

# Spermatozoon ultrastructure in seven South American species of *Tegula* Lesson, 1835 (Mollusca: Vetigastropoda) and the phylogenetic implications for the subfamily Tegulinae

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## Summary

This study presents results of the examination of the ultrastructure of the spermatozoon of seven South American species of *Tegula* using light and transmission electron microscopy. In all cases the spermatozoa were of the primitive or ect-aquaspermatozoon type, common in species which employ external fertilization as part of their reproductive strategy. The spermatozoon of *Tegula*, from anterior to posterior, are composed of: (a) a bullet-shaped head with an anterior acrosome and a basal nucleus, (b) a mid-piece with a prevalent number of five mitochondria plus proximal and distal centrioles, and (c) a flagellum with a 9+2 arrangement of microtubules. The ultrastructural dimensions of the spermatozoa and the characteristics of the acrosome and the nucleus of the *Tegula* species studied here, as well as those previously published in the literature, showed that each species produces male gametes having a species-specific morphology. The results also suggest that the ultrastructure of the spermatozoon could be potentially useful for recognizing subgenera within *Tegula*. The comparative study showed that some characters of the spermatozoon of the Tegulinae (e.g. length of the head, acrosome shape, acrosome length as percentage of total head length) agree with fossil, morphological and molecular evidence which suggest that members of this subfamily are more closely related to representative of the family Turbinidae.

**Key words:** Tegulinae, systematic, taxonomy, spermatozoon morphology, Chile

## Introduction

The subfamily Tegulinae Kuroda, Habe and Oyama, 1971 includes species which belong to the genera *Norrisia* Bayle, 1880, represented by the single North Pacific species *N. norrisi* Sowerby II, 1838, and *Tegula* Lesson, 1835, which contains a few more than

40 species inhabiting intertidal and subtidal environments of the Pacific basin, Caribbean, and Atlantic coast of South America (Keen, 1971; Abbott, 1974; Hickman and McLean, 1990; Hellberg, 1998).

The classification of *Tegula* species from the southern shores of South America has previously been

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based on shell morphology (e.g., Carcelles, 1945; Ramírez, 1981; Alamo and Valdivieso, 1987; among others). This typological nomenclature was the probable cause of variations in the number of species recognized by different authors. In fact, more than 50 species names for these taxa have been obtained from the literature (Collado, 2005). This suggests the limited value of relying on shell characteristics, opening this taxonomy to new types of characters which reflect on the systematics of the species promoting correct identification. Previous studies carried out on *Tegula* suggested that the morphology of the spermatozoon might be useful in the taxonomic definition of the species (Koike, 1985; Brown, 1992).

In the present study we analyze the ultrastructure of the spermatozoon from a number of South American *Tegula* species, and together with data available in the literature for other species of the genus, discuss its relevance at the specific level. Traditionally, the genus *Tegula* has been divided into five subgenera: *Agathistoma* Olsson and Harbison, 1953, *Chlorostoma* Swainson, 1840, *Promartynia* Dall, 1909, *Stearnsium* Berry, 1958 and *Tegula s.s.* Keen, 1971 (Keen, 1971; Abbott, 1974; Hickman and McLean, 1990), or into three groups based on the morphology of the radula (Fritchman II, 1965). The present study evaluates the potential taxonomic utility of the morphology of the spermatozoon in delineating the subgenera proposed for *Tegula*. Since up to the present the spermatozoon morphology of the species studied by Fritchman II (1965) has not been investigated, his groupings could not be used in comparison with the present study.

The subfamily Tegulinae has been difficult to assign to a specific taxon. According to Hickman (1996), the tegulinids represent an enigmatic taxon located somewhere between the Trochidae and Turbinidae. Hickman and McLean (1990) affirmed that “the place of Tegulinae in trochacean phylogeny is open to two very different interpretations”. The first, conservative view, includes the subfamily within the Trochidae based on plesiomorphic radular and ctenidial characters. The alternative interpretation is that they represent a turbinid group that lost the ability to form a calcareous operculum. The late appearance of *Tegula* in the fossil record, during the Miocene, the geographic distribution of the species, and the observation that the radula of the Tegulinae “is essentially a turbinid radula” favors the second alternative (Hickman and McLean, 1990). More recently, Bouchet et al. (2005) and Kano (2008) included the Tegulinae as a subfamily of the Turbinidae based on radular and DNA sequence data. However, considering that in these studies the anatomical characters were exclusively limited to the radula (Bouchet et

al. 2005), a new set of morphological data are needed to support this systematic arrangement.

Various studies have demonstrated the usefulness of spermatozoon morphology in taxonomic, systematic, and phylogenetic studies of the Vetigastropoda (Koike, 1985; Hodgson et al., 1990; Hodgson and Foster, 1992; Hodgson and Chia, 1993; Hodgson, 1995; Healy, 1988, 1989, 1990a; Healy and Harasewych, 1992; Healy et al., 1998; Collado and Brown, 2003; 2005; 2006; among others). The object of the present study was to assess potential usefulness in distinguishing South American species of *Tegula* and examining the systematic affinities of the Tegulinae using data on spermatozoon morphology.

## Materials and Methods

### *Choice and collection of taxa*

The present study examines the ultrastructure of the spermatozoa in each of the seven species of *Tegula* presently recognized in southern South America (Ramírez, 1976, 1981; Collado, 2005): *Tegula atra* (Lesson, 1830), *Tegula euryomphala* (Jones, 1844), *Tegula ignota* Ramírez, 1976, *Tegula luctuosa* (D’Orbigny, 1841), *Tegula patagonica* (D’Orbigny, 1835), *Tegula quadricostata* (Wood, 1828) and *Tegula tridentata* (Potiez and Michaud, 1838). Regarding the last species, Brown (1992) studied the morphology of the spermatozoon, but did not include its ultrastructural dimensions as obtained in the present study. Individuals of *T. atra*, *T. euryomphala*, *T. luctuosa*, *T. quadricostata* and *T. tridentata* were collected at La Herradura, Coquimbo, in Chile’s IV Region (29° 57.94’ S; 071° 21.15’ W). *T. ignota* was obtained at La Boca, in Chile’s VI Region (33°54’ S; 71°49’ W) and *T. patagonica* from Puerto Madryn, Argentina (42°42’ S; 65°06’ W). Voucher specimens of each species were deposited in the Museo Nacional de Historia Natural (MNHN) de Santiago, Chile, under the registration numbers 6148, 6145, 6146, 6150, 6147, 6144 and 6149, respectively.

### *Spermatozoon ultrastructure*

For transmission electron microscopy (TEM) the shell from one mature individual of each species was removed and 1 mm<sup>3</sup> pieces of the testis were obtained and fixed for two hours in 2% glutaraldehyde in 0.25 M pH 7.4 cacodylate buffer (with 17.5% sucrose) in a solution isosmolar to seawater. The samples were subsequently rinsed with the same buffer and postfixed for two hours in 2% osmium tetroxide, and buffer containing 25% sucrose. Fixation and rinsing were carried out at 4°C. The samples were dehydrated in a

series of ethanol, ethanol-acetone and acetone in ascending concentrations and embedded in Medcast hard-mixture resin. Ultrathin sections were obtained in a Reichert OM-U2 ultramicrotome, stained with uranyl acetate and lead citrate, and observed in a Zeiss 900 TEM at 80 Kv.

For morphometric data, a suspension of spermatozoa was obtained by agitating a small piece of tissue dissected from the testis and fixed in ALFAC (alcohol–formalin–acetic acid) solution. Samples of the spermatozoa suspension were deposited on glass microscope slides and stained with hematoxylin and erythrosine-orange G. Photographs of 20 spermatozoa of each species were taken in a light microscope at 1000× and ultrastructural measurements were made using the software program NIH 1.6 and given as the mean and standard deviation. The measurements included: the length of the head, length of the head plus the mid-piece, length and width of the nucleus, and length and width of the acrosome. To obtain the prevalence in the number of mitochondria, we scored these organelles in 10 to 25 midpiece per species observed in transverse sections at the TEM.

## Results

### Ultrastructure of the Tegula spermatozoon

The spermatozoa of seven South American species of the genus *Tegula* have been examined in the present study, and the overall ultrastructure of this gamete is given in this section. Species specific considerations then follow. Ultrastructural dimensions and proportions of the spermatozoa are given in Table 1. The spermatozoon of *Tegula*, as observed from the anterior to posterior portions, includes a bullet-shaped head with an anterior acrosome and a basal nucleus, a simple midpiece with mitochondria grouped around the proxi-

mal and distal centrioles, and a large flagellum with a 9+2 arrangement of microtubules which arise from the distal centriole (Fig. 1A–J). The acrosome of the spermatozoa occurs on the anterior extreme of the head, and is bell-shaped with a tendency to be conical elongated. With the exception of *T. patagonica*, the acrosomes of the spermatozoa of all species studied comprise more than 50% of the total head length (Table 1). The acrosome has a central subacrosomal space, which arises at its base and is directed toward the anterior of the organelle; its depth extends beyond the mid-zone of the acrosome. In some species the subacrosomal space houses an axial rod. The acrosomal contents are not differentiated. The nucleus of the spermatozoon is barrel-shaped, longer than wide, with condensed chromatin which sometimes presents nuclear lacunae. The nucleus has a weak U-shaped posterior invagination at the base while the anterior border presents small to well-developed invaginations. The midpiece possesses five mitochondria, rarely four or six (see below).

### Species-specific considerations

The ultrastructural details of the spermatozoon of *T. euryomphala* and *T. tridentata*, as representative of *Tegula* species, are shown in Fig. 1 (A–E and F–J, respectively). The spermatozoon head of *T. euryomphala* is 5.41 µm long × 1.39 µm in diameter (Table 1). The acrosome is bell-shaped (Fig 1A) and present granular internal content (Fig. 1A, B). The subacrosomal space extends beyond the mid-zone of the organelle. An axial rod was not observed in this species. Weak invaginations are observed on the anterior border and at the base of the nucleus (Fig 1A, C). The midpiece of contains four (n=1) or five (n=17) mitochondria (Fig. 1D). The flagellum has the typical 9 + 2 microtubule arrangement (Fig. 1E). The spermatozoon of *T. tri-*

Table 1. Ultrastructural dimensions of spermatozoa of *Tegula* spp. Measurements are given in µm (mean ± SD; n = 20 spermatozoa per species)

Species	Head length	H+mp <sup>a</sup>	Nucleus length	Nucleus breadth	Acrosomal length	Acrosomal breadth	A <sup>b</sup>	NL: NB <sup>c</sup>
<i>Tegula atra</i>	5.66	6.18 ± 0.11	2.17 ± 0.11	1.35 ± 0.04	3.49 ± 0.11	1.21 ± 0.07	61.7	1.6
<i>Tegula euryomphala</i>	5.41	5.88 ± 0.07	2.01 ± 0.08	1.39 ± 0.05	3.40 ± 0.11	1.27 ± 0.05	62.8	1.44
<i>Tegula ignota</i>	5.89	6.43 ± 0.07	2.27 ± 0.10	1.35 ± 0.05	3.62 ± 0.09	1.11 ± 0.05	61.5	1.68
<i>Tegula luctuosa</i>	5.63	6.13 ± 0.09	2.03 ± 0.10	1.45 ± 0.04	3.60 ± 0.15	1.39 ± 0.07	63.9	1.40
<i>Tegula patagonica</i>	4.53	5.08 ± 0.07	2.48 ± 0.07	1.27 ± 0.04	2.05 ± 0.12	1.10 ± 0.05	45.3	1.95
<i>Tegula quadricostata</i>	5.32	5.92 ± 0.04	2.56 ± 0.07	1.14 ± 0.05	2.76 ± 0.08	1.03 ± 0.04	51.9	2.24
<i>Tegula tridentata</i>	5.26	5.79 ± 0.07	2.09 ± 0.10	1.35 ± 0.03	3.17 ± 0.10	1.21 ± 0.05	60.3	1.54

<sup>a</sup>Length of head more mid-piece.

<sup>b</sup>Acrosome length as percentage of total head length.

<sup>c</sup>Ratio of length to breadth of nucleus.

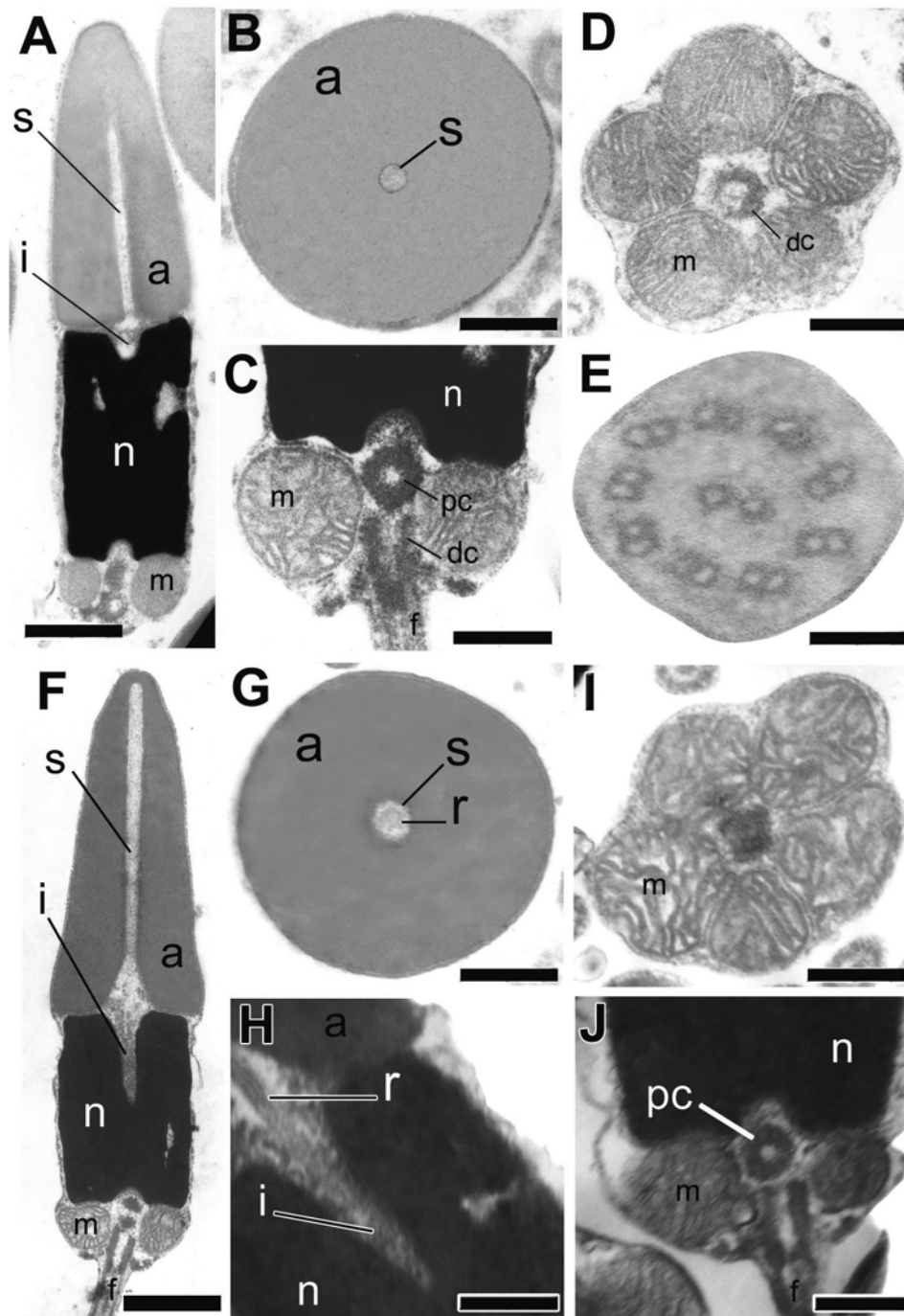


Fig. 1. TEM photomicrographs of longitudinal and transverse sections of the spermatozoon of *Tegula euryomphala* (A–E) and *Tegula tridentata* (F–J). A. Longitudinal section through acrosome, nucleus and midpiece of *T. euryomphala*. B. Transverse section through the acrosome. C. Longitudinal section through the mid-piece showing the nuclear basal invagination, mitochondria, centrioles and the proximal section of the flagellum. D. Transverse section through the midpiece showing the mitochondria and distal centriole. E. Transverse section through the flagellum. F. Longitudinal section through acrosome, nucleus and midpiece of *T. tridentata*. G. Transverse section through the acrosome. H. Longitudinal section through the base of the acrosome and anterior region of the nucleus showing the axial rod (distinct specimen from Fig. 1F). I. Transverse section through the midpiece showing the mitochondria. J. Longitudinal section through the midpiece showing the nuclear basal invagination, mitochondria, centrioles and the proximal section of the flagellum. a: acrosome; r: axial rod; dc: distal centriole; f: flagellum; i: nuclear anterior invagination; m: mitochondrion; n: nucleus; pc: proximal centriole; s: subacrosomal space. Scale bars = A: 1.0 μm; B–D: 0.5 μm; E: 0.3 μm; F: 1.0 μm; G–J: 0.5 μm.



Fig. 2. TEM photomicrographs of longitudinal sections of the spermatozoon of *Tegula* species. A. *T. quadricostata*, B. *T. patagonica*, C. *T. luctuosa*, D. *T. atra*, E. *T. ignota*. a: acrosome; i: anterior nuclear invagination; r: axial rod; m: mitochondrion; n: nucleus; s: subacrosomal space. Scale bar= 1  $\mu$ m.

*dentata* has a conical acrosome with a basal border curved toward the outside (Fig. 1F). The internal content of the acrosome is apparently homogenous (Fig. 1G). In this species the subacrosomal space penetrates deeply, nearly reaching the apex of the organelle, and bears a filament or axial rod (Fig. 1G, H). The nucleus of *T. tridentata* possesses the most pronounced U-shaped anterior invagination of the species studied (Fig. 1F, H). The midpiece contains four (n=1) or five (n=10) mitochondria (Fig. 1I). The posterior nuclear invagination is small (Fig. 1J). The flagellar axoneme has a 9+2 microtubule arrangement. In *T. quadricostata* (Fig. 2A) the acrosome is bell shaped with a concave border. An axial rod was not observed in this species. The midpiece contains five mitochondria (n=17). The sperm of *T. patagonica* (Fig. 2B) is the smallest of all the species studied, with a length of the head + midpiece of  $5.08 \pm 0.07 \mu\text{m}$  (Table 1). Also it is the only species in which the acrosome is smaller than the nucleus. The midpiece contains five (n=11) or six (n=1) mitochondria. Similarly to *T. tridentata*, the acrosome of *T. patagonica* has a basal border curved toward the outside; the subacrosomal space extends deeply toward the apex of the organelle and bears an axial rod. In *T.*

*luctuosa* (Fig. 2C) and *T. atra* (Fig. 2D) the acrosome of the sperm is bell shaped with a tendency toward being conical. In *T. atra* the subacrosomal space extends beyond the middle zone of the acrosome while in *T. luctuosa* its depth is less and it is narrower. The midpiece of *T. atra* and *T. luctuosa* contain five mitochondria (n=25 and n=22, respectively). In *T. ignota* (Fig. 2E) the average total length of the head + midpiece measured  $6.43 \pm 0.07 \mu\text{m}$ , being the largest measures among all the species studied (Table 1). In this species the acrosome is conical and pointed, with a deep subacrosomal space. The midpiece contains five mitochondria (n=12).

## Discussion

The descriptions of the spermatozoon of species belonging to the vetigastropods were summarized by Hodgson (1995). With the exception of the Skeneidae, which have dimorphic spermatozoa (Healy, 1990b), the vetigastropods produce a single type of spermatozoon, which is of the primitive (Franzén, 1955) or ect-aquaspermatozoon type (Jamieson, 1987), typical of species which employ external fertilization as a repro-

ductive strategy. All the species of *Tegula* examined in the present study had this type of gamete. Sperm dimorphism within Prosobranchia is limited to taxa with internal fertilization (Healy, 1990b).

The ultrastructural dimensions and the characteristics of the acrosome and nucleus of the spermatozoa of *Tegula* showed that each species has a gamete with unique morphology. This factor allowed distinguishing between species, as well as differentiating them from the Asiatic species *Tegula argyrostoma* (Gmelin, 1791) and *Tegula pfeifferi* (Philippi, 1846) studied by Koike (1985) (under the names *Chlorostoma argyrostoma lischkei* and *Omphalius pfeifferi pfeifferi*, respectively). The midpiece of the spermatozoon showed small variation when considering all the species of *Tegula*, similar to findings for other vetigastropods (Hodgson and Foster, 1992; Healy et al., 1998). It is important to know that the examination of spermatogenesis in the South American species of *Tegula* (unpublished data of authors) suggests that the spermatozoon of *T. pfeifferi* as reported by Koike (1985) probably represents a spermatid, and for this reason, we did not include this species in our subsequent discussion.

The ultrastructure of the spermatozoon of eight *Tegula* species is presently known, including the South American species *T. atra*, *T. euryomphala*, *T. ignota*, *T. luctuosa*, *T. patagonica*, *T. quadricostata* and *T. tridentata* (present study) and the Asiatic species *T. argyrostoma* (Koike, 1985). With the exception of *T. ignota*, a taxon until now not assigned to any of the subgenera proposed for the genus, all of these species have been assigned to the subgenus *Chlorostoma*. The characteristics of the spermatozoon of the species studied does not, however, appear to support the current subgeneric classification. For example, the spermatozoon of the Asiatic species *T. argyrostoma* is clearly distinguishable from the spermatozoa of the South American species due to the abrupt thinning observed in the middle portion of the acrosome, and its nucleus is much wider anteriorly (Koike, 1985; Fig. 3 in the present study). Observations on the spermatozoa allow division of the South American species into two groups. One group is made up of *T. patagonica*, *T. quadricostata* and *T. tridentata*; in these species the spermatozoa shows a similar pattern in the form of the acrosome and in the presence of a deep subacrosomal space. The second group includes *T. atra*, *T. ignota* and *T. luctuosa*; in these species the spermatozoa are larger, the acrosome is conical or bell-shaped, and the subacrosomal space is shallow. With the exception of the size, the spermatozoon of *T. euryomphala* is similar to the latter group. These phenetic groups are more consistent with recent molecular analyses (Hellberg, 1998; Collado, 2005) carried out on *Tegula*, which

revealed the existence of five monophyletic groups having common its geographic origin. The clades are made up of species from Asia, Chile, California, Baja California and a conglomerate of tropical and subtropical species of *Tegula* assigned mainly to the subgenus *Agathistoma*. Specifically considering the COI phylogeny of *Tegula* obtained by Collado (2005), in which the South American species are separated into two distinct clades (*T. patagonica*, *T. quadricostata* and *T. tridentata* within the *Agathistoma* clade, and *T. atra*, *T. euryomphala*, *T. luctuosa* and *T. ignota* in a Chilean clade), and in which *T. argyrostoma* appears to be strictly related to the Asiatic species, the pairing up of spermatozoon morphology and molecular analyses produces a more congruent result. This suggests a potential utility of the spermatozoon morphology to recognized subgenera within *Tegula*. It is clear that new data of spermatozoon ultrastructure of species of the genus belonging to different clades are required to further support this consideration. In vetigastropods, subgeneric divisions has been proposed by Gwo et al. (1997) in the genus *Haliotis* Linnaeus, 1758 regarding the length of the spermatozoon head, although these groupings have been questioned by Healy et al. (1998).

The data available on the ultrastructure of the spermatozoon also allow us to examine the phylogenetic affinity of the Tegulinae within Trochoidea. According to Hodgson (1995), the spermatozoan of the Trochidae has a barrel shaped nucleus with a length: width ratio of <4:1 and a U-shaped anterior invagination. The acrosome usually occupies 50% or less of the total length of the head, has a narrow subacrosomic space, and its contents may or may not show differentiation. Most of the species bear an axial rod. Hodgson (1995), reporting on the Turbinidae, indicated that the head of the spermatozoon has a nucleus with a length: width ratio of <1.5:1 and a wide anterior invagination. The acrosome is conical, representing more than 50% of the total head length. Also the acrosome had a differentiated internal content and a short and narrow invagination. The characteristics of the spermatozoon of the Turbinidae are apomorphic with respect to the Trochidae (Hodgson and Foster, 1992; Hodgson, 1995). Since the acrosomal contents of the Trochidae may or not be differentiated, this character cannot be used to evaluate the phylogenetic affinity of the Tegulinae, nor can the characteristics of the subacrosomal space, or presence/absence of an axial rod, since these characters vary among species and groups (see Hodgson and Foster, 1992). An example of the incongruence of characters within the Trochidae could be illustrated by the spermatozoa of *Gibbula umbilicalis* (da Costa 1778) and *Gibbula cineraria* (Linnaeus, 1758), considering that the first species presents an axial rod and differentiated acrosomal

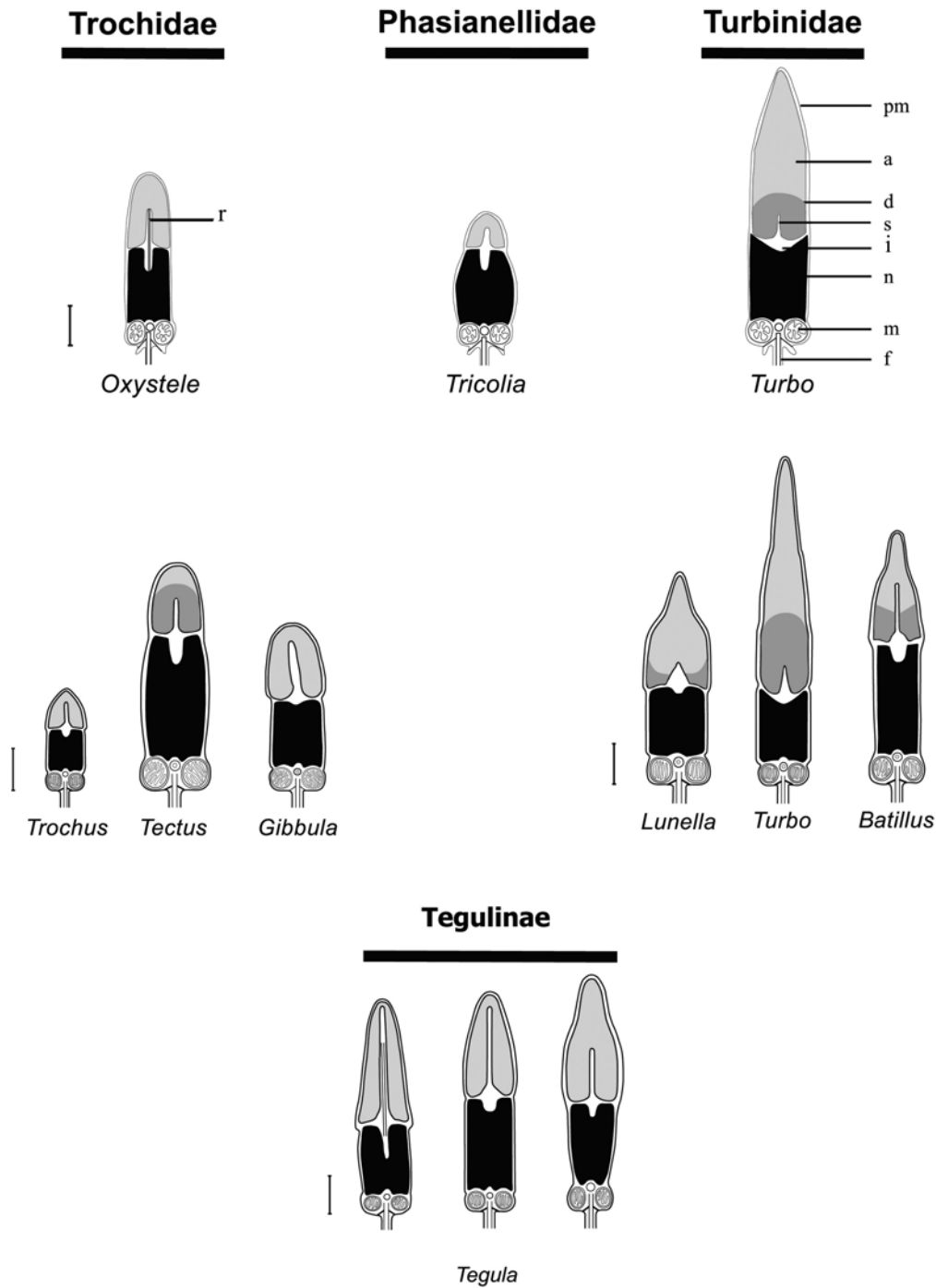


Fig. 3. Longitudinal sections through spermatozoa characteristics of three families of Trochozoa; modified from Hodgson and Foster (1992) and Hodgson (1995). The representative species of genera and families chosen by these authors were *Oxysteles sinensis* (Gmelin, 1791), *Tricolias capensis* (Dunker, 1846) and *Turbo sarmaticus* Linné, 1758. In the centre of the figure are shown other representatives of the family Trochidae and Turbinidae. Genera chosen as representatives of the Trochidae are, from left to right, *Trochus nigropunctatus* Reeve, 1861 after Hodgson and Foster (1992), *Tectus pyramis* (Born, 1778) after Koike (1985) and *Gibbula cineraria* after Sousa et al. (1995). Genera chosen as representatives of the family Turbinidae are, from left to right, *Lunella granulata* (Gmelin, 1791) after Koike (1985), *Turbo cidaris natalensis* Krauss, 1846 after Hodgson and Foster (1992) and *Batillus cornutus* Lightfoot, 1786 after Koike (1985). Beneath in the figure are shown, from left to right, three representatives of the subfamily Tegulinae: *T. tridentata*, *T. quadricostata*, present study, and *T. argyrostoma* from Koike (1985). a: acrosome; i: anterior nuclear invagination; r: axial rod; d: differentiated acrosomal content; f: flagellum; m: mitochondrion; n: nucleus; pm: plasma membrane; s: subacrosomal space. Scale bar = 1  $\mu$ m.

content (Azevedo et al., 1985); these features are absent in the second species (Sousa et al., 1995). Similarly, several species of Turbinidae lack of an axial rod (Hodgson and Foster, 1992), but it is present in *Turbo cornutus* Lightfoot, 1786 (Shiroya and Sakai, 1992). The quantitative characters length and breadth of the nucleus obtained in the present study are also inappropriate taxonomic characters, since in the Tegulinae this value ranges between 2.0 to 2.2 and 1.2 to 1.5  $\mu\text{m}$ , respectively (Table 1), a range which also may be found in Trochidae and Turbinidae (Hodgson and Foster, 1992). The same applies to character ratio of length to breadth of head.

We consider that the form of the acrosome of the spermatozoon of the tegulinids studied is a qualitative character that more closely resembles the Turbinidae when compared to other members of families existing within the Trochoidea (Fig. 3). Some quantitative characters also suggest a close affinity with turbinids: (1) length of the head; (2) length of the acrosome; (3) acrosome length as percentage of total head length (with the exception of *T. patagonica*, this proportion is greater than 50%, Table 1). The measures of length of the head and acrosome of *Tegula* species obtained in the present study and Koike (1985) are notably different from comparable values reported for representatives of the Trochidae (see Hodgson and Foster, 1992). These characters suggest that the Tegulinae, within the Trochoidea, could be phylogenetically more closely related to representatives of the Turbinidae, as is suggested by fossil record, distribution data of the species and some characters of the radula (Hickman and McLean, 1990). These data support the placement of the tegulinids within the family Turbinidae as was proposed by Bouchet et al. (2005) and Kano (2008). Apart of this consideration, the present study has shown that the morphology of the spermatozoa can be useful for discriminating among *Tegula* species. Additionally, the usefulness of the ultrastructure of the *Tegula* spermatozoon is promising for future research at the subgeneric taxonomic level, particularly considering that various subgenera have been proposed for *Tegula* only using (potentially variable) shell morphology.

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