

## Original Article

# Introduction of encysted metacercarial *Stephanostomum* sp. in Javanese ricefish (*Oryzias javanicus*) and bacterial diversity of encysts from mangrove swamps of Trang Province, Thailand

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**Abstract**

The genus *Stephanostomum* consists of digenean parasites that are found in many marine teleost fish. In this study, we aimed to describe the metacercarial cysts of *Stephanostomum* sp. in Javanese ricefish (*Oryzias javanicus*) collected from brackish water of mangrove swamps in Sikao District, Trang Province, Thailand. The morphology and tegumental histology of the worms were examined. Furthermore, molecular phylogenetic trees were established based on 16S, 12S, 18S and 28S ribosomal DNA (rDNA) sequences, including an analysis of the symbiotic bacteria of *Stephanostomum* cysts using a 16S rDNA sequencing method. Partial rDNA sequences of *Stephanostomum* were highly matched to *S. cf. cestillum* for percentage of similar identity from the nucleotide database of GenBank. Proteobacteria and Firmicutes were dominant on parasitic cysts with their environments. Our results contribute to the understanding of *Stephanostomum* from the Andaman Sea of Thailand.

**Keywords:** encysted *Stephanostomum*, *O. javanicus*, Andaman Sea of Thailand

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**1. Introduction**

*Stephanostomum* Looss, 1899, is a genus of digenean parasites that belong to the family Acanthocolpidae and are widely distributed throughout many regions of the world (Gibson, 2001). This acanthocolpid genus comprises more than 100 species (Cribb & Gibson, 2012); examples include *S. baccatum*, *S. caducum*, *S. africanum*, *S. aaravi*, and *S. lamothei* (Bray & Cribb, 2003, 2008; Fischthal & Williams, 1971; Nicoll, 1909; Srivastava, 1996). Regarding the *Stephanostomum* life cycle, gastropods, bivalves and small

fish in marine environments are intermediate hosts during the larva stage, and marine fish are a definitive host for adult development (Køie, 1978; Madhavi & Shameem, 1993; Martin, 1939). Many studies have reported teleosts from marine and brackish water as hosts of *Stephanostomum*, for example, *S. cloacum* found in short-nosed tripodfish (*Triacanthus biaculeatus*), *S. bicoronatum* found in brown meagre (*Sciaena umbra*), *S. kovalevae* found in Devil anglerfish (*Lophius vomerinus*), and *S. madhaviae* found in Giant trevally (*Caranx ignobilis*) (Bartoli & Bray, 2001; Bartoli & Cribb, 2003; Bray & Lothar, 2004; Madhavi & Shameem, 1993). Amongst fish of the genus *Oryzias* (Adrianichthyidae), Javanese ricefish or Java medaka (*O. javanicus*) are model vertebrates for several molecular biology, endocrinology, toxicology and physiology experi-

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ments (Imai, Koyama, & Fujii, 2005; Ismail & Yusof, 2011; Lee, Kim, & Nam, 2012; Miyanishi, Inokuchi, Nobata, & Kaneko, 2016). They are native to brackish and marine water in Indonesia, Singapore, Malaysia and South Thailand (Bleeker, 1854; Roberts, 1998). However, there are few data or records of digenean parasites in this species from aquatic environments in Thailand. Therefore, this study aimed to describe the metacercarial cysts of *Stephanostomum* sp. infected on Javanese ricefish from mangrove swamps of Trang Province, Thailand. Moreover, the phylogenetic trees for these worms were established via 16S, 12S, 18S and 28S ribosomal DNA (rDNA) sequencing. The symbiotic bacteria collected from the cysts were also examined using a 16S rDNA pyrosequencing method. The finding of this work may supply the knowledge of *Stephanostomum* and may help for the understanding of the improving prevention of incidence of these parasites.

## 2. Materials and Methods

### 2.1 Fish and parasite collection

In total, 41 Javanese ricefish were caught by hand net from mangrove swamps in the Sikao District of Trang Province on the Andaman coast of Thailand. The fish were anaesthetised in cold brackish water, and the metacercarial cysts of worms were gently separated beneath the fish skins using aseptic needles and scalpel blades under stereo-microscopy.

### 2.2 Morphological, scanning electron microscopic and histological observation

The cysts were moved into Petri dishes containing a saline solution. Some excysted metacercariae were fixed in AFA solution and stained with Semichon's aceto-carmin (Seo *et al.*, 2008). The metacercarial helminths were identified as *Stephanostomum* sp. according to the morphological descriptions from Gibson (1996), Kardousha (2005) and AL-Zubaidy (2011), and general structures were recorded by drawing using the optical aid of a camera lucida under a microscope. Prevalence percentage, mean abundance and mean intensity were calculated following the formula of Bush, Lafferty, Lotz, and Shostak (1997). Histological sections of the tegumental layers stained with haematoxylin and eosin were also analyzed. For scanning electron microscope (SEM) topography, 5 encysted samples were preserved in 2.5% glutaraldehyde fixative solution in 0.1 M Cacodylate buffer, pH 7.4, refixed with 1% osmium tetroxide solution, dehydrated via serial ethanol treatment, and dried using a critical-point drying apparatus. The specimens were coated with gold in a sputter coater and viewed using a SEM JSM-5410 LV.

### 2.3 Molecular phylogeny and pyrosequencing analysis

Total genomic DNA was extracted from two cysts using a DNeasy kit (Qiagen), and ribosomal DNA (rDNA) was amplified with Pfu DNA Polymerase Mastermix (Bioneer, Republic of Korea) and the following primer pairs: 5'-CCTTTTGCATCATGATTCCKCTGATGTGG-3' and 5'-GCTCTCGGGGTCTTTCCGTC-3' for 16S; 5'-CCTCGGG

GATAACTAGGTAAGAAG-3' and 5'-GGTTCCTCCCARCA TTACCATGTTACGAC-3' for 12S; 5'-CGCAGTCGGCCTT GTGTCGGCG-3' and 5'-GCGGTGTGTACAAAGGGCAGG GACG-3' for 18S; 5'-AGTAACGGCGAGTGAACAGGG-3' and 5'-GTCTTTCGCCCCCTATACTCAG-3' for 28S. The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 2 min. Final extension was at 72°C for 10 min. Following agarose gel electrophoresis, the PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen), and DNA sequencing was performed using an automated DNA sequencer (23 ABI 3730XLs) at MacroGen DNA Sequencing Service, Korea. DNA sequences were deposited into GenBank (<http://www.ncbi.nlm.nih.gov>) under accession numbers KY953189, KY953191, KY953190, KY953192, KY953189, KY953191, KY953190 and KY953192. For other trematodes and nematodes, nucleotide sequences were searched from the GenBank database. The resulting sequences were aligned with the Clustal Omega multiple sequence alignment programme (<http://www.genome.jp/tools/clustalw/>). For the molecular phylogenetic analyses, a maximum likelihood tree was constructed using the MEGA (Version 5.1) [Computer software] (Tamura *et al.* 2011).

For genetic and bioinformatic analyses, 5 cysts were pooled to form group 1, and 10 cysts were pooled to form group 2. DNA extraction, amplification with universal primers of 16S rDNA sequences in the V1-V3 hypervariable region (Huse *et al.*, 2008) and gel extraction were conducted following the methods described above. Sequencing bioinformatic analyses were carried out using the Illumina HiSeq2000/MiSeq system, and the operational taxonomic unit (OTU), Venn diagram, taxonomic rank and species phylogenetic tree were processed at Beijing Genomics Institute, BGI-Shenzhen, China.

## 3. Results

In total, 24 encysted *Stephanostomum* metacercaria were found in 12 fish out of the 41 sampled. The parasitic range, prevalence %, mean abundance and mean intensity were 1-4, 29.27, 0.58 and 2, respectively. The average cyst size was 0.46 mm (0.35-0.6 in range). The cysts were quite round, transparent and light yellow in color (Figures 1a-b). The larval body was curved within a thick cyst envelope. The oral sucker was located anteriorly, and the ventral sucker began mid-body. The excretory bladder was located posteriorly and was ovoid-shaped, enlarged and light brown (Figure 1c). In excysted larvae, the body was pear-shaped. Approximately 32-36 spines were observed around the oral sucker, and small spines were found at the end of the oral sucker. The oesophagus was present at a subterminal of the oral sucker, but the eye spots were difficult to observe. Caeca at the end of anterior region extended laterally to the ventral sucker and were directed posteriorly. The excretory bladder was an ovoid-shaped sac and was pale brown (Figure 1d). The surface of the cyst was roughly corrugated, and the tegumental surface of the *Stephanostomum* inside the cracked cyst near the oral sucker was bearded with numerous spines. These spines were stout and rod-shaped (Figures 1f-g). Upon histological analysis, a thick cyst wall covered the external and internal layers. The cyst wall and tegument were

separated by a gap in the ventral region (Figure 1h). The tegument, basal membrane, muscular layers and cell body were lined similarly to the ventral and dorsal surfaces. Spines were embedded in the tegumental layer and distributed throughout the body (Figures 1i-l). However, few spines were observed at the posterior end of the dorsal region (Figure 1m).

In a phylogenetic test based on 12S and 16S rDNA partial sequences, two *Stephanostomum* specimens belonging to Plagiorchiida (superfamily Lepocreadioidea) were related to *B. goliath* in the same superfamily and *D. dendriticum* of the same order (superfamily Gorgoderioidea) but were a sister group with *H. taichui* and *O. felineus* of Opisthorchiida (superfamily Opisthorchioidea). They were distantly related to the nematode *T. ovis* as an outgroup (Figure 2a). In the 18S and 28S rDNA sequences, two *Stephanostomum* specimens

formed a monophyletic group with *S. cf cestillum*, *S. bicoronatum*, *S. minutum* and *S. interruptum* and were closely related to *S. cf cestillum*. They formed a sister group with *B. goliath* and *D. dendriticum*, and were distantly related to *S. falcatum*, *M. yokogawai*, *H. taichui*, *P. varium* of Opisthorchiida and *Clinostomum sp.* of Strigeidida. The ingroup of trematodes was a distant relative to the nematode *P. decipiens* as an outgroup (Figure 2b).

Sequencing analysis, represented by OTUs, revealed 17 identified phyla (Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Elusimicrobia, Firmicutes, Fusobacteria, Nitrospirae, OD1, Planctomycetes, Proteobacteria, SBR1093, TM6, TM7, Tenericutes, Thermi and Verrucomicrobia); 32 classes (Acidobacteria, Alphaproteobacteria, Bacilli, Betaproteobacteria, Flavobacteria, Gamma-

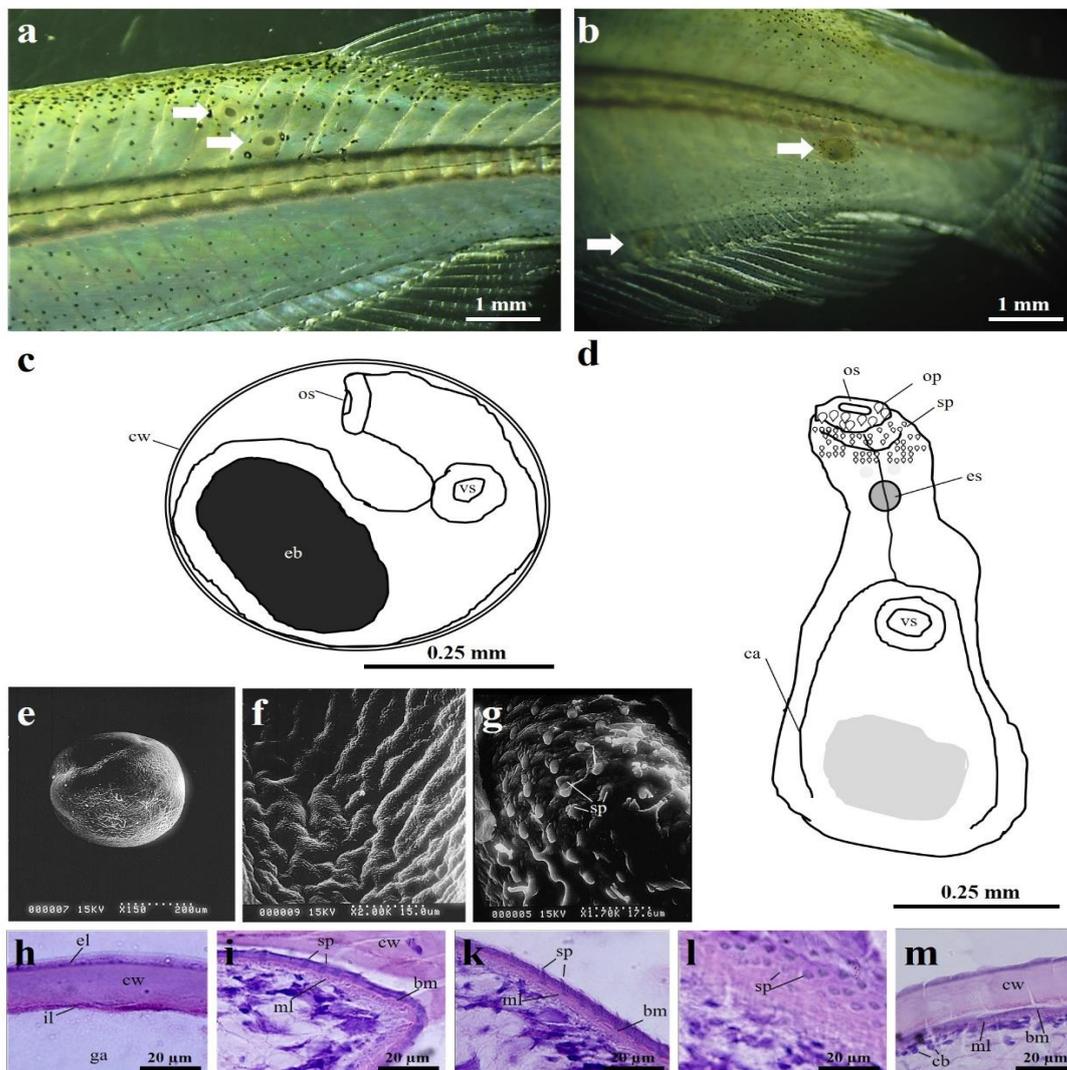


Figure 1. Encysted *Stephanostomum* sp. in musculature of Java ricefish (a and b). Drawing structure of encysted worm (c). Excysted worm (d). SEM topography of cyst surface (e), magnification of figure d (f) and tegument of metacercaria from a cracked cyst (g). Histological views of cyst (h), anterior area (i), middle area at ventral side (j), middle area at dorsal side (k) and posterior area (l). Arrows indicate encysted worm. basal membrane (bm), caeca (ca), cell body (cb), cyste wall (cw), excretory bladder (eb), external layer (el), gap area (ga), internal layer (il), muscular layers (ml), oesophagus (es), oral spine (op), oral sucker (os), spine (sp) and ventral sucker (vs).

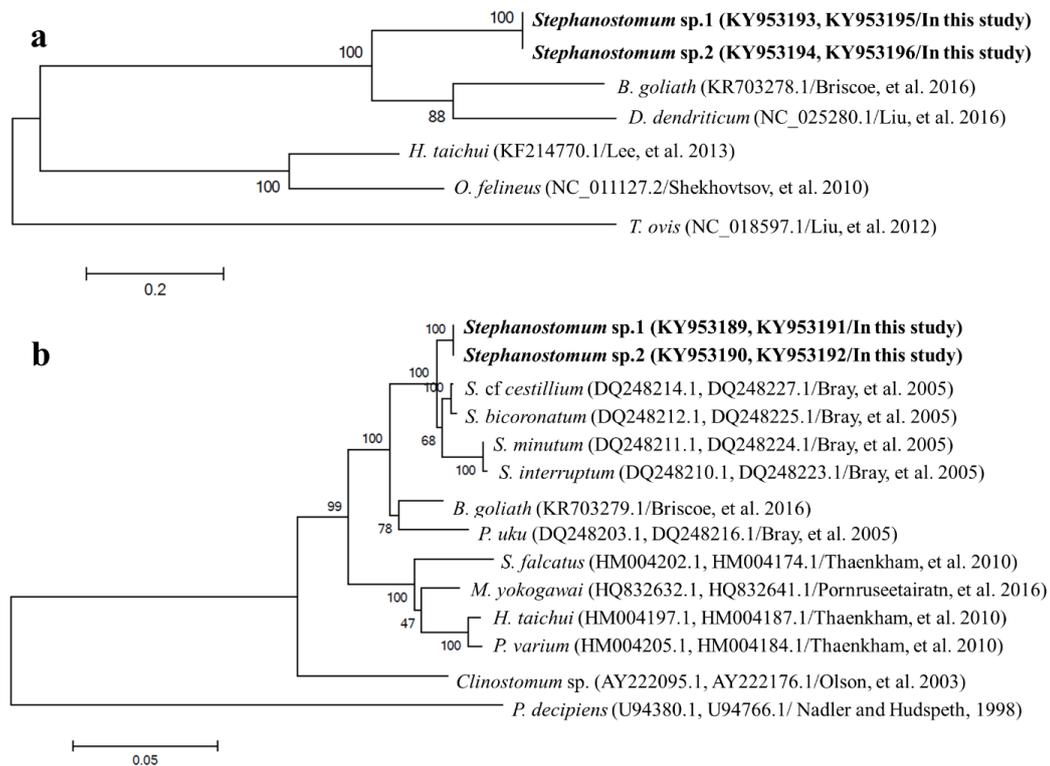


Figure 2. Maximum likelihood tree of 12S+16S (a) and 18S+28S (b) rDNA sequences for confirmation of *Stephanostomum* taxa.

proteobacteria, Mollicutes, Sphingobacteria and others (<0.5%); 61 orders (Actinomycetales, Bacillales, Burkholderiales, Caulobacteriales, Enterobacteriales, Entomoplasmatales, Flavobacteriales, Methylophilales, Neisseriales, Pseudomonadales, Rhizobiales, Rhodobacteriales, Sphingomonadales, Sphingobacteriales, Vibrionales, Xanthomonadales and others (<0.5%)); 99 families (Bacillaceae, Bartonellaceae, Brevibacteriaceae, Caulobacteraceae, Comamonadaceae, Corynebacteriaceae, Enterobacteriaceae, Flavobacteriaceae, Methylophilaceae, Microbacteriaceae, Micrococcaceae, Moraxellaceae, Neisseriaceae, Paenibacillaceae, Propionibacteriaceae, Pseudoalteromonadaceae, Pseudomonadaceae, Rhodobacteraceae, Sphingobacteriaceae, Sphingomonadaceae, Staphylococcaceae, Weeksellaceae, Xanthomonadaceae and others (<0.5%)); 138 genera (*Acidovorax*, *Acinetobacter*, *Bacillus*, *Brevibacillus*, *Brevibacterium*, *Chryseobacterium*, *Corynebacterium*, *Enhydrobacter*, *Flavobacterium*, *Hydrogenophaga*, *Luteimonas*, *Methylobacterium*, *Micrococcus*, *Paenibacillus*, *Pedobacter*, *Propionibacterium*, *Pseudomonas*, *Pseudoxanthomonas*, *Staphylococcus*, *Stenotrophomonas*, *Vibrio*, *Vogesella* and others (<0.5%)); and 237 species (*B. casei*, *B. firmus*, *B. fumarioli*, *B. reuszeri*, *B. conglomeratum*, *M. luteus*, *M. mobilis*, *P. acnes*, *P. lautus*, *S. geniculata*, *P. viridiflava*, *P. Mexicana* and others (<0.5%)). The OUT number for group 1 and 2 was 405 and 397, respectively (Figures 3a-g). Molecular phylogenetic tree analysis at the genus level revealed 66 genera that were classed as Proteobacteria, with the exception of proteobacteria of the *Desulfovibrio* genus. The results showed a close relationship to Actinobacteria, and 24 Actinobacteria genera were identi-

fied. *Planctomyces*, *Luteolibacter*, *Deinococcus*, *Thermus* and *Nitrospira* shared a common ancestor in this clade and were closely related to Bacteroidetes. In Firmicutes, three clades were identified. Clade 1 contained 14 genera that were related to *Rubrobacter* of Actinobacteria. Clade 2 comprised 2 genera that had a common ancestor with 3 genera of Fusobacteria. The four identified genera in clade 3 were far from clades 1 and 2 (Figure 4).

#### 4. Discussion

Recently, Nitta and Nagasawa (2017) reported monogenean infections of *Dactylogyrus oryzias* (Dactylogyridae) on the gill lamellae of Japanese medaka (*O. latipes*) from a freshwater canal in Japan. In digenean species, *Clinostomum complanatum* was found in the skin and muscle tissues of Thai ricefish (*O. minutillus*) collected from shallow ponds in Thailand (Ngamniyom, Manaboon, & Panyarachun, 2012). Umadevi and Madhavi (2000) also described the life cycle of *Procerovum varium* infected on the livers of Indian ricefish (*O. melastigma*) from fresh water in Visakhapatnam, India. According to the description of *Stephanostomum* as reported by Bartoli and Bray (2001), the characteristics of the *Stephanostomum* sp. in this study sound most similar to the *S. cestillum* species found in the digestive tract of the allmouth goosefish (*Lophius piscatorius*). However, our specimens lacked some of the reported internal morphology, and our measurement and ratio data included more specimens for identifying the correct species. In several studies, *Stephanostomum* spp. infected various tissues of teleost fish,

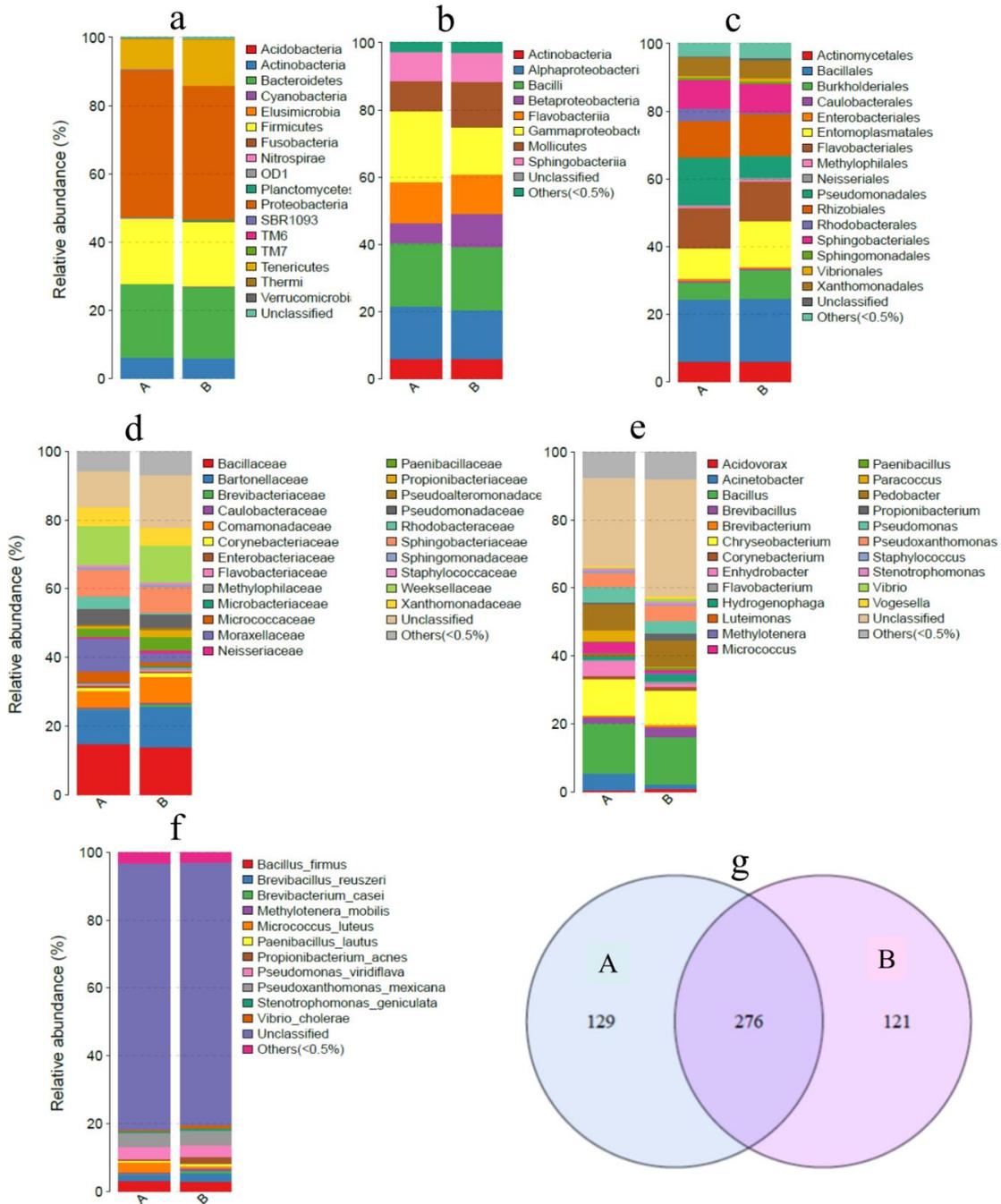


Figure 3. OTU based on 16S rDNA sequences in the V1-V3 hypervariable region represented Phyla (a), classes (b), orders (c), families (d), genera (e), species (f) and Venn diagram (g) of bacteria from encysted *Stephanostomum* sp. A and B indicate group A and B, respectively.

for example, *S. minutum* infection of the rectum of stargazer (*Uranoscopus scaber*), *S. kovalevae* infection of the intestine of monkfish (*L. vomerinus*), *S. murielae* infection of the digestive tract of Whitley (*Carangoides hedlandensis*), and *Stephanostomum* sp. infection of the body cavity of orangespotted trevally (*Carangoides bajad*) (AL-Zubaidy, 2011; Bartoli & Bray, 2001; Bray & Justine, 2011; Bray &

Reimer, 2004). Moreover, encysted metacercariae have been described in the fin, skin, muscle, gill, pericardium and spleen of fish species including *S. baccatum* and *S. tenue* (Gibson, 1996). In invertebrate, Pérez-Urbiola and Martínez-Díaz also reported that the *Stephanostomum* sp. was a serious cause of disease in catarina scallop (*Argopecten ventricosus*) in Baja California, Mexico. In this study, we describe platyhelminthic

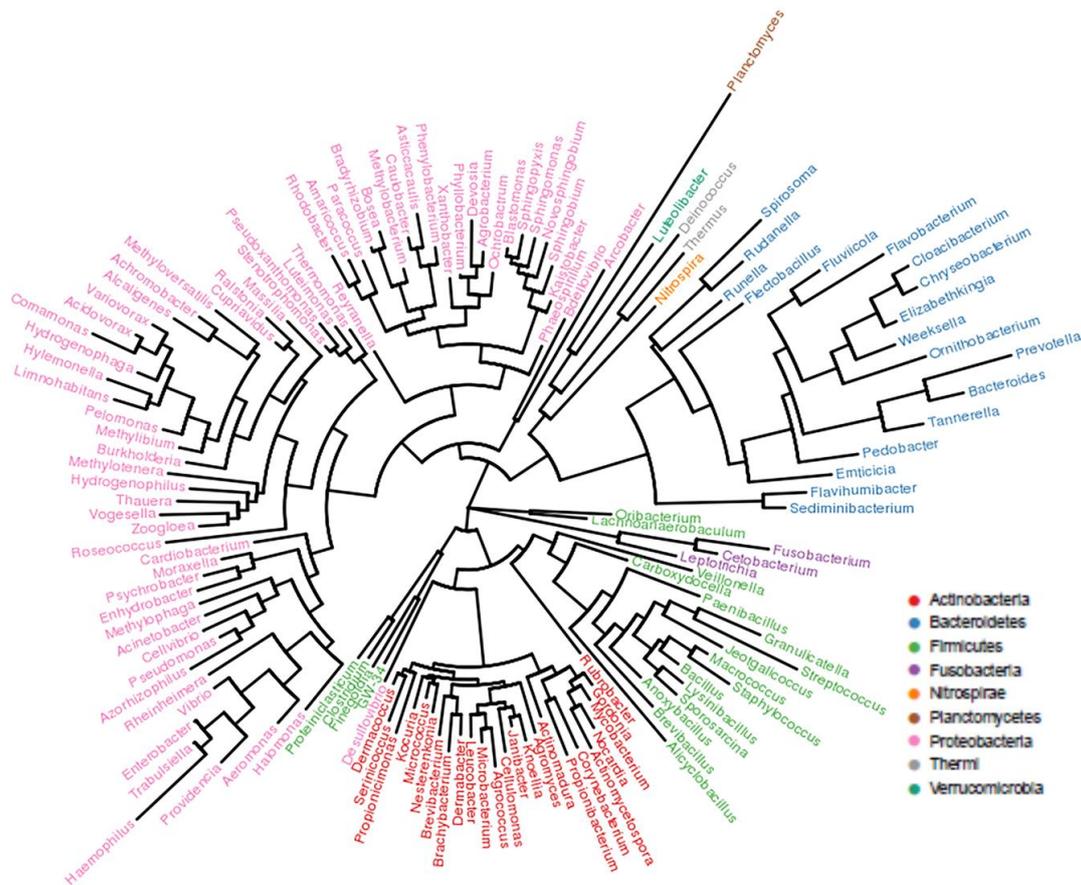


Figure 4. Genus level of molecular phylogenetic tree based on 16S rDNA sequences of bacteria from encysted *Stephanostomum* sp. Same colors indicate same phylum.

infection by *Stephanostomum* in *Oryzias* collected from brackish water in Thailand. For several studies of intermediate or definitive hosts above mentions included our report, the data may support and increase the understanding for improving prevention of the disease transmission or incidence infected by these trematodes.

In our molecular phylogenetic analysis, unidentified *Stephanostomum* sp. were confirmed to belong to the order Plagiorchiida based on 12S and 16S rDNA sequencing. Additionally, a close relatedness to *S. cf. cestillum* was determined based on morphological similarities to those described by Bray, Webster, Bartoli, and Littlewood (2005) and 18S and 28S rDNA sequencing. Thus, the nuclear and mitochondrial DNA analyses in this study showed that the *Stephanostomum* sp. were consistent with the morphological taxa of trematodes. Analysis of the bacterial community using 16S rDNA sequences in the V1-V3 hypervariable regions revealed little incongruence between bacterial taxon and molecular results at the genus level within Proteobacteria and Firmicutes for some species. Therefore, culture-based approaches may be an important method for the precise identification of the bacterial species in future studies. Recently, Xue, Xu, Wei, and Sun (2017) reported that Vibrionales and Flavobacteriales were the predominant microbial populations in fish diseases from marine fish aquaculture, including pathogenic strains (*V.*

*harveyi*, *V. rotiferianus*, *V. sinaloensis*, *V. brasiliensis*, *V. chagasii*, *V. fluvialis* and *A. salraonicida*). In this study, only *V. cholerae* were found in both groups. However, the relative abundance of unclassified species at the species level was high. Bacillales and Flavobacteriales were predominant in both groups, suggesting that Flavobacteriales may be assumed as the dominant group in *Stephanostomum*-mediated infectious disease.

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