Morphology of the Proboscis of *Hubrechella juliae* (Nemertea, Pilidiophora): Implications for Pilidiophoran Monophyly

Alexey V. Chernyshev,¹,²* Timur Yu. Magarlamov,¹ and James M. Turbeville³

1A.V. Zhirmunsky Institute of Marine Biology, Far East Branch, Russian Academy of Sciences, Vladivostok, Russia
2Far Eastern Federal University, Vladivostok, Russia
3Department of Biology, Virginia Commonwealth University, Richmond, Virginia 23284 USA

ABSTRACT. The proboscis of *Hubrechella juliae* was examined using transmission electron microscopy, scanning electron microscopy, and confocal laser scanning microscopy to reveal more features of basal pilidiophoran nemerteans for morphological and phylogenetic analysis. The proboscis glandular epithelium consists of sensory cells and four types of gland cells (granular, bacillary, mucoid, and pseudocnidia-containing cells) that are not associated with any glandular systems; rod-shaped pseudocnidia are 15–25 μm in length; the central cilium of the sensory cells is enclosed by two rings of microvilli. The cerebral sensory organ occupies a similar position relative to the brain and lateral blood vessel, and a connective tissue layer containing epidermal gland bodies between the epidermis and body-wall musculature is present (rudimentary in hubrechtiids). Historically, hubrechtiids were regarded as a transitional stage between Palaeonemertea and basal heteronemerteans (e.g., Bürger, 1895; Hylbom, 1957). Cantell (1969) reported a pilidium larva in their life history, a feature also characteristic of heteronemertean development, and Norenburg (1993) emphasized the pilidium larva as a synapomorphy linking *Hubrechella* and heteronemerteans. Recently, Chernyshev (2011) evaluated the available morphological characters, and his analysis also supported Pilidiophoran monophyly.

Molecular phylogenies have not definitively resolved their relationships, with one analysis supporting a clade (Pilidiophora) consisting of *Hubrechella* + Heteronemertea (Thollesson and Norenburg, 2003) and another revealing weak support for pilidiophora and only under certain optimality criteria and model parameters (Andrade et al., 2012). Although additional molecular data may help resolve the phylogeny of hubrechtiids, acquisition of complementary phylogenetically informative morphological characters is also warranted.

KEY WORDS: transmission electron microscopy; scanning electron microscopy; confocal laser-scanning microscopy; gland epithelium; pseudocnidia; musculature; endothelium; nervous plexus; homology

INTRODUCTION

The phylogenetic position of the hubrechtiid nemerteans (Hubrechtiidae s.l.) among the Nemertea has long been of great interest because of their mosaic of “palaeonemertean” and “heteronemertean” characters. For example, the studies of Bürger (1895), Hylbom (1957), and others revealed that in hubrechtiid and palaeonemerteans, the brain and lateral nerve chord are situated below the epidermis in the connective tissue, whereas in hubrechtiid and heteronemertean, the cerebral sensory organ occupies a similar position relative to the brain and lateral blood vessel, and a connective tissue layer containing epidermal gland bodies between the epidermis and body-wall musculature is present (rudimentary in hubrechtiids). Historically, hubrechtiids were regarded as a transitional stage between Palaeonemertea and basal heteronemerteans (e.g., Bürger, 1895; Hylbom, 1957). Cantell (1969) reported a pilidium larva in their life history, a feature also characteristic of heteronemertean development, and Norenburg (1993) emphasized the pilidium larva as a synapomorphy linking *Hubrechella* and heteronemerteans. Recently, Chernyshev (2011) evaluated the available morphological characters, and his analysis also supported Pilidiophoran monophyly.

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*Correspondence to: Alexey V. Chernyshev; A.V. Zhirmunsky Institute of Marine Biology, Far East Branch, Russian Academy of Sciences, Vladivostok, Russia. E-mail: nemertea@fromru.com

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Our knowledge of hubrechtiiid morphology is based almost exclusively on histological investigations (Bürger, 1895; Hyrbom, 1957; Gibson, 1979a, b). The only exceptions to date are a ultrastructural investigation of the epidermis (Norenburg, 1985) and confocal laser scanning microscopy study of the body-wall musculature (Chernyshev, 2010). The proboscis is an eversible muscular organ used to capture prey and is potentially character rich. Knowledge of the proboscis morphology of hubrechtiiids is based exclusively on light microscopic studies, and these data have been especially important for taxonomic considerations (e.g., Bürger, 1895; Hyrbom, 1957; Gibson, 1979a, b). However, this organ is also a promising source of characters for use in reconstructing nemertean phylogeny.

Here, we study the proboscis morphology of Hubrechtella juliae Chernyshev, 2003a using scanning (SEM) and transmission electron microscopy (TEM) as well as laser confocal microscopy to gauge the utility of its organization for placing the Hubrechtella within the nemerteans. Our observations show that the proboscis of H. juliae differs in some details from that of other nemerteans studied with TEM (e.g., Ling, 1971; Stricker and Clooney, 1983; Montalvo et al., 1996, 1998a, b; Junoy et al., 2000; Magarlamov and Chernyshev, 2010) and reveals a character potentially informative for reconstructing the phylogenetic position of the hubrechtiiids.

**MATERIAL AND METHODS**

Twenty six live specimens of H. juliae were collected in Vostok Bay (Peter the Great Bay) of the Sea of Japan in July–September 2008–2012. For TEM, the proboscides of H. juliae were anesthetized by 7% solution of magnesium chloride, fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS; pH 7.4) at +4°C, and postfixed with a 1% solution of osmium tetroxide in PBS for 1 h. The fixed material was dehydrated in an ethyl alcohol and acetone series and embedded in Epon-Araldite resin. Transverse and longitudinal thin (60–70 nm) sections were made with an Ultracut E (Reichert) and stained with 1% uranyl acetate and 0.35% lead citrate solutions. The sections were made with an Ultracut E (Reichert) and stained with 1% uranyl acetate and 0.35% lead citrate solutions. The examination of the proboscis pieces was transferred to a solution of antiserotonin-like immunoreactive (ibg) neurons, the proboscis pieces were transferred to a solution of antiserotonin (5-HT, polyclonal, rabbit, diluted 1:2000) primary antibody in PBS with 1% BSA. For observation of the catecholamin-lir nervous system, the proboscis fragments were placed in antityrosinehydroxylase (polyclonal, rabbit, diluted 1:2000, Immunostar) primary antibody in PBS with 1% BSA. In all cases the material was kept for 24 h at +4°C in the primary antibody, then rinsed in phosphate buffer and incubated for 2 h at room temperature in goat–antimouse Alexa Fluor 488 (Invitrogen) or goat–antirabbit Alexa Fluor 532 (Invitrogen) immunoglobulin G, diluted 1:800 in PBS. Four proboscides were fixed for 4 h at room temperature in 4% formaldehyde, rinsed in PBS, permeabilized for 1 h in 0.2% triton X-100 in PBS and stained for 1 h at room temperature with phalloidin–Alexa Fluor 633 (Invitrogen). All pieces of the proboscis were washed in PBS and immersed in Mowiol 4-88 (Aldrich) and mounted on glass slides. The specimens were examined with an LSM-510 Meta confocal microscope (Carl Zeiss, Germany). The obtained image series were analyzed with CLSM-510 Meta software. Images were further processed with Photoshop CS2 to adjust contrast and brightness and to create digital line drawings.

**RESULTS**

The anterior proboscis chamber of adult fixed H. juliae is up to 250 μm in diameter and consists of an outer glandular epithelium, basiepithelial nervous plexus, a subepithelial layer of extracellular matrix (ECM), both inner and outer layers of the muscle, a middle longitudinal muscle layer, an ECM, and an endothelium (peritoneum) with inner circular muscles and support cells (Fig. 1A). The peritoneum borders the proboscis coelom (rhynchocoel).

**Glandular Epithelium and Subepithelial ECM**

The proboscis gland epithelium consists of four types of gland cells: granular gland cells (type I), bacillary gland cells (type II), pseudocnidia-containing cells (type III), and mucoid gland cells (type IV). Sensory cells are distributed among these (Fig. 1A). The proboscis epithelium shows a dorsoventral differentiation (Fig. 2A); the epithelium of the ventral half is up to 72 μm thick and contains numerous pseudocnidiae and bacillary gland cells (other gland cells are rare here); the epithelium of the dorsal half is up to 30 μm thick and contains mainly granular and mucoid gland cells. The subepithelial ECM is up to 1.1 μm thick. Support cells with bundles of tonofilaments have not been detected. Adjacent gland and sensory cells are joined by an apicalateral zonula adherens junction. A basal process of each gland cell extends through the basiepithelial nervous plexus and attaches to basal ECM by tight junctions (Fig. 4H). The basal process contains numerous longitudinal intermediate filaments 16–20 nm thick.

Type I gland cells (granular gland cells; see Junoy et al., 2000) are club-shaped and have proximal, irregularly shaped bodies (Fig. 3A). The cell necks are narrow and enlarged distally forming bulbous protrusions (3–6 μm long) at the epithelial
surface (Figs. 2E,F, 3B). This apical dilation contains spherical granules (diameter 0.75 \(\mu m\)). The cortical layer of this dilation contains numerous thick filaments. Each secretory granule consists of a homogeneous core of moderate electron density surrounded by an area of more lightly stained finely granular material (Fig. 3B). Numerous cisternae of the rough endoplasmatic reticulum (RER) lie in the perinuclear cytoplasm and cell neck.

Type II gland cells (bacillary gland cells; see Junoy et al., 2000) are elongate and somewhat clavate (Fig. 3D). Cisternae of the RER are abundant in the cell cytoplasm. Central and distal (apical) parts of the cell contain long rod-shaped granules (0.2–0.6 \(\mu m\) wide, 2.5–12 \(\mu m\) long) (Figs. 3D,E). These secretory granules show a paracrystalline composition and consist of tightly packed longitudinal fibrils (up to 18 nm in diameter) with moderate electron density (Fig. 3E). A well-developed lining of longitudinal microtubules is present in the cortical layer of middle and apical portions of the cell (Figs. 3D inset, E). The apical surface of the cells forms 2–5 long finger-like protrusions (approximately 14 \(\mu m\) in length and about 1.3 \(\mu m\) in diameter) in which secretory granules are situated (Figs. 3E,F). These finger-like protrusions are raised above the surface of the glandular epithelium (up to 6 \(\mu m\); Figs. 2E,G). The central part or core of these protrusions contains tightly packed longitudinal microtubules (Fig. 3F).
Pseudocnidae-containing cells (type III gland cells) are spindle-shaped, tapering toward the cell neck distally and tapering proximally in an extension that reaches the ECM subjacent to the proboscial epithelium. These cells contain abundant RER, well-developed Golgi complexes, mitochondria, 2–4

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Fig. 3. *H. juliae*, transmission electron micrographs of transverse sections of the proboscis epithelium. (A) Panoramic view showing gland cell types I and IV and sensory cell (black arrow marks basal process extending from sensory cell body). (B) Apical dilation of the glandular cell of type I. High magnification shows cortical layer containing numerous thick filaments (arrow). (D) Panoramic view showing gland cells of the types I and II and sensory cell. High magnification shows the well-developed lining of longitudinal microtubules (arrow) located in the cortical layer of middle portion of the type II gland cell. (G) Pseudocnidae-containing cells. (H) Immature pseudocnidae. (I) Nearly mature pseudocnidae (arrow points to subapical extensions). (J) Nearly mature pseudocnidae with a rudimentary filament (arrow points to subapical extensions). (K) Granular (black arrows) and tubular components (white arrows) of the nearly mature pseudocnidae. (L) Longitudinal section of mature pseudocnidae. (M) Transverse section of the mature pseudocnidae. g1, g2, g4, gland cells of types I, II and IV; pc, pseudocnida; pcc, pseudocnidae-containing cell, sc, sensory cell.
Fig. 4. *H. juliae*, transmission electron micrographs of transverse sections of the proboscis. (A) Transverse section of the receptor process of the sensory cell. (B) The basal part of the receptor process with bundle of actin filament-like structures (arrow). (C) Ciliary rootlet (white arrow) of the sensory cell and anastomosis of the inner and outer micrivilli of the receptor process (black arrow). (D) The tip of the cilium of the sensory cell. (E) Ciliary basal body of the sensory cell (arrow). (F) Distal cytoplasm of sensory cell containing Golgi apparatus (white arrow) and ciliary rootlet (black arrow). (G) Axon of the sensory cell (arrow). (H) Basal extension (asterisk) of a gland cell passing through basiepithelial nervous plexus and attaching (arrow) to basal ECM. (I) Nerve trunk and connective nerves (arrow) of the wider half of the proboscis. (J) Nerve trunk of the narrow half of the proboscis. (K) High magnification of the nerve trunk. (L) Neuron body (asterisk) of the proboscis nerve. ecm, extracellular matrix; gle, glia-like cell; icm, inner circular musculature; idm, inner diagonal musculature; nt, nerve trunk; pn, proboscis nerve.
pseudocnidae in different stages of development (Fig. 3G), and residual bodies with homogenous or heterogenous contents. The nucleus contains a large nucleolus.

The immature pseudocnidae are rounded (6.5–8 μm in diameter; Figs. 1C1,C2, 3H). The cortex is bilaminar, consisting of a homogenous and moderately electron-clear outer layer (0.2–0.8 μm thick) and an inner layer of moderate electron density (0.9–2.5 μm thick) consisting of fibrous material. Granular material of high electron density fills the subcortical region or medulla (central region) of the pseudocnidae (Fig. 1C2). Two kinds of the granules are distinguished: 1) large granules 0.1–0.2 μm in diameter, 2) small granules up to 0.04 μm in diameter.

The nearly mature pseudocnidae (8–11 μm long and 5.0–5.5 μm in diameter; Figs. 1C3,C4, 3LJ) also have a bilaminar cortex with an outer homogenous, moderately electron-dense (0.28–0.5 μm thick) layer and an inner homogenous layer of high electron density (0.18–0.5 μm thick). Large (0.12–0.27 μm in diameter) and small (approximately 0.04 μm in diameter) granules fill the internal portion (medulla) of the pseudocnidae. A few pseudocnidae lack small granules and contain long tubules about 7 μm long and about 25 nm in diameter (Fig. 3K). A rudimentary filament (~1.2 μm long, ~0.55 μm in diameter) with a dense core is located at the anterior part (apex) of pseudocnida (Figs. 3LJ). Longitudinal sections reveal that the inner layer of the pseudocnida forms two short (~2 μm in length) subapical extensions, consisting of the fine granules containing content of high electron density (Figs. 3L,J).

Mature pseudocnidae are rod-shaped and 15–25 μm long and 3–5 μm in diameter (Figs. 1C5, 2C, 3GL,M). Apices of these mature pseudocnidae extend above the surface of the proboscis epithelium (Figs. 2C,G). The outer cortex (0.45 μm thick) is homogenous and moderately electron dense; the inner cortical layer is homogenous (0.26 μm thick) and exhibits high electron density. The cortex surrounds a medulla (thickness 0.15 μm), which consists of close-packed granular material of moderate electron density. A long tubular core (8–10 μm long, 0.65–0.75 μm in diameter) is situated acentrically and in the medulla. The tubular core consists of four concentric layers of different electron densities (Fig. 3M). The everted tubular core (filament) is very long (40–50 μm) and smooth (Figs. 2B,H). There are no subapical extensions in mature pseudocnidae.

Type IV or mucoid gland cells (see Junoy et al., 2000) are typically goblet-shaped and their cell bodies lie in the basal region of the epithelium. Each cell body extends to the epithelial surface via a cell neck that expands apically to form a globular papilla reaching about 4–6 μm above the epithelium surface (Figs. 2C,H). The gland cell necks are difficult to follow, because they are long and thin; they are often densely packed into the apical portion of the epithelium. Each papilla is filled with large numbers of granules having a spherical shape (1.2–3.5 μm in diameter). The content of each granule varies from loose-fibrous granules with heterogenous electron density to more compact granules with homogenous electron density within an individual gland cell (Figs. 3A,C). RER is abundant in the perinuclear cytoplasm.

Some glandular cells do not fall within any of the aforementioned categories. These contain a small number of secretory granules (up to 2 μm in diameter) with heterogeneous content and possess an electron-clear cytoplasm (Fig. 9A). Usually these cells are large and have irregular form. A nucleus is located in the basal part of the cell and has an irregular shape with a large amount of heterochromatin in the karyoplasm. There are a few mitochondria, short cisternae of ER, and 1–2 dictyosomes of Golgi apparatus in cytoplasm. The apical cytoplasmic membrane of the cells has numerous long microvilli (up to 6 μm), which form a dense mat on the surface of the glandular epithelium (Figs. 2D,E). Generally, these cells are found on the dorsal half of the proboscis.

Only one sensory cell type has been observed in the epithelium (Figs. 2E,I). These cells are relatively abundant and are scattered evenly on both sides of the proboscis epithelium. From the perikaryon, a single dendrite extends distally and terminates at the epithelium surface to form the receptor process (Fig. 3A). The receptor process consists of central cilium (length 6–9 μm) enclosed by two rings of microvilli (8 inner and 8 outer microvilli; Fig. 4A). Confocal laser scanning microscopy shows that the microvillar complex of the sensory cell is phalloidin-positive, 12–14 μm in length and 3.2–4.5 μm in maximal diameter (Figs. 5A, 6A). This positive reaction is attributable to each microvillus containing a bundle of actin filament-like structures 6–7 μm in diameter (Fig. 4B). Both inner and outer microvilli anastomose with each other in the basal part of the complex (Fig. 4C). The cilia are α-tubulin-positive (Figs. 5A,B); each cilium has the typical 9 × 2 + 2 axoneme arrangement. The tip of the cilium is expanded and modified to form a bulb-like structure approximately 0.7 μm in diameter (Fig. 4D). The axonemal microtubules terminate inside this globular tip and are enclosed by fibrous material. The ciliary basal body consists of granular electron dense material (Fig. 4E). 12–20 cross-striated rootlets extend from the basal body, which is surrounded by electron dense material (Fig. 4C). No accessory centrioles were observed. The cytoplasm of receptor cells contains small electron dense granules (up to 0.1 μm in diameter), which arise from a single Golgi complex of the receptor process (Fig. 4F). These granules are especially numerous in the basal portion of the axon (Fig. 4G). RER and autophagosomes.
are sparse in the sensory cell cytoplasm, which is denser than that of the gland cells.

**Nervous Plexus**

The nervous plexus lies in the basal part of the glandular epithelium outside ECM and includes nerve trunks that are surrounded by glia-like cell processes with numerous electron-dense oval granules (Figs. 1A, 4I,J). This layer shows α-tubulin immunoreactivity (Figs. 5A,D). About 26–33 catecholamin-lir and seroton-lir longitudinal nerve trunks irregularly anastomose and form the nervous plexus (Figs. 5E,F). The ventral half of the proboscis contains 18–20 nerves, whereas the dorsal half has 11–13 nerves. The nerve processes are filled with small spherical vesicles (60–75 nm in diameter) with electron-dense core and moderate electron-dense or semitranslucent periphery (Fig. 4K). Longitudinal nerve trunks are linked by connective nerves (commissures; Fig. 4I). Neuron bodies are located near longitudinal nerve trunks (Fig. 4L) and contain an irregular nucleus, numerous neurovesicles, separate cisternae of the RER,

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and mitochondria. The nervous plexus of the wider half is from 18 to 22 μm in thickness and nerve longitudinal trunks are 7–12 μm in diameter (Fig. 4I): the nervous plexus of the narrow half is up to 13 μm in thickness and nerve longitudinal trunks are up to 4 μm in diameter (Fig. 4J).

**Longitudinal and Diagonal Musculature**

The CLSM images show that the anterior proboscis includes four muscle layers: inner circular (endothelial), inner diagonal (subendothelial), longitudinal, and outer diagonal (Fig. 1D). An outer circular musculature is absent. The thickness of the other layers in the inverted (and everted) proboscis is as follows: inner circular layer 1.5–2.1 μm (2.0–2.8 μm in everted), inner diagonal 1.0–1.5 μm (1.4–2.0 μm in everted), longitudinal layer 4.0–4.6 μm (6.0–9.7 μm in everted), diagonal layer 1.5–1.8 μm (2.0–2.6 μm in everted). Both inner and outer diagonal muscles consist of noncrossing fibers (Figs. 6C,E,F). Diagonal muscles of the left and right proboscis halves are oriented in the opposite directions. The outer diagonal muscles of both halves overlap only on the ventral and dorsal sides of the proboscis (Figs. 6B,G). Irregular separate muscle fibers penetrate the longitudinal musculature, connecting the inner and outer diagonal musculature (Figs. 6D, 8E), but true muscle crosses are absent.

Transmission electron micrographs show that the longitudinal musculature is composed of two or three tightly packed layers of myocytes, and the inner and outer diagonal musculature of only one layer of myocyte protrusions (Fig. 8A). The bodies of both longitudinal and diagonal myocytes contain an irregularly shaped nucleus, many mitochondria and residual bodies (Fig. 8B). The central part of the contractile process has an actin–myosin complex up to 3 μm in thickness.

Fig. 6. *H. juliae*, confocal laser scanning micrographs of the proboscis labeled with phalloidin. (A) Z-projection of transverse sections of the everted proboscis. (B, C) 3D reconstruction of the fragment of the everted proboscis showing crisscrossed (B, arrow) and nonoverlapped (C) outer diagonal muscles. (D) Z-projection of transverse section of the everted proboscis stained with phalloidin (red) and DAPI (blue), high magnification image shows radial muscle fibers (arrows) between the outer and inner diagonal musculature. (E) Z-projection showing inner circular, inner diagonal (arrows) and longitudinal muscles. (F) Z-projection showing outer diagonal (arrows) and longitudinal muscles. (G) Z-projection showing crisscrossed outer diagonal muscles. odm, outer diagonal musculature; sc, sensory cells.
(Figs. 8A,E). The peripheral cytoplasm of the process contains wide transparent zones with the cisternae of sarcoplasmic reticulum and mitochondria. Well-developed dictyosomes of the Golgi apparatus and adjoining small vesicles up to 0.1 μm in diameter are situated in the peripheral cytoplasm of each contractile protrusion (Fig. 8C). The cytoplasmic membrane of each muscle cell process often forms a long noncontractile extension (“muscular arms” – see Stretton, 1976) up to 12 μm directed toward the subepithelial ECM (Fig. 8D). Noncontractile muscular arms extend through the ECM and form contacts (synapses) with longitudinal nerve trunks. Solitary or rare binate inner diagonal muscles adjoin the subendothelial ECM; a distance between adjacent diagonal muscles varies from 1 to 5 μm. The contractile protrusions of the outer diagonal muscles lie under the glandular subepithelial ECM and form small groups consisting of 4–10 cells (Fig. 8A). Connective tissue cells have not been found among muscular processes.

**Endothelium and Endothelial Musculature**

The endothelium (peritoneum) is organized as a myoepithelium and consists of two cell types: apically situated support cells (Fig. 7A) and subapical myocytes, which form the inner circular musculature of the proboscis (Figs. 1A, 7B). The shape of the support cells varies from cuboidal to squamous. The apical cytoplasmic membranes of these cells have sparsely scattered short microvilli. Only confocal laser-scanning microscopical observations of proboscis with anti-α-tubulin immunolabeling enabled the finding of sparse rudimentary cilia 2.0–10.6 μm in length (Fig. 5C). Adjacent support cells are joined by apicolateral junctions that resemble zonulae adherentes (Fig. 7C). The basal extensions of the support cells shows from 4 to 9 cytoplasmic sheets 1.5–3 μm in length, which spread over the myocytes and are anchored to the ECM (Fig. 7A). The perikaryon contains a nucleus of an irregular shape. There is a large nucleolus in
the karyoplasm. Irregularly shaped vacuoles occupy most of the cells. The Golgi complex develops generally in the perinuclear region, near the apical plasma membrane and contains a few flattened cisternae and small vesicles 40–50 nm in diameter. Smooth endoplasmatic reticulum and multivesicular bodies are commonly located near the Golgi complex. In some cells there are also autophagosomes and residual bodies (Fig. 7D). The endothelial myocytes are situated under the support cells and are separated from the underlying proboscis muscles by the subjacent ECM (Fig. 7B). The muscle fibers of the inner circular musculature are anchored to the subendothelial ECM by hemidesmosomes (Fig. 7E). Each cell consists of a cell body and peripheral myofilament-containing cytoplasmic processes (Fig. 7B). The cell body of each myocyte contains an irregular nucleus, relatively few granules with electron-dense material (~0.8 μm in diameter) and lucent vacuoles. The ECM underlying the basal surface of the endothelium consists of a densely packed fibrillar material (Fig. 7E). The thickness of the ECM varies from 0.1 to 0.5 μm. Confocal laser scanning micrographs show that the endothelial myocytes form a continuous circular muscle layer about 1–2 μm thick in the inverted proboscis (Figs. 6C,D). According to the SEM data, the endothelium surface has transverse rugosity (Fig. 2D).

**Proboscis of the Juvenile Specimens**

Transmission electron microscopy and CLSM show that the anterior proboscis of juvenile specimens is about 28 μm in diameter and includes three distinct layers, the endothelium with inner circular muscles, the longitudinal musculature, and the glandular epithelium (Figs. 1B, 9B).

The endothelium consists of an external layer of support cells and an internal layer of myocytes. (Figs. 9C,D). The support cells contain a large hyperchrome nucleus; a thin ring of the perinuclear cytoplasm shows few mitochondria and flattened cisternae of the RER (Fig. 9C). Thin apicolateral cytoplasmic sheets of each support cell fully or partially cover the myocytes (Fig. 9C black arrow). Each support cell is basally expanded and rests on the ECM (Fig. 9C white arrow). The cell soma of each myocyte basally protrudes into a
Fig. 9, *H. juliae*. Transmission electron micrographs of transverse sections of the proboscis of an adult (A) and juvenile (B–F) *H. juliae*. (A) Panoramic view of the dorsal half of the proboscis. (B) Panoramic view of the proboscis. (C) Support cells of the endothelium (asterisk), black arrow indicates apicolateral cytoplasmic sheets; white arrow indicates the cytoplasmic sheets extending from basal part of cell body; high magnification shows contractile process of myocyte (white arrowhead) lining on the ECM (black arrowhead). (D) Muscle layer of the proboscis. Black arrows indicate junctions of the subendothelial and subepithelial ECM between two muscle groups; white arrows indicate ECM surrounding muscle group. (E) Glandular epithelium with undifferentiated epithelial cells (white asterisks) and pseudocnidae-containing cells (black asterisks). High magnification micrograph shows cortex (arrowhead) and inner content (white asterisk) of immature pseudocnida. (F) Intercellular space of the glandular epithelium filling numerous granules (asterisk). en, endothelium; ge, glandular epithelium; icm, inner circular musculature; lc, large glandular cell; lm, longitudinal musculature; m, musculature; mv, microvilli; odm, outer diagonal musculature; pn, proboscis nerve.

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single myofilament-containing process up to 0.4 μm thick.

Delicate myofibrils of contractile processes consist of up to five rows of filaments (Fig. 9C inset). The ECM (0.08–0.21 μm thick) underlies the basal surface of the endothelial cells (Fig. 9D back arrow).

The longitudinal proboscis musculature lies under the endothelium and consists of one or two rows of muscle cells (Fig. 9D). This muscle layer is interrupted and divided into 7–8 groups about 1 μm thick (Fig. 1B). Each group contains 4–8 processes of myocytes. Adjacent groups are separated from each other by junctions between the subendothelial and subepithelial ECM (Fig. 9D). The glandular epithelium consists of large glandular cells resting on the basal ECM and measures approximately 0.21 μm thick. The large hyperchrome nucleus of the cells is surrounded by thin rings of electron-dense cytoplasm (Fig. 9E).

The intercellular space is filled with homogenous middle electron-dense matrix, which includes numerous granules (0.2–0.4 μm in diameter) with loosely packed fibrillar material (Fig. 9F). Pseudocnidae-containing cells are distinguished from the other epithelial cells by more lucent cytoplasm, which contains a single large spherical secretory granule per cell (future pseudocnida) up to 6 μm in diameter (Fig. 9E). The granule consists of a homogenous and moderately electron-dense cortex 0.1–0.35 μm thick. The inner content of the granule is formed from lightly packed fibrillar material and small granules up to 0.1 μm in diameter (Figs. 1C1, 9E–high magnification image in left corner).

About 11–12 flattened nerve trunks 2–5 μm in width lie at the basal part of glandular epithelium (Fig. 9B). Each trunk shows a few neurosecretory vesicles (Figs. 9C,D). Most nerve trunks adjoin the longitudinal muscles; the subepithelial ECM between nerve trunks and longitudinal musculature is often absent. Neither glia-like cells nor commissures are present between nerves.

**DISCUSSION**

**Glandular Epithelium**

The glandular epithelium of the nemertean proboscis contains a variety of secretory cells, support cells, and monolocated sensory cells. All of these cells are present in the proboscis of *Hubrechtella*, with the exception of tonofilament-containing support cells. It should be noted that the inverted proboscis of live *H. juliae* breaks at several points as soon as it is removed from the rhyynchocoele (unlike the everted proboscis, which can easily be removed intact). This fact may be attributed to the lack of support cells and tonofilaments in the proboscis of *H. juliae* and possibly mutable connective tissue such as that found in echinoderms (see Ribeiro et al., 2011).

The sensory cells occur in the proboscis of different palaeo- and heteronemertean species (Ling, 1971; Turbeville, 1991, 2006; Montalvo et al., 1996) and have similar morphology: bipolar cells bear a single cilium enclosed by a ring of six to eight prominent microvilli. A detailed description of these cells was provided only for *Riseriellus occultus* Rogers et al., 1993 (Montalvo et al., 1996). The sensory cells of *H. juliae* exhibit the following two prominent character differences from those of *R. occultus*: 1) two rings of the microvilli; 2) numerous (12–20) rootlet fibers. However, it is impossible to determine if these characters are specific for hubrechtiid nemerteans until comparative data for these cells are available for a broad range of nemertean taxa. Also of note is the phalloidin-positive reaction shown by the microvilli of sensory cells in the proboscis of *Hubrechtella* and those in some anoplan and enoplan nemerteans (unpublished observations). These results indicate that phalloidin staining can be a powerful method for investigating the proboscis sensory cells.

In the proboscis of *R. occultus* some gland cell types form two kinds (A and B) of adhesive glandular systems restricted to the ventral surface of the proboscis (Montalvo et al., 1998a). Montalvo et al. (1998a) assume that the type-A glandular systems have an adhesive function, and the type-B glandular systems may contribute to increasing the grip of the proboscis on the prey. The glandular proboscis cells of *Malacobella grossa* (Müller, 1776) (Magarlamov and Chernyshev, 2010) and *H. juliae* are not associated with any glandular systems, while *Tubulanus punctatus* (Takakura, 1898) has only the B type glandular system of (Magarlamov et al., 2011). However, *H. juliae*, similar to *R. occultus* and *T. punctatus*, exhibits dorsoventral differentiation of the proboscis glandular epithelium—pseudocnidae and most glands are restricted to the ventral surface of the proboscis; this surface comes in contact with the prey during its capture. Such differentiation of the proboscis gland cells apparently is the plesiomorphic state for nemerteans, as it is present in both palaeo- and heteronemertean, but is lacking in hoplonemerteans (except *Ototyphlonemertes valentinae* Chernyshev, 2003; see Chernyshev, 2003b).

Up to 10 different types of gland cells were found in the proboscis epithelium in *R. occultus* (Montalvo et al., 1998a; Junoy et al., 2000), whereas *H. juliae* and *Paranemertes peregrina* Coe, 1901 have only four types. Because of limited cytological complexity, homologizing proboscis gland cells is very difficult in nemerteans belonging to different groups. However, similar ultrastructure of pseudocnidae support the hypothesis that they are homologous among anoplan
nemerteans (Turbeville, 2006). According to light microscopy data, the proboscis of seven species of Hubrechtella contains numerous rod-like pseudocnidiae (Hylbom, 1957; Gibson, 1979; Senz, 2000). These structures measured 33 × 4–4.5 μm in H. queenslandica Gibson, 1979 (Gibson, 1979a) and 30 × 3–4 μm in H. malabarensis Gibson, 1979 (Gibson, 1979b). Most measured pseudocnidiae are 2–5 μm long (Turbeville, 2006), reaching 11 μm in some meiofaunal cephalothricids (Gerner, 1969), and in the heteronemertean Riserius pugetensis Norenburg, 1993 they are 0.5 × 13 μm. According to Gibson (1985), the proboscis of the heteronemertean Antarctolineus scotti (Müller and Scripcariu, 1964) contains very long “nematocysts,” up to 100 μm or more long and 2–3 μm wide, but these structures were described only from histological sections, and should not be considered pseudocnidiae with certainty. Therefore, the presence of very large pseudocnidiae is reliably established only for Hubrechtella species.

The proboscides of H. globostica Senz, 1993, H. kimuraorum Kajihara, 2006, and H. Jimai (Takakura, 1922) contain conspicuous acidophilic spherical bodies up to 10 μm in diameter (Senz, 1993; Kajihara, 2006), but it is unclear if they are pseudocnidiae. In the other three species, H. alba Gibson, 1997, H. saradrayayensis Kirsteuer, 1967, and H. sinimarinia Gibson and Sundberg, 1999, pseudocnidiae have not been detected in the proboscis (Kirsteuer, 1967; Gibson, 1997; Gibson and Sundberg, 1999). Hubrechtella is the only nemertean genus, in which the absence or presence of pseudocnidiae is relevant for species taxonomy. The absence of pseudocnidiae is confirmed for three nemertean groups: Carinoma, Baseodiscus, and all hoplonemerteans (Turbeville, 2006; Magarlamov and Chernyshev, 2011). Based on a recent molecular phylogeny of nemerteans (Andrade et al., 2012), this character can be interpreted as having been lost independently in these groups and possibly in some Hubrechtella species. However, a rigorous evaluation of the evolution of this character awaits a more strongly corroborated phylogeny of Nemertea (see also Turbeville, 2006).

The pseudocnidiae of H. juliae differ from the ones of other nemerteans by a peripheral position of the tubular core (filament). The pseudocnidiae of nemerteans studied at the ultrastructural level all have a central core, but differ in overall shape and in aspects of substructure. For example, pseudocnidiae of the palaeonemertean Cephalothrix cf. rufifrons and Tubulanus cf. pellucidus are clavate, whereas those of the palaeonemertean Carinomella lactea Coe, 1905 and the heteronemertean Zygeupolia rubens (Coe, 1895) are rod-shaped (Turbeville, 1991, 2006). Additionally, in contrast to those of latter two species, a lateral electron-dense bulbous process occurs on the pseudocnidiae of Cephalothrix cf. rufifrons and Zygeupolia rubens. The substructure also exhibits some variation in density and elaboration among these organelles (see Ling, 1971; Montalvo et al., 1998a; Turbeville, 2006, this paper). Comparative studies of these filament-containing structures in a broad sample of taxa will be necessary to fully evaluate their diversity.

SEM of the everted proboscis yielded additional relevant data. Previously, only the glandular epithelium of the hoplonemertean Paramenopomus peregrina Coe, 1901 proboscis was studied by SEM (Stricker and Cloney, 1983). In H. juliae SEM data provide a clear juxtaposition of different gland cell neck processes and reveal ultrastructure of the pseudocnidiae with extruded filaments (Figs. 2E,G,H). SEM investigations have long been used to evaluate the highly varied ultrastructure of the everted tubules (= threads) of cnidarian nematocysts (see Ostman, 2000). It is important to note that the extruded filament of H. juliae lacks any spines, fibers or other elaborations that would clarify function of the pseudocnidiae.

Previous investigators suggested that these structures serve to grip prey or puncture the prey’s body wall allowing entry of paralyzing toxins but no direct evidence was provided (see Jennings and Gibson, 1969; Montalvo et al., 1998a; Turbeville, 2006). Experimental investigations will be required to elucidate the function of these unique secretory structures.

Proboscis Nerves
Data from light microscopy suggest that in all Hubrechtella the proboscis possesses a neural sheath without distinct nerves. However, TEM and CLSM investigations demonstrate that the neural sheath of H. juliae is a nervous plexus consisting of 26–33 nerves (11 or 12 in the juvenile specimen). The nerve trunks and their irregular anastomoses are surrounded by glia-like cells and not separated from the glandular epithelium by the ECM. A similar nervous plexus was found in the proboscis of Baseodiscus delineatus (Delle Chiaje, 1825) (Magarlamov and Chernyshev, 2011). In hoplonemerteans from 7 to 10 proboscis nerves also form a plexus but it runs in the longitudinal muscle layer. Moreover, nerves in all hoplonemerteans are arranged radially, following the symmetry of the proboscis, whereas the proboscis nerves of H. juliae are arranged irregularly with numerous anastomoses. A single pair of proboscis nerves, present in all nemerteans, originates from the brain, but in most palaeonemerteans and many heteronemerteans these nerves extend the entire length of the proboscis. This is probably a plesiomorphic state for nemerteans. In Hubrechtella the two distinct proboscis nerves are recognizable only in the most anterior proboscis region (Gibson, 1979a).

All authors reported that the neural layer of the Hubrechtella proboscis lies between the glandular
epithelium and outer circular musculature, as with other palaonemerteans and many heterone-
miteans. However, TEM studies have demonstrated that in the adult and juvenile proboscis of H. juliæ the entire nervous plexus is situated at the base of the glandular epithelium. Obviously, the intraepithelial position of the nervous layer cannot be easily distinguished from the subepithe-
lium, and other species have an inner circular musculature (Gibson 1979a, p. 335). A second significant feature of the proboscis in Hubrechtella species (including type species, H. dubia Bergendal, 1902) as having an outer longitudinal and inner circular musculature; four species of this genus (H. globocystica, H. ijimai, H. kimuraorum, and H. queenslandica) also have an outer circular musculature in addition to these two layers. CLSM observations have shown an unusual arrangement of muscle layers in the proboscis of H. juliæ. The outer diagonal muscles lie under the glandular epithelium while the outer circular musculature is absent (Fig. 1D). In transverse histological sections this diagonal musculature looks like a thin layer of outer circular muscles. It is likely that the outer circular muscles of the four Hubrechtella species mentioned above are also outer diagonal muscles. We do not know with confidence, if H. dubia and other species have an outer diagonal muscle layer (= outer circular mus-
culature), but, as Gibson rightly stated, “it… is unlikely to have been missed in other species,” as suggested for the comparable situation with the inner circular musculature (Gibson 1979a, p. 335).

A second significant feature of the proboscis in Hubrechtella is the presence of inner diagonal muscles under the endothelium (Fig. 1D). This layer is very thin and lies so close to the endothelium that it is difficult to differentiate it from the inner circular musculature (endothelial musculature) in histological sections. Such inner (subendothelial) diagonal muscles were previously detected only in the species of the genus Baseodiscus (Fig. 1F), but the layer is thicker than the endothelial layer (Magarlamov and Chernyshev, 2011). Both inner and outer diagonal layers in H. juliæ consist of nonoverlapping muscles oppositely directed in left and right halves of the proboscis. The same arrangement of outer diagonal muscles was described in the proboscis of some lineids, but this diagonal layer forms one or two muscle crosses (Fig. 1G) (Chernyshev, 2010). There are no muscle crosses in the proboscis of H. juliæ, though outer diagonal muscles of the left and right sides overlap on the ventral and dorsal sides of the proboscis. Among other hubrechtiiid nemerteans a typical muscle cross was observed only in H. jimai (Kajihara, 2006).

The proboscis longitudinal musculature of the juvenile specimen of H. juliæ is divided into 7–8 groups (or bands). Such sectoring is distinct in the proboscis of most hoplonemerteans, in which poly-
radially arranged proboscis nerves interrupt the inner part of the longitudinal musculature. In some palaonemerteans (Callinera species) longi-
tudinal muscles of the first (most anterior) region of the proboscis are arranged in four bands (Rog-
ers et al., 1992; Chernyshev, 2002; Kajihara, 2006), and this state may be plesiomorphic for nemerteans. Unfortunately, there are no data on the morphology of the proboscis of other juvenile nemerteans; therefore, we cannot determine the formation of the proboscis musculature in other nemerteans.

Endothelium
According to TEM data, the endothelium of the proboscis in all studied nemerteans is organized as a pseudostratified myoepithelium and consists of two cell types: apically situated support cells and subapical myocytes. Usually, one layer of myocytes forms the inner circular musculature (3–5 μm thick) as in Riseriellus occultus (Montalvo et al., 1998b), Malacobdella grossa (Magarlamov and Chernyshev, 2010), and H. juliæ (present investiga-
tion). Likewise the inner circular endothelial musculature appears to be formed by one layer of myocytes in Carinoma tremaphoros and Tabula-
nus cf. pellucidus (Turbeville, 1991). The layer of the inner circular muscles in Baseodiscus delinea-
tus (Magarlamov and Chernyshev, 2011) and some reptantan nemerteans (unpublished observations) is 30–60 μm thick and consists of 4–7 layers of muscle cells. The subendothelial circular muscula-
ture has been found in nearly all nemerteans stud-
ted by TEM (Ling, 1971; Turbeville, 1991; Montalvo et al., 1998b; Magarlamov and Cherny-
shev, 2010, 2011) including H. juliæ. These facts suggest that the inner circular musculature is exclusively endothelial in origin.

The proboscis endothelium is exposed to the rhynchocoel fluid and is continuous with the lining of the rhynchocoel, which is considered a coelomic cavity (see Turbeville, 1991, 2002). Because the endothelium is likely derived from mesoderm and forms an extension of the rhynchocoel lining (peritoneum), this pseudostratified myoepithelium can

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be interpreted as a peritoneum. It is noteworthy that the coelom lining of regions of the coeloms of some annelids has a similar morphology (see Bartolomaeus, 1994; Rieger and Purschke, 2005).

**Phylogenetic Implications**

The genus *Hubrechtella* with a single species, *H. dubia*, was described by Bergendal (1902) as the second genus of the family Hubrechtiiidae Bürger, 1895 (or, erroneously, Hubrechtidae). By 2003, this family included 16 species from five genera: *Hubrechtia*, *Hubrechtella*, *Coeia*, *Tetramys*, and *Sundbergia*. Cladistic analysis separated the genus *Hubrechtia* from *Hubrechtella* and other hubrechtiiids (Sundberg and Hylbom, 2004), providing the basis for establishing of the family Hubrechtellidae (Chernyshev, 2003a).

Bürger (1895) considered Hubrechtiiidae an intermediate group between lower nemerteans (Tubulanidae) and heteronemerteans and assigned this family to order Protonemertea. The family Hubrechtiiidae s.l. was regarded as a part of the order Palaeonemertea in the 20th century, although different authors pointed out its close relation to the order Heteronemertea (see Hylbom, 1957). Cantell (1969) showed that *Hubrechtella dubia* has pilidium larvae and inferred that *Hubrechtella* may be considered a heteronemertean “which has, in the evolutionary process, lost its outer layer of longitudinal muscles” (p. 93).

Norenburg (1993) described the unusual heteronemertean *Riserius pugetensis*, which is very similar to *Hubrechtella*, and characteristics of both suggest a relationship with the baseodiscid heteronemerteans. He suggested considering *Hubrechtella* + Heteronemertea to be a conceivably monophyletic group based on sharing pilidium larvae. Molecular phylogenetic analysis carried out by Thollesson and Norenburg (2003), confirmed the monophyly of *Hubrechtella* + Heteronemertea and suggested a new name for this group, Plidiophora. Monophyly of Plidiophora is supported by a few morphological synapomorphies, including, a pilidium larva, caudal cirrus, special subepidermal layer (cutis), and dermal musculature (Chernyshev, 2011). A recent molecular phylogenetic analysis with additional data and taxa, but including only a single species of *Hubrechtella*, revealed only weak support for monophyly of Plidiophora with maximum likelihood and Bayesian analysis and no support with direct optimization analysis (Andrade et al., 2012). The authors considered plidiophoran monophyly neither supported nor refuted with present molecular data. Phylogenetic data for nemerteans are forthcoming (Andrade and Giribet, personal communication), but it will be very important to find and evaluate additional morphological characters common for heteronemerteans and *Hubrechtella*.

According to recent CLSM observations, the proboscis of all palaeonemerteans (Tubulanidae s.l., Carinomidae, and Cephalotrictidae) consists of four muscle layers: outer circular, outer diagonal, longitudinal, and inner (endothelial) circular (Fig. 1E). This arrangement of the muscle layers (palaeotype) has a more complicated morphology in most heteronemerteans (heterotype), owing to the presence of an additional outer longitudinal muscle layer and one or two muscle crosses (Fig. 1G).

Both outer circular and the outer diagonal musculature are absent from the proboscis of the heteronemerteans, *Riserius pugetensis*, *Valencinia longirostris* Quatrefages, 1846, *Cephalomastax brevis* Iwata, 1957, and species of the genus *Baseodiscus* (Fig. 1F; Iwata, 1957; Norenburg, 1993; Magarlamov and Chernyshev, 2011). Apart from these heteronemerteans, eight other species of *Hubrechtella*, including the type species, *H. dubia*, lack outer circular (= outer diagonal) muscles (judging from the descriptions). *H. juliae* has the outer diagonal musculature but lacks the outer circular one, which may be regarded as an intermediate state between “palaeotype” and the proboscis of *H. dubia*. However, available data indicate that in *Hubrechtella* and some heteronemerteans (including *Baseodiscus*) the outer circular and outer diagonal muscle layers were reduced independently. The proboscis of *Baseodiscus* (Fig. 1F) and, evidently, *Valencinia longirostris* possesses the inner (subendothelial) diagonal muscle layer (Norenburg, 1993; Magarlamov and Chernyshev, 2011), which is another similarity in the proboscis structure between them and *H. juliae*. The inner diagonal musculature is arranged in a unique way for *Baseodiscus*, *Valencinia*, and *Hubrechtella*, and thus its homology to the outer diagonal musculature of palaeonemerteans and other heteronemerteans is not supported. The inner diagonal musculature may be homologous in *Hubrechtella* and some lower heteronemerteans, but to test this hypothesis, the proboscis musculature should be investigated using CLSM methods in other related species.

The presence of one or two muscle crosses in the proboscis is the specific character of the heteronemerteans, although some species lack the crosses (e.g., all species of the genus *Baseodiscus*). There are no muscle crosses in the proboscis of *H. juliae*, but only irregularly distributed radial muscle fibers between the layers of outer and inner diagonal muscles (Fig. 6D). One muscle cross has been reported in *H. jimai* (Kajihara, 2006). In the lineid heteronemerteans two oppositely directed and laterally nonoverlapping diagonal layers are formed from the muscle crosses and in palaeonemerteans outer diagonal muscles have a crisscrossed arrangement (Chernyshev, 2010). The diagonal muscles of *H. juliae* do not overlap laterally, but they are crisscrossed dorsally and ventrally, that is, at the points, where the dorsal and ventral muscle crosses are located in lineid heteronemerteans. To sum up, the
probscisc musculature in *H. juliae* and most hetero-
nermecians is bilaterally arranged, which can be
considered a possible synapomorphy of Hubrechtellidae + Heteronemertea (= Pilidiophora), in addition
to the pildium larva, caudal cirrus, subepidermal layer (cutis) and dermal musculature (Norenburg, 1993; Kajihara, 2006; Chernyshev, 2010, 2011). According to Chernyshev (2011), both families, the monotypic Hubrechtellidae and Hubehtellieidae (see above), belong to a separate order Hubehtiformes of the superorder Pilidiophora. Further morphologi-
cal investigations of Hubrechtella, related species and basal heteronermecians should provide addi-
tional insight into the phylogenetic position of Hubrechtella.

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