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Morphology of a Larval Atlantic Footballfish *Himantolophus groenlandicus* Reinhardt, 1837 (Lophiiformes: Himantolophidae) Identified by Complete Mitochondrial DNA

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ABSTRACT A larva of the deep-sea angler fish, *Himantolophus groenlandicus* (2.2 mm BL), identified based on the complete mitochondrial DNA sequence, was collected at the surface of the western North Pacific. The postflexion stage larva had a round body, small teeth, incipient dorsal fin rays, eyes slightly recessed in the lower part, and melanophores on the gills and parietal and dorsal regions. These morphological features differ from a description of a larva reported as the same species with similar size (2.1 mm BL). The genetic and morphological information of our specimen should be useful for identifying larval *H. groenlandicus*.

Key words: Complete mitochondrial DNA, *Himantolophus groenlandicus*, larval fish, morphological description, species identification

INTRODUCTION

Himantolophus groenlandicus, belonged to Himantolophidae (Lophiiformes) comprising one genus and 22 species, is a deep-sea fish found in the Atlantic and Indo-Pacific Oceans (Pietsch, 1986; Priede, 2017). Larval *H. groenlandicus* is distributed below 50 m in the early stage and descends below 1,000 m during development and metamorphosis (Richards, 2005). Bertelsen (1951) described different developmental stages of larval *H. groenlandicus*. These descriptions have been cited in other larval fish identification guides (Richards, 2005; Fahay, 2007; Okiyama, 2014).

Morphological descriptions of larval fish are used to identify species (Leis and Carson-Ewart, 2000; Richards, 2005; Fahay, 2007; Okiyama, 2014), and the accuracy of species identification is enhanced by descriptions in similar size with the larval specimens (Powles and Markle, 1984). As they grow, the morphology of larval fish becomes similar to that of adults (Kendall *et al*., 1984). Unless larval fish are reared, their morphology according to developmental stages are difficult to be described (Hunter, 1984), especially for rare species (Leis *et al*., 2004).

Recently, the challenges of morphology-based species identification have been overcome by DNA barcoding (Hebert *et al*., 2003; Ko *et al*., 2013). Unlike the morphology changes during growth, DNA is maintained throughout the life of an organism (Avis, 1994). Partial mitochondrial DNA sequences like 12S, 16S, COI, and cytb are commonly used as DNA barcodes (Ward *et al*., 2005; Han *et al*., 2015; Miya *et al*., 2015; Choi *et al*., 2020). Species are determined by comparing intra- and interspecific genetic distances. However, there are genetic variations among different DNA barcodes (Viñas and Tudela, 2009; Choi *et al*., 2018). The complete mitochondrial DNA genome is helpful for searching specific DNA regions to distinguish species, and thus improve the accuracy of identification (Chagas *et al*., 2020).

In this study, we identified a larval fish (2.2 mm BL) collected from the surface of the north Western Pacific

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as *Himantolophus groenlandicus*, based on the complete mitogenome. Among the illustrations of larval *H. groenlandicus* of Bertelsen (1951), the smallest one (2.1 mm BL) was similar to our larval fish in size, but the morphological features differed markedly. Therefore, we report the morphology of our larval fish identified based on the mitogenome and compare it with the literature (Bertelsen, 1951).

MATERIALS AND METHODS

A larval fish was caught at 3 m depth of the western North Pacific (17.3686°N, 125.9984°E; Fig. 1) using a continuous underway fish egg sampler (300 μm mesh; C-100; Ocean Instruments, USA) mounted on R/V ISABU on 9 October 2019. The specimen was photographed using a digital camera attached to a stereomicroscope before and after being preserved in 95% ethanol. The specimen was stored in the Korea Institute of Ocean Science & Technology under the voucher name IB19C5L6.

Genomic DNA was extracted from the left eye of the larval fish following the MagListo^{TM} 5M Genomic DNA Extraction Kit protocol. First, we amplified the partial 16S rRNA region to determine the species using primers 16Sar (5′-CGCCTGTTTATCAAAAACAT-3′) and 16Sbr (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi, 1996). The PCR product was sequenced on a 3730xl DNA analyzer (Applied Biosystems, USA). The 16S rRNA sequence was searched for similar sequences

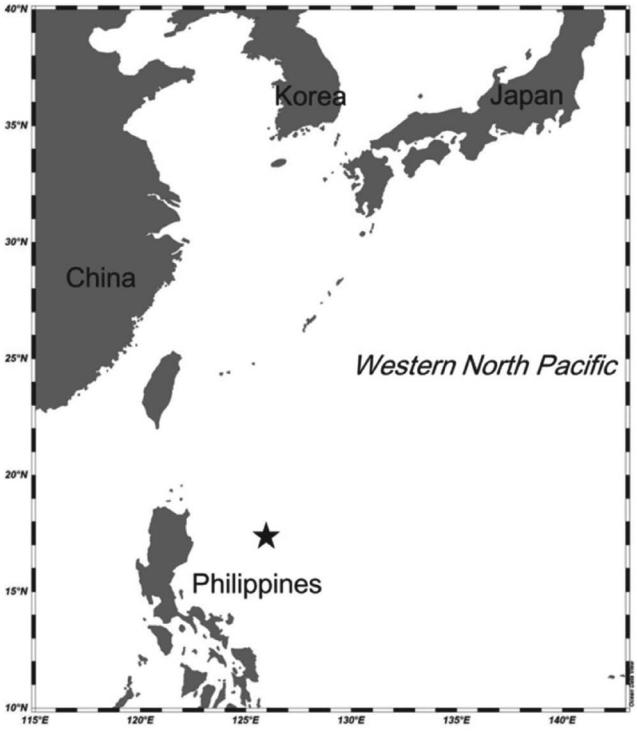


Fig. 1. Sampling station for a larval fish. Star indicates the station.

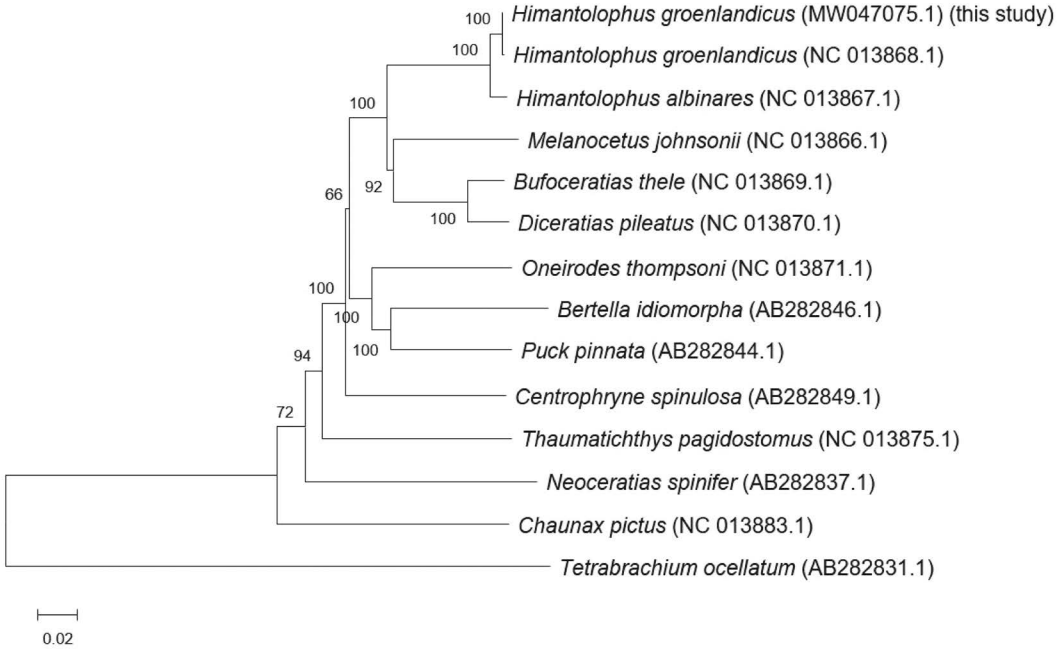


Fig. 2. Neighbor-joining tree constructed using concatenated sequences of 13 protein-coding genes and two rRNA genes from our larval fish and Lophiiformes. Numbers on the branches indicate bootstrap values (1,000 replicates).

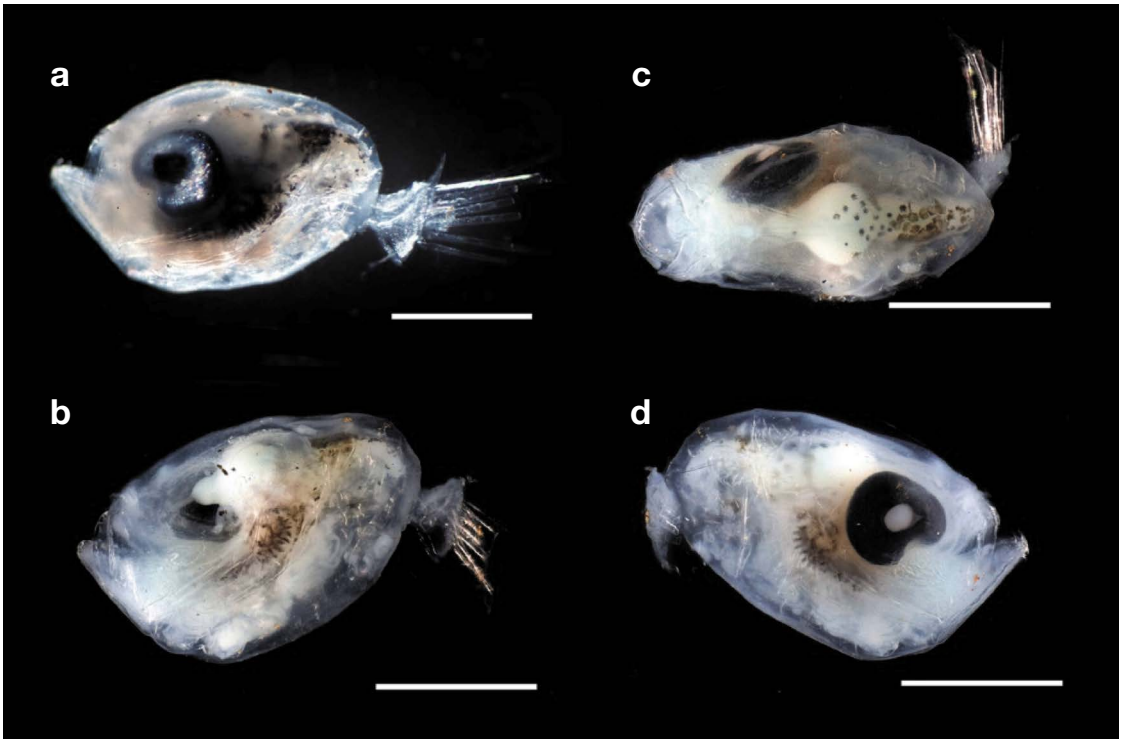


Fig. 3. Morphology of larval *Himantolophus groenlandicus*. a, left side of a fresh specimen; b∼d, left (b), dorsal (c), and right sides of the specimen after preservation in 95% ethanol. Scale bars, 1 mm.

using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi); this identified the Lophiiformes species *Himantolophus groenlandicus*, *H. albinares*, and *Linophryne bicornis*.

To identify larval fish at the species level using other DNA barcodes, we determined the complete mitochondrial DNA. To obtain sufficient DNA for sequencing, the larval fish gDNA was amplified using the REPLI-g mini kit (QIAGEN, Germany). The amplified product was purified with a MagListo^{TM} 5M PCR Purification Kit (Bioneer, Korea). For sequencing using a HiSeq 4000 instrument (2 × 150 bp; Illumina, USA), a sequencing library was prepared using the TruSeq Nano DNA kit. The library sample was run on the HiSeq 4000 instrument.

Using high-throughput sequencing, 83,146,602 raw reads were obtained. These were mapped to the *H. groenlandicus* reference sequence (GenBank acc. no. NC_013868.1; Miya *et al*., 2010) using Geneious (ver. 11.1.5; Kearse *et al*., 2012). A consensus sequence was then made with a mean coverage of 234×. The consensus sequence was extracted and annotated in Geneious. To verify the species, a neighbor-joining tree (Saitou and Nei, 1987) was constructed in MEGA-X (Kumar *et al*., 2018) using concatenated sequences of 13 protein-coding genes and two rRNA genes from the larval fish and Lophiiformes sequences obtained from NCBI/GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

The morphology of our larval fish was illustrated and compared with records of the same species in Bertelsen (1951), Richards (2005), Fahay (2007), and Okiyama (2014).

RESULTS

1. Mitogenome-based species identification

The complete mitogenome of the larval specimen was 16,438 bp long and comprised 13 protein-coding genes, 2 rRNAs, 22 tRNAs, and a control region (GenBank acc. no. MW047075). It was 27.7% A, 30.9% C, 17.0% G, and 24.3% T. The start codon of the protein-coding genes was ATG, except GTG for COX1 and ND4. There were five stop codons: TAA, TA, T, TAG, and AGA. The mitogenome was 99.9% identical to that of *H. groenlandicus* (NC_013868.1). The directions and positions of the genes were similar to those of Lophiiformes (Miya *et al*., 2010).

In the neighbor-joining tree based on concatenated sequences of 13 protein-coding genes and 2 rRNA genes from our larval fish and Lophiiformes, the larval fish formed a clade with *H. groenlandicus* (Fig. 2). Based on this, the larval fish was identified as *H. groenlandicus*.

2. Morphology

The larval fish identified as *H. groenlandicus* using the complete mitogenome had a round body with slightly inflated skin, and its notochord was bent (Fig. 3a). The fresh specimen had a body length (BL) of 2.5 mm. After preserving it in 95% ethanol, its morphology was somewhat deformed and the BL was decreased to 2.2 mm (Fig. 3b~d). The body depth was deep with 71% of the BL and the tail was short. The snout and head lengths were 15% and 60% of the BL, respectively. The eye diameter was 29% of the BL and the lower left part was dented. The mouth was oblique. The lower jaw had a pointed tip and the upper jaw had a small split in the center (Fig. 4). There were six teeth on the upper jaw and eight on the lower. There were small papillae on both sides of the upper jaw. Four incipient dorsal fin rays were under the epidermis. The first dorsal fin rays were located at 69% of the BL. The base of the pectoral fin was located in the middle, between the posterior eyes and first dorsal fin rays. The caudal fins were developed but damaged. The left caudal fin had eight rays. The lower part of the caudal fin was split. Melanophores were dense across the gills and dorsal surface and sparsely distributed in the parietal region.

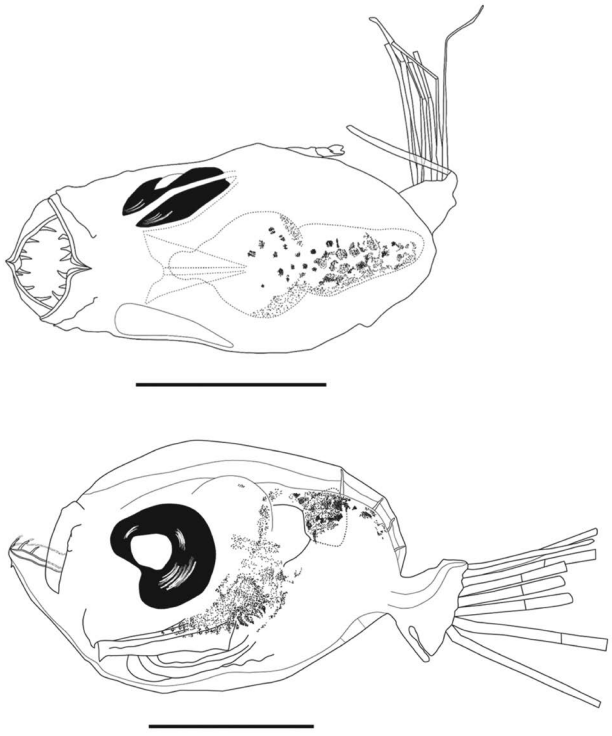
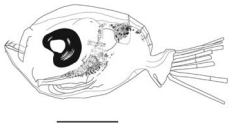
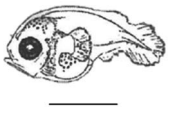


Fig. 4. Illustrations of the larval *Himantolophus groenlandicus* of this study. The body shape is based on the fresh specimen (Fig. 2a). Scale bars, 1 mm.

DISCUSSION

Larval fish provide information about spawning areas, nursery grounds, and population connectivity (Tsukamoto, 1992; Cowen and Sponaugle, 2009; Guerreiro *et al*., 2021). The first step in understanding fish ecology using larvae is species identification (Powles and Markle, 1984). The accuracy of morphological identification on

Table 1. Comparison of the morphological characteristics of larval *Himantolophus groenlandicus* between this study and references. Scale bars in morphology is 1 mm.

Characters	*H. groenlandicus* (this study)	*H. groenlandicus* (Bertelsen, 1951)
Morphology		
Body length (mm)	2.2 (preserved) 2.5 (fresh)	2.1
Developmental stage	Postflexion	Preflexion
Mouth angle	Oblique	Vertical
Shape of eye	Left lower part dented	Round
Teeth on upper jaw	6	0 (in description)
Teeth on lower jaw	8	0 (in description)
Melanophore distribution	Post parietal, dorsal, gill	Medullary, gill, gill cover peritoneum, peduncle, base of caudal fin
Incipient dorsal fin rays	4	0 (in description)
Caudal fin rays	Developed	Not developed

larval fish is affected by species descriptions, the condition of the larval specimen, and the researcher's experience (Rodriguez *et al*., 2017; Smith *et al*., 2018). The morphology-based identification can be verified by DNA barcoding (Ko *et al*., 2013; Wakabayashi *et al*., 2017). However, the DNA barcoding also has limitations due to the absence of sequences for comparison, unsuitability for distinguishing species, and instances where it was obtained from morphologically misidentified adults (Kim *et al*., 2008). Thus, a combination of morphological and genetic analyses is needed to improve the identification of larval fish (Leis, 2014).

The larval fish (2.2 mm BL) studied here was identified as *Himantolophus groenlandicus* based on the complete mitogenome. Before determining species using the complete mitogenome, a partial 16S rRNA of the larva was analyzed. However, the sequence was too similar to related taxa to distinguish them. Therefore, we used the concatenated sequence of 13 protein-coding genes and 2 rRNA genes from the complete mitogenome of our larval fish to determine the species, which had the highest similarity (99.9%) with that of *H. groenlandicus* (NC_013868.1; Miya *et al*., 2010) and formed a clade with the *H. groenlandicus* sequence in a neighbor-joining tree constructed with other Lophiiformes.

While there are illustrations of larval *H. groenlandicus*, we could not identify the species using them. The youngest larval fish (2.1 mm BL) described by Bertelsen (1951) based on specimen collected from the north west Atlantic was similar in size to our larval fish. However, there were significant differences in eye shapes, melanophore distribution, dorsal spines, tooth development, and caudal fin (Table 1). Above all, in the literature, it is estimated that the larval fish (2.1 mm BL) have just hatched. The earlier the developmental stage of larval fish, the fewer morphological traits available for species identification (Kendall *et al*., 1984). Considering the developmental stage and morphological differences, the classification of the larval *H. groenlandicus* (2.1 mm BL; Bertelsen, 1951) may need to be reexamined. Despite this ambiguity of the morphological descriptions, the larval fish of this study was identified into *H. groenlandicus* based on DNA sequences. The genetic and new morphological information of our specimen should be useful for identifying larval *H. groenlandicus*.

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미토콘드리아 전장 유전체로 동정한 아귀목 *Himantolophus groenlandicus* 자어의 형태적 특징

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요 약 : 북서태평양 표층에서 채집한 후기 자어(2.2 mm BL)의 미토콘드리아 전장 유전체를 근거로 *Himantolophus groenlandicus*로 동정하였다. 후기 자어는 둥근 몸통, 작은 이빨, 발달하기 시작한 등 지느러미, 아래 부분이 들어간 눈, 그리고 지느러미, 정수리, 등 부분에 분포하는 흑색소포를 가졌다. 이러한 특징은 크기가 유사한 동종으로 보고된 자어(2.1 mm BL)와 차이가 컸다. 본 연구 자어의 유전적, 형태적 특징은 유사종의 동정에 유용할 것이다.

찾아보기 낱말: 미토콘드리아 전장 유전체, 자치어, 종 동정, 형태 기재, *Himantolophus groenlandicus*