A set of new primers for COI (cytochrome oxidase subunit 1 mitochondrial gene) amplification in *Phyllodiaptomus tunguidus* (Copepoda, Calanoida, Diaptomidae)

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**ABSTRACT**

Although the traditional Folmer and Prosser primers enable successful amplification of COI ‘barcoding’ sequences in freshwater zooplankton species, many freshwater zooplankters including *Phyllodiaptomus tunguidus* are difficult to be amplified using traditional primers. *Phyllodiaptomus tunguidus* is an endemic calanoid copepod species, widely distributed in (sub)tropical China that plays an important role in freshwater ecosystems. In this study, we developed a new pair of primers for *P. tunguidus* with significantly higher amplification success rate. The new primers will facilitate the recovery of barcode data from this and probably other copepod species, and stimulate further studies of phylogeny and genetic diversity on *P. tunguidus*.

**Introduction**

Copepods are abundant and diverse in freshwaters, occurring as a major component of most planktonic, benthic and groundwater metazoan communities (Dussart and Defaye 2001, 2002). Copepods often dominate zooplankton communities, play a crucial role as primary and secondary consumers in aquatic ecosystems, and are a major food source for other aquatic predators. Calanoid copepods (Copepoda: Calanoida) in inland waters are principally represented by the family Diaptomidae (Huys and Boxshall 1991; Boxshall and Jaume 2000; Dussart and Defaye 2001). Many genera of Diaptomidae are endemic to particular continents or parts of continents. *Phyllodiaptomus* conforms to this pattern and has its highest species richness in Southeast Asia (Sanoamuang and Teeramaethee 2006). *Phyllodiaptomus tunguidus* (Copepod, Calanoida, Diaptomidae) is endemic to China, and widely distributes in reservoirs and ponds of (sub)tropical China. It is the dominant species of zooplankton in most reservoirs in southern China (Lin et al. 2003; Zhao and Han 2007). In spite of its importance, there is no published molecular information on this keystone species.

The mitochondrial COI gene fragment known as the barcoding sequence is a widely used DNA marker for species identification in the ongoing ‘barcoding of life’ project and proved to be a suitable marker for species identification in crustaceans (Hajibabaei et al. 2005; Costa et al. 2007). However, successful amplifications of COI are not always achieved in freshwater zooplankton species. Currently, ‘Folmer’ (Folmer et al. 1994) and ‘Prosser’ primers (Prosser et al. 2013) are the most frequently used universal primers for Copepods. Oddly, in *P. tunguidus* the gene appears to be more difficult to amplify than in most other copepods when using those universal primers. Therefore, our aim of this work was to develop a specific pair of primers to amplify the COI barcoding fragment of *P. tunguidus* reliably. This primer pair will greatly promote further studies in phylogeny and population genetics of *P. tunguidus*. Possibly, it may apply to other species as well.

**Material and methods**

**Primer design**

The complete mitochondrial genome of *P. tunguidus* was sequenced and uploaded to GenBank database (accession number: MN927223) in 2020, and the COI genes were excised and aligned with COI sequences of *Phyllodiaptomus* sp. (downloaded from GenBank database, accession number: JN183940) using BioEdit (Hall 1999). Potential primer regions were analyzed using Primer version 6.0 (Primer Biosoft Inst., Palo Alto, CA) with default parameters. A new primer set was then manually designed based on flanking the more polymorphic regions of the COI gene.

**DNA extraction, PCR and sequencing**

Whole genomic DNA was extracted from 24 specimens of *P. tunguidus* collected from the Qingshitan (25.30° N, 110.09° E) and Gaozhou reservoirs (22.23° N, 111.03° E) in southern China, using Chelex 100 (Bio-Rad Laboratories, CA, USA).
All collected specimens and extracts were stored at −20°C at the Institute of Hydrobiology, Jinan University, Guangzhou, China.

Two populations of *P. tunguidus* were used to test the effectiveness of the new primer pair, Gaozhou population from tropical and Qingshitan population from subtropical China. Of these two populations, DNA extracts from 12 specimens were amplified. Validation of DNA extraction were done with CYTB specific primers (L10319/H10648; Machida et al. 2004). In addition, we compared the success rate of the new pair of primers to two other pairs, Folmer (LCO1490/HCO2198) and Prosser (ZplankF1/ZplankR1) (Folmer et al. 1994; Prosser et al. 2013). PCR amplification was carried out in PCR solution with 3 μl 10 PCR buffer, 1.2 μl dNTPs, 0.5 μl of 20 μM solution of each primer, 21.5 μl ddH₂O, 0.3 μl Taq DNA polymerase, 3 μl template. The PCR conditions for amplification were as follows: initial denaturation 1 min at 95°C, followed by five cycles of 94°C (for 40 s, 45°C for 40 s, 72°C for 1 min), then 35 cycles of 94°C (for 40 s, 51°C for 40 s, 72°C for 1 min) and final extension of 72°C for 7 min.

PCR products were sequenced on an ABI 3730XL automated sequencer with both forward and reverse of *Phyllodiaptomus*-specific primers.

**Results**

The new primers are: forward PhylloF (5’-CCAATCGACAATAGCATAAA-3’) and reverse PhylloR (5’-AGATATGCGCTTGCTCGAAATC-3’). They were designed inside the most conservative region of the COI gene, and based on the completed mitochondrial genome of *P. tunguidus*.

To compare the efficacy of our new primer set to traditional Folmer primers and Zplank primers, we extracted DNA from 24 specimens of two different regions. All of these 24 samples from two populations generated positive PCR products with CYTB primers. The PCR success rate for traditional ZP primers was 29% and no positive band available for Folmer primers. As predicted, the new *Phyllodiaptomus*-specific primers were highly effective (100%) in amplifying the target region of COI (Figure 1). Sequences with 573 bp read length and of high quality (Figure 2). All sequences...
obtained were uploaded to the GenBank database (accession number: MN912844-MN912862).

Discussion

The COI appears to possess a greater range of phylogenetic signal than other mitochondrial genes (Hebert et al. 2003). Compared to other protein-coding genes, its third-position nucleotides show a high number of nucleotide substitutions, and evolved at a higher rate than (about three times greater) that 12S or 16S rDNA (Knowlton and Weigt 1998). DNA barcoding provides a unique and effective method for species identification, and is becoming a standard tool in taxonomical and population genetic studies. But, large-scale DNA barcoding studies of freshwater zooplankton are still too rare, mostly due to failures in COI amplification success rates. The success rate can range from 0% to 40%, while some exceptions (Elias-Gutiérrez et al. 2008; Jeffery et al. 2011). Success rates above 50% have rarely been reported. Compared with Folmer primer, primers for zooplankton can improve PCR success rate for Diaptomus. For population genetics studies, these low PCR success rates still represent a big impediment.

Our new Phylodiaptomus-based primers improve PCR success rates significantly when compared with traditional Folmer primers. We found that either the traditional primers could only amplify the nonspecific bands, or the target bands were accompanied by many nonspecific bands. Therefore, a combination of primer binding efficiency and primer specificity may explain the performance differences among the Folmer primer, zooplankton primer, and Phylodiaptomus-specific primer sets. This new primer set will therefore facilitate further studies, at least in calanoid copepods.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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