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ORIGINAL ARTICLE

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Basal chromodorid sperm ultrastructure (Nudibranchia, Gastropoda, Mollusca)

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Abstract The relationship between three genera considered basal in the Chromodorididae (*Cadlina*, *Tyrinna*, *Cadlinella*) has not yet been resolved by traditional morphological means. Here we examined the sperm ultrastructure of *Tyrinna nobilis*, *Tyrinna evelinae*, *Cadlina flavomaculata* and *Cadlina* cf. *nigrobranchiata*, with the expectation of finding phylogenetically informative characters. No *Tyrinna* or *Cadlina* species showed sperm similarities to *Cadlinella*. Both *Cadlina* species and *Tyrinna nobilis* (but not *T. evelinae*) exhibited coarse striations in the acrosomal pedestal. The putative fibers that occurred between the coarse striations of the pedestal are condensed into a layer in *Cadlina* and *Tyrinna*, but not in other species that also have coarse striations (*Gymnodoris*), and may constitute evidence for a close relationship. *Tyrinna evelinae* possessed fine acrosomal striations, which was shared with other Chromodorididae, Actinocyclidae and the cryptobranchs *Rostanga* and *Aphelodoris*. We also examined the sperm ultrastructure of '*Chromodoris*' *ambiguus*, an animal which has shown molecular affinities to species of *Cadlina*, and not *Chromodoris*. The sperm of '*C.*' *ambiguus* did not exhibit the typical *Cadlina* characteristics, but also showed important differences to other investigated *Chromodoris* species.

Keywords Chromodorididae · Phylogeny · Spermatozoa · Opisthobranchia

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Introduction

Tyrinna, *Cadlinella* and *Cadlina* are all considered to be basal genera associated with the Chromodorididae. Some workers were inclusive in their arrangement, placing these genera within the Chromodorididae (Thiele 1931; Boss 1982; Rudman 1984; Gosliner and Johnson 1999) while others maintained a distinct Cadlinidae (Bergh 1891; Odhner 1968; Bertsch 1977; Vaught 1989). Rudman (1984) made a morphological assessment of the Chromodorididae and suggested that *Cadlinella* was the most basal of the three genera. He hypothesized *Cadlina* and *Tyrinna* as each representing two divergent lineages within the Chromodorididae. Gosliner and Johnson (1999) presented a cladistic analysis of the family showing *Cadlina* as the sister taxon to *Tyrinna* + *Cadlinella*. This was also based on anatomical morphology, but the authors failed to provide any support indices for the hypotheses generated. A recent review of *Tyrinna* (Schrödl and Millen 2001) emphasized the uncertainty in our understanding of basal chromodoridid phylogeny.

To date, all studies have relied on traditional anatomical morphology, and no ultrastructural or molecular studies have specifically tested the relationships of basal taxa associated with the Chromodorididae. Considerable differences in sperm ultrastructure have been detected between *Cadlinella* and other chromodoridids (Wilson and Healy 2002a, b). While most chromodoridids possess a solid, conical acrosomal pedestal with fine internal striations, *Cadlinella ornatissima* (Risbec 1928) has a longitudinally inrolled, homogeneous acrosomal pedestal with an axial structure present within the pedestal cavity. Additional sperm ultrastructural work on the proposed sister group of the Chromodorididae, the Actinocyclidae, demonstrated that both groups (minus *Cadlinella*) share a finely striated conical acrosomal pedestal (Wilson 2005).

Our aim was to describe the sperm ultrastructure of both valid species of *Tyrinna*, and a northern and

southern hemisphere species of *Cadlina*, and determine if any can be unequivocally linked to *Cadlinella*. We have also included *'Chromodoris' ambiguus* Rudman 1987, as molecular evidence links this species with *Cadlina* (Wilson and Lee 2005). We believe that these new ultrastructural descriptions may contribute to resolving some of the present controversy surrounding the evolution of the Chromodorididae. Given the hypotheses outlined above, we might expect to see 1) either *Tyrinna* or *Cadlina* showing sperm ultrastructural similarities to *Cadlinella*; 2) *Tyrinna* showing more similarity to *Cadlinella* than *Cadlina*; and/or 3) '*Chromodoris' ambiguus* showing more similarity to *Cadlina* than other *Chromodoris* species.

Materials and methods

Specimens examined: *Cadlina* cf. *nigrobranchiata* Rudman 1985, Port Phillip Bay, Victoria, AUSTRALIA, 10 July 1999, 7 m, AM C376216; *Cadlina flavomaculata* MacFarland 1905, Royal Palms, Palos Verdes Peninsula, Los Angeles, California, USA, 15 June 2001, intertidal, AM C203860; '*Chromodoris*' *ambiguus* Rudman 1985, Port Phillip Bay, Victoria, AUSTRALIA, 1 January 2001, 7 m, SAM D19260; *Tyrinna evelinae* (Marcus 1958), Trindade, south of Rio, BRAZIL, ZSM 20040135; *Tyrinna nobilis* (Bergh 1898), Puerto Chacabuco, CHILE, 15 m, ZSM 20012200. Abbreviations: AM, Australian Museum, Sydney; SAM, South Australian Museum, Adelaide; ZSM, Zoologische Staatssammlung, München.

The two *Cadlina* specimens and '*C.*' *ambiguus* were fixed in chilled 3% glutaraldehyde in 0.1 M phosphate buffer (8% w/v sucrose), and the *Tyrinna* specimens were fixed in formaldehyde and transferred to ethanol. Glutaraldehyde fixation was carried out at 4°C for at least 12 h. Pieces of ampulla tissue were rinsed in buffer, further fixed in 4% osmium tetroxide (prepared in same buffer) and dehydrated through an ascending series of ethanol using an automated Lynx EL processor. These samples were embedded in Spurrs resin for ultra-thin sectioning. Ultrathin sections (60–90 nm) were collected on uncoated 200 μm mesh copper grids and stained with the modified uranyl acetate and lead citrate staining procedure of Daddow (1986). Grids were examined on a JEOL 1010 TEM operated at 80-kV. Measurements were taken with the aid of digital image analysis software Matrox Inspector 4. The pedestal measurement is given as the distance between the vesicle and the most anterior part of the nucleus. The overlap region of the pedestal and nucleus was measured separately. The variation in organelle measurements within an individual is represented by standard deviation. Mean midpiece length was estimated from light microscopy measurements with the mean TEM measurements for the acrosomal complex, nucleus and terminal region subtracted from whole sperm measurements.

Results

Sperm ultrastructure

In all specimens examined here the acrosomal complex consists of a rounded, membrane-bound vesicle, supported by a conical pedestal attached to the nuclear apex. The midpiece is elongate and helical, and attaches to the nucleus with a subnuclear ring. At the base of the nucleus, an invagination is filled by a bell-shaped centriolar derivative that is continuous with the axoneme/coarse fiber complex. The 9 + 2 axoneme and nine associated coarse fibers are surrounded by a mitochondrial derivative of matrix and paracrystalline materials. There is some material present where the axoneme/coarse fiber complex meets the most anterior part of the mitochondrial derivative. At least one glycogen helix is present within the midpiece, and terminates prior to the annulus. Posteriorly, there is an annular complex consisting of the annulus proper and an annular accessory body. A post annular glycogen deposit occurs in all specimens examined here.

Cadlina cf. *nigrobranchiata*

The acrosomal vesicle is ovoid (Fig. 1a), measuring 0.18 ± 0.01 μm in length (n = 3). The pedestal measures 2.11 ± 0.08 μm in length (n = 3). Longitudinal sections reveal alternating electron-lucent and electron-dense parallel bands. A thin layer of what appears to be fibrous material occurs in the mid-region of the lucent areas (Fig. 1a). Some longitudinal sections show a lateral region toward the posterior end of the pedestal to be only electron-dense (Fig. 1b). There is a region of overlap of 0.22 μm between the pedestal and the nucleus (Fig. 1b, e, i).

The nucleus measures 3.51 ± 0.13 μm in length (n = 5) (Fig. 1e) and shows some chromatin fibers (Fig. 1d), although is typically electron-dense (Fig. 1c). Two prominent keels were observed in the posterior half of the nucleus (Fig. 1e).

The coarse fibers are transversely striated, repeating at 46–48 nm (n = 2) (Fig. 1c). The midpiece measures approximately 447 μm. One secondary and one glycogen helix was observed (Fig. 1g, h).

The annulus is present at the posterior most region of the mitochondrial derivative, above and adjacent to the short annular accessory body (Fig. 1f). The axonemal microtubules extend beyond the annulus for a short way. A glycogen deposit persists beyond the annulus for 1.99 μm (Fig. 1f, j).

Cadlina flavomaculata

The acrosomal vesicle is ovoid, measuring 0.2 ± 0.02 μm in length (n = 6) (Fig. 2a). The pedestal measures 1.54 ± 0.1 μm in length (n = 6). Longitudinal

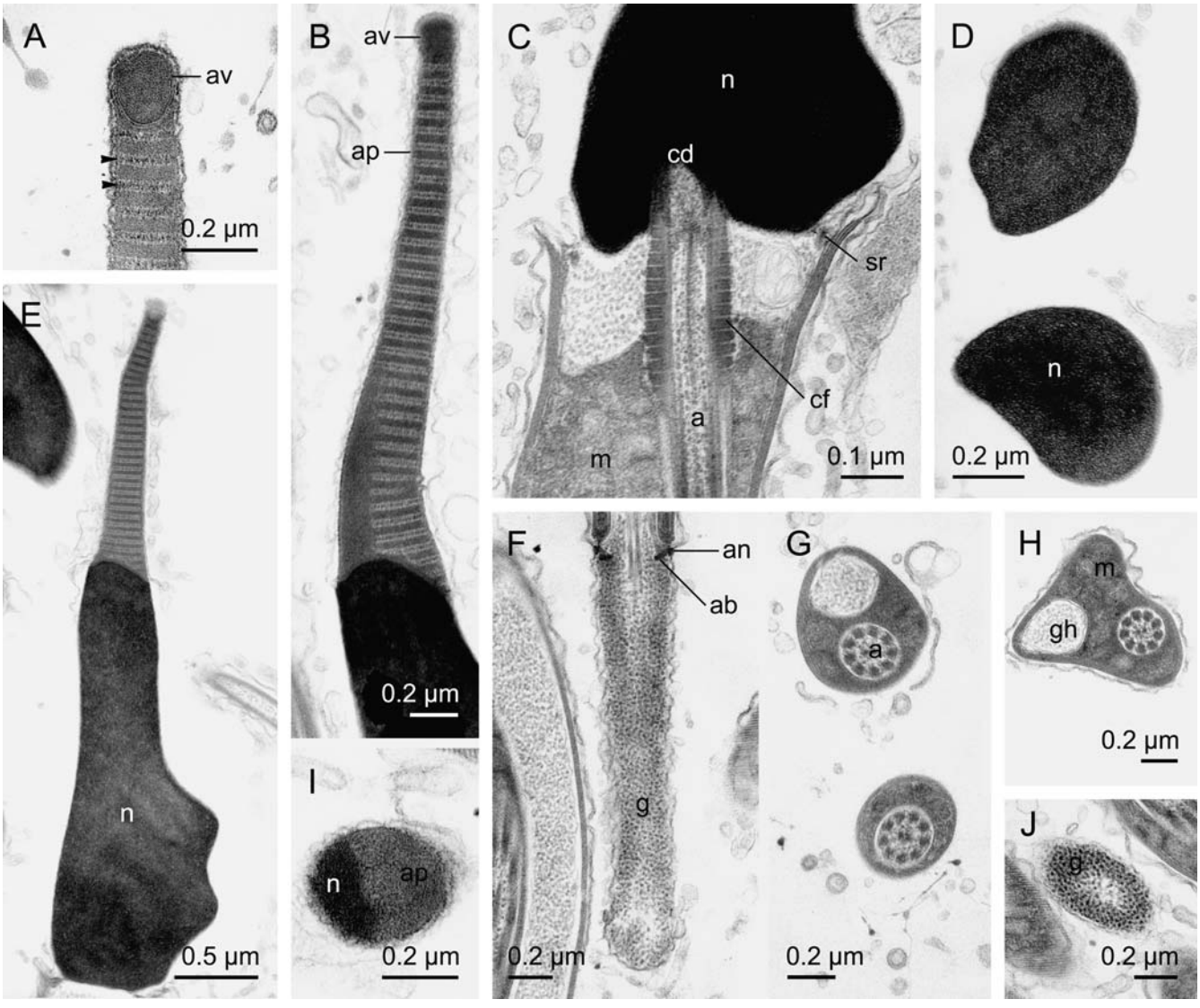

Fig. 1 Sperm ultrastructure of *Cadlina* cf. *nigrobranchiata* (glutaraldehyde, osmium fixation). **a** Longitudinal section (LS) through anterior part of acrosomal complex (acrosomal vesicle + acrosomal pedestal). Note coarse striations of pedestal. **b** LS acrosomal complex and nuclear apex. **c** LS of the nucleus/midpiece junction (neck region) showing the centriolar derivative (in nuclear invagination), subnuclear ring, striated coarse fibers and anterior region of mitochondrial derivative. **d** Transverse section (TS) through nuclei showing keels. **e** LS acrosomal complex and keeled nucleus. **f** LS posterior extremity of midpiece and entire terminal region. **g** TS midpiece with and without glycogen helix. **h** TS midpiece with both a glycogen and a secondary helix. **i** TS pedestal/nucleus overlap. **j** TS terminal glycogen deposit. *a* axoneme, *ab* annular accessory body, *an* annulus, *ap* acrosomal pedestal, *av* acrosomal vesicle, *cd* centriolar derivative, *cf* coarse fibers, *g* glycogen, *gh* glycogen helix, *n* nucleus, *sr* subnuclear ring

sections reveal alternating electron-lucent and electron-dense parallel bands. A thin layer of what appears to be fibrous material occurs in the mid-region of the lucent areas (Fig. 2b). Some longitudinal sections show a lateral region toward the posterior end of the pedestal to be only electron-dense (not illustrated). There is a region of overlap of 0.08 ± 0.03 µm (n = 6) between the pedestal and the nucleus (Fig. 2a, g).

The nucleus measures 5.05 ± 0.39 µm in length (n = 3), is highly electron-dense and shows longitudinally arranged chromatin fibers (Fig. 2c, d). No obvious helical shape or projecting keels were observed (Fig. 2d, f, g).

The coarse fibers are transversely striated, repeating at 40–46 nm (n = 2) (Fig. 2c). The midpiece length was not determined. There are no pronounced secondary helices present (formed by out-pocketing of the mitochondrial derivative) although the mitochondrial derivative was unevenly distributed around the axoneme, forming a slight rise in some parts of the midpiece (Fig. 2g). Only one glycogen helix could be detected.

The annulus is present at the posterior extremity of the mitochondrial derivative, above and adjacent to the short annular accessory body (Fig. 2e). The annular accessory body appears to consist of nine blocks (Fig. 2h). The axonemal microtubules extend beyond

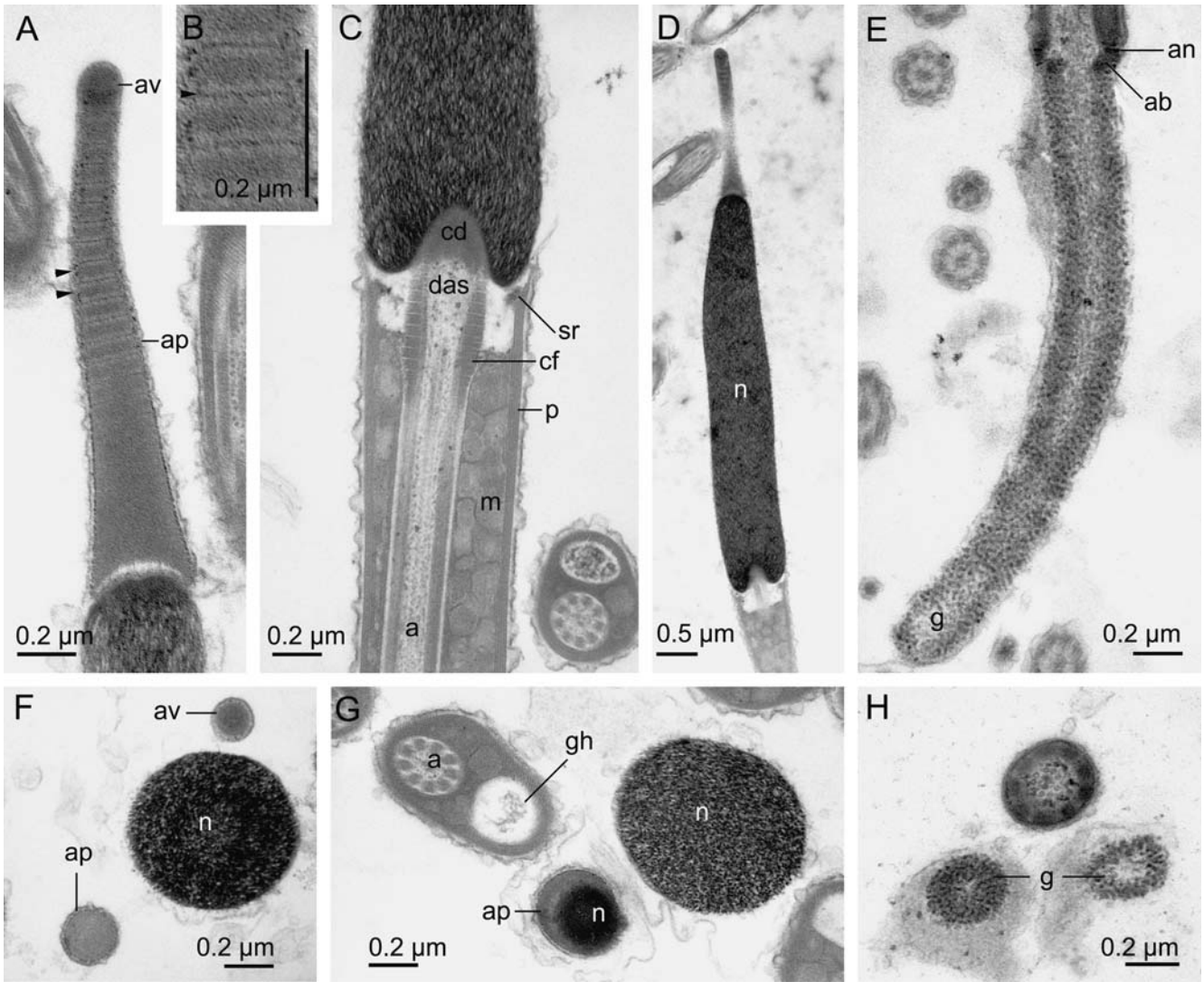

Fig. 2 Sperm ultrastructure of *Cadlina flavomaculata* (glutaraldehyde, osmium fixation). **a** Longitudinal section (LS) through acrosomal complex (acrosomal vesicle + acrosomal pedestal) and nuclear apex. Note coarse striations of pedestal. **b** Enlargement of LS acrosomal complex, showing detail of concentrated fibrous layer within electron-lucent region. **c** LS of the nucleus/midpiece junction (neck region). **d** LS acrosomal complex, nucleus and anterior extremity of midpiece. **e** LS posterior extremity of midpiece and entire terminal region. **f** Transverse section (TS) through acrosomal vesicle, nucleus and pedestal. **g** TS midpiece with uneven distribution of mitochondrial derivative, pedestal/nucleus overlap, and nucleus. **h** TS annular accessory body (nine blocks-upper) and terminal glycogen deposit (lower). *a* axoneme, *ab* annular accessory body, *an* annulus, *ap* acrosomal pedestal, *av* acrosomal vesicle, *cd* centriolar derivative, *cf* coarse fibers, *das* distal accessory sheath, *g* glycogen, *gh* glycogen helix, *n* nucleus, *p* paracrystalline, *sr* subnuclear ring

the annulus for a short way. A glycogen deposit persists beyond the annulus for 2.43 µm.

Tyrinna evelinae

The acrosomal vesicle is ovoid, measuring 0.11 ± 0.01 µm in length (n = 7) (Fig. 3a). The pedestal measures 0.17 ± 0.01 µm in length (n = 7). Longitudinal sections reveal fine parallel, transverse striations (Fig. 3a). There is a region of overlap of 0.08 ± 0.01 µm (n = 7) between the pedestal and the nucleus.

The nucleus measures 3.43 ± 0.47 µm in length (n = 6) and is typically electron-dense (Fig. 3b). No obvious sculpture or keels were observed (Fig. 3c).

The coarse fibers are transversely striated but were unable to be measured accurately (Fig. 3g). The midpiece length was also not determined. There were no secondary helices present but up to two glycogen helices were observed (Fig. 3d, e, h, i).

Details of the annular complex could not be discerned from the present material (Fig. 3f). The axonemal microtubules penetrate the full length of the glycogen deposit. A short glycogen deposit persists beyond the annulus for 0.59 ± 0.08 µm (n = 4) (Fig. 3f, i).

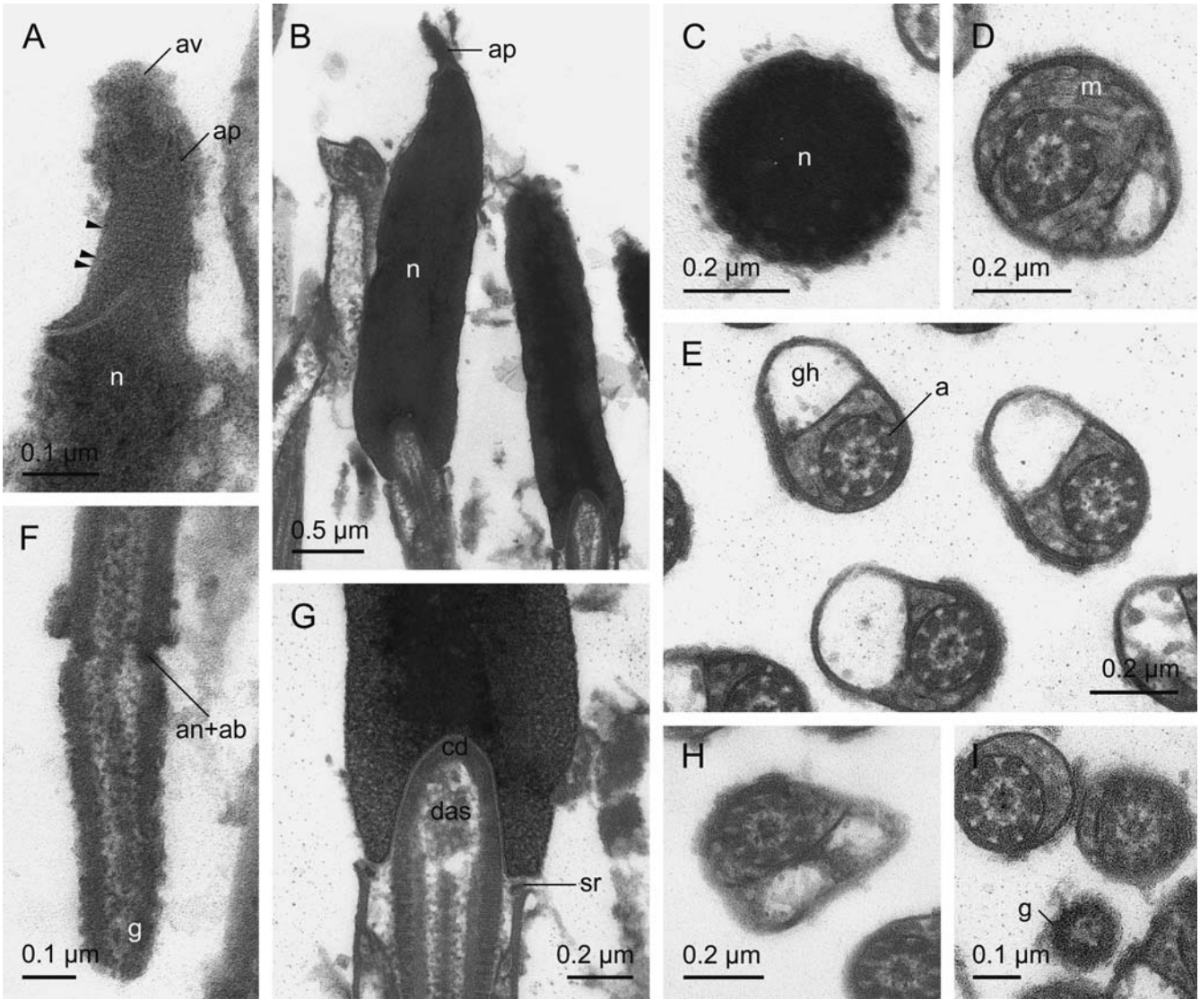

Fig. 3 Sperm ultrastructure of *Tyrinna evelinae* (formalin, ethanol, osmium fixation). **a** Longitudinal section (LS) through acrosomal complex (acrosomal vesicle + acrosomal pedestal) and nuclear apex. Note fine striations of pedestal. **b** LS acrosomal complex, nucleus and anterior extremity of midpiece. **c** Transverse section (TS) of nucleus. **d** TS midpiece showing mitochondrial derivative arranged in a lamellar pattern. **e** TS midpiece with single glycogen helix. **f** LS posterior extremity of midpiece and terminal region. **g** LS of the nucleus/midpiece junction (neck region). **h** TS midpiece with two glycogen helices. **i** TS posterior midpiece with no helices (upper) and terminal glycogen deposit (lower). *a* axoneme, *ab* annular accessory body, *an* annulus, *ap* acrosomal pedestal, *av* acrosomal vesicle, *cd* centriolar derivative, *das* distal accessory sheath, *g* glycogen, *gh* glycogen helix, *n* nucleus, *sr* subnuclear ring

Tyrinna nobilis

The acrosomal vesicle is ovoid, measuring 0.2 µm in length (Fig. 4a). The pedestal is long, and measures 1.43 ± 0.06 µm in length (n = 3). Longitudinal sections reveal alternating electron-lucent and electron-dense parallel bands (Fig. 4a). A thin layer of what appears to be fibrous material occurs in the mid-region of the lucent areas (Fig. 4g). Some longitudinal sections show an area toward the posterior end of the pedestal to be only electron-dense (Fig. 4g). There is a region of overlap of 0.25 ± 0.02 µm (n = 2) between the pedestal and the nucleus.

The nucleus measures 2.98 ± 0.32 µm in length (n = 4) and is typically electron-dense (Fig. 4b). No obvious sculpture or keels were observed (Fig. 4c).

The coarse fibers are transversely striated, repeating at 36 nm (Fig. 4i). The midpiece measures approximately 309 µm. There were no pronounced secondary helices present although the mitochondrial derivative was unevenly distributed around the axoneme, forming a slight rise in some parts of the midpiece (Fig. 4d). A maximum of three glycogen helices was observed (Fig. 4e, f, j).

The annulus is present at the posterior most region of the mitochondrial derivative, above and adjacent to the short annular accessory body (Fig. 4h). The axonemal

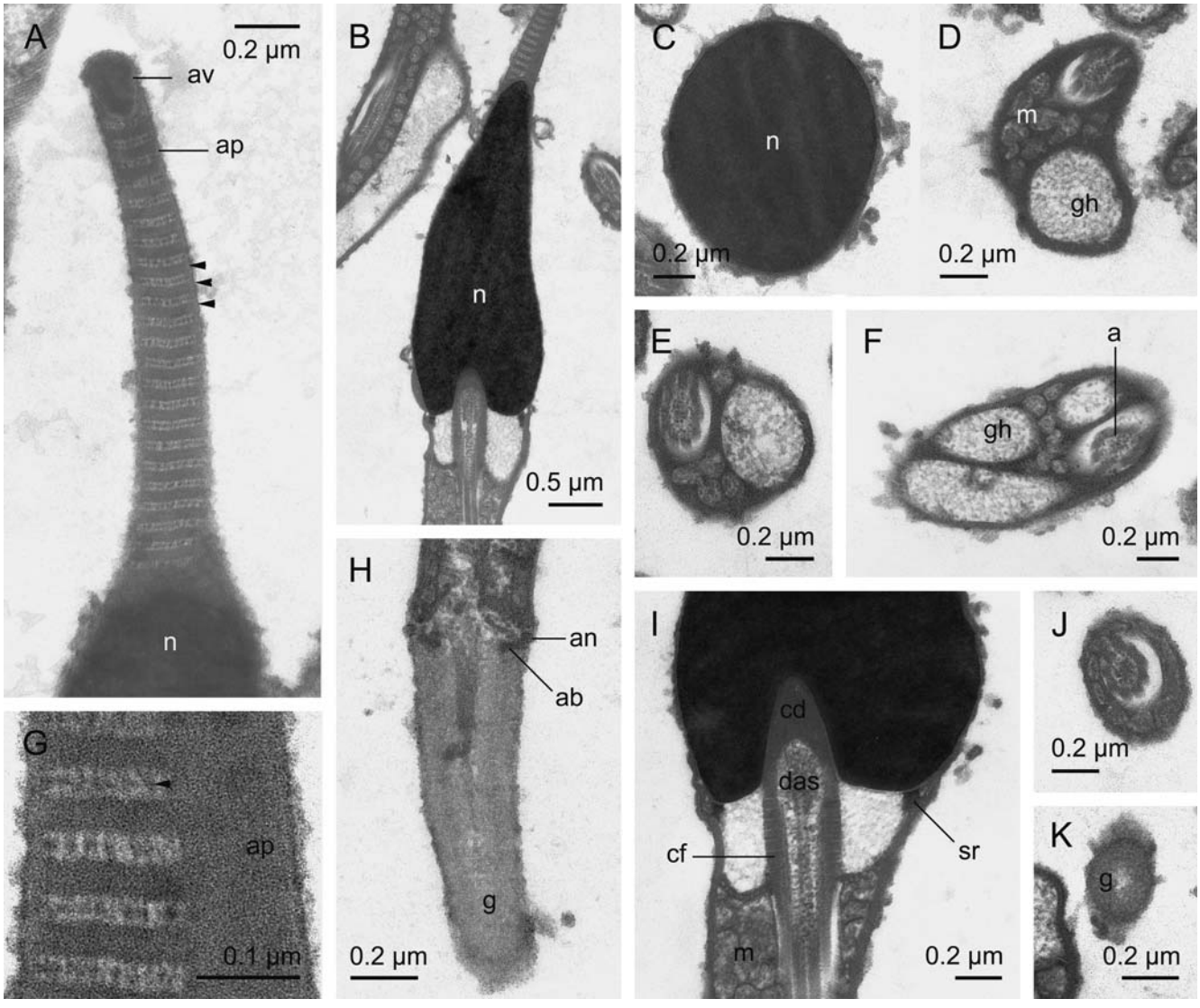

Fig. 4 Sperm ultrastructure of *Tyrinna nobilis* (formalin, ethanol, osmium fixation)**. a** Longitudinal section (LS) through acrosomal complex (acrosomal vesicle + acrosomal pedestal) and nuclear apex. Note coarse striations of pedestal. **b** LS nucleus. **c** Transverse section (TS) through nucleus. **d** TS midpiece with both a glycogen and uneven thickening of the mitochondrial derivative. **e** TS midpiece with glycogen helix. **f** TS midpiece with three glycogen helices. **g** LS detail of acrosomal pedestal, showing fibrous layer concentrated within electron-lucent region. **h** LS posterior extremity of midpiece and terminal region. **i** LS of the nucleus/midpiece junction (neck region). **j** TS posterior midpiece without helices. **k** TS terminal glycogen deposit. *a* axoneme, *ab* annular accessory body, *an* annulus, *ap* acrosomal pedestal, *av* acrosomal vesicle, *cd* centriolar derivative, *cf* coarse fibers, *das* distal accessory sheath, *g* glycogen, *gh* glycogen helix, *n* nucleus, *sr* subnuclear ring

microtubules appear to penetrate the length of the glycogen deposit. A glycogen deposit persists beyond the annulus for 1.07 ± 0.06 μm (n = 4) (Fig. 4h, k).

'Chromodoris' ambiguus

The acrosomal vesicle is ovoid, measuring 0.14 ± 0.01 μm in length (n = 5) (Fig. 5a). The pedestal measures 0.71 ± 0.08 μm in length (n = 5). Longitudinal sections reveal fine parallel, transverse striations in the pedestal (Fig. 5a, b). There is a region of overlap of 0.12 ± 0.01 μm (n = 3) between the pedestal and the nucleus.

The nucleus measures 3.98 ± 0.22 μm in length (n = 4) and shows some chromatin fibers, although can be electron-dense (Fig. 5c, f). At least one keel was observed in TS (Fig. 5c) but its positioning on the nucleus was impossible to determine on fibrous nuclei that lose sculpture.

The coarse fibers are transversely striated, repeating at 39–44 nm (n = 2) (Fig. 5g). The midpiece measures approximately 361 μm. There were no pronounced secondary helices present although the mitochondrial derivative was unevenly distributed around the axoneme, forming a slight rise in some parts of the midpiece (Fig. 5h). A maximum of three glycogen helices was observed (Fig. 5d, h, i).

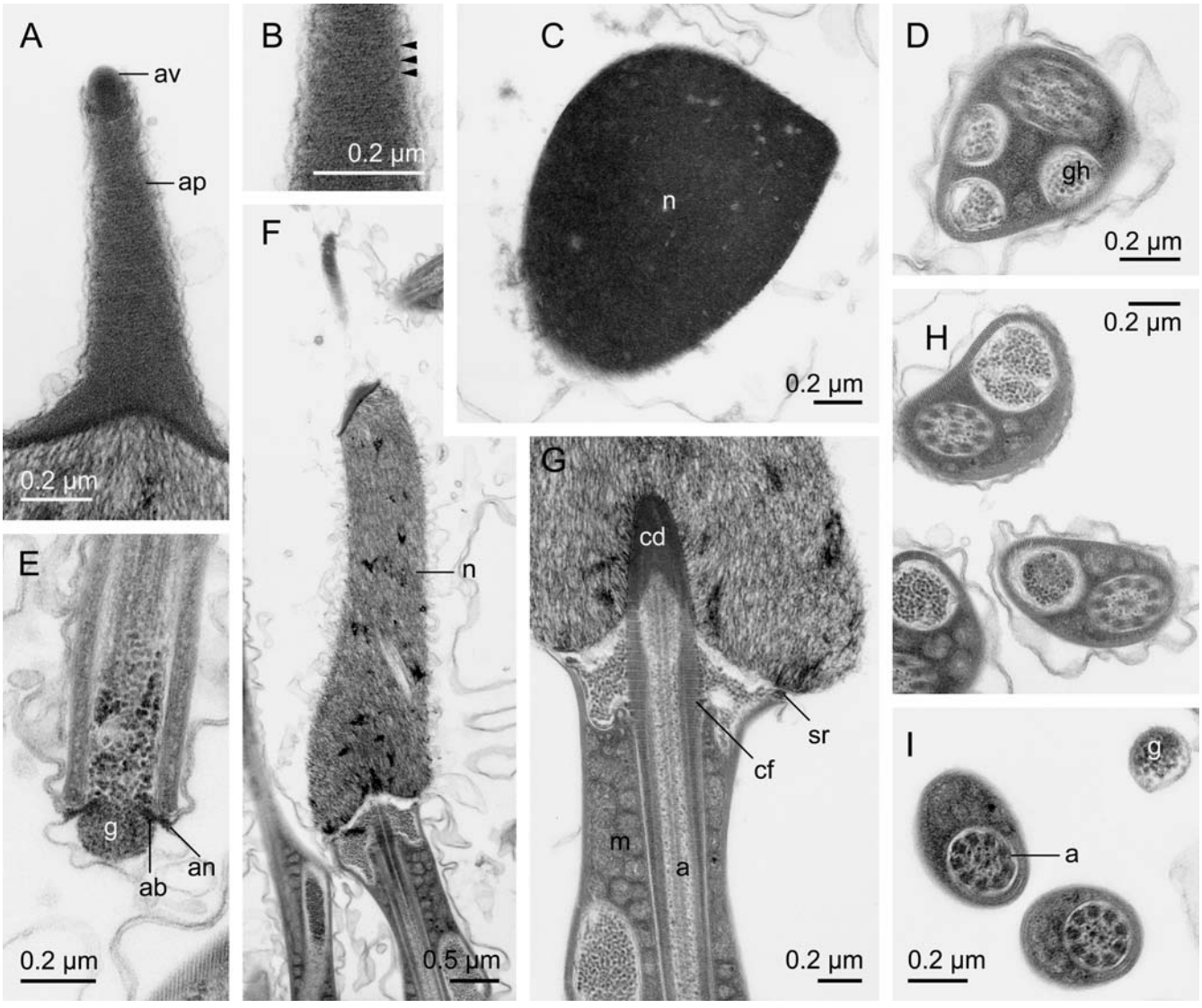

Fig. 5 Sperm ultrastructure of '*Chromodoris*' *ambiguus* (glutaraldehyde, osmium fixation)**. a** Longitudinal section (LS) through acrosomal complex (acrosomal vesicle + acrosomal pedestal) and nuclear apex. Note fine striations of pedestal. **b** Enlargement of pedestal striations. **c** Transverse section (TS) through nucleus showing keel. **d** TS midpiece with three glycogen helices. **e** LS posterior extremity of midpiece and entire terminal region. **f** LS acrosomal complex, nucleus and anterior extremity of mitochondrial derivative. **g** LS of the nucleus/midpiece junction (neck region). **h** TS midpiece (upper) with a glycogen and uneven thickening of the mitochondrial derivative, midpiece (lower) with glycogen helix. **i** TS posterior midpiece without helices (lower) and terminal glycogen deposit (upper). *a* axoneme, *ab* annular accessory body, *an* annulus, *ap* acrosomal pedestal, *av* acrosomal vesicle, *cd* centriolar derivative, *cf* coarse fibers, *das* distal accessory sheath, *g* glycogen, *gh* glycogen helix, *n* nucleus, *sr* subnuclear ring

The annulus is present at the posterior most region of the mitochondrial derivative, with the short annular accessory body adjacent to but slightly above the annulus (Fig. 5e). The axoneme appears to terminate before the annulus. A short glycogen deposit persists beyond the annulus for 0.16 ± 0.01 µm (n = 6) (Fig. 5e, i).

Discussion

Support for *Cadlina* + *Tyrinna nobilis*?

The sperm ultrastructure of the species examined here varied widely, and none showed any acrosomal similarities to *Cadlinella*. The two *Cadlina* species show consistent similarities in sperm morphology, despite hemispheric separation. Most *Cadlina* species are restricted to north or south temperate regions, essentially demonstrating a bipolar distribution for the genus (Rudman 1985). *Cadlina flavomaculata* is found from Alaska to California (Behrens 1991) whereas the *C. nigrobranchiata* complex is found on the southern coast of Australia (Rudman 1985; Wells and Bryce 1993, pers. obs). Both species exhibit coarse striations in the acrosomal pedestal, and a fibrous layer within the striations. *Cadlina* cf. *nigrobranchiata* exhibited both nuclear keels and secondary helices on the midpiece, neither of which were detected in *C. flavomaculata*.

The coarse acrosomal striations seen in *Cadlina* species were also found in *Tyrinna nobilis*, and are similar to those already known for the nudibranchs *Gymnodoris* sp. and *Kaloplocamus acutus* Baba 1949 (as *K. yatesi*) in Healy and Willan (1991). These same coarse striations also occur in at least one pleurobranch, *Pleurobranchus peroni* Cuvier 1804, and possibly also *Berthella ornata* (Cheeseman 1878) (Healy and Willan 1984). Given that the Pleurobranchoidea + Nudibranchia are now recognized as the monophyletic Nudipleura (Wägele and Willan 2000), the coarse form of striations is arguably a plesiomorphic state for the Nudipleura (the apomorphic condition having fine striations).

The putative fibers that occur in the electron-lucent layers occur longitudinally throughout the entire length of that layer in *Gymnodoris* species, but their distribution in species of *Pleurobranchus* and *Kaloplocamus* remains uncertain. In *Cadlina* spp., and *Tyrinna nobilis*, the fibers tend to be concentrated in the middle of the layer; in *Cadlina flavomaculata* the fibers are so concentrated that they almost appear as a single black line through the center of the electron-lucent layers (see Fig. 2b). If the condensing of the putative fibers within the coarse striations for *Tyrinna nobilis* and *Cadlina* sp. is a shared, derived feature, it constitutes evidence of a close relationship.

Is *Tyrinna* monophyletic?

The acrosomal pedestal of *Tyrinna evelinae* is finely striated, unlike the coarsely striated *T. nobilis*. Fine acrosomal striations are to date known to be present in the Chromodorididae (Medina et al. 1985; Healy and Willan 1991; Wilson and Healy 2002a), Actinocyclidae (Wilson 2005) and the dorid cryptobranchs *Rostanga* (Healy and Willan 1991) and *Aphelodoris* (Wilson 2003).

Schrödl and Millen (2001) assigned *Tyrinna* a monophyletic status, but clarified the possession of a vestibular gland in the two species. Although a vestibular gland has been reported for both species, its occurrence in *T. nobilis* was refuted (see Schrödl and Millen 2001). The results presented here add a suite of sperm ultrastructural differences between the two species (acrosome length and striations, nuclear length/width, glycogen deposit length), and raises the possibility of non-monophyly.

The acrosomal differences shown by *Tyrinna nobilis* and *T. evelinae* (the only two members of the genus) highlight an interesting situation. This disparity (in a conserved region of the sperm) indicates an abrupt change of character state within a genus, the extent of which would normally be associated with a change in fertilization biology (e.g., Popham 1974) or sperm storage (Jouin-Toulmond et al. 2002)- unlikely explanations in this instance. Both species fertilize their oocytes internally, and possess similar sperm storage receptacles. The change in morphology may be directly correlated with aspects of the egg envelope (Jamieson et al. 1983) but comparative investigations on mature oocytes in opisthobranchs are few. The notable exceptions are studies on already-encapsulated embryos by Eyster (1986) and Klussman-Kolb and Wägele (2001), who found conserved capsule ultrastructure across higher groupings, although neither study examined *Tyrinna*.

Should *'Chromodoris' ambiguus* be placed in *Chromodoris* or *Cadlina*?

'*Chromodoris' ambiguus* and '*C.*' *alternata* have recently been shown to have molecular affinities with *Cadlina*, rather than other *Chromodoris* species (Wilson and Lee 2005). The continued inclusion of those species in *Chromodoris* may render that genus paraphyletic. However, in the present study '*C.*' *ambiguus* displayed sperm ultrastructure that was more similar to *Chromodoris* than *Cadlina*, but some important differences were also noted. The acrosomal complex of '*Chromodoris' ambiguus* shows fine striations in the pedestal that are parallel to the transverse plane, rather than angular such as in other *Chromodoris* species (Healy and Willan 1991; Wilson and Healy 2002a). The same parallel, transverse striations are described for *T. evelinae*. The acrosome itself showed little overlap with the nucleus, a condition shared by *Cadlina* and *Tyrinna*. A moderate amount of nuclear overlap with the pedestal is usually characteristic of chromodoridids (see Wilson and Healy 2002a). The most important difference occurs in the posterior part of the sperm. All other *Chromodoris* species studied to date possess an annular accessory body that seals the terminal region of the sperm, and have no axoneme continuation or glycogen deposit beyond the annulus. Here, '*C.*' *ambiguus* has a short annular accessory body, no axonemal penetration beyond the annulus, but a very short stub of glycogen persists at the terminal region. The sperm ultrastructure of '*C.*' *ambiguus* does not necessarily support a close relationship with *Cadlina* as molecular data has suggested (Wilson and Lee 2005). However, a close relationship with *Chromodoris* is also not supported; the distinctive closed annular accessory body is lacking, and a glycogen deposit is present.

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