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Research article

# Contributions to the knowledge of *Pseudolevinseniella* (Trematoda: Digenea) and temnocephalans from alien crayfish in natural freshwaters of Thailand

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ABSTRACT

Redclaw crayfish (*Cherax quadricarinatus*) is a decapod species originating from Australian freshwater. For more than two decades, these crayfish have been re-distributing to environments in many countries, including Thailand. Moreover, they can carry endosymbionts and/or ectosymbionts into new environments. The aim of this study was to introduce a morphological description of *Pseudolevinseniella anenteron* as a metacercaria of the endoparasites of redclaw crayfish collected from natural water sources in Thailand. The occurrence of two ectosymbiotic temnocephalans (*Diceratocephala boschmai* and *Temnosewellia* sp.) in *C. quadricarinatus* was also reported. The internal morphology of *P. anenteron*, *D. boschmai* and *Temnosewellia* were described and discussed. The surface ultrastructure of the multidentate spines on the body and the metacercarial cyst wall of *P. anenteron* was investigated by scanning electron microscopy (SEM). By performing a search of the GenBank nucleotide database of partial sequences of 18S, 28S rDNA and cytochrome c oxidase subunit I (cox1), *P. anenteron* was found to be related to *Maritrema*, and *Temnosewellia* was found to be related to *T. fasciata*. However, according to the cox1 gene, *Temnosewellia* was found to be similar to *T. minor*. These results reveal that redclaw crayfish that inhabit natural freshwaters in Thailand may harbour endoparasites and ecto- and endosymbionts. Furthermore, these findings may be able to monitor invasive or non-invasive species in an ecosystem.

## 1. Introduction

In the order Decapoda, *Cherax* Erichson, 1846, which belongs to the Parastacidae family, are commonly known as crayfish or yabbies and are widespread in aquatic environments in Australian and Western New Guinea (Munasinghe et al., 2004; Longshaw and Stebbing, 2016; Lukhaup et al., 2018). In astaciculture, *Cherax* is a commercial, semi-commercial and pet markets in Australia, Europe and the southern United States (Holdich, 1993, 1999; Veselý et al., 2015); *Cherax* may be transferred from its origin to other countries (Holdich, 1993; Volonterio, 2009; Kawai et al., 2015). The redclaw crayfish (*C. quadricarinatus* von Martens, 1868) inhabits Australian freshwater and has been introduced to other countries, such as for aquaculture farming (New, 2003; Patoka et al., 2016), because it is tolerant to various environmental conditions (Lin et al., 1999; Jones et al., 2000). Redclaw crayfish are considered an invasive species in several counties, including Uruguay, Mexico, Puerto Rico, South Africa, Zimbabwe, Swaziland, Malaysia, Singapore and Indonesia (Williams et al., 2001; Ahyong and Yeo, 2007; Volonterio, 2009; du Preez and Smit, 2013; Vega-Villasante et al., 2015; Naqiuddin et al., 2016; Nunes et al., 2017; Marufu et al., 2018; Jiří et al., 2016). Moreover, Wanjit and Chaichana (2013) and Chaichana and Wanjit (2018) collected redclaw crayfish from a reservoir in Sa Kaeo Province, Thailand.

*Cherax* can carry pathogenic diseases as well as ecto-and/or endosymbionts (including protozoa, flatworms, bacteria and viruses) into new environments (Holdich, 1993; Edgerton et al., 2000; Longshaw and Stebbing, 2016). Jones and Lester (1992) found adult *Diceratocephala boschmai* on *C. quadricarinatus* to be an ectocommensal symbiont. Williams (1994) observed that *Temnosewellia minor* plays an ectocommensal role in crayfish. Volonterio (2009) described *D. boschmai* Baer (1953) collected from *C. quadricarinatus* on a Uruguayan crayfish farm. Ngamniyon et al. (2014) found *D. boschmai* on the bodies of *C. destructor* from local farms of freshwater crayfish in Thailand. Furthermore, Tavakol et al. (2016) reported excellent data regarding *D. boschmai*, *Craspedella pedum* and *Didymorchis* sp. in *C. quadricarinatus* from a natural river in Mpumalanga Province, South Africa. Among trematodes, *Pseudolevinseniella cheni*

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Tsai (1955) was found in *Macrobrachium nipponense* as the second intermediate host and *Anas platyrhynchos domesticus* as the definitive host (Belopol' skaya, 1963). Deblock and Pearson (1968) described *P. anenteron* in *M. australiense* and investigated its adult forms through experimental infection of mice. Nevertheless, the first intermediate host of *Pseudolevinseniella* has remained unknown (Kudlai et al., 2015).

The major aim of this study was to describe the metacercaria of *P. anenteron* in *C. quadricarinatus* collected from natural environments in Thailand. In addition, we report evidence of two Australian temnocephalans (*D. boschmai* and *Temnosewellia* Damborenea and Cannon, 2001) collected from *C. quadricarinatus*.

## 2. Materials and methods

### 2.1. Crayfish capture and flatworm collection

*Cherax quadricarinatus* were caught with fish traps, crab traps and purse nets between March 2015 and January 2017 from rivers, canals, ponds and reservoirs of central, western and eastern Thailand at the time of freshwater fish and crab surveys. For area sampling, thirty-five localities for capturing and trapping crayfish were in ten localities of the central part, nine localities for the western part, five for the northeastern part and eleven localities were in the eastern part. The captured crayfish were washed with distilled water, transferred to small aquariums containing freshwater with an air pump and then were transferred to the laboratory without feeding. Crayfish were identified as *C. quadricarinatus* or *C. destructor*. following Horwitz (1995). For *C. destructor*, there was not found any temnocephalans or trematodes. Flatworms were gently separated from several external parts of crayfish that were immediately killed by cutting the cervical groove according to the report of Tavakol et al. (2016). Temnocephalans were identified as *Temnosewellia* and *D. boschmai* by the key to the genera of Australian temnocephalans and species (Cannon and Sewell, 2001; Sewell, 2013; Sewell et al., 2006). Flukes were collected from the hepatopancreas and gill tissues and were identified as *P. anenteron* by the criteria of Deblock and Pearson (1968) and Bray et al. (2008). Specimens preserved in 70% ethanol were deposited in the Section of Zoological Exhibition, Botanical Learning Center, Faculty of Environmental Culture and Eco-tourism, Srinakharinwirot University, M.7 Rangsit-Nakhon Nayok Road Ongkharak, Nakhon Nayok 26120, Thailand, with voucher number ECE160560 for *P. anenteron*, ECE090860 for *Temnosewellia* and ECE111160 for *D. boschmai*.

### 2.2. Morphological observation, specimen fixation and staining

Two temnocephalans and *P. anenteron* were observed for general characteristics under a light microscope. To examine the internal morphology, *D. boschmai* and *Temnosewellia* sp. were fixed in hot 4% formaldehyde, moved to AFA solution, stained with carmine acetic acid, dehydrated by a serial grade of ethanol (70, 80 and 90%), re-stained with fast green in 95% ethanol, dehydrated with 100% ethanol, cleared with xylene and mounted in Canada balsam. To fix and stain *P. anenteron*, the metacercaria of flukes and cysts were processed as above without the hot formaldehyde fixation. Internal morphology of specimens (Faure's preparation for observation of temnocephalan cirrus) was recorded and measuring the organs with a Moticam digital microscope camera (Motic®, Hong Kong).

### 2.3. SEM investigations

In SEM, the fixation and dehydration of *D. boschmai* and *Temnosewellia* were followed by the method of Tavakol et al. (2016). Specimens were adhered by carbon conductive tape to a stub and then were coated with Pt/Pd using a sputter coater and were monographed using an SEM-HITACHI SU-8010. By a similar method, *P. anenteron* were processed without hot fixation. For surface observation of flukes in cysts or temnocephalans in eggs, cysts and eggs were cracked using a microneedle on stub-carbon conductive adhesive tape before metal coating. Eggs of *Temnosewellia* sp. or *D. boschmai* were separated from exoskeleton of crayfish among population of *Temnosewellia* sp. or *D. boschmai*, respectively.

## 3. Molecular confirmation

To confirm the identification of flatworms, total genomic DNA was extracted from whole bodies of *D. boschmai* or *Temnosewellia* and *P. anenteron* using a DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Two nuclear genes were amplified using *Taq* DNA polymerase (Takara, Tokyo, Japan) with the primers 5′-cgcagtcggccttgtgtcggcg-3′ and 5′-gcggtgtgtacaagggcagggacg-3′ for 18S rDNA (~1400 bp), 5′-agtaacggcgagtgaacaggg-3′ and 5′-gtctttcgcccctatactcacg-3′ for 28S rDNA (~1150 bp). Primers for cytochrome c oxidase subunit I (cox1) of temnocephalans were used according to Hoyal-Cuthill et al. (2016). Primer cox1 for fluke was 5′-gggwgctggkgttggttgrac-3′ and 5′-caggatcaccaccmccyaacggatc-3' (~400 bp). PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 35 s, extension at 72 °C for 60 s, and a final extension for 10 min. PCR products were purified by electrophoresis using a QIAquick Gel Extraction Kit (Qiagen). DNA sequencing analysis was performed by the Macrogen DNA Sequencing Service, Korea. The 18S, 28S rDNA and cox1 partial sequences were deposited into GenBank with the accession numbers MG725685-MG725696 and MK421403-MK421408. Sample sequences were identified using the Basic Local Alignment Search Tool (BLAST) (Mount, 2007) for the local similarity of 18S, 28S rDNA and cox1 from the nucleotide databases of The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). For other flatworms and outgroup species, nucleotide sequences were searched from these databases (accession numbers from Cribb et al., 2001; Lockyer et al., 2003; Van Steenkiste et al., 2013; Fraija-Fernandez et al., 2015; Vdacny et al., 2011; Laumer and Giribet, 2014; Ngamniyon et al., 2014; Tkach et al., 2003; Devi et al., 2010; Vdacny et al., 2012; Hoyal-Cuthill et al., 2016; Leung et al., 2009; Keeney et al., 2009; Ng et al., 2018). Sequences were aligned with the Clustal Omega multiple sequence alignment programme (http://www.genome.jp/tools/clustalw/), and phylogenetic trees inferred using the maximum-likelihood approach in MEGA 5.1 (Tamura et al., 2011).

Animal experiments were conducted under the National and Institutional Guidelines for the Animal Care and Use for vertebrates by Institute for Animals for Scientific Purpose Development (IAD) National Research Council of Thailand (NRCT) with a permission license (U1-02115-2558) and Biotechnology Safety of Laboratory SCBL14 Class BL2.

## 4. Results

Sixteen individuals of *C. quadricarinatus* [body length = 9.7 ± 2.9 cm (mean ± standard deviation), 7.5–15.5 cm (minimum – maximum)] and a dead specimen of *C. destructor* (body length = 7.8 cm) were captured for our work (Figure 1A). From redclaw crayfish, five samples harboured *D. boschmai* and two samples had *Temnosewellia* sp. Both populations of these temnocephalans were found in a single sample. In *P. anenteron*, metacercarial flukes were infected in two redclaw crayfish. In one sample of these crayfish, *D. boschmai* and *P. anenteron* were found. Total specimens of *D. boschmai* or *Temnosewellia*, *P. anenteron* and encysted were 43, 27, 56 and 36, respectively.

**Redescription of *Pseudolevinseniella anenteron*** Deblock and Pearson (1968)

**(Digenea: Microphallidae)**

*Host*: *C. quadricarinatus*, *tissue*: muscle, hepatopancreas and gills, *locality*: canals and reservoirs, *country*: Thailand, *life cycle*: metacercaria

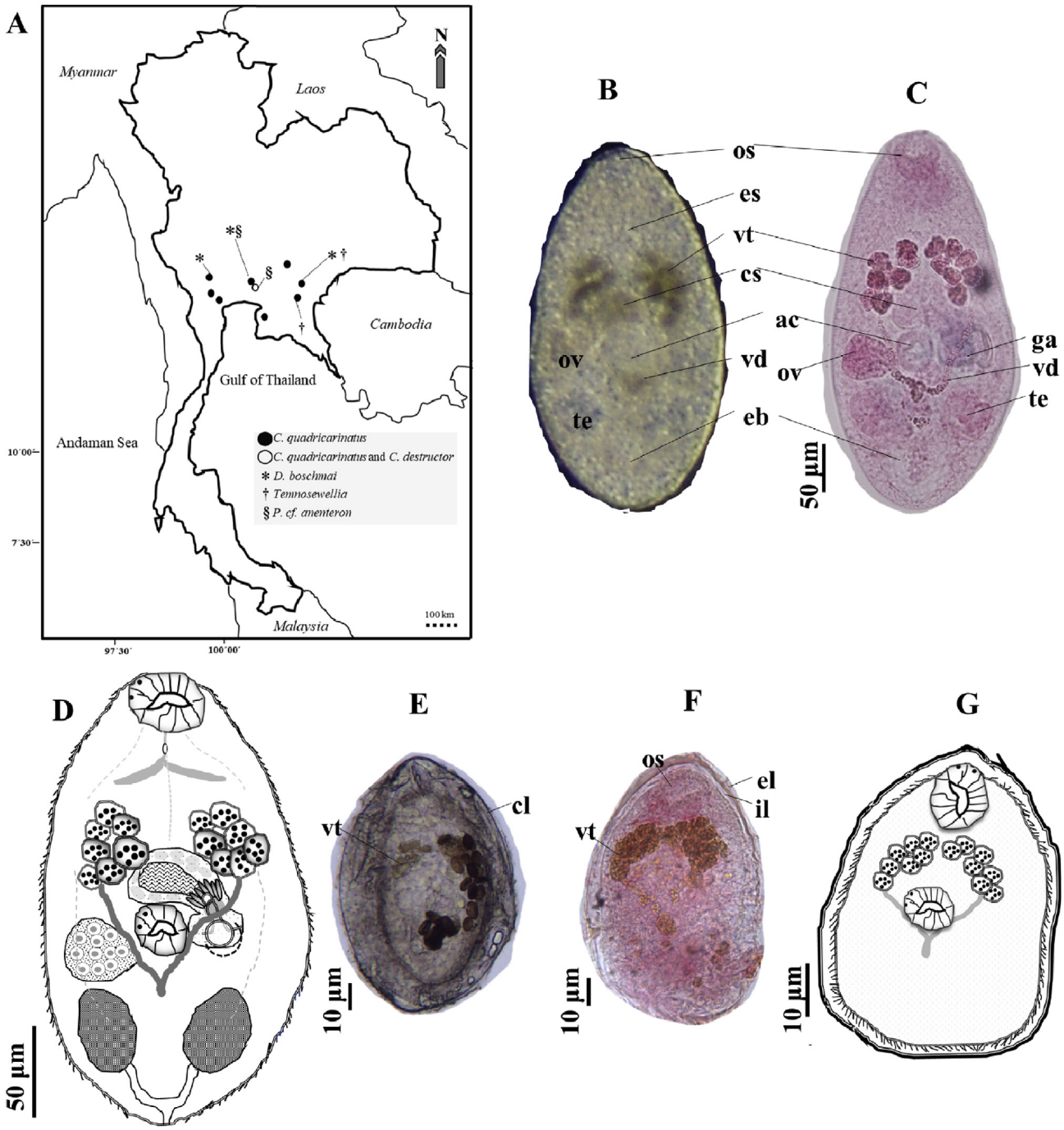


**Figure 1.** Map for finding crayfish and flatworm (A). Metacercaria of *P. anenteron* (B). Internal morphology (C). Line drawing of *P. anenteron* (D). Cyst of *P. anenteron* (E). Internal morphology of cyst (F). Line drawing of encyst *P. anenteron* (G) (ac: acetabulum, cl: cyst wall, cs: cirrus, eb: excretory bladder, el: external layer, es: esophagus, ga: genital aperture, il: internal layer, os: oral sucker, ov: ovary, te: testis, vd: vitelline duct, vt: vitelline follicle).

and cyst, *redescription*: In metacercaria, the worm body is small, typically flat, long-oval and yellowish to light brown. Body measurements were 354.8 ± 28.6 (mean ± SD) µm in length (minimum = 324.3 µm – maximum = 403.2 µm, number of specimens = 10) and 178.0 ± 22.0 µm in width (149.3–215.3, 10). Spines had a short ciliary shape and covered the surface body. The oral sucker (49.2 ± 6.7 µm in diameter, 38.9–59.5, 10) was positioned at the apical body, quite round with a thick muscle. Oesophagus is short and extended to two branches of short caeca. The caecum branched in a line divergently from the centre to the right and left laterally. The acetabulum was round, appearing almost in the centre of the body (40.0 ± 4.2 in diameter, 32.0–45.7, 10). The genital aperture (43.5 ± 5.1 µm, 37.9–56.7, 10) was slightly ovoid near the acetabulum sinisterly with thin muscles. The cirrus sac was large, invaginating and curving, transversely and superiorly to the acetabulum. Uteri were found on the upper hind body but were not clear. Vitelline follicles were two clusters that clearly predominated in which each cluster was composed of approximately seven follicles, quite round, with sub-caeca branches at the anterolateral part of the cirrus sac (22.0 ± 2.8, 18.6–26.2, 10). Two vitelline ducts were connected to each of the posterior ends of the follicle clusters, thick and direct to three-fourths of the body as a U-shape. The ovaries were slightly round and were sub-dextral to the acetabulum on the lateral side of the body (55.4 ± 13.1 in diameter, 35.7–67.1, 10). The

two testes were large, kidney-shaped, symmetrical and arranged transversely right and left (60.9 ± 4.3 μm in length, 54.9–68.4, 10 and 45.6 ± 8.4 μm in width, 33.5–57.5, 10). The right testis was post-ovarian, and the left was inferior to the genital aperture. The excretory vesicle was not long, was V-shaped and was present between the posterior part of both testes (Figure 1B-D).

In encysts, the body of *P. anenteron* was short subelliptical, transparent and light brownish (196.2 ± 20.4 μm for body length, 168.0–222.8, 10 and 124.3 ± 13.4 μm, 109.0–147.3, 10 for body width). Numerous spines with a ciliary shape covered the body surface. The oral sucker was bulky on the anterior part of the body (45.2 ± 7.2 μm in diameter, 33.2–53.5, 10). Two clusters of vitelline follicles were compacted densely and positioned symmetrically on the anterolateral body. Vitelline ducts were to some extent similar to those mentioned for metacercaria. The acetabulum appeared at the middle part of the fluke but was obscure (Figure 1E-G).

On SEM, the surface invaginates shallowly from an anteromiddle to a posteromiddle part of the ventral body. In the dorsal part, there was a hollow and like a reuleaux triangle shape at the posterior area. The fluke surface was occupied by spines throughout the whole body. Spines were multidentate (7 dentate lines), arranged on the dorsal and ventral parts (1.9 ± 0.3 μm in length, 1.4–2.3, 10, 1.6 ± 0.2 μm in base width, 1.4–1.9, 10). By contrast, the spines in the hollow were small (1.2 ± 0.1 μm in length, 1.0–1.3, 10, 1.0 ± 0.1 μm in base width, 0.8–1.2, 10) (Figure 2A-D). In encysts, the surface shell was rough. For the surface of *P. anenteron* removed from encysts, there was no invagination, and the multidentate spines were long and densely arranged (3.2 ± 0.4 μm in length, 2.7–3.7, 10, 1.1 ± 0.1 μm in base width, 0.9–1.3, 10) (Figure 2E-H).

**Description of *Temnosewellia* sp.** Damborenea and Cannon (2001)

***Temnocephala*** Blanchard et al., 1849

**(Rhabditophora: Temnocephalidae)**

*Host*: *C. quadricarinatus*, *position*: chelipeds, walking legs, carapace and branchial chamber, *locality*: canals and reservoirs, *country*: Thailand, *life cycle*: adult and egg, *description:* The anterior body bore five tentacles, and these tentacles were similar in shape (213.2 ± 61.3 μm in length, 104.3–318.6 in width, 30 from 6 specimens). The adhesive disk (178.3 ± 37.6 μm, 138.1–219.3, 6 in diameter) was thick and muscular, large with an adhesive disc and located at the posteroventral side of the body. The body was bumpy (858.9 ± 297.3 μm in length, 510.3–1313.2, 6 specimens from the margin of the adhesive disk to the base of the third tentacle and 411.5 ± 110.2 μm in width, 248.6–534.2, 6). Typical dorsal body pigment was observed. Haswell's cells were found about at the subpositions of the second to the fourth tentacles. Two eyes had black pigment near the base of Haswell's cells. The mouth (147.6 ± 34.0 μm in diameter, 106.5–183.4, 6) was muscular and round, in the mid-ventral part of body and under the paired eyes. The intestine sac was large at the middle part. Excretory ampullae were in the anterolateral part of the body. Rhabdite glands (33–24 in number, 21.2 ± 7.5 in diameter, 10.6–31.6, 18) were in the lateral side of the body, with sub-excretory ampullae that extended from the posterior mouth to the middle intestine

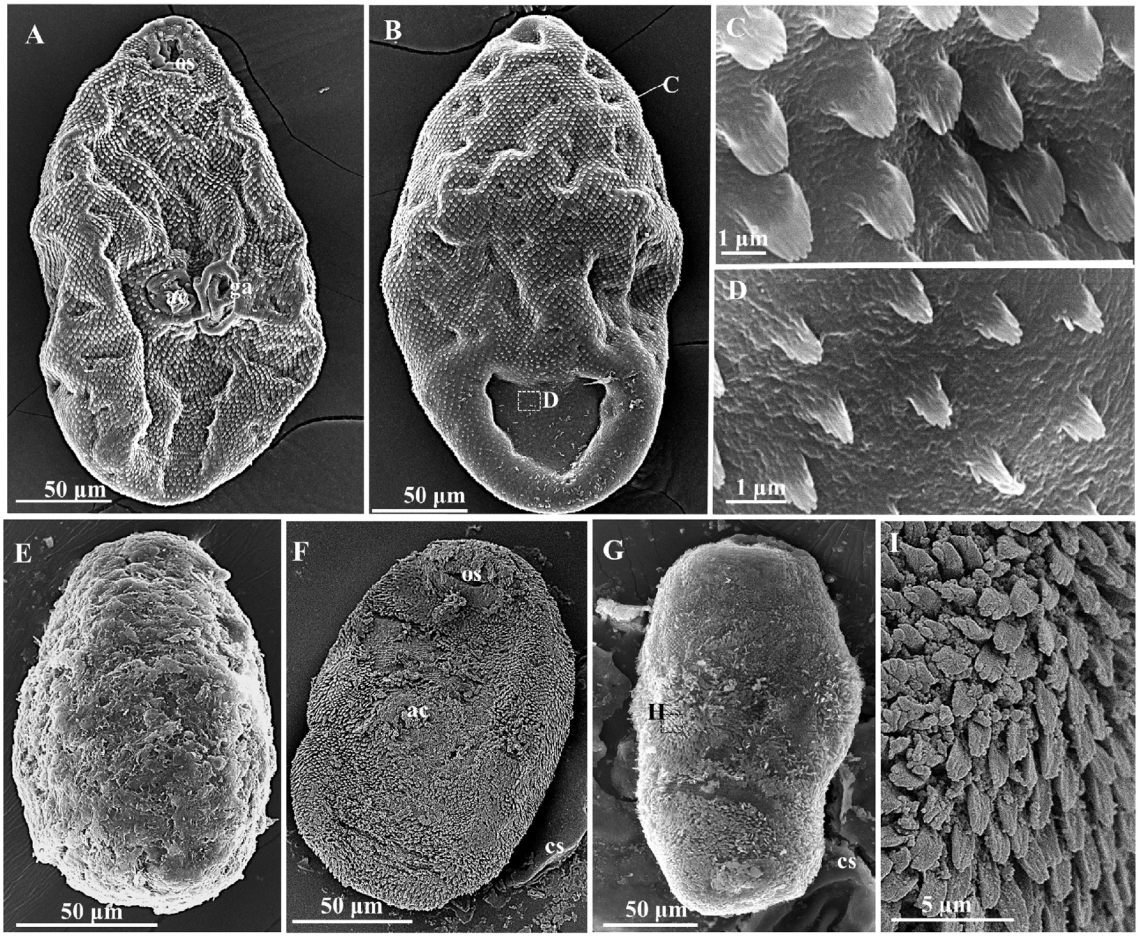


**Figure 2.** SEM micrograph of metacercaria of *P. anenteron*. Ventral side (A) and dorsal side (B). Magnification of dorsal spines (C) from and of posterior part (D) from Figure B. Encyst of *P. anenteron* (E). Ventral side (F) and dorsal side (G) of *P. anenteron* from the crackered cyst by using a microneedle. Magnification of spine area on the body of worm the crackered cyst (I) from H. (cs: cyst shell, ac: acetabulum, os: oral sucker).

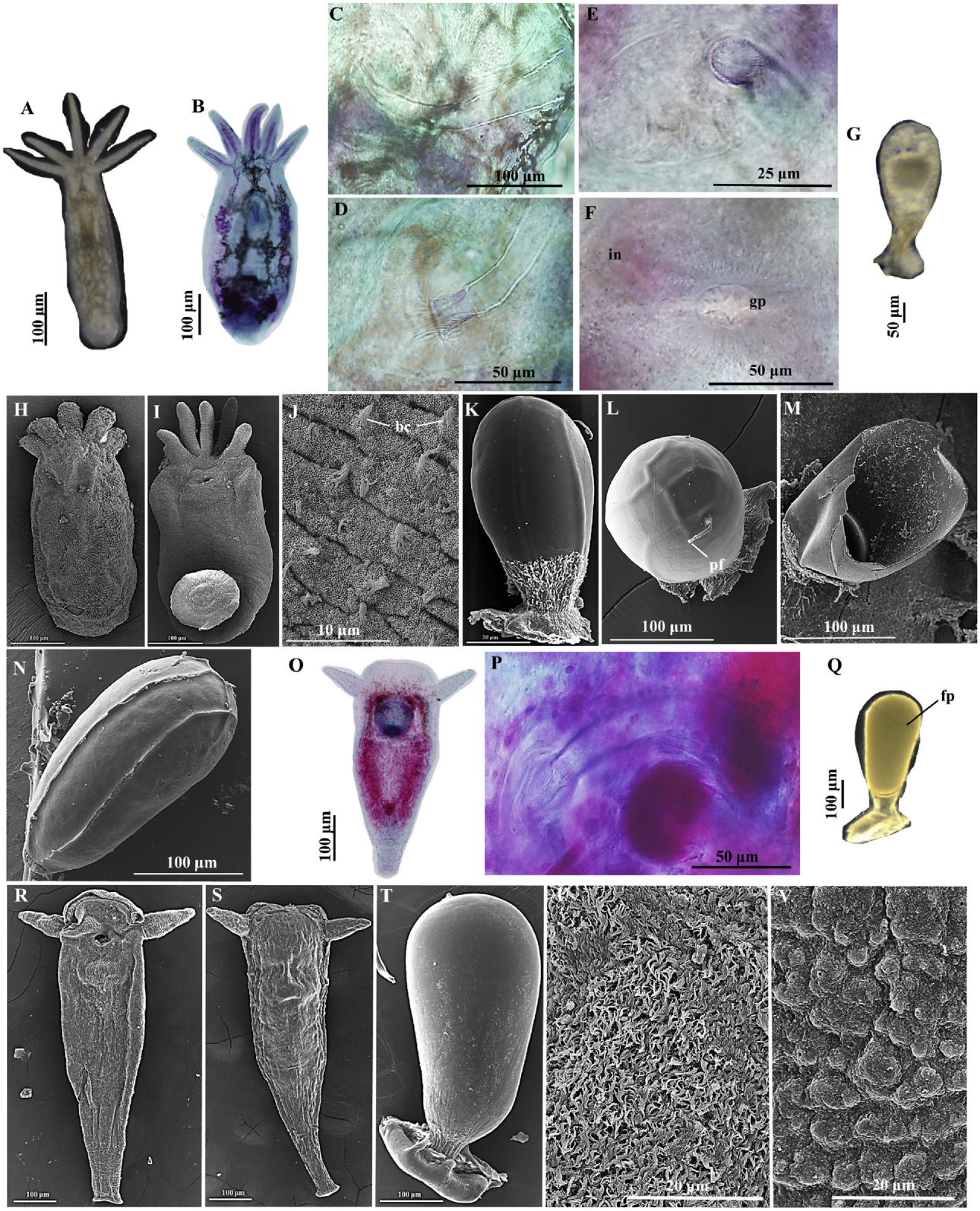


**Figure 3.** *Temnosewellia* sp. (A–N) and *D. boschmai* (O–V). Dorsal view of *Temnosewellia* sp. (A), ventral view of internal morphology (B), cirrus shaft (C), cirrus introvert (D), top of introvert (E) and genital aperture near the area of cirrus (F). Egg capsule (G). SEM reveals dorsal (H) and ventral surface (I). Magnification surface of tentacle (J). Surface of egg capsule (K), apical area of capsule with a single polar filament (L) and hatched egg with fracture plane on shells of crayfish (M). Egg in capsule (N). Ventral view of internal morphology (O), cirrus (P) and egg capsule (Q). SEM monograph of ventral (R) and dorsal surface (S). Surface of egg capsule and single polar filament on apical of capsule (T). Magnification surface of tentacle (U) and stalk V) (bc: bunch of cilia, fp: fracture plane, gp: genital aperture, in: introvert, pf: polar filament).

sac. Two testes were paired as the anterior and posterior testes at posterolateral of the rhabdite glands and the intestine sac. The ovary was ovoid near the right of the posterior testis. The cirrus was between the intestine sac and the anterior part of the attachment organ and curved to the antrum. Spined introvert cylinders, evens and cones with distal openings were not oblique (27.2 ± 5.8 μm in length, 23.9–39.23, 6, 19.7 ± 3.4 μm in base width, 12.6–18.2, 6). There was a thickened ring proximally, an unspined distal region (7.4 ± 1.1 μm, 6.6–9.0, 4), and the cone-shaped shaft was thick (204.0 ± 9.8 μm in length, 192.6–218.5, 6, 32.6 ± 6.2 μm in base width, 24.9–38.7, 6) (Figure 3 C-E). The gonopore was a strong muscle, over the antero-middle of the adhesive disk and opening out to the ventral body (Figure 3 F). The egg capsule was light yellow with two black eyes were observed inside, and the stalk was light brown (304 ± 29.3 μm from the apical egg capsule to the base of the stalk, 256.8–336.3, 5) (Figure 3G). On SEM, the surface body was not smooth and the tentacle surface was dominantly corrugated transversely. This epidermis was thin, covered by small spherical shapes and adorned with bunches of ciliated shapes. The egg capsule was a smooth, and there was a long peduncle with a single polar filament (37.9 ± 6.1, 33.5–42.2, 2) found on the apical egg (Figure 3H-L). Hatched egg capsules showed fracture planes in a diagonal line from the anterior to the middle zone (Figure 3M-N).

**Findings of *Diceratocephala boschmai* Baer (1953) (Diceratocephalidae) in crayfish.**

*Host*: *C. quadricarinatus*, *position*: pleopods, chelipeds, walking legs, carapace and branchial chamber, *locality*: shallow river, canals and reservoirs, *country*: Thailand, *life cycle*: adult and egg, *redescription:* Body (4.52 ± 0.75 mm in length, 1.3–6.6, 4) shaped obovate. Two tentacles (0.52 ± 0.19 mm in length, 0.3–0.71, 8) were separated by an intertentacular flange (0.8 ± 0.01 mm in length, 0.45–1.2, 4) in the anterolateral body. Eye (0.04 ± 0.19 mm, 0.03–0.06, 8) pairs were under the intertentacular flange. The mouth (0.44 ± 0.21 mm, 0.24–0.73, 4) was large with a strong muscle and posterior to the eyes. The excretory pores were dorsal, the mouth ventral, so the excretory vesicles were at the same level of the mouth. The gut was large in the middle part of the body, and vitellaria were found dorsal to the gut. The ovary (70 ± 30 μm, 40–100, 4) was slightly ovate near the posterior gut. The two testes (280 ± 150 μm in length, 120–490, 8) were large, lobate, kidney-shaped, and the right and left lobes were located on the posterolateral side of the body. A strong muscular adhesive disk (0.31 ± 0.16 mm, 0.16–0.53, 4) was located on the posterior part of the body (Figure 3O). The cirrus was curved to an antrum. The introvert had a crown of spines in the distal portion of the stylet (Figure 3P). The egg was yellow, and an opercular plate was observed at the anterior part (Figure 3Q). On SEM, the body surface was covered by cilia at the anterior part and tentacles. In the posterior part of the stalk, the surface was rough with a stone-wall texture. The egg surface was smooth with a short polar filament (Figure 3R-V).

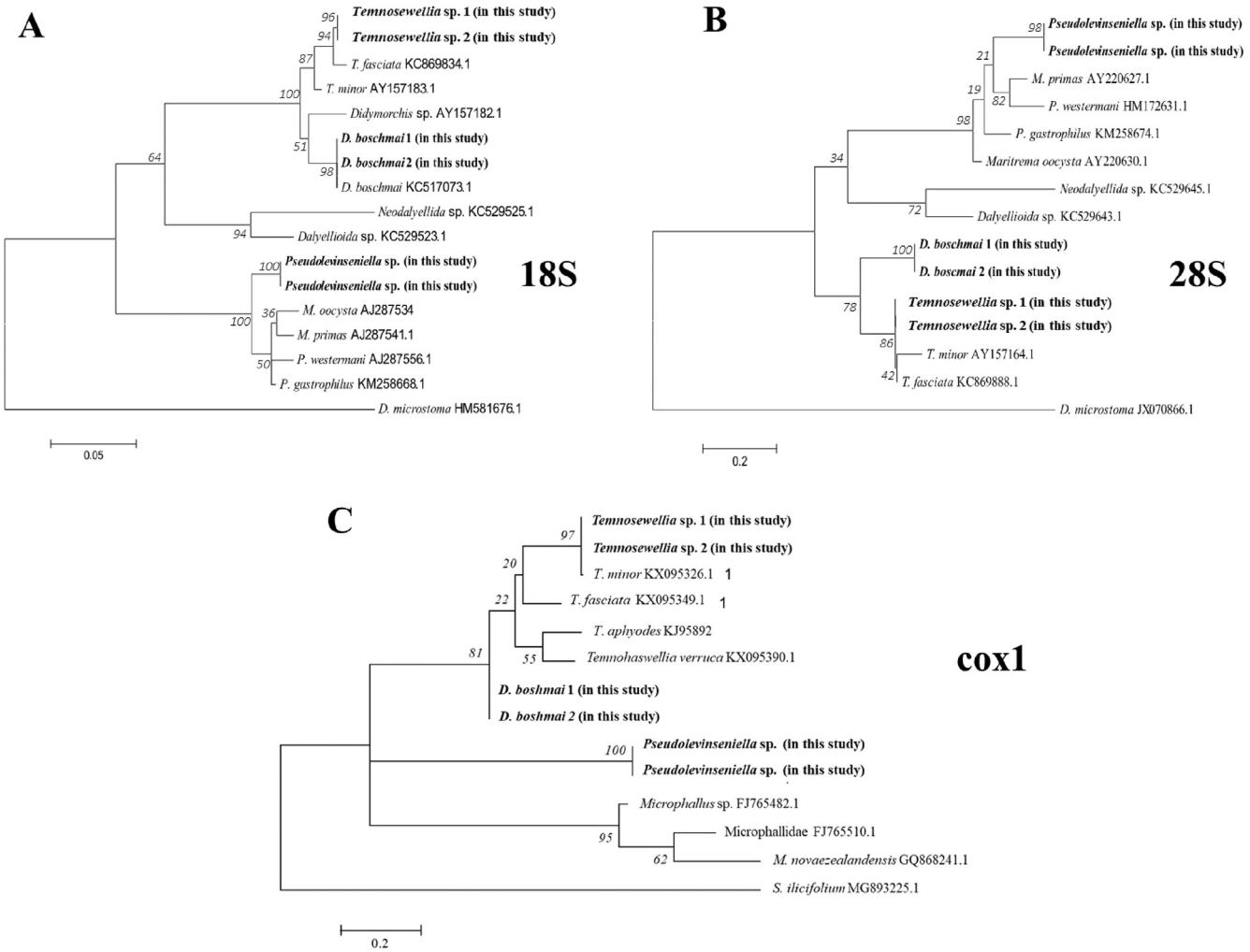


**Figure 4.** Maximum likelihood method shows phylogenetic confirmation for *P. anenteron* in Microphallidae and *Temnosewellia* and *D. boschmai* in Temnocephalida based on 18S (A) and 28S (B) and cox1 (C) partial rDNA sequences. The number in the branch of tree indicates bootstrap value (%).

### 4.1. Molecular analysis

In sequence alignments, there was partial rDNA sequences of *P. anenteron* matching those of *Maritrema oocysta* (AJ287534) with 94% identity and 28S *M. oocysta* (AY220630.1) with 89% identity from the nucleotide databases of GenBank. In our *Temnosewellia* sp., they were similar to that of *T. fasciata* (KC869834) with 98% identity for 18S rDNA sequences and to 28S *T. fasciata* (KC869888) with 99% identity. *D. boschmai* matched *D. boschmai* (KC517073) with 99% identity for 18S rDNA and *T. fasciata* (KC869888) with 96% identity for 28S rDNA. For cox1, *P. anenteron*, *Temnosewellia* and *D. boschmai* were 83% to *Tylodelphys* sp., and 99% to *T. minor* and 87% to *T. keras* (KX095360.1). *Temnosewellia* sp. were monophyletic to *T. minor* and *T. fasciata* based on 18S, 28S rDNA and cox1. The *D. boschmai* of this study was close to *Didymorchis* sp. (AY157182.1). *Temnosewellia* sp. and *D. boschmai* were far from *Neodalyellida* sp. (KC529645.1) and *Dalyellioida* sp. (KC529643.1). *P. anenteron* was in the Plagiorchiida group with *Microphallus*, *Maritrema*, *Paragonimus* and *Pholeter* (AY220627.1, AY220630.1, HM172631.1 and KM258674.1). In cox1, *Temnosewellia* sp. and *D. boschmai* shared common ancestor of temnocephalan (Figure 4A-C).

## 5. Discussion

In this study, crayfish *C. quadricarinatus* were captured from natural environments in Thailand as in Wanjit and Chaichana (2013) and Chaichana and Wanjit (2018), in which *C. quadricarinatus* was found in a reservoir in Eastern Thailand. These invasive crayfish occur as feral populations in several countries (Jones, 2011); in the excellent report by Tavakol et al. (2016), thirty-five individuals of *C. quadricarinatus* with three ecto-temnocephalan species were found in South Africa. In Southeast Asia, Belle and Yeo (2010) described specimens of redclaw crayfish approximately 17.6 cm in total body length that were caught from a lake in Singapore. In our study, however, the specimens of redclaw crayfish were few in number and small in size.

For endoparasites, tissues of *C. quadricarinatus* were infected by the metacercaria of the ex- and encysted forms of *P. anenteron*. The internal organ positions of these excysted specimens were similar to those of the metacercaria of *P. anenteron* and were 460–540 μm in length and 200–270 μm in width, as described by Deblock and Pearson (1968), for *M. australiense* as the host in Australia. For other related fluke species, *M. nipponense* was the intermediate host for *P. cheni* in China. Only the body size was different from that of *P. anenteron* in the previous report as our specimens were rather small. In *Cherax*, digenean infections were examined in previous studies, including *Microphallus minutus* and *Plagiorchis jaenschi* in *C. destructor* and *Opecoelus variabilis* in *C. depressus* and *C. dispar* (Johnston, 1948; Johnston and Angel, 1950; Cribb, 1985). Therefore, our findings may confirm that redclaw crayfish is an intermediate host of *P. anenteron*; nevertheless, it is unclear if infection transmitted from an alien host or a native host in Thailand.

In temnocephalans, in this study, *Temnosewellia* sp. was found to be similar to *T. fasciata* based on its 18S and 28S rDNA sequences, while the cox1 sequence is most similar to *T. minor*. It suggests that the relationship of *Temnosewellia* sp. and selected species may disagree between these nucleus genes and mitochondrial gene. However, there was no congruence between the molecular and morphological data showed that the cirrus introvert of *Temnosewellia* sp. from Thailand did not resemble that of *T. fasciata*, despite the similarities in rDNA sequences. Furthermore, *T. fasciata* attached on spiny crayfish (*Euastacus*) was common host (Cannon and Sewell, 2001). Examples of temnocephalidan symbionts of *C. quadricarinatus* included *T. chaeropsis*, *T. minor* and *T. punctate* (Cannon and Sewell, 2001; Damborenea and Cannon, 2001). Nevertheless, the introvert in our specimens was shorter than those from *T. chaeropsis* and *T. punctate*, although the morphology of the cirrus was similar. The sequence of the mitochondrial cox1 gene, *Temnosewellia* sp. from Thailand was nearly an exact match to that of *T. minor* described in Hoyal-Cuthill et al. (2016) from Australian environments. The introvert length of our *Temnosewellia* sp. was similar to that of *T. minor* (Cannon and Sewell, 2001); however, the morphology of its cirrus was different. In this study, cirrus structure of *Temnosewellia* sp. was incongruence between the morphological and molecular data.

In the Diceratocephalidae studied here, the organ positions were similar to those described by Ngamniyon et al. (2014) and were maintained in *C. destructor* from local farming of freshwater crayfish in Thailand. Nevertheless, we were not able to compare the morphometrics because the specimens of the previous report had become shrunken. In addition, the organ structures of our specimen corresponded to *D. boschmai* from Uruguay, as described by Volonterio (2009), and the surface ultrastructure was similar to that from South Africa described by Tavakol et al. (2016). Therefore, our report suggests that *D. boschmai* is present in the semi-astaciculture or natural water of Thailand.

## 6. Conclusion

This study describes the morphology and ultrastructure of ecto- and endosymbionts of flatworms in redclaw crayfish of natural waters in Thailand. The findings of this study show that alien crayfish with *P. anenteron* and invasive temnocephalans are present in these waters.

## Declarations

### Author contribution statement

Arin Ngamniyom: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Thayat Sriyapai, Pichapack Sriyapai, Busaba Panyarachun: Contributed reagents, materials, analysis tools or data.

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The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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