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ORIGINAL PAPER

Ultrastructure of protonephridia in *Xenotrichula carolinensis syltensis* and *Chaetonotus maximus* (Gastrotricha: Chaetonotida): comparative evaluation of the gastrotrich excretory organs

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Abstract In an attempt to obtain detailed information on the entire protonephridial system in Gastrotricha, we have studied the protonephridial ultrastructure of two paucitubulatan species, *Xenotrichula carolinensis syltensis* and *Chaetonotus maximus* by means of complete sets of ultrathin sections. In spite of some differences in detail, the morphology of protonephridia in both examined species shows a common pattern: Both species have one pair of protonephridia that consist of a bicellular terminal organ, a voluminous, aciliar canal cell and an adjacent, aciliar nephridiopore cell. The terminal organ consists of two monociliar terminal cells each with a distal cytoplasmic lobe. These lobes interdigitate and surround cilia and microvilli of the terminal cells. Where both lobes interdigitate, a meandering cleft is formed that is covered by the filtration barrier. We here term the entire structure composite filter. The elongated, in some regions convoluted protonephridial lumen opens distally to the outside via a permanent nephridiopore. A comparison with the protonephridia of other species of the Gastrotricha allows hypothesising the following autapomorphies of the Paucitubulata: The bicellular terminal organ with a composite filter, the convoluted distal canal cell lumen and the absence of cilia, ciliary basal structures and microvilli within the canal cell. Moreover, this comparative survey could confirm important characteristics of the protonephridial system assumed for the ground pattern of Gastrotricha like, for example, the single terminal cell with one cilium surrounded by eight microvilli.

Keywords Gastrotricha · Paucitubulata · Phylogenetic implications · Protonephridial system · Ultrastructure

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Introduction

Filtration nephridia remove wastes from the amino acid and nucleotide degradation as well as sometimes salt and water from the body. Elimination of these wastes is performed in a two step process, consisting of an initial ultrafiltration followed by modification of the ultrafiltrate. In protonephridia, the terminal region performs the filtration process and the canal cells modify the ultrafiltrate by endo- and exocytotic processes. The term protonephridium is basically defined by the structural integrity of the entire organ: Filtration and modification occur in the same organ, the terminal region is attached to the duct (Ruppert and Smith 1988; Bartolomaeus and Ax 1992). Protonephridia are often regarded as homologous throughout Bilateria and detailed hypotheses on their primary structure have been developed. According to these hypotheses, protonepridia primarily consisted of a single terminal cell, one canal and one nephridiopore cell. Gastrotricha is often regarded as sister taxon of the remaining Nemathelminthes which presumably represent one of

the three early bilaterian taxa [see Ax (2003) for discussion]. One therefore should expect that their protonephridia show several of the presumed plesiomorphic characters. However, despite their small size, Gastrotricha display a surprisingly high structural diversity in their protonephridial morphology. Their protonephridial system consists of one to several pairs in all Gastrotricha (Wilke 1954; Teuchert 1967; Mock 1979; Ruppert 1979) and may exceed three cells per organ in several species. Since the organs consist of a limited number of cells, reconstructions ought to be performed by using complete sets of ultrathin sections to guarantee a sufficiently detailed understanding of the morphology of these organs. Only the investigations of three species of Macrodasyida are satisfying in this respect (Teuchert 1973; Neuhaus 1987). They all clearly show that the terminal cell is monociliar and forms a perforated cytoplasmic hollow cylinder that surrounds the cilium and its peripheral circle of microvilli. There are only a few and even more scattered data from Paucitubulata (*Chaetonotus* sp., Brandenburg 1962, *Draculiciteria tesselata*, *Lepidodermella squammata*, Ruppert 1991) and *Neodasys* (*Neodasys* sp., Ruppert 1991). An extensive analysis of the protonephridial system of these taxa does not exist. Since Paucitubulata and *Neodasys* are regarded as highest-ranking sister taxa of the Chaetonotida, itself sister group to Macrodasyida, we here present a complete set of data on two species of the Paucitubulata. It is well known that one major problem in morphology research is terminology. It is therefore necessary to define the terms used before the actual description of the morphological structures. Criteria to recognise and to identify structures follow from these definitions and will be applied in the descriptive part.

In order to use the protonephridia for a new perspective on the early evolution of Bilateria, we first of all need the character states of protonephridia of the stem species of Gastrotricha. It is clear that it is not useful to analyse and reconstruct the phylogenetic relationships within Gastrotricha on the basis of a single organ system like protonephridia only. Therefore, we will evaluate our findings on the basis of an actually discussed phylogenetic system of Gastrotricha (Hochberg and Litvaitis 2000) with the aim to infer the protonephridial ground pattern of this taxon.

Materials and methods

The investigated specimens of the intertidal *Xenotrichula carolinensis syltensis* Mock, 1979 were sampled between October 16 and 18, 2002, at the beach of the islet Uthörn situated in the bay of Königshafen at the northern tip of the island Sylt, Germany. The specimens were extracted from the sandy sediment with the modified seawater-ice method according to Uhlig (1964) and Ruppert (1972).

Specimens of the freshwater gastrotrich *Chaetonotus maximus* (Ehrenberg, 1831) Zelinka, 1889 were sampled on April 4, 2002 in a muddy pond on the campus of the University of Bielefeld and on 9 September 2003 in the small brook Johannisbach near Hoberge-Uerentrup (Bielefeld, Germany). Extraction was carried out by a combination of suspending, decanting and sieving the samples through a plankton gauze (50 µm mesh size) corresponding to Balsamo and Todaro (2002).

For transmission electron microscopy (TEM) animals were fixed in 2.5% glutaraldehyde (a little ruthenium-red added) in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h (*C. maximus*) or 3 h (*X. carolinensis syltensis*) at 4°C. They were postfixed for 45 min at 4°C in 1% OsO_4, (buffered in 0.1 M sodium cacodylate solution, pH 7.2), dehydrated in an increasing acetone series and embedded in Araldite. Serial ultrathin sections (70 nm) were performed on a Reichert Ultracut S microtome and stained automatically with uranyl acetate and lead citrate (Leica EM Stain). They were observed on a Philips CM 100 at 60 kV and a Zeiss EM 109 at 50 kV. Digitized TEM micrographs were the basis for the graphical reconstruction.

Terminology

For an explicit communication and description of complex morphological structures, we will now give a clear explanation of the most important terms (Fig. 1a–e). It is the aim to use these terms as a standard in further investigations of the ultrastructure of protonephridia.

Protonephridial system All protonephridia represent the protonephridial system of an organism.

Terminal organ The terminal organ is the unit which enables the process of ultra-filtration by forming structures to (1) generate a negative pressure and (2) to provide a filtration barrier. The terminal organ can consist of (1) a single terminal cell or (2) of two or more aggregated terminal cells or (3) of a terminal cell and a following canal cell (thus forming a weir, Bartolomaeus and Ax 1992).

Simple filter The simple filter is the region of the terminal cell which forms the filtration barrier. It consists of a perforated cytoplasmic hollow cylinder that encloses a small compartment which contains cilia and microvilli of the terminal cell. The perforations might exhibit certain patterns of clefts or pores of various shape. Diaphragms that seal the clefts and pores represent the actual site of ultrafiltration. The simple filter is connected to the adjacent canal cell by cellular junctions.

Composite filter A composite filter is formed by more than one terminal cell. Distally oriented lobes of the terminal cells interdigitate and surround a common

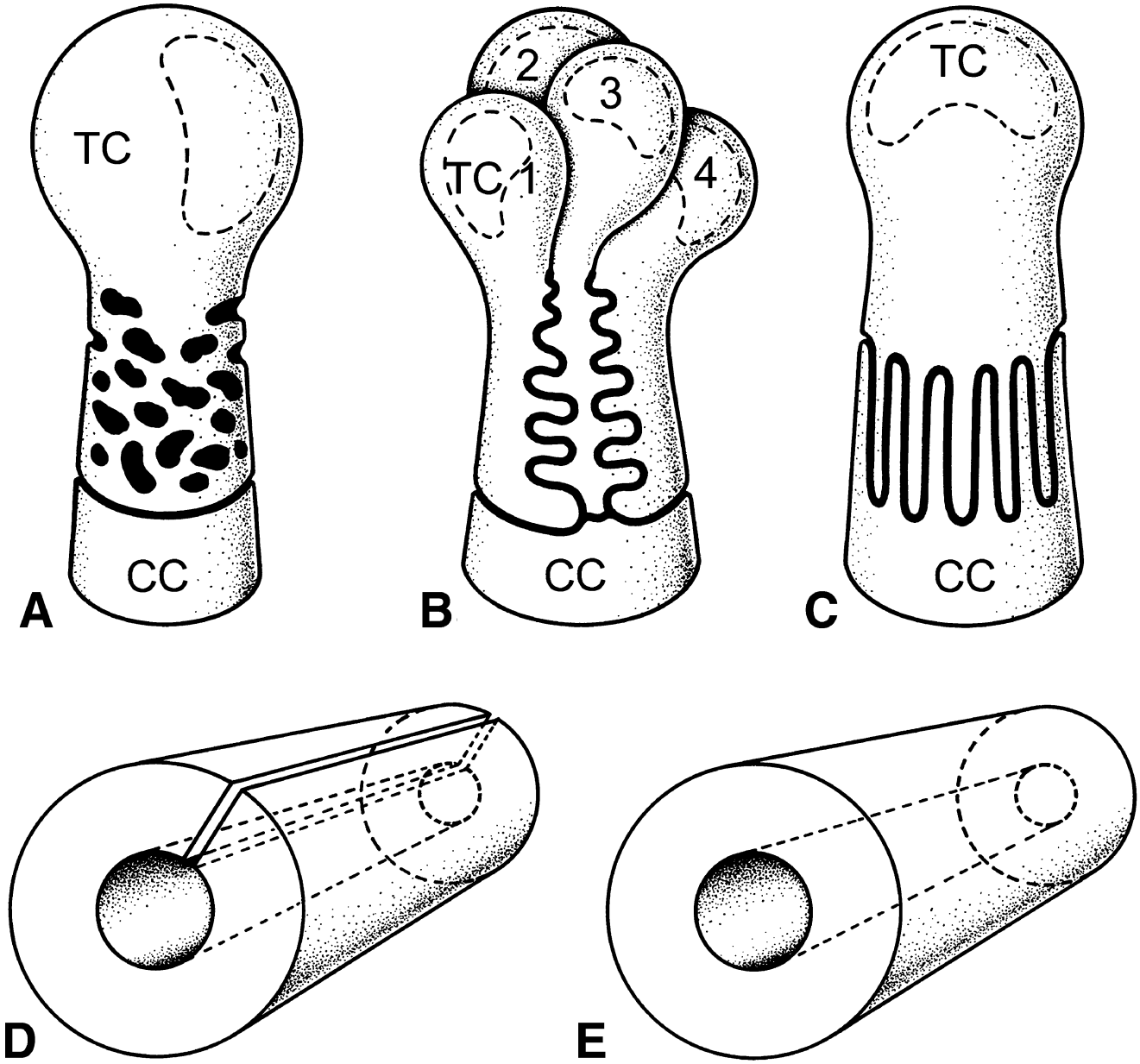

Fig. 1 Terminology of protonephridia. **a–c** Different types of the terminal organ and the filtration barrier. The nuclei (*broken line*) indicate the proximal pole, the adjacent canal cell (*CC*) indicates the distal pole of the terminal organ. **a** Single terminal cell (*TC*) with a simple filter perforated by irregular pores, **b** terminal organ consisting of four terminal cells (*TC1-4*) with a composite filter, **c** single terminal cell forms a weir with the adjacent canal cell. **d, e** Different types of the protonephridial lumen. **d** Enfolded lumen, **e** Enclosed lumen

compartment that houses the cilia and microvilli of the terminal cells. Where the lobes interdigitate, a meandering cleft is formed. This cleft is sealed off by a diaphragm which represents the actual site of filtration. The lobes are connected to the adjacent canal cell by cellular junctions.

Protonephridial lumen The protonephridial lumen is the sum of the terminal organ-, canal cell- and nephridiopore cell lumen. In most instances, the protonephridial lumen constitutes a continuous canal from the filter region to the nephridiopore. The protonephridial lumen, or parts of it, may have a straight or a convoluted passage through the whole organ or even sections.

Enfolded lumen In cross section, the cell membrane of the considered cell appears invaginated thus forming a lumen. The resultant cleft is sealed off by a belt desmosome and a septate junction (autodesmosome sensu Kristensen and Hay-Schmidt 1989).

Enclosed lumen In cross section of the considered cell, one can see a membrane enclosing the lumen and an outer cell membrane, each separated from the other.

Results

Ultrastructure of protonephridia of two members of Paucitubulata (d'Hondt, 1971)

Xenotrichula carolinensis syltensis *Mock, 1979*

Protonephridial system The nephridial system in *X. carolinensis syltensis* consists of a single pair of protonephridia, each situated in a lateral position posterior to the area of the pharyngeo-intestinal junction (Fig. 2a). Medially it is bordered by epithelium or musculature of the midgut, laterally by the testes (Figs. 5b, 6a).

The protonephridium consists of three distinct compartments: (1) a terminal organ with two terminal cells, (2) one canal cell and (3) one nephridiopore cell (Figs. 3a, b, 4a–c). The cells are linked by septate junctions and belt desmosomes (Fig. 5e, f). The continuous protonephridial lumen consists of the consecutive terminal organ-, canal cell- and nephridiopore cell lumen. Peripherally to the filter, the primary body cavity is enlarged (Fig. 5a, c–e). The extracellular matrix (ECM) within the filter region and other compartments of the protonephridium is very electron-lucent and flocculent. There is no distinct, perinephridial basal lamina present. The protonephridia measure 5.5–6 µm in frontocaudal length, whereas the highest dorsoventral extension is up to 10 µm. The lateral dimension varies from only 2–4 µm, in the area where the nephridiopore cell is situated it reaches 6 µm in diameter (Figs. 3a, 4a, b).

Terminal organ The terminal organ consists of two terminal cells forming one functional unit. Each terminal cell consists of one 8 µm long cilium, a basal body and 8 circumciliary microvilli (length: <8 µm, diameter: 75 nm) (Fig. 5c–e). An accessory centriole and ciliary rootlets, however, are absent. The microvilli are linked by flocculent ECM (Fig. 5c, e). Cilia and microvilli extend to the level of the canal cell and have a coaxial orientation throughout their whole length (Figs. 3a, 4a, 6a, c). Their longitudinal axis is oblique within the animal: It is oriented from frontodorsomedian to caudo-ventrolateral (Fig. 2a). Both terminal cells form a 2 µm long composite filter (Figs. 3b, 5c–e, g). A diaphragm bridges the meandering clefts (width: ~25 nm) between the cytoplasmic, podocyte-like processes (diameter: 75–100 nm). It appears flocculent and moderately electron-dense in tangential sections (Fig. 5g). The nucleus of each terminal cell is situated in a cytoplasmatic pocket proximal to the filter region (Figs. 3a, b, 5a–c, e).

Canal cell Within the canal cell, no cilia, basal bodies, accessory centrioles, ciliary rootlets or microvilli are present. The canal cell lumen is an enfolded lumen and subdivided into two distinct portions: A straight and 6 µm long proximal part and a highly convoluted distal part (Figs. 3a, 4a, b, 5b, 6a, b). The proximal canal cell lumen has a diameter of 1–1.5 µm and encloses cilia and microvilli of the terminal organ (Fig. 6b). It leads into the narrower distal canal cell lumen (diameter: 250 nm) via a characteristic curve (Fig. 6a). Mitochondria of christae-type are sparsely placed within the canal cell. A variety of membrane-bounded granules, vesicles and vacuoles are present: There are few large vacuoles ranging between 2 and 4 µm in diameter and a relatively high density of small, electron-lucent vesicles measuring 30–75 nm in diameter and vesicles of intermediate size with a diameter of 0.5–1 µm. Vacuoles and intermediate vesicles are filled with moderately electron-dense, fine-granular material (Figs. 5b, 6a, c, e). The small vesicles are located between the convolutions of the distal canal cell lumen, sometimes coalesced with the membrane of the vacuoles (Figs. 5b, 6a). The nucleus of the canal cell is placed near the proximal canal cell lumen (Figs. 3a, 4a, b, 6a–d).

Nephridiopore cell The nephridiopore cell is placed medially to the canal cell (Figs. 4a, b, 6c, d). There are no cilia, basal bodies, accessory centrioles, ciliary rootlets or microvilli present. The nephridiopore cell lumen (diameter around 50 nm) is an enfolded lumen and divided into a convoluted proximal (Figs. 4a, b, 6c, d) and a straight distal portion (Figs. 4a, 6d). The distal part of the nephridiopore cell pierces the ventral integument (Fig. 6c, d), but a direct connection between the nephridiopore cell lumen and the exterior was not seen. The nucleus is situated beneath the proximal nephridiopore cell lumen (Figs. 4a, 6c, d). Within the cytoplasm of the nephridiopore cell only a few organelles are present.

Chaetonotus maximus *(Ehrenberg, 1831)*

Protonephridial system *Chaetonotus maximus* has a single pair of protonephridia. They are situated in a lateral position and reach from the pharyngeo-intestinal junction to around halfway of the midgut (Fig. 2b). Medially, each protonephridium is bordered by the intestine, posteriorly the mature oocyte is the adjacent tissue. Laterally, the protonephridia are bordered by the body wall (Fig. 10a).

The protonephridium in *C. maximus* is built up of three distinct compartments: (1) a terminal organ consisting of two terminal cells, (2) a canal cell of high length and (3) a nephridiopore cell containing the nephridiopore (Figs. 7a, b, 8a, b, 9a–d). The cells are linked by septate junctions and belt desmosomes (Figs. 9d, 10d, 11d).

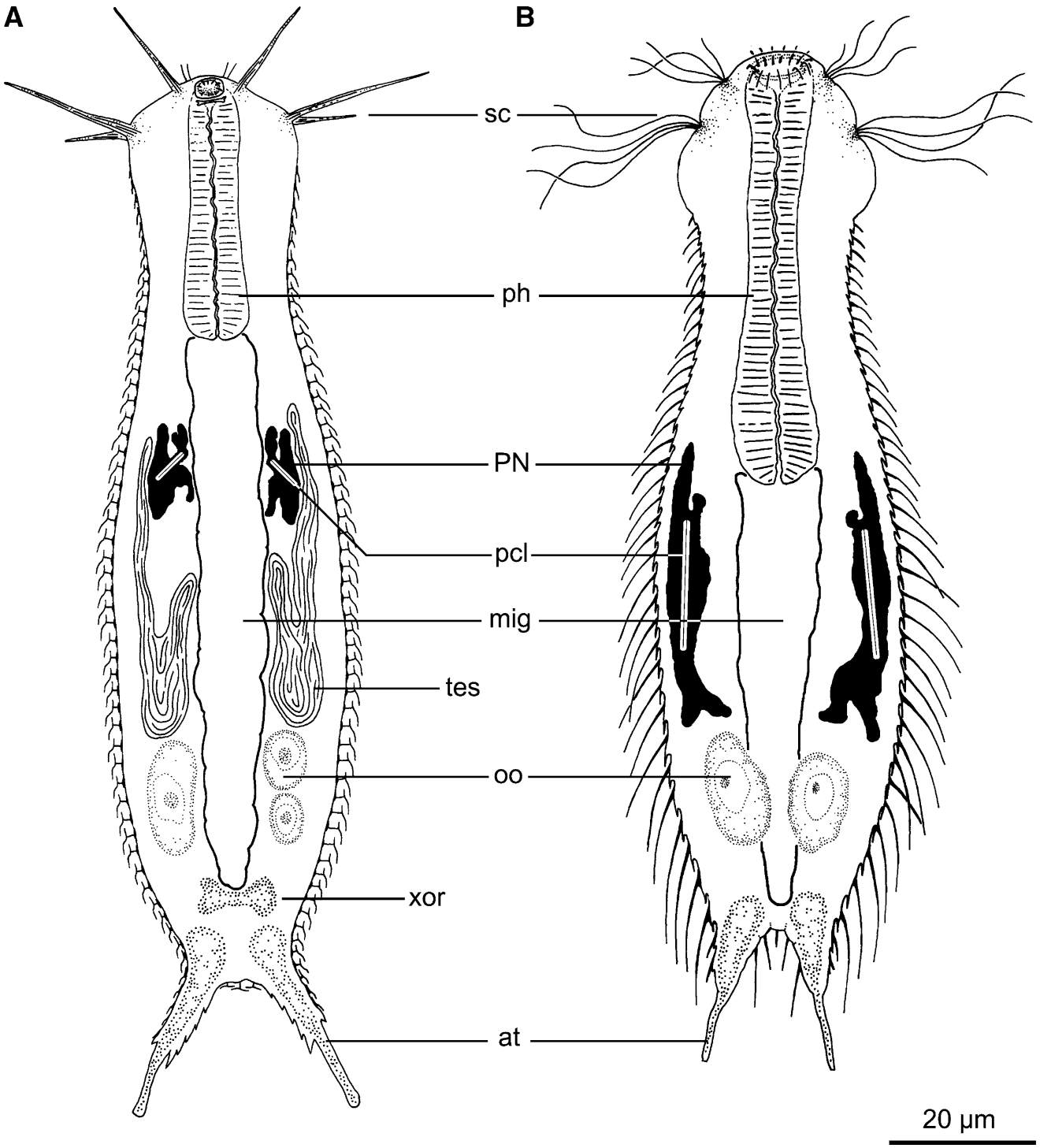

Fig. 2 Position of the protonephridia in the investigated species. **a** Schematic horizontal section of *Xenotrichula carolinensis syltensis* and **b** of *Chaetonotus maximus*. Note the different orientation of the proximal canal cell lumen in both species. *at* adhesive tubes, *mig* midgut, *oo* oocytes, *pcl* proximal canal cell lumen, *ph* pharynx, *PN* protonephridium, *sc* sensory cilia respectively cirri, *tes* testes, *xor* x-organ. After light microscopic observations

The continuous, highly elongated protonephridial lumen consists of the consecutive terminal organ-, canal cell- and nephridiopore cell lumen (Fig. 9d). The primary body cavity is enlarged in the filter region and forms a 0.5–1 µm wide compartment (Fig. 10a–d). It is filled with homogeneous, little electron-lucent ECM which is reduced to a thin layer (thickness: 20 nm) when lining other regions of the protonephridium (and other tissues).

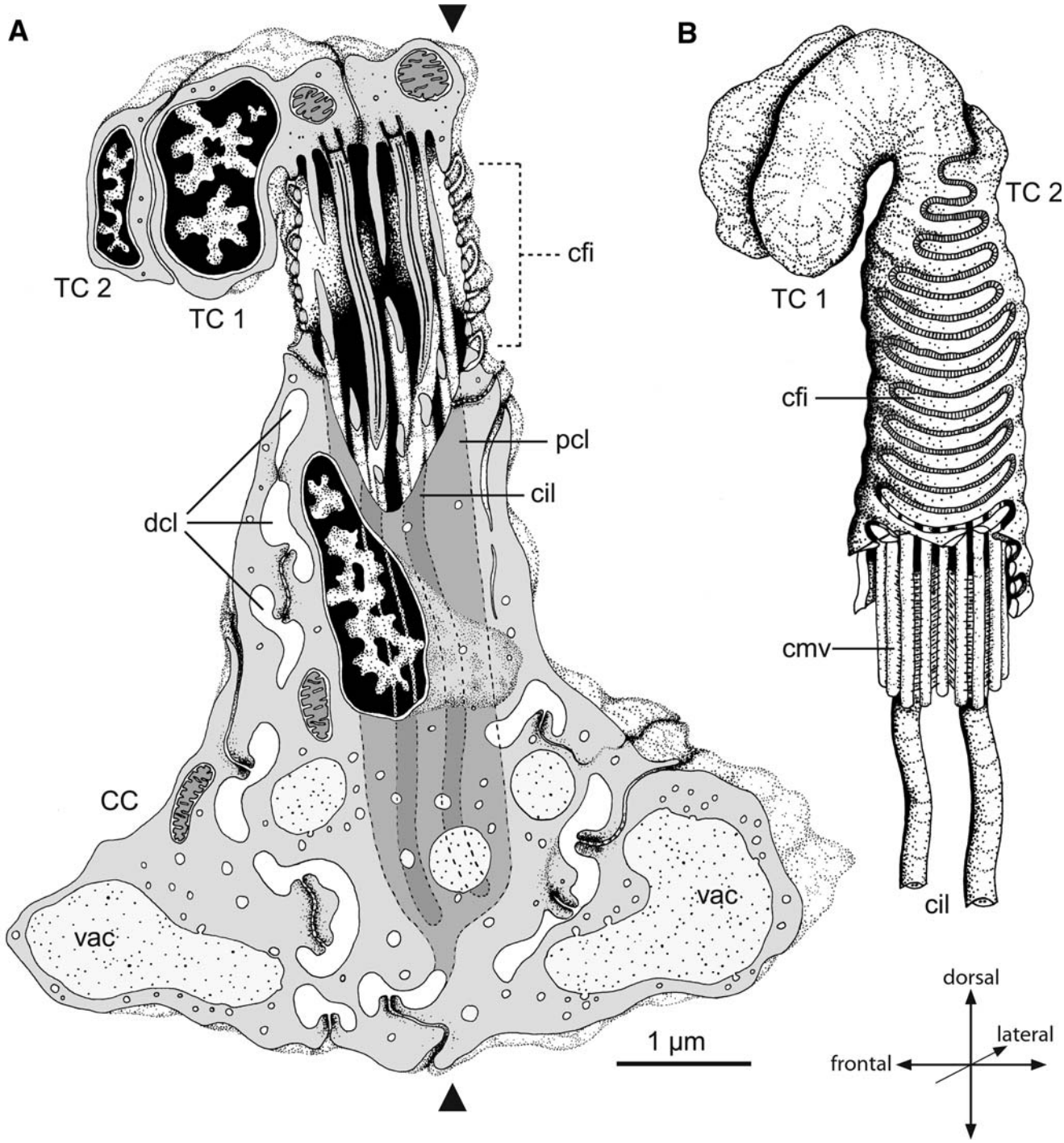

Fig. 3 *Xenotrichula carolinensis syltensis*. Schematic reconstruction of the protonephridium. **a** Longitudinal section of the protonephridium, nephridiopore cell not displayed. *Black triangles* indicate level of section plane for Fig. 4a. **b** External view of the terminal organ with the composite filter. *CC* canal cell, *cfi* composite filter, *cil* cilia, *cmv* circumciliary microvilli, *dcl* distal canal cell lumen, *pcl* proximal canal cell lumen, *TC1/2* terminal cells, *vac* vacuole

Each protonephridium reaches a longitudinal dimension of nearly 40 µm and a maximum dorsoventral extension of 10 µm. With its lateral diameter measuring 1–2 µm the protonephridium of *C. maximus* is laterally compressed.

Terminal organ Two adjacent terminal cells form the terminal organ in *C. maximus*. Each terminal cell consists of a single, 27 µm long cilium originating from a basal body and eight circumciliary microvilli (diameter: 100–150 nm,

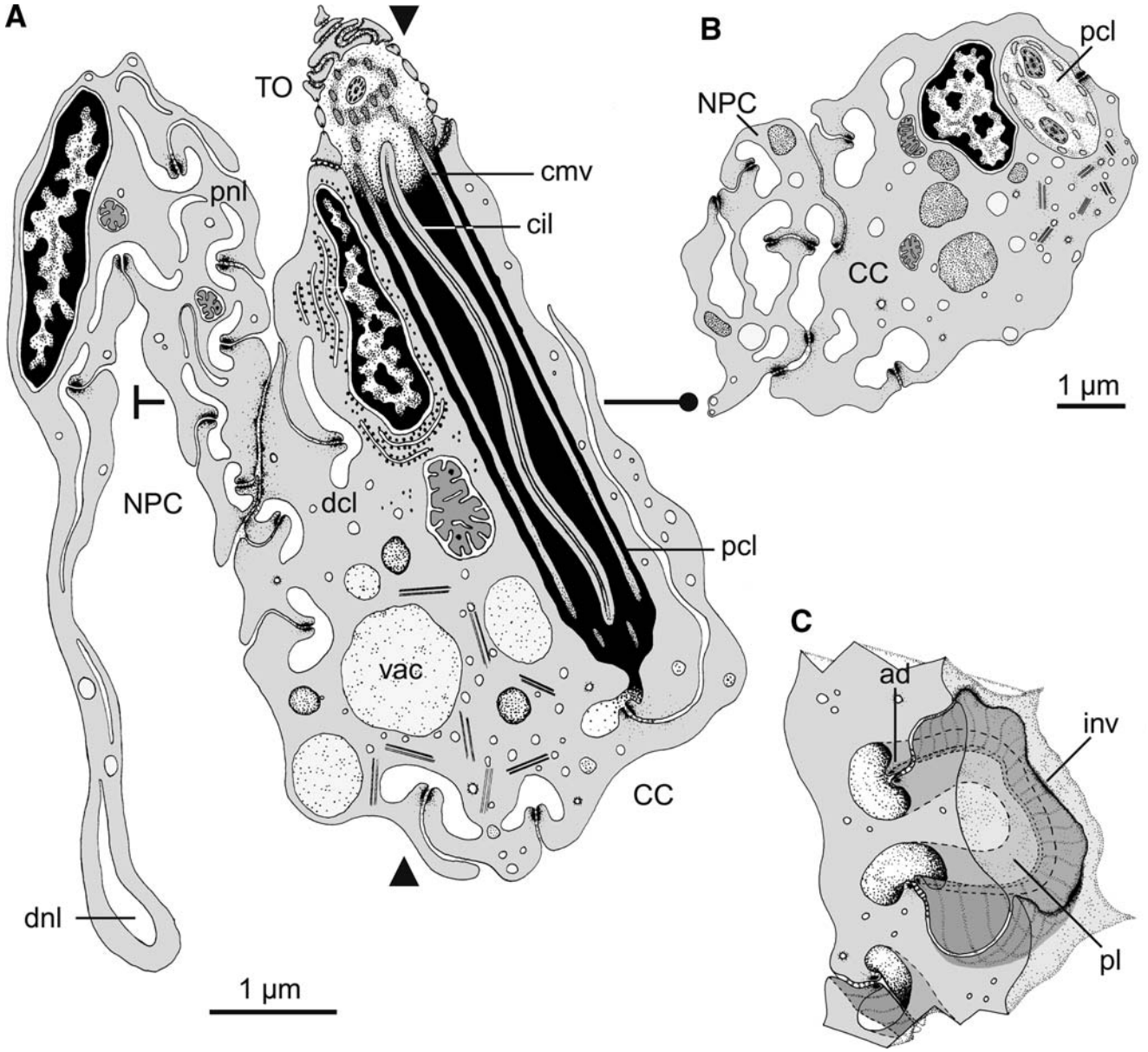

Fig. 4 *Xenotrichula carolinensis syltensis*. Schematic reconstruction of the protonephridium. **a** Cross section of the protonephridium showing terminal organ, canal- and nephridiopore cell. *Black triangles* indicate level of section plane for Fig. 3a. **b** Horizontal section of canal- and nephridiopore cell. Level of section plane indicated by bold line in a. **c** Scheme illustrating the convoluted, enfolded protonephridial lumen, which is an invagination of the cell membrane. *ad* autodesmosome, *CC* canal cell, *cil* cilia, *cmv* circumciliary microvilli, *dcl* distal canal cell lumen, *dnl* distal nephridiopore cell lumen, *inv* invagination of the cell membrane, *NPC* nephridiopore cell, *pcl* proximal canal cell lumen, *pl* protonephridial lumen, *pnl* proximal nephridiopore cell lumen, *TO* terminal organ, *vac* vacuole

length: 27 µm maximum) (Figs. 7a, b, 9b). An accessory centriole and ciliary rootlets are absent.

Circumciliary microvilli are linked by ECM showing an alternating, mirror-symmetrical pattern of electron-dark and -lucent bands in cross sections (Fig. 10c). Tangentially sectioned, this reticulate intermicrovillar ECM shows a pattern of small pores (diameter: 10–20 nm) with high level of regularity (Fig. 10c: inset).

Cilia and microvilli of the terminal organ are parallel throughout their whole length and project deeply into the proximal canal cell lumen (Figs. 7b, 8a, b, 9d). Their common axis is coaxial to the frontocaudal one of the whole animal (Fig. 2b).

The 5 µm long filter region in *C. maximus* consists of a composite filter (Fig. 7a). The 20 nm wide, meandering clefts between the podocyte-like processes (diameter around 60 nm) are bridged by a diaphragm (Fig. 10c).

The globular nucleus of one terminal cell is situated within a cytoplasmic pocket proximal to the filter (Figs. 7a, 9a). The second terminal cell also forms a cytoplasmic

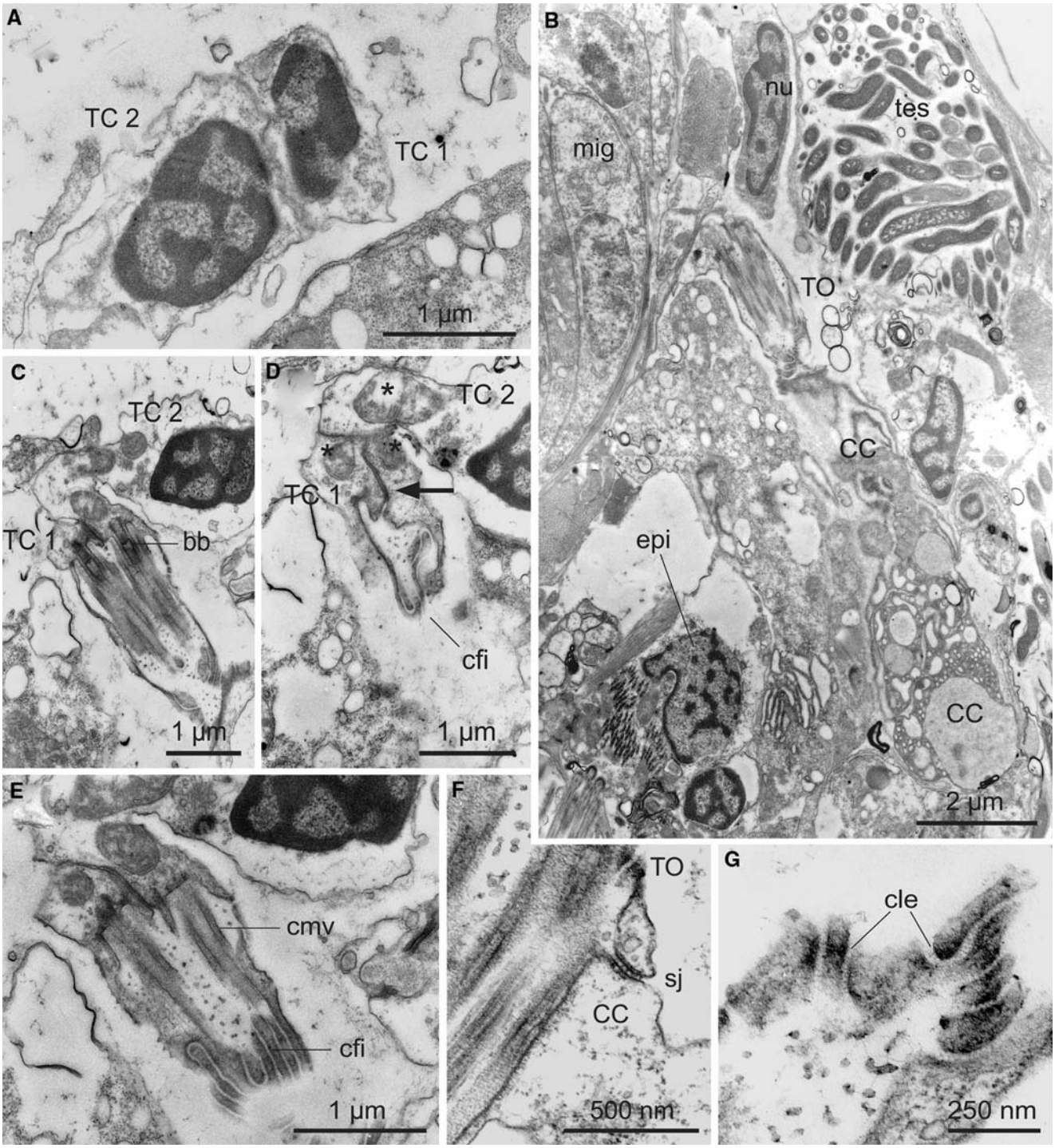

Fig. 5 *Xenotrichula carolinensis syltensis*. Ultrathin cross sections (TEM) of the protonephridium. **a** Cytoplasmic pockets of both terminal cells containing the nuclei (*TC1/TC2*). **b** Section of the terminal organ (*TO*) and the canal cell (*CC*) showing the adjacent tissues of the trunk region: testis (*tes*), midgut (*mig*) and epidermal cells (*epi*). The marked nucleus (*nu*) belongs to a terminal cell. **c** Terminal organ at the level of the basal bodies (*bb*). **d** Proximal region of the terminal organ with mitochondria (*asterisk*) and the composite filter (*cfi*). Note the border between both cells (*arrow*) lined by electron-dark material. **e** Tangential section of the composite filter (*cfi*) and some circumciliary microvilli (*cmv*). **f** Connection of the terminal organ (*TO*) and the canal cell (*CC*). Septate junction (*sj*) within the intercellular gap and electron-dense material along the cellular borders are clearly visible. **g** High magnification micrograph of the tangentially sectioned composite filter with the meandering cleft (*cle*) sealed by the diaphragm

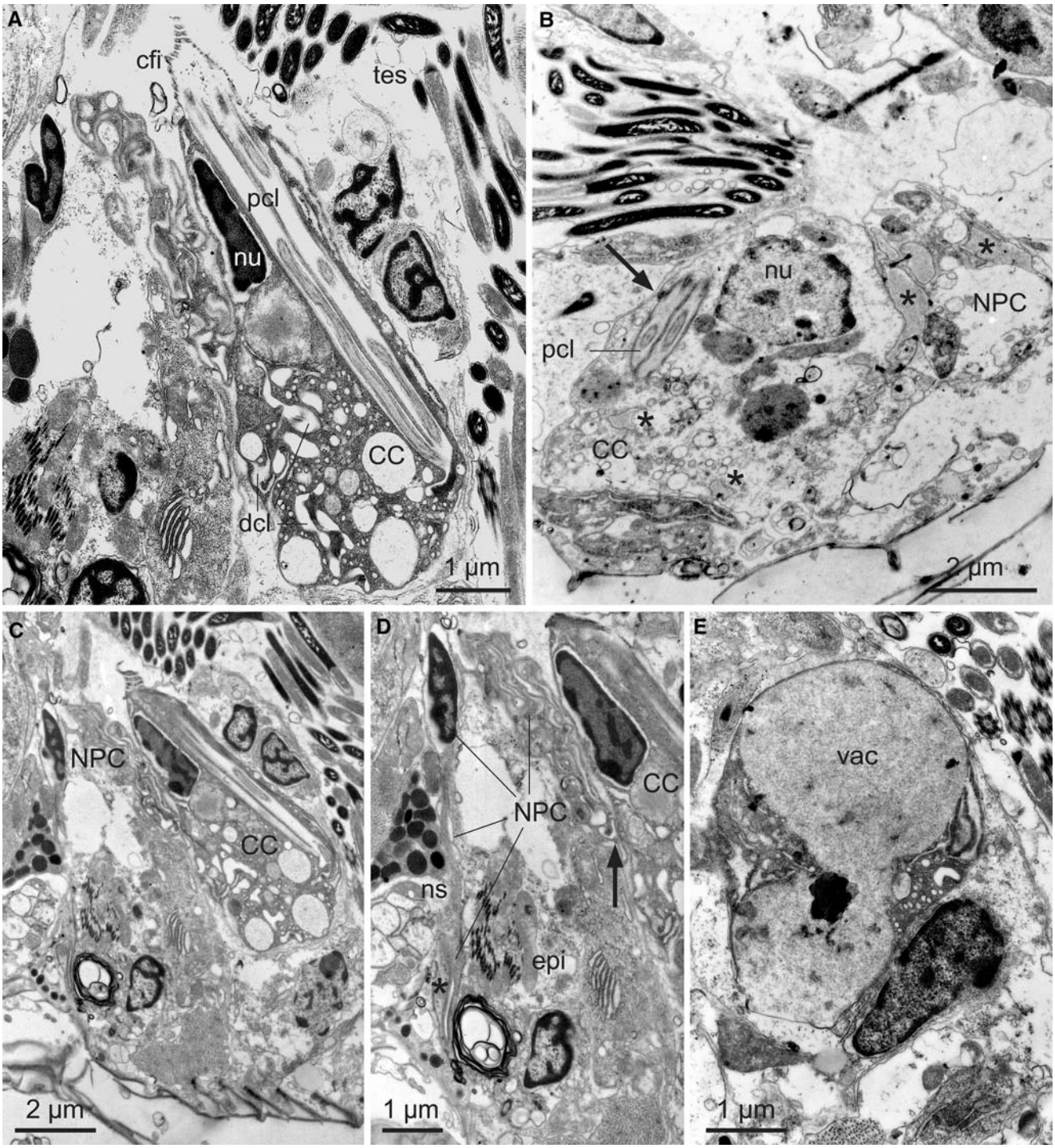

Fig. 6 *Xenotrichula carolinensis syltensis*. Ultrathin cross sections (TEM) of the protonephridium. **a** Canal cell (*CC*) with the proximal canal cell lumen (*pcl*) and both cilia of the terminal organ. At the proximal end of pcl you can see parts of the composite filter (*cfi*). Nucleus (*nu*) and sections of the distal canal cell lumen (*dcl*) situated medially to the pcl. Laterally to the protonephridium there are mature spermatozoa (*tes*). **b** Section rectangular to the axis of pcl showing CC and nephridiopore cell (*NPC*) with numerous sections of the distal canal cell lumen (*asterisk*) and the nucleus (*nu*) of the CC. **c** Transition of canal- and nephridiopore cell (*NPC*). **d** Close-up of the NPC. A thin cellular protrusion bears the distal nephridiopore cell lumen (*asterisk*) and lies beneath a ventral nerve cord (*ns*) and cells of the ventral epidermis (*epi*). Note the area of the connection of the canal cell lumen and the nephridiopore cell lumen (*arrow*). **e** Posterior tip of the canal cell showing a voluminous vacuole (*vac*)

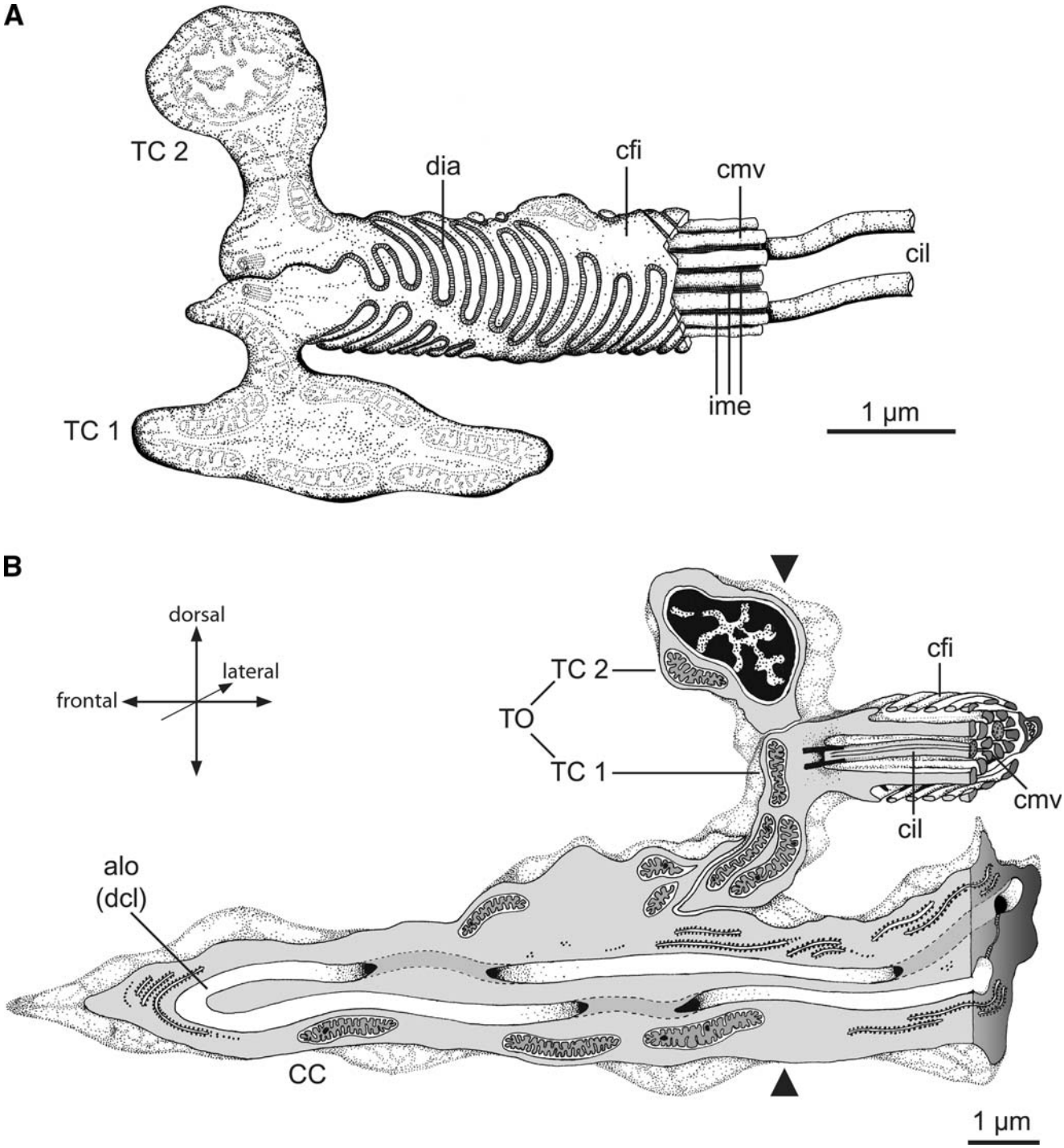

Fig. 7 *Chaetonotus maximus*. Schematic reconstruction of the protonephridium. **a** Exterior view of the terminal organ with the composite filter. **b** Sagittal section of the first third of the protonephridium showing parts of the canal cell and the terminal organ. *Black triangles* indicate level of section plane for Fig. 9a. *alo* anterior loop of the distal canal cell lumen, *CC* canal cell, *cfi* composite filter, *cil* cilia, *cmv* circumciliary microvilli, *dcl* dislat canal cell lumen, *dia* diaphragm, *ime* intermicrovillar ECM, *TC1/2* terminal cells, *TO* terminal organ

bulge proximal to the filter region (Figs. 7a, 10a) in which there are numerous mitochondria (christae-type). We could not detect the corresponding nucleus of this cell.

Canal cell The canal cell of *C. maximus* is strongly enlarged (it constitutes the whole total length of the protonephridium) but lacks cilia, basal bodies, accessory centrioles, ciliary rootlets and microvilli (Figs. 7b, 8a, b, 9d). The canal cell lumen is an enfolded lumen (Figs. 10e, 11a) and is subdivided into two distinct sections: A straight proximal part and a convoluted distal part (Figs. 9d, 10f, 11b, c).

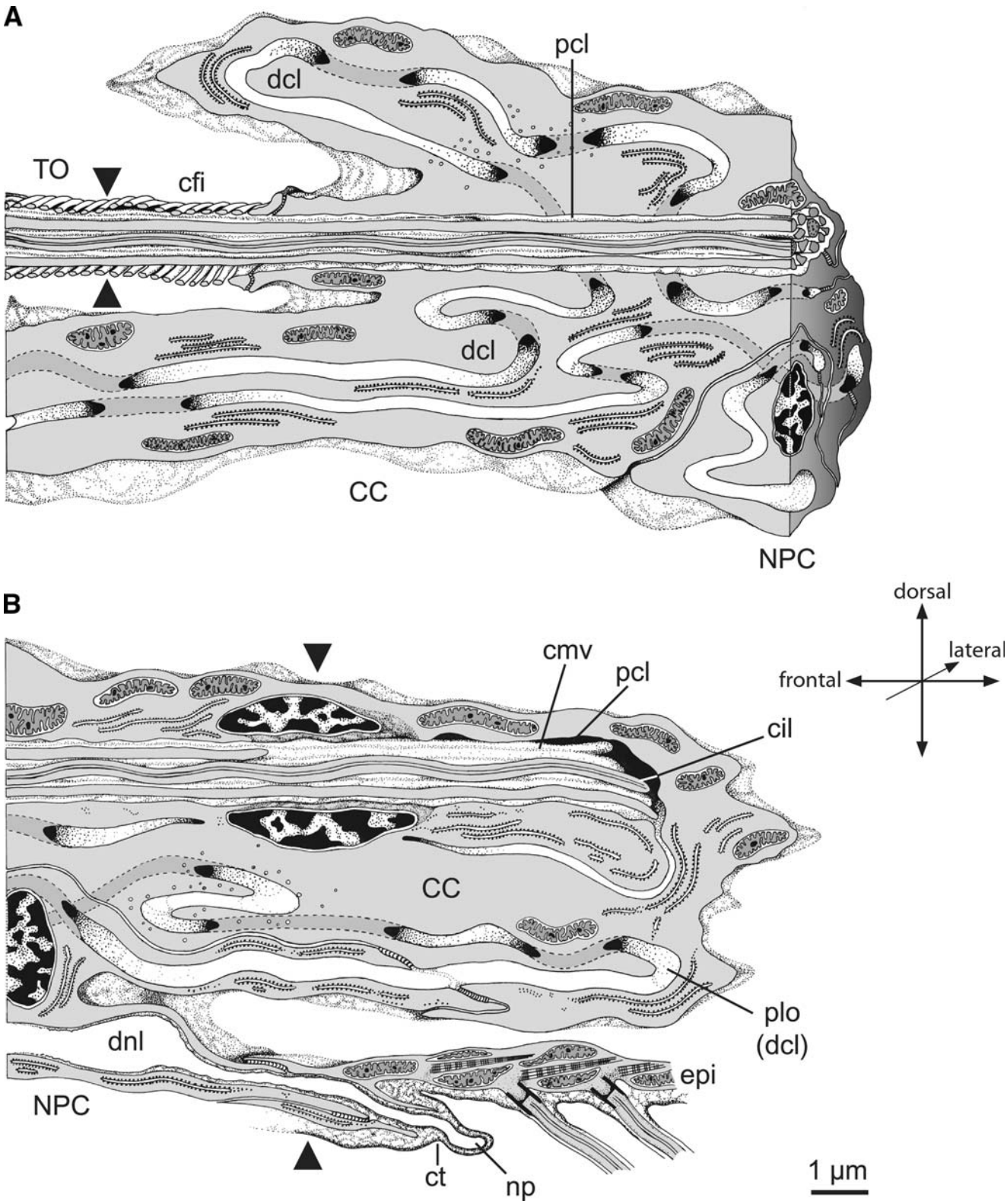

Fig. 8 *Chaetonotus maximus*. Schematic reconstruction of the protonephridium. **a** Sagittal section of the middle third of the protonephridium showing parts of the terminal organ, canal- and nephridiopore cell. **b** Sagittal section of the posterior third of the protonephridium showing parts of the canal- and nephridiopore cell. *Black triangles* in a and b indicate levels of section plane for Fig. 9b and c. *CC* canal cell, *cfi* composite filter, *cil* cilia, *cmv* circumciliary microvilli, *ct* cuticular tube, *dcl* dislat canal cell lumen, *dnl* distal nephridiopore cell lumen, *epi* epidermis, *np* nephridiopore, *NPC* nephridiopore cell, *plo* posterior loop of the distal canal cell lumen, *pcl* proximal canal cell lumen, *TO* terminal organ

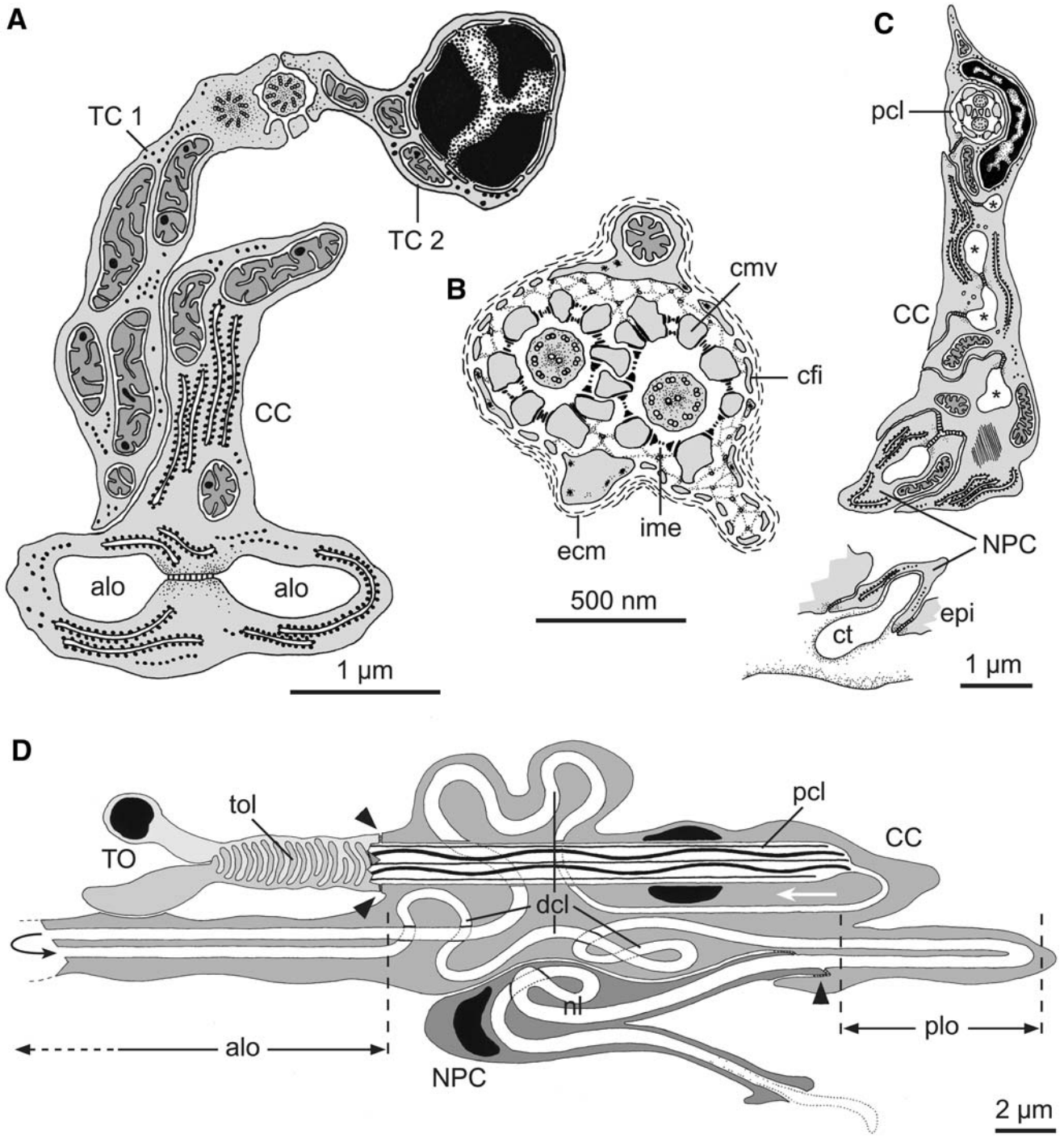

Fig. 9 *Chaetonotus maximus*. **a–c** Schematic cross sections of the protonephridium. Levels of corresponding section planes are indicated in Figs. 7b, 8a, b. **d** Simplified lateral view of the protonephridium, whole protonephridial lumen projected into one plane. Anterior tip of the canal cell is cut off. Cellular borders indicated by *black triangles*. *alo* anterior loop of the distal canal cell lumen, *CC* canal cell, *cfi* composite filter, *cmv* circumciliary microvilli, *ct* cuticular tube, *dcl* distal canal cell lumen (in **c** indicated by *asterisks*), *ecm* extracellular matrix, *epi* epidermis, *ime* intermicrovillar ECM, *nl* nephridiopore cell lumen, *NPC* nephridiopore cell, *pcl* proximal canal cell lumen, *plo* posterior loop of the distal canal cell lumen, *TC1/2* terminal cells, *TO* terminal organ, *tol* terminal organ lumen

Distally, the terminal organ lumen is continued by the straight, 20 µm long proximal canal cell lumen (diameter: 1 µm) housing cilia and microvilli of the terminal organ (Figs. 10d, 11a). Distally, the proximal canal cell lumen is linked to the distal canal cell lumen via a narrow duct (diameter around 25 nm) (Figs. 9d, 11a). The convoluted, distal canal cell lumen (diameter from 250 to 500 nm) forms two prominent curves: A 20 µm long anterior loop

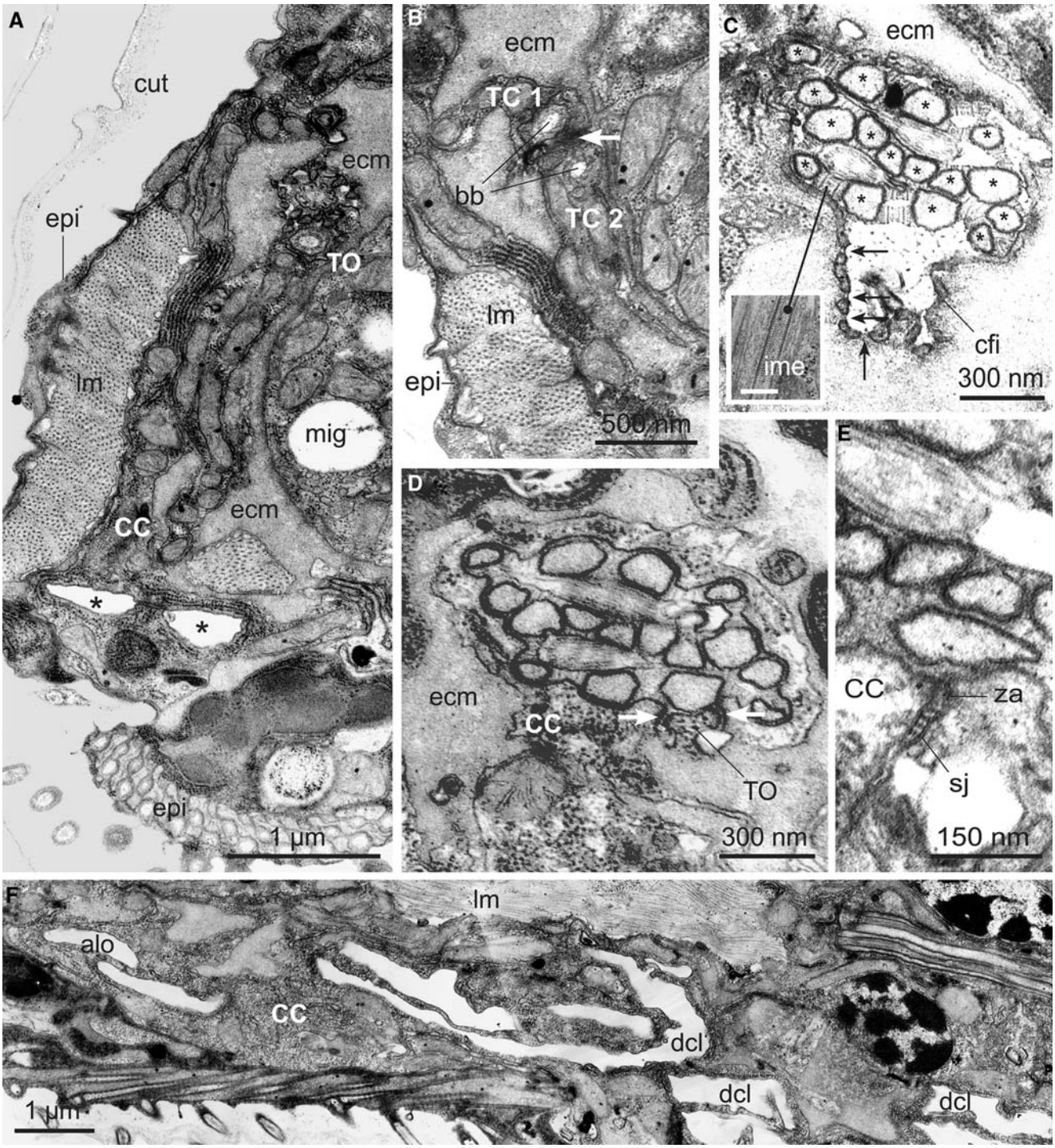

Fig. 10 *Chaetonotus maximus*. Ultrathin sections (TEM) of the protonephridium. **a** Terminal organ (*TO*) and the canal cell (*CC*) at the level of the ciliar bases. Sections of the anterior loop of the canal cell lumen are marked by *asterisks*. The whole organ is lined by an extracellular matrix (*ecm*), it is bordered by the midgut (*mig*), epidermis with cuticle (*epi, cut*) and longitudinal muscles (*lm*). **b** Basal bodies (*bb*) of the two terminal cells (*TC1, TC2*). Note the electron-dark material near the border of both cells (*arrow*). **c** TO with cilia, circumciliary microvilli (*asterisk*) and composite filter (*cfi*). Some sections of the meandering clefts covered with the diaphragm are marked (*arrows*). Embedded micrograph shows the ecm between the circumciliary microvilli (*ime*) in a tangential section (scale bar = 250 nm). **d** Transition from the terminal organ to the canal cell lumen. Two septate junctions (*arrows*) line the most distal tip of the TO. **e** High magnification of the septate junction (*sj*) and the belt desmosome (*za*) closing the enfolded proximal canal cell lumen. **f** Sagittal section of the CC with the anterior loop (*alo*) and some meanders of the distal canal cell lumen (*dcl*). **a**–**e** cross sections, **f** sagittal section

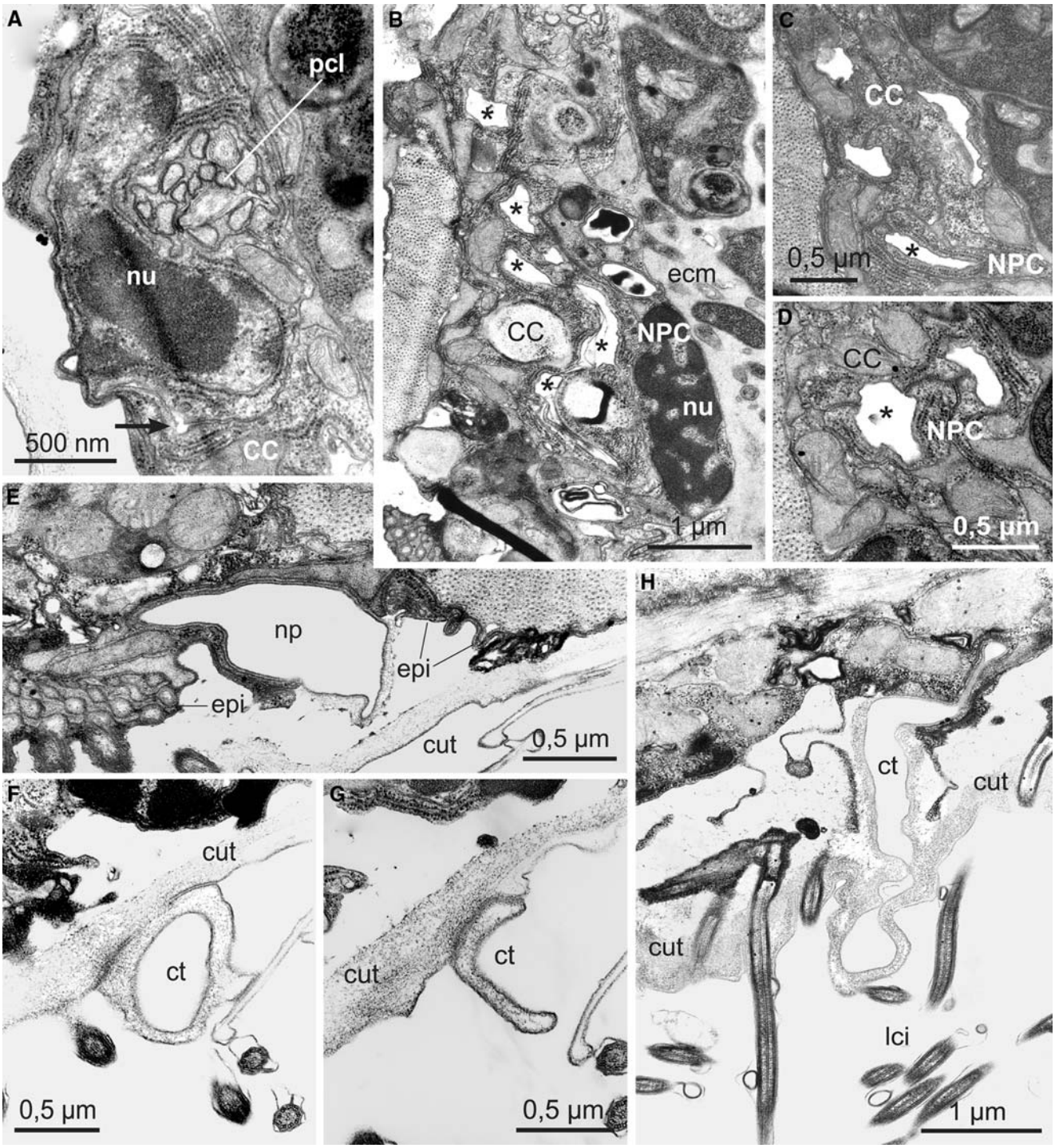

Fig. 11 *Chaetonotus maximus*. Ultrathin sections (TEM) of the Protonephridium. **a** Canal cell (*CC*) and proximal canal cell lumen (*pcl*) with the nucleus (*nu*) attached to it. Note the narrow duct of the canal cell lumen running back anteriorly (*arrow*). **b** Nephridiopore cell (*NPC*) and CC. Only canal cell lumen is marked (*asterisk*), the nucleus (*nu*) belongs to the NPC. The protonephridium is lined by extracellular matrix (*ecm*). **c, d** Transition from the CC to the NPC. In **c** the nephridiopore cell lumen is marked (*asterisk*), the marked lumen in **d** is surrounded by parts of the CC and NPC! **e** The NPC pierces the ventral epidermis (*epi*) and forms the nephridiopore (*np*). The cuticular lining of the distalmost portion of the nephridiopore cell lumen is inside the cuticle of the body wall (*cut*). **f, g** The cuticular tube (*ct*) has penetrated the cuticle of the body wall (*cut*). **h** Longitudinal view of the cuticular tube (*ct*) beneath locomotory cilia (*lci*). **a**–**g** cross sections, **h** sagittal section

(Figs. 7b, 9d, 10a, f) and a nearly 5 µm long posterior loop (Figs. 8b, 9d). One shank of the posterior loop of the distal canal cell lumen passes into the nephridiopore cell lumen (Figs. 9d, 11c, d).

The cytoplasm of the canal cell of *C. maximus* appears very electron-dark. Oblong mitochondria (cristae-type) are very abundant and there is a high density of rough endoplasmic reticulum (rER), often adjacent to the luminal membrane (Figs. 10a, f, 11a–d). Only in some sections small vesicles are visible (diameter from 20 to 50 nm). Globular vesicles of a second type (diameter around 1 µm) with electron-lucent content are situated sparsely within the canal cell (Fig. 11b). The nucleus of the canal cell is placed near the distal third of the proximal canal cell lumen (Figs. 8b, 9c, 11a).

Nephridiopore cell The nephridiopore cell of *C. maximus* is placed adjacent to the canal cell (Figs. 8a, b, 9c, 11b–d). There are no cilia, basal bodies, accessory centrioles, ciliary rootlets or microvilli present.

The convoluted, enfolded nephridiopore cell lumen (diameter around 300 nm) leads to the outside via a persistent nephridiopore (Fig. 11b–e). This "cellular nephridiopore" is lined and continued by cuticle increasing to a thickness equal to that of the body wall, forming a cuticular tube (diameter: 0.5 µm) (Figs. 8b, 9c, 11f–h). These cuticular tubes are positioned medially to the ventral rows of locomotory cilia (Fig. 11e–h).

The small nephridiopore cell is poor in organelles. You can see few mitochondria and cisternae of the rER next to the luminal membrane (Fig. 11c–e). The lentiform nucleus is situated beneath the nephridiopore cell lumen (Fig. 11b).

Discussion

Relationships within Gastrotricha

Since we have examined only a single organ system, we do not intend to generate a system depending on this character only. Rather, we here hypothesise ground pattern features on the basis of an already published phylogenetic hypothesis.

The reconstruction of character states in the ground pattern of basal stem species of a certain taxon depends in a high degree on the hypothesis of the phylogenetic relationships within this group. We have chosen a phylogenetic analysis of Gastrotricha based on morphological characters as the basis for our phylogenetic survey (Hochberg and Litvaitis 2000). Because this is the only investigation including all gastrotrichan genera, except for the recently described *Diuronotus* (Todaro et al. 2005), we are able to estimate whether the species used for our reconstructions of the ground pattern features have a basal or derived position. Hochberg and Litvaitis (2000) analysed the gastrotrichan relationships based on 59 genera and 81 morphological characters. The resulting consensus tree confirms Macrodasyida and Chaetonotida as basal sister taxa within Gastrotricha and *Neodasys* (Multitubulata sensu d`Hondt (1971)) and Paucitubulata within Chaetonotida (Fig. 12). The major clades of this hypothesis agree with former views of the gastrotrichan relationships which depend, for example, on the morphology of the gastrotrich pharynx or the organisation of the body wall (d'Hondt 1971; Ruppert 1982; Travis 1983).

It is important to refer to the position of the taxon *Musellifer*: In the phylogenetic hypothesis used, it has the most basal position within Paucitubulata. As there is no data available concerning the protonephridial system of *Musellifer* species, we will in fact only make assumptions for the stem species of Paucitubulata exclusive the taxon *Musellifer*. Implying the hypothesised basal position of the Xenotrichulidae within Paucitubulata (Manylov et al. 2004; Todaro et al. 2003; Travis 1983), we nevertheless think that our conclusions for the ground pattern of Paucitubulata are well founded. If further phylogenetic studies support the basal position of *Musellifer* within Paucitubulata or at least support a position as the sister group of Xenotrichulidae (Marotta et al. 2005), an investigation of the protonephridial system of *Musellifer* will be necessary for the reconstruction of the ground pattern of Paucitubulata.

Protonephridia within Gastrotricha

By comparing all available ultrastructural data of the species studied thus far, we hypothesise the ground patterns of *Neodasys*, the Paucitubulata and the Macrodasyida. In a second step, we compare these ground patterns to make at least some assumptions regarding the ancestral protonephridial organisation in Chaetonotida and Gastrotricha. In case of conflicting characters, an outgroup comparison with the related sister group is used. Yet undecided alternative character states of protonephridial substructures in gastrotrichs have to be in focus of subsequent studies.

Protonephridia of Paucitubulata (Table 1; Fig. 12, gp P)

The protonephridial system of all observed species of the Paucitubulata consists of one single pair of protonephridia in the central trunk region laterally to the midgut (Brandenburg 1962; Mock 1979; Remane 1936; Ruppert 1979, 1991; this study). The protonephridia are always embedded within a compact ECM in *Aspidiophorus* sp., *C. maximus*, *Chaetonotus* sp., *D. tesselata* (Renaud-Mornant, 1968), *Lepidodermella squamata* (Dujardin, 1841), *X. carolinensis syltensis* (Brandenburg 1962; Ruppert 1991; this study).

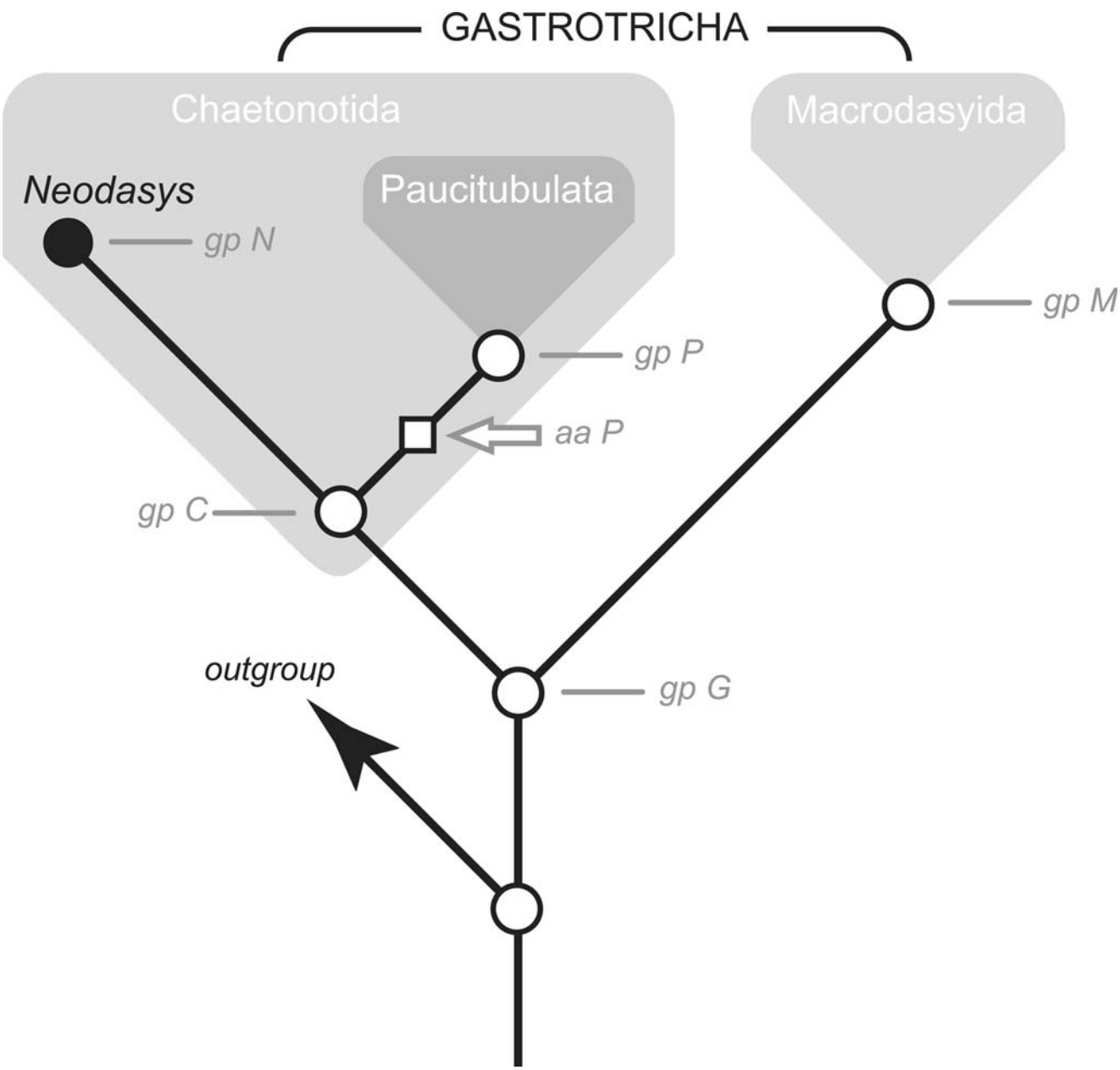

Fig. 12 Most basal clades within Gastrotricha corresponding to the phylogenetic hypotheses of Ruppert (1982), Travis (1983) and Hochberg and Litvaitis (2000). The ground patterns of the Gastrotricha (*gp G*) and of its subtaxa (*gp M, gp C, gp N, gp P*) are indicated. Within the stem lineage of Paucitubulata, we could detect some evolutionary transformations concerning the protonephridial system (*aa P*)

The protonephridia of *X. carolinensis syltensis* and *C. maximus* consist of a bicellular terminal organ, one canal- and one nephridiopore cell linked by septate junctions and belt desmosomes (zonula adhaerens). The figures shown by Ruppert (1991) allow to conclude that the same situation can also be found in *D. tesselata*.

Each terminal cell of the terminal organ consists of one cilium and eight circumciliar microvilli. The microvilli are connected by extracellular material (ECM) and represent a ground pattern characteristic of Paucitubulata. In *X. carolinensis syltensis* this ECM is flocculent, in *Chaetonotus* reticulate (Brandenburg 1962; our study). *X. carolinensis syltensis*, *C. maximus* and presumably *L. squamata* (Ruppert 1991) have a composite filter. A diaphragm covers the meandering clefts. In *Chaetonotus* sp. the filter region ("Plasmablase") is reported to be a hollow cytoplasmic cylinder, perforated by longitudinal slits (Brandenburg 1962). This is obviously a misinterpretation due to the lack of tangential sections of the filter region.

The canal cell lumen of *X. carolinensis syltensis*, *C. maximus* and very likely of *D. tesselata* and *Aspidiophorus* sp. (Ruppert 1991, Figs. 16, 101) is an enfolded lumen. It is

Table 1 Gastrotrich protonephridia. Character coding and hypothesised ground pattern

Taxon	Character number																														Reference
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	
Aspidophorus sp.	1	1	?	?	?	?	?	?	?	?	?	?	?	?	0	?	?	1	0	?	?	?	0	?	?	?	?	?	?	?	?	?	Ruppert (1991)
Chaetonotus sp.	1	1	?	1	2	?	1	?	1	1	?	?	8	1	?	0	1	1	0	?	?	?	?	?	?	?	?	?	?	?	?	?	Brandenburg (1962)
Chaetonotus maximus	1	1	1	1	2	1	2	1	1	1	0	0	8	1	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	this study
Draculiciteria tesselata	1	1	?	1	2	?	?	1	1	1	?	?	8	?	0	?	1	1	0	?	?	?	?	0	?	?	?	?	?	?	0	?	Ruppert (1991)
Lepidodermella squamata	1	?	?	1	2	1	2	?	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	Ruppert (1991)
Xenotrichula carolinensis syltensis	1	1	1	1	2	1	2	1	1	1	0	0	8	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	?	this study
Paucitubulata	1	1	1	1	2	1	2	1	1	1	0	0	8	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	
Neodasys sp.	?	1	?	1	1	0	2	1	1	1	?	?	7	0	0	0	1	0	1	1	?	?	1	?	?	?	?	?	?	?	?	?	Ruppert (1991)
Chaetonotida	?	1	?	1	1	0	?	1	1	1	?	?	8	0	0	0	1	0	1	1	?	?	1	?	?	?	?	?	?	?	?	1	
Dactylopodola baltica	2	1	1	1	1	0	0	1	1	1	1	0	8	0	0	0	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	Neuhaus (1987), Fischer (1994)
Mesodasys laticaudatus	11	1	1	1	1	0	0	1	1	1	1	1	8	0	1	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	1	1	Neuhaus (1987)
Turbanella cornuta	4	?	0	4	1	0	0	1	1	1	0	?	8	1	?	0	4	0	1	1	?	?	2	1	0	?	?	?	?	?	?	?	Teuchert (1973)
Macrodasyida	≥2	1	1	1	1	0	0	1	1	1	1	?	8	0	0	0	1	0	1	1	1	1	1	1	0	0	?	1	1	1	1	1	
Gastrotricha	?	1	?	1	1	?	?	1	1	1	?	?	8	0	0	0	1	0	1	1	?	?	1	?	?	?	?	?	?	?	?	1	

Protonephridial character states of different species and stem species of Gastrotricha reconstructed on the base of the phylogenetic analysis of Hochberg and Litvaitis (2000)

1–3. Protonephridial system. *1* Number of protonephridial pairs; *2* perinephridial ECM: absent (0), present (1); *3* continuous protonephridial lumen: absent (0), present (1). *4–14*. Terminal organ and cells. *4* Number of terminal organs per protonephridium; *5* number of terminal cells per terminal organ; *6* filter (see Fig. 1): simple (0), composite (1); *7* perforation of filter: slit-like (0), longitudinal slits (1), meandering (2); *8* filtration barrier (diaphragm): absent (0), present (1); *9* number of cilia per terminal cell; *10* ciliary basal body: absent (0), present (1); *11* accessory centriole: absent (0), present (1); *12* ciliary rootlet: absent (0), present (1); *13* number of circumciliary microvilli; *14* intermicrovillar ECM: Flocculent (0), reticulate (1); *15–23*. Canal cell. *15* Lumen type (see Fig. 1): enfolded (0), enclosed (1); *16* proximal portion passage: straight (0), convoluted (1); *17* number of proximal portions; *18* distal portion passage: straight (0), convoluted (1); *19* number of cilia per cell; *20* ciliary basal body: absent (0), present (1); *21* accessory centriole: absent (0), present (1); *22* ciliary rootlet: absent (0), present (1); *23* microvilli: absent (0), brush border (1), two solid rods (2). *24–32*. Nephridiopore cell. *24* Lumen type (see Fig. 1): enfolded (0), enclosed (1); *25* proximal portion passage: straight (0), convoluted (1); *26* distal portion passage: straight (0), convoluted (1); *27* Number of cilia per cell; *28* ciliary basal body: absent (0), present (1); *29* accessory centriole: absent (0), present (1); *30* ciliary rootlet: absent (0), present (1); *31* microvilli: absent (0), brush border (1); *32* Nephridiopore cell cuticle: absent (0), present (1)

fractioned into a straight and a highly convoluted portion as seen in several species of Paucitubualta (Zelinka 1889; Remane 1936; Ruppert 1979). The canal cell neither contains cilia nor microvilli. Investigations on the ultrastructural level provide that there are no basal bodies, accessory centrioles and rootlet-structures (this study). Compared to the terminal cells and the nephridiopore cell, the canal cell is very voluminous in *X. carolinensis syltensis* and *C. maximus*.

The nephridiopore cell resembles the canal cell but is less voluminous. The nephridopore cell of *X. carolinensis syltensis* and *C. maximus* and very likely of *D. tesselata* (Ruppert 1991) has no cilia and no microvilli, the enfolded lumen is as well convoluted in its proximal portion. A permanent, cuticle-lined nephridiopore is present in *C. maximus* and probable in *X. carolinensis syltensis* and *D. tesselata* (Ruppert 1991, Fig. 101).

According to this data the following composition of the protonephridia can be assumed for the paucitubulatan ground pattern (Fig. 12, *gp P*)

(a) two pairs of protonephridia in a lateral body position, embedded within a perinephridial ECM
(b) each protonephridium consists of four cells: two terminal-, one canal- and a subsequent nephridiopore cell. Cells are linked by septate junctions and belt desmosomes
(c) two monociliar terminal cells form the terminal organ and the composite filter. A diaphragm is present. There are eight long microvilli per terminal cell linked by ECM, but no accessory centrioles and ciliary rootlets
(d) the voluminous canal cell has an enfolded lumen with a straight proximal portion and a convoluted distal one. There are no cilia, basal structures and microvilli
(e) the nephridiopore cell has an enfolded lumen with a convoluted proximal portion and a straight distal one. There are no cilia, basal structures and microvilli. There is a permanent, cuticle-lined nephridiopore
(f) terminal organ-, canal cell- and nephridiopore cell form a continuous protonephridial lumen

Protonephridia of Neodasys *(Table 1, Fig. 12, gp N)*

Until now only data from *Neodasys* sp. of the taxon *Neodasys* is available (Ruppert 1991). At least one pair of protonephridia is present in *Neodasys* sp. but further pairs may exist (Ruppert 1991, p. 97). They lie in a lateral body position and are embedded within a compact ECM. The single terminal cell contains one cilium with seven circumciliary microvilli, which are linked by a flocculent ECM. The simple filter probably consists of interdigitating processes of the terminal cell (Ruppert 1991), a diaphragm is present. The canal cell has a single cilium which is encircled by a bundle of microvilli ("brush border", Ruppert 1991). The enfolded canal cell lumen has a straight passage, there are electron-dark fibres adjacent to the luminal membrane of the canal cell. There is no data concerning the nephridiopore cell.

Protonephridia of Macrodasyida (Table 1; Fig. 12, gp M)

Protonephridia are serially arranged in Macrodasyida. There are two to several pairs in a lateral position: Two pairs in *Dactylopodola baltica* (Remane, 1926) (Neuhaus 1987), *Dendrodasys gracilis* Wilke, 1954 and *Urodasys viviparus* Wilke, 1954 (Wilke 1954); three pairs in *Macrodasys caudatus* Remane, 1927 (Wilke 1954); four pairs in *Turbanella cornuta* Remane, 1925 (Teuchert 1967); six pairs in *Paradasys subterraneus* Remane, 1934 (Teuchert 1967) eleven pairs in *Mesodasys laticaudatus* Remane, 1951 (Neuhaus 1987).

The protonephridia of *D. baltica* and *M. laticaudatus*, both of which studied with TEM (Neuhaus 1987; Fischer 1994), vary within a few characters only. Characters shared by these two species and that of *T. cornuta* (Teuchert 1973) are hypothesised to be features of the ground pattern of Macrodasyida (Table 1). Therefore, the stem species of Macrodasyida contains serially arranged protonephridia (at least two pairs) which are covered by a perinephridial ECM. Each protonephridium consists of three cells: One terminal-, one canal- and one nephridiopore cell. The consecutive lumina of these three cells constitute a continuous protonephridial lumen.

The monociliar terminal cell possesses a basal body, an accessory centriole and eight circumciliary microvilli linked by flocculent ECM. The filter is perforated with irregular, slit-like openings, a diaphragm is present. The monociliar canal cell has a basal body, an accessory centriole, a cross-striated ciliary rootlet, and a bundle of microvilli surrounding the cilium. The enfolded canal cell lumen has a straight passage throughout its whole length through the canal cell. The nephridiopore cell contains a basal body, an accessory centriole, a cross-striated ciliary rootlet and a bundle of microvilli towards the enclosed and straight nephridiopore cell lumen. There are two conflicting character states for the ground pattern of Macrodasyida (Table 1): Is there a ciliary rootlet within the terminal cell and does the nephridiopore cell possess a cilium or not?

Due to the position of *Turbanella* within the phylogenetic analysis of Hochberg and Litvaitis (2000, 2001), we consider the aberrant characters of the protonephridium of *T. cornuta* as apomorphic. The most striking differences with respect to the protonephridia of *D. baltica* and *M. laticaudatus* are (1) up to four isolated terminal cells per protonephridium, (2) up to four isolated proximal canal cell lumina per canal cell, (3) the interrupted protonephridial lumen and (4) the two solid cytoplasmic rods of the canal cell (Teuchert 1973, Table 1).

Protonephridia of Chaetonotida (Table 1; Fig. 12, gp C)

Since there is only little data concerning the protonephridial system of *Neodasys*, the ground pattern of Chaetonotida is very fragmentary (Table 1). Furthermore, all data available for *Neodasys* sp. is not based on a complete reconstruction of the protonephridial system. Therefore, we will not give a detailed assumption of the ground pattern of Chaetonotida here.

However, in cases of conflicting characters between *Neodasys* sp. and the ground pattern of Paucitubulata, we had to compare the situation with that of Macrodasyida. Proceeding this way, we could polarise character states and discovered some apomorphic features for Paucitubulata (Fig. 12, *aa P*): The bicellular terminal organ with a composite filter, the convoluted distal canal cell lumen and the absence of cilia, ciliary basal structures and microvilli within the canal cell are hypothesised to be autapomorphies.

Protonephridia of Gastrotricha (Table 1; Fig. 12, gp G)

There is, however, only fragmentary data for the ground pattern of Gastrotricha (Table 1). Nevertheless, we could confirm some of the ground pattern features of Gastrotricha hypothesised earlier Remane, 1951 (Neuhaus 1987) when evaluating our new findings together with the structural data of Teuchert (1973), Neuhaus (1987) and Ruppert (1991). The most important points that have to be revised are (1) the number of protonephridial pairs which depends on the situation in *Neodasys* and (2) the morphology of the filter. The latter topic is of high interest as we think a filter perforated by slit-like openings in Macrodasyida species is very doubtful. It may well be that a meandering cleft running along the proximo-distal axis of the simple filter may represent the ancestral condition. To detect this, tangential sections of the filter or reconstructions carried out with the utmost care are necessary. Since these are lacking in the macrodasyid species studied, it may well be that a filter perforated in the mentioned way is a misinterpretation.

Alternative phylogenetic hypotheses

Evaluating protonephridial characters of the Gastrotricha on the basis of alternative phylogenetic hypotheses may result in character states that differ from the ground patterns presented here (Table 1). But there are, however, no major conflicts with these hypotheses.

An analysis based on molecular data (Todaro et al. 2003) shows no conflicts when only taking nodes with a high bootstrap support (>70%) into account: Problematic sister group relationships like *Neodasys ciritus* as an ingroup taxon of Macrodasyida or two *Lepidodasys* species as sister taxon of Paucitubulata disappear. Furthermore, the strict consensus tree of another molecular analysis (Manylov et al. 2004) implies no conflict with the topology used for our reconstructions. Even if one accepts the putative non-monophyly of Gastrotricha, our reconstructions of the ground pattern of Macrodasyida and the ground pattern of Paucitubulata would have validity. A representative of the important taxon *Neodasys*, however, is not included in that analysis.

The summary tree of the combined analysis of Zrzavý (2003) shows the Macrodasyida as a paraphyletic group consisting of successive off-branching clades from the stem lineage to the monophyletic Chaetonotida [*Neodasys* (=Multitubulata) + Paucitubulata]. However, this hypothesis does not conflict with our reconstructions of the ground pattern features, except for the stem species of Macrodasyida, which does not exist in this scenario. Especially with respect to the putative basal position of the Dactylopodolidae (Zrzavý 2003), a reconstruction of the protonephridial system in the ground pattern of Gastrotricha would lead to an equal result as given in Table 1.

To finish off, we shall point out that undecided conflicts will collapse after a complete investigation of the protonephridial system in *Neodasys* and a reinvestigation of the morphology of the filter in Macrodasyida species. If conflicts within the ground pattern of Gastrotricha persist (e.g. number of protonephridial pairs), we have to compare the data to the protonephridial system of putative sistertaxa of the Gastrotricha to make decisions. We also have to do this in order to polarise the features of the ground pattern of Gastrotricha. Such an outgroup comparison would be beyond the scope of this article as there are at least four putative sistergroups of Gastrotricha: Cycloneuralia (Ehlers et al. 1996), Plathelminthes (Giribet et al. 2000) Ecdysozoa (Schmidt-Rhaesa et al. 1998) or Gnathostomulida (Zrzavý et al. 1998).

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