RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2024.054

ScienceAsia 50 (2): 2024: ID 2024054: **1–9**

Male reproductive system and sperm ultrastructure of sesarmid crab *Episesarma singaporense* (Tweedie, 1936)

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Received 22 Aug 2023, Accepted 23 Apr 2024
Available online 25 May 2024

ABSTRACT: The popularity of sesarmid crab *Episesarma singaporense* (Tweedie, 1936) in Thai and Southeast Asian cuisine has put the species under pressure. However, sesarmids are important for nutrient cycling within mangrove ecosystems, and losses of the species due to overfishing could have a wide impact. Although the crab reproductive system has been well reported, our study aimed to describe the male reproductive system of *E. singaporense* in detail, using light and electron microscopy. We elucidated histological characters of the male reproductive tract, including the anterior, median and posterior vas deferens, showing the presence of droplet secretion and spermatophores, an accessory gland with basophilic secretion, and testes composed of active spermatogenesis and spermiogenesis. Five main phases of spermatogenesis were identified: spermatogonia (Sg), primary spermatocytes (PSc), secondary spermatocytes (SSc), spermatids (Sp), and spermatozoa (Sz). The Sg, the PSc, and the SSc stages could be further divided into substages of two Sg, six meiotic PSc, and two meiotic SSc substages, respectively. Since spermatozoa of sesarmid crabs are aflagellate, the substages of spermatid-spermatozoa cell differentiation could not be visualized by light microscope. Therefore, electron microscope was used to provide more detail of the successive changes in nuclear architecture, acrosome patterns, and perforatorium. It was also firstly noted that the anterior vas deferens (AVD) of *E. singaporense* comprised two parts: the proximal portion (AVDp) and the distal portion (AVDd). The study results increased our understanding of the effects of different seasonally changed nutrients on the reproductive development of *E. singaporense*.

KEYWORDS: mangrove, reproductive histology, *E. singaporense*, spermatogenesis, Thailand

INTRODUCTION

Mass production of crab seeds is one of the strategies used in the conservation and sustainable utilization of crab species. A detailed comprehension of crab's reproductive process, sexual maturation and life cycle is fundamental for hatchery and aquaculture purposes [1]. In addition, studies of the animal's reproductive system and sperm formation can provide an insight into reproductive maturation and fertilization processes [2, 3], promoting species propagation and helping population monitoring in natural environments [3, 4]. Several studies of the reproductive systems of brachyuran crabs have been carried out. The studied species included *Portunus pelagicus* [1], *Stenorhynchus seticornis* [4], *Callinectes sapidus* [5], and *Chionoecetes opilio* [6, 7].

The sesarmid crab *Episesarma singaporense* (Tweedie, 1936), or Singapore vinegar crab, is a member of the Family Sesarmidae [8]. It is one of the most common crabs in the Peninsular Malaysia, the Gulf of Thailand and the Andaman Sea [8, 9]; and it plays important roles in the intertidal zones and the mangrove ecosystems, particularly on the cycling of nutrients [10]. The *E. singaporense* is an economically important fishery product, and it is generally preserved with salt or fish sauce and then used as an ingredient of various dishes in South China and Southeast Asia [11, 12]. The huge consumer demand for the crab has led to overfishing and depletion of its populations in Thai mangrove forests [13]. The species is also faced with population depletion due to the increased destruction of mangrove in Southeast Asia [14]. So far, there have been no commercial hatcheries for the species; therefore, it is essential that natural populations should be restored, and the commercial aquaculture should be promoted. Given the increasing demand for *E. singaporense* and its potential as a candidate species for commercial culture, we

presented here a detailed study of the morphology and the histology of the male reproductive system throughout the sperm ultrastructure of *E. singaporense*.

MATERIALS AND METHODS

Sample collection

Twenty specimens of mature male *E. singaporense* (mean carapace width = 3.20 ± 2.02 cm and mean total weight = 24.15 ± 3.90 g) were collected from an estuarine area in Palian District, Trang Province, Thailand (7°08′58.12″ N, 99°40′10.11″ E) during October 2019 and January–February 2020. The collection site is surrounded by a large area of mangrove forests of around 540 km^2, and it is considered the most significant natural habitat for sesarmid crabs in Thailand. The collected specimens were identified following the taxonomic guidelines of Lee et al [8]. During specimen collection, the water temperature was around 25 °C to 28 °C, and the water salinity was 20–45 ppt. Specimens were packed in air-filled plastic bags and transported alive to the Marine Science Laboratory at the Department of Marine Science, Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya, Trang Campus, Thailand.

Morphological and histological observations

Adult male crabs with no signs of abnormalities or infestation were anesthetized by chilling [15]. The dorsal carapace was removed to gain access to the reproductive organs (testes and vas deferens) for morphological observation under a Canon EOS 200D Mark II (Canon Inc., Tokyo, Japan). Organs were removed and fixed in Bouin's solution for 24 h and then transferred to 70% ethanol (EtOH) (Northwest Scientific Inc., Billings, USA). Samples were dehydrated through a series of graduated alcohol solutions, cleared in xylene, and embedded in paraffin (Leica Biosystems, Deer Park, USA) [16, 17]. Paraffin blocks were sectioned at 4 µm thickness with a rotary microtome. The tissue ribbons were mounted on glass slides and stained with Harris's hematoxylin and eosin (H&E), Masson's trichrome (MT), and Periodic acid-Schiff reaction (PAS). The reproductive system structure was examined and photographed under a light microscope equipped with a 3DHISTECH Panoramic Viewer (3DHISTECH Ltd., Budapest, Hungary).

Ultrastructure study of spermatozoa

A small piece of the vas deferens was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4 °C. The sample was subsequently post-fixed in 1% osmium tetroxide (OsO_4) (Sigma Company, St. Louis, USA) for 2 h, dehydrated through a graded series of acetone, and embedded in Araldite® (Sigma Company). Semithin sections were stained with toluidine blue and examined under a light microscope. Ultrathin sections of 80 nm in thickness were doubled-stained with uranyl acetate and lead citrate [18]. The specimens were analyzed with electron microscopy (Philips/FEI Tecnai G2 F20, FEI Co., Eindhoven, Netherlands). General descriptions of spermatozoa were made from the innermost to the outermost regions of the cell. Detailed illustrations were produced using the Adobe® Illustrator® CS5 (Adobe Inc., San Jose, USA).

RESULTS

Morphology of male reproductive system

E. singaporense is distinguished by the presence of entirely red chela and reticulated pterygostomial regions. Morphologically, the structure of the reproductive system of the male *E. singaporense* resembled the letter H (Fig. 1A–C), in which the upper region was the anterior lobe composed of a pair of tubular testes; and the lower region was the posterior lobe composed of male reproductive, or testicular, ducts. The tubular testes were bridged by a transversal commissure. The testicular ducts were coiled tubules with different degrees of convolution; and, based on morphology and histology, could be divided into two major regions: the vas deferens and the accessory gland. The vas deferens comprised three distinct parts: the anterior vas deferens (AVD), the median vas deferens (MVD), and the posterior vas deferens (PVD) (Fig. 1A–C). The histological observations of each reproductive region were described as follows.

Histology of male reproductive system

Stages of spermatogenesis

Based on light microscopy, germ cells within the seminiferous tubules were categorized into five histologically distinct developmental types according to cytological features that included nucleus morphology and nuclear/cytoplasm staining. The five types of germ cells were spermatogonia (Sg), primary spermatocytes (PSc), secondary spermatocytes (SSc), spermatids (St), and spermatozoa (Sz) (Fig. 2A).

Sg could be separated into two types, Type A Sg (SgA) and Type B Sg (SgB), based on the pattern of heterochromatin/nuclei inside the nucleoplasm. As shown in Fig. 2B, the SgA was single cell located at the basement membrane. The SgA had a very large nucleus compared with the cell size and thin cytoplasm, the nucleus contained three nucleoli and some heterochromatin at the nuclear periphery. The similarity of SgA to common embryonic stem cells with big nuclei containing several nucleoli suggested an active state of cell proliferation. Along the basement membrane in close proximity to the SgA cells, there was SgB consisting of two cells. The SgB cells displayed a thin cytoplasm and distinct nuclear characters, including less-stained nucleoplasm, indicating the presence of euchromatin, heterochromatin with deep nuclear staining around the nuclear periphery, and an

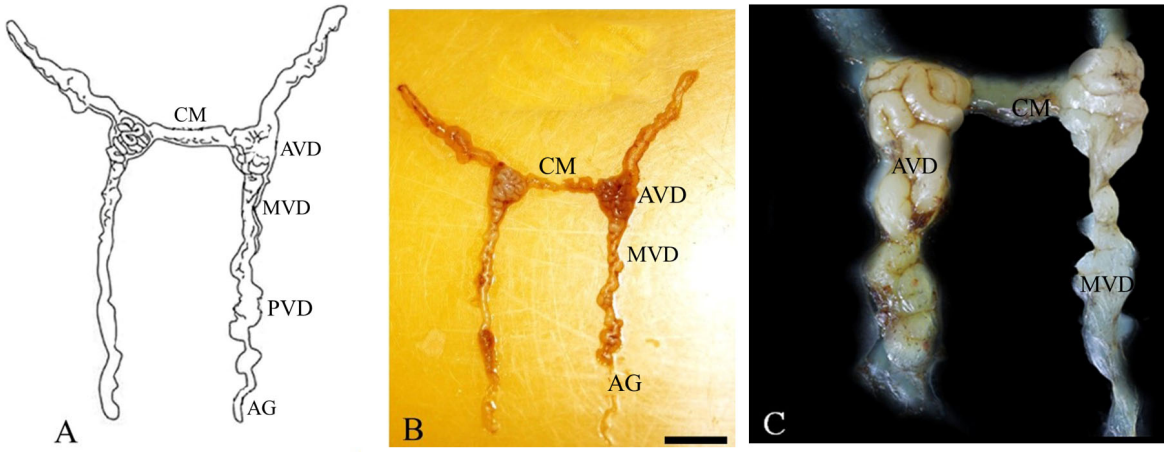

Fig. 1 Morphological figures and photographs of male reproductive system of *E. singaporense*. (A–B), Male reproductive system, showing a pair of tubular type-testes and four distinct regions of the reproductive tract: the anterior vas deferens (AVD), median vas deferens (MVD), posterior vas deferens (PVD), and accessory gland (AG) with the left and the right sides of the testis and reproductive tract connected by a transversal commissure (CM); (C), high magnification photograph showing the coiled tubules of AVD. Scale bar = 1 cm.

absence of nucleoli (Fig. 2B). The presence of more euchromatin in SgB implied active transcription, probably important in the later stages of spermatogenesis. Each SgB cell also had a smaller companion nurse cell, which had a nucleus full of mostly heterochromatin.

PSc was spherical cell with a large round nucleus occupying the majority of the cell. The nucleus contained condensed heterochromatin with stage-specific characteristics undergoing meiosis I (Fig. 2D–G). The PSc could be further categorized under the light microscope into six distinct sub-stages, PSc1–6, based on chromatin patterns and the presence of a nuclear envelope versus cytoplasmic staining of no stain or pink stain (Fig. 2D–G). The PSc1 exhibited clear cytoplasm with a central round nucleus full of thin and long fiber-like chromatins (Fig. 2D), indicating the leptotene stage of meiosis I. The PSc2 and the PSc3 had pink stained cytoplasm and displayed clear changes to chromatin architecture within the nucleus (Fig. 2F,G). PSc2 and PSc3 showed different patterns of condensed chromosomes. While PSc2 presented aligned chromosome pairs, indicating the zygotene-pachytene stage of prophase I (Fig. 2E,F), PSc3 showed denser chromosomes that were starting to separate and cross over, indicating the diplotene (Fig. 2G) and the diakinesis (Fig. 2H) stages of prophase I. PSc4 and PSc5 both had no nuclear envelope. PSc4s showed non-aligned condensed chromosomes scattered around the center of the cells, while PSc5 showed aligned chromosomes at the center of the metaphase plate and some cells that were starting to show chromosome separation (Fig. 2I). Therefore, PSc4 and PSc5 could be placed in pro-metaphase I and metaphase I-anaphase I stages, respectively. PSc6 showed decondensed chromosomes and nuclear envelope reformation. PSc6 nuclei were smaller than the nuclei of PSc1–3 (Fig. 2J). Notably, PSc4–PSc6 also showed pink cytoplasmic staining. PSc6 was the last stage of the PSc series and could be placed in telophase I, close to the secondary spermatocyte stage.

SSc was a spherical cell relatively smaller than PSc. Early SSc showed a nucleus surrounded with scattered heterochromatin, and the nucleus of late SSc was completely filled with deeply stained heterochromatin (Fig. 2J). Located close to the early-stage SSc were cells with a smaller, faintly stained nucleus, which could indicate the beginning of division into early St (Fig. 2K).

St was a tiny spherical cell located near the lumen of seminiferous tubules. The St nucleus was homogenous due to the complete de-condensation and uniform dispersion of chromatin filaments (Fig. 2K).

Sz was aflagellate with clear nuclear staining, and acrosomes was surrounded with light yellow stain. The division of acrosomes into inner and outer zones as described [20], could be seen as dark orange/brown and light purple regions, respectively. Within the testis, the levels of nucleus enclosing the central acrosome were diverse, showing different sub-stages of sperm differentiation (Fig. 2L).

Anterior vas deferens

This anterior region of the reproductive duct was divided into two parts: the proximal anterior vas deferens (AVDp) and the distal anterior vas deferens (AVDd) (Fig. 3A). The wall of the AVDp consisted of simple squamous epithelium and a thin layer of muscle. Free spermatozoa were found within the AVDp (Fig. 3B).

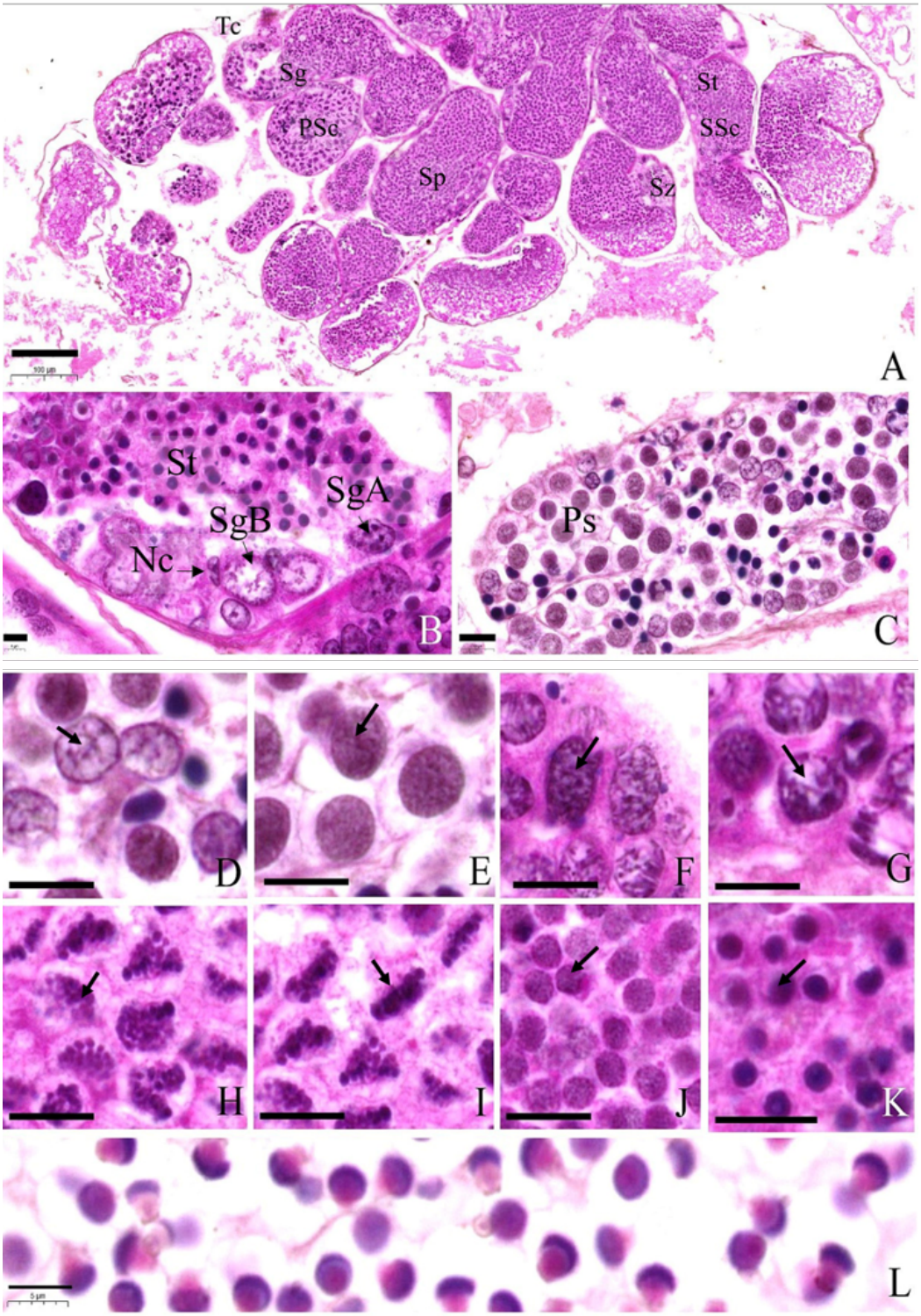

Fig. 2 Histology of male reproductive system of *E. singaporense*. (A), The testis surrounded with the testicular capsule (Tc) with seminiferous tubule (Sp) and the differentiated stages of sperm cells, including spermatogonia (Sg), primary spermatocyte (PSc), secondary spermatocyte (SSc), spermatid (St), and spermatozoa (Sz); (B), high magnification images showing the detailed structure of spermatogonium A (SgA) and spermatogonium B (SgB); (C), primary spermatocytes (PSc) comprising; (D), Ps leptotene [arrow]; (E), Ps diplotene [arrow]; (F), Ps pachytene [arrow]; (G), Ps zygotene [arrow]; (H), Ps diakinesis [arrow]; (I), Ps metaphase [arrow] substages; (J), secondary spermatocyte [arrow]; (K), spermatid [arrow]; and (L) spermatozoa. Abbreviation: Nc = nurse cell, arrows D–K = nuclear membrane.

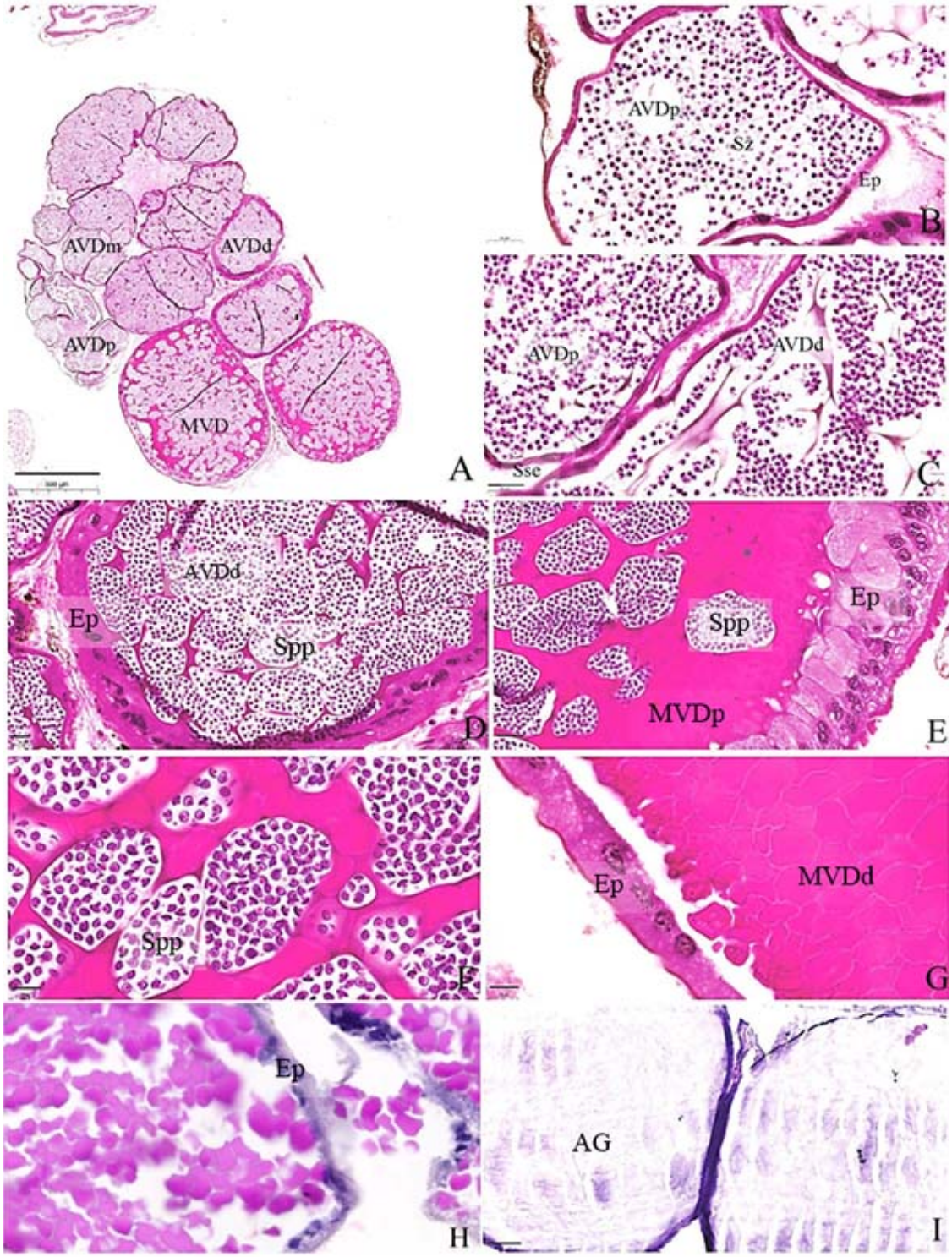

Fig. 3 Histology of male reproductive tract of *E. singaporense*, stained with the H and E method. (A), Overall male reproductive system; (B), proximal region of anterior vas deferens (AVDp); (C), middle region of anterior vas deferens (AVDm); (D), distal region of anterior vas deferens (AVDd); (E–F), proximal region of median vas deferens (MVDp) carrying several spermatophores (Spp) and pinkish-stained droplets of secretion; (G), distal region of median vas deferens (MVDd), showing a large amount of secretion; (H), posterior vas deferens (PVD) containing densely packed secreting granules (asterisk) and droplets of secretion (AS); and (I), PVD connected to the accessory gland (AG) containing basophilic secretion within the lumen. Abbreviation: Ep = epithelium.

The squamous epithelium of the AVDd was covered by a thin muscular layer. The distinguishing feature of this part was the presence of well-developed spherical spermatophores, with a few acidophilic secretions (Fig. 3C).

Median vas deferens

Distinguishable characters of the MVD were enlarged tubules, whitish in color and less coiled than tubules of the AVD. Wall structures of the MVD and the AVDd were similar (Fig. 3D). Another distinguishing characteristic of the MVD was the presence of numerous spermatophores and pinkish-stained drops of secretion around the spermatophores (Fig. 3E–G).

Posterior vas deferens

The PVD comprised the long duct of the vas deferens. The distinctive character of this part was a simple cubic epithelium lining and irregular nuclei. A few spermatophores were observed in the lumen along with weakly pinkish-stained droplets of secretion (Fig. 3H).

Accessory glands

The accessory glands were blind tubes located at the end of each side of the male reproductive tract. Pseudostratified epithelium lay within the wall of the accessory gland and a purple stained basophilic secretion were found within the lumen (Fig. 3I).

Spermatid differentiation and sperm ultrastructure

The spermiogenesis of *E. singaporense* exhibited a series of changes in St cell morphology. In particular, the shape of the nucleus and the emergence of acrosomes were notable. The changes enabled the categorization of three phases of cell differentiation, including early, middle and late St stages. Based on TEM observations, the acrosome vesicle morphology changed to the perforatorium development. At first, the perforatorium was observed on one side of the acrosomal vesicle as an invagination into the vesicle. As the immature spermatozoa developed, the invagination pointed inward to form a cylindrical shape. At this point, the acrosomal vesicles became cup-like. The morphology of the nucleus changed in the following ways. In the early stage, the nucleus enclosed the entire acrosomal vesicle; and once the perforatorium developed and the cup-like acrosome formed, the nucleus became C-shaped. From these changes in the nucleus and the acrosome, the formation of spermatozoa could be divided into three phases. Early spermatozoa (Sz1) contained the initial perforatorium with loose fibrous materials oriented inward toward acrosomal vesicles (Fig. 4A,B). Intermediate spermatozoa (Sz2) showed a more advanced perforatorium forming cylindrical shapes, and at this stage, the acrosome exhibited a division of dense and loose materials (Fig. 4C,D). At the late stage, the mature sperms formed as spherical aflagellate cells. The fully developed perforatorium was a cylindrical acrosomal core invaginating into the acrosomal vesicle. The nucleus of the mature sperm was C-shaped, while the cytoplasm was very thin (Fig. 4E,F and Figs. 5 and 6).

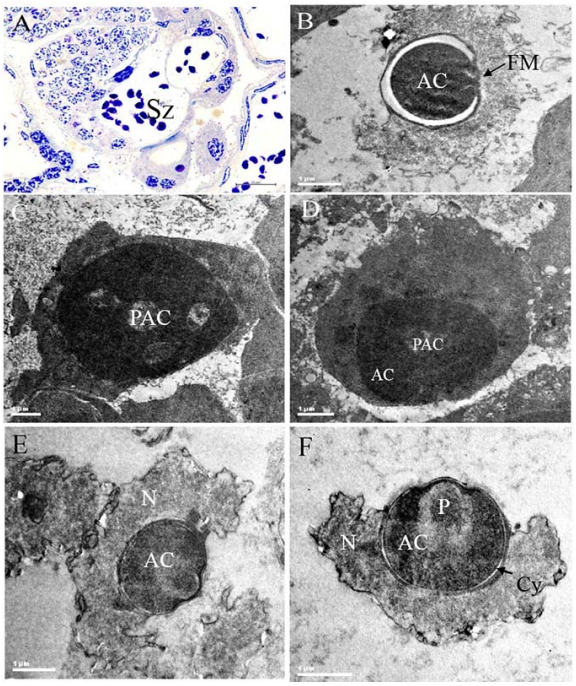

Fig. 4 Light microscopy and ultrastructure of spermatozoa of *E. singaporense*. (A), Spermatozoa (Sz); (B–D), early spermatozoa; and (E–F), intermediate spermatozoa. Abbreviations: AC = acrosomal cap, N = nucleus, PAC = the perforatorium chamber, P = perforatorium, FM = loose fibrous materials.

DISCUSSION

The reproductive system of brachyurans has been well established in the literature. Studied species include the mangrove tree crab (*Goniopsis cruentata*; Family Grapsidae), the mangrove land crab (*Ucides cordatus*; Family Ocypodidae), the swimming crab (*Arenaeus cribrarius*; Family Portunidae), and the blue land crab (*Cardisoma guanhumi*: Family Gecarcinidae) [19–22]. Another sesarmid crab that has been studied is *Armases rubripes* [23, 24], whose male reproductive system very closely resembles the reproductive system of *E. singaporense* in the present study. Hence, the H-pattern of the male gonads is a conserved trait found across Brachyura, comprising a pair of testes and a pair of vasa deferentia at the upper and the lower ends of the H, respectively. The vas deferens of *E. singaporense* could be classified into anterior, median and posterior regions, similarly to the vas deferens of *Scyllarus chacei* [25]. Stewart et al [26] also divided the vas deferens into three regions; others divided

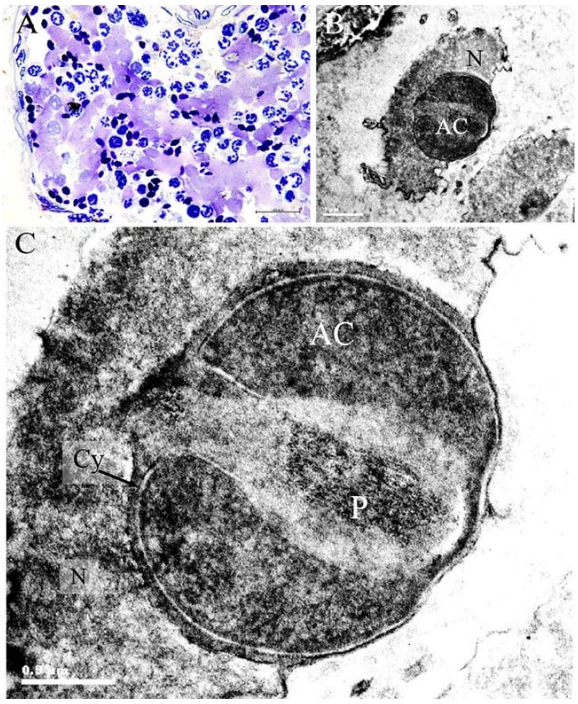

Fig. 5 Light microscopy and ultrastructure of spermatozoa of *E. singaporense*. (A), The appearance of late spermatozoa among basophilic secretion; (B–C), spermatozoa ultrastructure. Abbreviations: AC = acrosomal cap, Cy = cytoplasm, N = nucleus, P = perforatorium development.

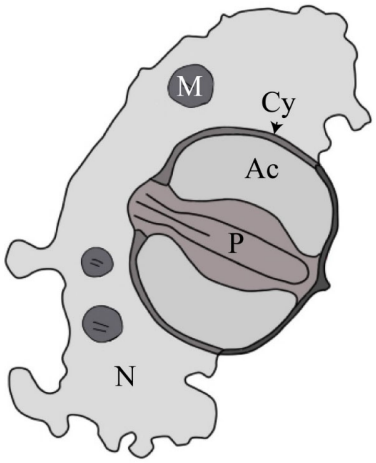

Fig. 6 An illustration showing details of mature spermatozoa of *E. singaporense*. Abbreviations: AC = acrosomal cap, Cy = cytoplasm, M = mitochondria, N = nucleus, P = perforatorium.

the vas deferens of decapods differently. Garcia and Silva [19] described only a posterior region in *G. cruentata*, while four parts were described by Hinsch and McKnight [25], including most of the distal ejaculatory duct, in *Scyllarus chacei*. We observed that once the sperm mass was inside the vas deferens, sperm cells were encapsulated within spermatophore surrounded by pinkish stained droplets. A similar development was described in the blue swimming crab [26]. The pinkish droplets were called substance I in [26], while there was no evidence of substance II or different categories of secretion types along the whole vas deferens of *Alainodromia timorensis* [27].

The processes of spermatogenesis and cell differentiation to produce spermatozoa in *E. singaporense* took place within seminiferous tubules, as in *Telmessus cheiragonus*; while spermatogonia were found at the periphery of the tubules [28]. More differentiated cells within the tubules were located away from the periphery. In *Maja brachydactyla* [29], the testes were divided into three zones: germinal, transformation and evacuation zones. Although, in our study, spermatogenesis zone was divided into five regions based on histological observation, the regions were similar to the zones observed in *M. brachydactyla*. The Sg region was in the germinal zone, the Ps/Ss/St zones were in the transformation zone, and the Sz region was in the evacuation zone. Thus, the pattern of spermatogenesis of *E. singaporense* was considered a tubular type where cell division and differentiation progressed along the length of the testis. This type of testis was also found in *S. seticornis* [4], *Pachygrapsus* spp. [15], and primitive crabs [27]. Other studies have observed different types of testes in Brachyurans; for example, testicular cysts or lobes were found in *P. pelagicus* [1] and *G. cruentata* [19]. Based on an in-depth phylogenetic analysis of decapods [30], these Families of crab represent early (Dromiidae) to later (Inachidae) splits in evolution and possess tubular testes connected to a long vas deferens; while Grapsid members present either tubular or lobular testes within the same family. This pattern of variation suggested that the tubular model of spermatogenesis is conserved across Brachyura, while the lobular model probably emerged independently. Further investigations into the evolutionary progression of all families of Brachyura would highlight the conservation and the divergence within their reproductive systems.

Our histological observations revealed the division of germ cells of *E. singaporense* into five major stages and the subcategorization of PSc into six substages based on unique nucleus-cytoplasm characters. Other studies of crab species have classified male germ cells differently based on different techniques and markers, e.g., four distinct zones (Sg, Sc, St, and Sz) [24], a germinal zone and a spermatid zone [1]. The histological characteristics of Sg, Spc, St, and spermiogenesis

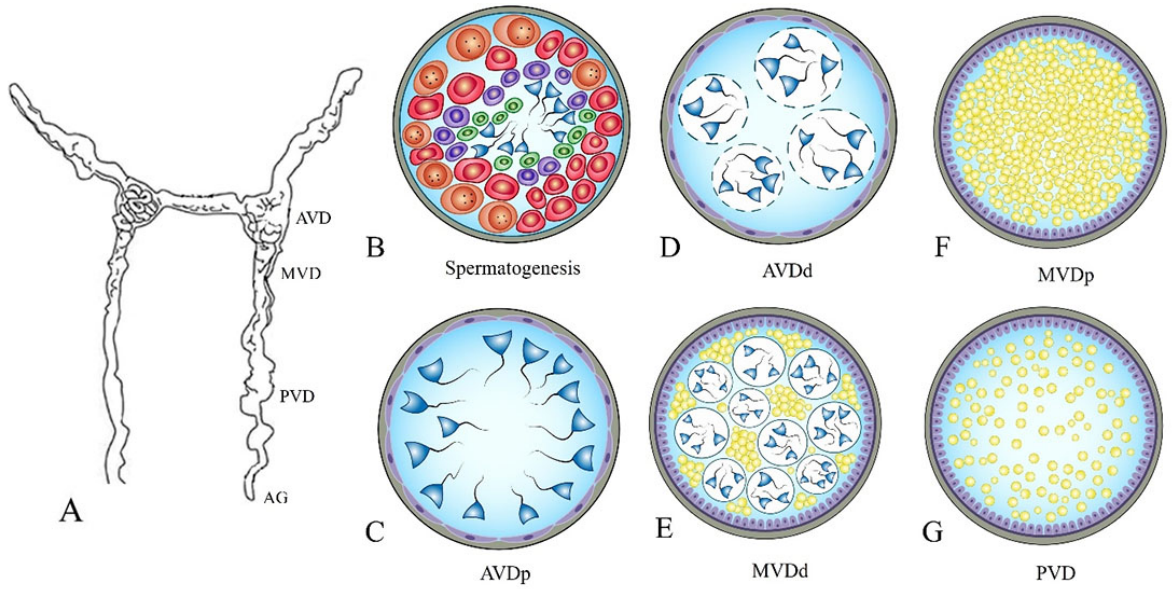

Fig. 7 An illustrated diagram of male reproductive system of *E. singaporense*. (A), The reproductive system; (B), the spermatogenesis; (C), the proximal region of the anterior vas deferens (AVDp); (D), the distal region of the anterior vas deferens (AVDd); (E), the proximal region of the median vas deferens (MVDp); (F), the distal region of the median vas deferens (MVDd); and (G), the posterior vas deferens (PVD).

in this study were in good agreement with other well-established studies of Brachyura [15, 22], with detailed structures of classified SgA and SgB in *E. singaporense*. However, the understanding of their roles was limited. Some investigations suggested that SgA is an undifferentiated Sg or spermatogonial stem cells, and only SgB might be differentiated and transformed into different germ cell types [31, 32].

Our ultrastructural analysis confirmed the typical Brachyuran aflagellate morphology of the Sz of sesarmid crabs (Fig. 6) although the apical button and zonation in acrosomes of *E. singaporense* sperm were not obvious. This morphology has also been attributed in the literature to other Families of crabs, such as Ocypodidae [33, 34]. This study used a histological approach unravelling the reproductive morphology and sperm ultrastructure of male *E. singaporense*, revealed that sesarmid crabs closely shared traits with other brachyurans and decapods.

CONCLUSION

The findings of this study demonstrated the similarities between *E. singaporense* and other decapod crustaceans in terms of the morphology and the spermatogenesis of male reproductive system (Fig. 7), highlighting the conservation of characteristics among Brachyura. We confirmed for the first time that the anterior vas deferens of *E. singaporense* could be separated into two parts: the proximal portion and the distal portion. Further investigations of the reproductive systems of different crab families would provide insights into their evolutionary diversity and conservation.

Acknowledgements: This research is financially supported by Rajamangala University of Technology Srivijaya, Thailand with a research fund grant (Project Title: Reproductive biology of the sesarmid crab, *Episesarma singaporense* in Trang mangrove forest). We also would like to thank the Department of Marine Science and Environment, Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya, Trang Campus, for laboratory support. We also thank to Dr. Tommy Coyne for his check and correction the grammatical structure. Ethical approval for the work was provided by the Animal Care and Use Committee of Rajamagala University of Technology Srivijaya (ID#IAC 02-01-62).

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