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Little genetic difference between controversial Japanese codling species *Physiculus japonicus* and *P. maximowiczi*

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Abstract

Two Japanese codling *Physiculus japonicus* Hilgendorf, 1879 and *P. maximowiczi* (Herzenstein, 1896) have been suspected to be conspecific. Many Japanese literatures have described phenotypic differences between these two morphs; *P. japonicus* possessing larger eye (eye diameter/snout length larger than 2/3) and pale brown body is distributed deep seafloor (150–650m) in south of Tokyo Bay, while *P. maximowiczi* possessing smaller eye (eye diameter/snout length less than 2/3) and dark brown body is distributed shallow seafloor (≤ several tens of meters) in south of Hokkaido. We collected 44 *Physiculus* individuals (13 from shallow area and 31 from deep area) from northern to central Japan and performed nucleotide sequence analysis on the mitochondrial COI, 16S rRNA and Dloop regions. Body color largely varied, but dark brown individuals were abundant in the shallow group and pale brown individuals were abundant in the deep group. Ratio of eye diameter to snout length also largely varied, but the average was larger in the deep group than in the shallow group. In contrast, genetic difference among individuals and between shallow and deep groups was very small, and phylogenetic analysis indicated all individuals analyzed to be conspecific. These indicate that *P. maximowiczi* is a junior synonym of *P. japonicus* and phenotypic differences are intraspecific variation probably in response to the environmental diversity.

Key words: mtDNA; morphology; *Physiculus japonicus*; *P. maximowiczi*; synonym

Introduction

Morid fish of the genus *Physiculus* Kaup, 1858 (Gadiformes) can be distinguished from other genera of the family Moridae, by having ventral light organ, chin barbel and toothless vomer (Paulin 1989). Two Japanese codling species, *Physiculus japonicus* Hilgendorf, 1879 (“Chigodara” in Japanese) and *Physiculus maximowiczi* (Herzenstein, 1896) (“Ezoisoainame” in Japanese), are common in the shallow to deep seafloor along Japanese coastal area. Cohen (1979) compared the holotypes and strongly suggested these to be the same species, although some differences were recognized. After examining several specimens collected in Japan, Paulin (1989) noted the morphological variations observed between the holotypes to fall within local variation and proposed *P. maximowiczi* to be a junior synonym of *P. japonicus*. Yu and Ho (2012) also observed large morphological variation among *P. japonicus* or *P. maximowiczi* in Taiwan, supporting conclusion by Paulin (1989). *P. maximowiczi* was on a checklist of the fishes of Taiwan (Shao et al. 2008) but not included in more recent database (Shao and Liao 2015). Furthermore, *P. maximowiczi* is no longer listed in

catalogue of world gadiform fishes (Cohen et al. 1990). However, almost all literatures published in Japan have treated these two as distinct species (see Abe 1963; Okamura 1982, 1984a,b; Sakai 1986; Taki et al. 2005; Amaoka et al. 2011; Nakabo and Kai 2013). The literatures all described differences in the distribution, eye size and body color between these two species; *P. japonicus* is distributed deep seafloor (150–650m) in south of Tokyo Bay, possessing larger eye (eye diameter/snout length larger than 2/3) and pale brown body, while *P. maximowiczi* is distributed shallow seafloor (≤ several tens of meters) in south of Hakodate, Hokkaido, possessing smaller eye (eye diameter/snout length less than 2/3) and dark brown body. In contrast, Kitagawa and Nagahora (1983) considered all codlings collected wide depth range (50-330m) along the coastal area of Iwate Prefecture, Japan, to be *P. maximowiczi*. Recently, Nakabo (2018) raised question on the specific status of *P. maximowiczi*, since no distinct morphological difference has been found between these two forms. In order to resolve these confusions, we performed molecular genetic analysis on *Physiculus* samples collected in the waters from northern (Hakodate, Hokkaido) (near the type locality of *P. maximowitczi*) to central (Yokosuka, Kanagawa) (near the type locality of *P. japonicus*) Japan.

Materials and Methods

Collection locations and descriptions of the Japanese codling samples used in this study are presented in Table 1 and Fig. 1. All individuals landed were frozen and transferred to the laboratory. After thawing, fishes were photographed, and body length, snout length, and eye diameter were measured. All individuals were identified to be *P. japonicus* or *P. maximowiczi* according to Nakabo and Kai (2013). Body color considerably varied as shown in Fig. 2, but we subjectively determined four classes; dark (d) (Fig. 2A), half dark (h) (Fig. 2B), brown (b) (Fig. 2C), and pale brown (p) (Fig. 2D). Individuals determined to be "half dark" possessed dark anterior part and brown posterior part. A small piece of muscle was dissected and preserved in 80 % ethanol. These individuals (n = 44) were deposited in the Seikai National Research Institute, Fish Specimens Collection, National Fisheries Research Agency, Nagasaki, Japan (catalog No. of SNFR21531, 21532, 21543–21562, 21581–21600, 21714, 21715).

DNA samples of the muscle were extracted using a DNA extraction kit (Genomic Prep Cell and Tissue DNA Isolation Kit, Amersham Bioscience), which were used to PCR amplification. Primer pairs used to amplify partial sequences of the mitochondrial cytochrome oxidase subunit I (COI) and 16S rRNA (16S) genes and Dloop region (CR) are presented in Table 2. These three regions were amplified as described previously (Chow et al. 2017). Amplicons were treated with ExoSAP-IT (Amersham Biosciences) to remove primers and subjected to direct nucleotide sequencing using the PCR primers. Nucleotide sequence data determined for COI (576–668 bp, n = 44), 16S (539–561 bp, n = 40), and CR (474–503 bp, n = 44) are available in DNA database of Japan (DDBJ), European Molecular Biology Laboratory (EMBL) and GenBank under accession numbers of LC487998–LC488125. Calculation of Kimura's

two parameter (K2P) distance between sequences and construction of phylogenetic tree were performed using MEGA 6 (Tamura et al. 2013).

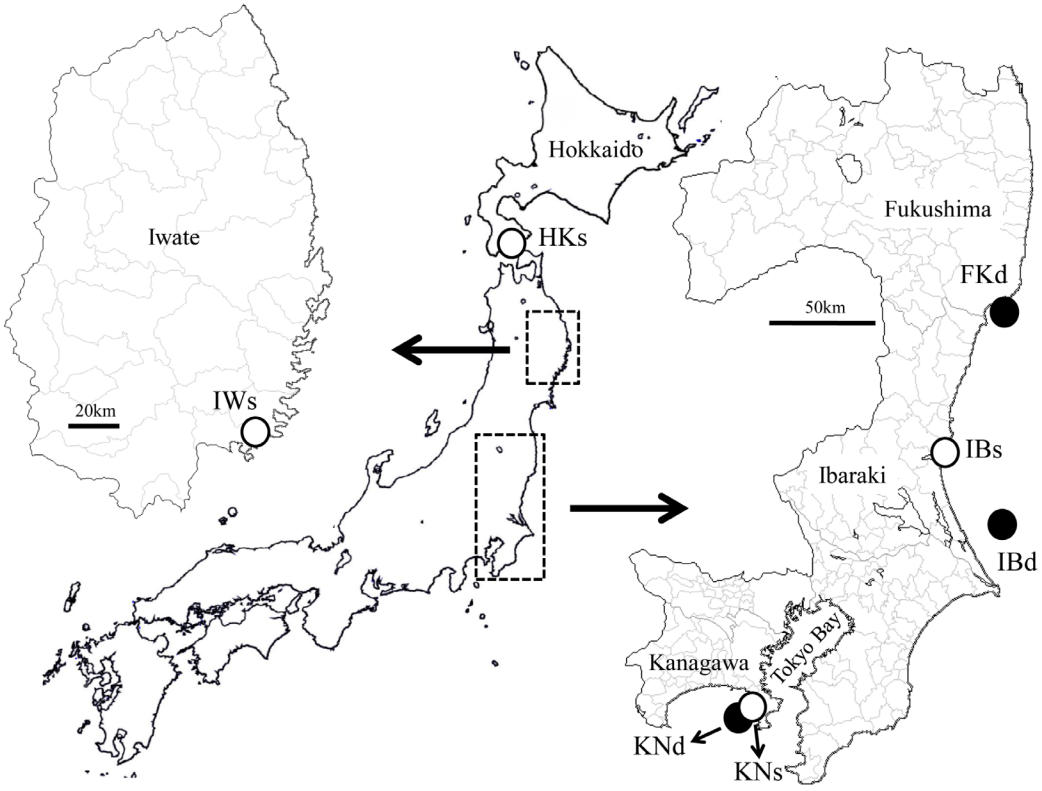

Fig. 1. Map showing collection locations of codling samples of the genus *Physiculus* in Japan. Open and closed circles represent samples collected at shallow seafloor (≤ 80 m) and deep seafloor (≥ 200 m), respectively. See Table 1 for information of these local samples.

Table 1. Seven local codling samples (*Physiculus*) used in this study.

Sample[a]	n	date	Depth (m)	Gear[b]	SL (mm)[c]	% E/S[d]	d/h/b/p[e]	SNFR No.
HKs	2	February 2018	~10	PL	264.8±9.5	65.3±7.9 (59.7–70.9)	2/0/0/0	21714, 21715
IWs	6	November 2016	~80	SN	251.0±19.3	76.2±4.5 (70.1–81.2)	0/0/6/0	21543–21548
FKd	7	November 2016	240	BT	319.1±34.5	69.0±5.5 (60.9–78.9)	0/1/6/0	21581–21587
IBs	3	February 2017	~10	PL	205.2±47.3	77.5±7.0 (70.5–84.5)	2/0/1/0	21598–21600
IBd	10	January 2017	350–450	BT	222.6±24.3	80.8±8.9 (66.8–93.2)	0/3/4/3	21588–21597
KNs	2	July 2017	~30	SN	190.3±14.6	82.7±8.6 (76.7–88.8)	0/0/2/0	21531, 21532
KNd	14	February 2017	~200	BC	247.5±16.1	81.0±5.9 (67.1–90.2)	0/0/14/0	21549–21562

[a]See Fig. 1 for catch location. [b]PL: pole and line; SN: set net; BT: bottom trawl; BC: bottom trap cage. [c]standard length ± S.D. [d]percent of eye diameter to snout length ± S.D. and the range (parenthesis). [e]number of individuals possessing different body color; d: dark; h: half dark; b: brown; p: pale.

Table 2. Primers used to amplify three partial mitochondrial DNA regions.

Region	Primer	Sequence (5'-3')	Source
COI	L5956	CACAAAGACATTGGCACCCT	Liu et al. (2006a)
	H6558	CTCAGAATGACATTTGTCCTGA	Liu et al. (2006a)
16S rRNA	16Sar-L	CGCCTGTTTATCAAAAACAT	Palumbi et al. (1991)
	16Sbr-H	GGTCTGAACTCAGATCACGT	Palumbi et al. (1991)
control region	A	TTCCACCTCTAACTCCCAAAGCTAG	Lee et al. (1995)
	B	ACGCTGGAAAGAACGCCCGGCATGG	Lee et al. (1995)

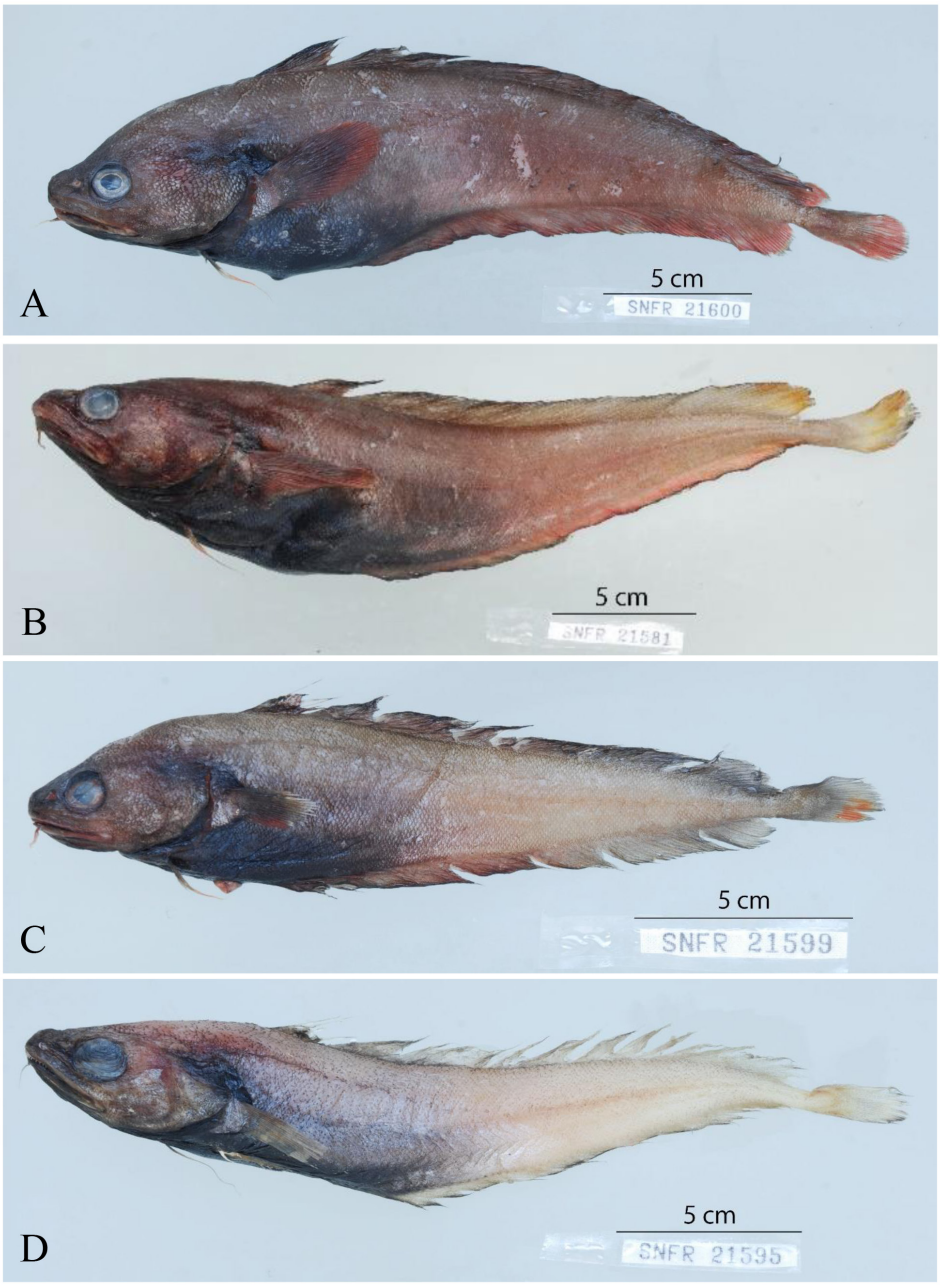

Fig. 2. Photographs showing color variation in *Physiculus* individuals. A: dark; B: half dark, C: brown; D: pale brown. A (SNFR21600: Ibaraki, shallow), B (SNFR21581: Fukushima, deep), C (SNFR21599: Ibaraki, shallow), D (SNFR21595: Ibaraki, deep).

Result

Phenotypic variation

Based on the catch depth, four samples (HKs, IWs, IBs, and KNs) (n = 13) were supposed to be *P. maximowiczi* (shallow group) and three (FKd, IBd, and KNd) (n = 31) to be *P. japonicus* (deep group) (Table 1). The ratio of eye diameter to snout length (E/S) considerably varied among individuals. Based on the E/S criterion determined previously, only three individuals (SNFR 21585, 21587, and 21715) collected in the shallow area of Hokkaido (HKs) and deep area of Fukushima Prefecture (FKd) corresponded to *P. maximowiczi*. E/S of the shallow group ranged from 59.7 to 88.8 % with an average of 75.8 ± 7.4 % S.D., and that of the deep group ranged from 60.9 to 93.2 % with an average of 78.2 ± 8.4 % S.D., in which the average in the shallow group was slightly smaller than that in the deep group but not significantly different (Mann-Whitney U test, p = 0.322). Body color also varied considerably (Fig. 2, Table 1), in which individuals collected in the shallow area were not always dark and those in the deep area were not always pale. However, frequencies of four body color classes were significantly heterogenous between the shallow and deep groups (χ^2 test, p = 0.007) (Table 1), indicating dark individuals were abundant in the shallow group and pale brown individuals were abundant in the deep group.

Genetic variation

Mean ± S.E. K2P distances among all individuals were 0.5 ± 0.1 % for COI, 0.1 ± 0.1 % for 16S and 1.9 ± 0.3 % for CR, and those between the shallow and deep groups were 0.6 ± 0.1 % for COI, 0.1 ± 0.1 % for 16S and 1.9 ± 0.3 % for CR. A total of 36 COI sequences of 10 *Physiculus* species were available in the database, which were incorporated into our COI sequence alignment. Phylogenetic relationships for these COI sequences were assessed using a maximum likelihood (ML) analysis under HKY+G+I as the best fit model selected by MEGA6 (Fig. 3). All of our sequences with five *P. japonicus* and one *P. maximowiczi* sequences derived from database formed a distinct cohesive clade (designated as Japanese codlings) substantially divergent from the other group (designated as other codlings). Two *P. japonicus* (KU943148 and KU943149) and one *P. maximowiczi* (KP266809) sequences (boxed) derived from database were clustered in the other codling species, which may be due to misidentification. One *P. rastrelliger* (KF918891) clustered with *P. nematopus* may be also due to misidentification (asterisk). Mean intraspecific K2P distances of COI in *P. capensis*, *P. fulvis*, and *P. natalensis* were 0.6 ± 0.2 %, 0.4 ± 0.1 %, and 0.2 ± 0.1 %, respectively, comparable with that in our codling sample. In the Japanese codling clade, no distinct clustering of depth groups or local samples was observed.

Discussion

Genetic distances among individuals and between depth groups in *Physiculus* samples used in the present study are very small and fall well within the range of intraspecific divergence in the fish COI (Ward et al. 2005; Kochzius et al. 2010; Chang et al. 2017), 16S (Kochzius et al. 2010; Cawthorn et al. 2012), and CR (Nomura et al. 2004; Liu et al. 2006a,b; Grant et al. 2012; Gwak

et al. 2015; Gu et al. 2016; Mansourkiaei et al. 2016). Phylogenetic analysis also indicates that all samples are conspecific, corroborating implications of Cohen (1979) and Nakabo (2018) that *P. maximowiczi* may be a junior synonym of *P. japonicus*. The eye size and body color may differ between depth groups, but it is likely that these characteristics may be intraspecific variation probably in response to the environmental diversity. Also, variations of these characters were gradual

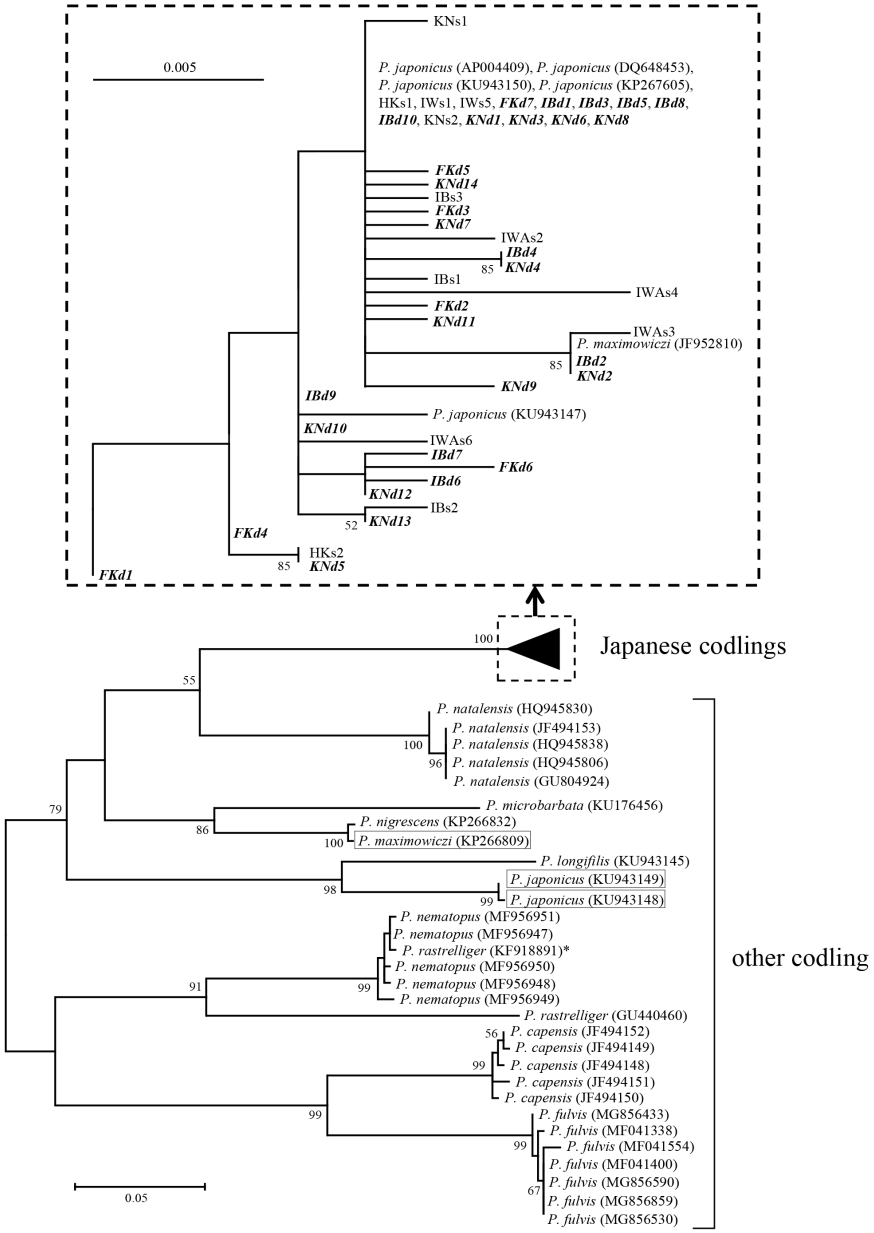

Fig. 3. A maximum-likelihood (ML) tree of the cytochrome oxidase subunit I (COI) sequences of 44 individuals of *Physiculus* determined in the present study and 36 individuals of 10 *Physiculus* species derived from database. Values on branches indicate bootstrap probability with 1,000 replications. Sequences boxed or with asterisk are probably from misidentified individuals. In the Japanese codling clade, sample names in bold italic are individuals from deep seafloor (≥ 200 m) and those in plain text are from shallow seafloor (≤ 80 m).

and continuous, but not clearly separable character states. This indicates that these characters are invalid to separate species. The results of our molecular and morphological analyses support that *P. maximowitczi* is a junior synonym of *P. japonicus* as pointed by Paulin (1989), accordingly the Japanese standard name "Ezoisoainame" for *P. maximowitczi* should be unused. The distribution range of *P. japonicus*, therefore, must be redefined to be wider in the horizontal dimension (south of Hokkaido) and in the vertical dimension (shallow coastal area to the deep ~650 m or more) than previously determined.

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チゴダラ *Physiculus japonicus* とエゾイソアイナメ *P. maximowiczi* 間には遺伝的差異がほとんどない

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チゴダラとエゾイソアイナメ間には顕著な形態差がなく同種の可能性が指摘されてきたものの本邦ではこれらを別種として扱ってきた。その根拠として、前者は東京湾以南の深海（150–650m）に分布し、眼径が大きく体色が淡褐色であること、後者は函館以南の浅海（数 10m 以浅）に分布し、眼径が小さく体色が濃褐色であること、が挙げられている。本研究では北海道から神奈川県の範囲で 44 個体のチゴダラ類標本を採集し、上記の表現型とともにミトコンドリア DNA の 3 領域（COI、16S rDNA、Dloop）の塩基配列を分析した。体色は個体間変異が大きいものの浅場標本（80m 以浅）では濃褐色個体が多く、深場標本（200m 以深）では淡褐色個体が多かった。眼径/吻長も個体間変異が大きく深場標本と浅場標本の平均値間に有意差はなかった。個体間および浅深標本間の遺伝的差異は非常に小さく種内個体間レベルの範囲であることが示された。データベースより入手したチゴダラ属他種の配列を加えた系統樹解析においても、本研究で分析した個体は全て独立したクレードに属し同種と考えられた。以上のことからチゴダラとエゾイソアイナメは同種であり、体色や眼径の変異は種内個体間差であることが示された。

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