PREVALENCE AND GENETIC IDENTIFICATION OF BUCEPHALID CERCARIAE FROM THE GOLDEN MUSSEL (*Limnoperna fortunei*) IN NORTHERN VIETNAM

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ABSTRACT

A total of 1,453 specimens of the golden mussel *Limnoperna fortunei* were collected by handpicking from cultured areas of channel catfish (*Ictalurus punctatus*) in Son La, Hoa Binh, Thac Ba reservoirs, and from the Duong River, and examined for trematode infection. Of these, 178 specimens were found to shed bucephalid cercariae. The infection rate in the Duong River was 3.14 times higher than that of the reservoirs (p = 0.005), while no significant differences were observed among the reservoirs. Based on *28S* rDNA sequence analysis, three trematode species were identified, including *Dollfustrema bagarii* and two species of the genus *Prosorhynchoides*.

Keywords: *28S* rDNA, phylogenetic analysis, Bucephalidae, *Dollfustrema*, *Prosorhynchoides*.

INTRODUCTION

The family Bucephalidae Poche, 1907 comprises trematodes that mainly parasitize marine and freshwater fish. In their life cycle, mollusks, fish, and piscivorous fish serve as the first, second, and definitive hosts, respectively (Saito et al., 2025). In Vietnam, several bucephalid species have been recorded, including *Dollfustrema bagarii*, *Prosorhynchus vietnamensis*, and *Metadena bagarii*, etc. (Moravec & Sey, 1989). These trematodes were described from native fish species, such as catfish *Bagarius bagarius* (Sisoridae), *Pseudobagrus vachelli* (Bagridae), and gobi *Saurogobius dobryi* (Gobiidae). Most species have been identified based on the morphology of mature worms.

Recently, metacercariae of *Dollfustrema bagarii* have been frequently reported in the introduced channel catfish *Ictalurus punctatus* (Kim et al., 2022; Vu et al., 2024). This species has become an economically valuable aquaculture fish in Vietnam since successful trials of its culture and breeding were conducted in 2010 (Kim et al., 2022; Vu et al., 2024). Since then, *I. punctatus* has been widely farmed across most Northern provinces. As a newly introduced cultured species, only a few diseases have been reported in channel catfish in Vietnam, for example, bacterial infections caused by *Aeromonas veronii*, and *Edwardsiella ictaluri*, as well as fungal infections caused by *Saprolegnia* spp. (Hoai et al., 2019). To date, only one parasitic disease, caused by metacercariae of the bucephalid trematode *Dollfustrema bagarii* (known as "off-white grub"), has been documented (Kim et al., 2022). Molecular data of *D. bagarii* (both metacercariae and adult stages) have been deposited in GenBank (Kim et al., 2022). However, there is no information on the sources of infection or the first intermediate host of *D. bagarii*.

Le et al. (2025b) identified the golden mussel *Limnoperna fortunei* (Syn. *L. lacustris*) as the first intermediate host of *D. bagarii*. This discovery is important because it fills a critical gap in understanding the life cycle of the species and provides a basis for mapping the distribution of "off-white grub" disease. In the present study, we collected golden mussels, *L. fortunei,* from different locations and investigated whether cercariae of *D. bagarii* and other bucephalids shed from mussels in different geographic regions exhibit molecular divergence.

MATERIALS AND METHODS

Mussel sampling and examination

The golden mussel *L. fortunei* was collected from aquaculture cage areas in Thac Ba (Yen Binh commune, Yen Bai province), Son La (Quynh Nhai commune, Son La province), Hoa Binh (Muong Hoa commune, Phu Tho province), as well as from the Duong (Đuống) River (Bac Ninh province) in the Northern region of Vietnam. Mussels were collected by the same person in all sites (from cage nets) during the morning hours, with each collection lasting 30 minutes per site. Specimens were handpicked, placed in plastic containers, and transported alive to the laboratory. Mussels were identified to species level using the keys of Thanh & Hai (2017).

The mussels were examined for digenean infection using the crushing method described by Frandsen & Christensen (1984). The morphological characteristics of cercariae were observed under an Olympus BX53 light microscope (Olympus Corp., Tokyo, Japan). Identification of cercariae were based on the keys of Yamaguti (1975). Cercariae randomly collected from mussels at each site were subsequently used for polymerase chain reaction (PCR) and sequencing to confirm species identity.

Molecular analysis

Genomic DNA from bucephalid cercariae (two cercarial samples obtained from two different mussel specimens in each reservoir/river) was extracted using the DNA Mini Kit QIAamp® (cat. no. 51304, Qiagen Inc., Valencia, California) following the manufacturer's instructions. A fragment of the *28S* rDNA (~1.6 kb) was amplified using primers LSU-5 (5'−TAGGTCGACCCGCTG

AAYTTAAGCA−3', forward; Littlewood et al., 2000) and 1500R: (5'−GCTATCCTGAG GGAAACTTCG−3', reverse; Tkach et al., 2003). PCR reactions were carried out in a 25 μL total volume containing 12.5 μL PCR Master Mix (2 ×) (cat. no. K0171, Thermo Scientific™, USA), 1 μL (10 pM) of each primer, 1 μL DNA, and 9.5 μL nuclease-free water. Amplifications were performed on an Eppendorf Master Cycler with both positive and negative controls.

PCR products were purified using the ExoSAP-IT enzymatic cleanup reagent (cat. no. 78201.1.ML, Thermo Scientific™, USA) and directly sequenced with the LSU-5 and 1500R primers. Sequence similarity searches were performed using the NCBI BLAST service (http://blast.ncbi.nlm.nih.gov/) (Camacho et al., 2010).

Pairwise p-distances were calculated, and phylogenetic analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) in MEGA version 7.1 (Kumar et al., 2016). Statistical support for the UPGMA dendrogram was assessed using the bootstrap method with 1,000 replicates (Felsenstein, 1985).

Statistical analyses

Mussel infection data were entered into an Excel database (Microsoft Corporation, Redmond, Washington) and analyzed using STATA/IC 12 (StataCorp 2011). The prevalence of infection was compared between mussels collected from different locations and between those from reservoirs versus the river. using a binomial logistic regression with adjustments made for clustering within collection sites. Differences were considered statistically significant at P-values below 0.05.

RESULTS

Trematode infection

A total of 1,453 specimens of *L. fortunei* (Fig. 1) were collected, including 669 specimens from reservoirs and 784 specimens from the Duong River. Among them, 178 specimens were found to shed bucephalid cercariae (Fig. 2). The infection rate did not differ significantly among the reservoirs (Table 1); however, the prevalence in the Duong River was 3.14 times higher than that in the reservoirs (p = 0.005).

Figure 1. The golden mussel *Limnoperna fortunei* collected in the Hoa Binh reservoir (Scale bar: 0.5 cm)

Table 1. Infection prevalence of *Limnoperna fortunei* across study sites

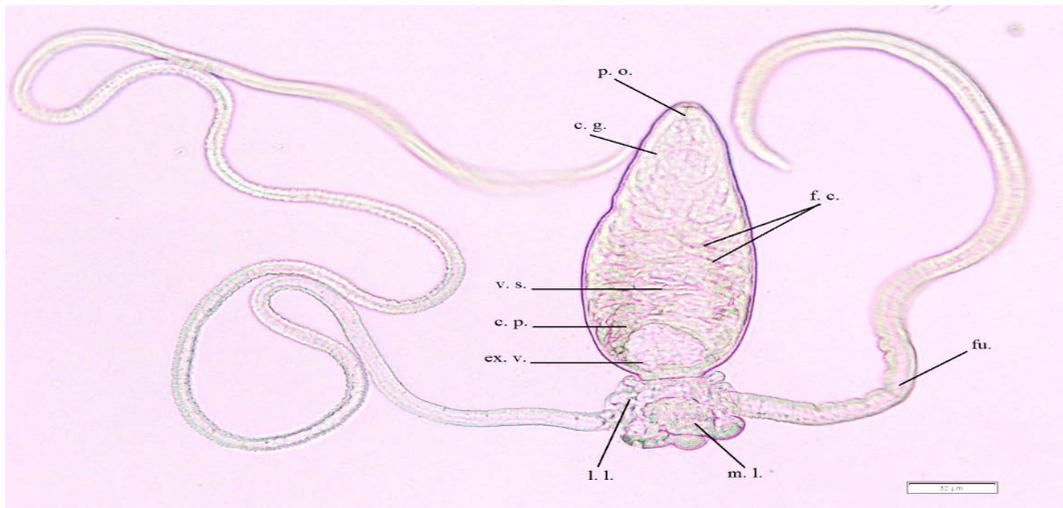| Study sites	No. collected samples	No. infected samples	Prevalence
Son La	116	9	7.75
Hoa Binh	256	15	5.86
Thac Ba	297	20	6.73
Duong River	784	134	17.09
Total	1,453	178	12.25

Figure 2. Bucephalid cercaria shed from the golden mussel *Limnoperna fortunei*. (c.g. cephalic grands, c.p. cirrus pouch, ex.v. excretory vesicle, f.c. flame cells, fu. furca, l.l. lateral lobe, m.l. median lobe, p.o. penetration organ, v.s. ventral sucker)
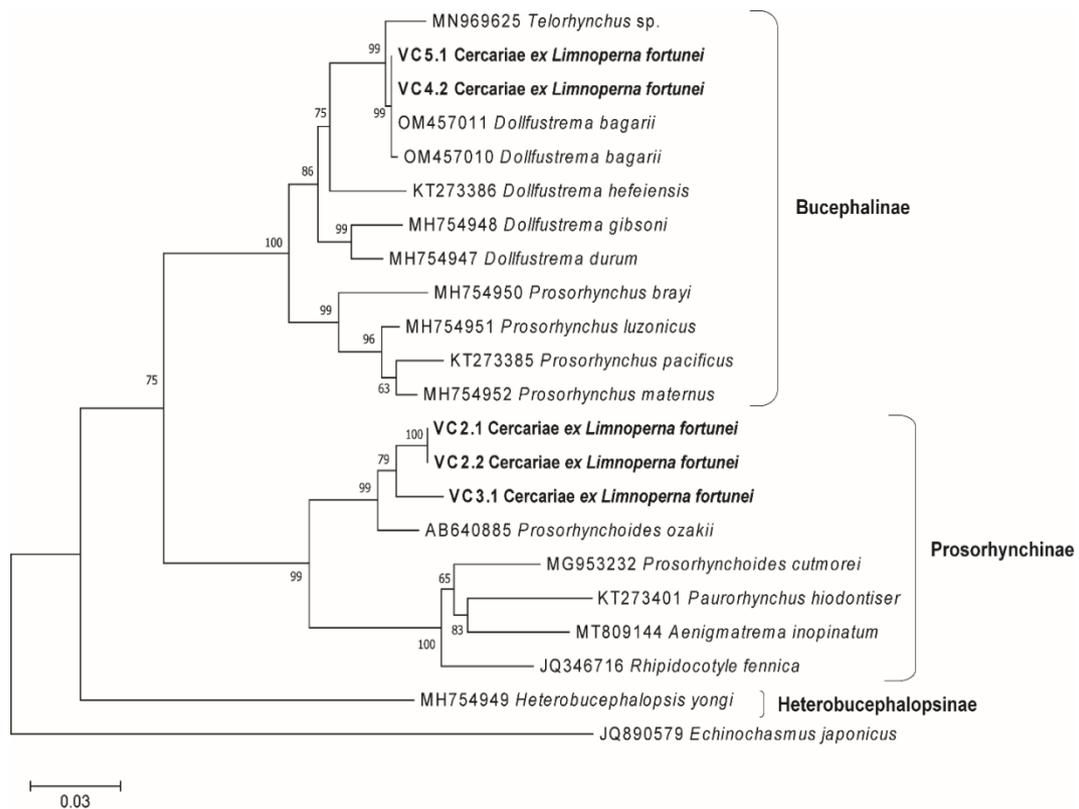

Figure 3. Phylogenetic tree inferred from *28S* rDNA sequences using the unweighted pair group method with arithmetic mean (UPGMA) algorithm. GenBank accession number precedes species name. The newly generated sequence is in bold. *Echinochasmus japonicus* was used as an outgroup. Bootstrap confidence values are expressed as a percentage based on 1,000 replicates

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Molecular data

The *28S* rDNA sequences of two furcocercous cercariae isolates, VC4.2 (from *L. fortunei* in Duong River) (GenBank accession numbers PZ149712) and VC5.1 (from *L. fortunei* in Thac Ba reservoir) (GenBank accession numbers PZ149713), showed 100% similarity to the sequence from *D. bagarii* (GenBank accession numbers OM457010, OM457011). No intraspecific variation was detected. UPGMA analysis based on *28S* rDNA sequences confirmed that these cercariae from our material were conspecific with *D. bagarii* (Fig. 3).

Similarly, the *28S* rDNA sequence of three furcocercous cercariae isolates from the present study, VC2.1 and VC2.2 (from *L. fortunei* in Hoa Binh and Son La reservoirs) (GenBank accession numbers PZ149709, PZ149710) and VC3.1 (from *L. fortunei* in Hoa Binh reservoir) (GenBank accession numbers PZ149711) formed a monophyletic clade, and are sister to other species of the genus *Prosorhynchoides*. Sequences VC2.1 and VC2.2 were identical (100% identity), with no intraspecific variation detected, whereas both showed 97.7% identity with VC3.1. This result indicates that VC2.1 and VC2.2 represent cercariae of one *Prosorhynchoides* species, while VC3.1. represents a different species of the same genus (Fig. 2).

In the phylogenetic tree inferred from the *28S* rDNA gene, all bucephalids formed a single lineage, excluding the outgroup taxon *Echinochasmus japonicus* (GenBank accession number JQ890579). Within the bucephalid lineage, all *Dollfustrema* species clustered within the Prosorhynchinae clade, and all *Prosorhynchoides* species clustered within the Bucephalinae clade. Both clades were more closely related to each other than to the Heterobucephalopsinae.

DISCUSSION

In the life cycle of bucephalids, mussels serve as the first intermediate host for several known species. For instance, the golden mussel *L. fortunei* has previously been reported as the first intermediate host of *Dollfustrema vaneyi* (based on experimental infection) (Wang & Wang, 2000), and *Dollfustrema invadens* (based on natural infection) (Saito et al., 2025). In this study, *L. fortunei* was found to be infected with *D. bagarii*, and two other species of *Prosorhynchoides*, with high prevalence observed across all surveyed sites. Since *L. fortunei* is invasive worldwide and channel catfish (*Ictarulus punctatus*) has been cultured globally, the distribution of bucephalid trematodes may not be confined to Vietnam and have the potential to spread worldwide, especially because *L. fortunei* commonly forms dense aggregations on cage nets, which could help explain the high infection rates in cultured fish (Thuy & Buchmann, 2008; Le et al., 2025b). The prevalence of infection in *L. fortunei* collected from the river was significantly higher than in reservoirs, which could be explained by differences in environmental conditions, such as the distance of cultured cages from the banks of reservoirs/rivers and the speed of water.

According to Nguyen et al. (2022), the identification of digenean larvae based on morphological characters is difficult. For example, by morphological characteristics alone, the authors identified only 10 morphotypes of cercariae, whereas molecular data revealed 13 species. Therefore, identification should be supported by experimental infection or DNA sequencing. In the present study, we recognized only furcocercous cercarial morphotypes with several morphological features typical of bucephalids, for example, two long furcal rami originating from the tail stem; a penetration organ at the anterior end of the body, containing a rhynchus with tiny, spine-like structures; flame cell formula: $2 \times [(3 + 3 + 3) + (3 + 3 + 3)]$; and the presence of intestine saccular, ventral sucker, testicular and ovarian anlage of particular size and position, and a cirrus sac. Corner et al. (2020) reported that integrated data, including both morphological and molecular characteristics, are needed to resolve systematic and phylogenetic

relationships within Bucephalidae. Therefore, the identification of bucephalid cercariae should combine both morphological and molecular analyses.

In the phylogenetic tree, several worms identified as members of different genera clustered together, suggesting that they may actually belong to the same genus. For example, in Figure 3, *Telorhynchus* sp. (GenBank accession number MN969625) is more closely related to *D. bagarii* (GenBank accession numbers OM457010, OM457011, PZ149712, and PZ149712) than to other species of *Dollfustrema*, such as *Dollfustrema hefeiensis* (GenBank accession number KT273386), *Dollfustrema gibsoni* (GenBank accession number MH754948), and *Dollfustrema durum* (GenBank accession number MH754947). Therefore, the identification of *Telorhynchus* sp. based on morphology should be carefully re-examined.

Previous studies in Hoa Binh, Son La, and Thac Ba reservoir reported trematodes only from freshwater snails, with no records from bivalves (Nguyen et al., 2021; Nguyen, 2022; Le et al., 2025a). Because mussels in our study were collected from cage nets in cultured areas, future faunal surveys should include both mollusks and bivalves, particularly where fish are cultured.

D. bagarii was first described from the dwarf goonch catfish *Bagarius bagarius* (Sisoridae) in Hoa Binh province (Moravec & Sey, 1989), and later found in channel catfish (*I. punctatus*) cultured in nine provinces in Northern Vietnam (Le et al., 2025b), while *Prosorhynchoides ozakii* has been reported in southern provinces from the river catfish *Pangasianodon hypophthalmus* (Thuy & Buchmann, 2008), and *Pangasianodon karvei* in the marine fish Spottail needlefish *Strongylura strongylura* in Halong Bay (Atopkin et al., 2022). The present study provides the first record of *Prosorhynchoides* in a freshwater environment in Northern Vietnam.

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