

Morphological and Molecular Confirmation of *Parvatrema duboisi* Metacercariae in the Manila Clam *Ruditapes philippinarum* from Gochang-gun, Korea

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Abstract: Gymnophallid metacercariae found in the Manila clam *Ruditapes philippinarum* ('Banjirak' in Korean) from Gochang-gun, Jeollabuk-do, Korea were morphologically and molecularly confirmed to be *Parvatrema duboisi* (Dollfus, 1923) Bartoli, 1974. The metacercariae were morphologically characterized by having a large oral sucker, small ventral sucker, genital pore some distance anterior to the ventral sucker, no ventral pit, and 1 compact or slightly lobed vitellarium, which were all compatible with *P. duboisi*. Some of the metacercariae were experimentally fed to mice, and adult flukes were recovered at day 7 post-infection. The morphology of the adult flukes was basically the same as that of the metacercariae except for the presence of uterine eggs; the uterus was filled with up to 40 eggs. The nucleotide sequences (1,193 bp) from ITS regions (ITS1, 5.8S rDNA, and ITS2) of the metacercariae showed 99.7% identity with *P. duboisi* and 75.7% identity with *Gymnophalloides seoi* deposited in GenBank. These results confirmed the presence of *P. duboisi* metacercariae in the Manila clam *R. philippinarum* in an estuary region of Gochang-gun, Korea.

Key words: *Parvatrema duboisi*, gymnophallid, Manila clam, Gochang-gun, Korea

In gymnophallid trematodes (family Gymnophallidae Odhner, 1905), 5 genera are acknowledged to be valid; *Gymnophalloides* Fujita, 1925 (syn. *Lacunovermis* Ching, 1965), *Parvatrema* Cable, 1953 (syn. *Meiogymnophallus* Ching, 1965), *Gymnophallus* Odhner, 1900 (syn. *Paragymnophallus* Ching, 1973), *Pseudogymnophallus* Hoberg, 1981, and *Bartolius* Cremona, 2001 [1]. The adult flukes generally occur in the intestine, gall-bladder, and bursa Fabricii of marine birds, including shore birds and diving ducks [1]. However, they also occur rarely in mammals, for example, in 2 species of *Gymnophalloides*; *G. seoi* in humans and cats [2,3] and *G. heardi* in rats [4].

With regard to *Parvatrema*, the adult flukes are found exclusively in shore birds and diving ducks [1]. In the Republic of Korea (= Korea), the presence of the life cycle of several gymnophallids have been reported, including *G. seoi* [2,5], *Parva-*

trema macrostoma n. comb. (syn. *Gymnophallus macrostoma* Yamaguti, 1939) [6], *Parvatrema duboisi* (Dollfus, 1923) Bartoli, 1974 (syn. *Parvatrema timondavidi* Bartoli, 1964) [7], *Parvatrema chaii* Sohn et al., 2007 [8], *Parvatrema homoeotectum* James, 1964 [9], and *Parvatrema sinonovaculae* n. comb. (*Meiogymnophallus sinonovaculae* Chai et al., 2007) [10].

In Korea, the presence of *P. duboisi* metacercariae was first documented by Yu et al. [7]. They detected gymnophallid metacercariae in the Manila clam, *Ruditapes philippinarum* (syn. *Tapes philippinarum*), purchased from a fishery market in Seoul and obtained adult flukes after experimental infection to ICR mice. However, the locality of the clam collected was unknown [7]. Subsequently, Sohn et al. [11,12] found metacercariae of *Parvatrema* spp. (including *P. duboisi*) in *R. philippinarum* collected from various areas of Gyeongsangnam-do, Jeollanam-do, and Chungcheongnam-do. In addition, Chung et al. [9] recovered adult flukes of *P. duboisi* from the intestine of great knots *Calidris tenuirostris* caught from a coastal area of Gunsansi (City), Jeollabuk-do. However, in Gochang-gun, Jeollabuk-do, where the Manila clam, *R. philippinarum*, is abundantly produced, there have been no reports on gymnophallid meta-

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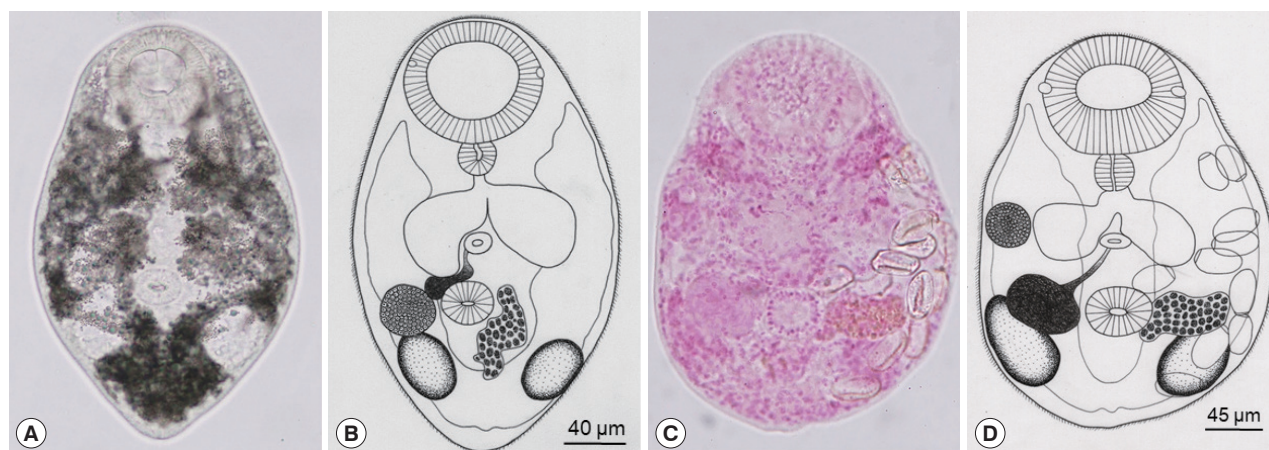


Fig. 1. *Parvatrema duboisi* metacercariae and adults originating from Gochang-gun, Korea. (A) A living metacercaria isolated from a Manila clam, *Ruditapes philippinarum*. The oral sucker, ventral sucker, genital pore, and 2 testes are recognizable. (B) Line drawing of the metacercaria showing the internal organs, including the oral sucker, ventral sucker, genital pore, seminal vesicle, ovary, 2 testes, slightly lobed vitellarium, and V-shaped excretory vesicle. (C) An adult fluke recovered from an experimental mouse at day 7 post-infection. The seminal vesicle is well developed and the uterus with eggs is seen. (D) Line drawing of Fig. 1C showing the oral sucker, ventral sucker, ceca, genital pore, seminal vesicle, ovary, 2 testes, vitellarium, and eggs.

cercariae, including *P. duboisi*. Thus, the present study was performed to determine the existence of the life cycle of gymno-phallid flukes, in particular, *P. duboisi*, in coastal regions of Gochang-gun, Korea.

The Manila clam, *R. philippinarum*, was collected from an estuary of Gochang-gun and also purchased from a fishery market in Seoul (those clams having the known origin). The shell of the clam was removed, and the animal was cut into several pieces using a pair of scissors. The animal pieces were incubated at 37°C in artificial gastric juice (pepsin-HCl solution) for 5 min and washed several times with 0.85% saline. The sediment was examined for gymno-phallid metacercariae using a stereomicroscope. Some metacercariae were fixed in 70% ethanol for molecular studies, and some others were fixed in 10% formalin for morphological studies.

In order to obtain the adult flukes, laboratory mice (ICR) were experimentally fed the metacercariae isolated from the clam and killed at day 7 post-infection. The animal experiment was performed according to the guidelines of the Committee on the Ethics of Animal Experiments at Seoul National University, Seoul, Korea. The intestines of the mice were resected and examined for the presence of adult flukes. The flukes collected were fixed with 10% formalin and stained with Semichon's acetocarmine.

Genomic DNA was extracted from metacercariae (8 specimens) using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). The internal transcribed spacer (ITS) re-

gions of rDNA, including ITS1, 5.8S rRNA, ITS2, were amplified using the standard PCR protocol with eukaryotic universal primers, 18d (5'-cacaccgccctgctactaccgattg-3') and 28cc (5'-actgccgttactgaggaatcctggttag-3') [13]. The amplified product was processed as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, and extension at 72°C for 1.3 min, followed by a final elongation at 72°C for 5 min. The PCR products were purified and directly sequenced by Macrogen Inc. (Seoul, Korea). The phylogenetic relationships of our sample with other gymno-phallid species available in GenBank were analyzed using the maximum-likelihood method.

Based on the morphological (Fig. 1) and molecular data (Fig. 2), the gymno-phallid metacercariae found in the Manila clam from Gochang-gun have been confirmed to be *P. duboisi*. The brief morphologies of the metacercariae and adults of *P. duboisi* were as follows:

The metacercariae ($n = 10$) were 315 (295-330) μm long and 205 (190-220) μm wide (Fig. 1). The oral sucker was large and well developed with 2 small lateral projections on the lip. The pre-pharynx was absent; the pharynx was round and muscular. The ceca were inflated forming oval sacs. The ventral sucker was small, round in shape, and located posterior to the equatorial line. Two testes were round to elliptical in shape, and located laterally to the ventral sucker. The ovary was in front of the right testis, slightly smaller than the testes. The genital pore was a wide slit-like opening, located some distance anterior to

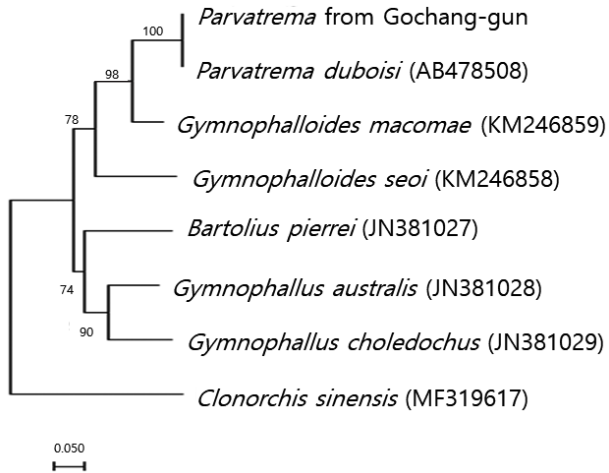


Fig. 2. Phylogenetic tree showing a relationship between *Parvatrema* from Gochang-gun and members of the family Gymnophallidae available in GenBank analyzed by maximum-likelihood method using *Clonorchis sinensis* as an outgroup. Bootstrap values of 1,000 replicates are shown on nodes. GenBank accession numbers are indicated for reference species. Scale bar indicates number of nucleotide substitutions per site.

the ventral sucker. The seminal vesicle was unipartite, and connected to the genital pore. The vitellarium was only 1 compact or slightly lobed mass, located nearby the left or postero-sinistral side of the ventral sucker. The excretory bladder was V-shaped with 2 arms reaching to the level of the oral sucker.

The adult flukes ($n=10$) were 317 (298-330) μm long and 208 (190-230) μm wide (Fig. 1). The oral sucker was characteristic with 2 small lateral projections on the lip. The pharynx was well developed. The ventral sucker was small, round in shape, and located in the posterior field of the body. Two testes were slightly elliptical in shape, and located laterally to the ventral sucker, vitellarium, and seminal vesicle. The ovary was some distance anterior to the right testis. The genital pore was well developed, located anterior to the ventral sucker. The seminal vesicle was large and unipartite, and connected to the genital pore. The vitellarium was 1 compact or slightly lobed mass, located at postero-sinistral side of the ventral sucker. The excretory bladder was V-shaped with 2 arms reaching to the level of the oral sucker. The uterus was equipped with many loops and a small number of eggs; the egg size was 28 (27-29) μm long and 19 (17-21) μm wide.

The sequences of the ITS region of our specimen revealed only 3 nucleotide substitutions (1,190 of 1,193 sites were identical) from the known sequences of *P. duboisi* deposited in GenBank (no. AB478508). The sequence identity was 99.7%.

Meanwhile, our sample showed a sequence homology of only 75.7% in comparison with *G. seoi*. The phylogenetic tree (Fig. 2) showed that our sample was genetically closest to *P. duboisi* and quite close to *Gymnophalloides macomae* n. comb. Scholz, 2002 [syn. *Lacunovermis macomae* (Lebour, 1908) Loos-Frank, 1970] and *G. seoi*, but far from *Bartolius pierrei*, *Gymnophallus australis*, and *G. choledochus*.

The taxonomy of gymnophallid flukes has been highly complex and confusing. Ching [14] created 2 new genera, *Lacunovermis* (wide genital pore, ventral pit present) and *Meiogymnophallus* (small genital pore, no ventral pit). However, Yamaguti [15] and Scholz [1] synonymized *Lacunovermis* with *Gymnophalloides* (small genital pore, ventral pit present), both having a ventral pit, but because the size of the genital pore could not be a significant character to differentiate the 2 genera. Scholz [1] also synonymized *Meiogymnophallus* with *Parvatrema* because the difference between the 2 genera given by Ching [14,16] was only the size of the genital pore, small in the former and wide in the latter. Ching [17] also created a new genus *Paragymnophallus* to accept those gymnophallids having a wide genital pore situated at a distance anterior to the ventral sucker. However, this genus was synonymized with *Gymnophallus* by Scholz [1].

Therefore, currently in the genus *Parvatrema*, about 24 species are included; *P. affinis* Jameson and Nicoll, 1913, *P. borealis* Stunkard and Uzmann, 1958, *P. borinquena* Cable, 1953, *P. bushi* Ching, 1995, *P. chaii* Sohn et al., 2007, *P. donacis* Hopkins, 1958, *P. duboisi* (Dollfus, 1923), *P. fossarum* Bartoli, 1965, *P. homoeotecnium* James, 1964, *P. isostoma* Belopolskaja, 1966, *P. jamesoni* (Bowers, 1965), *P. lintoni* (Linton, 1928), *P. macrostomus* (Yamaguti, 1939), *P. margaritense* (Ching, 1982), *P. minutus* (Cobbold, 1859), *P. obscurum* Ching, 1960; *P. ovoplenum* (James and Nicoll, 1913), *P. polymesoda* Ching, 1995, *P. rebecqui* Bartoli, 1983, *P. rebunense* Shimazu, 1975, *P. sinonovaculae* Chai et al., 2007, *P. skrjabini* Ryschikov, 1963, *P. somateriae* (Levinsen, 1881), and *P. strigatus* Lebour, 1908 [1,18].

Our specimens (*P. duboisi*) from Gochang-gun, Korea had only 1 compact or slightly lobed vitellarium. Thus, they differed from *P. affinis*, *P. fossarum*, *P. jamesoni*, *P. macrostomus*, *P. minutus*, *P. obscurum*, *P. rebecqui*, *P. sinonovaculae*, *P. skrjabini*, *P. somateriae*, and *P. strigatus* which have a paired group of vitellaria and previously assigned to *Meiogymnophallus* [14,18]. Among the others, 7 species, including *P. borinquena*, *P. bushi*, *P. chaii*, *P. donacis*, *P. duboisi*, *P. polymesoda*, and *P. rebunense*, have a single compact vitellarium and were comparable with

our specimens. Their differential points from our specimens were as follows. *P. borinqueña* is characterized by its small egg size (12-19 µm long) and the presence of gland cells around the oral sucker [4]. *P. bushi* has a small delicate body with fewer body spines, smaller lateral papillae, smaller pharynx, and shallower genital atrium [4]. *P. chaii* is characterized by smaller-sized eggs (18-20 µm long), the presence of an anterior arch of 16-17 sensory papillae on the genital pore, and club-shaped seminal vesicle [8]. *P. donacis* is distinct having a large body size and does not have an esophagus [19], whereas our specimens have a smaller body and a short esophagus. *P. polymesoda* is characterized by a large body size, large lateral papillae, large ceca, but a small vitellarium [4]. *P. rebunense* differs from our samples in the shape of its ovary which is slightly lobed and elongated oval [20]. Based on these differential points, our specimens could be assigned as *P. duboisi*.

The morphological diagnosis of our samples was supported by the results of sequencing of the ITS region. The homology with *P. duboisi* was 99.7%, while the homology with *G. seoi* was 75.7%. In GenBank, there are nucleotide data of only 2 *Parvatrema* sp., *P. duboisi* and *Parvatrema* sp., so that comparison of our specimens with other species of *Parvatrema* could not be done.

In 1923, Dollfus [21] described *Gymnophallus duboisi* with the metacercariae obtained from the marine mussel *Mytilus galloprovincialis* in France. In 1963, Bartoli [22] described a new species of *Parvatrema* (*P. timondavidi*) based on metacercariae from *M. galloprovincialis* collected from the Gulf of Marseille, France. However, in 1974, Bartoli [23] found that the worm described by him in 1963 was identical with those of *Gymnophallus duboisi* and named it as *Parvatrema duboisi* synonymizing *P. timondavidi* with *P. duboisi*. Separately from this, in 1944, Ogata [24] obtained gymnophallid metacercariae from a bivalve *Ruditapes philippinarum* in Japan and adults from an experimental rat and described them as *Gymnophallus bursicola* which was originally reported by Odhner in 1900. Endo and Hoshino [25] in 1974 found that these metacercariae and adults were different from *G. bursicola* and should be assigned to *P. timondavidi*. In 1982, Shimura et al. [26] detected the same metacercariae from *R. philippinarum* in the Lake Hamana and determined them as *P. duboisi*. Later, Yanagida et al. [13] discovered the larval stages of this gymnophallid, including the sporocyst containing cercariae and free metacercariae in the Manila clam in Ariake Sea, Japan and obtained adult flukes in experimental mice and rats.

In conclusion, the present study confirmed the presence of the life cycle of *P. duboisi* in an estuary area of Gochang-gun using the Manila clam *R. philippinarum* as (the first and) the second intermediate host. The possibility of human infection with this gymnophallid should be ruled out in Korea where people like to consume the Manila clams.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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