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Research Article

Morphological and molecular identification of *Diceratocephala boschmai* Baer, 1953 and *Decadidymus* sp. Cannon, 1991 on wild and cultured environment of *Cherax quadricarinatus* in Malaysia

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Abstract

The introduction of Australian *Cherax quadricarinatus* into Malaysia as an aquaculture species has resulted in wild populations in several Malaysian states, and it is now considered an invasive species. The introduction coincidentally co-introduced Diceratocephalid, flatworms that externally inhabit *C. quadricarinatus*. Thirty-three wild *C. quadricarinatus* were caught alive in Tasik Ayer Keroh, Melaka; while 32 cultured *C. quadricarinatus* were bought in Bandar Tenggara, Johor. Two species of ecto-symbiont (*Diceratocephala boschmai* and *Decadidymus* sp.) were morphologically identified and genetically 18S rDNA sequenced. Currently, only one 18S rDNA sequence is available for *Decadidymus* sp. in the GenBank, isolated from redclaw crayfish, *C. quadricarinatus* in Australia. This *D. boschmai* 18S rDNA phylogenetic analysis was consistent with the data from previous studies.

Key words: cultured *Cherax* sp., Diceratocephalidae, morphology, 18S rDNA, wild *Cherax* sp.

Introduction

Cherax quadricarinatus is a highly cultivated native species of freshwater parastacid crayfish in Australia. The species has been exported live both for aquaculture and as an ornamental species to other countries (Douthwaite et al. 2018). This species of crayfish is easily cultured primarily in earth ponds, concrete ponds, and canvas ponds. However, *C. quadricarinatus* is recognised as an invasive species in many countries where it was introduced, including Indonesia (Patoka et al. 2018), Malaysia (Naqiuddin et al. 2016), Martinique (Baudry et al. 2020), Mexico (Vega-Villasante et al. 2015), Brazil (Amato et al. 2005, 2010), Singapore (Ahyong and Yeo 2007), and United States of America (Morningstar et al. 2020). Uncontrolled culture activity, poor hatchery management, and irresponsible releases of this species resulted in the creation of wild populations and their spread through

Figure 1. Sampling location of *C. quadricarinatus* in Tasik Ayer Keroh, Melaka (red label) and Bandar Tenggara, Johor (blue label), Malaysia. The map was created with Google Maps.

the waterways of the countries where they were introduced. In addition, introduced non-native species can carry pathogens that could infect native species (Reyda et al. 2020).

Although, Ihwan et al. (2016) reported the presence of *Temnocephala* sp. on *Johora punicea* in Malaysia, no records of Diceratocephalidae from this country have been present in the literature. Reports of feral *C. quadricarinatus* in Malaysian waters (Naqiuddin et al. 2016) and occurrence of temnocephalid (Ihwan et al. 2016) led us to identify the temnocephalid in Malaysia in both wild and cultured *C. quadricarinatus*.

Materials and methods

Sampling

Wild *C. quadricarinatus* (n = 33) were sampled live with fishnets, or crab pots from Tasik Ayer Keroh, Melaka, and live cultured *C. quadricarinatus* (n = 32) were bought from a local crayfish farm in Bandar Tenggara, Johor, in January 2020 (Figure 1). All *C. quadricarinatus* were maintained and transported to the laboratory live in aquaria. Diceratocephalidae and their eggs were gently separated from *C. quadricarinatus* under stereomicroscope (Nikon, Japan), rinsed in saline solution and preserved in 70 % ethanol for morphological description and molecular analysis.

Identification and specimen staining

Diceratocephalidae were stained in acetic alum carmine (AAC) staining solution, dehydrated in ethanol series, and mounted in Canada Balsam.

Photomicrographs were taken with Advance Research Microscope (Nikon Eclipse 80i, Japan) and drawings with a camera. Images were prepared using Inkscape software. Measurements are in micrometres (µm). For SEM, the fixed Diceratocephalidae were cleaned using an ultrasonic bath, post-fixed in osmium tetroxide, rinsed in sodium cacodylate buffer, dehydrated in ethanol, air-dried with hexamethyldisilazane (HMDS), mounted onto a stub, coated with gold using a JEOL JFC 1600 sputter coater (Jeol, Japan), and examined with Tabletop Electron Microscope (Hitachi TM 1000, Japan) following method of Ngamniyom et al. (2014).

Temnocephalids molecular analysis

Diceratocephalidae were also identified by 18S rDNA sequence analysis. Analysis of *Diceratocephala boschmai* and *Decadidymus* sp. symbionts were performed separately using a method adapted from Ngamniyom et al. (2014, 2019). Total genomic DNA was extracted from the entire sample body with a DNeasy tissue kit (Qiagen, Germany) and eluted in 50 µL AE buffer. Then, the DNA fragments were amplified with 18S rDNA universal primers; UniEP-F (5'-CGAATTCAACCTGGTTGATCCTGCCAGT-3') and UniEP-R (5'- CCGGATCCTGATCCTTCTGCAGGTTCA-3') (Cyronak et al. 2014). For the PCR thermal cycling the protocol followed a denaturation for 2 min in 95 °C, then 35 cycles (45 s in 92 °C, 45 s in 92 °C, 1 min 30 s in 72 °C) and 5 min in 72 °C. The process finished by cooling PCR products in 4 °C. PCR products were verified with 1.5% agarose gel under ultraviolet (UV) light stained with SYBR® safe stain (Thermo Fisher Scientific, Canada). All PCR product purification and forward and reverse Sanger sequences were performed by APICAL Scientific Sequencing Service, Malaysia.

18S rDNA partial sequences were deposited into GenBank accession numbers MZ475304.1, MZ475305.1 and MZ457909. A Basic Local Alignment Search Tool (BLAST) search determined 18S rDNA similarities from the National Centre for Biotechnology Information (NCBI) nucleotide database. Sequences were aligned and corrected using Molecular Evolutionary Genetics Analysis (MEGA X). 18S rDNA pairwise distances were calculated using the Tamura 3-parameter model. A phylogenetic tree was constructed based on Neighbour-Joining (NJ) with 1000 bootstrap replicates. *Mariplanella frisia* (Trigonostomidae) (AJ012514.1) was chosen as an outgroup. Genetic distances were determined using Tamura 3-parameter model matrix.

Results

Diceratocephala boschmai Baer, 1953

Host: Red claw crayfish, *C. quadricarinatus* (von Martens, 1868).

Locality: Wild; Tasik Ayer Keroh, Melaka: 2.2741°N; 102.3021°E and cultured; Bandar Tenggara, Johor: 1.8457°N; 103.6404°E.

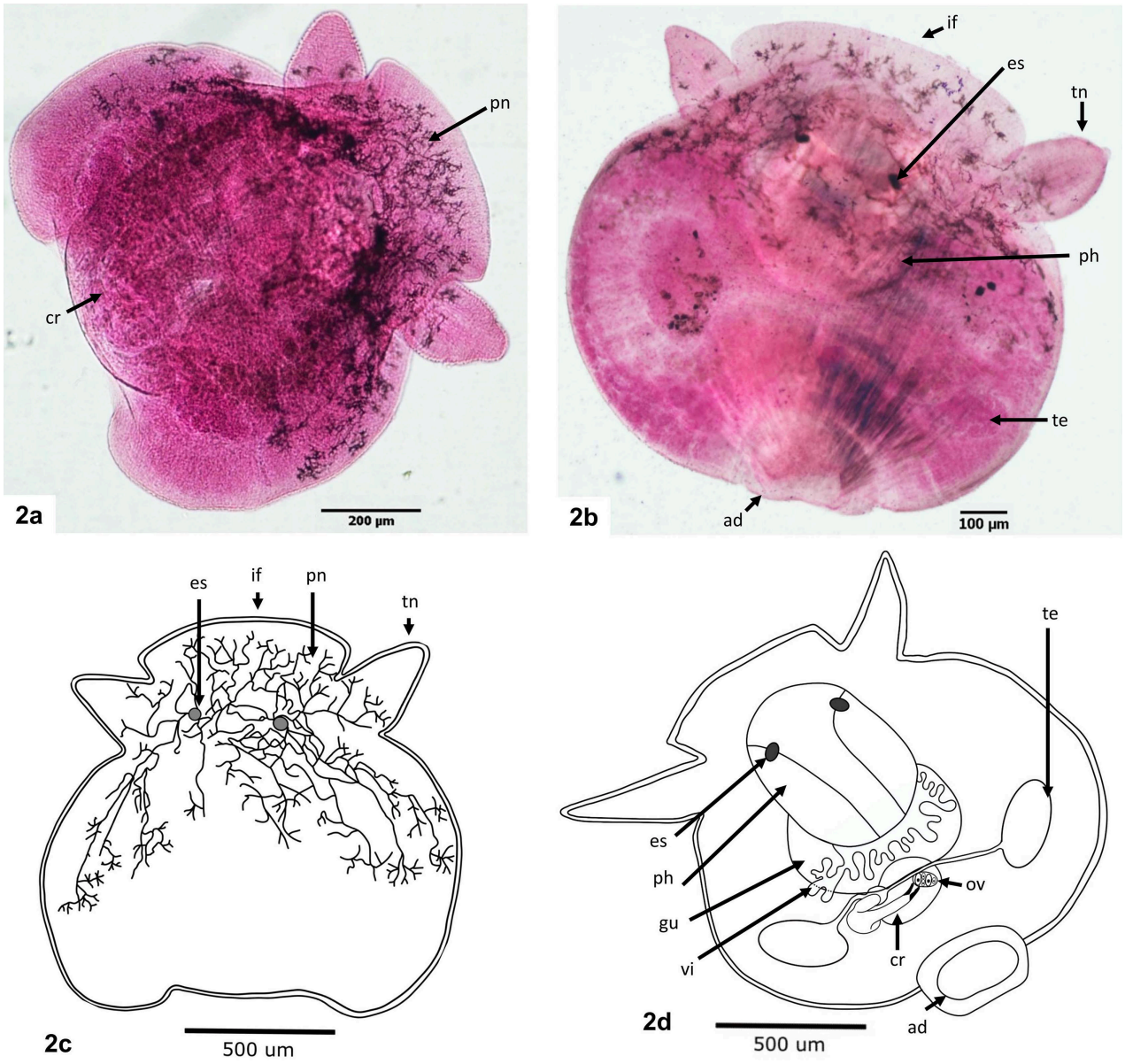

Figure 2. General adult *D. boschmai* morphology. (2a) stained adult *D. boschmai*, dorsal view; (2b) stained adult, ventral view; (2c) diagram of adult *D. boschmai*, subepidermal pigmented network; (2d) diagram of adult *D. boschmai*, internal structure. ad: adhesive disc, cr: cirrus, es: eyespot, gu: gut, if: intertentacular flange, ov: ovary, ph: pharynx, pn: subepidermal pigmented network, te: testes, tn: tentacle. vi: vitellaria. Photomicrographs by Norhan N. Azri-Shah.

Microhabitat: Host antenna, antennule, antennal scale, eye region, carapace, rostrum, mouth region, chelipeds, walking legs, swimming legs, abdomen, telson and uropod.

Life cycle: Adult and egg.

Description: *D. boschmai* body length (without tentacles) was 816.46 ± 185.86 µm long and 818.42 ± 217.06 µm wide. Body was lingulate obovate, with two anterolateral tentacles (211.17 ± 55.02 µm long) separated with a broad intertentacular flange (485.21 ± 114.47 µm in length) (Figures 2, 5a and 5b). Two oval shaped eyespots were 184.68 ± 40.40 µm posteriorly from the intertentacular flange and 162.64 ± 38.75 µm apart from each other. Eyespots were associated with a dorsal subepidermal pigmented network, distributed anteriorly from eyes to the intertentacular flange,

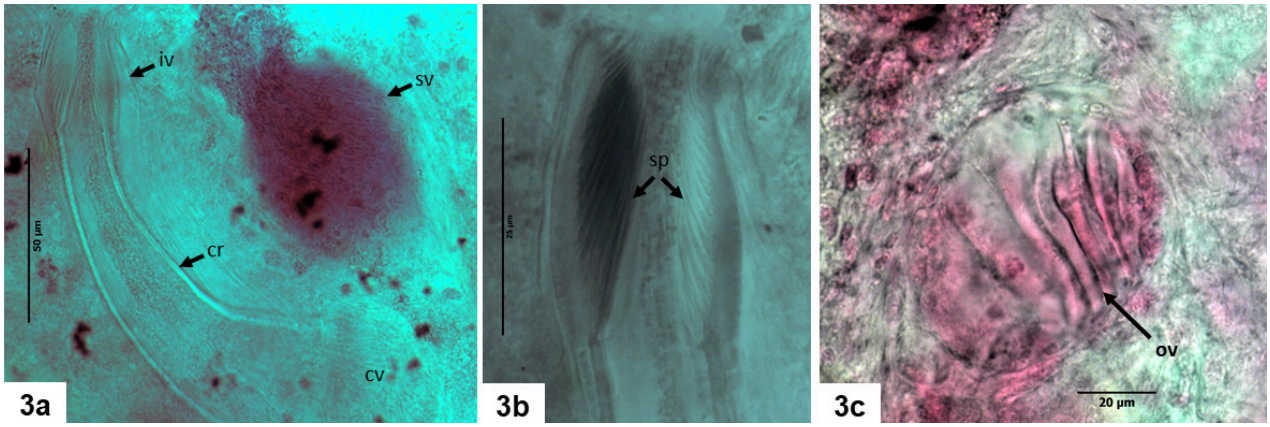

Figure 3. Stained adult *D. boschmai* reproductive. (3a) cirrus. (3b) male introvert with spines (3c) ovary. cr: cirrus, cv: contractile vesicle, iv: introvert, ov: ovary, sp: spines, sv: seminal vesicle, te: testes. Photomicrographs by Norhan N. Azri-Shah.

laterally to tentacles, and posteriorly covering the pharynx and gut region (Figure 2a and 2c). Small pharynx (287.61 ± 98.22 µm long and 296.47 ± 85.49 µm wide) was positioned anteriorly to equator and ventrally to eyes. Posterior muscular adhesive disc 321.09 ± 108.53 µm was present at end of the terminal muscular stalk (Figure 2b and 2d).

Testes (161.79 ± 37.70 µm long and 81.97 ± 20.84 µm wide), kidney-shaped, lobated and posterolateral located to gut (Figure 2b and 2d). Vas deferens extended from testis and connected to pear-shaped seminal vesicle (60 ± 13.38 µm long and 34.91 ± 8.13 µm wide). Seminal vesicle and ejaculatory sac connected to contractile vesicle that delivers to the cirrus. Cirrus compromises a shaft and an introvert. Cirrus medial length 138.86 ± 25.35 µm; cone and curve-shaped shaft with average proximal opening 15.86 ±2.05 µm; introvert with cylindrical-shaped, oblique, curved 19–20 crown of spines at distal part of cirrus (Figure 3a and 3b). Single ovoid-shaped ovary width 49.62 ± 17.10 µm long and 41.62 ± 12.48 µm wide (Figure 3c).

Unhatched eggs with embryos elliptical-shaped, yellowish, attached directly to host body surface via a stalk, particularly abundant near the crevices (Figure 4a, 4b and 4d). A short terminal filament was observed at apical end; stalk at the basal end of the egg capsule. Length for an unhatched egg; with stalk 489.79 ± 58.83 µm; without stalk 420.34 ± 45.06 µm; width 253.06 ± 37.48 µm. A large opercular plate was positioned on egg capsule transverse plane (near anterior end), giving a horizontal fracture to an unhatched egg (Figures 4c, 4e, 5g and 5h). Hatched egg was half-elliptical-shaped, no embryo present and a horizontal fracture. Length for hatched egg; with stalk 387.13 ± 70.50 µm; without stalk 322.93 ± 34.93 µm; was 268.87 ± 13.27 µm. The *D. boschmai* eggs are easily distinguished from *Decadidymus* sp. eggs, which are more elliptical-shaped and are absent from gills and gill covers.

The SEM images have confirmed that *D. boschmai* anterolateral tentacles are separated from the intertentacular flange (Figure 5a and 5b). The anterolateral tentacles and entire *D. boschmai* body were covered with wrinkles (Figure 5f) attributable to contraction and expansion. A semi-closed

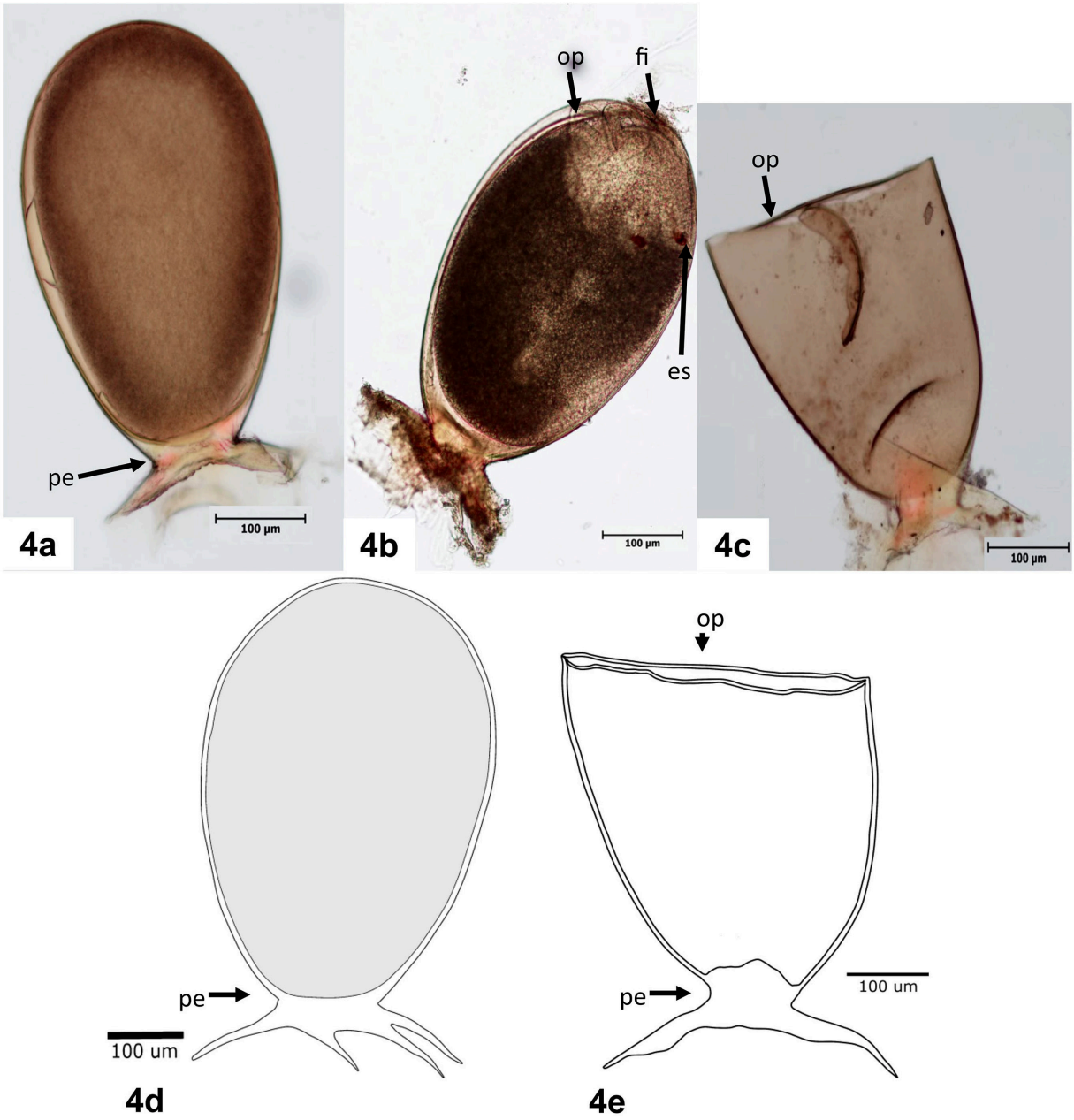

Figure 4. General hatched and unhatched *D. boschmai* egg capsule morphology. (4a–b) unstained unhatched egg capsule; (4c) unstained hatched egg capsule; (4d) diagram of unhatched egg capsule; (4e) diagram of hatched egg capsule. es: eyespot, fi: filament, op: opercular plane, pe: peduncle. Photomicrographs by Norhan N. Azri-Shah.

mouth with two 'lips' (dorsal and ventral) was positioned posterior to the intertentacular flange on the ventral body surface (Figure 5c and 5d). Once opened, the mouth was revealed as a sub-terminal, large muscular sphincter with an opening in the middle and small papillae around the mouth margin with the pharynx structure at the interior of the mouth. The *D. boschmai* posterior revealed a contractible stalk between the flatworm body and a circular-shaped adhesive disc (Figure 5e).

Molecular analysis: The *D. boschmai* DNA sequences were isolated and amplified from two localities: Melaka isolate (1812 base pair; MZ475304.1) and Johor isolation (1572 base pair; MZ475305.1). These DNA sequences were compared with a Thailand sequence, *D. boschmai* (KC517073.1).

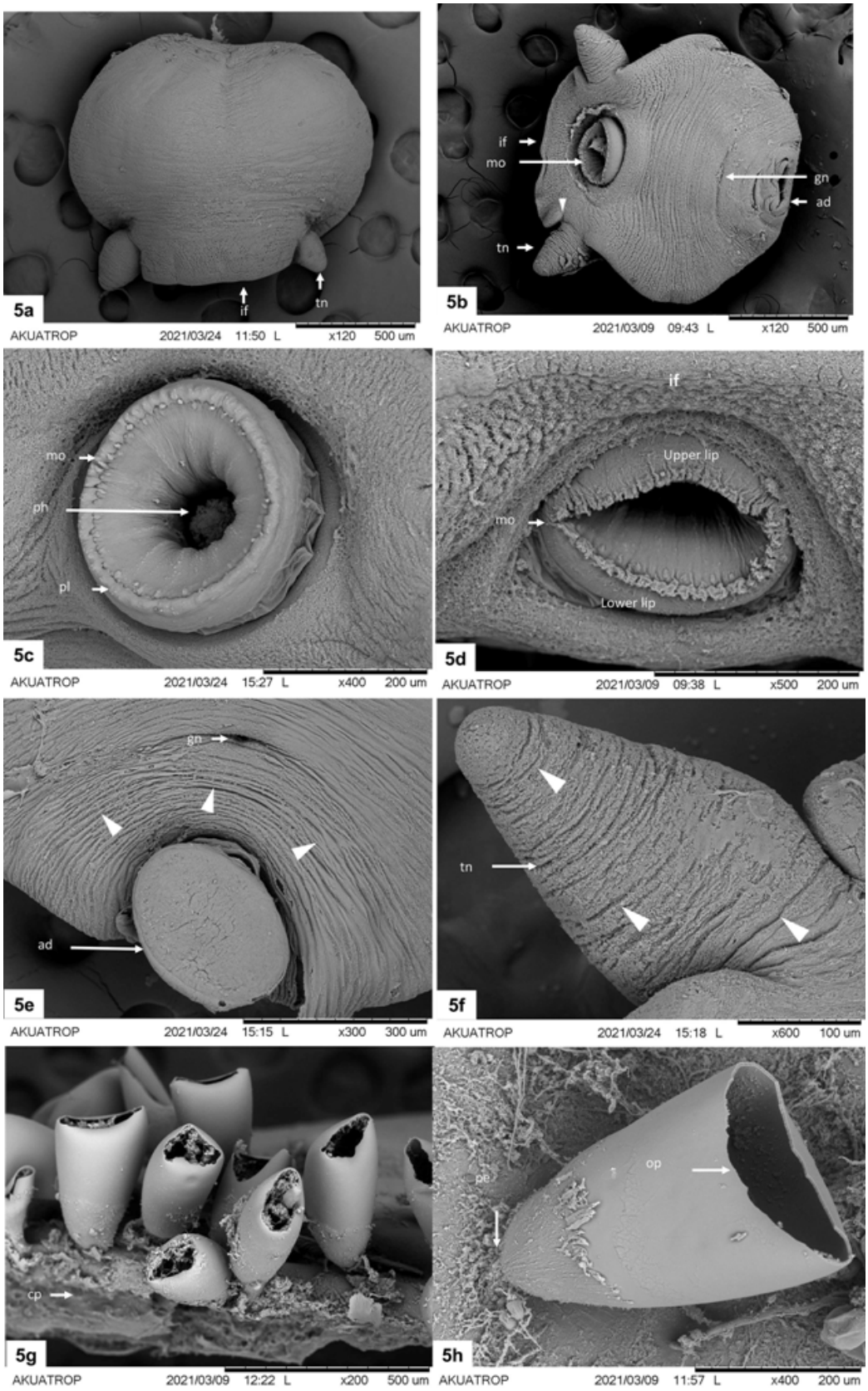

Figure 5. Scanning electron micrograph of adult *D. boschmai*. (5a) dorsal view (5b) ventral view, arrowhead showing the separation between tentacle and intertentacular flange; (5c) detail of mouth with small papillae near the margin; (5d) anterior view of mouth showing upper and lower lip; (5e) ventral view of adhesive disc at the posterior end, arrowhead showing wrinkles on ventral surface; (5f) detail of tentacle, arrowhead showing wrinkles on tentacle; (5g) egg capsules attached on carapace; (5h) surface of hatched egg capsule. ad: adhesive disc, cp: carapace, gn: gonophore, if: intertentacular flange, mo: mouth, op: opercular plane, pe: peduncle, pl: papillae, tn: tentacle. Photomicrographs by Norhan N. Azri-Shah.

The BLAST analysis indicated a 99.65% of molecular similarity (Query cover: 97%) between Melaka sequence and *D. boschmai* (KC517073.1). Meanwhile, 99.22% of molecular similarity (Query cover: 95%) was indicated between Johor isolate and *D. boschmai* (KC517073.1).

***Decadidymus* sp. Cannon, 1991**

Host: Red claw crayfish, *C. quadricarinatus* (von Martens, 1868)

Locality: Wild; Tasik Ayer Keroh, Melaka: 2.2741°N; 102.3021°E and cultured; Bandar Tenggara, Johor: 1.8457°N; 103.6404°E.

Microhabitat: Gill chambers.

Life cycle: Adult and egg.

Description: *Decadidymus* sp. heavy-bodied, lingulate, 1298.94 ± 613.20 µm long, 913.71 ± 254.79 µm wide. Two long, thick anterolateral tentacles measuring 789.18 ± 111.88 µm, with 'false' intertentacular flange (Figure 6a). A pair of eyespots were positioned posteriorly from the "false" intertentacular flange and 214.44 ± 36.95 µm apart (Figure 6e). Eyespots were associated with a dorsal subepidermal pigmented network (Figure 6d) that spread anteriorly from the eyes to the "false" intertentacular flange, laterally to the tentacles, and to posterior of worm body, covering pharynx, gut, and reproductive organs. Large pharynx (442.46 ± 109.73 µm long and 401.62 ± 74.84 µm wide) positioned anteriorly to the equator and ventrally to eyespots. A non-pedunculated muscular adhesive disc 301.20 ± 71.79 µm wide was positioned at worm posterior (Figure 6e).

Ten pairs of compact ovoid-shaped testes (52.16 ± 43.51 µm long 56.70 ± 39.52 µm wide) were serially joined and positioned lateral to gut and posterior to excretory vesicle on both sides of trunk (Figure 6b). Vas deferens extended from each testis to a pear-shaped seminal vesicle and then to a copulatory bulb. Cirrus 174.29 ± 18.23 µm long, compromising a funnel, curved-shaped shaft and a cylindrical, non-spineous, oblique non-curved introvert (Figure 6c). Single ovoid-shaped ovary 110.46 ± 0.00 µm long and 101.55 ± 0.00 µm wide.

Unhatched and hatched eggs were observed attached to the base of host gills and margin of gill covers. Unhatched egg with embryo ovoid, yellowish 484.81 ± 38.27 µm long, 375.29 ± 27.12 µm wide (Figure 7a). Short terminal filament at apical end, large opercular plate positioned on egg capsule transverse plane (near anterior end) and a stalk at basal end of egg capsule (Figures 7b and 8d). Hatched egg an empty capsule with horizontal fracture.

The SEM image of *Decadidymus* sp. showed that the anterolateral tentacles were merged with a "false" intertentacular flange, and its body was covered with wrinkles (Figure 8a and 8b). A semi-closed mouth with two "lips" (dorsal and ventral) was position posterior to the "false" intertentacular flange on the ventral body surface (Figure 8c). An opened mouth was revealed

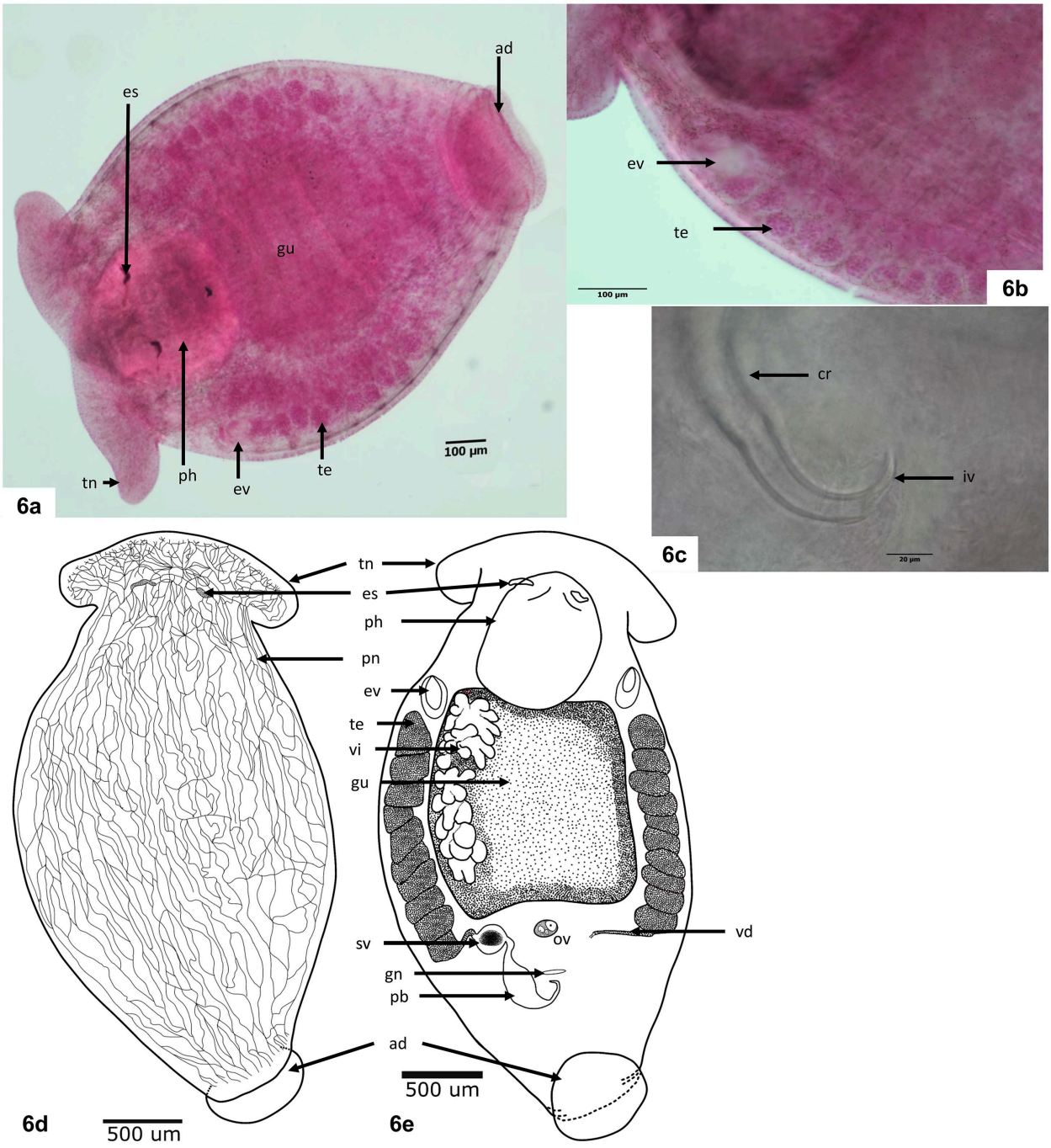

Figure 6. General adult *Decadidymus* sp. morphology. (6a) stained adult, ventral view; (6b) stained adult, magnified view of ten testes (left side); (6c) cirrus; (6d) diagram of adult *Decadidymus* sp., subepidermal pigmented network; (6e) diagram of adult *Decadidymus* sp., internal structure. ad: adhesive disc, cr: cirrus, es: eyespot, ev: excretory vesicle, gn: gonophore, gu: gut, iv: introvert, pb: prostatic bulb, pe: peduncle, ph: pharynx, pn: subepidermal pigmented network, sv: seminal vesicle, te: testes, tn: tentacle. Photomicrographs by Norhan N. Azri-Shah.

as a sub-terminal, large muscular sphincter with an opening in the middle and small papillae around the mouth margin with a pharynx structure at the interior of the mouth.

Molecular analysis: *Decadidymus* sp. DNA was only isolated and amplified from Johor (1902 base pair; MZ457909). The DNA sequence was compared to an Australian sample, *Decadidymus* sp. (MG345101.1). The BLAST analysis

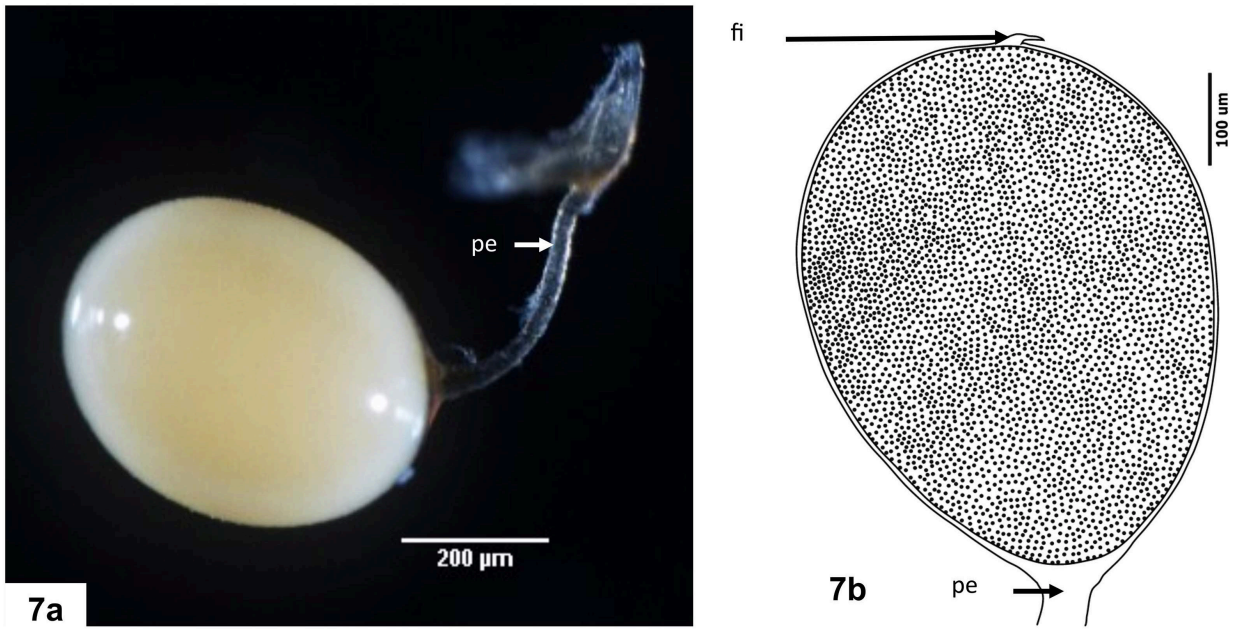

Figure 7. General unhatched *Decadidymus* sp. egg capsule morphology. (7a) unhatched *Decadidymus* sp. egg capsule with attached peduncle under Advance Sterozoom Microscope (7b) diagram of unhatched *Decadidymus* sp. egg capsule. fi: filament, pe: peduncle. Photomicrographs by Norhan N. Azri-Shah.

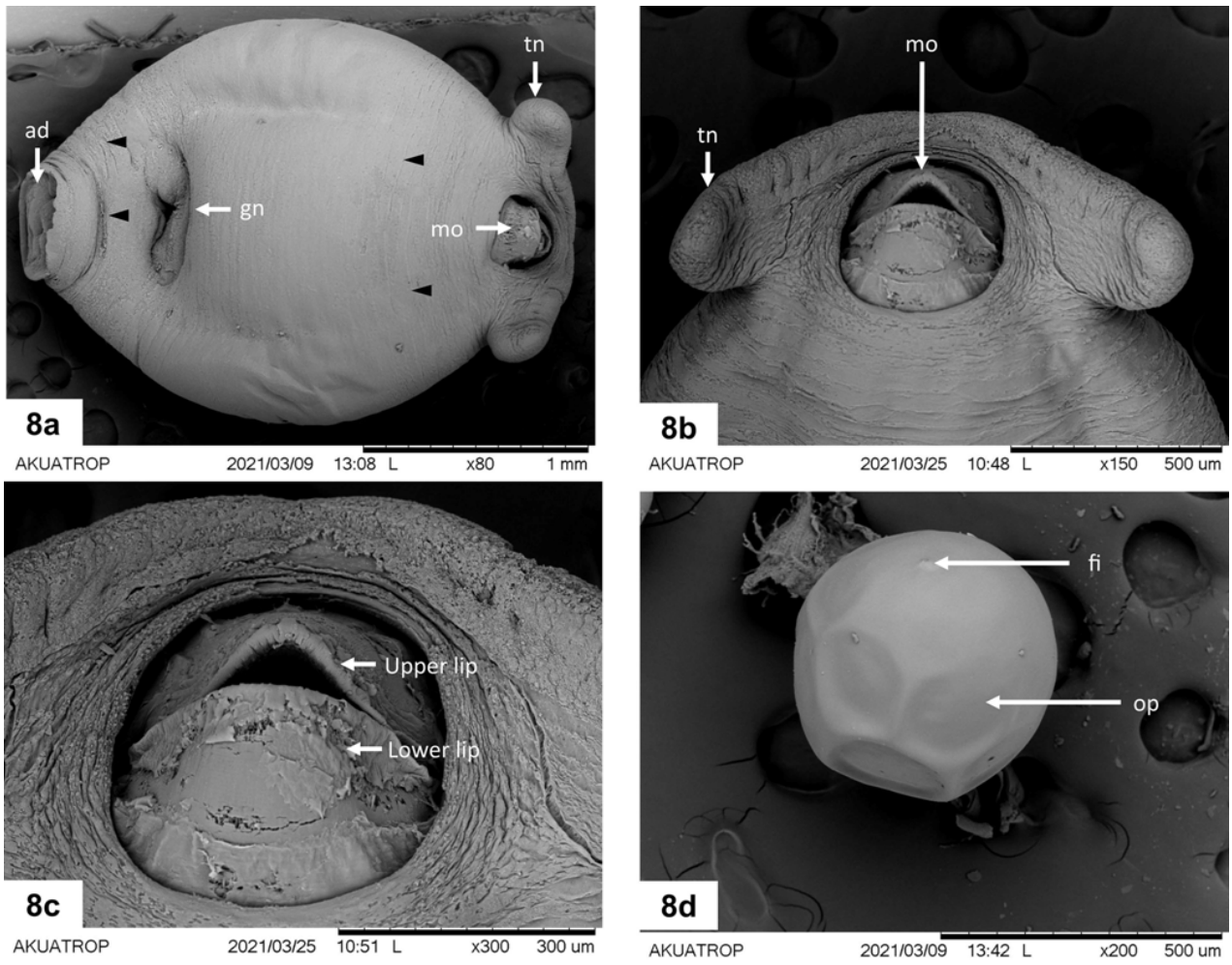

Figure 8. Scanning electron micrograph of adult *Decadidymus* sp. (8a) ventral view, arrowhead showing wrinkles on ventral surface; (8b) detail of anterior end; (8c) detail of mouth region; (8d) unhatched egg capsule, anterior view. ad: adhesive disc, gn: gonophore, fi: filament, mo: mouth, tn: tentacle. Photomicrographs by Norhan N. Azri-Shah.

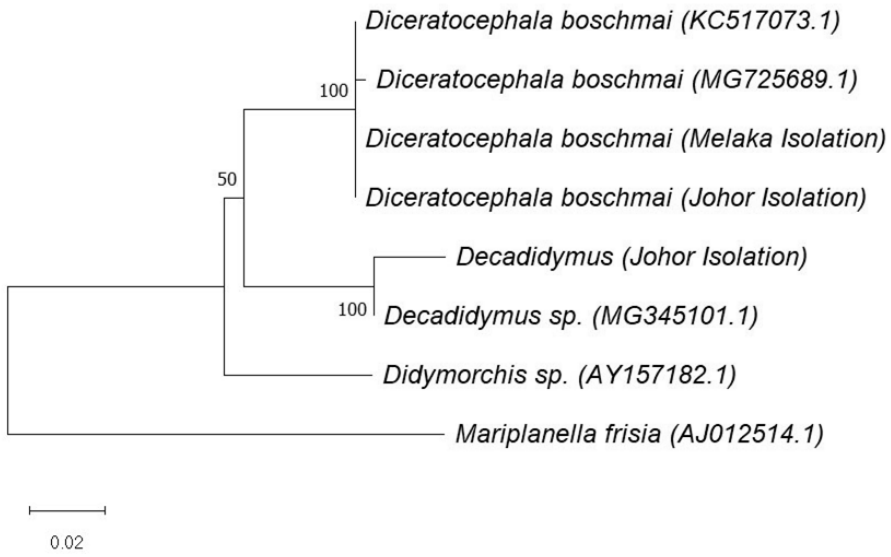

Figure 9. Phylogenetic relationship of *D. boschmai* and *Decadidymus* sp. and other closely related Temnocephalida species identified by BLAST. The tree was inferred in MEGA X using Neighbour-Joining (NJ) analysis of 18S rDNA sequence and Tamura 3-parameter model. GenBank accession numbers are shown before the species name. Numbers shown close to nodes indicate the bootstrap value. *Mariplanella frisia* was used as an outgroup.

indicated a 97.37% molecular similarity (Query cover: 95%) between Johor sequence and *Decadidymus* sp. (MG345101.1).

Phylogenetic analysis: The phylogenetic relationship (Figure 9) exhibited only one available 18S rDNA sequence for the genus *Decadidymus*, Australian sequence, *Decadidymus* sp. (MG345101.1). The genetic distance between *Decadidymus* sp. Johor sequence and MG345101.1 was valued at 0.0190. There were two 18S rDNA sequences for the genus *Diceratocephala*; a Thailand sequence (MG725689) and (KC517073.1). The genetic distance between *D. boschmai* Melaka sequence and MG725689 was valued at 0.0029, and for KC517073.1 was valued at 0.0000. As for *D. boschmai* Johor isolation, the differences to MG725689 were valued at 0.0029 and KC517073.1 was valued at 0.0000. The genetic distance between *D. boschmai* Melaka sequence and the Johor sequence, it was valued at 0.0000. The genetic distance is the difference between two individuals, a higher genetic distance value suggests greater differences between them. The Johor isolate, the Melaka isolate and the Thailand sequence are therefore from the same species. All species were highly distant from *Mariplanella frisia* with genetic distance valued at 0.2000.

Discussion

Temnocephaloidea Baer, 1953 consisted of four families: Actinodactylellidae, Diceratocephalidae, Didymorchiidae, and Temnocephalidae all primarily ectocommensal on freshwater parastacid crayfish (Sewell 2013). Temnocephalids have been reported from other hosts including palaemonid shrimps, reptilians and gastropods (Martín et al. 2005; Seixas et al. 2010; Volonterio 2010; Zivano et al. 2020). Temnocephalids are rhabdocoel turbellarians, hermaphrodite organisms that share similar morphological

characteristics including paired eyespots and tentacles. The family Diceratocephalidae Joffe, Cannon, and Schockaert, 1998 includes two genera: *Diceratocephala* Baer, 1953 and *Decadidymus* Cannon, 1991 (Joffe et al. 1998). The presence of Diceratocephalidae on freshwater crayfish is easily identified as the adult and egg capsule that are visible to the naked eye. According to Tavakol et al. (2016), the Diceratocephalidae were observed externally on *Cherax quadricarinatus* introduced into South Africa. The Diceratocephalids occurred on carapaces and branchial chambers with a high infestation rate and three temnocephalan species, were identified as *Craspedella pedum*, *Diceratocephala boschmai* and *Didymorchis* sp.

Previous studies have shown that *Diceratocephala boschmai* is widely distributed in Australia (Cannon 1991; Jones and Lester 1992), Uruguay (Volonterio 2009), and Thailand (Ngamniyom et al. 2014, 2019). *Diceratocephala boschmai* infestation was documented in the Czech Republic on *Cherax* sp. imported from Indonesia two months after being cultivated in a closed recirculation system there (Ložek et al. 2021). *Decadidymus* sp. is currently only reported to be present in Australian waters (Brand 2017; Cannon 1991).

This work reports that the wild and cultured *C. quadricarinatus* were highly infested with Diceratocephalidae. To the best of our knowledge, this is the first report of Diceratocephalidae on wild and cultured *C. quadricarinatus* in Malaysia. Prior to this study, Thailand was the only country in Southeast Asia that had reported the presence of *D. boschmai* (Ngamniyom et al. 2014, 2019) and there are no Asian records for *Decadidymus* sp. The Diceratocephalidae in this study were morphologically and molecularly consistent with *D. boschmai* (Jones and Lester 1992; Ložek et al. 2021; Ngamniyom et al. 2014, 2019; Tavakol et al. 2016, 2021) and *Decadidymus* sp. (Brand 2017).

The number of tentacles possessed by Diceratocephalidae is a prominent morphological characteristic that distinguishes it from other families of Temnocephaloidea Baer, 1953 (Sewell 2013). In this study, other similar observable morphological characteristics shared by *D. boschmai* and *Decadidymus* sp. included a tongue-like body shape, a pair of anterolateral tentacles, and a pair of eyespots associated with a dorsal subepidermal pigmented network and a posterior muscular adhesive disc. These morphological characteristics are consistent with *D. boschmai* recovered from *C. quadricarinatus* (du Preez and Smit 2013; Jones and Lester 1992; Ngamniyom et al. 2019; Volonterio 2009), *C. destructor* (Ngamniyom et al. 2014) and other *Cherax* sp. (Ložek et al. 2021). As for *Decadidymus* sp. it was reported by Brand (2017) and Cannon (1991).

Three significant morphological characteristics differentiated *D. boschmai* from *Decadidymus* sp. First is the true intertentacular flange that separates the anterolateral tentacles. This was observed only on *D. boschmai*. In contrast to *Decadidymus* sp., a false intertentacular flange was observed

merged with the anterolateral tentacles. The second morphological characteristic is the number of paired testes. *Decadidymus* sp. has ten paired testes, whereas *D. boschmai* has only one pair. The third notable morphological characteristic are the locomotory cilia, used for mobility and only present on *D. boschmai*. These similarities and differences were described in Cannon (1991) and Sewell (2013).

Phylogenetic analysis highlighted that *D. boschmai* and *Decadidymus* sp. were sister taxon and shared a recent common ancestor. This was congruent as both species are in the Diceratocephalidae family. The phylogenetic analysis for *D. boschmai* and *Decadidymus* sp. was analysed based on a synapomorphic trait, the number of tentacles. Both possessed two tentacles and a circular adhesive disc, located posteriorly. The autapomorphic trait that was presented only in *Decadidymus* was ten pairs of testes, whereas *D. boschmai* has only one pair of testes. This autapomorphic trait supported and confirmed our hypotheses based on one of three significant morphological characteristic differences, the number of paired eggs. To the genus *Didymorchis* sp., also has locomotory cilia but its autapomorphic trait is the absence of tentacles. Sewell (2013) noted that *Didymorchis* sp. only used locomotory cilia for movement. Hence, it was taxonomically categorized in Didymorchiidae family. The *D. boschmai* 18S rDNA phylogenetic analysis was consistent with the data in previous studies (Ngamniyom et al. 2014; 2019). Based on the genetic distance value, all *D. boschmai* isolates in Melaka, Johor, and Thailand sequence were categorized as similar and shared a more recent common ancestor in the phylogenetic analysis.

In conclusion, this study has identified the species of ectosymbiont found in the wild and cultured *C. quadricarinatus* in Malaysia, both of which belong to the Diceratocephalidae family. These species were identified morphologically and molecularly as *Diceratocephala boschmai* and *Decadidymus* sp., are both known to infest *Cherax* sp. in Australia within its native range.

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Authors' contribution

Marina Hassan is the leader who initiated the project, monitored the authors during the experiment and data analysis, and was greatly contributed in writing the manuscript. Norhan N. Azri-Shah conducted all the experiments, data analysis and writing the manuscript. Meanwhile, Mohd Ihwan Zakariah, Nor Asma Husna Yusoff, Farizan Abdullah, Wahidah Wahab, Ahmad Najmi Ishak, Norainy Mohd Husin, and John Brian Jones were involved in data analysis and writing the manuscript.

References

Ahyong ST, Yeo DCJ (2007) Feral populations of the Australian Red-Claw crayfish (*Cherax quadricarinatus* von Martens) in water supply catchments of Singapore. *Biological Invasions* 9: 943–946, https://doi.org/10.1007/s10530-007-9094-0

Amato JFR, Amato SB, Seixas SA (2005) Temnocephala Monticelli (Platyhelminthes, Temnocephalida) ectosymbiont on two species of *Latreille* (Crustacea, Decapoda, Trichodactylidae) from southern Brazil. *Revista Brasileira de Zoologia* 22: 1085–1094, https://doi.org/10.1590/S0101-81752005000400038

Amato JFR, Amato SB, Seixas SA, Fonseca M, Ilário RJ (2010) *Temnocephala pignalberiae* Dioni, 1967 (Platyhelminthes, Temnocephalida) from two allopatric populations of *Dilocarcinus pagei* Stimpson, 1861 (Crustacea, Decapoda) - First record for Brazil. *Zootaxa* 2613: 15–28, https://doi.org/10.11646/zootaxa.2613.1.2

Baudry T, Becking T, Goût JP, Arqué A, Gan HM, Austin CM, Delaunay C, Smith-Ravin J, Roques JAC, Grandjean F (2020) Invasion and distribution of the redclaw crayfish, *Cherax quadricarinatus*, in Martinique. *Knowledge and Management of Aquatic Ecosystem* 421: 50, https://doi.org/10.1051/kmae/2020041

Brand J (2017) The association of a novel *Decadidymus* species (Temnocephalida) with Australian redclaw crayfish (*Cherax quadricarinatus*); impacts and management. PhD Thesis, Southampton University, Southampton, United Kingdom

Cannon LRG (1991) Temnocephalan symbionts of the freshwater crayfish *Cherax quadricarinatus* from northern Australia. *Hydrobiologia* 227: 341–347, https://doi.org/10.1007/BF00027620

Cyronak T, O'Reilly E, Lee PA, DiTullio GR (2014) In situ determination of cellular DMSP and pigment quotas in a *Prorocentrum minimum* bloom near the Falkland Islands. *Advances in Oceanography and Limnology* 5: 123–140, https://doi.org/10.1080/19475721.2014.968620

Douthwaite RJ, Jones EW, Tyser AB, Vrdoljak SM (2018) The introduction, spread and ecology of redclaw crayfish *Cherax quadricarinatus* in the Zambezi catchment. *African Journal of Aquatic Science* 43: 353–366, https://doi.org/10.2989/16085914.2018.1517080

du Preez L, Smit N (2013) Double blow: Alien crayfish infected with invasive temnocephalan in South African waters. *African Journal of Aquatic Science* 109: 1–4, https://doi.org/10.1590/sajs.2013/20130109

Ihwan MZ, Ambak MA, Wahidah W, Nurul-Ulfah K, Fakhrulddin IM, Marina H (2016) A new Temnocephalid Trematode report from freshwater crustaceans of the East Coast of Peninsular Malaysia. *Academy of Agriculture Journal* 1: 53–55, https://doi.org/10.15520/aaj.v1i3

Joffe BI, Cannon LRG, Schockaert (1998) On the phylogeny of families and genera within the Temnocephalida. *Hydrobiologia* 383: 263–268, https://doi.org/10.1023/A:1003438321560

Jones TC, Lester RJG (1992) The life history and biology of *Diceratocephala boschmai* (Platyhelminthes; Temnocephalida), an ectosymbiont on the redclaw crayfish *Cherax quadricarinatus*. *Hydrobiologia* 248: 193–199, https://doi.org/10.1007/BF00006147

Ložek F, Patoka J, Bláha M (2021) Another hitchhiker exposed: *Diceratocephala boschmai* (Platyhelminthes: Temnocephalida) found associated with ornamental crayfish *Cherax* spp. *Knowledge and Management of Aquatic Ecosystems* 422: 25, https://doi.org/10.1051/kmae/2021023

Martín PR, Estebenet AL, Burela S (2005) Factors affecting the distribution and abundance of the commensal *Temnocephala* (Platyhelminthes: Temnocephalidae) among the southernmost populations of the apple snail *Pomacea canaliculata* (Mollusca: Ampullariidae). *Hydrobiologia* 545: 45–53, https://doi.org/10.1007/s10750-005-1825-6

Morningstar CR, Daniel WM, Neilson ME, Yazaryan AK (2020) The first occurrence of the Australian redclaw crayfish *Cherax quadricarinatus* (Von martens, 1868) in the contiguous United States. *Bioinvasions Records* 9: 120–126, https://doi.org/10.3391/bir.2020.9.1.16

Naqiuddin AS, Rahim KAA, Long SM, Nicholas FFF (2016) The spread of the Australian redclaw crayfish (*Cherax quadricarinatus* von Martens, 1868) in Malaysia. *Journal of Sustainability Science and Management* 11(2): 31–38

Ngamniyom A, Sriyapai T, Silprasit K (2014) *Diceratocephala boschmai* (Platyhelminthes: Temnocephalida) from crayfish farms in Thailand: Investigation of the topographic surface and analysis of 18S ribosomal DNA sequences. *Turkish Journal of Zoology* 38: 471–478, https://doi.org/10.3906/zoo-1304-49

Ngamniyom A, Sriyapai T, Sriyapai P, Panyarachun B (2019) Contributions to the knowledge of *Pseudolevinseniella* (Trematoda: Digenea) and temnocephalans from alien crayfish in natural freshwaters of Thailand. *Heliyon* 5: e02990, https://doi.org/10.1016/j.heliyon.2019.e02990

Patoka J, Wardiatno Y, Mashar A, Yonvitner D, Wowor R, Jerikho M, Takdir L, Purnamasari M, Petrtýl L, Kalous A, Kouba Bláha M (2018) Redclaw crayfish, *Cherax quadricarinatus* (von Martens, 1868), widespread throughout Indonesia. *Bioinvasions Records* 7: 185–189, https://doi.org/10.3391/bir.2018.7.2.11

Reyda FB, Wells SM, Ermolenko AV, Zetara MS, Lumme JI (2020) Global parasite trafficking: Asian *Gyrodactylus* (Monogenea) arrived to the U.S.A. via invasive fish *Misgurnus*

anguillicaudatus as a threat to amphibians. *Biological Invasions* 22: 391–402, https://doi.org/10.1007/s10530-019-02097-4

Seixas SA, Amato JFR, Amato SB (2010) First report of *Temnocephala haswelli* (Platyhelminthes: Temnocephalida) from *Pomacea canaliculata* (Mollusca: Ampullariidae) outside Uruguay - Description update based on specimens from the state of Rio Grande do Sul, Brazil. *Zoologia* 27: 820–828, https://doi.org/10.1590/S1984-46702010000500019

Sewell KB (2013) Key to the genera and checklist of species of Australian temnocephalans (Temnocephalida). *Museum Victoria Science Reports* 17: 1–13, https://doi.org/10.24199/j.mvsr.2013.17

Tavakol S, Luus-Powell WJ, Smit WJ, Baker CB, Hoffman A, Halajian A (2016) First introduction of two Australian Temnocephalan species into Africa with an alien host: double trouble. *Journal of Parasitology* 102: 653–658, https://doi.org/10.1645/15-936

Tavakol S, Blair D, Morgan JAT, Halajian A, Luus-Powell WJ (2021) Molecular characterization of two Australian temnocephalans (Temnocephalida, Platyhelminthes) introduced with alien crayfish (Parastacidae, Decapoda) into South Africa. *Aquaculture Research* 10: 1–6, https://doi.org/10.1111/are.15294

Vega-Villasante F, Ávalos-Aguilar JJ, Vargas-Ceballos MA, Chong-Carrillo O, Ruiz-Núñez MF, Nolasco-Soria H, Bortolini-Rosales JL, Morales-Hernández JC (2015) Wild populations of the invasive Australian red claw crayfish *Cherax quadricarinatus* (Crustacea, Decapoda) near the northern coast of Jalisco, Mexico: A new fishing and profitable resource. *Latin American Journal of Aquatic Research* 43: 781–785, https://doi.org/10.3856/vol43-issue4-fulltext-17

Volonterio O (2009) First report of the introduction of an Australian Temnocephalidan into the new world. *Journal of Parasitology* 95: 120–123, https://doi.org/10.1645/GE-1726.1

Volonterio O (2010) Two new species of *Temnocephala* (Platyhelminthes, Temnocephalida) from the South American snake-necked turtle *Hydromedusa* (Testudines, Chelidae). *Zoological Science* 27: 965–970, https://doi.org/10.2108/zsj.27.965

Zivano A, Brusa F, Damborenea C (2020) Integrated morphological and ecological traits to identify *Temnocephala digitata* Monticelli, 1902 (Platyhelminthes, Temnocephalida) and neotype designation. *Zoological Anzeiger* 287: 31–44, https://doi.org/10.1016/j.jcz.2020.04.005