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The Complete Mitochondrial Genome and Molecular Phylogeny of the Flathead *Platycephalus cultellatus* Richardson, 1846 from Vietnam (Teleostei; Scorpaeniformes)

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ABSTRACT The family Platycephalidae is a taxonomic group of economically important demersal flathead fishes that predominantly occupy tropical or temperate estuaries and coastal environments of the Indo-Pacific oceans and the Mediterranean Sea. In this study, we for the first time analyzed the complete mitochondrial genome (mitogenome) of the flathead *Platycephalus cultellatus* Richardson, 1846 from Vietnam by Next Generation Sequencing method. Its mitogenome was 16,641 bp in total length, comprising 13 protein-coding genes (PCGs), two ribosomal RNA genes, and 22 transfer RNA genes. The gene composition and order of the mitogenome were identical to those of typical vertebrates. The phylogenetic trees were reconstructed based on the concatenated nucleotide sequence matrix of 13 PCGs and the partial sequence of a DNA barcoding marker, *cox1* in order to determine its molecular phylogenetic position among the order Scorpaeniformes. The phylogenetic result revealed that *P. cultellatus* formed a monophyletic group with species belonging to the same family and consistently clustered with one nominal species, *P. indicus*, and two *Platycephalus* sp. specimens. Besides, the *cox1* tree confirmed the taxonomic validity of our specimen by forming a monophyletic clade with its conspecific specimens. The mitogenome of *P. cultellatus* analyzed in this study will contribute valuable information for further study on taxonomy and phylogeny of flatheads.

Key words: Flathead fish, mitochondrial genome, phylogeny, *Platycephalus cultellatus*, Scorpaeniformes, Vietnam

INTRODUCTION

Flathead fishes belonging to the family Platycephalidae are predominantly found in tropical or temperate estuaries and coastal environments of the Indo-Western Pacific oceans and the eastern Mediterranean Sea (Cheng *et al*., 2019). As an ambush predator with sandy and muddy habitats, they have high economic value in countries of Indo-Pacific and East Asia and were commercially targeted by the Australian trawling industry owing to their high-quality flesh (Cheng *et al*., 2019). This family consists of approximately 70 valid species of 18 genera, but their phylogenetic relationship remains contentious due to the dearth of information and the confinement of the morphological method (Imamura *et al*., 1996; Bray, 2020).

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The bottom-dwelling flathead, *Platycephalus cultellatus* Richardson, 1846 was first discovered in Canton, Guangdong province, China (Richardson, 1846; Chen *et al*., 2018). However, there was very little information about this species, until a comprehensive revision had been made by Chen *et al*. (2018) using morphology and DNA barcoding approach. In Vietnam, its appearance was first recorded and described by Imamura *et al*. (2006) in Nha Trang based on just the morphological method. Thus, future revision of *P. cultellatus* is required to provide more reliable knowledge not only to solve its taxonomic problems, but also its phylogenetic relationship among the flatheads.

Mitochondrial genomes (mitogenomes) of vertebrates are typically small, double-stranded, and circular DNA molecules with multiple copies in a mitochondrion (Meyer, 1993). Its size ranges from 15 to 20 kb, consisting of a single non-coding control region (D-loop) responsible for DNA replication and RNA transcription; 13 genes code for proteins; two genes code for ribosomal RNAs (rRNAs); and 22 genes code for transfer RNAs (tRNAs) (Boore, 1999). Owing to typical characteristics such as conservation of gene content and order, maternal inheritance, rapid evolution, absence of recombination events, and so forth, it has increasingly been recognized as a powerful marker in phylogenetic analyses (Satoh *et al*., 2016; Cui *et al*., 2017). In research of phylogenetic estimates using various genetic markers, Duchêne *et al*. (2011) pointed out that mitogenomic data provide the most reliable result with highly supported topologies, clock-like behavior, and low saturation compared to single genes. Although its superiority has been proven in concordance with the advent of next-generation sequencing (NGS) technology, a cost-effective, high-throughput, and accurate method in generating huge amount of genetic data (Metzker, 2009; Briscoe *et al*., 2016), the available mitogenomic sequences of the Platycephalidae remains scarce in the GenBank database with just four out of at least 70 identified species to date.

In the present study, we presented the complete mitogenomic sequence of the flathead *P. cultellatus* from Vietnam in order to fill the gap of genetic data. Besides, the phylogenetic trees based on the concatenated nucleotide sequence matrix of protein-coding genes (PCGs) and the partial *cox1* sequence of *P. cultellatus* were reconstructed together with the members in the order Scorpaeniformes to confirm the taxonomic validity of our specimen and provide information on the phylogenetic relationship among scorpaeniform species.

MATERIALS AND METHODS

1. Sampling and genomic DNA extraction

A specimen of *P. cultellatus* was collected from a local fisheries market at Can Tho, Vietnam (10°01′40.6″N, 105°47′13.0″E) in 2019. The voucher specimen was deposited at the National Marine Biodiversity Institute of Korea (https://www.mabik.re.kr/) under voucher number, MABIK Lot No. 0016931. Its genomic DNA (gDNA) was extracted from a piece of the pelvic fin. The excised sample was incubated with 600 μL of TNES-urea buffer (10 mM Tris-HCl, pH 8.0; 125 mM NaCl; 10 mM EDTA, pH 8.0; 0.5% SDS; 6 M urea) and 10 μL proteinase K (20 μg/mL) at 60°C for more than 12 h, followed by the phenol : chloroform : isoamylalcohol (25 : 24 : 1) extraction and ethanol precipitation (Asahida *et al*., 1996). The extracted gDNA was resuspended in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) and stored at −20°C until ready to be used. Its concentration was confirmed using the VICTOR^{3TM} Multilabel Plate Reader (Perkin Elmer Inc., Shelton, CT, USA) after staining with the Quant-iT^{TM} PicoGreen^{TM} dsDNA Assay Kit (Molecular Probes Inc., Eugene, OR, USA).

2. NGS library preparation and analysis

For Next Generation Sequencing (NGS), a library was constructed with 1 μg of the gDNA according to the manufacturer's instruction of MGIEasy Universal DNA Library Prep Set (MGI Tech Co. Ltd., Shenzhen, China). The library with an average insert size of *ca*. 409 bp was generated and subjected to a NGS analysis using Genetic Sequence MGISEQ-2000 (MGI Tech Co. Ltd.). Approximately, 10.8 gigabyte (Gb) of raw data was obtained by 150 bp paired-end sequencing. The raw data was trimmed with cutadapt v1.9.1 (Martin, 2011), and 9.5 Gb of raw data was finally obtained with 64.3 million reads. By using Geneious Prime v2020.2 (Biomatters Ltd, Auckland, New Zealand), the mitogenomic sequence was mapped with a 98% homology option and 10 times in iterations. A total of 159,013 reads were used to assemble the complete mitogenome of *P. cultellatus* with ×1,378 average coverage (standard deviation ×311.8). Its gene content and order were further confirmed through a fish mitogenome database, MitoFish (http://mitofish.aori.u-tokyo.ac.jp/) (Wataru *et al*., 2013). The mitogenomic sequence analyzed in this study was deposited in the GenBank database under the accession number OK136111.

3. Molecular phylogeny

A total of 69 mitogenomic sequences of the Scorpaeniformes available in the GenBank database along with that of *P. cultellatus* newly analyzed in this study were used to investigate their phylogenetic relationship at the family level. Two species belonging to the order Perciformes, i.e., *Percalates novemaculeata* (GenBank accession number NC_024850), and *Siniperca knerii* (NC_015987) were assigned as the outgroups. After aligning and refining manually to correct obvious misalignments using Clustal W in BioEdit 7.2.5 (Hall, 1999), the concatenated nucleotide sequence matrix of 13 PCGs was used to reconstruct Bayesian inference (BI) tree by MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and maximum likelihood (ML) tree by RAxML 7.0.4 (Alexandros, 2006). The synonymous gene labels of Boor (1999) were used consistently throughout this manuscript (Table 1).

For the BI analysis, General Time Reversible (GTR) model (Tavaré, 1986) with gamma-distributed rate variation across sites (Γ) and a proportion of invariable (I) was selected as the best model for the nucleotide sequence matrix of PCGs using jModelTest 2 (Darriba *et al*., 2012) based on the Bayesian Information Criterion (BIC). Four independent Markov chains were simultaneously used at 1,000,000 generations with sampling every 100 generations, and the first 25% were discarded as burn-in. Stationarity was considered to be reached, when the average standard deviation of split frequencies was much less than 0.01. For the ML analysis, the RAxML search was executed in one single program run (the "-f a" option), instead of the default maximum parsimony-starting tree. The best-scoring ML tree thorough ML analysis was determined under the GTRMIXI model. Statistical support was evaluated with 1,000 non-parametric bootstrap inferences. The resultant trees of both BI and ML analyses were illustrated using TreeView 1.6.6 (Page, 1996).

In addition, a phylogeny tree was reconstructed using the 22 representative *cox1* sequences of all *Platycephalus* species and *P. cultellatus* available from the GenBank database including that of *P. cultellatus* in this study. Two species belonging to the genus *Onigocia*, i.e., *Onigocia macrolepis* (JX488197) and *Onigocia pedimacula* (GU67 3208) were assigned as the outgroups. After manual correction using BioEdit 7.2.5, the nucleotide matrix was partitioned into three according to three positions of codon triplets. The final nucleotide matrix with 579 bp in length was prepared to reconstruct the ML tree using RAxML 7.0.4 as previous described. The resultant tree was illustrated using TreeView 1.6.6.

RESULTS AND DISCUSSION

1. Organization and structure of mitogenome

The complete mitogenome of *P. cultellatus* MABIK Lot No. 0016931 from Vietnam analyzed in this study was a circular molecule of 16,641 bp in length, which is proximately similar to those of other *Platycephalus* species, i.e., *Platycephalus indicus* (AP006783) and two specimens of *Platycephalus* sp. (MT584655 and MK344191), available in the GenBank database to date. Its gene content consisted of 13 PCGs, two rRNA genes (*rns* and *rnl*), 22 tRNA genes, and a D-loop. Apart from *nad6*, and eight tRNA genes (*trnQ*, *trnA*, *trnN*, *trnC*, *trnY*, *trnS1*, *trnE*, and *trnP*), which were distributed on the light (L) strand, most of the other genes encoded in the heavy (H) strand (Table 1). This is consistent with those of typical vertebrates, as illustrated in Fig. 1.

All PCGs initiated by ATG except for *cox1* using GTG as the start codon. Eight out of 13 PCGs (*nad1*, *nad2*, *cox1*, *atp8*, *atp6*, *cox3*, *nad4l*, and *nad5*) were terminated with the stop codon, TAA, while the others by TAG (*nad3* and *nad6*) and an incomplete stop codon, T (*cox2*, *nad4*, and *cob*) (Table 1). Twenty-two tRNA genes ranged from 68 bp (*trnF*) to 75 bp (*trnN*), and potentially tended to fold into a stem-loop secondary structures. The two rRNA genes, *rns* and *rnl* were 954 bp and 1695 bp

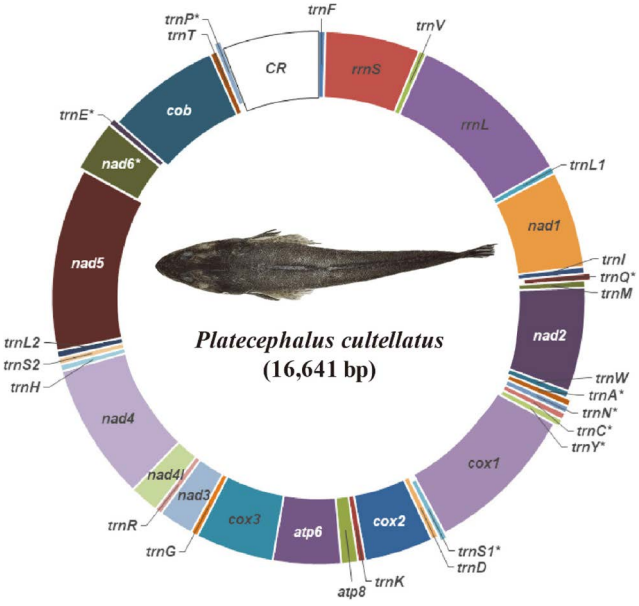

Fig. 1. The circular map of the complete mitochondrial genome of the flathead fish, *Platycephalus cultellatus*. Gene names including asterisk (*) denote encoding sequence in the light strand and those without asterisk denote encoding sequence in the heavy strand.

Table 1. Gene contents and positions of the mitochondrial genome of the flathead *Platycephalus cultellatus*

Full gene name	Gene	Strand[1)]	Position[2)]	Length (bp)	Start/Stop Codon
tRNA-Phe	*trnF*	H	1–68	68	
12S ribosomal RNA	*rns*	H	69–1022	954	
tRNA-Val	*trnV*	H	1023–1095	73	
16S ribosomal RNA	*rnl*	H	1096–2790	1695	
tRNA-Leu 1 (UAA)	*trnL1*	H	2791–2864	74	
NADH dehydrogenase subunit 1	*nad1*	H	2865–3839	975	ATG/TAA
tRNA-Ile	*trnI*	H	3844–3911 (+4)	67	
tRNA-Gln	*trnQ*	L	3911–3981 (−1)	71	
tRNA-Met	*trnM*	H	3981–4049 (−1)	69	
NADH dehydrogenase subunit 2	*nad2*	H	4053–5099 (+3)	1047	ATG/TAA
tRNA-Trp	*trnW*	H	5099–5169 (−1)	71	
tRNA-Ala	*trnA*	L	5171–5239 (+1)	69	
tRNA-Asn	*trnN*	L	5241–5313 (+1)	75	
tRNA-Cys	*trnC*	L	5352–5421 (+38)	70	
tRNA-Tyr	*trnY*	L	5421–5492 (−1)	72	
Cytochrome *c* oxidase subunit I	*cox1*	H	5494–7044 (+1)	1551	GTG/TAA
tRNA-Ser 1 (UGA)	*trnS1*	L	7045–7115	71	
tRNA-Asp	*trnD*	H	7119–7191 (+3)	73	
Cytochrome *c* oxidase subunit II	*cox2*	H	7199–7889 (+7)	691	ATG/T
tRNA-Lys	*trnK*	H	7890–7963	74	
ATP synthase F_0 subunit 8	*atp8*	H	7965–8132 (+1)	168	ATG/TAA
ATP synthase F_0 subunit 6	*atp6*	H	8123–8806 (−10)	684	ATG/TAA
Cytochrome *c* oxidase subunit III	*cox3*	H	8806–9591 (−1)	786	ATG/TAA
tRNA-Gly	*trnG*	H	9591–9661 (−1)	71	
NADH dehydrogenase subunit 3	*nad3*	H	9662–10012 (+1)	351	ATG/TAG
tRNA-Arg	*trnR*	H	10011–10079 (−2)	69	
NADH dehydrogenase subunit 4L	*nad4l*	H	10080–10376	297	ATG/TAA
NADH dehydrogenase subunit 4	*nad4*	H	10370–11750 (−7)	1381	ATG/T
tRNA-His	*trnH*	H	11751–11819	69	
tRNA-Ser 2 (GCU)	*trnS2*	H	11820–11887	68	
tRNA-Leu 2 (UAG)	*trnL2*	H	11891–11963 (+1)	72	
NADH dehydrogenase subunit 5	*nad5*	H	11964–13802	1838	ATG/TAA
NADH dehydrogenase subunit 6	*nad6*	L	13799–14320 (−4)	522	ATG/TAG
tRNA-Glu	*trnE*	L	14321–14389	69	
Cytochrome *b*	*cob*	H	14395–15535 (+5)	1141	ATG/T
tRNA-Thr	*trnT*	H	15536–15607	72	
tRNA-Pro	*trnP*	L	15608–15677	70	
Control region	CR	H	15678–16641	964	

[1)]H and L refer to genes transcribed in the heavy and the light strand, respectively.
[2)]The number in the parenthesis indicates nucleotide base(s) of the intergenic spacer (positive number) or overlap (negative number).

in length, respectively, located between *trnF* and *trnL1* and separated by *trnV*. The D-loop was 964 bp in length blanked by *trnP* and *trnF*. There were twelve intergenic spacers ranging from 1 to 38 bp, in which the largest one was located between *trnN* and *trnC*. Among them, the intergenic spacer between *trnT* and *trnP* (T-P spacer), presumably associated with phylogenetic relationship in the evolution by its molecular complexity (Jørgensen *et al*., 2014), comprised 0 bp in this study. Besides, the mitogenome of *P. cultellatus* comprised ten overlaps scattered from 1 to 10 bp, in which the largest one lied between *atp8* and *atp6*.

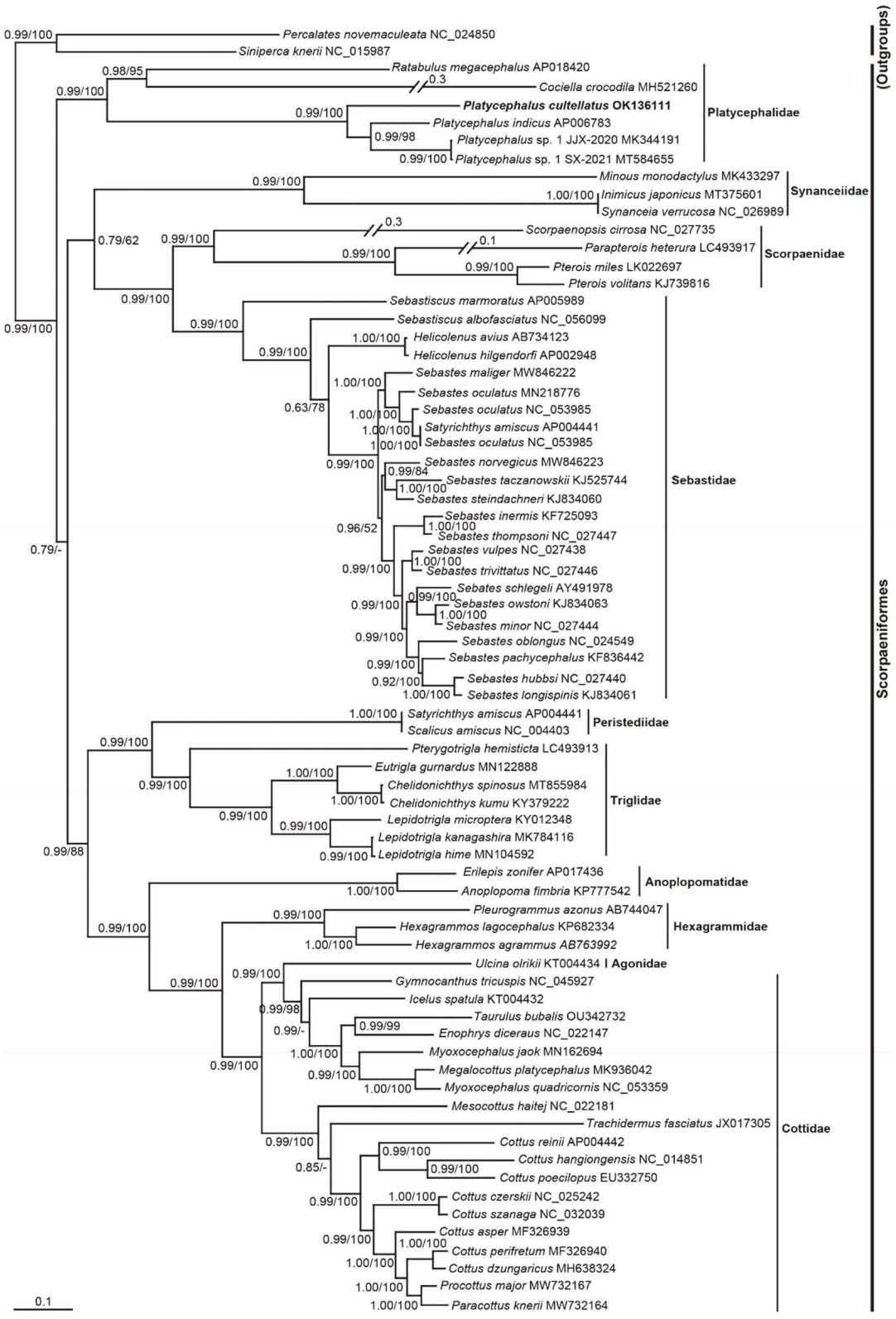

Fig. 2. A phylogenetic tree based on the concatenated nucleotide sequence matrix from the 13 protein coding-genes of all species belonging to the order Scorpaeniformes including the flathead fish, *Platycephalus cultellatus* newly analyzed in this study, using Bayesian inference (BI) and maximum likelihood (ML) methods. Two perciform species were assigned as the outgroups. Posterior probability values above 0.70 in the BI analysis and bootstrap values above 50% in the ML analysis are indicated at each node, respectively. The flathead fish, *Platycephalus cultellatus* in this study was highlighted in bold.

2. Molecular phylogeny

The bottom-dwelling flathead, *P. cultellatus* was comprehensively revised by Imamura *et al*. (2006) and Chen *et al*. (2018). According to their descriptions on basically shared traits, the prominent taxonomic characteristics distinguishing *P. cultellatus* from other closely-related species is its possession of 3−6 horizontal blackish bars on the caudal fin and lack of yellow blotch in the middle of the caudal fin when fresh. However, due to the limitation of the traditional taxonomy and the complexities of morphological characters among the flathead fishes, *P. cultellatus* was often mistakenly classified to other species, e.g., *Platycephalus indicus*, or an undetermined *Platycephalus* sp. (Imamura *et al*., 2006; Chen *et al*., 2018, 2020).

Our BI and ML tree based on 13 PCGs in this study showed a consistency of a well-resolved tree topology as opposed to the outgroups (Fig. 2). It also illustrated that *P. cultellatus* formed a monophyletic group with species belonging to the same family and consistently clustered with one nominal species, *P. indicus*, and two unidentified specimens, *Platycephalus* sp. 1 SX-2021 (MT584655) (Zhang *et al*., 2021) and JJX-2020 (MK344191) (Luo *et al*., 2019) with the high bootstrap (BS) and posterior probability (PP) values. In general, their general topologies were highly congruent with those of Cui *et al*. (2019). However, the Platycephalidae alone emerged as the most basal taxon in the Scorpaeniformes in contrast to Cui *et al*. (2019), in which both Synanceiidae and Platycephalidae were placed at the basal position. The difference could be resulted from including more mitogenomic sequence data of synanceiid and platycephalid species available in the GenBank database together with that of *P. cultellatus* from this study. Besides, our tree agreed with previous studies (Cui *et al*., 2017; Xu *et al*., 2019) by addressing the closest relationship of the Synanceiidae, Scorpaenidae, and Sebastindae. Further phylogenetic research is necessary to revise the relationship among these families in the Scorpaeniformes.

In addition, the ML tree was reconstructed based on the gold standard *cox1* for DNA barcoding to genetically identify our *P. cultellatus* specimen and to elucidate its phylogenetic relationship among the congeneric representative specimens for each species retrieved from the GenBank database (Fig. 3). The resultant tree revealed well-resolved tree topologies that separated all *Platycephalus* species as opposed to the outgroups. The result supported the taxonomic validity of our specimen from Vietnam by forming a strongly supported monophyletic clade with its conspecific specimens including two specimens from Indonesia (HQ564511 and GU673453) as an effort of International

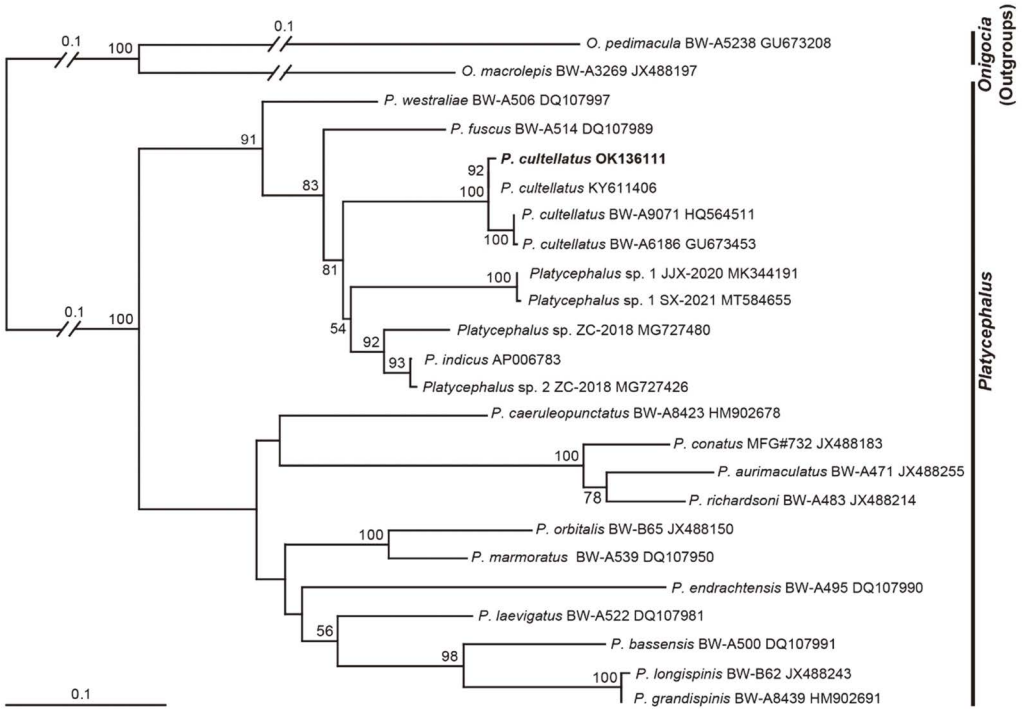

Fig. 3. A maximum-likelihood tree inferred from the partial *cox1* sequences of the genus *Platycephalus* including two *Onigocia* species assigned as the outgroups. The bootstrap values above 50% are marked on each node. The flathead fish, *Platycephalus cultellatus* newly analyzed in this study was highlighted in bold.

Barcode of Life (iBOL) and one specimen from China (KY611406) (Chen *et al*., 2018). Furthermore, our tree was consistent with previous studies in general topologies by grouping *P. cultellatus* together with one nominal species, *P. indicus* (AP006783), and other undescribed specimens, *Platycephalus* sp. ZC-2018 (MG727480), *Platycephalus* sp. 1 JJX-2020 (MK344191), *Platycephalus* sp. 1 SX-2021 (MT584655), and *Platycephalus* sp. 2 ZC-2018 (MG727426) as an elucidation for their closest relationship opposing to the other species, *Platycephalus fuscus* BW-A514 (DQ107989) and *Platycephalus westraliae* BW-A506 (DQ107997) (Cheng *et al*., 2019; Puckridge *et al*., 2019).

Previous studies (Chen *et al*., 2018, 2020; Cheng *et al*., 2019) agreed with the morphological similarities of *P. cultellatus* and other congeners, i.e., *P. indicus*, *Platycephalus* sp. ZC-2018, *Platycephalus* sp. 1 JJX-2020 and SX-2021, and *Platycephalus* sp. 2 ZC-2018 by sharing almost all morphological and meristic characteristics. There are few distinct features to morphologically identify them. For example, *P. indicus* is the only one with a yellow blotch on the caudal fin; coloration of *Platycephalus* sp. 1 JJX-2020 and SX-2021 is uniquely orange-brown and becomes dark after being frozen; *Platycephalus* sp. 2 ZC-2018 has very small black spots on its pectoral fins (Qin *et al*., 2013; Chen *et al*., 2018). However, *P. cultellatus* is not supposedly distinguishable from *Platycephalus* sp. by solely comparing a single morphological character (Chen *et al*., 2020). In this study, the *cox1* tree was highly efficient for discriminating *P. cultellatus* from its congeners by forming an independent branch clearly separated from them.

The mitogenomic sequence of *P. cultellatus* in this study not only validates its presence in Vietnam, but also contributes valuable information for further phylogenetic studies of the Platycephalidae.

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베트남 *Platycephalus cultellatus* Richardson, 1846 (Teleostei; Scorpaeniformes)의 전장 미토콘드리아 유전체와 분자계통

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요 약 : 양태과는 경제적으로 중요한 저서성 바닷물고기로써 인도태평양과 지중해의 열대 또는 온대지역의 하구역에 서식한다. 이번 연구에서 우리는 차세대염기서열분석법을 이용하여 flathead의 일종인 *Platycephalus cultellatus* Richardson, 1846의 전장 미토콘드리아 유전체를 최초로 분석하였다. 그 총 길이는 16,641 bp이었고, 단백질암호화 유전자 13개, 리보솜 RNA 유전자 2개, 전량 RNA 유전자 22개로 구성되었다. 그 유전자의 구성과 배열은 전형적인 척추동물과 같았다. 단백질암호화 유전자 13개를 바탕으로 작성된 분자계통수에서 *P. cultellatus*는 같은 과에 속하는 종들과 단계통군을 형성하였고, *P. indicus*를 비롯하여 *Platycephalus* sp.로 등록된 표본들과 함께 분기하였다. 또한 DNA 바코딩 분자마커로 널리 사용되는 *cox1* 유전자를 바탕으로 작성된 분자계통수에서 우리의 표본은 같은 종에 속하는 표본들과 단계통군을 형성하여 그 분류학적 위치가 명확하게 밝혀졌다. 이번 연구에서 새롭게 분석된 *P. cultellatus*의 미토콘드리아 유전체는 이후 flatheads의 분류와 분자계통을 위한 중요한 기초정보로 활용될 것이다.

찾아보기 낱말 : Flathead, 미토콘드리아 유전체, 분자계통, *Platycephalus cultellatus*, 쏨뱅이목, 베트남