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Short Communication

Integrating Morphology, Breeding Ground and Mitochondrial COI Gene Analysis for Species Identification of *Bellamya lithophaga* (Gastropoda: Viviparidae) in China

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Abstract

Background: *Angiostrongylus cantonensis* is a zoonotic public health concern that causes human severe eosinophilic meningitis in Southeast Asia and China. As a medically important intermediate host of *A. cantonensis*, *Bellamya lithophaga* (Gastropoda: Viviparidae) is often confused with other morphologically similar sibling species of genus *Bellamya*, such as *B. aeruginosa* and *B. purificata* in the past. Hence, the aim of the present study was to investigate evidences to discriminate these equivocal *Bellamya* species.

Methods: This study was carried out by getting *Bellamya* snail samples from Fujian Province in the South-East of China. The snail morphological features, breeding grounds and phylogenetic relationship according to mitochondrial cytochrome *c* oxidase subunit I (COI) gene marker were analyzed.

Results: Based on external morphology, radular shape and cusp formula, as well as major breeding environment, *B. lithophaga* could be distinguished from *B. aeruginosa*, *B. purificata*. The phylogenetic tree also unconfirmed that *B. lithophaga* belongs to a different genetic clade from other morphologically similar species.

Conclusion: Our findings demonstrate the significant differences in *B. lithophaga* and other sibling species, which supports the traditional species delimitation in the genus *Bellamya*.

Introduction

Angiostrongylus cantonensis is the pathogen of zoonotic angiostr-ongyliasis, which causes human severe eosinophilic meningitis in Southeast Asia and China. *Bellamya lithophaga*, a newly recorded freshwater molluscan intermediate host for *A. cantonensis*, is widely distributed in Fujian Province, China (1). The other morphologically similar species of the genus *Bellamya*, such as *B. aeruginosa* and *B. purificata*, also inhabit the freshwater benthic environments (2), and it is not easy to distinguish these genetic closely related snails in field survey.

In this study, integrating morphological features, major breeding grounds and mitochondrial cytochrome *c* oxidase subunit I (COI) gene marker, we differentiated and characterized *B. lithophaga*.

Materials and Methods

Traditional species identification of Bellamya lithophaga

Bellamya lithophaga samples were collected from Xixia village, Minhou County, Fujian province of China. The snails were digested with 5% NaOH to observe the radulae, and their dimensions were precisely measured by an electronic digital display caliper. The snail's morphological features and its major breeding grounds were studied with comparison to other closely related *Bellamya* species. The topotype and paratype specimens of *B. lithophaga* were deposited in the parasite specimen room of Fujian province center for disease control and prevention.

PCR reagents

Ex-Taq DNA Polymerase, PCR buffer, $MgCl_2$ and dNTPs (TaKaRa Biotech Co., Ltd., Dalian, China) ; Protease K (Merck, USA) ; Wizard™ DNA Clean-up System (Promega, USA).

Mitochondrial COI gene analysis

Bellamya lithophaga were collected separately from four counties: Longhai, Lianjiang, Minhou and Changle in Fujian province. *B. aeruginosa* and *B. purificata* were both collected from Shaowu County. Each snail was crushed and repeatedly washed with distilled water. The cephalopedal region of treated snail was cut into small pieces, and then 300ul digestive solution (100mM NaCl, 10mM Tris-HC1 (pH 8.0), 25mM EDTA ((pH 8.0), 1% SDS and 5g/L proteinase K) was added to the tissue. After sufficient overnight digestion in a constant temperature incubator at 37℃, DNA extraction of the digested tissue suspension was proceeded with a Wizard ™ DNA Clean-up System kit following the manufacturer's instruction. The obtained DNA sample was stored at -20℃ until used for PCR amplification.

Two conserved primers, JB3 (forward): 5'-TTTTTTGGGCATCCTGAGGTTTAT-3' and JB4.5 (reverse): 5'-TAAAGAAAGAACATAATGAAAATG-3' (3), were used to amplify the mtDNA coding for partial COI gene. PCR was performed using 0.25 µl of Taq DNA polymerase (5 U/µL), 2.5 µl of 10× PCR buffer, 3.5 µl of $MgCl_2$, 2 µl of dNTPs (25 mM) , 0.25 µl of each primer (50 pmol/µL) and 1 µl of template DNA in a 25 µl final volume of reaction under the following protocol: a hot start at 94 °C for 5 min, then 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55°C, 30 s extension at 72 °C, followed by a final extension at 72 °C for 5 min. A 5µl aliquot of amplicons was checked by 1% agarose gel electrophoresis to validate the amplification efficiency.

The purified PCR products were sent to Shanghai Invitrogen Biotechnology Co., Ltd. for sequencing. Sequences were aligned with COI gene sequences of other related species retrieved from GenBank™ (Table 1) using ClustalX version 1.81.

The genetic distances between these sequences were calculated using MEGA version 4, with *Tricula pingi* (Tp) as outgroup. The phylogenetic trees of *B. lithophaga* and its sibling species were built using three methods, namely, neighbor joining (NJ) implemented in Mega version 4.0, maximum likelihood (ML) implemented in Puzzle version5.2, and maximum parsimony (MP) implemented in Phylip version 3.67. Reliability of the phylogenetic tree was estimated using bootstrap values run for 1000 iterations. Sequence homology was analyzed using the Megalign program of DNAstar version 5.0.

Table 1: Mitochondrial COI gene sequences of *Bellamya* spp. and *Tricula pingi* available in GenBank

Species/ abbreviation	GenBankTM accession No.
Bellamya monardi(Bm)	HQ012800
Bellamya robertsoni(Br)	HQ012796
Bellamya costulata(Bco)	HQ012712
Tricula pingi(Tp)	EF394901

Results

Characterization of Bellamya lithophaga topotype and paratype

Topotype (FJ521): 24.03 mm in shell height, 15.95 mm in shell width, 9.98 mm in body whorl height, 8.92 mm in aperture height, 11.73 mm in aperture width, and height of shell 2.4 times that of body whorl.

Twenty paratypes: 19.94~28.05 mm in shell height, 12.61~17.97 mm in shell width, 8.53~10.61 mm in aperture height, 10.57~12.75 mm in aperture width.

Morphological description: Medium-sized conical shape, thick and hard shell, ashen-grey surface and coarse growth line, ladder-like spiral whorl with 5 whorls, broadly conical spire. The earlier spiral whorls, especially the first three protoconch whorls, are often severely corroded.

Every 7 tongue teeth ranked as horizontal line on the U-shape grooved radula ribbon. One central tooth is in the bottom grooved radula, characterized with an incisor at the center and 4 cusps on both sides. Cusp formula: 5-1-5·4-1-4·3-1-3·10 (Fig. 1).

Fig.1: Radula of *Bellamya lithophaga*, left to right showing: central teeth, lateral teeth, inner marginal teeth, outer marginal teeth

Comparison of morphology and breeding grounds of Bellamya snails

The snail *B. lithophaga* is a new record species in Fujian province. In the past, it was often confused with *B. aeruginosa* or *B. purificata* (Fig. 2). Their morphological features and major breeding grounds were compared (Table 2).

Comparison of mtDNA COI gene between Bellamya lithophaga and other sibling species

About 500 bp fragments were successfully amplified from six snail samples, which conformed to the expected fragment size of target COI gene, without nonspecific band (Fig. 3).

The sequence data of smtDNA COI gene were submitted to GenBank and compared with the existing other *Bellamya* COI gene sequences. The phylogenetic tree was constructed, which showed that *B. lithophaga* and *B. monardi* had a close genetic relationship, and were clustered in a monophyletic clade. *B. aeruginosa* and *B. robertsoni* were grouped in another clade, and there were obvious genetic differences between them and *B. purificata*. *B. purificata* and *B. aeruginosa* were grouped into a lower clade for their closer genetic similarity (Fig. 4).

Fig. 2: *Bellamya lithophaga* (left), *B. aeruginosa* (middle) and *B. purificata* (right)

Table 2: Comparison of morphological features and major breeding grounds between *Bellamya lithophaga*, *B. aeruginosa* and *B. purificata*

Sail species	*B. lithophaga*	*B. aeruginosa*	*B. purificata*
Shell shape	Conical shape, broadly conical spire with five whorls, blunt and bald apex.	Elongated, conical shape, sharp conical spire with six or seven whorl, relatively sharp apex.	Conic shape, broadly conical spire with six or seven whorls, relatively sharp apex.
Aperture	Apertural margin and inner margin featured with black rims, with an obtuse angle on the upper side.	Only apertural margin featured with black rims.	Only apertural margin featured with black rims.
Shell surface	Severely corroded surface in ashen-grey colour, 5~6 obvious ribs on body whorl.	Aeruginous or green-brown surface, about 3 ribs on body whorl.	Green-brown surface, 3~4 ribs on body whorl or penultimate spiral whorl.
Shell height: shell width	1.50:1	1.77:1	1.57:1
Shell height: body whorl height	2.40:1	2.51:1	2.26:1
Breeding grounds	Irrigation ditch, pit ditch.	Lake, river and pond.	Lake, river and pond.

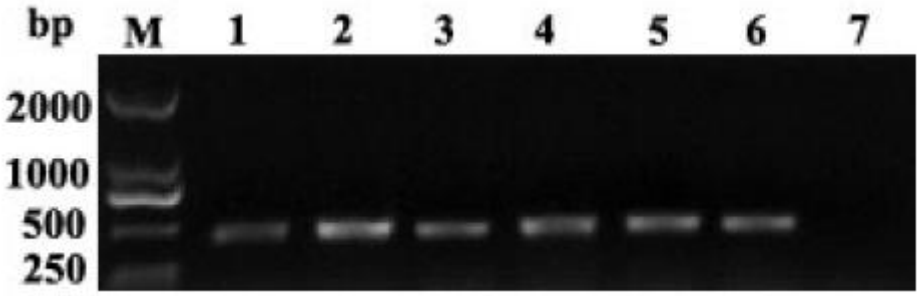

Fig. 3: Analysis of PCR-amplified mtDNA COI gene from *Bellamya* spp. by agarose gel electrophoresis. M: DL-2000 DNA marker; 1-4 *Bellamya lithophaga*; 5 *B. aeruginosa*; 6 *B. purificata*; 7 Negative control

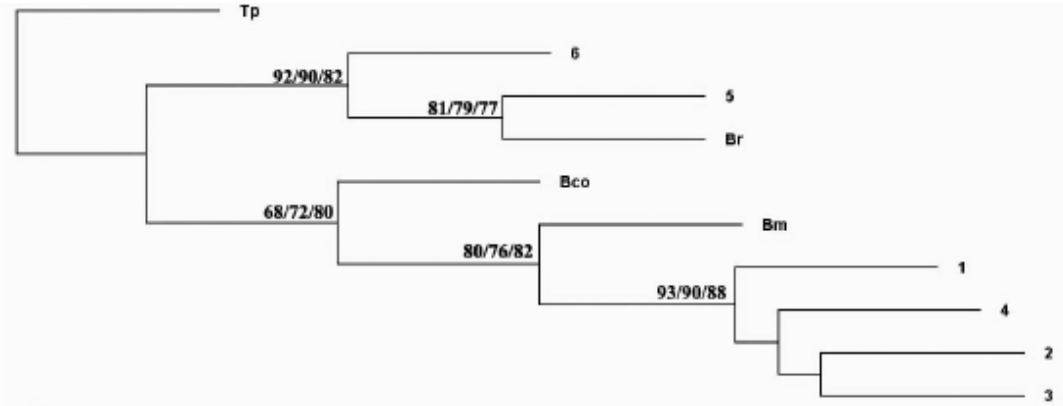

Fig. 4: The bootstrap consensus phylogram of the stationary tree for *Bellamya* spp. reconstructed by Maximum parsimony (MP) using the COI gene sequences, with *Tricula pingi* as outgroup. 1-4 *Bellamya lithophaga*; 5 *B. aeruginosa*; 6 *B. purificata*. Note: Numbers at nodes indicate bootstrap values (%) resulting from MP/NJ/ML

Discussion

Since 1980s, mitochondrial DNA analysis has been widely applied in animal evolutionary genetics, population genetic structure, genetic diversity, species and strain identification, molecular ecology, etc. (4, 5). Mitochondria belong to maternal inheritance and recombination does not occur between mitochondrial genes, so mitochondrial gene can reflect evolutionary history of the maternal line. For a long time, mitochondrial DNA COI gene with its extensive polymorphism between species, intra-species, intrapopulation and interpopulation, was used as a genetic marker for species identification (6). In this study, COI gene marker was firstly employed to create phylogenetic tree of freshwater snails of genus *Bellamya*.

Based on external morphology, radular shape and cusp formula, as well as major breeding ecological environment, *B. lithophaga* can be distinguished from *B. aeruginosa*, *B. purificata* in Fujian province of China. The major breeding grounds of *B. lithophaga* are irrigation ditch and pit ditch, while *B. aeruginosa* and *B. purificata* mainly inhabit in lake, river and pond. COI gene analysis also confirmed that *B. lithophaga*, *B. aeruginosa* and *B. purificata* are three independent species and their genetic differences are obvious. Our research will shed light on further studies about the biology of *Bellamya* snail, which, in turn, has implications for the effective control of zoonotic angiostrongyliasis it may transmits.

Conclusion

The phylogenetic tree being constructed according to partial COI gene sequence data confirmed that *B. lithophaga* belonges to a different genetic clade from other two sibling species, *B. aeruginosa* and *B. purificata*, which supported the traditional biological species delimitation in the genus *Bellamya* based on morphological features and breeding grounds.

Acknowledgements

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