JOURNAL OF MORPHOLOGY 270:175–193 (2009)

Ultrastructure of Spermatozoa and Spermatophores of Old World Freshwater Crabs (Brachyura: Potamoidea: Gecarcinucidae, Potamidae, and Potamonautidae)

Sebastian Klaus,[1]* Christoph D. Schubart,[2] and Dirk Brandis[3]

[1]*Institut für Zoologie, Universität Heidelberg, INF 230, 69120 Heidelberg, Germany*
[2]*Biologie I, Universität Regensburg, Universitätsstr. 31, 93053 Regensburg, Germany*
[3]*Zoologisches Museum, Universität Kiel, Hegewischstr. 3, 24105 Kiel, Germany*

ABSTRACT We investigated the ultrastructure of spermatozoa and spermatophores of 19 palaeotropical freshwater crab species [12 species of the Gecarcinucidae, 6 of the Potamidae (Potamiscinae), and 1 species of the Potamonautidae (Deckeniinae: Hydrothelphusini)]. The investigated Potamiscinae have densely packed coenospermic spermatophores with the exception of *Thaiphusa sirikit* and *Johora singaporensis* that exhibit cleistospermia. In contrast, in the Gecarcinucidae the spermatozoa are loosely embedded in a mucous matrix. The gecarcinucid and potamiscine sperm differ, furthermore, in acrosomal structure and size. The acrosome in the Gecarcinucidae is much smaller and spherical, while the larger acrosome in the Potamiscinae has the tendency to be depressed. In the Potamiscinae, an additional middle acrosomal zone evolved between the acrosome ray zone and the outer acrosomal zone. Within the Gecarcinucidae, a differentiation into two groups (Gecarcinucinae and Parathelphusinae) is not supported by the present spermatological data. The sperm morphology of *Hydrothelphusa* aff. *madagascariensis* (Potamonautidae: Deckeniinae) differs from *Potamonautes sidneyi* (Potamonautidae: Potamonautinae) in acrosomal size and shape, and in the absence of a periopercular rim. A closer relationship of Deckeniinae and Gecarcinucidae cannot be confirmed by spermatology. J. Morphol. 270:175–193, 2009.

KEY WORDS: spermatozoa; spermatophores; freshwater crabs; Brachyura; Potamoidea

Brachyuran sperm cell morphology has been investigated for more than 100 years (reviewed in Felgenhauer and Abele, 1991; Jamieson and Tudge, 2000). Electron microscopy studies especially improved our understanding of the morphology and function of brachyuran sperm cells. The acrosomal reaction of the complex brachyuran sperm cell during fertilization was resolved by electron microscopic studies (Brown, 1966). Spermatological investigations revealed both a conserved ground pattern of sperm cell morphology within the Brachyura, as well as variability between groups, mainly at the family level and above (Felgenhauer and Abele, 1991; Jamieson, 1994; Jamieson et al., 1995). Brachyuran spermatozoal characters were used in several cladistic studies (Jamieson, 1991, 1994; Jamieson et al., 1995) and largely support the system of classification of the Brachyura as suggested by Guinot (1978), that is, the grouping into Podotremata, Heterotremata *s.lat.*, and Thoracotremata.

Brachyuran spermatozoa, as with decapod sperm cells in general, are aflagellate and immotile. The acrosome is spherical and consists of a central perforatorial chamber (also called a "perforatorium") that contains microtubule-like structures and is surrounded by several acrosomal zones (see Fig. 1). We will term the electron-lucent zone surrounding the perforatorial chamber the "inner acrosomal zone" (as per Jamieson, 1994). This zone is usually surrounded externally by the "acrosome ray zone" in the investigated freshwater crabs (but claimed to be absent in *Potamon fluviatile*, *P. ibericum*, and *Potamonautes sidneyi* by Guinot et al., 1997). The ray zone is defined by its distinct, coarse pattern and the potentially homologous structure in podotreme crabs is called the "fingerprint zone" (Guinot et al., 1998). Between the acrosome ray zone and the prominent "outer acrosomal zone," an additional zone can be distinguished in some species. We term this the "middle acrosomal zone." Apically, the acrosome is capped with an electron-dense operculum that contacts the oocyte during a successful fertilization. Beneath the operculum the subopercular material separates operculum, inner acrosomal material, and perforatorial chamber, respectively. The acrosome is embedded in a cup-like nucleus, while between acrosome and nucleus a thin layer of cytoplasm remains, often accompanied by membraneous structures that are interpreted as vestigial

*Correspondence to: Sebastian Klaus, Institut für Zoologie, Universität Heidelberg, INF 230, D-69120 Heidelberg, Germany.
E-mail: sebastian.klaus@zoo.uni-heidelberg.de

Published online 22 October 2008 in
Wiley InterScience (www.interscience.wiley.com)
DOI: 10.1002/jmor.10678

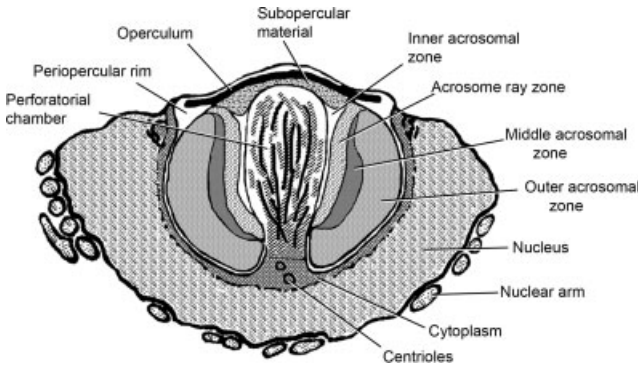

Fig. 1. Diagrammatic drawing of a freshwater crab spermatozoon with nomenclature of its morphology.

mitochondria (Jamieson, 1993). The nucleus has several to many lateral arms that, in the Brachyura, mostly are without microtubules but contain chromatin.

Brachyuran spermatophores are considered to be one of the most simple type in decapod crustaceans (Subramoniam, 1991). They are spherical and consist of sperm masses that are enclosed by a mucopolysaccharide matrix. In some brachyurans (e.g., *Libinia emarginata*, Pisidae and *Carcinus maenas*, Portunidae), the spermatophores were reported to consist of two distinct layers with the outer layer consisting of chitinous material (Hinsch, 1991; Subramoniam, 1991). The formation of the spermatophores takes place in the anterior vasa deferentia, and both apocrine and exocrine secretion of the epithelia are described (Hinsch, 1991). After copulation, when the spermatophores are transferred to the female spermathecae, they are dissolved and the spermatozoa released. Potentially, the seminal fluids may also play a role in sperm plug formation (e.g., *Ovalipes ocellatus*, Portunidae, Hinsch, 1986, 1991).

Several advantages of the mucopolysaccharide-enveloping of the spermatozoa were suggested, among them are mechanical protection, prevention of dehydration, an antimicrobial function and nutrition of the sperm (Hinsch, 1991; Subramoniam, 1991). Based on the fact that the spermatophores are dissolved after copulation in the genus *Geryon* (Geryonidae), Hinsch (1988) proposed that the spermatophores are mainly a packaging device for sperm transfer. A more complex function was proposed by Beninger et al. (1993). They observed that in *Chionoecetes opilio* (Majidae), the spermatophores perform a "differential dehiscence." In this process, free spermatozoa from the initially dehisced spermatophore are available for fertilization, while the still intact spermatophores store the sperm in the female spermathecae. The spermatophore pellicle was proposed to prevent excessive acrosome reactions and keeps the sperm for future fertilizations.

Although freshwater crabs represent one of the most diverse groups within the Brachyura, only a few studies on their spermatozoal and spermatophore morphology have been conducted (*Potamonautes sidneyi*: Jamieson, 1993; *Potamon fluviatile*: Tudge and Justine, 1994; *P. fluviatile* and *P. ibericum*: Guinot et al., 1997; *Potamiscus beieri*: no description, but depicted in Brandis, 2000 as *Potamiscus* sp.; *Sinopotamon yangtsekiense*: Du et al., 1999; Wang et al., 1999; *Austrothelphusa transversa*: no description, but depicted in Jamieson and Tudge, 2000 as *Holthuisana transversa*). These previously investigated species belong to the African family Potamonautidae, subfamily Potamonautinae (*P. sidneyi*) and to the Eurasian–North African Potamidae, subfamily Potaminae (*P. fluviatile, P. ibericum*) and Potamiscinae (*P. beieri*, *S. yangtsekiense*), and to the Gecarcinucidae (*sensu* Klaus et al., 2006; *A. transversa*). Within the Old World freshwater crabs, spermatological data on the African-Madagascan Deckeniinae (the Deckeniidae *sensu* Klaus et al., 2006) are still lacking. Also the spermatozoa and spermatophores of the neotropical Pseudothelphusoidea and the Trichodactylidae (*Dilocarcinus septemdentatus*: Matos et al., 1996) still remain largely unexplored.

Freshwater crabs are well adapted to their limnic environment, which also affects their mode of reproduction. They show direct development with relatively few but large, lecithotrophic eggs. Earlier studies on freshwater crab spermatozoa could not detect correlations of sperm morphology with their limnic habitat (Guinot et al., 1997). Nevertheless, it was proposed that the occurrence of cleistospermia (spermatophores that contain only a single spermatozoon) could be an adaptation to reduce polyspermy, and therefore prevent wastage of eggs (Guinot et al., 1997).

We understand the Old World freshwater crabs as one superfamily Potamoidea (as already kept as an option by Klaus et al., 2006) that includes the here investigated species. This taxonomic approach is supported by the proposed potamoid monophyly (von Sternberg et al., 1999; Daniels et al., 2006; Klaus et al., 2006; Cumberlidge et al., 2008), the recognition of just two Asian families (the Gecarcinucidae and the Potamidae; Klaus et al., 2006), and the still unresolved phylogenetic relationship between the three potamoid families (Gecarcinucidae, Potamidae, and Potamonautidae; Daniels et al., 2006; Cumberlidge et al., 2008).

In this study, we describe spermatozoal and spermatophore morphology of potamoid freshwater crabs with the focus on the Asian Gecarcinucidae (12 species), but also including representatives of the Potamidae (the Asian subfamily Potamiscinae, six species) and one species of the Madagascan Hydrothelphusini (Potamonautidae: Deckeniinae: *Hydrothelphusa* aff. *madagascariensis*). Spermatological data for the Australian Gecarcinucidae, the

TABLE 1. Specimens used for preparation of the vas deferens

Species	Provenance
Gecarcinucidae	
Geithusa pulchra (Ng, 1989)	Malaysia, Pulau Redang
Heterothelphusa fatum (Ng, 1997)	Singapore, Aquarist
Oziothelphusa ceylonensis (Fernando, 1960)[a]	Sri Lanka, Aquarist
Oziothelphusa sp.	South India, Aquarist
Parathelphusa convexa (De Man, 1879)	Indonesia, Java, Garut
Parathelphusa aff. *maindroni* (Rathbun, 1902)	Indonesia, S-Sumatra, near Lampung
Phricotelphusa gracilipes (Ng and Ng, 1987)	Malaysia, Pulau Langkawi
Sartoriana spinigera (Wood-Mason, 1871)	Nepal, Mechi province
Sayamia bangkokensis (Naiyanetr, 1982)	Thailand, Aquarist
Siamthelphusa improvisa (Lanchester, 1901)	Malaysia, Pulau Langkawi
Somanniathelphusa sp.	Thailand, Aquarist
Terrathelphusa kuhli (De Man, 1883)	Indonesia, Java, Cibodas
Potamidae: Potamiscinae	
Geothelphusa albogilva (Shy, Ng and Yu, 1994)	Taiwan, Aquarist
Johora singaporensis (Ng, 1986)	Singapore
Larnaudia beusekomae (Bott, 1970)	Thailand, Aquarist
Malayopotamon cf. *brevimarginatum* (De Man, 1892)[b]	Indonesia, S-Sumatra, Danau Ranau
Pudaengon thatphanom (Ng and Naiyanetr, 1995)	Thailand, Aquarist
Thaiphusa sirikit (Naiyanetr, 1992)	Thailand, Aquarist
Potamonautidae: Deckeniinae: Hydrothelphusini	
Hydrothelphusa aff. *madagascariensis* (A. Milne-Edwards, 1872)	Madagascar, Aquarist

[a]Oziothelphusa ceylonensis: both vas deferens (ultrastructure) and spermatheca (histology).
[b]Malayopotamon cf. *brevimarginatum*: only spermatheca for ultrastructure.

Potamonautidae, subfamily Potamonautinae and the Potamidae, subfamily Potaminae are available through the studies of Jamieson (1993), Jamieson and Tudge (2000), and Guinot et al. (1997).

MATERIALS AND METHODS

The investigated freshwater crab species were purchased at aquarists or collected on field trips to Malaysia, Singapore, and Indonesia during 2005 (Table 1). For transmission electron microscopy, vasa deferentia and spermathecae (*Malayopotamon* cf. *brevimarginatum*) were fixed in 4% glutaraldehyde, either phosphate- or cacodylate-buffered (both pH 7.4). After several washing steps with cacodylate buffer, the tissue was postfixed with 1% osmium tetroxide for 2 h. Cacodylate and maleate buffer (pH 5.2) washing steps were followed by en-bloc staining with 1% uranyl acetate overnight. After dehydration through a graded series of ethanol, the tissue was infiltrated and embedded with Spurr's resin. Thin-sections (75 nm) were cut with a diamond knife, collected on copper grids (200 mesh, coated with a Formvar® support film if required). The sections were poststained with aqueous lead citrate for 1 min. Electron micrographs were taken on a Zeiss EM10 transmission electron microscope. Acrosomal measurements were taken with a sliding caliper from the negative.

For light microscopy, a spermatheca of *Oziothelphusa ceylonensis* was fixed with a mixture of formalin, acetic acid, mercuric chloride, and trichloroacetic acid ("SuSa" according to Heidenhain, 1917), dehydrated through a series of ethanol and after treatment with methyl benzoate embedded in Paraplast®. Sections of 8 μm thickness were cut on a sliding microtome and stained trichromatically according to Goldner (1938).

RESULTS

Especially for the vasa deferentia that were fixed under tropical field conditions, the ultrastructural analysis did not yield well-resolved images, notably at higher resolution (beyond 10,000×). The outer spermatozoal cell membranes and the chromatin were often already in a state of decay. Nevertheless, the structure of the acrosome was always sufficiently preserved for morphological comparison. Measurements of acrosomal length and width and opercular width and height are given in Figures 2 and 3, respectively.

Potamidae: Potamiscinae

Diagrammatic drawings of a longitudinal sagittal section of potamiscine spermatozoa are given in Figure 4. The acrosomes of the Potamiscinae are slightly depressed in shape, the acrosomal length to width (AL : AW) ranging from 0.7 to 0.8. The smallest acrosome is found in *Malayopotamon* cf. *brevimarginatum* with a mean acrosomal width (AW) of 3.4 ± 0.3 μm and a mean acrosomal length (AL) of 2.4 ± 0.2 μm (n = 7) (Fig. 2, no. 2). At the upper limit of acrosomal size ranges is *Thaiphusa sirikit* with AW = 5.2 ± 0.3 μm and AL = 4.0 ± 0.3 μm (n = 5) (Fig. 2, no. 6). The perforatorial chamber is of moderate diameter, ranging approximately from 1/4 to 1/5 of the total AW (*M*. cf. *brevimarginatum*: 1/3; *T. sirikit*: 1/11).

The acrosomes of the investigated Potamiscinae have a complex zonation, with the acrosome ray zone always externally adjacent to a middle and an outer acrosomal zone. The operculum and acrosome ray zone are linked via a "tongue and groove" connection (Fig. 5F). A low circular ridge at the border between outer and inner acrosomal zone interdigitates with a circular groove on the basal side of the operculum. This kind of connec-

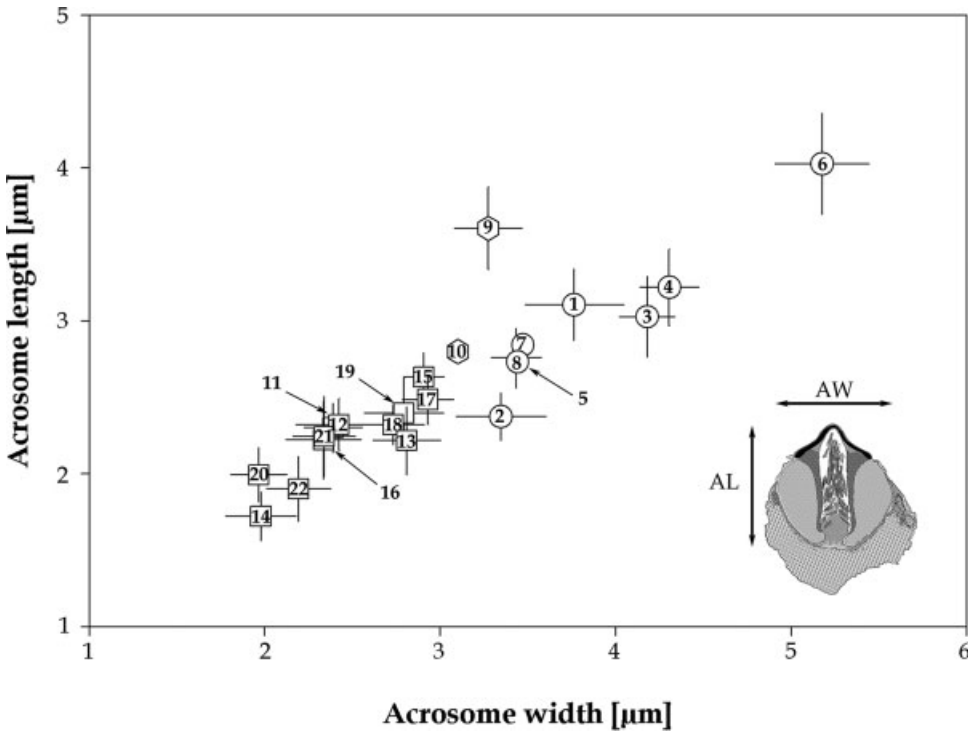

Fig. 2. Acrosome length plotted against acrosome width. 1, *Larnaudia beusekomae*; 2, *Malayopotamon* cf. *brevimarginatum*; 3, *Geothelphusa albogilva*; 4, *Pudaengon thatphanom*; 5, *Johora singaporensis*; 6, *Thaiphusa sirikit*; 7, *Potamon fluviatile*; 8, *Potamon ibericum*; 9, *Hydrothelphusa* aff. *madagascariensis*; 10, *Potamonautes sidneyi*; 11, *Phricotelphusa gracilipes*; 12, *Sartoriana spinigera*; 13, *Oziothelphusa ceylonensis*; 14, *Oziothelphusa* sp.; 15, *Terrathelphusa kuhli*; 16, *Parathelphusa convexa*; 17, *Parathelphusa* aff. *maindroni*, 18, *Geithusa pulchra*; 19, *Heterothelphusa fatum*, 20, *Siamthelphusa improvisa*; 21, *Somanniathelphusa* sp.; 22, *Sayamia bangkokensis*. Circles, Potamidae; squares, Gecarcinucidae; hexagons, Potamonautidae. Measurements of *P. sidneyi*, *P. fluviatile*, and *P. ibericum* are taken from the literature, standard deviation not indicated.

tion is found in all investigated potamiscine spermatozoa, although shallowly developed in *Johora singaporensis* and *Thaiphusa sirikit*.

Coenospermic spermatophores are always densely packed with several sperm cells and irregularly shaped, while cleistospermic spermatophores (only one sperm cell per spermatophore) are spherical (see Guinot et al., 1997). Both types of spermatophores occur within the Potamiscinae. The spermatophores are enclosed by a distinct pellicle that consists of several layers (see Fig. 6). Coenospermic spermatophores have a pellicle with three layers, first an electron-dense layer (Fig. 6A, no. 1), followed by electron-lucent material (Fig. 6A, no. 2) and a third, denser layer (Fig. 6A, no. 3). In *Larnaudia beusekomae*, diffuse electron-dense appendages are visible on the surface of layer one (Fig. 6B, arrows). The cleistosperm spermatophores of *Johora singaporensis* and *Thaiphusa sirikit* show a more complex pellicle. A thin electron-dense layer (Fig. 6B, no. 1) is followed by a thicker layer (Fig. 6B, no. 2), resembling the first electron-dense layer in *L. beusekomae*. As in *L. beusekomae*, this layer is followed by more electron-lucent material (Fig. 6B, no. 3). Basal to this layer a second thin, electron-dense layer is situated (Fig. 6B, no. 4), followed by a broader layer of undefined material (Fig. 6B, no. 5). Where the spermatophore pellicle of *T. sirikit* is disrupted, the layers two, three, and five extrude, while the layers one and four seem to stay intact (Fig. 6B, star). This argues for the extruding layer being viscous while the thin electron-dense layers are probably more rigid.

Larnaudia beusekomae (Figs. 7A–C, 5F, 4, and 6A)

The acrosome of *Larnaudia beusekomae* is slightly depressed with an acrosome length to width ratio of 0.8 ± 0.1 (n = 7). The operculum is imperforate and more convex than in the other potamiscines (Fig. 3, no. 1). A periopercular rim is absent. The perforatorial chamber is surrounded by a thin inner acrosomal zone and a cylindrical acrosome ray zone that connects apically to the operculum. The ray zone is externally adjacent to a very thin electron-dense middle acrosomal zone. The outer acrosomal zone is prominent and homogeneous. The nuclear arms are situated laterally. *L. beusekomae* exhibits coenospermia.

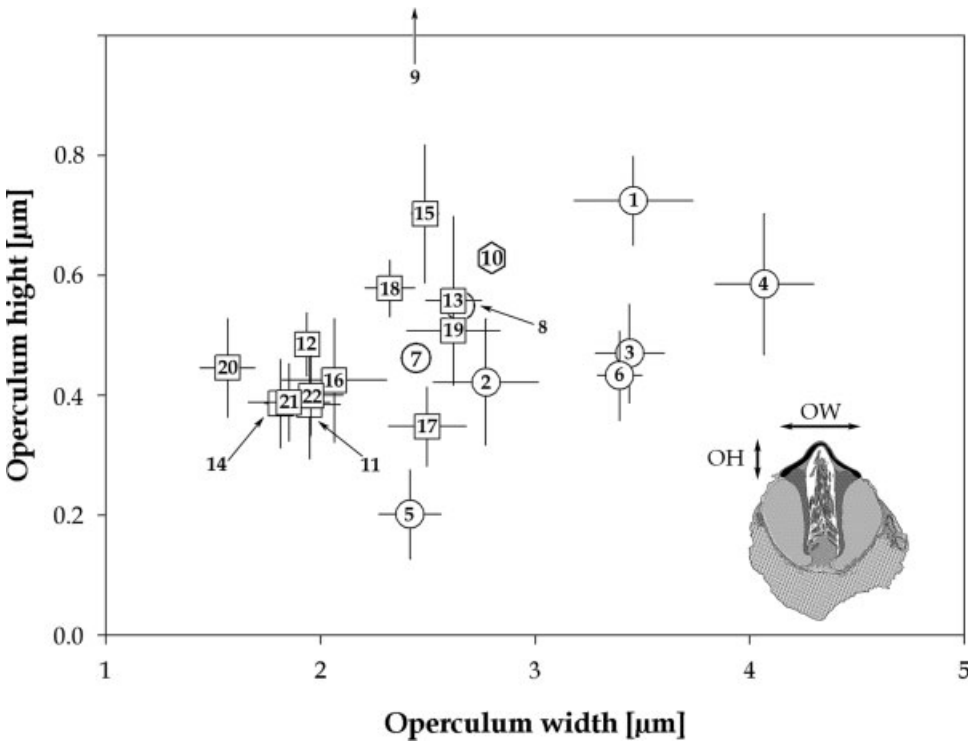

Fig. 3. Operculum height plotted against operculum width. 1, *Larnaudia beusekomae*; 2, *Malayopotamon* cf. *brevimarginatum*; 3, *Geothelphusa albogilva*; 4, *Pudaengon thatphanom*; 5, *Johora singaporensis*; 6, *Thaiphusa sirikit*; 7, *Potamon fluviatile*; 8, *Potamon ibericum*; 9, *Hydrothelphusa* aff. *madagascariensis*; 10, *Potamonautes sidneyi*; 11, *Phricotelphusa gracilipes*; 12, *Sartoriana spinigera*; 13, *Oziothelphusa ceylonensis*; 14, *Oziothelphusa* sp.; 15, *Terrathelphusa kuhli*; 16, *Parathelphusa convexa*; 17, *Parathelphusa* aff. *maindroni*, 18, *Geithusa pulchra*; 19, *Heterothelphusa fatum*, 20, *Siamthelphusa improvisa*; 21, *Somanniathelphusa* sp.; 22, *Sayamia bangkokensis*. Circles, Potamidae; squares, Gecarcinucidae; hexagons, Potamonautidae. Measurements of *P. sidneyi*, *P. fluviatile*, and *P. ibericum* are taken from the literature, standard deviation not indicated.

Malayopotamon cf. *brevimarginatum* (Figs. 7D,E and 4)

Of the investigated potamid spermatozoa, those of *Malayopotamon* cf. *brevimarginatum* from south Sumatra have the smallest acrosome. It is depressed (AL/AW = 0.7 ± 0.06, n = 7), the operculum is imperforate, and a perioperculum rim is absent. The acrosome ray zone is broad and cylindrical. A prominent, more electron-dense outer acrosomal zone and a middle acrosomal zone can also be distinguished. The middle acrosomal zone does not attach to the operculum and surrounds the acrosome ray zone like a ring. In the outer acrosomal zone, patches of more electron-dense material can frequently be observed and are probably not an artifact. There are several lateral nuclear arms. As the investigated spermatozoa originate from a female spermatheca, spermatophores, if present, would have been dissolved.

Geothelphusa albogilva (Figs. 7F–H and 4)

The acrosome of *Geothelphusa albogilva* is like in *Malayopotamon* cf. *brevimarginatum*, remarkably depressed (AL/AW = 0.7 ± 0.06, n = 7), and the operculum only slightly bulging centrally (Fig. 3, no. 3). Beneath the outer rim of the operculum a less electron-dense zone is situated, probably a vestigial perioperculum rim. The middle acrosomal zone is thin and cylindrical and attaches directly to the operculum as any subopercular material seems to be absent. The acrosome ray zone is prominent, surrounding cylindrically the wide perforatorial chamber, and apically attaching to the operculum. As in *M.* cf. *brevimarginatum*, the outer acrosomal zone can be distinguished into an inner lighter area and an outer denser one. The inner area is outwardly convex and reaches apically the vestigal perioperculum rim. At the base of the acrosome, the perforatorial chamber bulges laterally toward the outer acrosomal zone. The nuclear arms are situated laterally. *Geothelphusa albogilva* exhibits densely packed coenospermia.

Pudaengon thatphanom (Figs. 7I–K and 4)

The acrosome of *Pudaengon thatphanom* is also depressed (AL/AW = 0.7 ± 0.05, n = 5). The acrosome ray zone is broad but contacts, in contrast to *Geothelphusa albogilva*, the overlying operculum

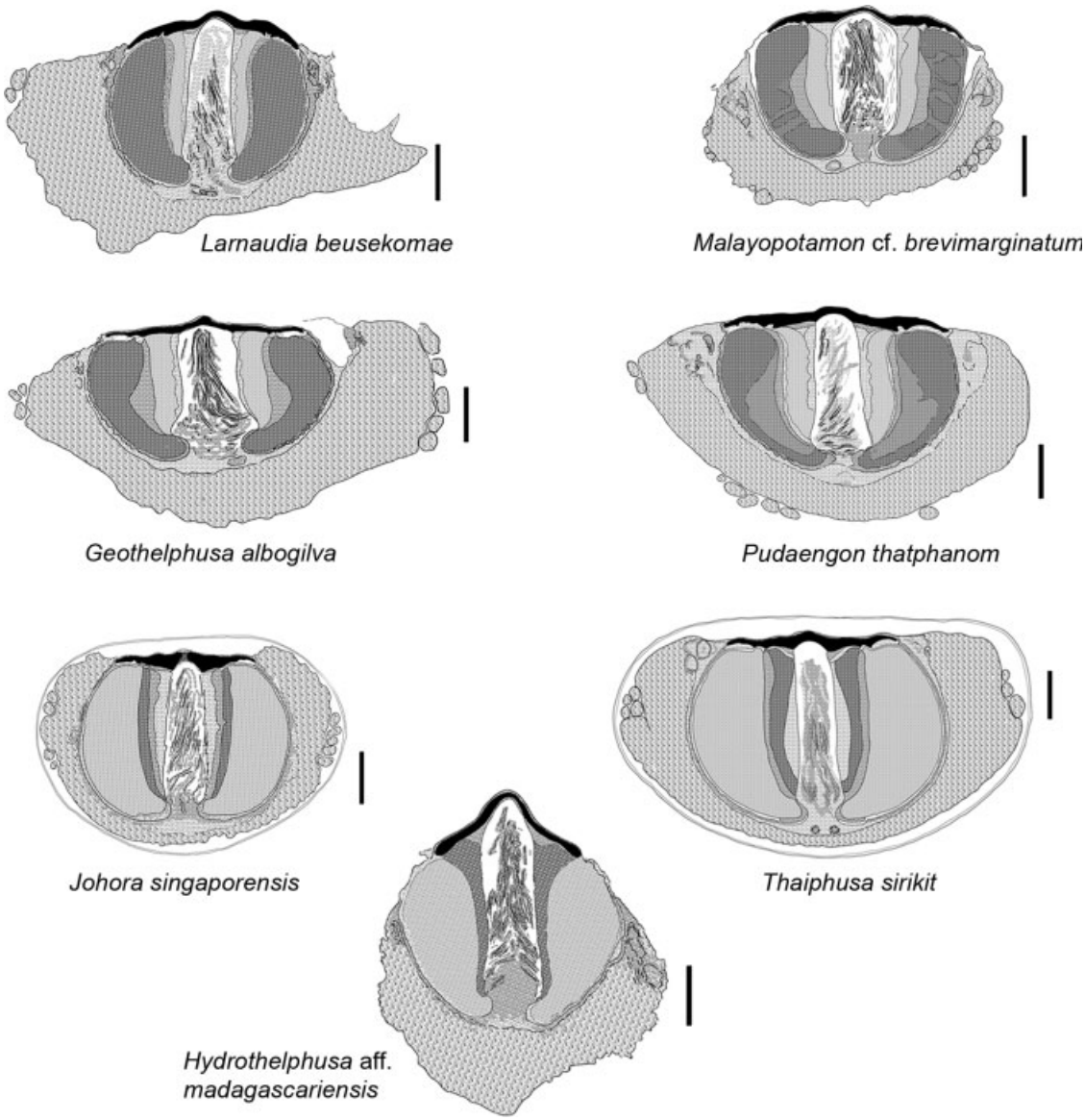

Fig. 4. Diagrammatic drawings of potamid and potamonautid (*Hydrothelphusa* aff. *madagascariensis*) spermatozoa (longitudinal sagittal view). Scale bar = 1 μm.

only marginally. The acrosome ray zone loses electron density, outwardly, toward the middle acrosomal zone, thus forming an intermediate zone absent in the other investigated potamiscine spermatozoa. The middle acrosomal zone is very thin and cylindrical. Within the outer acrosomal zone, an inner, less electron-dense area can be identified that is outwardly convex and attaches to the middle acrosomal zone. *Pudaengon thatphanom* also exhibits densely packed coenospermia.

Johora singaporensis (Figs. 5A–C and 4)

The spermatozoa of this potamiscine crab are of a slightly depressed shape (AL/AW = 0.8 ± 0.04, n = 11). The nuclear arms are situated equatorially in a very regular way. The acrosome of *Johora singaporensis* also shows a complex zonation. The inner acrosomal zone is more prominent than in the previously described species. The acrosome ray zone and the middle acrosomal zone are cylindrical, similarly thick, and much thinner than the prominent outer acrosomal zone. Also, in contrast to the previously described potamiscines, the outer acrosomal zone is less electron dense than the inner one. The operculum is depressed and is the only one of the investigated species with an apical perforation (Fig. 5C). Opercular width and height are smallest among the Potamiscinae (Fig. 3, no. 5). A periopercular rim is missing. *Johora singaporensis* exhibits only cleistospermia, with every single spermatozoon being encapsulated by a thin double membrane.

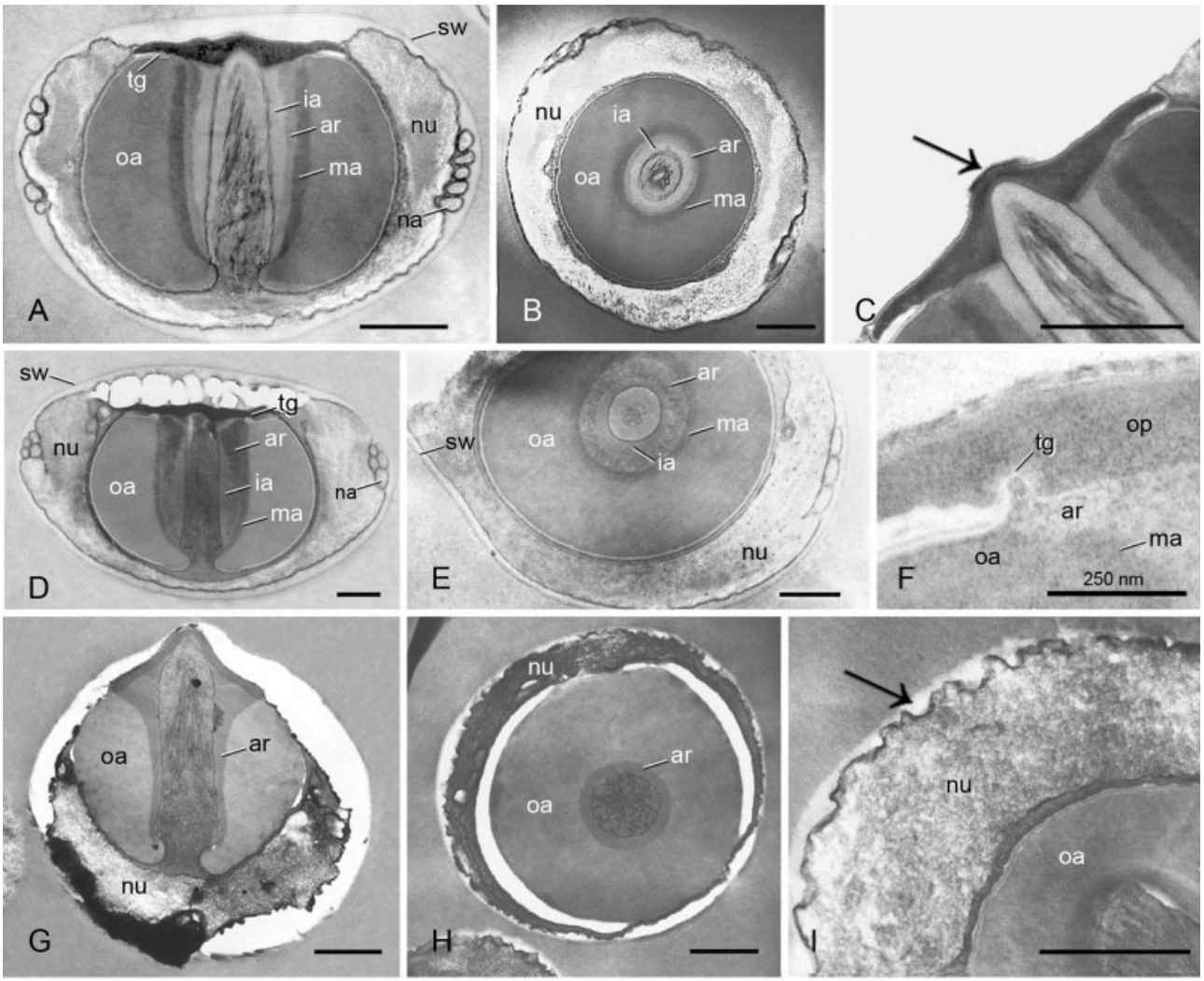

Fig. 5. Potamid (**A–F**) and potamonautid (**G–I**) spermatozoa and spermatophores. TEM. (A–C) *Johora singaporensis*. (D–F) *Thaiphusa sirikit*. (G–I) *Hydrothelphusa* aff. *madagascariensis*. First column longitudinal sagittal section, second column cross section. (C) Perforated operculum of *Johora singaporensis* (arrow). (F) *Larnaudia beusekomae*. (I) Corrugated surface of *Hydrothelphusa* aff. *madagascariensis* spermatozoon with electron-lucent corona (arrow). ar, acrosome ray zone; ia, inner acrosomal zone; ma, middle acrosomal zone; nu, nucleus; na, nuclear arm; oa, outer acrosomal zone; op, operculum; sw, spermatophore wall; tg, "tongue and groove" connection of operculum and outer acrosomal zone. Scale bar = 1 μm or as indicated.

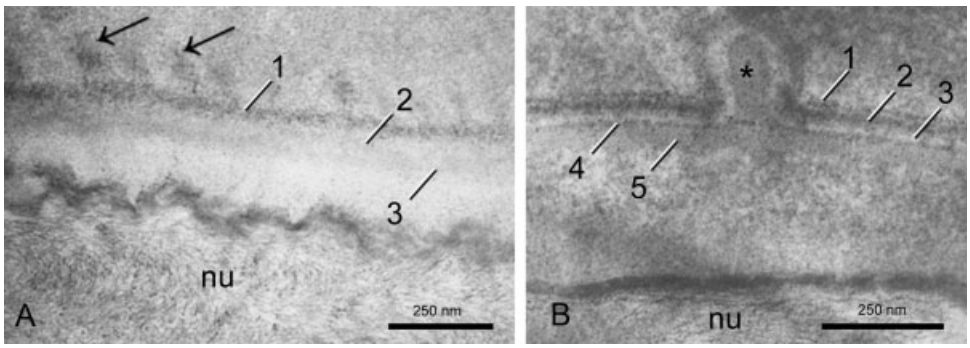

Fig. 6. The potamiscine spermatophore pellicle. TEM. (**A**) *Larnaudia beusekomae* (coenospermia). 1, outer electron-dense layer; 2, electron-lucent material; 3, inner electron-dense layer; arrows, extraspermatophoral appendages. (**B**) *Thaiphusa sirikit* (cleistospermia). 1, outer thin electron-dense layer; 2, thick electron-dense layer; 3, electron-lucent material; 4, inner thin electron-dense layer; 5, undefined electron-dense material; star, extrusion of layers 2, 3, and 5; nu, nucleus of sperm cell.

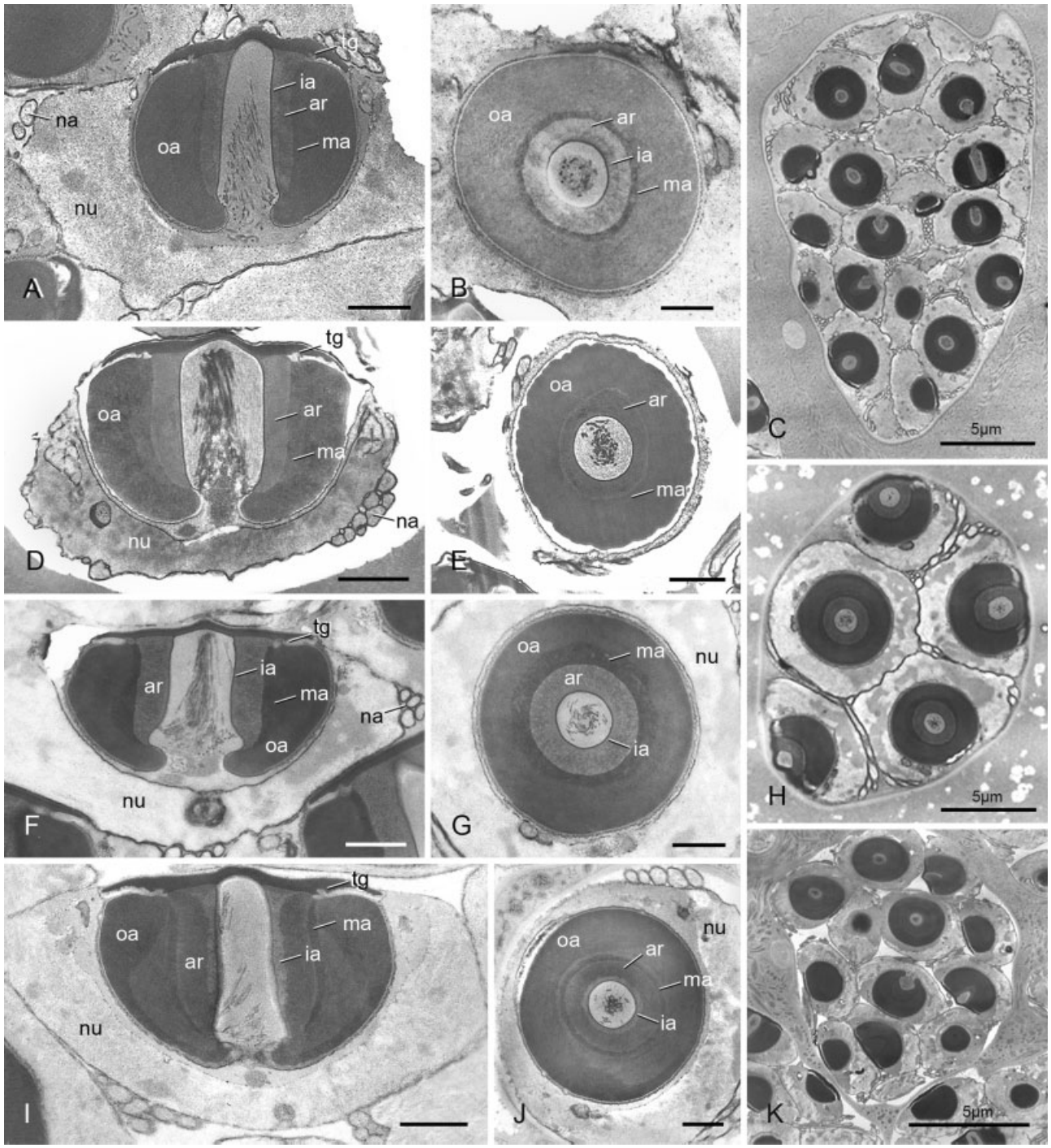

Fig. 7. Potamid spermatozoa and spermatophores. TEM. (**A–C**) *Larnaudia beusekomae*. (**D**, **E**) *Malayopotamon* cf. *brevimarginatum*. (**F–H**) *Geothelphusa albogilva*. (**I–K**) *Pudaengon thatphanom*. First column longitudinal sagittal section, second column cross section, and last column spermatophores. ar, acrosome ray zone; ia, inner acrosomal zone; ma, middle acrosomal zone; nu, nucleus; na, nuclear arm; oa, outer acrosomal zone; tg, "tongue and groove" connection of operculum and outer acrosomal zone. Scale bar = 1 µm or as indicated.

Thaiphusa sirikit (Figs. 5D–F, 4, and 6B)

The spermatozoa of *Thaiphusa sirikit* closely resemble those of *Johora singaporensis*, but exceed all other investigated spermatozoa in size (Fig. 2, no. 6). Its acrosome is slightly depressed (AL/AW = 0.8 ± 0.03, n = 5), the operculum is as depressed as the operculum of *J. singaporensis* but is imperforate. The larger opercular height in *T. sirikit* compared to *J. singaporensis* is the result of a thicker operculum, and not because of shape dif-

ferences (Fig. 3, no. 6). A periopercular rim is absent. The acrosome ray zone is slightly convex to the outside and is attached to the perforatorial chamber anteriorly and posteriorly. In contrast to *J. singaporensis*, the ray zone is more electron dense than the middle acrosomal zone. The latter is very thin. Rudiments of the thickened ring surround the posterior opening of the perforatorial chamber. A pair of parallel situated centrioles could also be detected. The nuclear arms are situated equatorially. As in *J. singaporensis*, only cleistospermia could be identified in *T. sirikit*.

Potamonautidae: Deckeniinae: Hydrothelphusini

Hydrothelphusa aff. *madagascariensis* (Figs. 5G–I and 4)

The spermatozoa of this Madagascan species are large, as indicated by acrosomal measurements (Figs. 2 and 3, no. 9). The acrosome proportions are slightly elongate (AL/AW = 1.1 ± 0.09, n = 9), and the operculum is strongly apically projected (Fig. 3, no. 9). No evidence for the existence of nuclear arms could be detected, although they have been found in all other brachyurans with the exception of some podotreme crabs (Jamieson, 1994). The outer acrosomal zone is prominent and, in contrast to the other here investigated spermatozoa, is inwardly convex. The more electron-dense, tubule-like, structured acrosome ray zone between the outer acrosomal zone and the perforatorium broadens apically and contacts the outer rim of the operculum. Inner and middle acrosomal zones are absent, and the acrosome ray zone continuously attaches to the perforatorial chamber, the subopercular zone and the outer acrosomal zone. A periopercular rim could not be identified. Any evidence for spermatophores, either of cleisto- or coenospermic type, is lacking. Nevertheless, around the corrugated outer margin of the spermatozoa a thin and light zone is situated that could be interpreted as vestigal spermatophore matrix (Fig. 5I).

Gecarcinucidae

Diagrammatic drawings of longitudinal saggittal sections of many gecarcinucid spermatozoa are given in Figure 8. The acrosomal shape of the Gecarcinucidae is spherical or slightly depressed (AL/AW = 0.8–1.0). *Oziothelphusa* sp. from India has the smallest acrosome of the investigated gecarcinucids (AW = 1.98 ± 0.2 μm, AL = 1.72 ± 0.2 μm, n = 9; Fig. 2, no. 14) and *Parathelphusa* aff. *maindroni* the largest (AW = 2.93 ± 0.2 μm, AL = 2.49 ± 0.2 μm, n = 12; Fig. 2, no. 17). The perforatorial chamber is of moderate relative size (the diameter approximately one-third of the total AW). Spermatophores, if present, are irregular or spherical (cleistospermic) and always consist of a mucous matrix in which the spermatozoa are embedded. A complex spermatophore pellicle, as in the potamiscines, is absent. Only in *Phricotelphusa gracilipes*, *Terrathelphusa kuhli*, and *Siamthelphusa improvisa* (Fig. 9G) could a pellicle consisting of two thin, electron-dense layers be identified. Although in the other gecarcinucids a comparable structure was not identified, its absence can be attributed to the poor preservation.

Phricotelphusa gracilipes (Figs. 8 and 10A–C)

The spermatozoa are of relatively small size and the acrosomes are spherical in shape (AL/AW = 1.0 ± 0.03, n = 12). The perforatorial chamber is broad in diameter, about one-third of the AW. The acrosomal ray zone is outwardly convex, its structure being very distinct and more granular-like than the typical "fingerprint" style. A middle acrosomal zone is absent. The operculum is imperforate and bulges out, and a thin periopercular rim can be identified. The nuclear arms are very small and distributed over the whole surface of the nucleus, in some cases only apically around the operculum. Several spermatozoa are loosely embedded in a coenospermic homogeneous matrix.

Sartoriana spinigera (Figs. 8 and 10D–F)

The spherical spermatozoa of *Sartoriana spinigera* (AL/AW = 1.0 ± 0.07, n = 9) are of larger size than in *Phricotelphusa gracilipes*. The acrosome ray zone is relatively thin and cylindrical. A middle acrosomal zone is absent, the outer acrosomal zone is homogeneous and less electron dense than the ray zone. The periopercular rim is very prominent and situated more under the edge of the operculum than surrounding it. Nuclear arms are found laterally and basally. Only cleistospermic single spermatozoa that are enveloped by a thin membrane were found.

Oziothelphusa ceylonensis (Figs. 8 and 10G–I) and *Oziothelphusa* sp. (Figs. 8 and 10J–L)

Of the genus *Oziothelphusa* two species were investigated, *O. ceylonensis* from Sri Lanka and one undetermined species from India. The spermatozoa of both species are very similar in their structure, but not in their size. The acrosome of *Oziothelphusa* sp. is the smallest of the here investigated spermatozoa (mean AW = 1.98 ± 0.2 μm, AL = 1.72 ± 0.2 μm, n = 9; Fig. 2, no. 14) and significantly smaller than *O. ceylonensis* (mean AW = 2.82 ± 0.2 μm, AL = 2.22 ± 0.2 μm, n = 5; Fig. 2, no. 13). The acrosome of *O. ceylonensis* is slightly depressed (AL/AW = 0.8 ± 0.04, n = 5), while it is

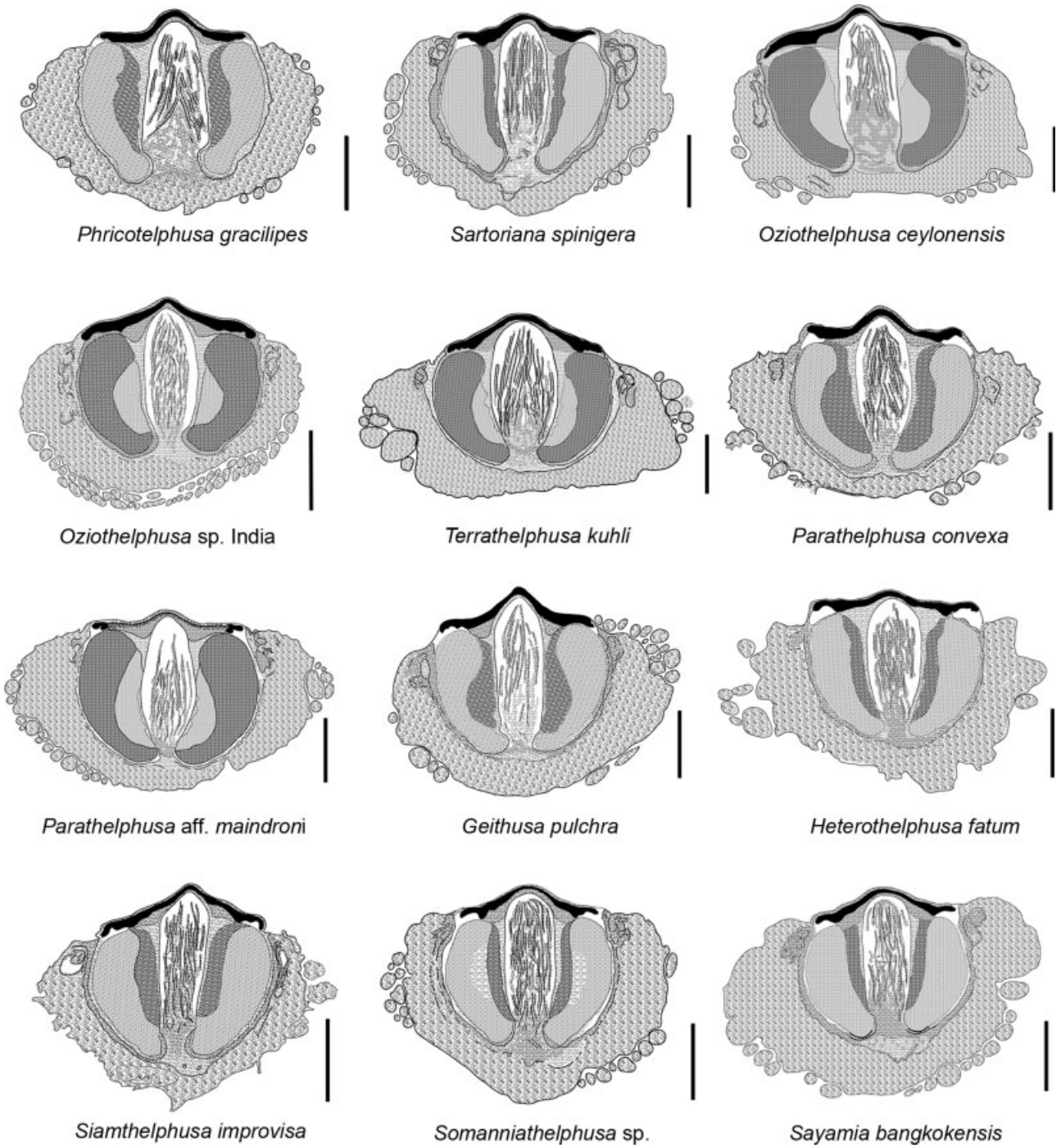

Fig. 8. Diagrammatic drawings of gecarcinucid spermatozoa (longitudinal sagittal view). Scale bar = 1 μm.

nearly spherical in *Oziothelphusa* sp. (AL/AW = 0.9 ± 0.09, n = 9). The acrosome ray zone is of the fingerprint-type in both species, outwardly convex and does not reach the subopercular zone. A middle acrosomal zone is absent. The outer acrosomal zone is more electron dense than the ray zone. The operculum is gently and centrally bulging. There is no peripercular rim. The nuclear arms are arranged at the basal side of the spermatozoon. In *Oziothelphusa* sp. they have a small diameter, occur in high number, and appear in longitudinal sagittal section as if arranged in two rows. In both species, the coenospermic spermatozoa are packed in a homogeneous matrix.

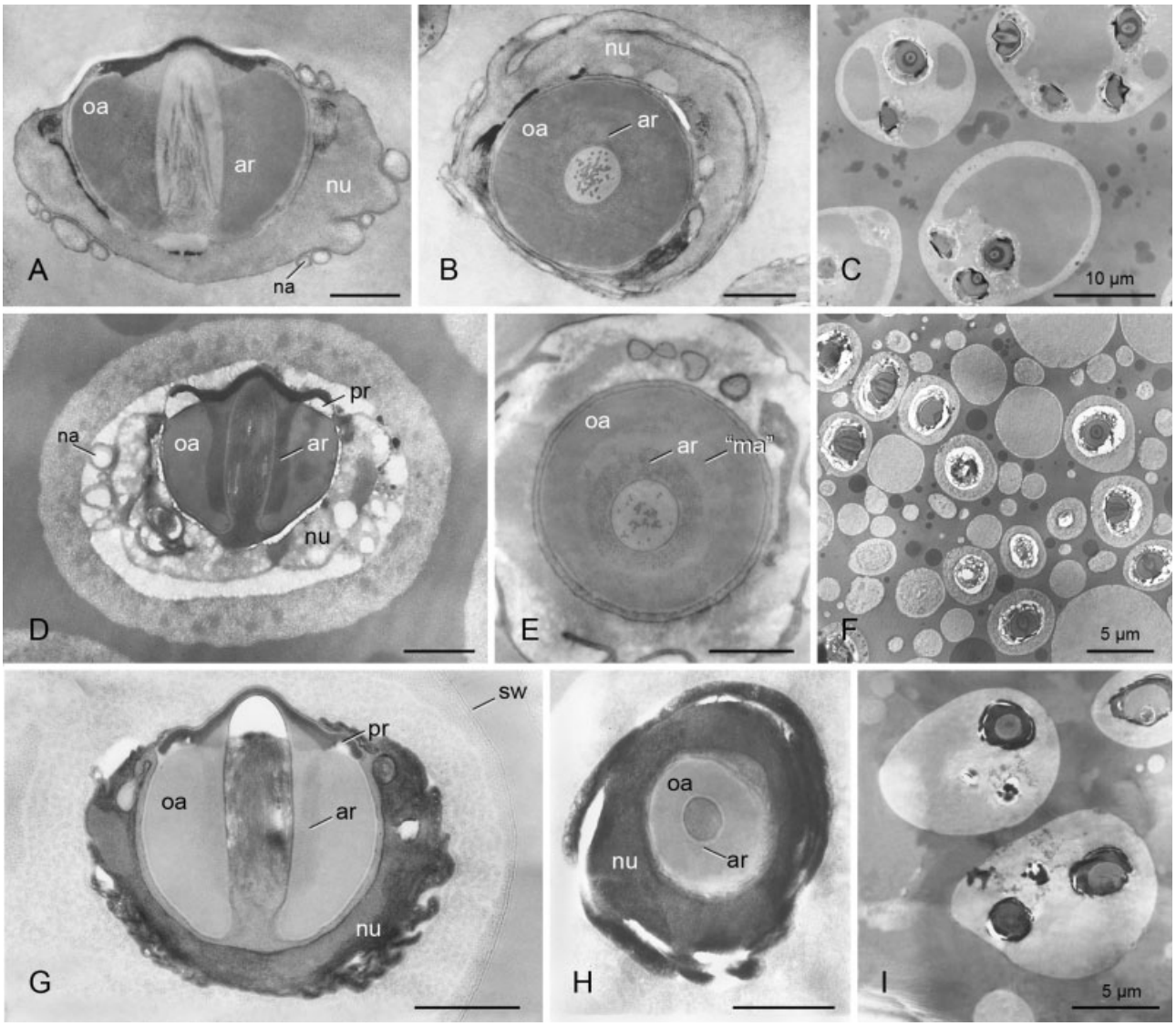

Fig. 9. Gecarcinucid spermatozoa and spermatophores. TEM. (**A–C**) *Geithusa pulchra*. (**D–F**) *Heterothelphusa fatum*. (**G–I**) *Siamthelphusa improvisa*. First column longitudinal sagittal section, second column cross section, and last column spermatophores. ar, acrosome ray zone; "ma," middle acrosomal zone-like layer; nu, nucleus; na, nuclear arm; oa, outer acrosomal zone; pr, perioperacular rim; sw, spermatophore wall. Scale bar = 1 µm or as indicated.

Terrathelphusa kuhli (Figs. 8 and 11A–C)

The spermatozoa of *Terrathelphusa kuhli* are nearly spherical (AL/AW = 0.9 ± 0.07, n = 13). The zoning of the acrosome is similar to *Oziothelphusa ceylonensis*. the acrosome ray zone is outwardly convex, and the outer acrosomal zone homogeneous. In contrast to the spermatozoa of the genus *Oziothelphusa*, the perforatorial chamber has a larger diameter. The operculum is strongly convex but not particularly apically bulging (Fig. 3, no. 15). A perioperacular rim is absent. The nuclear arms are situated laterally often having a large diameter. The many coenospermic spermatozoa are situated in a homogeneous matrix. Within the spermatophores, clusters of spherical electron-lucent, globular structures with an electron-dense core are visible.

Parathelphusa convexa (Fig. 11D–F) and *Parathelphusa* aff. *maindroni* (Fig. 11G–I)

Spermatozoa of two closely related species of the genus *Parathelphusa* were investigated, *P. convexa* from Java and *P.* aff. *maindroni* from south Sumatra. The acrosomal structure in both species is very similar. The acrosome ray zone is outwardly convex, apically reaching the subopercular material in *P. convexa* but not in *P.* aff. *maindroni*. A middle acrosomal zone is absent. The operculum bulges centrally (more distinct in *P. convexa*). The operculum

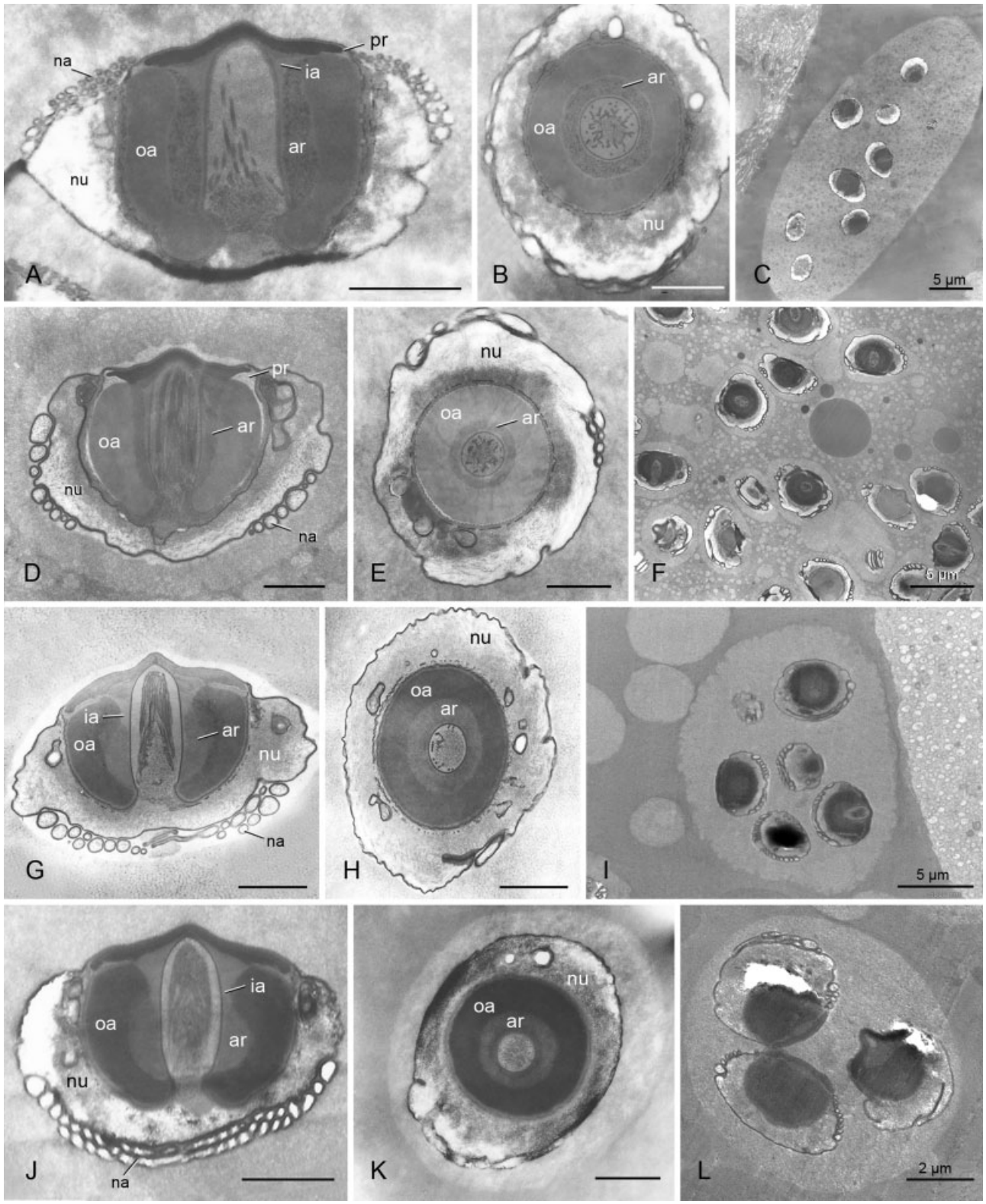

Fig. 10. Gecarcinucid spermatozoa and spermatophores. TEM. (**A–C**) *Phricotelphusa gracilipes*. (**D–F**) *Sartoriana spinigera*. (**G–I**) *Oziothelphusa* sp. India. (**J–L**) *Oziothelphusa ceylonensis*. First column longitudinal sagittal section, second column cross section, and last column spermatophores. ar, acrosome ray zone; ia, inner acrosomal zone; nu, nucleus; na, nuclear arm; oa, outer acrosomal zone; pr, periopercular rim. Scale bar = 1 µm or as indicated.

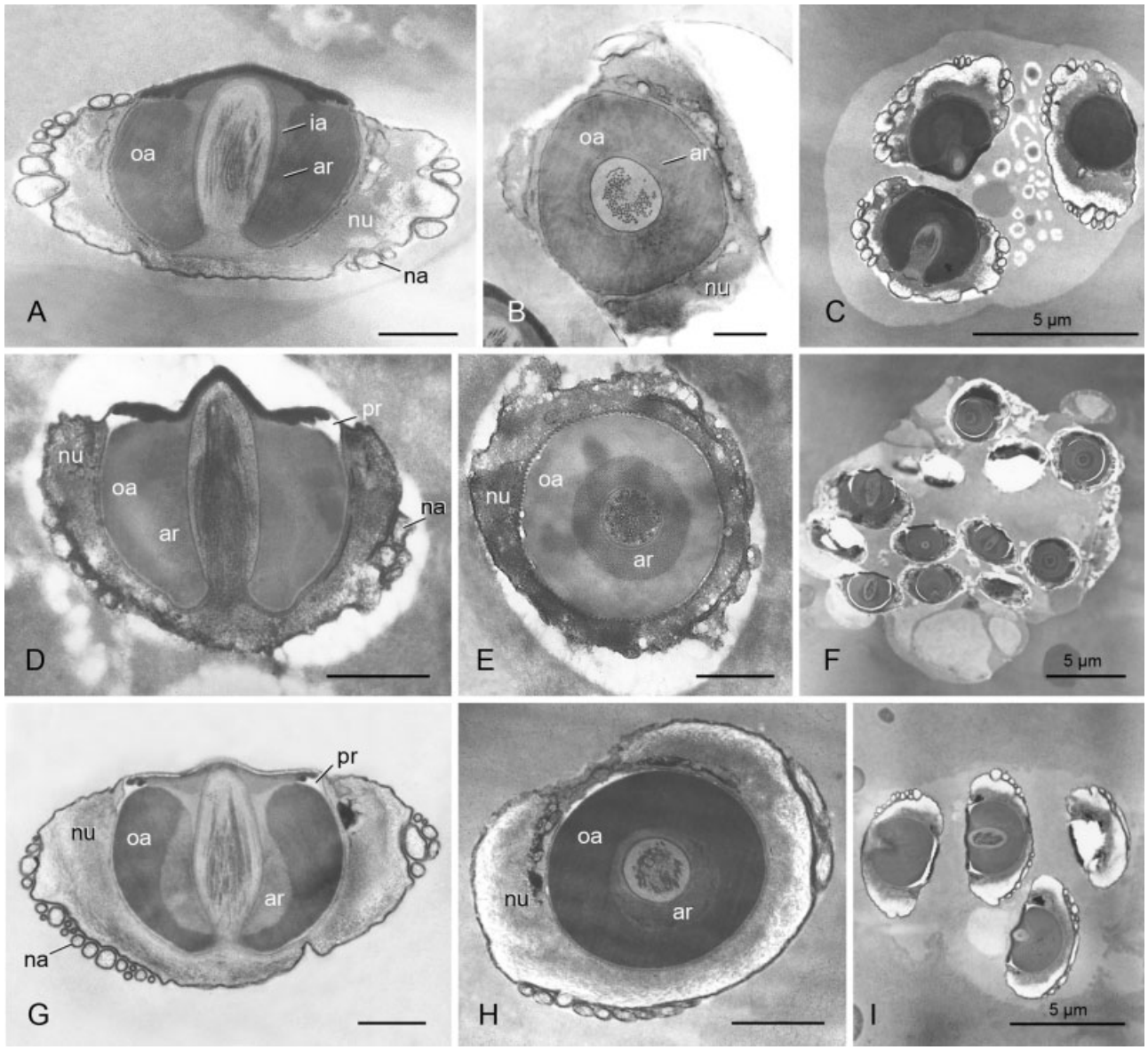

Fig. 11. Gecarcinucid spermatozoa and spermatophores. TEM. (**A–C**) *Terrathelphusa kuhli*. (**D–F**) *Parathelphusa convexa*. (**G–I**) *Parathelphusa* aff. *maindroni*. First column longitudinal sagittal section, second column cross section, and last column spermatophores. ar, acrosome ray zone; ia, inner acrosomal zone; nu, nucleus; na, nuclear arm; oa, outer acrosomal zone; pr, periopercular rim. Scale bar = 1 µm or as indicated.

in *P.* aff. *maindroni* shows a comparatively thin electron-dense zone that is connected to the outer spermatozoal membrane by columnar structures, not detected in the other investigated freshwater crabs. A periopercular rim is present. Nuclear arms are many in both species and are situated laterally. The spermatozoa are of similar size, with the acrosome of *P. convexa* being spherical (AL/AW = 1.0 ± 0.05, n = 7), while the acrosome of *P.* aff. *maindroni* is slightly depressed (AL/AW = 0.8 ± 0.04, n = 12). The spermatozoa of both species are coenospermically packed in a homogeneous matrix.

The following five species are closely related within the Gecarcinucidae. We will term this assemblage the "*Somanniathelphusa*-group" (see Klaus et al., 2009), and their close phylogenetic relationship is reflected by a very similar sperm morphology.

Geithusa pulchra (Figs. 8 and 9A–C)

The acrosome of the spermatozoa of *Geithusa pulchra* is spherical (AL/AW = 1.0 ± 0.07, n = 9). In contrast to all other investigated species of the "*Somanniathelphusa*-group," the operculum distinctly bulges out (Fig. 3, no. 18). A periopercular rim is only weakly developed, and there is a prominent subopercular zone. The acrosome ray zone is,

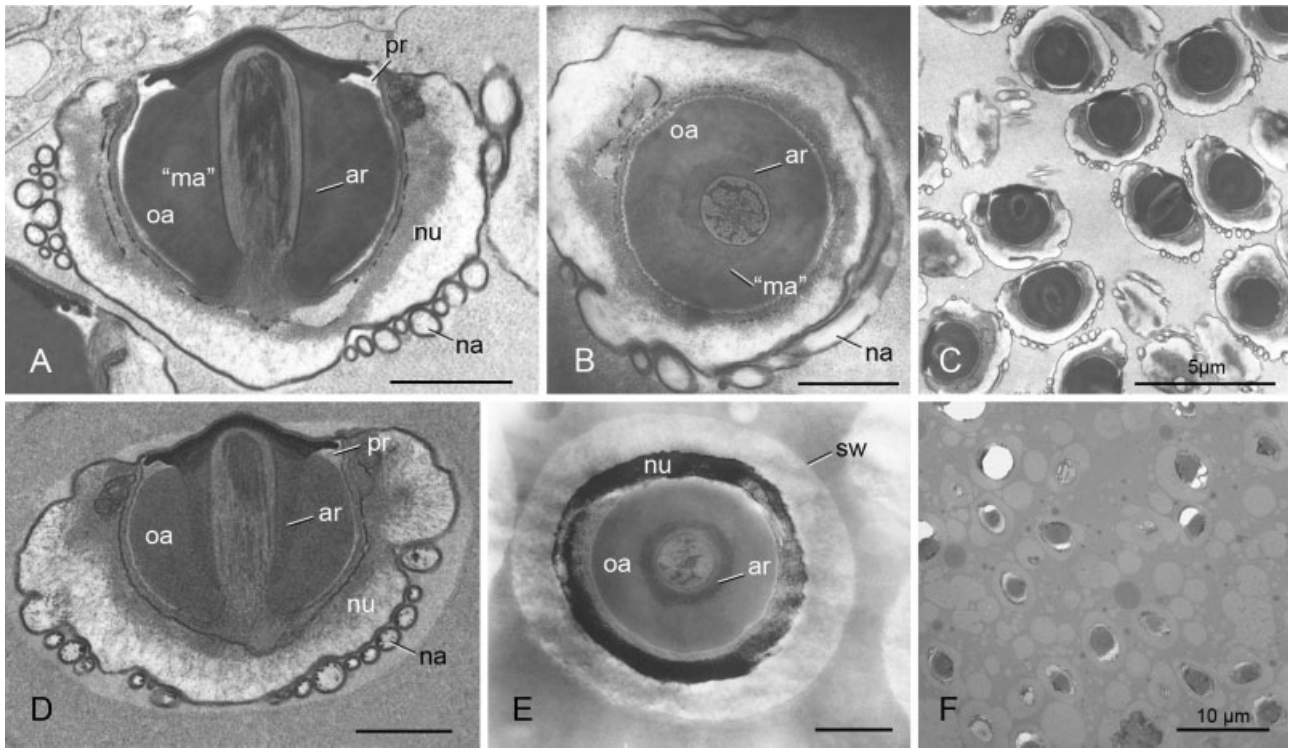

Fig. 12. Gecarcinucid spermatozoa and spermatophores. TEM. (**A–C**) *Somanniathelphusa* species. (**D–F**) *Sayamia bangkokensis*. First column longitudinal sagittal section, second column cross section, and last column spermatophores. ar, acrosome ray zone; "ma," middle acrosomal zone-like layer; nu, nucleus; na, nuclear arm; oa, outer acrosomal zone; pr, periopercular rim. Scale bar = 1 μm or as indicated.

also in contrast to the other spermatozoa of this group, not cylindrical but outwardly convex. A middle acrosomal zone is absent. There are many nuclear arms that are located on the apical, lateral, and basal side of the spermatozoon. *Geithusa pulchra* exhibits coenospermia with the spermatophores showing a strong zonation with a less electron-dense outer zone containing the spermatozoa and a more electron-dense central sperm-free zone.

Heterothelphusa fatum (Figs. 8 and 9D–F)

The spermatozoa of *Heterothelphusa fatum* are characterized by their prominent periopercular rim. The acrosome is nearly spherical (AL/AW = 0.9 ± 0.08, n = 9), and the operculum is less bulging than in *Geithusa pulchra* (Fig. 3, no. 19). The acrosome ray zone is cylindrical in shape. Its granular structure is clearly visible. Only in cross-section can the differentiation into a more electron-dense outer and a less electron-dense middle acrosomal zone be identified (Fig. 9E). Nuclear arms are present, although poorly preserved. The spermatozoa are packed into spherical individual cleistospermic spermatophores with an inner electron-lucent zone encapsulated by a thick and more electron-dense layer.

Siamthelphusa improvisa (Figs. 8 and 9G–I)

The spermatozoa of *Siamthelphusa improvisa* are spherical (AL/AW = 1.0 ± 0.07, n = 11). The acrosome ray zone reaches the subopercular material and is cylindrical. A middle acrosomal zone is absent and a periopercular rim is only weakly developed. Two small parallel centrioles can be identified at the base of the perforatorium. The nuclear arms are poorly preserved; they are situated laterally and at the basal side of the sperm. Like in *Geithusa pulchra*, the coenospermic spermatophores are differentiated into zones. Each spermatozoon is situated in a granular matrix that fuses with the matrix of the other spermatozoa within the spermatophore. This aggregate is embedded in more electron-dense material (Fig. 9G). The whole spermatophore is encapsulated by a clearly visible membrane.

Somanniathelphusa sp. (Figs. 8 and 12A–C) and *Sayamia bangkokensis* (Figs. 8 and 12D–F)

The spermatozoa of these two species are very similar as expected by the close relationship of the two genera (Naiyanetr, 1994). Nevertheless, as observed within the genus *Oziothelphusa*, acrosomal sizes differ, with the acrosome of *Sayamia*

bangkokensis being smaller (*Somanniathelphusa* sp.: AW = 2.3 ± 0.2, AL = 2.2 ± 0.3, n = 12; Fig. 2, no. 21; *S. bangkokensis*: AW = 2.2 ± 0.2 μm, AL = 1.9 ± 0.2 μm, n = 10; Fig. 2, no. 22). The shape of the acrosome is in both species spherical or nearly spherical (*Somanniathelphusa* sp.: AL/AW = 1.0 ± 0.07, n = 12; *S. bangkokensis*: AL/AW = 0.9 ± 0.06, n = 10). The thin acrosome ray zone is cylindrical. The operculum is only gently and centrally bulging and operculum measurements are broadly overlapping (Fig. 3, nos. 21 and 22). The periopercular rim and the subopercular material are prominent. The only structural difference is a light middle acrosomal zone in *Somanniathelphusa* sp. that could not unequivocally be identified in *Sayamia bangkokensis*. There are many nuclear arms situated laterally and at the base of the nucleus. *S. bangkokensis* shows cleistospermia while in *Somanniathelphusa* sp. no evidence of spermatophores could be identified, with the spermatozoa floating uncoated in the vas deferens.

DISCUSSION

Spermatophore Morphology

There are basic differences in sperm packing between the investigated Potamidae (Potamiscinae) and the Gecarcinucidae. Although in the Gecarcinucidae the spermatozoa are irregularly embedded in a mucous matrix, they are densely packed in the Potamiscinae and surrounded by a complex pellicle consisting of several layers. In the second subfamily of the Potamidae, the Potaminae, only cleistospermic spermatophores are described (Guinot et al., 1997), but also consisting of several layers. The mucous type of spermatophore was biochemically analyzed by Jeyalectumie and Subramoniam (1987) for *Spiralothelphusa hydrodroma* (Gecarcinucidae, their misspelled *Paratelphusa hydrodromous*) and showed to contain protein, free carbohydrates, and lipids. The morphological differentiation in more and less electron-dense areas within mucous spermatophores (identified in *Geithusa pulchra* and *Siamthelphusa improvisa*) points to a biochemically different composition of these areas. This could be due to different primary functions of the matrix types, for example, nutrition or protection. Such a morphological differentiation of the spermatophore matrix was already described in marine brachyurans like *Scylla serrata* (Portunidae, see Uma and Subramoniam, 1979) and *Chaceon fenneri* (Geryonidae, see Hinsch, 1991). The densely packed spermatophores of the Potamiscinae, leaving very little space not only between the individual spermatozoa but also between spermatozoa and spermatophore wall, argue for a main function as a transfer device. At least a nutritional function can most probably be excluded, in contrast to the gecarcinucid spermatophores. The potamiscine spermatophores also question the assumption that in all brachyurans the spermatophores consist of sperm masses that are merely surrounded by seminal fluid (Hinsch, 1991).

It is shown that the occurrence of cleistospermia is not an apomorphy uniting the genera *Potamon* (Potamidae: Potaminae) and *Potamonautes* (Potamonautidae: Potamonautinae) as taken into consideration by Guinot et al. (1997). It is a very variable character and both cleisto- and coenospermia occur within the investigated potamids and gecarcinucids. Even within the monophyletic "*Somanniathelphusa*-group" in the Gecarcinucidae both types of spermatophores occur, cleistospermic spermatophores in *Sayamia bangkokensis* and *Heterothelphusa fatum* and coenospermic in *Siamthelphusa improvisa* and *Geithusa pulchra*. The early separation of *G. pulcher* from the investigated species of the "*Somanniathelphusa*-group" (Klaus et al., 2009) argues for coenospermia to be the plesiomorphic character state. The endpoint of this reductive evolution could be the spermatozoa of *Somanniathelphusa* sp., where any evidence of a spermatophore envelope is absent. The reduction of spermatophores in the "*Somanniathelphusa*-group" might be correlated with a change in the mechanism of sperm transfer, as all species except *G. pulchra* have males with reduced second gonopods. However, this possibility would not explain the occurrence of coenospermia in *S. improvisa*. Based on the frequent occurrence of coenospermia in the Gecarcinucidae and Potamiscinae, this character state most probably is plesiomorphic in both groups, although they are probably not homologous. The exclusive presence of coenospermic spermatophores containing only two spermatozoa in *Potamiscus beieri* (depicted in Brandis, 2000) possibly represents an intermediate between coeno- and cleistospermia. Unfortunately, the phylogenetic relationship between the potamoid families (Gecarcinucidae, Potamidae, and Potamonautidae) and the identity of their marine sister group remains elusive, preventing an outgroup comparison for spermatophore morphology.

In *Oziothelphusa ceylonensis* (Gecarcinucidae), intact spermatophores were found in the female gonoduct that leads from the gonopore to the spermatheca (Fig. 13A). The spermatophore masses are situated centrally in the gonoduct and are surrounded by an amorphous substance, probably seminal fluids. The diameter of the sperm masses (about 80 μm; gonoduct diameter about 150 μm) can be correlated with the diameter of the groove of the male second gonopod (about 50 μm in *O. ceylonensis*, data not shown). Possibly, the spermatophores in the female gonoduct serve together with the hardened seminal fluids as a sperm plug preventing a successive fertilization by male competitors (Diesel, 1991). A few millimeters upward in the corresponding spermatheca only free spermato-

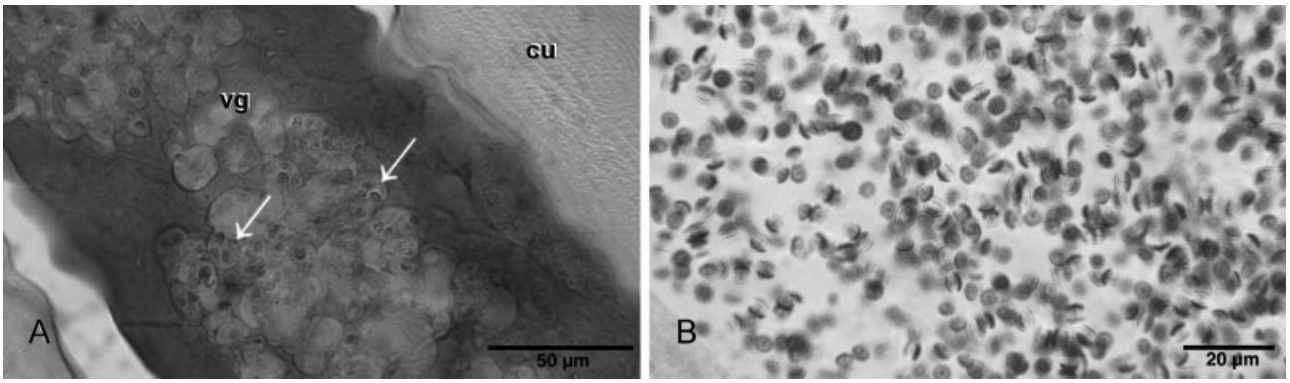

Fig. 13. Histology of the female reproductive apparatus of *Oziothelphusa ceylonensis* (Goldner staining). (**A**) Section through the vagina, with intact spermatophores (arrows). vg, vagina; cu, vaginal cuticle. (**B**) Free sperm in the spermatheca of the same specimen.

zoa could be found (Fig. 13B). This argues for an immediate dissolution of the spermatophores that enter the spermatheca as it was reported for *Libinia emarginata* (Pisidae, see Hinsch, 1991). A "differential dehiscence" as observed in *Chionoecetes opilio* (Majidae) by Beninger et al. (1993) can be excluded, at least for *O. ceylonensis*. The occurrence of free spermatozoa in the spermatheca and intact spermatophores in the vagina points to spermathecal fluids eliciting spermatophore disintegration in *O. ceylonensis*, and not external triggers (Diesel, 1991).

Sperm Morphology

Sperm morphology is another character the Potamiscinae and the Gecarcinucidae can be distinctly separated on. Morphometric differences are most obvious. The spermatozoa of the potamiscines are much larger, as indicated by measurement of acrosome width and length. The size difference between gecarcinucid and potamiscid spermatozoa is definitely independent of body size. Possibly, there is a relationship between sperm size (and therefore a relationship with spermatophore size) and the diameter of the second gonopod structures for sperm transfer. These structures consist of a small groove in the Gecarcinucidae and a larger tube in the Potamidae. Nevertheless, this would not explain size differences in cleistospermia and in gecarcinucid species that have a reduced groove. The ratio of AL to width shows that the Gecarcinucidae have more spherical acrosomes (AL/AW = 0.9–1.0), while the acrosomal shape in the Potamiscinae is slightly depressed (AL/AW = 0.7–0.8). In relation to operculum width, the gecarcinucids have higher, more bulging operculae than the potamiscines (see Fig. 3). Moreover, in the Potamiscinae operculum height is also strongly affected by operculum thickness and therefore by overall sperm size and not by operculum shape.

A major difference between Gecarcinucidae and Potamiscinae is the complexity of the acrosome. In the Potamiscinae, in addition to the acrosome ray zone, the spermatozoa can always be distinguished by a thin middle and prominent outer acrosomal zone. This seems to be an apomorphy of the Potamiscinae, as in the Potaminae, the Potamonautidae, and the Gecarcinucidae this character is absent. The faint middle acrosomal zone of *Heterothelphusa fatum* and *Somanniathelphusa* sp. is not homologous to the potamiscine character state as these two species nest deeply within the Gecarcinucidae (Klaus et al., 2009). Also the "tongue and groove" connection between operculum and outer acrosomal zone only occurs in the Potamiscinae and is a potential apomorphy of this group. Both characters, the middle acrosomal zone and the "tongue and groove" connection, are also absent in the investigated Potaminae so far (see Guinot et al., 1997) and therefore separate, as diagnostic characters, the two subfamilies of the family Potamidae.

The spermatozoa of the Gecarcinucidae can be characterized, besides their smaller size, by the occurrence of a prominent, electron-lucent periopercular rim. The periopercular rim of the Gecarcinucidae extends beneath the rim of the operculum. Therefore, it is probably not homologous to the periopercular rim described in *Potamonautes sidneyi* (Potamonautidae: Potamonautinae) that is situated on the shoulder of the acrosome (Jamieson, 1993). In *Hydrothelphusa* aff. *madagascariensis* (Potamonautidae, Deckeniinae), no equivalent structure to a periopercular rim can be found. In the Potamiscinae and in the Potaminae (Guinot et al., 1997), a kind of vestigal periopercular rim is present.

Hydrothelphusa aff. *madagascariensis* differs from all other potamoid spermatozoa investigated in this article by its slightly elongated acrosome and the inwardly convex outer acrosomal zone. The shape of the operculum is similar to that of *Potamonautes sidneyi*, but more centrally bulging

(Jamieson, 1993). Acrosomal proportions and the shape of the acrosome ray zone are distinctly different from *Potamonautes*, although both species were assigned to the same family (Cumberlidge et al., 2008). The absence of a periopercular rim is in contrast to *Potamonautes sidneyi* (see Jamieson, 1993). These differences probably reflect a long independent evolutionary history and may justify the assignment to different families (but see Cumberlidge et al., 2008). A close relationship of the Deckeniinae and the Gecarcinucidae (the "Gecarcinucoidea" *sensu* Klaus et al., 2006) is definitely not supported by spermatozoan morphology, which is consistent with the studies of Daniels et al. (2006), the molecular data in Klaus et al. (2006), and the taxonomic reappraisal of Cumberlidge et al. (2008). Further investigations are necessary to evaluate probable synapomorphies of the Deckeniinae, especially concerning the dubious absence of nuclear arms (that would be unique within the Brachyura) and spermatophore structure. More insight into sperm morphology of the Potamonautinae would be preferable, too.

Three spermatozoal characters were claimed to be synapomorphies uniting *Potamonautes* and *Potamon* by Guinot et al. (1997): the elongation of the two centrioles, their parallel disposition, and the reduction of the thickened ring that surrounds the basal opening of the perforatorial chamber (the latter occurring also in the Grapsidae and the Gecarcinidae). Unfortunately, the centrioles are hardly visible in the presently investigated species, probably due to fixation problems in tropical environments. Only in *Thaiphusa sirikit*, *Siamthelphusa improvisa*, and *Sartoriana spinigera* can their parallel arrangement be identified. It cannot be excluded, that this character state also occurs in the other gecarcinucid and potamid species and therefore might represent a synapomorphy for the Potamoidea. The thickened ring is reduced in all investigated species. A vestigal thickened ring can be identified in *T. sirikit, Pudaengon thatphanom, Johora singaporensis, S. spinigera*, and *Sayamia bangkokensis*. This character could be a second spermatological synapomorphy uniting the Old World freshwater crabs.

Furthermore, Guinot et al. (1997) proposed two potentially synapomorphic characters of *Potamonautes* and *Potamon* (besides the occurrence of coenospermia that we contested as a synapomorphy above): a wide inner acrosomal zone and the absence of a definite acrosome ray zone. We disagree with the interpretation of these characters. We recognize their "inner acrosomal zone" as the acrosome ray zone because of its distinct granular or tubuliform pattern. This zone can be identified so far in all freshwater crabs including the genera *Potamon* and *Potamonautes*. Here we follow Jamieson (1993), who already described this zone in *Potamonautes sidneyi* as the acrosome ray zone. It can also be confirmed, that the nuclear arms are wrapped around the spermatozoon in freshwater crabs (with the doubtful exception of *Hydrothelphusa* aff. *madagascariensis*) and that the nuclear membrane is simple and not multilamellar (see Guinot et al., 1997).

Within the Potamiscinae, *Johora singaporensis* and *Thaiphusa sirikit* have very similar spermatozoa, due to the situation of the nuclear arms, the shape of the acrosome and operculum, the zonation of the acrosome, and the occurrence of cleistospermia. The only differences are in size, the ray zone in *T. sirikit* attaching to the perforatorial chamber apically and the operculum in *J. singaporensis* being perforate. Also the spermatozoa of *Geothelphusa albogilva*, *Pudaengon thatphanom*, and *Malayopotamon* cf. *brevimarginatum* show distinct similarities. The acrosomal ray zone is much broader in these species, the outer acrosomal zone is outwardly convex, the operculum bulges centrally and they always have coenospermic spermatophores. *Larnaudia beusekomae* shows an intermediate morphology between *J. singaporensis–T. sirikit* and the other investigated Potamiscinae. The acrosome shows *Johora*-like features, as the prominent outer acrosomal zone and the middle acrosomal zone and acrosome ray zone are both cylindrical and thin. In contrast to *J. singaporensis* and *T. sirikit*, *L. beusekomae* has densely packed coenospermia, a centrally bulging operculum that is not planar but curved downward laterally. As detailed phylogenetic data on the Potamiscinae, either morphological or molecular, are still lacking, it is difficult to evaluate the phylogenetic information of these spermatological similarities concerning their apo- or plesiomorphic character. Most probably, *J. singaporensis* and *T. sirikit* are more closely related than to the other investigated Potamiscinae. The spermatozoa of *Potamiscus beieri* (see Brandis, 2000) seem to resemble closely those of *Pudaengon thatphanom* in acrosome morphology especially concerning the shape of operculum and perforatorial chamber. The spermatozoa of *Sinopotamon yangtsekiense* show potamiscine characters like the depressed acrosomal shape, the middle acrosomal zone, and a shallow "tongue and groove" connection (see Du et al., 1999). The wide acrosome ray zone attaching the operculum and the bulging operculum of *S. yangtsekiense* resemble strongly the morphology of *Geothelphusa albogilva*.

The investigated spermatozoa of the Gecarcinucidae are very similar. Apart from their comparatively small size, the bulging operculum, the periopercular rim, the relatively broad outwardly convex ray zone, and the absence of a middle acrosomal zone belong to their ground pattern. The acrosome ray zone repeatedly changes from outwardly convex to cylindrical shape (in *Sartoriana spinigera* and the "*Somanniathelphusa*-group" excluding *Geithusa pulchra*). Also the periopercular rim is reduced several times. It is present but weak in

Phricotelphusa gracilipes, belonging to the sister group of all other gecarcinucids (Klaus et al., 2009), but absent in the genus *Oziothelphusa*, and in *Terrathelphusa kuhli* and *Austrothelphusa transversa* (see Jamieson and Tudge, 2000). As *T. kuhli* and *A. transversa* belong to the same phylogenetic lineage within the Gecarcinucidae (see Klaus et al., 2009), the reduction of the periopercular rim could represent an apomorphy for this group.

The spermatozoa of the investigated congeners are of very similar shape. Surprisingly, both in *Oziothelphusa* and *Parathelphusa* spermatozoa of congeners differ profoundly in size. In *Parathelphusa*, the differences in the opercular structure are also distinct. Moreover, the electron density of the ray zone and the overlying outer acrosomal zone varies in longitudinal sagittal section in both species.

In contrast to the Potamidae and the Potamonautidae, a differentiation of the Gecarcinucidae is not supported by spermatology. This applies both for the approach of Klaus et al. (2006) based on the second gonopod (two subfamilies: Gecarcinucinae and Parathelphusinae) and on approaches based on character states of the frontal triangle (Bott, 1970, three families: Gecarcinucidae, Parathelphusidae, and Sundathelphusidae).

Spermatozoal morphology clearly carries phylogenetic information within the Old World freshwater crabs. This is evident especially for the Potamiscinae, which show several character traits that are probably apomorphic. Acrosomal size and shape differences are also of phylogenetic significance, as shown by the differences between the Gecarcinucidae and the Potamidae. In contrast, within these groups, acrosomal size does not reflect phylogenetic relationship. The Potamonautinae and Potamidae seem to overlap in acrosomal size, while within the Potamonautidae the two subfamilies Deckeniinae and Potamonautinae can preliminarily (as we investigated only one species of the Deckeniinae) be separated.

A prerequisite for spermatological studies in freshwater crabs, as probably for studies in Brachyura in general, is a larger sample size. Investigating just one species per group highly increases the probability of false assumptions on the homology for spermatozoal character states.

ACKNOWLEDGMENTS

The authors thank Prof. P.K.L. Ng, National University of Singapore, C. Lukhaup, Stuttgart and Aquarium Glaser, Offenbach for providing specimens. C. Kempendorf and A. Lautenschlager (Heidelberg) gave laboratory support and G. Adam (Heidelberg) assisted with the photographic work. They are also grateful to Dr. C.C. Tudge, American University, Washington D.C., and to an anonymous reviewer, who kindly improved their English and gave valuable comments on the manuscript.

LITERATURE CITED

Beninger PG, Lanteigne C, Elner RW. 1993. Reproductive processes revealed by spermatophore dehiscence experiments and by histology, ultrastructure and histochemistry of the female reproductive system in the snow crab *Chionoecetes opilio* (*O. Fabricius*). J Crust Biol 13:1–16.

Bott R. 1970. Die Süßwasserkrabben von Europa, Asien, Australien und ihre Stammesgeschichte. Abh Senckenb Naturforsch Ges 526:1–338.

Brandis D. 2000. The taxonomical status of the freshwater crab genus *Potamiscus* Alcock 1909 (Decapoda, Brachyura, Potamidae). Senckenb Biol 80:57–100.

Brown GG. 1966. Ultrastructural studies of sperm morphology and sperm-egg interaction in the decapod *Callinectes sapidus.*. J Ultrastruct Res 14:425–440.

Cumberlidge N, von Sternberg R, Daniels SR. 2008. A revision of the higher taxonomy of the Afrotropical freshwater crabs (Decapoda: Brachyura) with a discussion of their biogeography. Biol J Linn Soc Lond 93:399–413.

Daniels SR, Cumberlidge N, Pérez-Losada M, Marijnissen SAE, Crandall KA. 2006. Evolution of Afrotropical freshwater crab lineages obscured by morphological convergence. Mol Phylogenet Evol 40:227–235.

Diesel R. 1991. Sperm competition and the evolution of mating behavior in Brachyura with special reference to spider crabs (Decapoda, Majidae). In: Bauer RT, Martin JW, editors. Crustacean Sexual Biology. New York: Columbia University Press. pp 145–163.

Du NS, Lei W, Chen LQ, Xue LZ, Li TW, Wang L. 1999. Studies on the comparative ultrastructure of crab spermatozoa (Crustacea, Decapoda, Reptantia, Brachyura). Zool Res 20:372–378 [In Chinese].

Felgenhauer BE, Abele LG. 1991. Morphological diversity of decapod spermatozoa. In: Bauer RT, Martin JW, editors. Crustacean Sexual Biology. New York: Columbia University Press. pp 322–341.

Goldner J. 1938. A modification of the Masson trichrome technique for routine laboratory purpose. Am J Pathol 14:237–243.

Guinot D. 1978. Principe d'une classification évolutive des Crustacés Décapodes Brachyoures. Bull Biol Fr Belg 112:211–292.

Guinot D, Jamieson BGM, Tudge CC. 1997. Ultrastructure and relationships of spermatozoa of the freshwater crabs *Potamon fluviatile* and *Potamon ibericum* (Crustacea, Brachyura, Potamidae). J Zool Lond 241:229–244.

Guinot D, Jamieson BGM, Richer de Forges B, Tudge CC. 1998. Comparative ultrastructure of the three dromiacean families exemplified by *Homolodromia kai* (Homolodromiidae), *Sphaerodromia lamellata* (Dromiidae), and *Dynomene tanensis* (Dynomenidae) (Podotremata: Brachyura). J Crust Biol 18:78–94.

Heidenhain M. 1917. Über neuere Sublimatgemische. Z Wiss Mikrosk 33:232–234.

Hinsch GW. 1986. A comparison of sperm morphologies, transfer and sperm mass storage between two species of crab, *Ovalipes ocellatus* and *Libinia emarginata*. Int J Invert Reprod Dev 10:79–87.

Hinsch GW. 1988. Ultrastructure of the sperm and spermatophores of the golden crab *Geryon fenneri* and a closely related species, the red crab *G. quinquedens*, from the eastern Gulf of Mexico. J Crust Biol 8:340–345.

Hinsch GW. 1991. Structure and chemical content of the spermatophores and seminal fluid of reptantian decapods. In: Bauer RT, Martin JW, editors. Crustacean Sexual Biology. New York: Columbia University Press. pp 290–307.

Jamieson BGM. 1991. Ultrastructure and phylogeny of crustacean spermatozoa. Mem Queensl Mus 31:109–142.

Jamieson BGM. 1993. Ultrastructure of the spermatozoon of *Potamonautes perlatus sidneyi* (Heterotremata, Brachyura, Crustacea). S Afr J Zool 28:40–45.

Jamieson BGM. 1994. Phylogeny of the Brachyura with particular reference to the Podotremata: Evidence from a review of

spermatozoal ultrastructure (Crustacea, Decapoda). Philos Trans R Soc Lond B Biol Sci 345:373–393.

Jamieson BGM, Tudge CC. 2000. Crustacea–Decapoda, Part 1. In: Jamieson BGM, editor. Progress in Male Gamete Ultrastructure and Phylogeny, Vol. 9, Part C of Adiyodi KG, Adiyodi RG, series editors. Reproductive Biology of the Invertebrates. Chichester: Wiley and Sons. pp 1–95.

Jamieson BGM, Guinot D, Richer de Forges B. 1995. Phylogeny of the Brachyura: Evidence from spermatozoal ultrastructure (Crustacea, Decapoda). In: Jamieson BGM, Ausio J, Justine J-L, editors. Advances in Spermatozoal Phylogeny and Taxonomy. Mem Mus Nat Hist Nat, Ser A Zool 166:265–283.

Jeyalectumie C, Subramoniam T. 1987. Biochemical composition of seminal secretions with special reference to LDH activity in the reproductive tissues of the field crab, *Paratelphusa hydrodromous* (Herbst). Exp Biol 46:231–236.

Klaus S, Schubart CD, Brandis D. 2006. Phylogeny, biogeography and a new taxonomy for the Gecarcinucoidea Rathbun, 1904 (Decapoda: Brachyura). Org Divers Evol 6:199–217.

Klaus S, Brandis D, Ng PKL, Yeo DCJ, Schubart CD. 2009. Phylogeny and biogeography of Asian freshwater crabs of the family Gecarcinucidae (Brachyura: Potamoidea). In: Martin JW, Crandall KA, Felder DL, editors. Decapod Crustacean Phylogenetics. Crustacean Issues (in press).

Matos E, Matos P, Corral L, Azevedo C. 1996. Ultrastructural and morphological aspects of the spermatozoon of *Dilocarcinus septemdentatus* Herbst, 1783 (Crustacea, Decapoda, Trichodactylidae) of the northern sea-coast of Brazil. Braz J Morphol Sci 13:31–35.

Naiyanetr P. 1994. On three new genera of Thai ricefield crabs allied to *Somanniathelphusa* Bott, 1968 (Crustacea: Decapoda: Brachyura: Parathelphusidae). Raffles Bull Zool 42:695–700.

Subramoniam T. 1991. Chemical composition of spermatophores in decapod crustaceans. In: Bauer RT, Martin JW, editors. Crustacean Sexual Biology. New York: Columbia University Press. pp 308–321.

Tudge CC, Justine J-L. 1994. The cytoskeletal proteins actin and tubulin in the spermatozoa of four decapod crabs (Crustacea, Decapoda). Acta Zool 75:277–285.

Uma K, Subramoniam T. 1979. Histochemical characteristics of spermatophore layers of *Scylla serrata* (Forskal) (Decapoda: Portunidae). Int J Invert Reprod Dev 1:31–40.

von Sternberg R, Cumberlidge N, Rodríguez G. 1999. On the marine sister groups of the freshwater crabs (Crustacea: Decapoda: Brachyura). J Zoolog Syst Evol Res 37:19–38.

Wang L, Du NS, Lai W. 1999. Studies on spermiogenesis of a freshwater crab *Sinopotamon yangtsekiense* (Crustacea Decapoda). Acta Hydrobiol Sin 23:31–33 [In Chinese].