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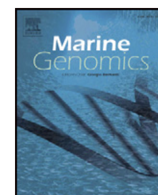

The complete mitochondrial genome sequence of the network pipefish (*Corythoichthys flavofasciatus*) and the analyses of phylogenetic relationships within the Syngnathidae species

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ABSTRACT

This study determined the mitochondrial genome sequence of the network pipefish (*Corythoichthys flavofasciatus*) (Gasterosteiformes: Syngnathidae). The mitogenome was a circular molecule consisting of 16,961 nucleotides, including 13 protein-coding genes, 22 tRNA genes, two rRNA genes and a control region. The nucleotide composition of the genome was biased toward A + T content at 59.3%. All tRNA genes had typical cloverleaf secondary structure except for $tRNA^{Ser\ (AGY)}$, in which the dihydrouridine arm was missing. The *C. flavofasciatus* control region of 1130 bp contained several features common to other teleost, including conserved sequence blocks. We also performed comparative analysis of the network pipefish mitogenome to the available mitogenome sequences of other Syngnathidae species, and phylogenetic relationship of the Syngnathidae species was constructed based on the data sets including all the concatenated nucleotide sequences of the mitogenomes except the third codon positions. Partitioned Bayesian inference and maximum likelihood analyses showed that all seahorse species formed a monophyletic group of *Hippocampus* with 100% PPs and BPs, but the pipefish species did not form a monophyletic group. *Corythoichthys flavofasciatus* was placed as a sister relationship to the *Hippocampus* clade by strong node-supporting values. The availability of mitogenome of the network pipefish will shed light on the molecular systematics, biogeography and genetic differentiation in this species.

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1. Introduction

The network pipefish *Corythoichthys flavofasciatus* is a member of the subfamily Syngnathidae (Gasterosteiformes: Syngnathidae). Syngnathidae is an assemblage of pipefishes, seahorses and seadragons. In China, this species is distributed in the areas of East China Sea and South China Sea. The network pipefish mainly lives in shallow reef areas from the tide pool to 25 m deep, and feeds on small zooplankton (Dawson, 1985). Their evolutionary biology has been of continuing interest due to their shape diversity and interesting behaviors.

The typical vertebrate mitochondrial genome includes a set of 13 protein-coding genes (PCGs), 2 ribosomal RNA genes (12S rRNA and 16S rRNA) and 22 tRNA genes. Additionally, there are two major non-coding regions, including the light-strand (L-strand) origin of replication (O_L) and the control region (CR) (Boore, 1999). Recent work showed that large scale reorganizations of gene orders had occurred in some fishes (Inoue et al., 2003), while the organization of gene order in short-tail pipefish (*Microphis brachyurus*) looked normal (Kawahara et al., 2008).

The mitochondrial DNA (mtDNA) is generally considered as a useful molecular marker for phylogenetic analyses and species identification. Recently, mtDNA has also been used for DNA barcoding for identification on the level of species and population (Ko et al., 2013). However, short mtDNA gene fragments exhibit limitations in resolving complicated phylogenetic relationships in many fish lineages (Steplen and Kocher, 1997). The additional informative sites from mitogenomes allow these deeper branches to be better resolved (Miya and Nishida, 2000). Hence, the mitogenomes provided in this study will help resolving the evolutionary relationships with Syngnathidae.

To date, only several pipefish's mitogenomes had been completely sequenced (Kawahara et al., 2008) (Song et al., 2014). To better understand the structure of the pipefish mitogenome and its classification status, we sequenced the complete mitogenome of *C. flavofasciatus* and used the results in a phylogenetic analysis of Syngnathidae.

2. Materials and methods

2.1. Genomic DNA extraction

An adult pipefish of *C. flavofasciatus* was collected from area of South China sea in August 2013 and was directly frozen in liquid nitrogen and stored in the laboratory at −80 °C. Total genomic DNA was extracted

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from this single sample using TIANGEN marine animal DNA kit (TIANGEN, China) according to the manufacturer's instruction. The DNA integrity was checked by agarose gel electrophoresis, and the purity was established by calculating the ratio of the absorbance readings at 260 and 280 nm. Then the DNA was used for PCR amplification.

2.2. Primer design, PCR amplification and sequencing

The degenerated primers (Table S1) for fragment amplifications were designed based on conserved nucleotide sequences of *Hippocampus kuda* and *Microphis brachyurus* mitochondrial genome, using DNAssist 2.2 and Primer Premier 5.0 software (Singh et al., 1998).

All PCR reactions were performed in a T-100 thermocycler (BIO-RAD, USA) using TaKaRa LA Taq polymerase under the following conditions: denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 50–55 °C for 30 s and 68 °C for 2–4.5 min. The reaction was further incubated for 10 min at 68 °C. The PCR amplification products examined by 1.5% agarose gel were purified using E.Z.N.A. Gel Extraction Kit (Omega BioTek, USA) and ligated into the pMD18-T vector (TaKaRa, Japan). Three different individual positive clones per each fragment were sequenced on the ABI 3730 sequencer (Applied Biosystems, USA).

2.3. Sequence assembly, annotation and analysis

The mtDNA sequences from overlapping fragments were proofread and assembled using Bioedit 7.0.1 software. The majority of tRNA genes were identified by the program tRNAscan-SE1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/) (Lowe and Eddy, 1997). The rest of tRNA genes, PCGs and ribosomal RNA genes were identified by comparison with homologous mtDNA sequence of previously published short-tailed pipefish (Kawahara et al., 2008). The tRNA secondary structures were predicted by iRNA structure 4.5 (Reuter and Mathews, 2010). The map of mitogenome was generated by CGview (http://stothard.afns.ualberta.ca/cgview_server/) (Stothard and Wishart, 2005).

Nucleotide composition of the complete mitochondrial genome of *C. flavofasciatus* was calculated using MEGA 4.0 (Tamura et al., 2007). The skews analysis was used to describe the base composition of nucleotide sequences. The relative number was calculated using the formulas: AT skew = [A − T] / [A + T] and GC skew = [G − C] / [G + C] (Lee and Kocher, 1995).

2.4. Phylogenetic analysis

To examine phylogenetic relationships of Syngnathidae in Gasterosteiformes, thirteen mitogenomes of Syngnathidae were downloaded from GenBank (Table S2). Those species were obtained from the pipefish and seahorse family. *Solenostomus paradoxus* and three-spined stickleback (*Gasterosteus aculeatus*) were selected as outgroup for this phylogenetic analysis, for Solenostomidae is the most closely related family to Syngnathidae (Song et al., 2014). The mitogenome sequences of 13 PCGs were aligned with ClustalX 2.0

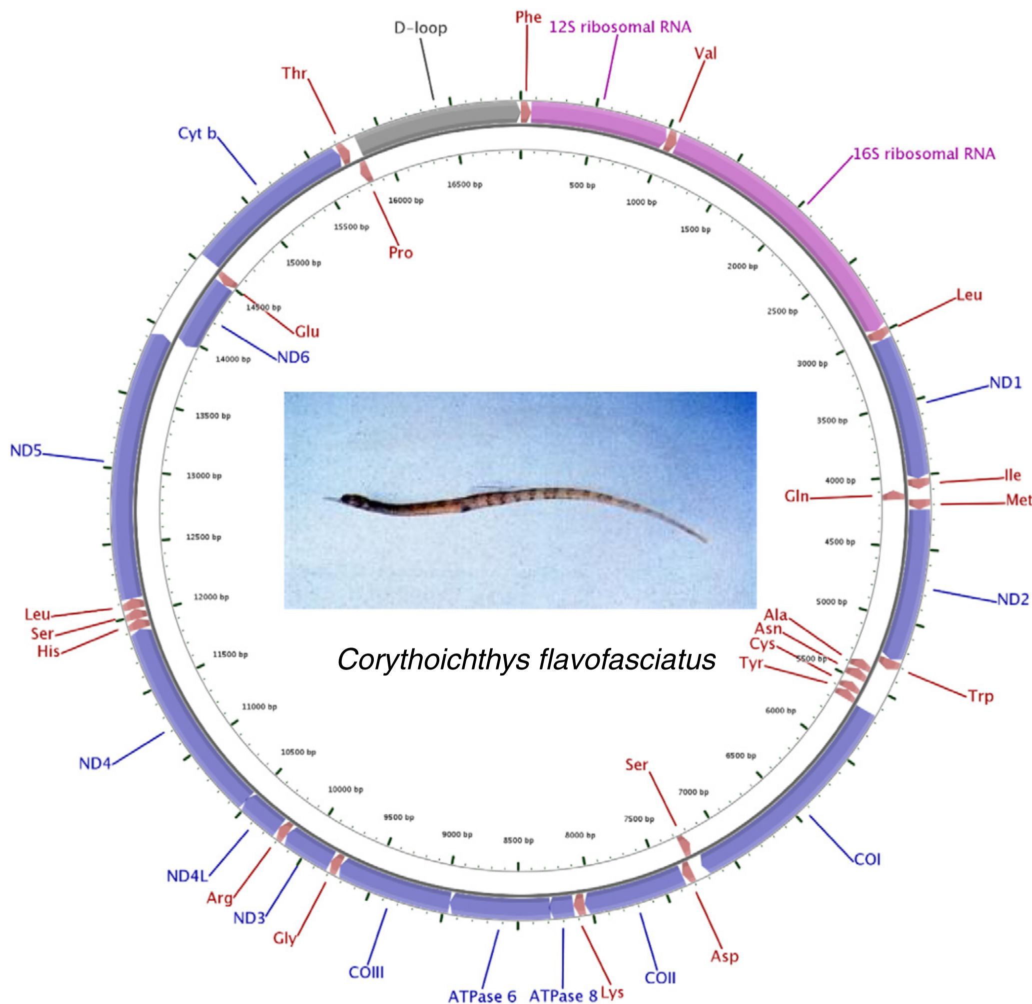

Fig. 1. Genetic map of the *C. flavofasciatus* mitochondrial genome. Annotation arrows indicate orientation of genes; COI-III indicates cytochrome oxidase subunits I–III; ATP 6/8, ATPase subunit 6/8; ND1-6/4L, NADH dehydrogenase 1-6/4L, and Cyt b, Cytochrome b.

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