COMPARATIVE SPERM ULTRASTRUCTURE IN FIVE GENERA OF THE NUDIBRANCH FAMILY CHROMODORIDIDAE (GASTROPODA: OPISTHOBRANCHIA)

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ABSTRACT

Sperm ultrastructure is examined in representatives of five genera of the nudibranch gastropod family Chromodorididae: (*Chromodoris*, *Hypselodoris*, *Glossodoris*, *Risbecia* and *Pectenodoris*) and the results compared with previous work on other gastropods, especially other nudibranchs. As chromodoridid phylogeny is still incompletely understood, this study partly focuses on the search for new and as yet untapped sources of informative characters. Like spermatozoa of most other heterobranch gastropods, those of the Chromodorididae are elongate, complex cells composed of an acrosomal complex (small, rounded acrosomal vesicle, and columnar acrosomal pedestal), a condensed nucleus, subnuclear ring, a highly modified mid-piece (axoneme + coarse fibres surrounded by a glycogen-containing, helically-coiled mitochondrial derivative) and terminally a glycogen piece (or homologue thereof). The finely striated acrosomal pedestal is a synapomorphy of all genera examined here, but interestingly also occurs in at least one dorid (*Rostanga arbutus*). Substantial and potentially taxonomically informative differences were also observed between genera in the morphology of the nucleus, the neck region of the mid-piece, and also the terminal glycogen piece. The subnuclear ring is shown for the first time to be a segmented, rather than a continuous structure; similarly, the annular complex is shown to consist of two structures, the annulus proper and the herein-termed annular accessory body.

INTRODUCTION

The use of transmission electron microscopy (TEM) in the study of spermatozoa has led to significant improvements in our understanding of phylogenetic relationships in many phyla (for review, see Jamieson, Ausio & Justine, 1995). Comparative spermatozoal ultrastructure has proved useful in resolving relationships within the Mollusca, particularly among caenogastropod prosobranchs and among the various heterogastropod groups (= Allogastropoda + Opisthobranchia *sensu stricto* + Pulmonata; see Haszprunar, 1988) (Thompson 1973; Healy & Willan 1984; Healy, 1988a, 1993a, 1996). Sperm morphology is most conservative and, therefore, of most taxonomic and phylogenetic value, in groups exhibiting the same or similar environments of fertilization (e.g. all aquatic or all internal fertilizers; Franzén, 1955; Giusti, 1971; Thompson, 1973; Healy, 1983, 1988a, 1996; Kohnert & Storch, 1984; Koike, 1985; Hodgson & Bernard, 1988; Hodgson & Foster, 1992). Fortunately, this is the case in most of the 'higher' Gastropoda (all being fully internal fertilizers, though the precise mode of sperm transfer may vary), but in groups showing transitions between aquatic and internal fertilization, such as the marine Trochoidea, enormous variation in sperm structure is encountered (J. M. Healy, unpublished data).

Thompson (1966) was the first worker to examine sperm ultrastructural features in a nudibranch (*Archidoris pseudoargus* Rapp, 1827) and found strong similarities to the complex, helical spermatozoa already known to occur in the Pulmonata (André, 1956, 1962). Later he reported that some of these features, such as the intricate mitochondrial derivative, were probably general for the 'Euthyneura' (= Opisthobranchia + Pulmonata; Thompson, 1973), as the earlier light microscopic work of Retzius (1906) and Franzén (1955) had hinted. Single species studies of nudibranch sperm ultrastructure or development (Schmekel, 1971; Holman, 1972; Eckelbarger & Eyster, 1981; Medina, Moreno & Lopez-Campos, 1985, 1986; Medina, Moreno & Garcia-Herdungo, 1988a; Medina, Griffond, Garcia-Gomez & Garcia, 1988b) have been aimed at resolving cytological rather than taxonomic issues. With this in mind, Healy & Willan (1991) presented the first comparative study of nudibranch sperm ultrastructure, covering 27 species, and all four suborders (Doridina, Dendronotina, Arminina, and Aeolidina). While they were unable to recognize any synapomorphies unifying the Nudibranchia (and differentiating this group from other heterobranchs), they did demonstrate that the acrosomal complex was clearly the most taxonomically useful character and that other sperm components (nucleus, mid-piece, and glycogen piece) were also worthy of investigation. Healy & Willan recognized four subgroups of Doridina and two groups of Aeolidina based on sperm similarities, but also discerned considerable heterogeneity among the Dendronotina. Despite the wide scope of the Healy & Willan account, many genera and several families within the Nudibranchia remain unstudied or incompletely studied as regards sperm morphology, including some of the most speciose and ecologically important families such as the Chromodorididae and Polyceridae.

Although a large proportion of data from spermatozoal ultrastructure has been employed at relatively high taxonomic levels in the Gastropoda (for reviews and literature see Healy, 1988a, 1993a, 1996), family-level studies have also proven of considerable systematic and phylogenetic use (Hodgson & Bernard, 1988; Jamieson, Hodgson & Bernard, 1991; Hodgson & Foster, 1992). The present study aims to investigate the taxonomic and phylogenetic importance of spermatological characters between chromodorid genera, particularly in relation to

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the internal structure and morphology of the acrosomal complex. In addition to the five species examined herein, reference is also made to the four previously studied species of Chromodorididae: *Hypselodoris orsini* Verany 1876 [as *H. tricolor* (Cantraine, 1835) (Medina *et al.*, 1985, 1986, 1988a)], *Hypselodoris fontandrani* Pruvot-Fol, 1951 [as *H. messinensis* (Ihering, 1880) (Medina *et al.*, 1988b)], *Chromodoris annae* Bergh, 1877 (Healy & Willan, 1991) and *Glossodoris atromarginata* (Cuvier, 1804) (Healy, 1984).

MATERIALS AND METHODS

Type species of five chromodorid genera were collected for this study. Collection was typically carried out with the use of SCUBA and took place at various sites along the east coast of Australia. Voucher specimens of the five species examined for ultrastructure have been deposited in the Australian Museum (Sydney): *Chromodoris magnifica* (Quoy & Gaimard, 1832), one specimen, Orpheus Island, QLD, 6 m, (AM C.379387); *Glossodoris pallida* (Rüppell & Leuckart, 1828) one specimen, Orpheus Island, QLD, 13.5 m, (AM C.379391), one specimen, Heron Island, QLD, 13 m; *Hypselodoris obscura* (Stimpson, 1855) one specimen, North Stradbroke Island, QLD, 5.5 m, (AM C.379393); *Pectenodoris trilineata* (Adams & Reeve, 1850), two specimens, Heron Island, QLD, inter-tidal, (AM C. 386779); *Risbecia tryoni* (Garrett, 1873) one specimen, Orpheus Island, QLD, 12.5 m, (AM C.379392).

Specimens were anaesthetized by chilling in seawater to at least 3°C for 10–20 minutes, depending on the size of the animal. In each species, the sperm-filled ampulla was dissected out, diced into small portions and placed in 3% glutaraldehyde in 0.1M sodium phosphate buffer, pH 7.2 and 10% w/v sucrose at 4°C. Some specimens were preserved whole in the field. In these cases, the dorsum was sliced open to allow adequate penetration of the fixative and the ampulla dissected out later. The initial preservation always took place at 4°C for at least 12 hours.

Ampulla portions were processed using a Lynx EL microscopy tissue autoprocessor. This schedule involved the following steps at 4°C. First, the tissue was rinsed thoroughly in 0.1 M sodium phosphate buffer containing 10% w/v sucrose (three changes of 15 minutes). It was then transferred to a 1% osmium tetroxide solution (prepared in sucrose-adjusted sodium phosphate buffer) for 80 minutes and rinsed as above in buffer. This was followed by dehydration through ascending ethanols (20–100%). At 90% ethanol, the processor returned to room temperature. After dehydration, the tissues were infiltrated and embedded in Spurr's epoxy resin. The resin blocks were allowed to polymerize overnight in a 60°C oven. Semi-thin sections (approximately 1 μm thick) were cut using a glass knife, while ultra-thin sections (approximately 60–90 nm thick) were cut using a Microstar diamond knife (2.1 mm, 4°), both on an LKB 2088 Ultratome V. The ultra-thin sections were collected on 200-μm uncoated mesh copper grids, and stained with uranyl acetate and lead citrate according to the modified double lead staining procedure of Daddow (1986). The sections were then examined with a Hitachi 300 transmission electron microscope (TEM) operated at 75 kV.

The number of ultrastructural measurements and their ranges are given throughout. In some instances only a few perfect longitudinal sections were observed. Mid-piece length was determined by taking the maximum length of 10 measured spermatozoa under a light microscope, and then subtracting the combined maximum lengths of the acrosome, nucleus, and glycogen piece.

RESULTS

General sperm features of Chromodorididae

All five chromodorids investigated shared several spermatological characteristics, which are summarized below. In anterior-posterior sequence, the spermatozoon consists of four main regions—the acrosomal complex, the nucleus, the mid-piece, and the annular complex, all enclosed by the plasma membrane (refer to Figures 1–5 for detail).

Acrosomal complex. This region is divisible in to an apical, membrane-bound, acrosomal vesicle, and a non-membrane bound, column-shaped structure, the acrosomal pedestal, which supports the acrosomal vesicle and also attaches to the nuclear apex. The internal structure of the pedestal exhibits periodic striations relative to the transverse plane. In chromodorids there is a short region of overlap between the acrosomal pedestal and the nuclear apex.

Nucleus and neck. The mature nucleus contains the condensed chromatin, and under TEM always appears as the most intensely electron dense organelle. Like other sperm components, the length and shape of the nucleus are taxon-specific. A shallow invagination at the base of the nucleus is filled by a bell-shaped centriolar derivative that is continuous with the axoneme/coarse fibre complex. Dense material almost always obscures the micro-tubular structure of the axoneme where it penetrates the basal invagination of the nucleus. Slight overlap occurs between the base of the nucleus and the thin anterior extremity of the mitochondrial derivative. An electron dense, segmented subnuclear ring is present where the mitochondrial derivative flares out to meet the nucleus.

Mid-piece. The mid-piece is composed of an axoneme/coarse fibre complex and an enveloping mitochondrial derivative. Immediately posterior to the nucleus, the coarse fibres that surround the 9 + 2 axoneme are thick and transversely banded. They are surrounded by dense glycogen deposits and beyond that, by the thin anterior extremity of the mitochondrial derivative. As the coarse fibres progressed further into the mid-piece, their periodic substructure became less evident. The diameter of the fibres initially decreased, but then remained relatively constant down the remaining length of the mid-piece. The mitochondrial derivative consisted of paracrystalline and matrix materials that enclosed:

(1) the glycogen or primary helix (helical compartment containing glycogen granules);
(2) the axoneme/coarse-fibre complex.

The helical, lattice-like internal structure of the paracrystalline material was more clearly visible in oblique sections through the mid-piece.

Annular complex and glycogen piece. Immediately posterior to the mid-piece is an annular complex, consisting of the annulus proper (a simple, highly electron dense ring attached to the inside surface of the plasma membrane at the end of the mid-piece), and a cylindrical or funnel-shaped body, which is attached to the annulus proper and projects into the glycogen piece (herein termed the annular accessory body). Typically, the terminal region of the spermatozoon exhibits a discrete glycogen piece.

Description of sperm from TEM observations

Genus *Chromodoris* Alder & Hancock, 1855

***C. magnifica* (Quoy & Gaimard, 1832) (Figure 1)**

Acrosomal complex. The acrosomal vesicle rests in a shallow depression at the anterior end of the acrosomal pedestal (Figure 1B). The vesicle is ovoid (ranging from 0.1–0.13 μm in length, $n = 3$; Figure 1B,E). The pedestal is long and conical (0.88–0.97 μm long, $n = 3$; Figure 1B). Longitudinal sections through the pedestal often reveal very fine parallel striations orientated at approximately 21° to the transverse plane (Figure 1B).

Nucleus and neck: The nucleus is 5.29 μm long ($n = 1$) and shows a single, angular helical keel (Figure 1A). Where the axoneme penetrates the nuclear invagination, the doublets always remain distinct from the coarse fibres, but in contact with them (Figure 1F).

Mid-piece. The mid-piece measures approximately 299 μm in length. The coarse fibres surrounding the axoneme exhibited bands at a periodicity of 42 nm (Figure 1C). The matrix component of the mitochondrial derivative forms a secondary helix (Figure 1G,I). Progressing posteriorly, the secondary helix is gradually lost (Figure 1H,J). Similarly, the glycogen helix reduces in size along the length of the mid-piece, until it too disappears (Figure 1D).

Annular complex. The terminal region of the mid-piece consists of the annulus and the annular accessory body, which seals off the distal end of the spermatozoan (Figure 1D). The axoneme appears to terminate a short distance from the annular complex. Prior to the annular accessory body the mid-piece is filled with glycogen granules, although no glycogen piece exists beyond it.

Genus *Glossodoris* Ehrenberg, 1831

***G. pallida* (Rüppell & Leuckart, 1828) (Figure 2)**

Acrosomal complex. The acrosomal vesicle is ovoid (0.1 μm in length, $n = 2$; Figure 2B). The pedestal is conical and short (0.23 μm, $n = 1$). Longitudinal sections through the pedestal often reveal striations parallel (but slightly concave) to the transverse plane (Figure 2B). The periodicity of the acrosomal pedestal striations is 13 nm.

Nucleus and neck. The nucleus is elongate (11.56 μm, $n = 1$) and shows strong helical keeling (Figure 2A,B,D). Where the axoneme penetrates into the nuclear invagination, the doublets remain distinct from the coarse fibres, although usually in contact with them (Figure 2E).

Mid-piece. The length of the mid-piece was not ascertained from this specimen, but a second animal was later collected, which had a mid-piece length of 198 μm. The periodicity of the coarse fibres measured 56 nm (Figure 3C). No secondary helices are formed by the matrix component of the mitochondrial derivative (Figure 2G,I). Progressing posteriorly, the glycogen helix remains similar in size until it reaches the terminal region (Figure 2F).

Annular complex and glycogen piece. The annular accessory body incompletely seals the disrupted axoneme (Figure 2H). There is a small glycogen piece (measuring 0.5–0.53 μm), which appears partially penetrated by the most distal part of the axoneme (Figure 2H,J).

Genus *Hypselodoris* Stimpson, 1855

***H. obscura* Stimpson, 1855 (Figure 3)**

Acrosomal complex. The acrosomal vesicle is spherical (ranging from 0.1 to 0.12 μm in diameter, $n = 3$; Figure 3B,C). The pedestal is conical and short (0.23–0.35 μm, $n = 2$; Figure 3B,C). Longitudinal sections through the pedestal often reveal striations (periodicity of 14 nm) parallel to the transverse plane (Figure 3B,C).

Nucleus and neck. The nucleus is short (4.07–4.33 μm, $n = 2$), and possesses two or possibly three, rounded, helical keels (Figure 3A,E). The doublets remain distinct from the coarse fibres and appear to cluster around the distal accessory sheath (Figure 3F).

Mid-piece. The mid-piece in *Hypselodoris obscura* measures approximately 476 μm in length. The banding periodicity of the coarse fibres is approximately 50 nm (Figure 3G,L). The matrix component of the mitochondrial derivative forms a secondary helix in the section of the mid-piece that is immediately post-nuclear (Figure 3G). Progressing posteriorly, the secondary helix is gradually lost (Figure 3H,J,N). Similarly, the glycogen helix diminishes in size along the length of the mid-piece, until it too is lost (Figure 3I,K,N). In one spermatozoan, there were two glycogen helices visible (Figure 3J).

Annular complex and glycogen piece. The annular accessory body incompletely seals the disrupted axoneme (Figure 3M). A small glycogen piece persists beyond the annulus proper for 0.4 μm (Figure 3M). The axoneme appears to terminate at the annular accessory body.

Genus *Pectenodoris* Rudman, 1984

***P. trilineata* (Adams & Reeve, 1850) (Figure 4)**

Acrosomal complex. The acrosomal vesicle is elongate, ovoid and shows some lateral constriction (0.13 μm in length, $n = 1$; Figure 4B,C). The pedestal is long, slender, and cylindrical (0.95 μm, $n = 1$; Figure 4B). Longitudinal sections through the pedestal often reveal striations parallel to the transverse plane, repeating at 12 nm.

Nucleus and neck. The nucleus is 7.1 μm long ($n = 1$), and shows no helical keeling (Figure 4A,D,G). The doublets remain distinct, but aligned with the coarse fibres (Figure 4F). The subnuclear ring appears to be segmented (Figure 4F,I).

Mid-piece. The mid-piece measures approximately 242 μm in length. The banding periodicity of the coarse fibres could not be determined as no longitudinal sections through the neck region were observed. No secondary helices could be detected in the small amount of tissue available for study, although the presence of such structures remains possible (Figure 4E). Up to three glycogen helices are present, all of which decrease in size as they progress posteriorly (Figure 4H,J).

Annular complex and glycogen piece. The terminal region of the mid-piece was not observed.

Genus *Risbecia* Odhner, 1934

***R. tryoni* (Garrett, 1873) (Figure 5)**

Acrosomal complex. The acrosomal vesicle is spherical (0.08–0.09 μm in diameter, $n = 2$). The pedestal is cylindrical and short (0.28 μm, $n = 1$; Figure 5B). Longitudinal sections through the pedestal often reveal striations parallel to the transverse plane (Figure 5C). The periodicity of these pedestal striations is 12 nm.

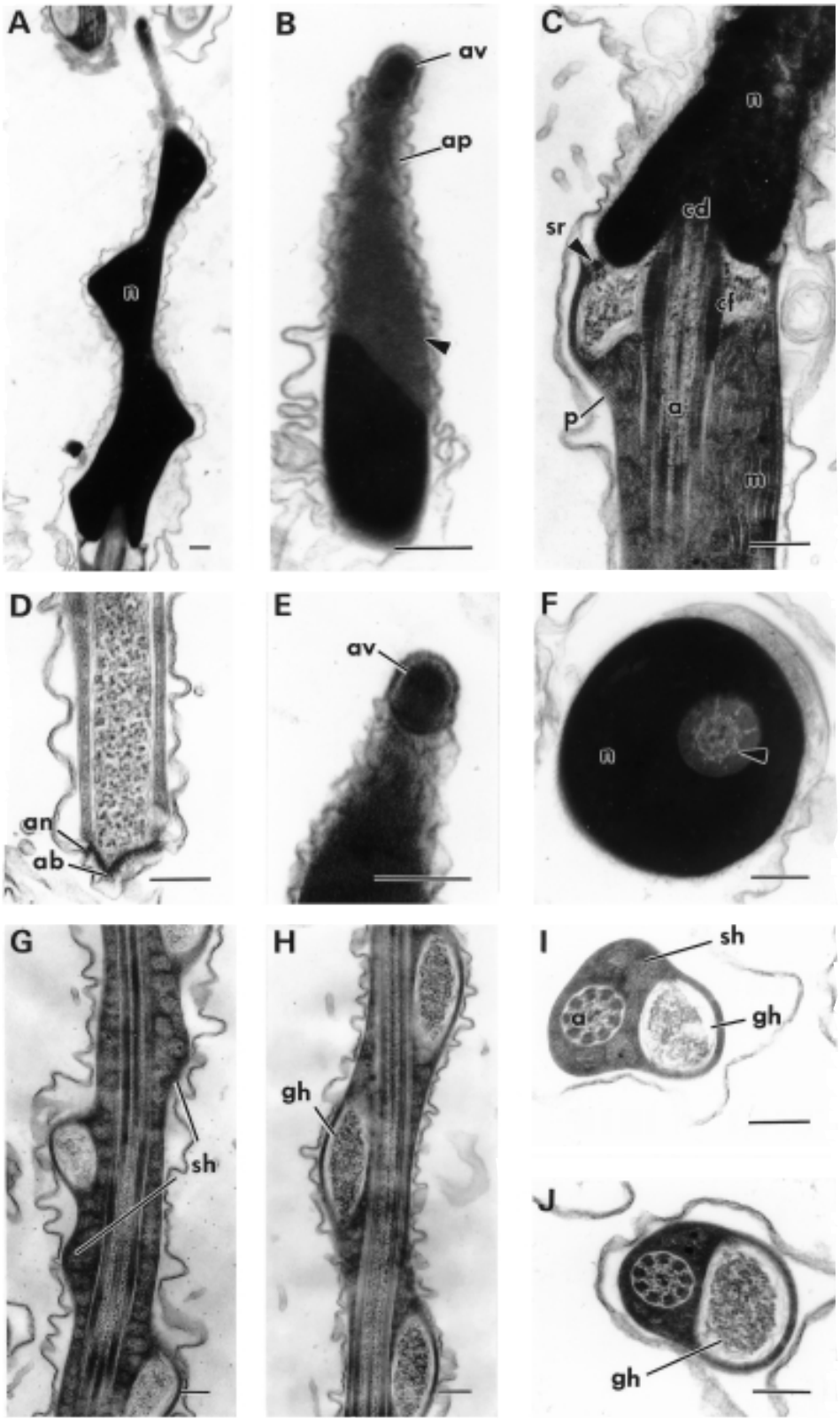

Figure 1. Sperm ultrastructure of *Chromodoris magnifica*. Scale bar = 0.2 μm in all. (A) Longitudinal section (LS) through the acrosomal complex and nucleus, showing helical keel. (B) LS through the acrosomal complex, note fine striations. (C) LS of the junction between the nucleus and neck region of mid-piece. (D) LS through terminal region of spermatozoon. (E) LS of the acrosomal vesicle. (F) Transverse section (TS) of anterior part of nucleus/mid-piece junction. (G,H) LS through mid-piece. (I,J) TS through mid-piece. Abbreviations: a, axoneme; ab, annular accessory body; an, annulus; ap, acrosomal pedestal; av, acrosomal vesicle; cd, centriolar derivative; cf, coarse fibres; gh, glycogen helix; m, matrix material; n, nucleus; p, paracrystalline material; sr, subnuclear ring.

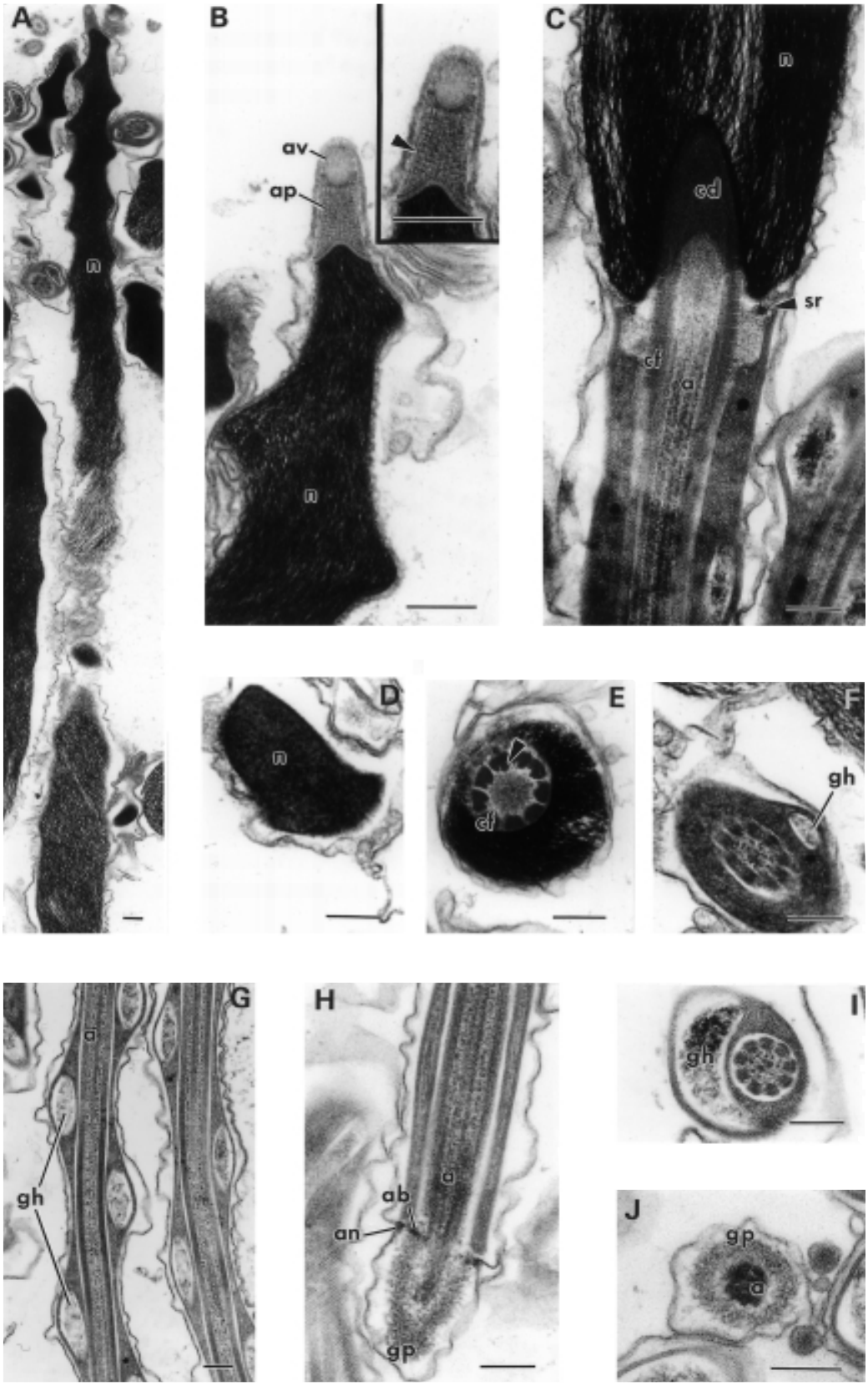

Figure 2. Sperm ultrastructure of *Glossodoris pallida*. Scale bar = 0.2 μm in all. (A) Longitudinal section (LS) through the acrosomal complex and nucleus. (B) LS of the acrosomal complex and nuclear apex, note fine striations on pedestal. Inset: enlarged LS acrosomal complex. (C) LS of the nucleus/mid-piece junction. (D) Transverse section (TS) through nucleus, note keel. (E) TS of nucleus/mid-piece junction, note microtubules aligned on interior of coarse fibres. (F) TS of posterior region of mid-piece. (G) LS through mid-piece. (H) LS of terminal region of mid-piece and glycogen piece. (I) TS of mid-piece. (J) TS of the glycogen piece showing axonemal penetration. Abbreviations: a, axoneme; ab, annular accessory body; an, annulus; ap, acrosomal pedestal; av, acrosomal vesicle; cd, centriolar derivative; cf, coarse fibres; gh, glycogen helix; gp, glycogen piece; n, nucleus; sr, subnuclear ring.

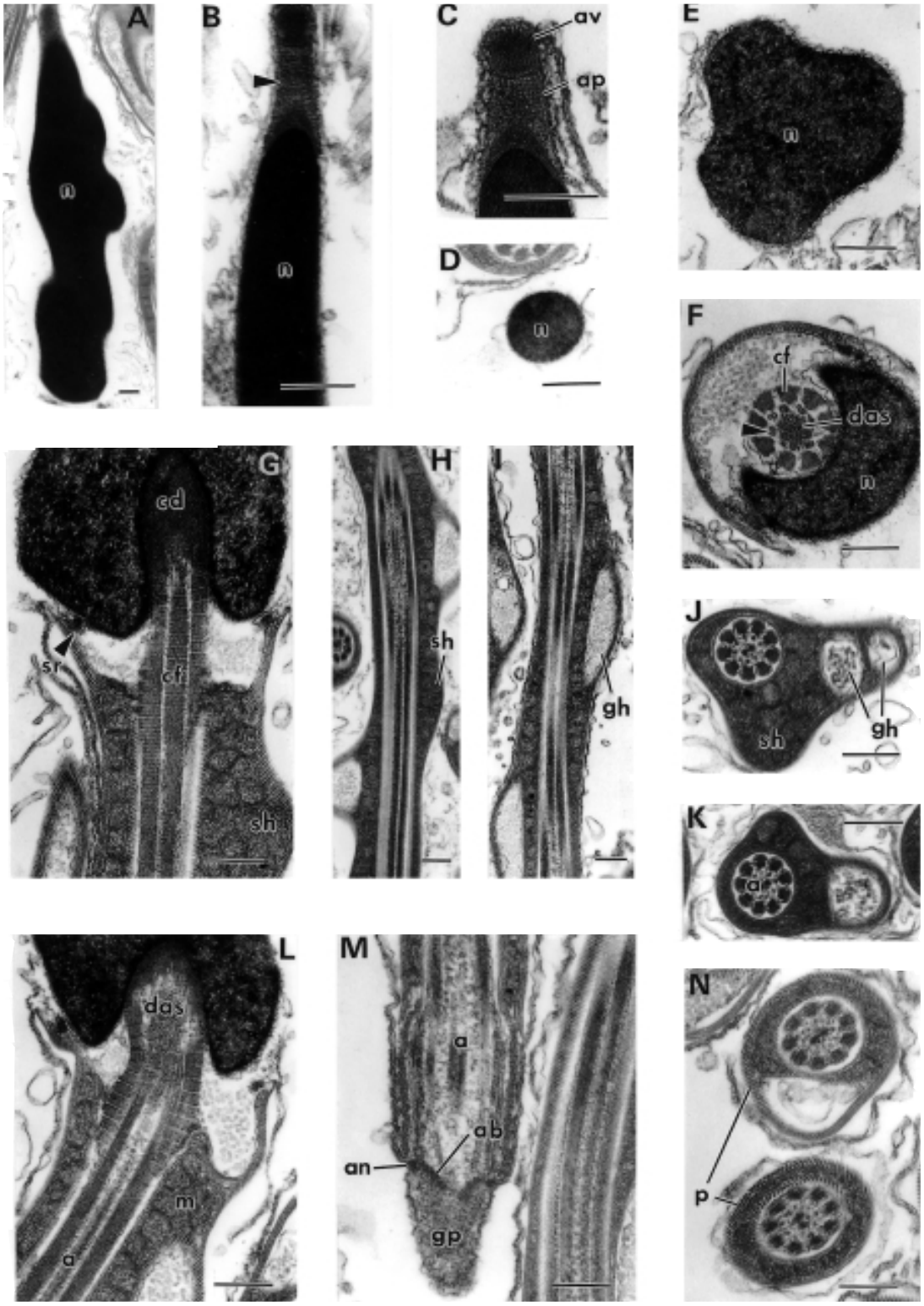

Figure 3. Sperm ultrastructure of *Hypselodoris obscura*. Scale bar = 0.2 μm in all. (A) Longitudinal section (LS) through the nucleus. (B) LS of the acrosomal complex and nuclear apex, note fine striations on pedestal. (C) LS of the acrosomal complex and nuclear apex. (D,E) Transverse section (TS) through nucleus. (F) TS through nucleus and neck region of the mid-piece, note microtubules between coarse fibres and distal accessory sheath. (G) LS of nucleus/mid-piece junction. (H,I) LS through mid-piece. (J,K) TS through mid-piece. (L) LS of nucleus/mid-piece junction. (M) LS through terminal region of mid-piece. (N) TS through mid-piece. Abbreviations: a, axoneme; ab, annular accessory body; an, annulus; ap, acrosomal pedestal; av, acrosomal vesicle; cd, centriolar derivative; cf, coarse fibres; das, distal accessory sheath; gh, glycogen helix; gp, glycogen piece; m, matrix material; n, nucleus; p, paracrystalline material; sh, secondary helix; sr, subnuclear ring.

Nucleus and neck. The nucleus is 6.45 μm long (n = 1), and shows no helical keeling (Figure 5A). The penetration of the axoneme/coarse fibre complex into the nucleus appeared typical and the doublets remain distinct but in contact with the coarse fibres (Figure 5D,E). The subnuclear ring appears segmented (Figure 5E).

Mid-piece. The mid-piece in *Risbecia tryoni* measures approximately 361 μm in length. The coarse fibre periodicity was 50 nm (Figure 5D). A secondary helix is formed by the matrix component of the mitochondrial derivative (Figure 5H,G). Progressing posteriorly, the secondary helix is gradually lost (Figure 5H,K,L). Similarly, the glycogen helix eventually disappears (Figure 5I,K,L). In one spermatozoon, a second glycogen helix was visible (Figure 5F).

Annular complex and glycogen piece. An annular accessory body is present at the posterior region of the disrupted axoneme (Figure 5J). A glycogen piece persists beyond the annulus proper for 1.13 μm (Figure 5J). The axoneme appears to terminate at the annular accessory body.

The comparative acrosomal morphology of these five chromodorids is illustrated in Figure 6.

DISCUSSION

Structural comparisons

Acrosomal complex. All five investigated species showed marked similarities in acrosomal morphology, but also some potentially useful differences. The length of the acrosomal vesicle was very similar in all species (Figure 6), although the shape shows some variation among taxa. The vesicle was found to be almost spherical in *Risbecia tryoni* and *Hypselodoris obscura*, ovate in *Chromodoris magnifica* and *Glossodoris pallida* and slightly elongate-ovate with a lateral constriction in *Pectenodoris trilineata* (see Figure 6 for comparison). The spherical shape of the acrosomal vesicle observed by us in *Hypselodoris obscura* was similar to that of *H. fontandrani* illustrated by Medina et al. (1988b, as *H. messinensis*). This contrasted with the results presented by Medina *et al.* (1985), which clearly indicates an ovate acrosomal vesicle in *Hypselodoris orsini* (as *H. tricolor*). It may be that minor differences in acrosomal vesicle shape are caused by preservation differences or that vesicle shape is variable between species, as well as genera.

The length of the pedestal was variable within the Chromodorididae, ranging from 0.23 μm (*Hypselodoris obscura* and *Glossodoris pallida*) to 0.97 μm (*Chromodoris magnifica*). Interestingly, measurements taken from micrographs of Medina *et al.* (1985, 1988b) indicate a pedestal length of approximately 0.51 μm for *Hypselodoris orsini* and 0.67 μm for *H. fontandrani*—substantially longer than the length observed in *H. obscura*. These differences may be explained by the presence of two distinct clades in the genus *Hypselodoris* (Gosliner & Johnson, 1999). *H. obscura* is one of the more highly derived members of the Indo-Pacific clade, while presumably *H. fontandrani* and *H. orsini* would belong in the Atlantic and Eastern Pacific clade with their Mediterranean counterparts. The pedestal length recorded previously for *Chromodoris annae* (0.8 μm; Healy & Willan, 1991) is similar to the length measured for *C. magnifica* (0.88–0.97 μm).

The pedestal of *Chromodoris* exhibits very fine, angularly inclined striations. Although the striations in *Chromodoris magnifica* could not be accurately measured, those of *C. annae* (from Healy & Willan's 1991 study) exhibited a periodicity of 12 nm. Examined species of *Glossodoris*, *Pectenodoris*, *Hypselodoris*, and *Risbecia*, all exhibited striations of similar periodicity (12–14 nm). A periodicity of 12 nm is evident for the striations in micrographs of Medina *et al.*, (1985, 1988b) for *Hypselodoris orsini* and *H. fontandrani*. Thus, the periodicity of all acrosomal pedestal striations in all investigated Chromodorididae varies within a very small range. It is worth noting that the dorid *Rostanga arbutus* (Angas, 1864) shows very similar acrosomal pedestal morphology and striations to those of the Chromodorididae (especially to *Chromodoris*), but that other known examples of striations within the Nudibranchia are all of much coarser and more complex types (Dorididae—*Jorunna pantherina* (Angas, 1864); Polyceridae—*Kaloplocamus acutus* Baba, 1949; Gymnodoridae—*Gymnodoris* sp., see Healy & Willan, 1991). Coarse striations are also observed in at least one notaspidean (*Pleurobranchus peroni*, Cuvier, 1804—Healy & Willan, 1984). The function of the periodic striations remains unknown, but assuming homology of the pedestal with the basal plate and/or axial rod of prosobranch sperm, or axial rod of bivalve sperm, then it is possible that the striations represent some form of ordered actin (e.g. Lewis, Leighton & Vacquier, 1980; Shiroya, Hosoya, Mabuchi & Sakai, 1986; Tilney, Fukui & DeRosier, 1987). Nothing is as yet known of the acrosome reaction in heterobranchs or caenogastropods, and current understanding of the process rests almost entirely on studies of externally fertilizing bivalves (e.g. Hylander & Summers, 1977). As striated pedestals occur in only some nudibranchs, it seems likely that the striations are only required in specialized circumstances—perhaps interacting with a certain type of egg surface architecture. The transverse periodic striations are not to be confused with the striated acrosomal vesicles of certain cephalopods (the octopod genera *Octopus* and *Opisthoteuthis*—Galangau & Tuzet, 1968; Longo & Anderson, 1970; Healy, 1993b) and certain polychaetes (the genera *Phragmatopoma* and *Idanthyrus*—Jamieson & Rouse, 1989). In these examples the striations are believed to reflect ordering of egg-interacting enzymes contained within the vesicle.

Nucleus

The nuclei of *Chromodoris* and *Glossodoris* both possess a single, very strong and angular helical keel, whereas in *Hypselodoris* two or possibly three rounded keels are present. These observations are the first reports of nuclear keels in the suborder Doridina. Smooth, unkeeled nuclei are characteristic of both *Risbecia* and *Pectenodoris*. Strong, angular nuclear keels are known to be present in some Aeolidina, and at least some members of the Dendronotina and Arminina (Healy & Willan, 1991). Thompson's (1966, 1973) work on the sperm of *Archidoris pseudoargus* concluded that keels on a spermatozoon convert uni-planar flagellation into helical progression, particularly in a viscous medium (glass models towed through glycerol). This strongly suggests that keels may enhance sperm movement, but additional experimental and especially *in vivo* observations are now required to test this idea.

The fibrous appearance of some nuclei in this study (*Glossodoris pallida* and *Pectenodoris trilineata*) is identical to that observed previously within the Nudibranchia (Healy & Willan, 1991 and references therein). The phenomenon appears to be related to factors such as spermatozoon age, differences in chemical composition and possibly fixation, although there is no conclusive evidence to support any of these suggestions.

In externally fertilizing bivalves (Franzén, 1983) and some polychaetes (Gibbs, 1971) nuclear elongation has been correlated with larger, yolky eggs. In the present study, observations on both nuclear length and egg size were only recorded for *Pectenodoris trilineata*. This species indeed showed a relatively long nucleus compared to the other species examined ultrastructurally, and also had a large egg size ($\bar{x}$ = 204.64 ± 11.08, n = 8; N. G. Wilson, unpublished data). The longest recorded nucleus in this study was that of *Glossodoris pallida*, whose egg size and developmental type remain unrecorded. Interestingly, *G. atromarginata,* also has an elongate nucleus (length 9.3 μm;

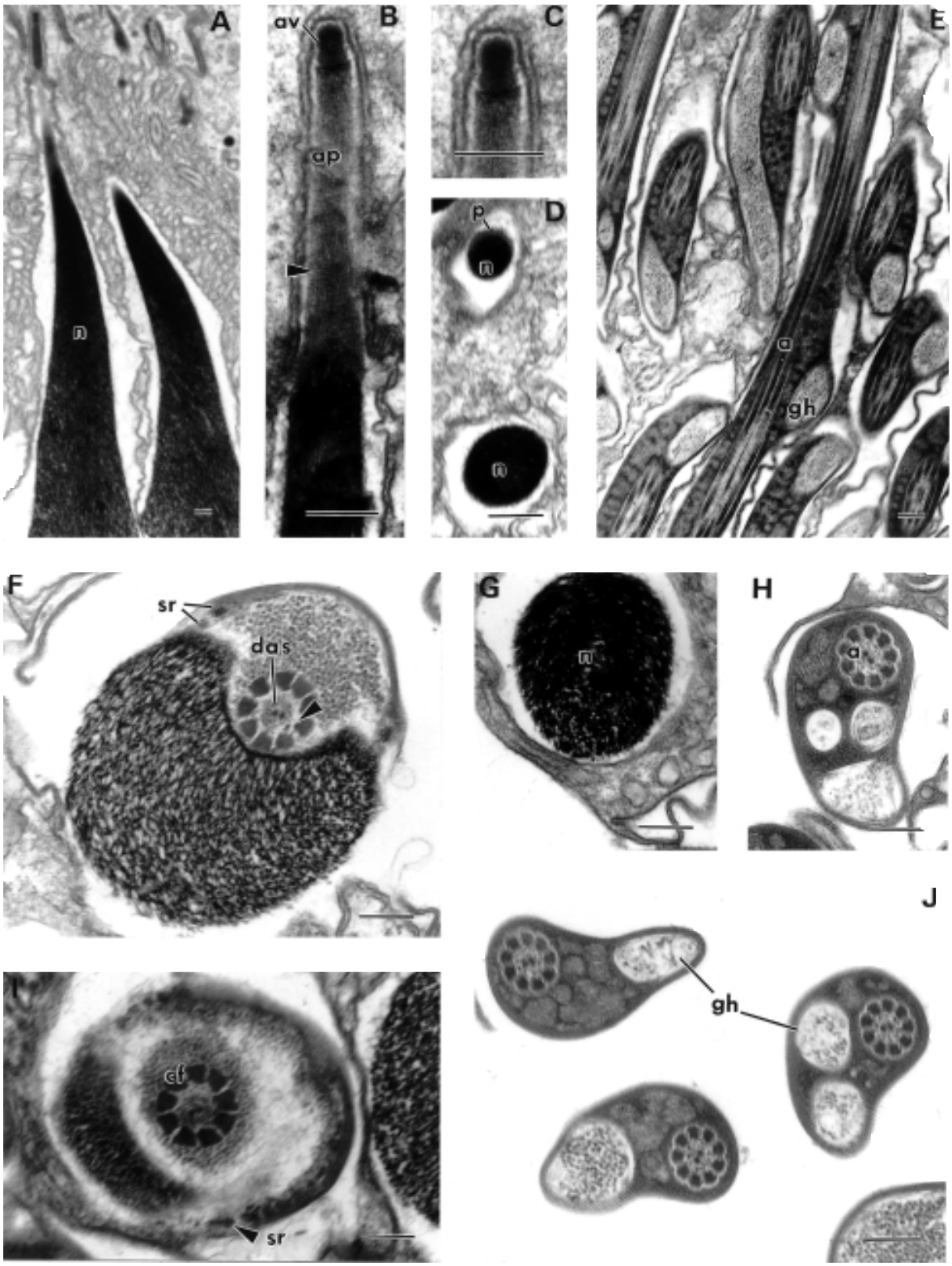

Figure 4. Sperm ultrastructure of *Pectenodoris trilineata*. Scale bar = 0.2 μm in all. (A) Longitudinal section (LS) through the acrosomal complex and nucleus. (B) LS of the acrosomal complex and nuclear apex, note fine striations on pedestal. (C) LS through acrosomal vesicle. (D) Transverse section (TS) of pedestal/nucleus junction and nucleus. (E) LS of mid-piece and oblique sections. (F) TS through nucleus/mid-piece junction. (G) TS of nucleus. (H) TS of mid-piece. (I) TS through nucleus/mid-piece junction. (J) TS of mid-piece. Abbreviations: a, axoneme; ap, acrosomal pedestal; av, acrosomal vesicle; cf, coarse fibres; das, distal accessory sheath; gh, glycogen helix; n, nucleus; sr, subnuclear ring.

Healy, 1984), and its egg size and developmental type are also unrecorded. Given that egg size is correlated with developmental type (Hadfield & Switzer-Dunlap, 1984), and that different developmental types are often found in closely related species (Hadfield & Miller, 1987; Rudman & Avern, 1989), nuclear elongation may prove of more taxonomic and phylogenetic significance at lower, rather than higher levels (for example, within genera). Having said this, larval development (and thus egg size) is also subject to phylogenetic constraints (Hadfield & Miller, 1987), and thus nuclear length should remain in consideration as a potential indicator of broader relationships, though in conjunction with other characters.

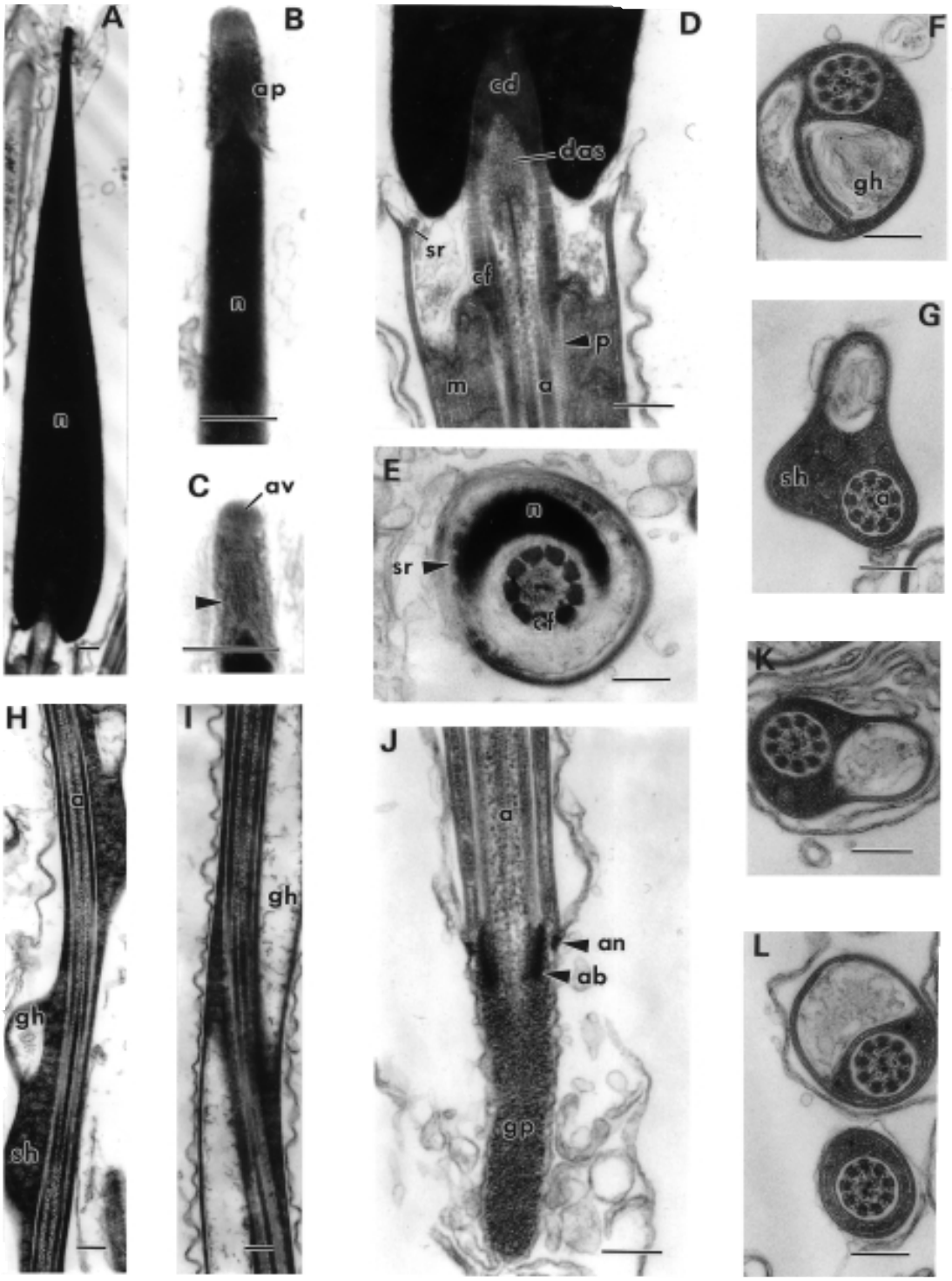

Figure 5. Sperm ultrastructure of *Risbecia tryoni*. Scale bar = 0.2 μm in all. (A) Longitudinal section (LS) through the acrosomal complex and nucleus. (B) LS through acrosomal complex and nuclear apex. (C) LS through acrosomal complex and nuclear apex, note fine striations on the pedestal. (D) LS of the nucleus/mid-piece junction. (E) Transverse section (TS) through nucleus/mid-piece junction. (F,G) TS through mid-piece. (H,I) LS of mid-piece. (J) LS of terminal region of mid-piece. (K,L) TS of mid-piece. Abbreviations: a, axoneme; ab, annular accessory body; an, annulus; ap, acrosomal pedestal; av, acrosomal vesicle; cd, centriolar derivative; cf, coarse fibres; das, distal accessory sheath; gh, glycogen helix; gp, glycogen piece; m, matrix material; n, nucleus; p, paracrystalline material; sh, secondary helix; sr, subnuclear ring.

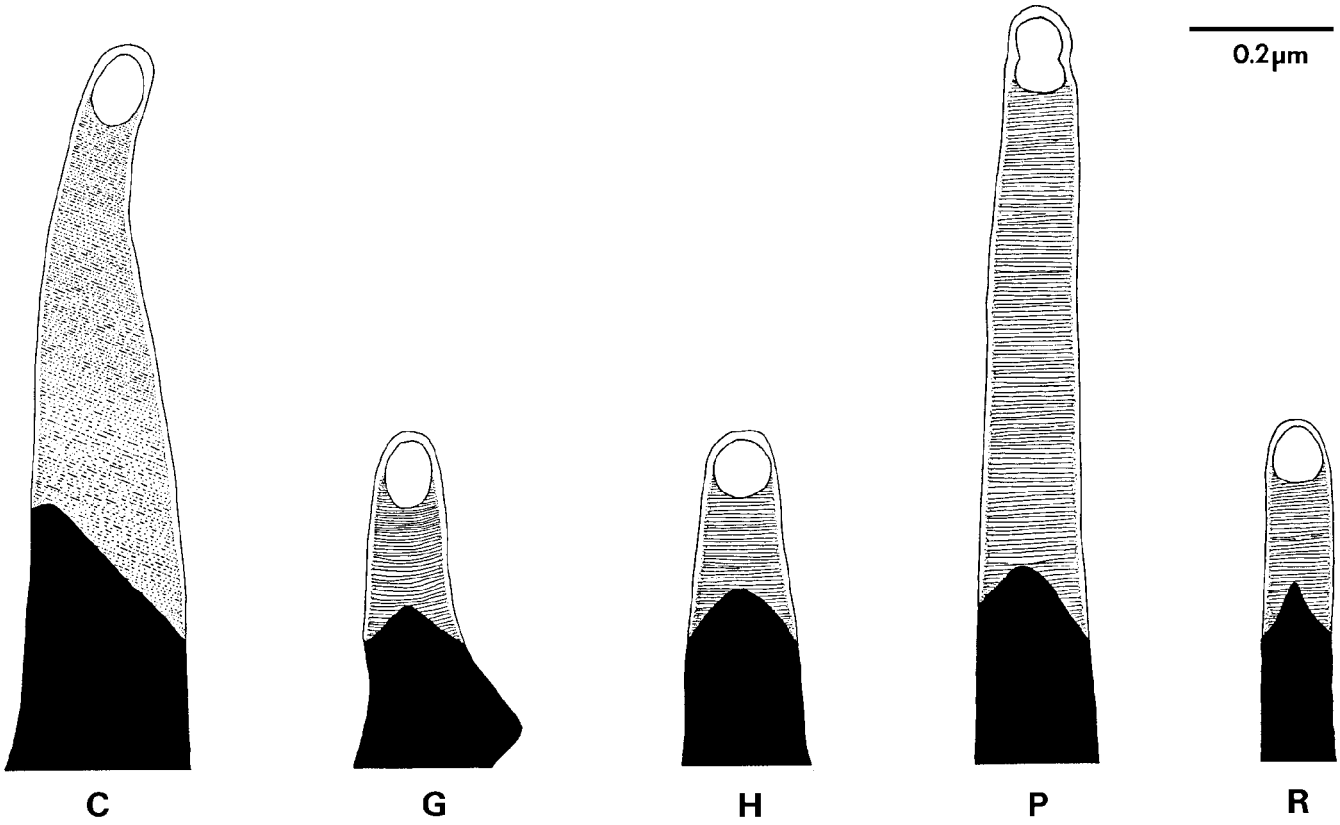

Figure 6. Comparative acrosomal morphology of five chromodorid species. C, *Chromodoris magnifica*; G, *Glossodoris pallida*; H, *Hypselodoris obscura*; P, *Pectenodoris trilineata*; R, *Risbecia tryoni*.

Neck region

Within the neck region axonemal doublets become associated with the coarse fibres to form a complex that projects anteriorly into the base of the nucleus. Typically in heterobranch gastropods, the doublets penetrate the centre of the thick, coarse fibres. However, these doublets can also be aligned on the inner face of the coarse fibres. In four species examined in the present study, the axonemal doublets lie on the inner face of the coarse fibres, rather than penetrating them (*Chromodoris magnifica, Glossodoris pallida, Pectenodoris trilineata*, and *Risbecia tryoni*), while in *Hypselodoris obscura* the doublets were arranged around the distal accessory sheath. The doublets have been known to terminate before the neck region in the pulmonate *Discus rotundus* (Müller, 1774) (Maxwell, 1976), but this did not occur in any genera studied here.

The banding periodicity of the coarse fibre complex appears to be consistent throughout all chromodorid genera examined and ranged from 41 to 56 nm (*Chromodoris* 42 nm, *Hypselodoris* 50 nm, *Glossodoris* 56 nm, and *Risbecia* 50 nm). This is in agreement with the results of Healy & Willan (1991), who recorded coarse fibre periodicity within all four suborders of the Nudibranchia (Doridina 37–54 nm, Aeolidina 40–55 nm, Dendronotina 48–54 nm, and Arminina 52 nm). Interestingly, pulmonate spermatozoa, while very similar to those of nudibranchs (and opisthobranchs in general), exhibit much greater variation in coarse fibre periodicity (20–75 nm; Healy & Jamieson, 1989).

All the chromodorids examined here showed a short basal invagination of the nucleus, consistent with other nudibranchs and the majority of other opisthobranchs (Healy & Willan, 1991). A subnuclear ring, distal accessory sheath, and bell-shaped centriolar derivative continuous with the axoneme/coarse fibre complex were present in all genera examined herein, and all nudibranchs previously studied (Thompson, 1966, 1973; Holman, 1972; Eckelbarger & Eyster, 1981; Medina *et al.*, 1986, 1988a; Healy & Willan, 1991) as well as most other opisthobranchs and all basommatophoran pulmonates (for literature see Healy, 1983; Healy & Willan, 1984). The subnuclear ring is visibly segmented in *Pectenodoris trilineata* and *Risbecia tryoni*. Although this is the first time such segmentation has been demonstrated, it is possible that this condition applies to all heterobranch spermatozoa exhibiting a subnuclear ring. Certainly, the segmentation would explain why the ring is not always observed in longitudinal sections. The distal accessory sheath is also present in stylommatophoran and some basommatophoran pulmonates, and possibly plays a role in determining motility of the axial complex (Anderson & Personne, 1967, 1976).

Mid-piece

The spermatozoal mid-piece of opisthobranchs and pulmonates consists of a single structure, the mitochondrial derivative, which is composed of fused mitochondria that have undergone substantial metamorphosis during spermiogenesis to form discrete paracrystalline and matrix layers (André, 1956, 1962; Thompson, 1973; Anderson & Personne, 1976; Dan & Takaichi, 1979; Eckelbarger & Eyster, 1981; Healy, 1983, 1988a,b, 1996; Maxwell, 1983; Medina *et al.*, 1988a). In most heterobranchs, there is also one or more glycogen-filled, helical compartments (the primary or glycogen helices) enclosed by paracrystalline layers within the mid-piece, although this may be accompanied by a secondary helix composed of mitochondrial derivative (Healy & Willan, 1991). Up to four glycogen helices have been reported in some cephalaspid opistho-

branchs and basommatophoran pulmonates (Thompson, 1973; Healy, 1983), and two have been noted in some notaspideans (Healy & Willan, 1984). Prior to the work of Healy and Willan, it was believed that secondary helices were absent in all nudibranch spermatozoa (Thompson, 1973; Maxwell, 1983). Healy and Willan (1991) reported a maximum of one glycogen helix and two secondary helices in *Chromodoris annae*, with the exception of the aeolid *Aeolidiella indica* Bergh, 1888, which occasionally showed duplication of the axoneme and glycogen helices (justifiably interpreted by them as the likely result of aberrant development). In the present study *Chromodoris magnifica* was the only species to exhibit one glycogen helix and one secondary helix. *Hypselodoris obscura* and *Risbecia tryoni* both showed a maximum of two glycogen helices and one secondary helix. A pair of glycogen helices was observed only once in each of these two species. This may be the result of an aberrant spermatozoan in each case, or that the portion of the mid-piece with two glycogen helices is quite short and, therefore, seldom seen in transverse section. *Glossodoris pallida* had one glycogen helix and no secondary helices. The material available for *Pectenodoris trilineata* was minimal due to the small size of the ampulla (whole adult length less than 5 mm) and there were very few longitudinal sections visible. Three glycogen helices were observed and, while no secondary helices were observed, their presence in this species cannot be ruled out. Axonemal duplication was only observed once in *Glossodoris pallida* and *Hypselodoris obscura*, and not in any of the other three species investigated. This suggests that this type of aberrancy is quite rare.

Annular complex and glycogen piece

Results of the present study have demonstrated that the 'annulus' of chromodorids actually consists of two structures—the annulus proper and the annular accessory body (both easily observed in Figure 5J). In *Chromodoris magnifica*, and *C. annae* (Healy & Willan, 1991), the annular accessory body actually forms the posterior extremity of the spermatozoon. The annular accessory body appears to be present in other nudibranchs and in siphonariid basommatophorans (see Healy, 1983, Sumikawa & Funakoshi, 1984; Azevedo & Corral, 1985; Healy & Willan, 1991; Hodgson *et al.*, 1991), but due to its proximity to the annulus proper and comparable electron density, it has not been previously recognized. In most cases the axoneme appears to terminate at the annular complex, except in *Glossodoris pallida*, where it appeared to partially penetrate the small glycogen piece, and *Chromodoris magnifica*, where it terminated shortly before the annular accessory body.

In all investigated chromodorids the glycogen piece is either poorly developed, ranging in length from 0.4 μm in *Hypselodoris obscura* to 1.13 μm in *Risbecia tryoni*, or absent, as in *Chromodoris magnifica* and *C. annae* (Healy & Willan, 1991). Poor development of the glycogen piece occurs throughout the Nudibranchia (Healy & Willan, 1991) and has been observed in several species of other heterobranch groups including the Pyramidelloidea (Healy, 1988b), Notaspidea (Healy & Willan, 1984) and stylommatophoran and some basommatophoran pulmonates (Healy, 1983, 1986; Selmi *et al.*, 1988). In stylommatophoran pulmonates the glycogen piece is absent in all investigated species (for discussion and literature see Healy & Jamieson, 1989; Giusti *et al.*, 1991). Incorporation of substantial deposits of glycogen within the mitochondrial derivative of most heterobranch spermatozoa has undoubtedly been the driving force behind the regression and eventual loss of the glycogen piece (Thompson, 1973; Healy & Willan, 1991). This situation contrasts with that observed in caenogastropods and some allogastropod heterobranchs (Architectonicoidea, Valvatoidea), where glycogen is almost wholly restricted to the post-mitochondrial glycogen piece (Kohnert & Storch, 1984; Koike, 1985; Healy, 1993a, 1996). Thompson (1973) described glycogen deposits within opisthobranch and pulmonate spermatozoa as 'endogenous food reserves'. Although experimental data pertaining to the function of these deposits are still required (but see Anderson & Personne, 1976, for a summary of enzymatic pathways), there appears to be general agreement that stored glycogen in heterobranch sperm is involved in the maintenance of sperm viability before and/or after copulation (that is, within the hermaphrodite duct prior to mating, and subsequently within the partner's seminal receptaculum; see Healy, 1996 for further literature).

Taxonomic and phylogenetic implications

According to Gosliner & Johnson (1999) the phylogeny of the Chromodorididae is far from being completely resolved. Examination of the acrosome in five genera associated with the Chromodorididae has revealed a sperm synapomorphy for the family in the form of fine striations in the internal structure of the acrosomal pedestal. These striations (present in *Chromodoris*, *Glossodoris*, *Hypselodoris*, *Pectenodoris*, and *Risbecia*) are also observed in a single species of Doridiidae (*Rostanga arbutus*, but not in some other Doridiidae; see Healy & Willan, 1991). This suggests the need for additional information on acrosomal morphology in both the Chromodorididae and it's proposed sister taxon, the Actinocyclidae (Gosliner & Johnson, 1994), as well as other Doridiidae.

The monophyly of *Hypselodoris* + *Risbecia* was first advanced by Rudman (1984) in his generic revision of the Chromodorididae. This relationship is supported by a recent cladistic analysis (Gosliner & Johnson, 1999) and by some of the available sperm ultrastructural data (see Figure 6). Considerable similarity in acrosomal morphology exists between *Hypselodoris*, *Risbecia*, and *Glossodoris*, and is suggestive of a reasonably close relationship between these three taxa (the morphology of the acrosomal complex correlating strongly with supraspecific taxa within the Nudibranchia and other heterobranchs; Healy, 1988, 1993a, 1996; Healy & Willan, 1991). It is anticipated that investigation of acrosomal morphology in genera falling between *Glossodoris* and *Hypselodoris* + *Risbecia* (particularly *Verconia, Noumea, Ceratosoma, Thorunna*, and *Mexichromis*) will shed further light on the precise relationship between these three genera. While it is true that acrosomal features, especially the shape and internal structure of the pedestal, have been shown to be taxonomically informative or at least potentially so (Healy & Willan, 1991), the high degree of divergence in nuclear morphology between all five investigated chromodorid genera suggest that nuclear length and sculpture may prove equally valuable once data for additional (and ideally all) genera are available. Structural diversity in other chromodorid sperm features, e.g. the number and type of helices present in the mid-piece, the configuration of axonemal microtubules, and the shape and degree of development of the annulus and glycogen piece may also provide information on the relationships within the Chromodorididae.

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