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Phylogenetic estimation of Mytilidae in the East China Sea inferred from mitochondrial genes and nuclear DNA sequence variation

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ABSTRACT

We performed a phylogenetic estimation of the family Mytilidae in the East China Sea based on nuclear internal transcribed spacer (*ITS*) genes and two mitochondrial genes (*COI* and *16S RNA*). Analysis of five mytilid species based on each of the three genes resulted in mostly congruent trees, although there were some discrepancies in the classification of these species. We combine the results obtained from the three separate analyses to provide a phylogenetic estimation of Mytilidae. We found that the Mytilidae was divided into two major lineages: in one clade, *Mytilus galloprovincialis* was grouped with *Mytilus coruscus*; in the second clade, *Septifer bilocularis* was placed at the basal position in an individual clade, and *Perna viridis* and *Musculista senhousia* were recovered as a monophyletic group. Although these finding provide important insights into the taxonomic relationships among the Mytilidae, many aspects of Mytilidae phylogeny remain unresolved. Further analysis based on more molecular information and extensive taxon sampling is necessary to elucidate the phylogenetic relationships among the major lineages within the Mytilidae.
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1. Introduction

The Mytilidae are a family of small to large marine bivalve mollusks in the order Mytilidae, commonly referred to as mussels. Species in the family Mytilidae are found worldwide, and are one of the most widely distributed group of marine invertebrates. Mytilid species are found in all high latitudes oceans and world's major seas, although they tend to be more abundant in colder seas, where they often form uninterrupted beds on rocky shores in the intertidal zone and shallow subtidal areas. Mytilidae has become an important model group for studying the evolution and differentiation of marine species (Boore, 1999).

Mussel species are important in marine aquaculture as they are noted for their tender nutrient-rich meat, which has been widely consumed by humans from early times. In recent years, mussel aquaculture has become a prominent industry with respect to marine renewable resources (De Groot, 1973). However, the development of mussel farming has had a detrimental impact on wild living Mytilidae, and has led to a reduction in the genetic variation in wild populations (Wang and Yang, 2000).

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In previous studies of the Mytilidae, researchers have mainly focused on investigating physiology (Navarro, 1988), aquaculture technology (Carton et al., 2007), morphological classification (Semenikhina et al., 2008), and ecology (Creese et al., 1997; Gosling, 1992). However, to the best of our knowledge, the phylogenetic relationships of Mytilidae around the coasts of China have not been reported.

Previous molecular studies on Mytilidae phylogeny have relied primarily on mitochondrial DNA (mtDNA) sequences, which have provided little resolution. To avoid the limitations of mtDNA (e.g., maternal inheritance) (Bensasson et al., 2001), internal transcribed spacer (*ITS*) sequences were used in the present study. *ITS* sequences are nuclear genes that have three advantages in phylogenetic studies (Sang et al., 1995). First, universal primers can be used across a wide taxonomic range. Second, the sequences can be easily determined by comparing either their exon or intron sequences depending on the genetic distance between the taxa. Third, they have both exon and intron fragments, which are useful for examining intraspecific and interspecific genetic variations, which is particularly helpful for studying complex species.

In the present study, we performed a phylogenetic analysis of the Mytilidae of the East China Sea based on nuclear *ITS* sequences and two mitochondrial (Mt) genes (*CO1* and *16S RNA*). To our knowledge, the present study is the first to investigate the phylogenetic relationships in Mytilidae based on combined analyses of Mt genes and nuclear *ITS* genes. For these analyses, we used a 673-bp fragment of the *COI* gene, a 1113-bp fragment of the *16S RNA* gene, and 797-bp *ITS* sequences. We propose that the molecular phylogenetic trees obtained provide a novel estimation of the phylogeny of Mytilidae.

2. Material and methods

2.1. Sample collection and DNA extraction

Mytilidae species were collected from aquaculture fibrous ropes (Table 1). We initially used morphological features and the *COI* Mt gene to identify five different species (*Mytilus galloprovincialis*, *Mytilus coruscus*, *Perna viridis*, *Septifer bilocularis*, and *Musculista senhousia*). Tissue samples from the collected specimens were preserved in 95% ethanol for molecular analysis. Voucher specimens were fixed in 10% formalin, and later transferred to 75% ethanol for preservation. We used the phenol-chloroform method to extract whole genomic DNA from the adductor muscle tissue of individual specimens. Genomic DNA samples were stored at −20 °C for later analysis.

2.2. PCR amplification and sequencing

ITS sequences were amplified using the previously developed primers (Wood et al., 2007), 5′-GGAAG TAAAA GTCGT AACAA GG-3′ and 5′-CGCCG TTACT AGGGG AATCC TTGTA AG-3′. Polymerase chain reaction (PCR) was performed using the following conditions for all five species: 3 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 2 min at 72 °C; and a single final extension period of 10 min at 72 °C. The PCR products were stored at 12 °C. We used generic invertebrate primer pairs to amplify the MT genes: *COI*, 5′-GGTCA ACAAA TCATA AAGAT ATTGG and 5′-TAAAC TTCAG GGTGA CCAAA AAATCA-3′(Folmer et al., 1994); *16S RNA*, 5′-TGAGCG TGC TAAGG TAGC-3′ and 5′-AGCCA ACATC GAGGT CGC-3′ (Lydeard et al., 1996). The amplifications were performed in a PTC-200 thermal cycler with the following conditions: denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 2 min; and final extension at 72 °C for 10 min. TaKaRa Taq™ DNA Polymerase (Code: R001B) was used in all amplifications. The PCR products were separated on a 1% agarose gel to check integrity and visualized using the Molecular Imager Gel Doc XR system (BioRad, USA). The PCR products were purified using a QIAEX II Gel Extraction Kit (Qiagen) and then sequenced using an ABI 3730 automated sequencer, for which the same PCR primers were used.

2.3. Data analyses

Sequences were assembled using Geneious 4.5.3 (Biomatters; http://www.geneious.com). BioEdit 7.0 (Hall, 1999) was used for sequence alignment. DNAsp5.0 was used to calculate the genetic variation parameters (Librado and Rozas, 2009), and JModeltest was used to select the optimal mode (Posada, 2008). The maximum likelihood (ML) method (Rzhetsky and Nei, 1992) was used to construct phylogenetic trees. The ML trees were obtained with 1000 bootstrap replications using MEGA 5.0 (Tamura et al., 2011). The sampling interval was 1000.

Table 1
Identification and origins of Mytilidae samples.

Species	Genus	Abbreviation	Quantity	Collection sites	Collection date
Mytilus galloprovincialis (Lamarck, 1819)	*Mytilus*	MG	10	Zhoushan	2016/4/10
Mytilus coruscus (Gould, 1861)	*Mytilus*	MC	10	Zhoushan	2016/4/10
Perna viridis (Linnaeus, 1758)	*Perna*	PV	10	Zhoushan	2016/4/10
Septifer bilocularis (Linnaeus, 1758)	*Septifer*	SB	10	Zhoushan	2016/4/10
Musculista senhousia (Benson, 1842)	*Musculista*	MS	10	Xiamen	2016/5/2

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