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Original article

Phylogenetic investigations of *Lingula anatina* among some northwestern Pacific populations, based on mitochondrial DNA cytochrome *c* oxidase subunit I gene

Seong-Geun Kim, Mustafa Zafer Karagozlu, Chang-Bae Kim*

Department of Life Science, Sangmyung University, Seoul, Republic of Korea

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ABSTRACT

In this study, five tailed mussels were collected from the western coastal area of South Korea, and their DNA analyses were assessed by sequencing the partial mitochondrial cytochrome *c* oxidase subunit I (COI) gene. All DNA sequences were identified as *Lingula anatina*. These are the first COI record of the *L. anatina* from South Korea. Furthermore, the COI gene sequences of *L. anatina* reported from China, Japan, and Hong Kong were retrieved from GenBank, and phylogenetic relationships and genetic distances of the organisms were analyzed. Phylogenetic analysis and genetic distances suggest that the most related population with *L. anatina* of the present study is the Chinese population. The Japanese population diverged early from a linkage that includes Korean and Chinese populations. The Hong Kong population and one Japanese specimen examined were the most distantly related to other populations. This study provides additional data for phylogenetic study of *L. anatina*.

Introduction

Lingula, which is known as tailed mussel, is a brachiopod genus of the family Lingulidae and regarded as one of the most primitive genera in brachiopods (Luo et al 2015a). They continue to represent them in sandy coastal seafloors from the West African coast to Australia (Emig 2008). For genera of Lingulidae, morphological variation and evolutionary changes can be easily distinguishable, especially in terms of the inner structures of species (Emig 2003). There are 11 different species in the genus *Lingula* registered in the Brachiopod Database (BrachNet), and among them eight species have morphological identification.

Among the species of *Lingula*, *Lingula anatina* is easily distinguishable because of its inner structures such as wide triangular ventral valve or dorsal valve with narrow internal central ridge (Biernat and Emig 1993). For a long time, researchers believed that *L. anatina* keep these morphological characteristics without changing from their first appearance during the Cambrian period. However, deep morphological examinations of *L. anatina* show that there are evolutionary changes in the morphology of *L. anatina* (Emig 2003). Molecular studies also showed similar results. DNA barcoding studies on cytochrome *c* oxidase subunit I (COI) and elongation factor 1 alpha (EF-1α) genes of *L. anatina* showed variations in the length of gene and amino acid sequences between the specimens (Endo et al 2005; Reyment et al 2007; Yang et al 2013). A recent research on the complete genome of *L. anatina* showed that it has the highest mutational rate of various gene families in brachiopods (Luo et al 2015b). Rapid changes in genotype cannot be observed phenotypically, and this situation might result in difficulties in terms of species identification. Previously, the specimens collected from Hong Kong (GenBank accession no. AB056461) have been designated as *L. anatina* despite the authors' reservations (Endo et al 2001), and similar doubts were also noted during a further investigation based on both of COI and EF-1α (Yang et al 2013). These findings led to the necessity of investigating whether the records under the name of *L. anatina* are actual *L. anatina* or they belong to a different *Lingula* species.

In the present study, our aim was to provide additional data to understand the evolutionary history of *L. anatina*. For this aim, five individuals of *L. anatina* were collected from Incheon, South Korea, and their DNA analyses were assessed by sequencing the partial mitochondrial (COI) gene. Furthermore, COI gene sequences of *L. anatina* reported from different locations were retrieved from

* Corresponding author. Tel.: +82 222875288.
E-mail address: evodevo@smu.ac.kr (C.-B. Kim).

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GenBank, and phylogenetic relationships and genetic distances of the organisms were analyzed.

Materials and methods

Sample collection

The specimens were collected in February 2016 from Incheon (37°26′53″ N, 126°22′13″ E), which is located in the western coastal area of South Korea. The collected specimens were preserved in 97% ethanol until the DNA extraction, and a total of five individual samples were analyzed (Figure 1). The specimens were deposited in the Department of Life Science, Sangmyung University, Seoul, South Korea.

Isolation of DNA

Total genomic DNA was extracted from the whole body using the E.Z.N.A. Mollusc DNA extraction kit (Omega Bio-Tek Inc., Norcross, GA, USA). All extraction products were stored at −20°C. The mitochondrial COI gene was amplified by using the primers LCO1490 5′-GGT-CAA-ATC-ATA-AAG-ATA-TTG-G-3′ (forward) and HCO2198 5′-TAA-ACT-TCA-GGG-TGA-CCA-AAA-AAT-CA-3′ (reverse) to obtain a sequence of 658 bp (Folmer et al 1994). For polymerase chain reaction (PCR) reaction, Pure M-Taq Polymerase kit (Smarteome Co Ltd., Gyeonggi, South Korea) was used. PCR reactions of 50 μL contained the following: 100 ng template DNA; 20 pmol from each primer; 10mM dNTP mix; 10 μL PCR enhancer solution; 10 μL buffer solution; 1 μL mTaq polymerase and the remaining volume of distilled water. PCR was performed by a thermal cycler (Takara Bio Inc., Kusatsu, Japan) with 5 minutes denaturation step at 94°C and 35 cycles of 94°C for 30 seconds, 45°C for 45 seconds, and 72°C for 1 minute, followed by a 7-minute extension at 72°C. The amplified genes was analyzed by electrophoresis in 1% (w/v) agarose gels in tris-acetate buffer, and the gel bands were visualized using Midori Green (Nippon Genetics Europe GmbH, Dueren, Germany).

DNA Sequencing and data analysis

The purified PCR products were directly sequenced. DNA sequence data sets were refined using Geneious software version 9.1.3 (Kearse et al 2012), and consensus sequences were extracted from forward and reverse sequences. The consensus sequences were deposited at GenBank (KY091121, KY091122, KY091123, KY091124, and KY091125). For investigation of phylogenetic relationships, entire COI coding sequences of *L. anatina* and *Lingula adamsi* were obtained from GenBank. Additionally, records from different subphyla species—*Novocrania japonicas* and *Pictothyris picta*—were retrieved for the outgroup (Table 1). Furthermore, retrieved sequences were aligned using ClustalW for phylogenetic analysis. For reconstruction of the phylogenetic tree, the maximum likelihood method was used with the Kimura two-parameter model, and the bootstrap method with 1000 replicates was used to determine the statistical support. Phylogenetic and evolutionary analyses were conducted using MEGA 6 (Tamura et al 2013).

Figure 1. One of the specimens of *Lingula anatina*, which was examined in this study.

Results and discussion

The obtained sequences, which are 580 bp of the COI gene, were analyzed in terms of nucleotide composition. The sequences show more than 98% similarity between the five specimens. The maximum distance within the Korean specimens is 0.01, and the average distance within the Korean population is 0.01 (Table 2). These data suggest that all five specimens belong to the same species, and the specimens show a 98% match with the records of *L. anatina* at GenBank. The COI gene-based phylogenetic tree showed that genetically the closest population to the Korean population is the Chinese population (Figure 2). The five specimens show a 97% similarity with the Chinese record. The maximum genetic distance between Korean specimens and Chinese specimens is 0.02, and the average distance between the Chinese population and the South Korean population is also 0.02. The average distance between the Korean population and the Japanese population (except KP881498) is 0.11. However, the average distance between the Korean population and the Japanese specimen (KP881498) is 0.33. This value increases to 0.35 between the Chinese population and KP881498, and increases up to 0.42 in comparison with *L. adamsi* records. Moreover, the maximum genetic distance between the Korean population and *L. adamsi* is 0.36. These results suggest that the record KP881498 does not belong to *L. anatina.* Similarly, the maximum genetic distance between the Korean

Table 1. Data retrieved from GenBank for this study.

Species	Location	Country	Accession Number	Reference
Lingula anatina	Fukuoka	Japan	AB056460	Endo et al (2001)
L. anatina	Fukuoka	Japan	AB178773	Endo et al (2005)
L. anatina	N/A	Hong Kong	AB056461	Endo et al (2001)
L. anatina	Qinhuangdao	China	GU056040	Wu et al (2010)
L. anatina	Qinhuangdao	China	GU056041	Wu et al (2010)
L. anatina	N/A	Japan	AB026520	Saito et al (2000)
L. anatina	Amami Island	Japan	KP881498	Luo et al (2015b)
Lingula shantungensis	N/A	Japan	AB056459	Endo et al (2001)
L. adamsi	Muan	South Korea	AB128063	Sato et al (2004)
L. adamsi	Muan	South Korea	AB128062	Sato et al (2004)
L. adamsi	Muan	South Korea	AB128061	Sato et al (2004)
L. adamsi	Muan	South Korea	AB128060	Sato et al (2004)
L. adamsi	Muan	South Korea	AB128059	Sato et al (2004)
L. adamsi	Muan	South Korea	AB128058	Sato et al (2004)
L. adamsi	Muan	South Korea	AB128057	Sato et al (2004)
L. adamsi	Muan	South Korea	AB128056	Sato et al (2004)
L. adamsi	Muan	South Korea	AB128055	Sato et al (2004)
L. adamsi	Muan	South Korea	AB128054	Sato et al (2004)
Pictothyris picta	N/A	Japan	AB026506	Saito et al (2000)
Novocrania japonicus	N/A	Japan	AB026519	Saito et al (2000)

NA = no information.