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# Amended diagnosis of the genus *Provitellotrema* (Digenea: Haplosplanchnidae) with description of a new species from vietnamese mullets

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ABSTRACT

Morphological and molecular data for a new representative of the genus *Provitellotrema* (Haplosplanchnidae) from the mullets of the coastal waters of Vietnam were provided in the present study. Morphologically, the trematodes described here are similar to representatives of *Haplosplanchnus* and *Pseudohaplosplanchnus* and demonstrate the most closeness to *Haplosplanchnus purii* by most of the metric parameters. Results of molecular phylogenetic analysis show that the trematodes we found were closely related to *Provitellotrema crenimugilis,* and *H. purii* was in the same clade. Genetic distance values between trematodes from new material and *P. crenimugilis* were 0.69 ± 0.22 % and 10.78 ± 1.01 % by 28S rDNA and mitochondrial COI gene sequence data, respectively. Morphologically, the adult worms of *P. crenimugilis* and trematodes from our study can be discriminated by several characters, including the distribution of the vitelline follicles, pharynx, and egg size. Based on the results, we conclude that trematodes from our study belong to the new species of the genus *Provitellotrema*, *P. halongensis* n. sp. The difference in vitellaria arrangement between new species and *P. crenimugilis* can be considered a species-specific characteristic within the genus *Provitellotrema*. An amended diagnosis for the genus *Provitellotrema* was provided.

## 1. Introduction

According to Madhavi [1], the sparse digenean family Haplosplanchnidae Poche, 1926, contains four subfamilies: Haplosplanchninae Poche, 1926, Haplosplanchnoidinae Yamaguti, 1971, Hymenocottinae Yamaguti, 1971, and Schikhobalotrematinae Skrjabin & Guschanskaja, 1955. Representatives of these subfamilies are intestinal parasites of mainly marine, estuarial, and freshwater fish species. Of these, Haplosplanchninae Poche, 1926, is the most numerous group, which includes five genera; the other three subfamilies are represented by one or two genera [1]. The results of recent taxonomical studies of Haplosplanchnidae bring about two new genera being included in the family: *Trigonocephalotrema* Huston, Cutmore, Cribb, 2018, parasites of Australian marine fishes, and *Pseudohaplosplanchnus* Atopkin, Besprozvannykh, Ha, Nguyen, Nguyen, 2020, from Vietnamese mullets [2,3]. Huston [2] notified that the genus *Trigonocephalotrema* requires the erection of a new subfamily based on both morphological and molecular data. However, these authors did not recognise this status for the trematodes they described because they found little value in subfamily-level division within a clade with *Trigonocephalotrema*, containing so few genera and species. Representatives of the genus *Pseudohaplosplanchnus* are similar to trematodes of the genus *Haplosplanchnus* Looss, 1902 morphologically, but differ from species of this genus by molecular data at a level of subfamily. Nevertheless, this genus was not recognised as a distinct subfamily because of disagreements about morphological and molecular data. These results uncovered the problem of discrepancy in taxonomical interpretations of morphological and molecular data for different species of the family Haplosplanchnidae. This arises from the fact that there is not enough knowledge on the species diversity of this trematode group. [2–5]. There is a little knowledge on trematodes of the genus *Provitellotrema*. Before our present study, this genus was monotypic, which included the only type species, *Provitellotrema crenimugilis*

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Pan, 1984, from the intestines of Chinese brackish-water mullets. [1] In the present study, we provide morphological and molecular data for a new species of the genus *Provitellotrema* Pan, 1984 (Haplosplanchnidae) from the mullets of the coastal waters of Vietnam.

## 2. Material and methods

### 2.1. Sample collection

Adults of haplosplanchnids were collected from the intestines of bluespot mullet *Crenimugil seheli* (Fabricius, 1775) (Mugilidae) from the coastal waters of Cat Ba Island, Vietnam. Worms were separately rinsed in saline, killed with hot distilled water, and preserved in 70 % ethanol. After fixation, worms were replaced with 96 % ethanol. Before slide preparation, all detected worms were previously defined under a microscope, Nicon Eclipse E200. All trematodes detected in the intestines of five of nine exemplars of *C. seheli* were sorted into two groups. The first group of five specimens from two hosts was morphologically identified as *Haplosplanchnus pachysomus* (Eysenhardt, 1829). The second group consists of 59 worms with identical general morphology, including vitellarium formed from small follicles of irregular forms, located near the ventral sucker. In the first view, these trematodes were the putative new species of Haplosplanchninae. Whole mounts were made by staining specimens with alum carmine, dehydrating the worms in a graded ethanol series, and clearing in clove oil. The clove oil treatment was followed by mounting the specimens in Canada balsam under a coverslip on a glass slide. All measurements are given in micrometers.

### 2.2. DNA extraction, amplification and sequencing

Six adult trematodes of putative new species and one specimen of *Haplosplanchnus pachysomus* were fixed in 96 % ethanol for molecular analysis (Table 1). Total DNA was extracted from flukes using a Hot Shot technique [6]. Nuclear 18S rDNA and 28S rDNA and mitochondrial COI gene fragments were amplified using a polymerase chain reaction technique. Then, 18S rDNA was amplified with the following primers: 18S-E (5' CCG AAT TCG TCG ACA ACC TGG TTG ATC CTG CCA GT 3′) and 18S—F (5' CCA GCT TGA TCC TTC TGC AGG TTC ACC TAC 3′), described earlier [7].

The initial PCR reaction was performed in a total volume of 20 μl containing 0.25 mM of each primer pair, 25 ng of total DNA in water, 5× Taq buffer, 1.25 mM dNTPs, 1.5 mM magnesium, and 1 unit of Taq polymerase. Amplification of a 2000-bp fragment of the 18S rRNA gene was performed in a GeneAmp 9700, Applied Biosystems, with a 5-min denaturation at 96 °C, 35 cycles of 1 min at 96 °C, 20 s at 58 °C, and 5 min at 72 °C, and a 10-min extension at 72 °C. Negative and positive controls using both primers were used. 28S ribosomal DNA (rDNA) was amplified with the primers DIG12 (5′-AAG CAT ATC ACT AAG CGG-3′) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) [8] with an annealing temperature of 55 °C. Mitochondrial COI gene partial sequences were amplified with PCR primers JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) and COI-R Trema (5'-CAA CAA ATC ATG ATG CAA AAG G-3′) [9] with an annealing temperature of 48 °C.

PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA), as recommended by the manufacturer, with the internal sequencing primers described by Tkach [8] for 28S rDNA and Littlewood and Olson [7] for 18S rDNA. Mitochondrial COI gene fragment sequences were generated with primers used for amplification. PCR product sequences were analysed using an ABI 3500 genetic analyser at the Federal Scientific Centre of the East Asia Terrestrial Biodiversity FEB RAS. Sequences were submitted to the GenBank database (NCBI).

### 2.3. Alignments and phylogenetic analysis

Ribosomal DNA sequences were assembled with SeqScape v.2.6 software, provided by Applied Biosystems. Alignments and estimations

**Table 1**
List of taxa incorporated in the molecular analysis of the superfamily Haplosplanchnoidea with the number of DNA sequences given in parentheses. * $n = 3$ for COI gene sequence dataset; ** - $n = 2$ for for COI gene sequence dataset. ***The article is not available for a cite. GenBank accession numbers shown in bold were newly obtained in this study.

| Species | Author | GenBank accession numbers | | |
|---|---|---|---|---|
| | | 18S | 28S | COI |
| **Haplosplanchnoidea** | | | | |
| ***Haplosplanchnus pachysomus* (n = 1)** | **This study** | **PP809843** | **PP809857** | **PQ218972** |
| *Haplosplanchnus pachysomus* (*n* = 4)* | [4] | LK932143- LK932146 | LK932149- LK932152 | **PQ220047-PQ220049** |
| *Haplosplanchnus pachysomus* (n = 1) | Unpublished*** | FJ211224 | FJ211241 | – |
| *Haplosplanchnus pachysomus* (*n* = 1) | [5] | – | KY852458 | – |
| *Haplosplanchnus pachysomus* (n = 1) | [20] | – | MZ618878 | – |
| *Haplosplanchnus purii* (n = 1) | Unpublished*** | FJ211225 | FJ211242 | **SUB15099870** |
| *Hymenocotta mulli* (n = 1) | [23,24] | AJ287524 | AY222239 | – |
| *Hymenocotta mulli* (n = 2) | [3] | MT280021, MT280022 | MT280023, MT280024 | – |
| ***Provitellotrema halongensis* n. sp. (*n* = 6)** | **This study** | **PP809843-PP809848** | **PP809850-PP809856** | **PQ218966- PQ218971** |
| *Provitellotrema crenimugilis* (n = 2) | [4] | LK932147- LK932148 | LK932153- LK932154 | **PQ220051- PQ220052** |
| *Provitellotrema crenimugilis* (n = 1) | **This study** | – | **PP809857** | **PQ220050** |
| *Pseudohaplosplanchnus catbaensis* (n = 5)** | [3] | MT298956- MT298957 | MT298961- MT298962 | **PQ220053- PQ220054** |
| *Schikhobalotrema sparisomae* (n = 1) | Unpublished*** | FJ211223 | FJ211240 | – |
| *Schikhobalotrema* sp. (n = 1) | [23,24] | AJ287574 | AY222238 | – |
| *Trigonocephalotrema euclidi* (n = 1) | [2] | MG386254 | MG386255 | – |
| *Trigonocephalotrema hipparchi* (n = 1) | [2] | MG386257 | MG386258 | – |
| *Trigonocephalotrema sohcahtoa* (n = 1) | [2] | MG386260 | MG386261 | – |
| *Trigonocephalotrema* sp. (n = 1) | [2] | MG386263 | MG386264 | – |
| Outgroup | | | | |
| Echinostomatoidea | | | | |
| *Psilochasmus oxyurus* | [24,25] | AY222135 | AF151940 | – |
| Pronocephaloidea | | | | |
| *Catatropis indicus* | [24] | AY222114 | AY222220 | – |
| *Lankatrema mannarense* | [24] | AY222116 | AY222222 | – |
| Strigeoidea | | | | |
| *Rhipidocotyle galeata* | [24] | AY222119 | AY222225 | – |

of the number of variable sites and sequence differences were performed using the MEGA 7.1 software [10]. The values of genetic p-distances were calculated for the 28S ribosomal DNA fragment and mitochondrial COI gene fragment.

Phylogenetic relationships were obtained using only the 28S ribosomal rRNA gene, concatenating complete 18S rRNA and partial sequences of the 28S rRNA gene and mitochondrial COI gene fragment sequence datasets. Phylogenetic analysis was performed using the Maximum Likelihood (ML) and Bayesian Inference (BI) algorithms with the PhyML 3.1 and the MrBayes v. 3.1.2 software, respectively [11,12]. The best-fit model of sequence evolution for the phylogenetic analysis was estimated based on Akaike's information criterion (AIC) [13] using jModeltest version 2.1.5 software [14]. The best nucleotide substitution model, the GTR + I + G [15], was estimated for all used ribosomal DNA sequence datasets. The TPM1uf + I + G model [16] was estimated as optimal for the partial COI gene sequence dataset.

Bayesian analysis was used with the respective set of parameters for the evolutionary model chosen; the main priors, including frequencies of nucleotide substitutions, nucleotide composition, gamma shape, and amount of invariant sites, were introduced as fixed values. The Markov Chain Monte Carlo (MCMC) algorithm was performed with the following parameters: ngen = 10,000,000 via two independent runs (nruns = 2), four simultaneous Markov chains (nchains = 4) with every 100th tree saved (samplefreq = 100), and the standard deviation of split frequencies at 0.0099. Summary parameters and the phylogenetic tree were calculated with a burn-in of 25 % of generations.

The significance of phylogenetic relationships was estimated with posteriori probabilities [17] for the BI algorithm and an approximate likelihood-ratio test [18] for the ML algorithm. Accession numbers, authority, and supporting information about rDNA sequences from GenBank used for the phylogenetic analyses are provided in Table 1. We do not include the species *Schikhobalotrema minuta* in our analysis because too short sequences of 28S rDNA (600–782 bp) are available. The accession numbers of newly generated sequences are provided in Table 1.

## 3. Results

### 3.1. Taxonomy of the new species

**Family Haplosplanchnidae Poche, 1926.**
**Genus *Provitellotrema* Pan, 1984.**
*Provitellotrema halongensis* n. sp. (Fig. 1).
**Type host.** *Crenimugil seheli* (Fabricius, 1775).
**Site:** Small intestine.
**Prevalence:** 5 of 9 (56 %) *C. seheli.*
**Intensity of infection:** 10–15 worms per fish.
**Type locality:** Cat Ba Island, Ha Long Bay, northern Vietnam (20°88′40″N, 10°68′590″E).
**Type material:** holotype whole mature worm (Registration number: 256-Tr), paratypes whole 4 worms, same data as holotype, (Registration number: 257–260-Tr) are deposed in the parasitological collection of the Zoological Museum (Federal Scientific Center of the East Asia Terrestrial Biodiversity Far Eastern Branch of Russian Academy of Sciences, Vladivostok, Russia; e-mail: petrova@ibss.dvo.ru).
**ZooBank registration:** LSID: urn:lsid:zoobank.org:act:F5DE6E15-19E6-4713-BA67-3EE9C5C57C06
**Etymology:** The species name originates from the name of Halong Bay, Vietnam, from where mullets infected with this trematode were caught.

### 3.2. Examination and description of samples (Fig. 1)

Based on 5 mature worm specimens. Body elongate, with narrowed anterior and posterior end. Spines were not found on the body. Eyespot pigment dispersed in forebody. Oral sucker subterminal. Ventral sucker at border of anterior and middle third of body. Prepharynx short. Pharynx transversally oval. Oesophagus short. Caecum single, reaching level posterior border of ventral sucker. Testis single, in middle or posterior third of body, round, or oval. Seminal vesicle tubular, reaches to border middle and posterior third of body. Prostatic part indistinct, short, prostatic cells not numerous. Hermaphroditic duct very short. Genital pore median, at middle between oral and ventral suckers. Ovary spherical, pre-testicular at small distance from testis or contiguous with testis. Seminal receptacle round, dextral contiguous to ovary. Uterus in middle third of body, with several loops, posterior reaching level of ovary. Eggs not numerous, large, operculated, containing miracidium, with eyespot. Vitellarium in two lateral fields formed from small follicles of irregular forms, extending between level of anterior border of ventral sucker and level middle of distance between ventral sucker and ovary. Excretory bladder, I-shaped with terminal pore. Posterior end of excretory bladder, surrounded by numerous elongated cells (Fig. 1).

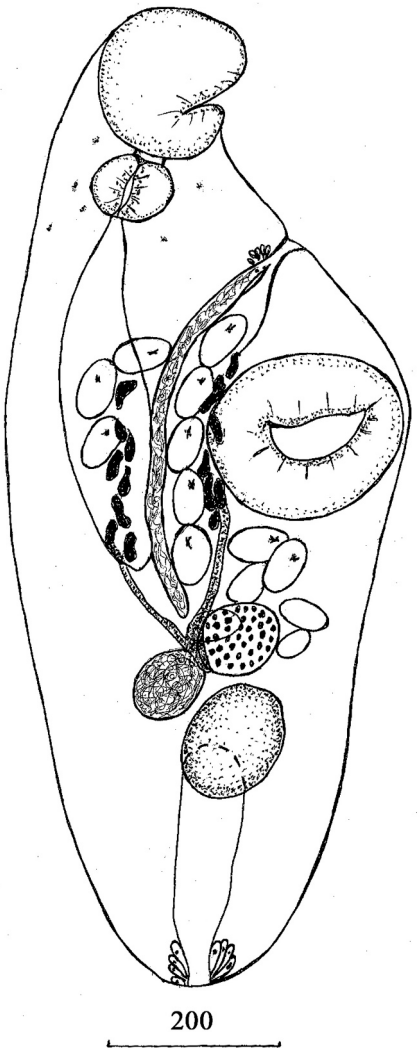


**Fig. 1.** Holotype *Provitellotrema halongensis* n. sp. Scale: 200 μm.

### 3.3. Molecular results

The complete nucleotide sequences of the 18S rRNA gene (1785 bp in length) and the partial 28S rRNA gene (1249 bp in length) were generated for six specimens of new species and one of *H. pachysomus*. Additionally, we generated a 28S rDNA sequence for one specimen of *Provitellotrema crenimugilis* from our earlier material, provided in [4]. The one and three variable sites were detected for the 18S rRNA gene

and the 28S rRNA gene of *P. halongensis* n. sp., respectively.

Results of phylogenetic analysis based on the 28S rDNA sequence dataset show that the Haplosplanchnidae appears as a highly supported monophyletic clade on both ML and BI trees (Fig. 2). Within this clade, genera *Provitellotrema* and *Haplosplanchnus* were closely related to each other, as were genera *Pseudohaplosplanchnus* and *Hymenocotta*. Genera *Schikhobalotrema* and *Trigonocephalotrema* formed distinct, highly supported clades; the clade "*Schikhobalotrema*" which was sister to the clade, contained closely related *Provitellotrema* and *Haplosplanchnus*. The new species, *Provitellotrema halongensis* n. sp., was closely related to the poorly supported subclade, which contains *P. crenimugilis* and *H. purii.* All three species created the highly supported clade "*Provitellotrema,*" which was closely related to *Haplosplanchnus pachysomus* (clade "*Haplosplanchnus*") with high values of posterior probabilities. The p-distance value between 28S rDNA sequences of *P. halongensis* n. sp. and *P. crenimugilis* was 0.69 ± 0.22 %, representing 13 variable sites, including 7 with fixed substitutions. Between these two species and specimens denoted as *Haplosplanchnus purii* under accession number FJ211242, the p-distance values were 0.87 ± 0.27 % (12 variable sites) and 1.01 ± 0.3 % (13 variable sites), respectively (Table 3); fixed substitutions in this case cannot be revealed because a single sequence of *H. purii* is available. Overall, for Haplosplanchnidae, intergeneric p-distance values based on the 28S rDNA sequence dataset between all member genera of this family ranged from 3.3 ± 0.5 % (*Provitellotrema/Haplosplanchnus*) to 23.26 ± 2.1 % (*Provitellotrema/Hymenocotta*). Between nucleotide sequences of representatives of *Provitellotrema* and *H. pachysomus,* 35 fixed genus-specific substitutions were revealed; of these, 24 transitions and 11 transversions were observed. Between 28S rDNA sequences of representatives of the *Provitellotrema* and *Hymenocotta* genera, there were 171 fixed genus-specific nucleotide substitutions; of these, 100 transitions and 71 transversions were observed.

The ML and BI phylogenetic tree topologies based on concatenated complete 18S rDNA and partial 28S rDNA were highly similar to that based on 28S rDNA (Figs. S1, S2) and differ from each other by a few internal differences for clades "*Haplosplanchnus*" and "*Trigonocephalotrema.*" *Provitellotrema halongensis* n. sp. was closely related to *Provitellotrema crenimugilis* with high support, and *H. purii* appears as a sister species to [*P. halongensis* n. sp. / *P. crenimugilis*] with poor support (Fig. S1, S2). *Haplosplanchnus pachysomus* formed a sister clade relative to the "*Provitellotrema*" clade.

The mitochondrial COI gene fragment 765 bp in length was generated for all species available in our material, including new species, *Provitellotrema crenimugilis*, *Haplosplanchnus pachysomus*, *Pseudohaplosplanchnus catbaensis,* and *Hymenocotta mulli*. The COI gene fragments of 6 specimens of new species in pairwise comparison contained from 0 to 5 variable sites, representing 0–0.65 ± 0.29 % of divergence.

Results of phylogenetic analysis based on a mitochondrial partial COI gene sequence dataset show that the new species, *Provitellotrema halongensis* n. sp., is closely related to *Provitellotrema crenimugilis*, and *H. pachysomus* was a sister relative to *Provitellotrema* (Fig. 3). The genetic p-distance mean value between new species and *P. crenimugilis* was 10.78 ± 1.01 %, representing 86 variable sites, including 80 sites with fixed species-specific substitutions. Between *Provitellotrema* and *H. pachysomus,* the p-distance values ranged from 18.91 ± 1.3 % to 20.83 ± 1.33 %, representing 165/146–174/158 variable/parsimony-informative sites for *P. crenimugilis/H. pachysomus* and *P. halongensis* n. sp. */H. pachysomus*, respectively. Between COI gene partial sequences of representatives of the *Provitellotrema* and *Haplosplanchnus* genera, there were 66 fixed genus-specific nucleotide substitutions; of these, 32 transitions and 34 transversions were observed. It is notable that the new specimen of *H. pachysomus* ex *Planiliza* sp., collected in 2023 on Cat Ba Island coastal waters, differs from specimens of this species from *Liza engeli* (=*Osteomugil engeli* (Bleeker, 1858)) from the same location, collected in 2014 [4]. The genetic distance value was 5.32 ± 0.72 %, representing 38 nucleotide substitutions.

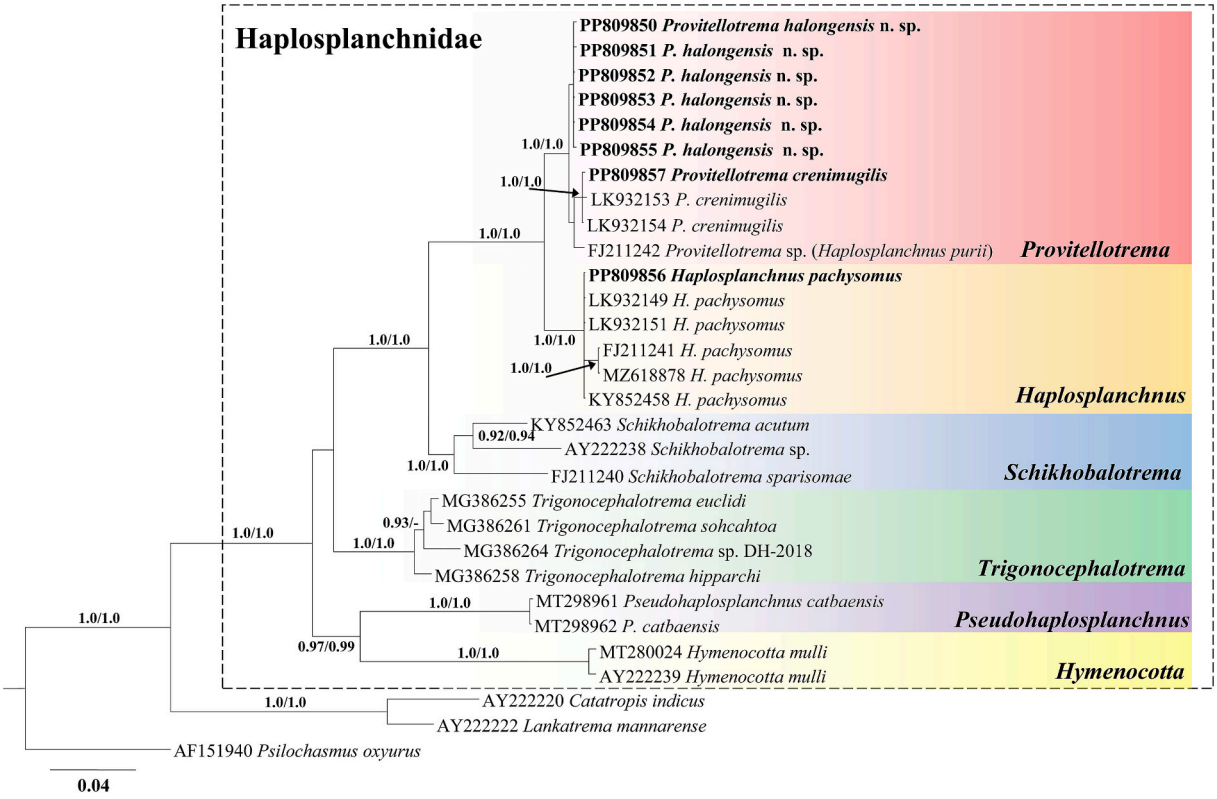


**Fig. 2.** Phylogenetic tree of the family Haplosplanchnidae based on the analysis of 28S rRNA (partial) gene sequences 1249 bp in length; nodal numbers indicate posterior probabilities for ML / BI algoritms. **Sequences from the present study are bolded.**

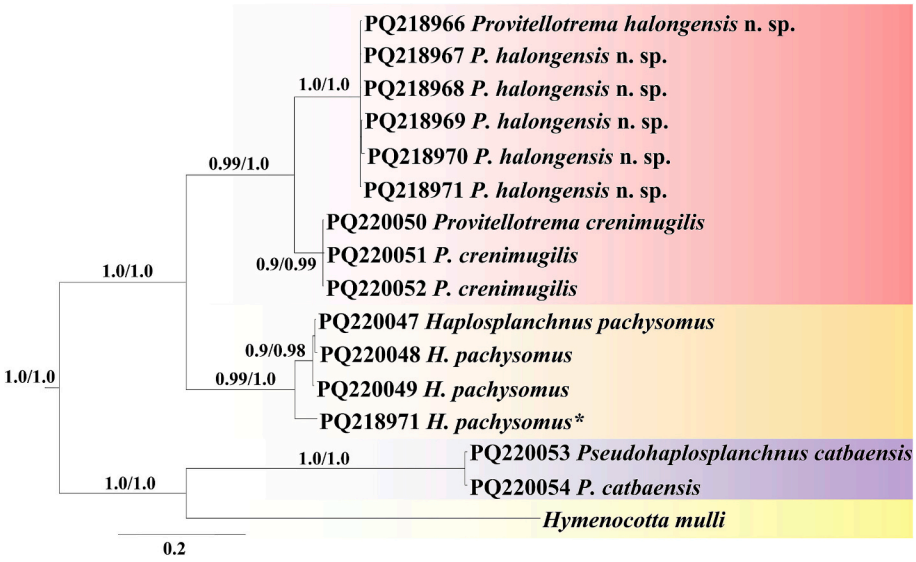


**Fig. 3.** Phylogenetic tree of the family Haplosplanchnidae based on the analysis of mitochondrial COI gene partial sequences 765 bp in length; nodal numbers indicate posterior probabilities for ML / BI algorithms. **Sequences from the present study are bolded.** * - a specimen of *H. pachysomus* ex *Planiliza* sp., collected in 2023 on Cat Ba Island coastal waters.

### 3.4. Remarks

The new worms demonstrate the most similarity in morphometric parameters with *Haplosplanchnus purii* Srivastava, 1939, and *Pseudohaplosplanchnus catbaensis* Atopkin, Besprozvannykh, Ha, Nguyen, Nguyen, 2020, from mullets of the Bay of Bengal and Ha Long Bay, respectively [3,19]. The detected new trematodes differ from these two species morphometrically, namely by pharynx and egg size (Table 2). Morphologically, trematodes from our material differ from two species mentioned above by the vitellarium arrangement that extends between the level of the anterior border ventral sucker and the level of the middle distance between the posterior border ventral sucker and ovary vs. the posterior border of the ventral sucker to the level of the ovary or middle testis, respectively.

## 4. Discussion

Despite the agreement of morphological characteristics of new trematodes to diagnoses of the genera *Haplosplanchnus* or *Pseudohaplosplanchnus,* molecular data show that new trematodes are closer to *Provitellotrema crenimugilis* and the trematode denoted as *H. purii* (accession No. FJ211242) in the GenBank database (Fig. 2, S1, S2; Table 3). Unfortunately, morphological data for the last specimen are not available, and we cannot carry out comparative morphological analysis of this specimen and two species from the same clade (Fig. 2, S1, S2). *Provitellotrema crenimugilis* and new trematodes have no considerable differences in most metric characteristics, excluding egg size (Table 2). Morphologically, these species differ from each other only in the arrangement of the vitellarium: vitelline follicles form two clumps in

**Table 2**
Metric parameters (in micrometers) of the trematodes of the genus *Provitellotrema*, and *Haplosplanchnus purii*.

| Characteristic | *Provitelotrema halongensis* n. sp. $n = 5$ | | | *Haplosplanchnus purii* (Nahhas et al., 1997) | *Pseudohaplosplanchnus catbaensis* (Atopkin et al., 2020) | *Provitellotrema crenimugilis* | |
|---|---|---|---|---|---|---|---|
| | Holotype | Range | Mean | | | (Besprozvannykh et al., 2016) | (Pan, 1984) |
| Body length | 1155 | 1032–1328 | 1156 | 725–1425 | 785–1278 | 986–1000 | 1062 |
| Body width | 462 | 462–554 | 499 | 400–650 | 431–554 | 390–493 | 434 |
| Body length/ width % | 40.0 | 40.0–47.8 | 43.2 | – | – | – | – |
| Forebody length | 431 | 231–431 | 355 | – | 316–539 | 300–316 | – |
| Body/forebody length ratio | 37.3 | 19.0–39.4 | 30.7 | – | 37.0–44.9 | – | – |
| Oral sucker length | 169 | 154–227 | 177 | 110–190 | 166–212 | 158–193 | 184 |
| Oral sucker width | 173 | 173–212 | 193 | 150–200 | 166–212 | 177–193 | 184 |
| Ventral sucker length | 204 | 173–250 | 196 | 250–350 | 193–277 | 162–185 | 172 |
| Ventral sucker width | 227 | 177–250 | 209 | 170–230 | 193–277 | 185–189 | 184 |
| Ventral/oral sucker length ratio | 1.21 | 1:0.76–1.43 | 1.11 | 1:1.5 | 1:1.08–1.31 | – | – |
| Ventral/oral sucker width ratio | 1.31 | 1:0.91–1.31 | 1.08 | 1:1.6–1.8 | 1:1.08–1.31 | – | – |
| Pharynx length | 77 | 73–77 | 74 | 60–80 | 116–135 | 65–69 | – |
| Pharynx width | 92 | 81–104 | 92 | 60–80 | 119–158 | 81–89 | – |
| Ovary length | 81 | 65–96 | 75 | 100–160 | 54–116 | 96–116 | 104 |
| Ovary width | 100 | 68–100 | 82 | 100–120 | 73–92 | 81–116 | 88 |
| Testis length | 135 | 127–139 | 127 | 110–260 | 154–200 | 116–139 | 172 |
| Testis width | 116 | 116–154 | 125 | 100–200 | 154–173 | 112–116 | 152 |
| Posterior end of testis | 223 | 135–250 | 202 | – | – | 100–135 | – |
| Eggs length | 65–73 | 65–73 | – | 42–58 | 135–142 | 50–54 | 60 |
| Eggs width | 35–46 | 35–46 | – | 25–33 | 92–104 | 27–35 | 28 |

the forebody vs. vitelline follicles form two lateral rows not extending anterior to the margin of the anterior edge of the ventral sucker. Taking into account the closeness of new trematodes from our study and *P. crenimugilis* by molecular data, we conclude that trematodes from our study belong to the genus *Provitellotrema*, *P. halongensis* n. sp. The difference in vitellaria arrangement between the new species and *P. crenimugilis* can be considered a species-specific characteristic within the genus *Provitellotrema*. Earlier, the same results were obtained for the trematodes *Pseudohaplosplanchnus catbaensis*: the general morphology of this species agrees with the diagnosis for the genus *Haplosplanchnus*, but based on molecular data, this species is closely related to *Hymenocotta mulli* Manter, 1961, the species that highly differs from *P. catbaensis* morphologically [3]. Additionally, the specimen of *Haplosplanchnus purii* provided in the GenBank that was not validated morphologically belongs to the genus *Provitellotrema* based on phylogenetic reconstructions from the present study; this specimen is proposed to be denoted as *Provitellotrema* sp. Unfortunately, the mitochondrial COI gene sequence data are unavailable for this specimen of '*H. purii*,' hampering the consolidation of this interpretation.

Our discussion of molecular characteristics for taxonomical conclusions is based mainly on the data provided in Table 3. The p-distance values between *P. halongensis* n. sp., *P. crenimugilis,* and *Provitellotrema* sp. correspond to the minimal boundary of the interspecific divergence level known for representatives of Haplosplanchnidae based on the 28S rDNA dataset [2,5,20], as well as for the mitochondrial COI gene dataset [21]. In the study carried out by Pérez-Ponce de León [21], the divergence level for haplosplanchnid trematodes *Schikhobalotrema acutum* (Linton, 1910) Skrjabin and Guschanskaja, 1955, was shown to be 0.1–0.3 % based on 28S rDNA and 3.2–7.4 % based on mitochondrial COI gene sequence datasets, which is almost 2–3 times lower than the values between *P. halongensis* n. sp., *P. crenimugilis,* and *Provitellotrema* sp. for both molecular markers. Thus, we believe that our data is circumstantial evidence that the 0.69 ± 0.22 %–1.01 ± 0.3 % 28S rDNA sequence differentiation of *Provitellotrema* trematodes from the nearest Far Eastern regions is interspecific. These results indicate that *P. halongensis*, *P. crenimugilis*, and *Provitellotrema* sp. should be treated as distinct species.

Genetic distance values between these three species and the sister clade, which contained specimens of *H. pachysomus*, ranged from 3.3 ± 0.5 % to 3.92 ± 0.61 % and from 18.91 ± 1.3 % to 20.83 ± 1.33 % based on 28S rDNA and COI gene sequence datasets, respectively. These data show that two genera, *Provitellotrema* and *Haplosplanchnus*, are well separated from each other at the molecular level and support their taxonomical consistency with the current species content used for phylogenetic analysis. Additionally, our data confirm that the trematode specimen denoted as *H. purii* in the GenBabk database (accession No. FJ211242), which is closely related to *Provitellotrema* species, do not belong to the genus *Haplosplanchnus*.

In our opinion, the intraspecific differences revealed in our study for *H. pachysomus* based on 28S rDNA sequence data should be discussed as well. The difference of 0.6 ± 0.2 % in 28S rDNA was observed between specimens from geographically distant regions, European and Far Eastern territories, whereas identical sequences were observed between specimens from the nearest regions, for example, Spain and the Russian coast of the Black Sea, as well as different trematode specimens from Vietnam. It is notable that the differentiation within *H. pachysomus* based on the partial COI gene sequence dataset was 5.32 ± 0.72 % for specimens of *H. pachysomus* from the same location but from different hosts of Vietnam; these specimens have identical 28S rDNA. Despite the fact that this value of COI gene sequence differentiation corresponds to the intraspecific variation level for representatives of Haplosplanchnidae [21], we cannot exclude that different species of Haplosplanchnus or at least the species *H. pachysomus* species complex will be revealed for Vietnamese mullets.

The p-distance values based on the 28S rDNA sequence dataset between other haplosplanchnid genera are considerably higher than the intergeneric values between *Provitellotrema* and *Haplosplanchnus* (Table 3), as well as between different subfamilies in the other groups of trematodes of marine fish species. For example, these features were reported for trematodes of the family Haploporidae, collected from mullets from coastal waters of Vietnam [22]. These results still lead us to adhere to the viewpoint stated by Huston [2] on disclaiming the subfamily concept within Haplosplanchnidae that was additionally supported in our recent studies [3]. However, based on p-distance values and the results of phylogenetic analysis, we do not exclude the possibility that this family will be separated into several different families. Nevertheless, the haplosplanchnid species diversity is still poorly uncovered and cannot be interpreted unambiguously in this respect. Based on new species-specific morphological characteristics we recognise within the *Provitellotrema*, we provide an amended diagnosis for this genus. The species diagnosis provided here is based on our original data from this study, the original description of the type species *Provitellotrema crenimugili* given in [26], and our previous data, available in [4]. In the last referred study [4], authors denoted the presence of eyespot pigment and embryonated eggs for *P. crenimugili*. Eyespot pigment can be seen well or not seen that possibly can be related to the development and ageing of adult worms. The presence of eggs with miracidia within the uterus in trematodes of the type species of *Provitellotrema* was validated in the study of Besprozvannykh et al. [4]. For this reason, we believe that the presence of unembryonated eggs is not necessary to be included in the generic diagnosis for *Provitellotrema*.

**Family Haplosplanchnidae Poche, 1926.**

*Provitellotrema* Pan, 1984.

*Diagnosis***.** Body elongate. Eyespot pigment dispersed in forebody or absent. Oral sucker subterminal. Prepharynx short. Pharynx transversally oval. Caecum single, reaching level of posterior end of ventral sucker or anterior border of ovary. Ventral sucker, larger than oral sucker. Testis single, in posterior third of body, round or oval. Seminal vesicle tubular, reaching to level posterior border of ventral sucker or ovary. Hermaphroditic duct short. Genital pore median, close to anterior border of ventral sucker. Ovary spherical, pre-testicular or contiguous with testis. Seminal receptacle round, dorsal to ovary and testis. Uterus in middle third of body. Vitellarium in two lateral fields formed from follicles of irregular forms, extending in forebody or predominantly on

**Table 3**
P-distance values (d, %) between representatives of Haplosplancnoidea based on 28S rRNA gene sequence dataset. Values between new species *Provitellorema halongensis* n. sp. and other trematodes are bolded. The d values are below diagonal, the standard error values - above diagonal. *P*-value distances represent the average values for each taxonomic group.

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | ***Provitellotrema halongensis* n. sp.** | | **0.22** | **0.27** | **0.50** | **0.86** | **1.26** | **1.41** | **1.70** |
| 2 | *Provitellotrema crenimugilis* | **0.69** | | 0.30 | 0.56 | 0.89 | 1.30 | 1.42 | 1.69 |
| 3 | *Provitellotrema* sp. *(H. purii)* | **0.87** | 1.01 | | 0.61 | 1.06 | 1.50 | 1.78 | 2.11 |
| 4 | *Haplosplanchnus pachysomus* | **3.30** | 3.79 | 3.92 | | 0.88 | 1.27 | 1.49 | 1.73 |
| 5 | *Schikhobalotrema* spp. | **10.50** | 11.07 | 12.64 | 10.79 | | 1.02 | 1.28 | 1.48 |
| 6 | *Trigonocephalotrema* spp. | **13.96** | 14.38 | 16.13 | 13.87 | 12.30 | | 1.30 | 1.37 |
| 7 | *Pseudohaplosplanchnus catbaensis* | **16.36** | 16.71 | 19.43 | 17.05 | 15.69 | 13.48 | | 1.44 |
| 8 | *Hymenocotta mulli* | **19.75** | 19.58 | 23.26 | 20.25 | 18.68 | 15.18 | 15.75 | |

level of ventral sucker. Eggs large, operculated, contain miracidium with eyespot. Intestinal parasites of mugilids in the West Pacific Oceans. Type-species *Provitellotrema crenimugilis* Pan, 1984.

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## Ethics approval

Sample collection and procedures were approved by Committee of Bioethics of Federal Scientific Center of Biodiversity FEB RAS.

## CRediT authorship contribution statement

**Dmitry M. Atopkin:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Yana I. Ivashko:** Writing – review & editing, Investigation. **Nguyen Van Ha:** Investigation, Funding acquisition. **Hoang Van Hien:** Investigation. **Vladimir V. Besprozvannykh:** Writing – review & editing, Methodology, Investigation, Conceptualization.

## Declaration of competing interest

The authors declare no competing interests.

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## Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request. Molecular data will be available in GenBank, NCBI database (https://www.ncbi.nlm.nih.gov/).

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