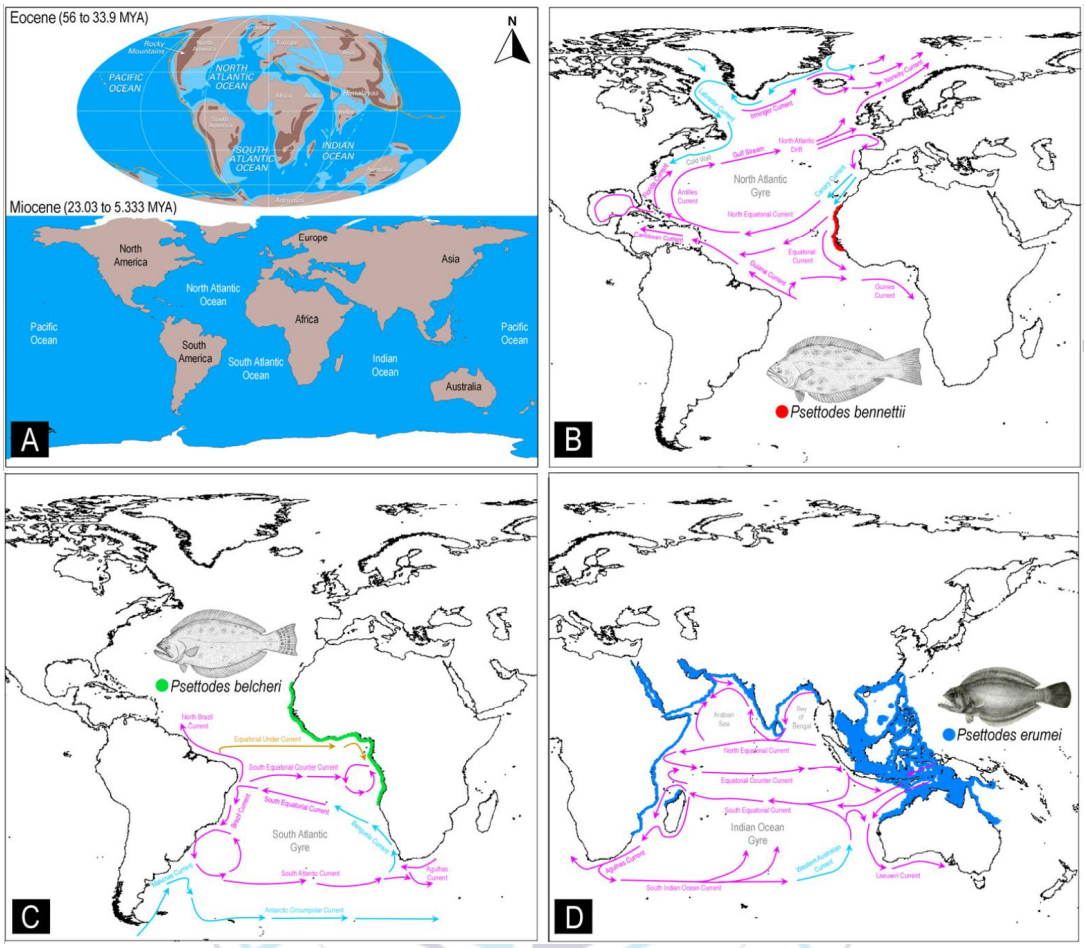

Figure 9. (A) Maps displaying the maritime environments during the Eocene and Miocene periods (source: Encyclopædia Britannica, Inc.). Schematic representation of the major current systems in the North Atlantic, South Atlantic, and Indo-West Pacific regions. This section also explores the potential diversification and colonization of: (B) *Psettodes bennettii*, (C) *Psettodes belcheri*, and (D) *Psettodes erumei*. The illustration was sourced from the free media repository (Wikimedia Commons), Carpenter et al. (2016), and O'Brien et al. (2017). Violet arrows indicate the warmer currents, while the blue arrows signify the cooler currents.

The Indian Ocean, known for the Arabian Sea and the Bay of Bengal, and the presence of warm and cold currents such as the Western Australian cold current, creates the Indian Ocean Gyre. The separation of Psettodidae from other Pleuronectiformes during the late Paleocene to early Eocene implies that these early flatfishes might have originated in the Eastern Atlantic Ocean. The oceanic currents and salinity levels in the North and South Atlantic Oceans could have restricted the distribution of the two Psettodidae species, *Psettodes belcheri* and *Psettodes bennettii*, to the continental shelves of western Africa. Another related species, *Psettodes erumei*, likely evolved during the early Miocene and expanded its territory from the Red Sea to the Indo-West Pacific Ocean following the formation of the Antarctic circumpolar current (Figure 9D). Notably, the Agulhas current might have functioned as an obstacle to the movement of *Psettodes erumei* between the Indian Ocean and the Atlantic Ocean. Despite the variations in hydrography, the high saline discharge from the Red Sea to the Indian Ocean results in comparable characteristics of these two marine environments, promoting similar communities of flatfish. However, the Indian Ocean and Western Pacific Ocean are seismically active because of the presence of tectonic plate boundaries. Such ecological scenarios are often used to elucidate these patterns, although distinguishing between ecological adaptation and allopatric speciation remains challenging (Teske et al., 2019). The migration of *Psettodes erumei* from the Indian Ocean to the West Pacific Ocean might have been stimulated by the Equatorial counter current of the Indian Ocean Gyre. These ecological factors significantly influence the evolution and adjustment of various flatfish species, including *Psettodes*, potentially

leading to increased endemism in certain demersal marine ecosystems. An interesting instance of rapid ecological speciation has been observed in Baltic flounder species, characterized by the development of a new ecological niche through demersal spawning behaviour, representing the quickest speciation event recorded for a marine vertebrate (Carpenter, 2016). Consequently, the distinctive spawning behaviour attributes of *Psettodes* flatfishes, along with ecological factors that could lead to reproductive isolation, suggest a process of ecological speciation.

Conclusion

In this study, we generated the complete mitochondrial genome sequence of *Psettodes belcheri* (OR231239) obtained from Cameroon, Africa. The total length of this mitochondrial genome was found to be 16,747 base pairs, encompassing 37 genes. The arrangement of genes conforms to the common pattern observed in vertebrates, with 28 genes located on the heavy strand, while the light strand carries eight tRNAs and the NAD6 gene. Bayesian phylogenetic analysis distinctly differentiated Psetodidae as a monophyletic cluster from other flatfish species within the Pleuronectiformes order. This genetic data played a crucial role in unravelling the evolutionary trajectory of *Psettodes* flatfish, yielding valuable insights into their genetic composition and potential adaptations within the East Atlantic and Indo-West Pacific Oceans. Additionally, our study revealed significant genetic divergence between *Psettodes belcheri* and *Psettodes bennettii* in the mitochondrial COI gene, indicating reproductive isolation despite their shared habitat. We also estimated the phylogenetic relationship, divergence times, and patterns of diversification, shedding light on the origin and dispersion of *Psettodes* species across various oceanic regions. In summary, our analysis of the mitogenome and evolutionary history of *Psettodes* flatfish provides valuable insights into their genetic characteristics and potential ecological adaptations in the East Atlantic and Indo-West Pacific Oceans.

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is for his beloved wife, Omega Raya Simarangkir. Thank you for being the biggest encouragement and motivation while he studied in Korea.

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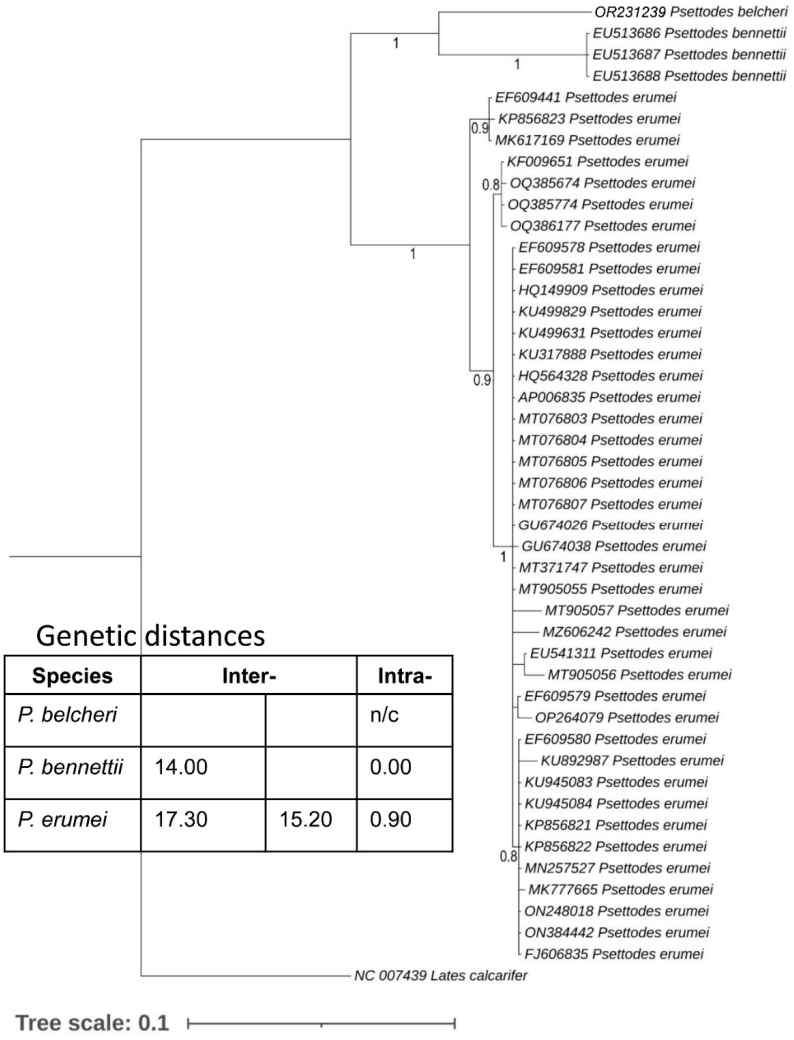
Appendix

Appendix Figure S1. Mitochondrial COI based Bayesian phylogeny clearly discriminate three *Psettodes* species with high posterior probabilities branch supports. The K2P genetic distances of three *Psettodes* species were overlaid on the topology.



Appendix Table S1. List of species dataset within Pleuronectiformes order including the species outgroup in this study.

Sub Order	Family	Species	Accession Number	Size (bp)	Reference
Psettodoidei	Psettodidae	*Psettodes belcheri*	OR231239	16,747	This study
		Psettodes erumei	FJ606835	17,315	GeneBank
		Psettodes erumei	AP006835	16,683	GeneBank
Pleuronectoidei	Achiridae	*Trinectes maculatus*	JQ639070	16,553	GeneBank
	Bothidae	*Bothus myriaster*	KJ433563	16,873	Gong et al., 2016
	Citharidae	*Lepidoblepharon ophthalmolepis*	AP014592	16,805	Campbell et al., 2014
	Cyclopsettidae	*Cyclopsetta fimbriata*	AP014590	16,506	Campbell et al., 2014
	Cynoglossidae	*Symphurus orientalis*	KP992899	17,498	Shi et al., 2015
		Cynoglossus gracilis	KT809367	16,565	Wei et al.2016
	Paralichthyidae	*Pseudorhombus dupliciocellatus*	KJ433562	16,621	Si et al., 2017
	Pleuronectidae	*Verasper moseri*	EF025506	17,588	He et al., 2008
	Rhombosoleidae	*Neoachiropsetta milfordi*	AP014593	16,623	Campbell et al., 2014
	Samaridae	*Samariscus latus*	KF494223	18,706	Shi et al., 2014
	Scophthalmidae	*Scophthalmus maximus*	EU419747	17,583	GeneBank
	Soleidae	*Zebrias crossolepis*	KJ433564	16,734	Gong et al., 2016
	Centropomidae	*Lates calcarifer**	DQ010541	16,535	Lin et al., 2005

*outgroup

Appendix Table S2. Comparison of the intergenic nucleotides of two *Psettodes* species mitogenomes.

Genes	SPECIES		
	P. belcheri (OR231239)	*P. erumei* (FJ606835)	*P. erumei* (AP006835)

tRNA-Phe (F)	0	0	0
12S rRNA	0	0	0
tRNA-Val (V)	26	0	0
16S rRNA	0	0	0
tRNA-Leu (L2)	0	0	0
ND1	4	4	4
tRNA-Ile (I)	1	3	3
tRNA-Gln (Q)	-1	-1	-1
tRNA-Met (M)	0	0	0
ND2	0	0	0
tRNA-Trp (W)	2	1	1
tRNA-Ala (A)	1	1	1
tRNA-Asn (N)	38	37	38
tRNA-Cys (C)	0	0	0
tRNA-Tyr (Y)	1	1	1
COI	0	0	0
tRNA-Ser (S2)	8	10	10
tRNA-Asp (D)	8	8	8
COII	0	0	0
tRNA-Lys (K)	1	1	1
ATP8	-7	-10	-10
ATP6	2	0	0
COIII	2	0	0
tRNA-Gly (G)	0	0	0
ND3	1	0	0
tRNA-Arg (R)	0	0	0
ND4L	-4	-7	-7
ND4	0	0	0
tRNA-His (H)	0	0	0
tRNA-Ser (S1)	6	6	6
tRNA-Leu (L1)	0	0	0
ND5	-1	-4	-4
ND6	0	0	0
tRNA-Glu (E)	5	5	5
Cyt b	0	0	0
tRNA-Thr (T)	-1	-1	-1
tRNA-Pro (P)	0	0	0
Control region			

Appendix Table S3. Start and stop codons of all 13 PCGS in two *Psettodes* species mitogenomes.

PCGs	*Psettodes belcheri* (OR231239)		*Psettodes erumei* (FJ606835)		*Psettodes erumei* (AP006835)	
	START	**STOP**	**START**	**STOP**	**START**	**STOP**
ND1	ATG	AGG	ATG	AGG	ATG	AGG
ND2	ATG	T--	ATG	T--	ATG	T--
COI	GTG	TAA	GTG	TAA	GTG	TAA
COII	ATG	T--	ATG	T--	ATG	T--
ATP8	ATG	TAA	ATG	TAA	ATG	TAA
ATP6	ATG	TA-	ATG	TA-	ATG	TA-
COIII	ATG	TA-	ATG	TA-	ATG	TA-
ND3	ATG	T--	ATG	T--	ATG	T--
ND4L	ATG	TAA	ATG	TAA	ATG	TAA
ND4	ATG	T--	ATG	T--	ATG	T--
ND5	ATG	TAA	ATG	TAA	ATG	TAA
ND6	ATG	TAG	ATG	TAG	ATG	TAG
Cyt b	ATG	T--	ATG	T--	ATG	T--

Outcomes Publication

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Insights into the Mitochondrial Genetic Makeup and Miocene Colonization of Primitive Flatfishes (Pleuronectiformes: Psettodidae) in the East Atlantic and Indo-West Pacific Ocean

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Simple Summary: The present research enriches our comprehension of the mitogenomic genetic features, genetic diversity, evolutionary past, and conservation prerequisites of *Psettodes* flatfishes on a global scale. This study focuses on the matrilineal evolutionary path of these primitive groups, with a specific emphasis on the complete mitogenome of the *Psettodes belcheri* and casting light on its genetic composition, structural traits, and evolutionary chronicle. Exploring genetic variations and phylogenetic relationships uncovers the intricate evolutionary links between *Psettodes* species and their broader context within the Pleuronectiformes species. The complex interplay of hydrographic conditions, ocean currents, and ecological factors emerges as pivotal in shaping the evolutionary landscape of these flatfishes. Given the potential consequences for conservation, this study highlights the necessity for a holistic comprehension of marine environments and the ramifications of climate change and human interventions on flatfish species.