



Molecular Identification of Parasitic Copepods Using Cytochrome *c* Oxidase Subunit 1 from Chinese Marine Waters

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ABSTRACT

It has been reviewed that less attention has been given to parasitic copepods of mollusc species found in Chinese coastal waters and meager information is available about their morphological and molecular based taxonomy. Therefore, this study is designed to understand the taxonomic status of parasitic copepods of molluscs inhabiting Chinese coastal waters using cytochrome *c* oxidase subunit 1 (COX1) molecular marker. The results revealed seven parasitic copepods i.e. *Conchyliliurus quintus*, *Lichomolgus similis*, *Modiolicola bifida*, *Mytilicola orientalis*, *Trochicola japonica*, *Ostrincola koe* and *Pseudomyicola spinosus*. All the sequences were submitted to NCBI, under accession numbers OQ725274-OQ725305. Evolutionary distance within families and between families ranged from 0.007 to 0.113 and 0.299 to 0.350 respectively. Pairwise distance ranged from 0.186 to 0.360. The neighbor joining phylogenetic tree described three main clades of these species. The identification of seven parasitic species of copepods found in Chinese marine waters is confirmed by the current investigation. The present study will be useful for future taxonomic investigations.

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Authors' Contribution

DX: Writing the original manuscript, designed the experiment, generated funds, review and editing, visualization, resources, validation, formal analysis, data curation. XX, ZC, ZZ and LZ: Participated in the writing, review and editing the manuscript. CMS: Participated in the writing, editing and review the manuscript, designed the experiment, data curation, conceptualization, methodology and data analysis.

Key words

Parasitic copepods, Molluscs, Chinese coastal waters, COX1

INTRODUCTION

To date approximately 280 parasitic copepods of mollusc species have been reported as parasites or associates of molluscs (Boxshall and O'Reilly, 2015), and more than 430 species of molluscs were known to be hosts of copepods, with most of them being bivalves (Humes, 1994; Kim, 2004). The study of the marine mollusc parasitic copepod fauna from China seas is relatively limited and short of systematic taxonomy.

So far, 14 species of copepod parasitic species have

been found with mollusc hosts (Humes and Lee, 1985; Humes and Boxshall, 1988; Ho and Zheng, 1994; Lin and Ho, 1999; Ho *et al.*, 2012; Du *et al.*, 2018; Du and Sun, 2022), from the Yellow Sea, including 9 species (*Ostrincola koe*, *Octopicola huanghaiensis*, *Conchyliliurus quintus*, *Herrmannella soleni*, *Lichomolgus similis*, *Modiolicola bifida*, *Pseudomyicola spinosus*, *Mytilicola orientalis* and *Trochicola japonica*), of which 5 species (*O. koe*, *C. quintus*, *L. similis*, *M. bifida* and *T. japonica*) were reported from the Bohai Sea, 4 species (*Anthessius mytilicolus*, *O. koe*, *L. similis* and *Myicola formosanus*) were known from the East China Sea and 3 species (*A. mytilicolus*, *A. pinnae* and *Panjakus platygyrae*) were recorded from the South China Sea. Two methods of taxonomic identification have been used up till now. The morphological based characteristic and molecular marker based identification. The DNA barcode-based identifications are valued around the world, especially for all kinds of morphologically complicated species, because traditional morphological identifications are significant but raise accuracy concerns.

The complex morphological characteristics confuses the identification of species, therefore, it becomes intuitive

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to identify the species accurately to understand the diversity, determine the reference basis of resources and assess potential diversity resources of the region using modern techniques like the DNA barcode-based identifications are appreciated worldwide especially for the animals of similar morphological characteristics. The mitochondrial DNA (mtDNA) are potential markers because of maternal inheritance, fast evolutionary rate. Therefore, cytochrome *c* oxidase subunit 1 has been used in present study.

The aim of the present study was to identify parasitic copepod of molluscs applying molecular approaches supported by morphological features. To our knowledge, it is a first attempt to identify the parasitic copepods using molecular based approach in China.

MATERIALS AND METHODS

Sample collection

A total of 32 species and 8628 individuals of bivalves were sampled from Shandong, Jiangsu and Liaoning provinces along the coasts of Yellow Sea and Bohai Sea (Du and Sun, 2022). After being washed with filtered water, each mollusc was opened in a petri dish containing a small amount of filtered sea water. The parasitic copepods were isolated under a dissecting microscope (Zeiss Stemi, 508). Some copepod specimens were isolated from the hosts that were sampled from seven localities along the coasts of Yellow Sea and Bohai Sea and then fixed and stored in 75% ethanol until study (Fig. 1). The parasitic species were first identified using major morphological taxonomic characters (Huys and Boxshall, 1991; Boxshall and Halsey, 2004).

Molecular procedures

Genomic DNA from each of 32 samples was extracted using a Column Genomic DNA Isolation Kit (Shanghai Sangon, China) according to the manufacturer's instructions. The COX1 region was amplified by polymerase chain reaction (PCR) using the forward primer LCO-1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and the reverse primer HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.*, 1994). The total volume of 20 μ l of PCR reaction mixture comprised 5.8 μ l of distilled water, 2 μ l of DNA template, 1 μ l each of the forward and reverse primers (Diluted 20 times for using), 2 \times reaction mix 10 μ l and golden DNA polymerase (Beijing, Tiangen, China) 0.2 μ l. The thermo-cycling profile was 5 min at 94 $^{\circ}$ C for initial denaturation, 30 sec at 94 $^{\circ}$ C for denaturation, 30 sec at 50 $^{\circ}$ C for annealing, 1 min at 72 $^{\circ}$ C for extension, and 7 min at 72 $^{\circ}$ C for final extension. The thermal cycle from denaturation to extension was repeated 35 times.

PCR products were checked on Gold View-stained 1.5% agarose gels. Sanger dideoxy sequencing of each sample was carried out on both strands. The DNA sequences obtained herein were deposited in the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>) and compared (using the Clustal W) with those available in the GenBank database. The accession number of each sequence is submitted under accession numbers OQ725274-OQ725305 in Table I.

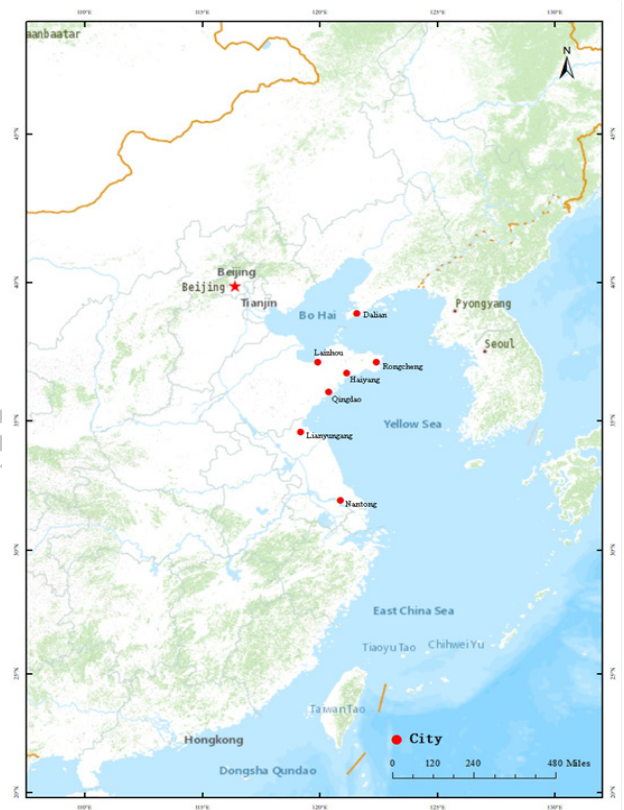


Fig. 1. The map showing the collection areas mainly samples were collected from Bohai Sea and Yellow Sea area of Shandong, Jiangsu and Liaoning provinces, respectively.

RESULTS

Seven bivalves including *Ruditapes philippinarum*, *Meretrix petechialis*, *Macra chinensis*, *Mytilus galloprovincialis*, *Macra quadrangularis*, *Leukoma jodoensis*, and *Crassostrea gigas* hosts were found to harbor seven parasitic copepods of the order Cyclopoida (Table I). The parasitic species were first identified using taxonomic characters. The molecular identification using 621 bp of COX1 molecular marker sequence revealed

Table I. The hosts, geographical locations, gene bank accession numbers of cyclopoid copepods parasitic in molluscs.

Order/Family/Species	Host	Locality	Gene bank accession number
Order: Cyclopoida			
Family: Clausidiidae			
<i>Conchylurus quintus</i>	<i>Ruditapes philippinarum</i>	Yellow Sea	OQ725274, OQ725275
Family: Lichomolgidae			
<i>Lichomolagus similis</i>	<i>Meretrix petechialis</i>	Bohai Sea	OQ725276
<i>Modiolicola bifida</i>	<i>R. philippinarum</i> , <i>Mactra chinensis</i> , <i>Mytilus galloprovincialis</i> , <i>Mactra quadrangularis</i> , <i>Leukoma jedomensis</i>	Yellow Sea	OQ725277, OQ725278, OQ725279, OQ725280, OQ725281, OQ725282, OQ725283, OQ725284, OQ725285, OQ725286
Family: Mytilicolidae			
<i>Mytilicola orientalis</i>	<i>M. galloprovincialis</i> , <i>Crassostrea gigas</i>	Yellow Sea	OQ725287, OQ725288, OQ725289, OQ725290
<i>Trochicola japonica</i>	<i>R. philippinarum</i>	Yellow Sea	OQ725302, OQ725303, OQ725304, OQ725305
Family: Myicolidae			
<i>Ostrincola koe</i>	<i>M. petechialis</i> , <i>M. quadrangularis</i>	Bohai Sea and Yellow Sea	OQ725291, OQ725292, OQ725293
<i>Pseudomyicola spinosus</i>	<i>M. galloprovincialis</i>	Yellow Sea	OQ725294, OQ725295, OQ725296, OQ725297, OQ725298, OQ725299, OQ725300, OQ725301

Table II. Average evolutionary distance within the family.

Name of family	Average distance
Clausidiidae	0.007
Lichomolgidae	0.064
Mytilicolidae	0.110
Myicolidae	0.113

Table III. Average evolutionary distance between families.

Name of family	Clausidiidae	Lichomolgidae	Mytilicolidae	Myicolidae
Clausidiidae	-			
Lichomoldidae	0.309	-		
Mytilicolidae	0.330	0.350	-	
Myicolidae	0.310	0.299	0.304	-

seven parasitic species: *Conchylurus quintus*, *Lichomolagus similis*, *Modiolicola bifida*, *Mytilicola orientalis*, *Trochicola japonica*, *Ostrincola koe* and *Pseudomyicola spinosus*. Twenty-three sequences of COX1 of these species submitted in NCBI under accession numbers OQ725274-OQ725305. The NCBI had no early record for *Trochicola japonica*, and first-time sequences for this species under the accession numbers OQ725302, OQ725303, OQ725304 and OQ725305 were submitted. The average evolutionary distances within families were

ranging from 0.007 to 0.113 (Table II), whereas those between families ranged from 0.299 to 0.350 (Table III). The pairwise distance ranged from 0.186 to 0.360 (Table IV). The neighbor joining results revealed three major clades and certain sub-clades belongs to four families (Fig. 2). Only the individuals of family Lichomolgidae showed differentiated clades whereas the rest of the families showed sub-clades within a single major clade.

DISCUSSION

According to Ho and Kim (1991), the edible bivalve species are commercially important, and their populations experience mass mortalities as a result of various problems including parasitic copepods as causative agent of such mass mortality. Consequently, there are instability in the bivalve species' culture. Parasitic copepods are one of the many factors that contribute to mass mortality, like in the case of the *Meretrix meretrix* mass mortality in Jiangsu's southern shore (Ho and Zheng, 1994). *Meretrix lusoria* had mass mortality in the early 1970s, which contributed to the abrupt fall in annual productivity (Ho and Kim, 1991). The parasitic species like *C. quintus*, *M. bifida*, *O. koe*, and *P. spinosus* depends on variety of bivalve species, and these have negative impact on numerous host species which are significant from an economic perspective (Tanaka, 1961; Ko et al., 1962; Humes, 1968; Ko and Yoshikoshi, 1974; Do and Kajihara, 1986; Yoosukh, 1991; Ho and Zheng, 1994; Ho and Kim, 1991). According to Ho (1992), there are 51 different species of bivalves worldwide that contain *P. spinosus*.

Table IV. Pairwise distance (K2P) of COX1 sequences of all species of present study.

Name of species	1	2	3	4	5	6	7
<i>Conchylurus quintus</i>	-						
<i>Lichomolgus similis</i>	0.359	-					
<i>Modiolicola bifida</i>	0.303	0.325	-				
<i>Mytilicola orientalis</i>	0.329	0.360	0.340	-			
<i>Ostrincola koe</i>	0.301	0.315	0.289	0.317	-		
<i>Pseudomyicola spinosus</i>	0.313	0.327	0.297	0.283	0.252	-	
<i>Trochicola japonica</i>	0.337	0.354	0.353	0.186	0.327	0.296	-

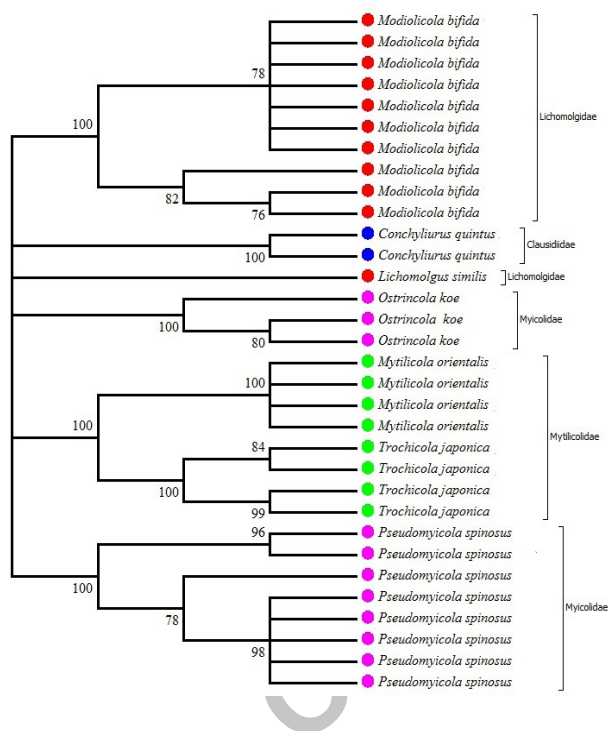


Fig. 2. Neighbor joining tree (NJ) based on thirty-two COX1 sequences of seven parasitic copepods of order Cyclopoida belongs to four families. The colors are representing the families, respectively.

Fundamentally, copepod identification is based on anatomical and morphological characters (Huys and Boxshall, 1991; Boxshall and Halsey, 2004), yet, molecular identification of these significant microscopic organisms has received relatively less attention. Therefore, in order to understand the relationships between the parasitic copepod molecular-based identification of copepods and their phylogenetic studies are of utmost relevance. The traditional method of identification and associated investigations are being quickly replaced by molecular techniques (Khodami *et al.*, 2019). Parasitic copepods can find a wide variety of hosts in marine environments,

practically in every phylum (Boxshall and Halsey, 2004).

In China, the molecular based identification of parasitic copepods associated with marine bivalve species have not been reported yet, however, the parasites of fish have, such as the work by Song *et al.* (2008). The traditional based identification can cause confrontations because of complex structure and way of understanding of morpho-features, usually the taxonomic used certain character keys, and these keys some time being misinterpreted and misunderstood, therefore, the present study designed to identify the parasitic copepods of bivalve species using COX1.

The outcome of the present study consists of seven species which are re-confirmed using molecular marker such as, *C. quintus*, *L. similis*, *M. bifida*, *M. orientalis*, *O. koe*, *P. spinosus* and *T. japonica*. Earlier investigations on parasitic copepods of mollusc focused the identifications based on the morphological taxonomic characters (Humes and Lee, 1985; Humes and Boxshall, 1988; Ho and Zheng, 1994; Lin and Ho, 1999; Ho *et al.*, 2012; Du *et al.*, 2018; Du and Sun, 2022). To complement the study of the phylogenetic relationships among parasitic copepods of molluscs based on the morphological characters, a different approach employing molecular data is necessary (Elsner *et al.*, 2010; Feis *et al.*, 2019).

It has been said that partial COX1 region in 600 bp in length is valuable to identify out a cryptic species but might not be possible to conclude the phylogenetic relationship in certain copepods (Machida and Tsuda, 2010; Blanco-Bercial *et al.*, 2011, 2014; Aarbakke *et al.*, 2014). Nevertheless, several publications on different copepods provides profound importance of COX1 in taxonomy of species. Bucklin *et al.* (2010) detailed as the sequence for determining the difference between species, Laakmann *et al.* (2013) used proteome finger printing and DNA sequencing for comparison of calanoid copepods and the authors used several markers like 18S rDNA, COX1 etc. As mentioned above, COX1 molecular marker also has profound importance for phylogenetic studies for

diversified taxa, such as Aarbakke *et al.* (2014) investigated the five sibling species of *Pseudocalanus* demography and phylogeny. Kozol *et al.* (2012), used multigene approach to find the divergence between two *Calanus* species. The phylogenetic study of present investigation denoted three main groups along with sub-clades.

In the current investigation, these seven parasitic copepod species were reported on seven different bivalve hosts (Table I). The present research confirms the identification of seven copepod parasite species using molecular marker.

CONCLUSION

Rare are the molecular-based investigations of copepods from Chinese waters. The identification of seven parasite species of copepods found in Chinese marine waters is confirmed by the current investigation. This exploratory research will be useful for taxonomists as they plan future investigations of parasitic copepods in many scientific areas. Additionally, molecular studies based on DNA sequences are of immense power in the analysis of phylogenetic relationships of parasitic copepods in molluscs, when new parasitic copepods or two similar copepods are appeared, molecular data may provide useful information to determine the phylogenetic affiliation of the species, and a period of profound change is to be expected.

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Ethical statement

All the individuals in this research work we handled carefully, we neither harm nor damage to the animal. The study was conducted in accordance with the Declaration of Hebei Agriculture University, China and the protocol was approved by the Ethics Committee of Hebei Agricultural University.

Competing declaration of research interest

We all the authors declare that the following financial interests and personal relationships which may be considered as potential interest: Du Xin reports

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Statement of animal welfare

In this research work, we had not involved any harm or threat to animal.

Statement of conflict of interest

The authors have declared no conflict of interest.

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