Co-infection of a novel *Chlamydia*-like organism and *Henneguya* sp. (Myxosporea, Myxobolidae) in snakeskin gourami *Trichopodus pectoralis* (Regan 1910), in Thailand

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Running head: *Chlamydia*-like organism and *Henneguya* sp. in snakeskin gourami

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request.

CONFLICT OF INTEREST

The authors declare no conflict of interest

Snakeskin gourami, *Trichopodus pectoralis*, is native to Asia and commonly found in the Mekong and Chao Phraya basins of Cambodia, Thailand, Southern Vietnam, and Laos (Paepke, 2009). In Thailand, snakeskin gourami is a highly economic species and an important aquaculture freshwater species (FAO, 2020). Parasitic and bacterial diseases are major challenges in farming of this species (Kanchan, Imjai, Kanchan, & Chaiyara, 2020). However, studies of disease occurrence in snakeskin gourami are still very scarce (U.S Fish & Wildlife Service, 2019).

Myxosporeans are diverse, common parasites causing serious damage to wild and cultivated fish species worldwide (Lom & Dyková, 2006). The genus *Henneguya* comprises more than 200 species and is among the most specious myxosporean genera in the family *Myxobolidae* (Eiras & Adriano, 2012). *Henneguya* sp. infection usually produce cyst-like structures on gill filaments leading to their destruction, resulting in respiratory distress (Lom & Dyková, 2006; Molnar, 2002). Other commonly known pathogens causing gill cysts in fish are bacteria from the *Chlamydiales* order, typically causing epithelial cysts (epitheliocystis) in skin and gills (Nowak & LaPatra, 2006; Pawlikowska-Warych & Deptula, 2016). Chlamydial infections reveal distinctive pathology which possibly depend on the chlamydial species, infected host, and affected tissue (Borel, Polkinghorne, & Pospischil, 2018). To date, lack of culture methods for chlamydial pathogens is a major setback to *in vitro* studies (Borel et al., 2018; Nowak & LaPatra, 2006). In the present study, we describe firstly, the systemic pathology caused by a novel *Chlamydia*-like organism (CLO) and comment on the gill parasite *Henneguya* sp. (Myxosporea, Myxobolidae) infecting the same fish, based on molecular analysis and histopathology observations.

In March 2021, two grow-out ponds in central Thailand recorded above-normal mortality involving snakeskin gourami fingerlings. Regarding case history, snakeskin gourami fingerlings (0.3g ± 0.05) produced using traditional method, were purchased from a hatchery.

Male and female broodstock were mated naturally in earthen pond containment. Eggs were spawned in natural bubble nests made by male gourami within the same pond. Offspring were harvested at the size of 3.0 cm (weight 0.2-0.3g) and 150,000 fish were delivered to two mentioned ponds. Fingerlings were contained within $5m^2$ net in the pond and fed daily with 28% protein commercial feed (Betagro, Thailand) at 3% body weight. Mortality was noticed on day 2 after fingerlings introduction. Daily mortality was around 20, 50, 150, 200, and 400 fish between 1 and 5 days after disease onset (dao). Affected fish exhibited lethargy, gasping at the water surface, and appetite loss followed by mortality. At 3 dao, sea salt was added to the pond daily (200 kg in total), though no reduction of mortality was observed. Disease diagnosis performed on-site at 6 dao revealed no external lesions on the body surface of fish (Figure 1a). Wet mounts of skin mucus and gills showed no external parasites, though formation of multiple characteristic cyst-like was prevalent in gill filament (Figure 1b). This observation initially led us to tentative diagnosis of an "*epitheliocystis*" case. Afterwards, oxytetracycline (OTC) (200 mg/g active ingredient, Pharmatech, Thailand) was continually given to fish via feed admixture (5g OTC per 1 kg feed) for 7 days. After OTC treatment, decrease in daily mortality was obvious from 150, 50, and 16 fish at 7, 8, and 9 dao, consecutively. No mortality was found after OTC treatment was applied for 4 days (10 dao). Due to the effective antibiotic treatment, we assumed the disease might have been primarily caused by CLO. Indeed, OTC considerably declined mortalities from chlamydia infection, as recorded previously (Goodwin, Park, & Nowak, 2005), and further supported this treatment regime for future outbreaks.

Representative 10 fingerling fish were sampled at 6 dao for further investigation under approval from Kasetsart University's Institutional Animal Care and Use Committee (ID: ACKU63-FIS-009). Swabs from brain, liver, and kidney samples were streaked onto tryptic soy agar (Himedia, India) and incubated at 28°C for bacterial isolation. Samples of whole-

body fish *(n=5)* and separated gills were fixed in 10% neutral buffered formalin, routinely processed and stained with hematoxylin and eosin, and examined by light microscope. Genomic DNA was isolated from infected gill using Tissue Genomic DNA Kit (Geneaid, Taiwan) according to manufacturer's instructions. Presence of chlamydial DNA was screened firstly using primers 16SIGF (5'-CGGCGTGGATGAGGCAT-3') and 16SIGR (5'-TCAGTCCCAGTGTTGGC-3') (Everett et al., 1999). All positive samples were subjected to another PCR with *Chlamydiales*-specific primers 16SIGF (5′-CGGCGTGGATGAGGCAT-3′) and 806R (5′-GGAC TACCAGGGTATCTAAT-3′) (Relman 1993). PCR assays were performed as previously described by Sood et al (2019). Expected amplicons of first and second PCR methods were 300 bp and 766 bp, respectively. Amplified PCR products (766 bp) were purified and sequenced (U2Bio, Thailand). The multiple sequence alignment of partial 16S rRNA gene sequence of the *T. pectoralis* chlamydial agent from 4 infected fish indicated that the sequences were identical and consensus sequence was deposited in GenBank (accession number MW832782). BLASTn query against available nucleotide sequences deposited in GenBank database determined taxonomic identity. Phylogenetic tree was constructed using the neighbor-joining method with 1,000 bootstraps, after multiple alignments using ClustalW in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018).

All collected samples were positive after repeated PCR amplification of *Chlamydiales* 16S rRNA gene (Figure S1). Our sequence respectively showed 93.5% and 92.3% similarity with 98% query coverage to the uncultured bacterium (accession no. LN612734.1, unpublished) and *Candidatus* Piscichlamydia sp. associated with epitheliocystis infection in cyprinid fish (accession no. KY380090.1, Sellyei, Molnár, & Székely, 2017). These bacteria formed a clade as an unclassified family belonging to the genus *Candidatus* Piscichlamydia within the order *Chlamydiales* (Figure 4) and also shared >90% sequence similarity with other members of the family *Ca.* Piscichlamydiaceae, suggesting that these agents belonged to the same

105 family (Everett et al., 1999). Since the causative agent shared <95% similarity with other
106 previously reported *Chlamydia*-like 16S rRNA sequences, the sequenced bacteria are novel
107 from the order *Chlamydiales* (Everett et al 1999). Therefore, the name *Candidatus*
108 Piscichlamydia trichopodus was proposed to describe the novel CLO in snakeskin gourami.
109 The findings are consistent with remark of Stride et al (2014) that each new piscine host has
110 revealed the presence of a phylogenetically unique and novel chlamydial pathogen.

111 Histological examination revealed colonization of intracellular bacteria in several organs
112 including gill filaments (Figure 2a, b), submucosa of the intestine (Figure 2c, d), caudal fin
113 tissue (Figure 2e, f). Obviously, the bacteria dominantly infects primary gill filaments more
114 than secondary gill filaments. There were dense, rounded to oval-shaped intracellular bacteria
115 observed at cartilaginous junction of primary and secondary gill filaments, resulting in
116 disconnection of the two components (Figure 2d). Similar changes were observed at
117 cartilaginous junction of the caudal fin (Figure 2f). These findings suggested that this bacteria
118 might target cartilaginous tissues. However, no culturable bacteria were isolated from internal
119 organs on nutrient agar plates, which combined with positive PCR results, suggests that the
120 observed bacteria probably are intracellular unculturable CLO. Interestingly, histological
121 analyses showed that “cysts” found in infected fish were not epitheliocystis as tentatively
122 diagnosed and later identified as plasmodia of a myxosporean. Presence of numerous
123 plasmodia was observed at gill filaments (Figure 3a). Myxospores and plasmodia
124 demonstrated asynchronous development with young, rounded plasmodium, enveloped by a
125 wall formed by epithelium cells of gill filaments (Figure 3b). When plasmodium grew, the
126 envelope ruptured and released myxospores into adjacent tissue (Figure 3c). Myxospores
127 possessed typical features of the genus *Henneguya* with two equal polar capsules, sporoplasm
128 located at the posterior pole of spore, and two long superimposed tail processes (Figure 3d).
129 Histological and morphological changes may relate to the observed clinical signs. Mortalities

were likely associated with concurrent infection of CLO with parasitic infestation by *Henneguya* sp. Generally, diagnosis of CLO agents is primarily by histopathology figuring of epithelial cysts, mainly found in fish gills (Pawlikowska-Warych & Deptula, 2016); however, histopathological changes associated with infection of a novel intracellular bacteria showed massive bacterial colonization in the infected tissue but no obvious epithelial cysts observation. This histopathological feature should be considered for presumptive diagnosis of this novel pathogen. Previous reports also found mixed infections of CLO along with gill parasites in red sea bream *Pagrus major* (Nowak & LaPatra, 2006), African catfish *Clarias gariepinus* (Steigen et al., 2013), striped catfish *Pangasius hypophthalmus* (Sood et al., 2018), and rohu *Labeo rohita* (Sood et al., 2019), which suggested some link between parasitic infestation and CLO, involved in their causation (Nowak & LaPatra, 2006). Our initial observation revealed no abnormal mortality in other fishes sharing the same aquatic environment with the affected farms. It is unclear whether this novel bacterium has potential to cause significant impact on snakeskin gourami aquaculture. Further investigation on its host range, prevalence, disease risk factors, and transmission route may provide better understanding on biology of this pathogen and prevent its widespread.

Summarily, presence of pathogenic potential of mixed infection of a novel intracellular CLO (*Candidatus* Piscichlamydia trichopodus), and a gill parasite *Henneguya* sp. in snakeskin gourami in Thailand is firstly reported. This study expands knowledge on snakeskin gourami pathology, an important fish species in Asian aquaculture, and contributes to better understanding of diseases in this fish species.

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Figures

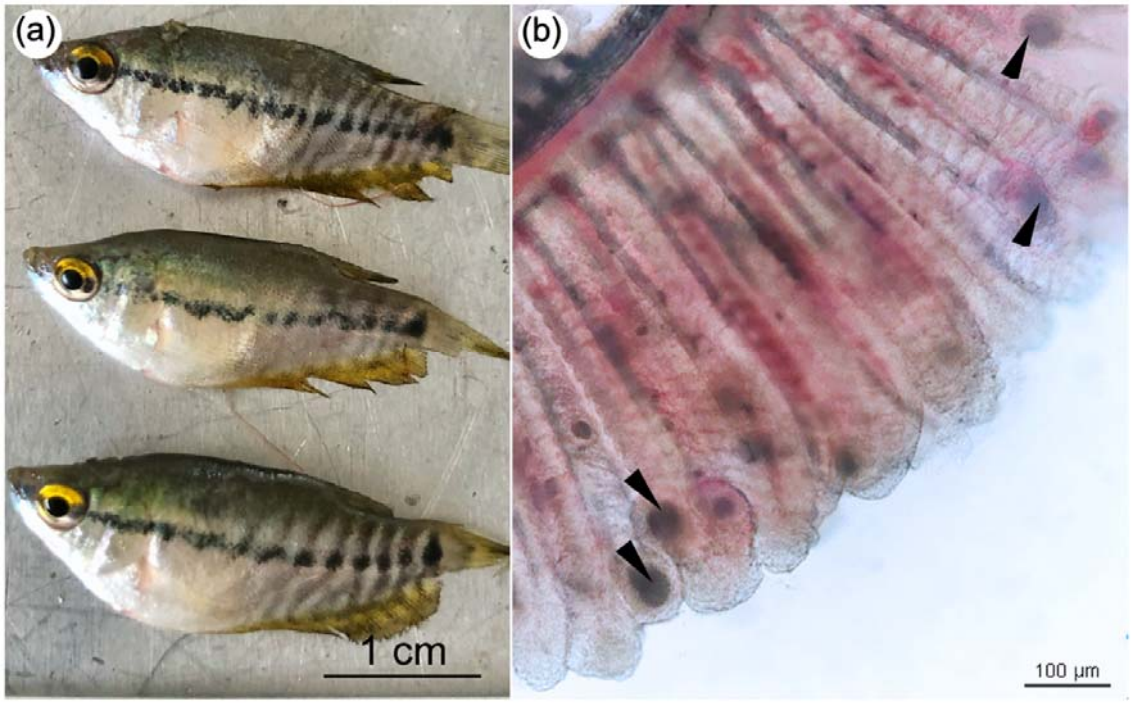


FIGURE 1 (a) Snakeskin gourami (*Trichopodus pectoralis*), no external lesions on the body surface of fish. (b) Wet mount preparations of infected fish gill showing numerous morphological characteristics of "cysts" (arrowheads). The scale bars are shown in the pictures.

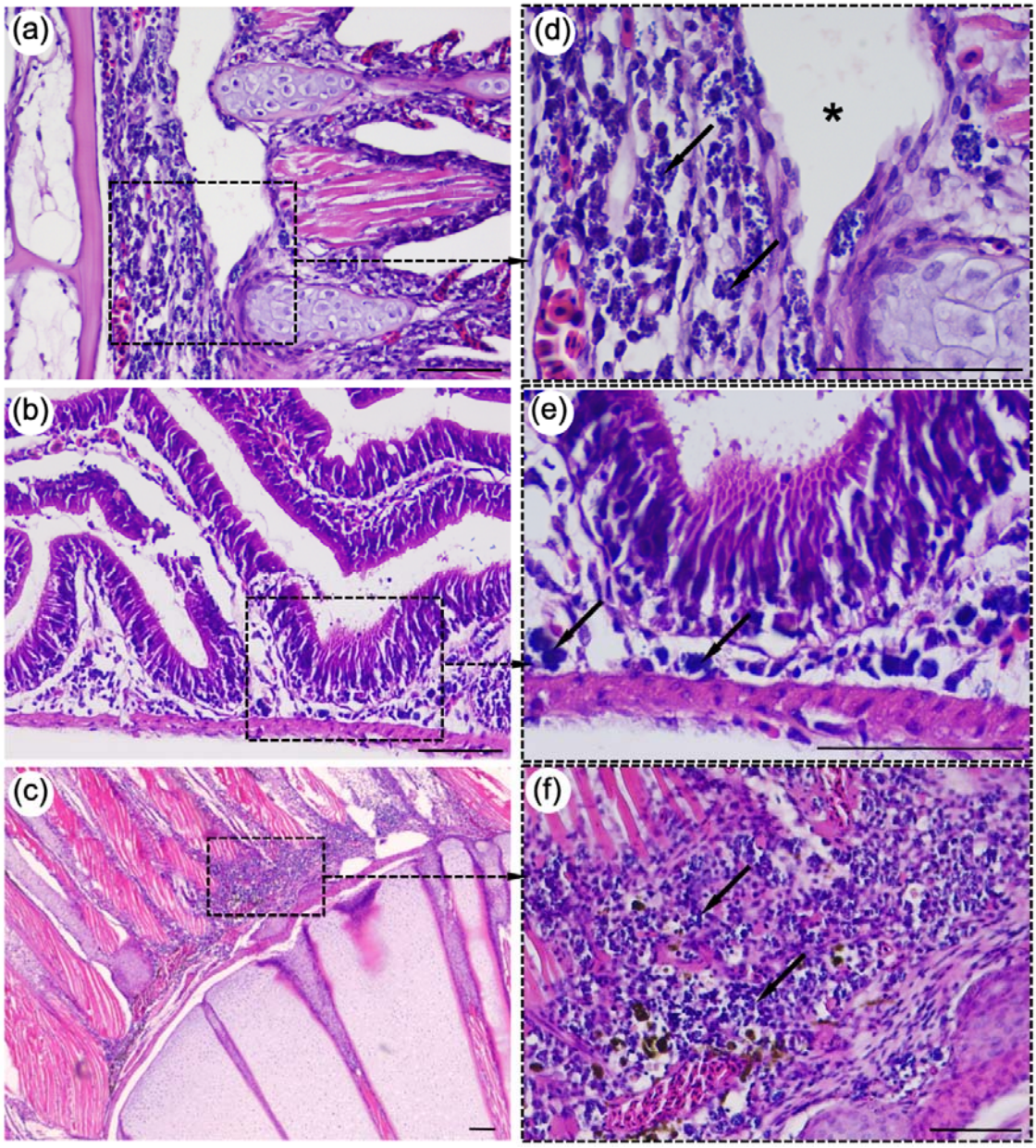


FIGURE 2 Infected fish showed presence of a novel intracellular bacteria in the gill (a), submucosa of the intestine (b), and tail/caudal fin (c). Higher magnification indicated the colonization of dense, rounded to oval-shaped intracellular bacteria stained with blue color (arrows in Figure d, e and f). The bacteria were observed near the cartilaginous junctions of the primary and secondary gill filaments (d) and caudal fin (f) resulting in tissue disconnection (asterisk Figure d). The slides were stained with hematoxylin and eosin (H&E) and scale bars = 50 µm.

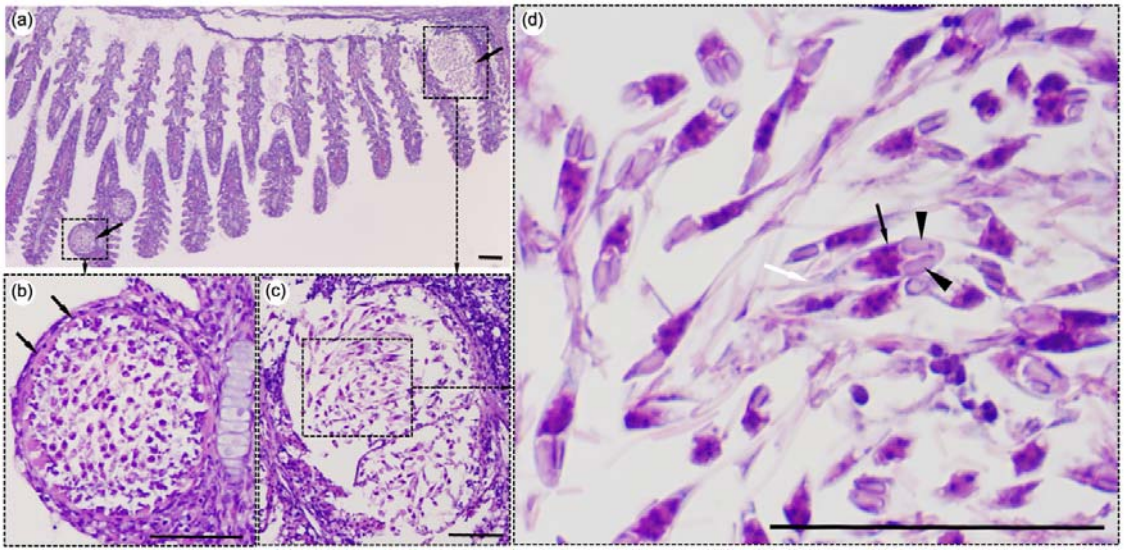


FIGURE 3 Histological lesions in snakeskin gourami (*Trichopodus pectoralis*) gill infected with *Henneguya* sp. (a) Plasmodia (arrows) with different developmental stages. (b) young plasmodium was rounded and enveloped by a wall formed by epithelium cells (arrows), immature myxospores located peripherally within plasmodia and mature myxospores located centrally. (c) A grown plasmodium ruptured the envelope. (d) Myxospores possess typical features of a *Henneguya* sp., including two equal polar capsules (arrowheads), sporoplasm located at the posterior pole of spore (black arrow), and two long superimposed tail processes (white arrow). The slides were stained with hematoxylin and eosin (H&E) and scale bars = 50 µm.

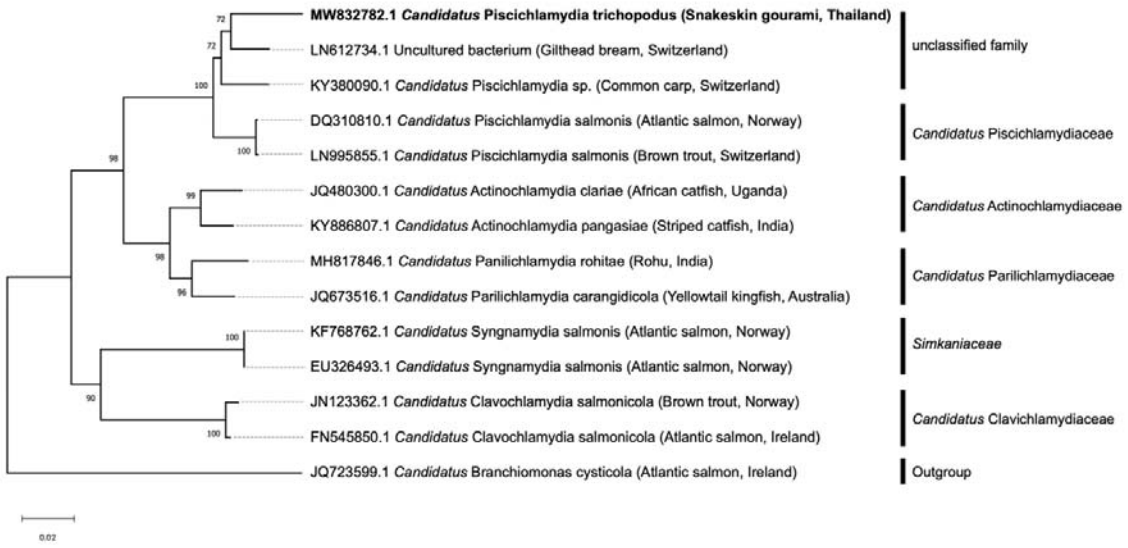
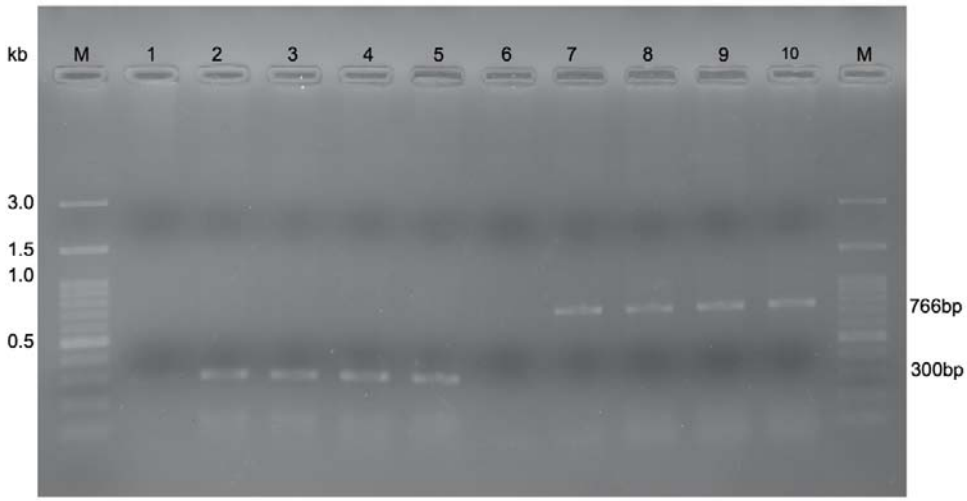


FIGURE 4 Phylogenetic tree was constructed based on partial 16S rDNA sequence (766 bp) from snakeskin gourami in this study (MW832782) and closely related species. The accession numbers and taxonomic identities, as well as the host origins, of the organisms included in this phylogenetic analysis, are demonstrated. *Candidatus* Branchiomonas cysticola was selected as an out group. The tree was constructed using neighbor-joining method. The scale bar represents 0.02 - nucleotide substitution per site, whereas the number at the node of the tree indicates bootstrap value in percentage.

253 **Supplementary data**



254

255 **Figure S1** PCR testing results confirmation under the agarose gel electrophoresis of samples
256 from four representative fish. Lane 1 and 6 were amplified without DNA template from the
257 primer set 1 and 2 as negative controls, respectively. Lane 2, 3, 4 and 5 were amplified with
258 DNA template from the primer set 1 (300bp). Lane 7, 8, 9 and 10 were amplified with DNA
259 template from the primer set 2 (766bp). Lane M was a DNA marker (Himedia, India).