

Rapid Communication**First evidence of *Craspedella pedum* (Cannon and Sewell, 1995) (Platyhelminthes: Rhabdocoela) infesting alien red swamp crayfish with white spot syndrome virus infections collected from Thailand**

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OPEN ACCESS**Abstract**

Red swamp crayfish (*Procambarus clarkii*) is natively distributed in the tropical fresh water of the Southern Mississippi River, the United States, and Northeastern Mexico. This crayfish is also transported to many countries through commercial trade. The aim of this study was to determine the presence of temnocephalid flatworm (*Craspedella pedum*) on red swamp crayfish collected from the natural waters of Thailand. At the 34 collection sites, only three *P. clarkii* were caught in traps from two locations in the north-central Thailand region near the Nan River. A total of 23 *C. pedum* individuals were found in two *P. clarkii* individuals collected at the same site. White spot syndrome virus (WSSV) was detected in the gill tissue of those two crayfish and *C. pedum* using PCR analysis. Morphological and molecular *C. pedum* data are provided. The result is the first report of temnocephalans with viral infection in alien host from the natural environment of Thailand. Furthermore, it is warning that the invasive crayfish and non-native temnocephalans may be already established in natural freshwater of Thailand.

Key words: *P. clarkii*, temnocephalans, WSSV, Nan River**Introduction**

Freshwater cambarid crayfish, *Procambarus clarkii* Girard, 1852, is commonly known as the red swamp crayfish or red swamp crawfish (Crandall 2010; Loureiro et al. 2015). *Procambarus clarkii* is native to the fresh waters of the drainage region of southern Mississippi River, South-central United States and North-eastern Mexico (Hobbs et al. 1989). The species has been transported and introduced to many countries including Spain, Italy, Portugal, the Netherlands, Egypt, China, and Japan (Huner 1988; Gutiérrez-Yurrita and Montes 2001; Cecchinelli et al. 2012; Sousa et al. 2013; van der Wal et al. 2013; Abdallah et al. 2018; Yi et al. 2018). Red swamp crayfish is considered an invasive species that impacts the ecological environment (Yue et al. 2010; van der Wal et al. 2013; Nishijima et al. 2017). It has the ability to adapt to a wide range of aquatic environments (Shen et al. 2014). In Thailand, *P. clarkii* and the red claw crayfish (*Cherax quadricarinatus* Von Martens, 1868) were introduced in

1987 as ornamental aquatic pets (both *P. clarkii* and *C. quadricarinatus*) and for astaciculture or alternative food production (*C. quadricarinatus* only) (Chaichana and Wanjit 2018).

In a study on temnocephalan ectosymbionts, Damborenea and Cannon (2001) reported that *Procambarus digueti* Bouvier, 1897 was infected with *Temnosewellia mexicana* Vayssiere, 1898, in Mexico. Recently, the ectoparasite *Temnosewellia minor* Haswell, 1888, was found on alien *P. clarkii* collected from ponds in Central Italy (Mazza et al. 2018). During these infections, white spot syndrome virus (WSSV) is one of the major pathogens in crustacean species including *P. clarkii* (Pace et al. 2016; Jiang et al. 2019). WSSV is a well-known cause of large economic losses of shrimp and crayfish farms (Fan et al. 2016; Xia et al. 2014). Therefore, the aim of our study was to 1: confirm presence of *P. clarkii* in Thailand waters as well as to investigate its ectosymbionts with viral detections collected from the Thai aquatic environment.

Materials and methods

All captured crayfish were obtained from a survey of invasive crustaceans in natural habitats alongside the middle part of the Nan River to the middle part of the Chao Phraya River from January to October 2018. They were captured with crayfish traps (Yahkoub et al. 2019), fish traps, crab traps, and purse nets from rivers, canals, ponds, and reservoirs. Crayfish were maintained in a small aquarium containing fresh water and an air pump, and transferred to a laboratory without feeding. Crayfish that were identified as *P. clarkii* were killed by cervical groove cutting according to Tavakol et al. (2016).

Temnocephalans were gently moved from the external body of *P. clarkii* and identified to the species level following the key to the Craspedellinae species (Cannon and Sewell 1995). Some specimens of *Craspedella pedum* (Cannon and Sewell, 1995) were preserved in hot 4% formaldehyde and re-preserved in 70% ethanol.

Craspedella pedum individuals were observed for general characteristics under a light microscope. To examine the internal morphology, *C. pedum* were fixed in hot 4% formaldehyde solution, moved to AFA solution, stained with carmine acetic acid, dehydrated with a serial grade of ethanol, cleared with xylene, and mounted in Canada balsam. Internal morphology (Faure's preparation for observation of cirrus) was recorded by line drawing and measuring the organs (*n*, number of specimens; mean, $\mu\text{m} \pm$ standard deviation; minimum–maximum (μm)) with a Moticam digital microscope camera (Motic®, Hong Kong) under a microscope. Calculations of prevalence, abundance, and mean density followed those of Margolis et al. (1982).

For SEM, the fixation and dehydration of *C. pedum* followed the protocol reported by Tavakol et al. (2016). Dried specimens were adhered

to a stub using carbon conductive tape and then coated with Pt/Pd using a sputter coater. They were viewed using a SEM-HITACHI SU-8010.

The terminology and measurements for temnocephalans in this study followed the standard terms and methods of Tavakol et al. (2016), Cannon and Sewell (1995), and Volonterio (2009).

For molecular analysis, genomic DNA was extracted from whole bodies of living *C. pedum* or gill tissue of crayfish using a DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Primers paired for cytochrome *c* oxidase subunit I (CO1) by Hoyal Cuthill et al. (2016) and for 18S ribosomal DNA (18S rDNA) by Norén and Jondelius (1999) were used for gene amplification of *C. pedum* with *Taq* DNA polymerase (Takara, Tokyo, Japan). For viral detection, primers were selected to amplify the targets of WSSV (Yang et al. 2006), *Penaeus monodon* Nudivirus (PmNV) (Belcher and Young 1998), and infectious hypodermal hematopoietic necrosis virus (IHHNV) (Yang et al. 2007). The steps followed in PCR were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 40 s, extension at 72 °C for 60 s, and a final extension for 10 min. PCR products were purified by electrophoresis using the QIAquick Gel Extraction Kit (Qiagen). DNA sequencing analysis was performed by the Macrogen DNA Sequencing Service, Korea. The CO1 and 18S rDNA partial sequences were deposited into GenBank with the accession numbers MN073837 and MN073838. The hypothetical proteins of the virus accession number are MN078153 and MN078154. Specimens 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 (voucher numbers ECE150461 and ECE150461). Sample sequences were identified using the Basic Local Alignment Search Tool (BLAST) to determine the local similarity of *co1* and 18S rDNA from the nucleotide databases of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>). Sequences were aligned with the Clustal Omega multiple sequence alignment programme (<http://www.genome.jp/tools/clustalw/>), and the basic neighbor joining and minimum evolution phylogenetic trees were created using MEGA 6.

Results

A total of 23 adult individuals of *Craspedella pedum* were found on the walking legs, cheliped, carapace, and branchial chamber of two *P. clarkii* from three red swamp crayfish (5–9 cm in length) collected from a large pond near the Nan River in Thailand (Figure 1A–B). Prevalence (%), abundance, and mean density were 66.7, 7.7, and 11.5, respectively. Bodies ($n = 5$) from the posterior end to the tip of the tentacle measured 498.2 ± 193.3

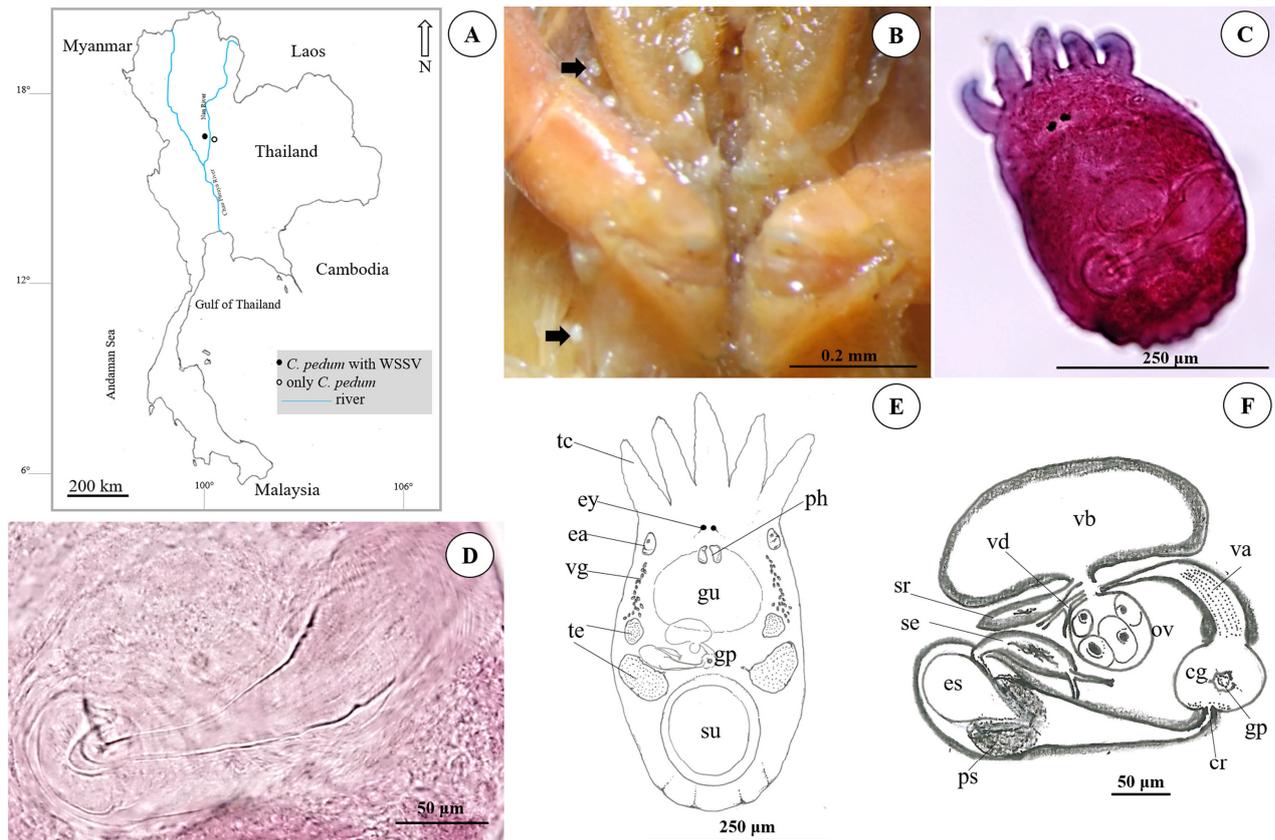


Figure 1. (A) Map of the captured *Procambarus clarkii* and the *Craspedella pedum* found with white spot syndrome virus (WSSV). (B) Living *C. pedum* (arrow) on *P. clarkii*. (C) External and internal morphology and (D) cirrus of *C. pedum*. (E) An illustration of the internal organs and (F) reproductive system. cg, common genital atrium; cr, cirrus; ea, excretory ampallae; es, ejaculatory sac; ey, eye; gp, genital pore; gu, gut; ov, ovary; ph, pharynx; ps, prostate gland; sr, seminal receptacle; se, seminal vesicle; su, adhesive disc; tc, tentacle; te, testis; va, vagina; vb, vesicular resorbiens; vd, vitelline duct; vg, vitelline gland. Photos by Arin Ngamniyom.

(Mean \pm SD) μm , ranging from a minimum length of 263.1 μm to a maximum of 710.3 μm , and widths were $244.5 \pm 89.9 \mu\text{m}$, with a range of 101.9 to 351.8 μm . Adhesive discs ($n = 5$) were $140 \pm 32.3 \mu\text{m}$ (range 96.1 to 174.1 μm) in diameter. Eyes ($n = 10$) were $9.3 \pm 1.1 \mu\text{m}$ (7.5–10.8 μm) in cross-section. We observed a pharynx below the two eyes, and excretory ampullae lateral to the pharynx. The gut was large and located in the middle, anterior to the equator. The female reproductive complex was positioned in the posteromedial part. The gonopore was positioned in the ventroposterior third of the body (Figure 1C–E). Vitelline glands were scattered and surrounded the gut. The vesicular resorbiens ($n = 5$) were $108 \pm 13.2 \mu\text{m}$ (90–121 μm) in length. The ovary cross-sections ($n = 5$) were $35.4 \pm 11.7 \mu\text{m}$ (24–51 μm). We observed one seminal receptacle digitiform. Anterior testes ($n = 10$) and posterior testes ($n = 10$) were $46 \pm 5.1 \mu\text{m}$ (39–54 μm) in length and $35.8 \pm 8.3 \mu\text{m}$ (26–47 μm) in width, and $70.7 \pm 7.2 \mu\text{m}$ (62.7–81.4 μm) in length and $34.9 \pm 5.8 \mu\text{m}$ (28.8–44.9 μm) in width. The cirri ($n = 5$) were $115.5 \pm 43.8 \mu\text{m}$ (66.7–184.3 μm) in length with a funnel-shaped shaft, introvert eversion, and curved shape. The spinal plates were not prominent (Figure 1F).

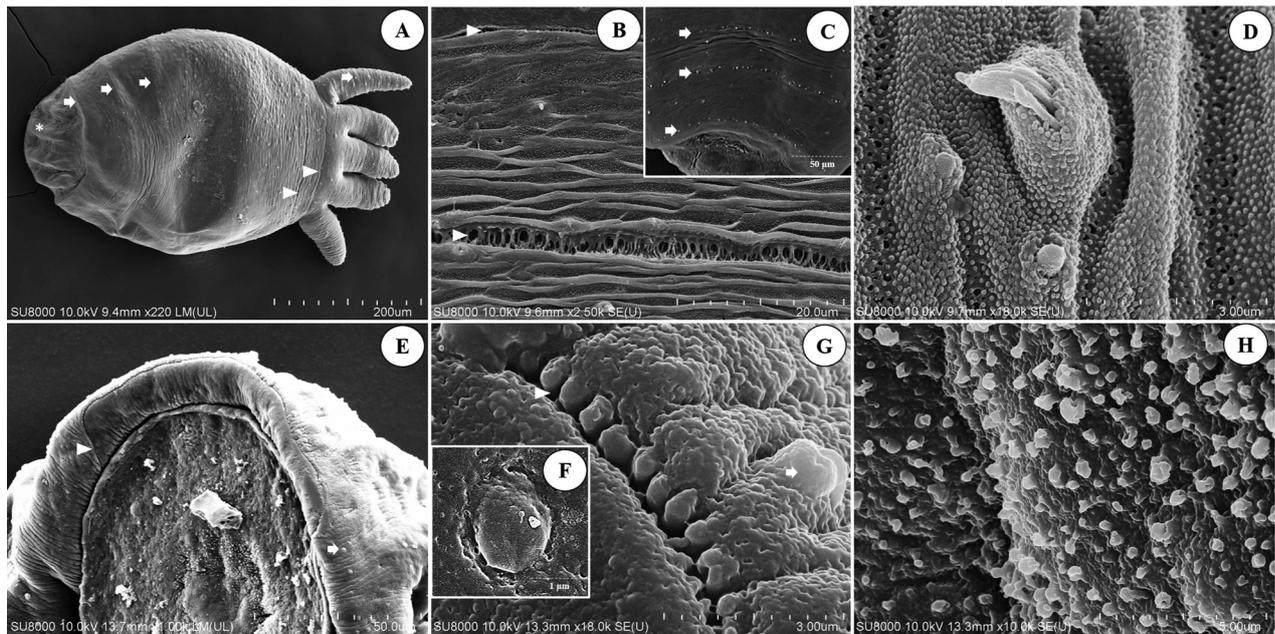


Figure 2. SEM monograph of the *C. pedum* surface. (A) Dorsal view of the body. (B) Radial ridge (asterisk) and magnification of the epidermal grooves (arrowhead) in the anterior body. (C) Magnification of the transverse ridges showing papillae (arrow). (D) Ciliated papillae on the tentacle. (E) Posterior epidermal grooves near the adhesive disc. (F) Magnification of the posterior papillae and (G) epidermal grooves. (H) Papillae on the adhesive disc. Photos by Arin Ngamniyom.

SEM ($n = 8$) observation showed that the dorsal part of *C. pedum* had two transversal lines of epidermal grooves at the tentacle bases. Three transverse ridges had raised papillae and four radial ridges at the dorsoposterior part (Figure 2A, B). The tentacle surfaces had short papillae and ciliated papillae (Figure 2C). At the posterior end of the worms, the epidermal groove was observed at the posterior attachment of the organ (Figure 2D). The papillae near the posterior epidermal groove were slightly flat and plump (Figure 2E–F). The surface of the attachment organ shows crowded papillae in the center as a small rounded projection (Figure 2G–H).

Examining the relationship among temnocephalans based on the CO1 partial sequences, revealed that *C. pedum* was closer to *Temnohaswellia comes* Haswell, 1893 and *T. alpina* Sewell, Cannon and Blair, 2006 than to *T. verruca* Sewell, Cannon and Blair, 2006 and *T. batiola* Sewell, Cannon and Blair, 2006, and clearly separated from *Macrostromum* as an outgroup. In the tree based on 18S rDNA partial sequences, *C. pedum* individuals related to *T. minor* Haswell, 1888 more than *Diceratocephala boschmai* Baer, 1953, *Didymorchis* sp. Haswell, 1900, *Castrella truncata* Abildgaard, 1789, and uncultured Rhynchomesostominae. In the hypothetical proteins, the DNA sequences of hypothetical proteins of WSSV from all specimens of *C. pedum* and gill tissues closely matched to four WSSV infections from nucleotide data sources (Figure 3).

Discussion

Although *Procambarus* and *Cherax* were introduced to Thailand almost 30 years ago, only *C. quadricarinatus* has been found in reservoirs in Sa Kaeo

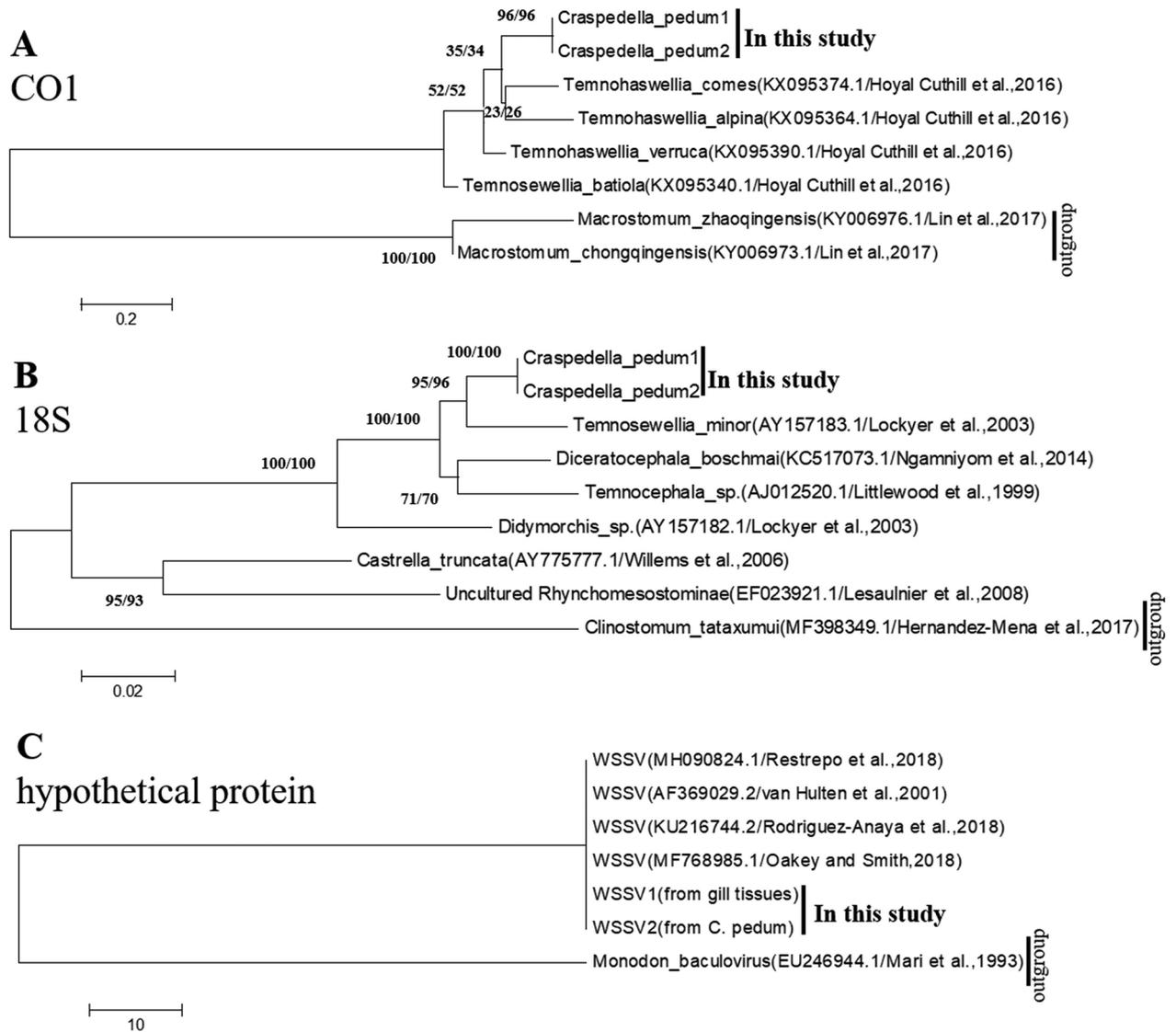


Figure 3. Phylogenetic analysis (the bootstrap value of neighbor joining/minimum evolution tree) based on the partial sequences of (A) CO1, (B) 18S, and (C) hypothetical protein with sequences of other species or other viruses from the nucleotide NCBI databases.

Province in the north-eastern Thailand region (Wanjit and Chaichana 2013) until now. However, several studies have reported the occurrence of invasive *P. clarkii* in natural environments such as in Kenya, Malta, and Brazil (Hofkin et al. 1991; Vella et al. 2017; Loureiro et al. 2018). In this study, three *P. clarkii* were found in natural ponds near the Nan River in North-central Thailand. This finding may be of concern to those monitoring the adaptability of the alien red swamp crayfish to the Thai ecosystem.

Regarding ectosymbionts, many species of temnocephalans have been recognized to infest Australian *Cherax* and *Euastacus* (Cannon and Sewell 1995; Edgerton et al. 2002; Sewell et al. 2006; Longshaw and Stebbing 2016). In Thailand, *Diceratocephala boschmai* were found in *C. quadricarinatus* as a host of this flatworm in crayfish farms (Ngamniyom et al. 2014). For procambarid crayfish, *T. mexicana* and *T. minor* were found to infest *Procambarus digueti* in Mexico and *P. clarkii* in Italy (Damborenea and

Cannon 2001; Mazza et al. 2018). In this study, we increase the available data about alien red swamp crayfish with the ectosymbiont *C. pedum* collected from the aquatic freshwater resources of Thailand.

The morphology and structure of *C. pedum* were described by Cannon and Sewell (1995) and ultrastructural data from Australia crayfish were provided by Tavakol et al. (2016). Our results, where we investigated the general morphology and ultrastructure of *C. pedum* from *P. clarkii*, are consistent with those descriptions. However, the number of temnocephalans in red swamp crayfish specimens of this study was very low compared with the report of Tavakol et al. (2016). In addition, we examined the characteristics of papillae between the anterior and posterior parts.

In the *C. pedum* used in this study, the mitochondria and nucleus genes were similar to those of *Temnohaswellia* and *Temnosewellia* for CO1 and *Temnosewellia*, *Diceratocephala*, and *Didymorchis* for 18S rDNA, as determined by the genetic resources available from GenBank. This confirmed that *C. pedum* is genetically grouped into the Temnocephalida. This finding adds to the available molecular data, increasing our knowledge of temnocephalans.

In the *C. pedum* with viral infection, WSSV was detected in the total genomic materials, which corresponded to the results of viral signals in the gill tissues of *P. clarkii*. Although WSSV results were positive, whether the viral transmission was from a native or non-native organism remains unknown. Yan et al. (2007) demonstrated WSSV transmission from rotifer inoculum to red swamp crayfish. This may be of interest for further study of viral transmission from temnocephalans to crayfish. Whether WSSV transmits from *C. pedum* to *P. clarkii* should be determined.

Du Preez and Smit (2013) and Tavakol et al. (2016) suggested that the invasive temnocephalans in an alien crayfish can be attributed to crayfish that brought temnocephalans to new environments. In this study, the evidence of *C. pedum* in *P. clarkii* was the first record in natural water of Thailand. We not only found alien *P. clarkii* bared ectosymbionts, but WSSV was also discovered in both specimens.

The results of this study may be of interest for those involved in the monitoring and surveillance of red swamp crayfish and their ectosymbionts or viral pathogens in natural environments of Thailand.

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