ANATOMY AND AFFINITIES OF *MICROPILINA MINUTA* WARÉN, 1989 (MONOPLACOPHORA: MICROPILINIDAE)

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ABSTRACT

The anatomy of the tiny (maximum shell length 1.5 mm) monoplacophoran limpet *Micropilina minuta* Warén, 1989 was studied by means of semithin serial sections with subsequent 3D computer analysis and visualization (interactive 3D model in the online version). As in other monoplacophorans there are eight pairs of shell muscles ('sectors' *A*–*H*). The species has four pairs (unique for monoplacophorans, sectors *D*–*G*) of small gills, four pairs of kidneys (sectors *A*, *D*–*F*) which also have gamete-releasing function (sectors *D*, *E*), and one (or two) pair(s) of gonadal sacs. Eggs are yolk-rich, but there are no signs of retention of eggs in the mantle cavity for brooding. Only some of these characters, particularly those connected with miniaturization, are shared with *Micropilina arntzi*. Two characters (absence of a heart and absence of sector *G* kidneys) are interpreted as synapomorphies of these two species. Based on these new data the supraspecific systematics of *Micropilina* and Micropilinidae are reevaluated. We also discuss the implications for the evolution of the serial arrangement of organs in the Neopilinoidea.

INTRODUCTION

Over half a century after the discovery of the first extant Monoplacophora (Lemche, 1957) these animals remain a key taxon for understanding the evolution and phylogeny of the Mollusca. There are 30 described extant species; 29 are mentioned in reviews by Haszprunar (2008) and Lindberg (2009), to which can be added *Neopilina starobogatovi* described by Ivanov & Moskalev (2007). Although knowledge of hard parts and external morphology has substantially increased (Warén & Gofas, 1996) and molecular data have led to new phylogenetic hypotheses (Wilson *et al.*, 2009), more knowledge of anatomy is still desirable. In addition, new methods of analysis and visualization of anatomy (Ruthensteiner, 2008), such as interactive 3D-models in PDF publication versions (Ruthensteiner & Heß, 2008), can improve the results substantially.

Currently, detailed anatomical information on extant monoplacophorans (Tryblidiida, Neopilinoidea) are restricted to four species: *Neopilina galatheae* Lemche, 1957 and *Vema ewingi* (Clarke & Menzies, 1959) (see Lemche & Wingstrand, 1959; Wingstrand, 1985), *Laevipilina antarctica* Warén & Hain, 1992 (see Haszprunar *et al.*, 1995; Healy, Schaefer & Haszprunar, 1995; Schaefer & Haszprunar, 1997a, b) and *Micropilina arntzi* Warén & Hain, 1992 (see Haszprunar & Schaefer, 1997a, b). The characters of the tiny and partly paedomorphic *M. arntzi* have shed light on the enduring controversy about the segmented *vs* serial body plan of tryblidiidans, favouring the latter (Haszprunar & Schaefer, 1997a, b; Haszprunar, 2008; Lindberg, 2009). One outstanding problem is the explanation of the considerable differences in nearly all major organ systems between *M. arntzi* and all remaining neopilinoids. Although the assumption of paedomorphosis could explain some of these differences, questions remain about the evolutionary events leading to such an aberrant species.

Warén (1989) described the minute monoplacophoran, *Micropilina minuta* (type species of *Micropilina*), from deep water (770–900 m) off southern and eastern Iceland, on the basis of scanning electron micrographs of empty shells. Unknown to Warén, living animals of this species had already been found in 1976 in deep water (900 m) south of the Faeroe Islands (Killeen & Smith, 1994). These samples were deposited in the National Museum of Scotland (Edinburgh), and have been loaned to the authors for anatomical study. As will be shown, the anatomy of this second species of *Micropilina* partly bridges the morphological gap between *M. arntzi* and the remaining neopilinoids, and provides new evidence for the evolutionary history of the extant monoplacophorans.

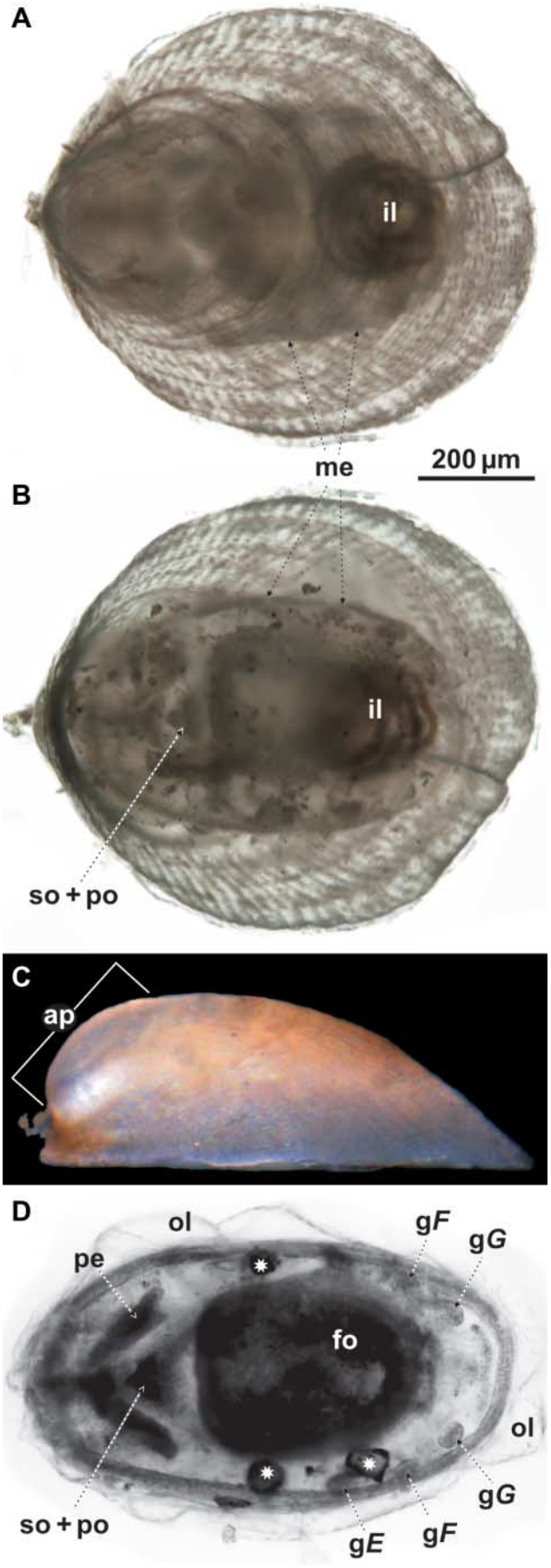
MATERIAL AND METHODS

A single sample of *Micropilina minuta* Warén, 1989 consisting of four live-collected specimens has been investigated: NMSZ (National Museum of Scotland, Zoology) 1993188.00002; *Challenger* Stn 73: 60°10′N, 08°12′W, 900 m; 03.07.1976.

One specimen (spm 4) was kept intact in ethanol and examined by light microscopy using various lighting setups.

Three specimens (spms 1–3) were processed for anatomical study by means of semithin serial sectioning. Specimens originally fixed in a formalin solution were transferred to 70% ethanol. For shell decalcification, animals were immersed in Bouin's fixative fluid, followed by thorough rinsing in 80% ethanol. After dehydration in an ethanol series, specimens were embedded in Araldite M via propylene oxide as a transitional solvent. Ribboned serial cross-sections (2 μm thickness) were cut with Ralph glass knives. Sections were stained with methylene blue-azurII dye. Except for Figure 1C all photographs of whole specimens and sections were taken with a Leica DMRBE compound microscope.

One of the sectioned specimens (spm 1) was computer-graphically analysed with the software *AMIRA* (versions 4.x, 5.x). Every other section was used for reconstruction, resulting in a 3D-stack with a voxel resolution of *x*: 1,555, *y*: 844, *z*: 170 (*x*, *y* = section image resolution in pixels, *z* = number of section images).



Both histological and surface rendering treatment followed the procedures outlined by Ruthensteiner (2008). In that account the specimen reconstructed in the present study was used as an example (Ruthensteiner, 2008: figs 10, 13) for certain 3D-graphical procedures. Preparation of the PDF-3D-model largely followed the procedure described in Ruthensteiner & Heß (2008) using the 3D components of Adobe Acrobat 9 Pro Extend software.

Killeen & Smith (1994) stated that the 'live-collected animals are not well preserved'. Nevertheless, preservation was found to be sufficient to reconstruct gross anatomy, although not all histological details could be resolved. For example not all nerves that were expected to be present could be discerned. Other organ systems, such as the musculature, yielded full information. The current analysis has been restricted to those parts of the anatomy with particular value for comparative purposes.

As in previous anatomical descriptions (Wingstrand, 1985; Haszprunar & Schaefer, 1997a, b; Schaefer & Haszprunar, 1997a) we use the term 'sector' (with capital letters *A–H*) to describe the relative position of various organ systems. The anterior border of each sector is defined by the anterior edges of the main shell muscle insertion areas (Haszprunar & Schaefer, 1997a, b).

RESULTS

Shell and external morphology

The shell of the smallest individual investigated (Fig. 1A–C) is transparent, almost circular and shows concentrically arranged radial ribs, which differ slightly from the type material, where they are denser (present specimen: 29, paratype of Warén (1989): 36; ribs counted from, Warén, 1989: fig. 2E, in inner shell area that corresponds in size to present specimen) and growth lines. The apex (Fig. 1C) has a diameter of *c.* 350 µm. It lies inside the shell periphery and is free of ribs and growth lines. Shell dimensions: length (i.e. maximum diameter) = 815 µm, width = 710 µm, height = 280 µm.

In sections the decalcified shell (Fig. 1D; ol) is represented by two layers: a layer (*c.* 1 µm thickness) above the mantle, obviously representing the organic matrix of the foliated (nacreous) layer of the calcareous shell (Figs 2A, C, D, 3B; os); and outside this the periostracum, which is more corrugated and thinner (Fig. 2A, C, D) and is continuous with the free periostracum laterally (see below).

In a ventral view of the soft parts (Figs 1D, 4C) the large head bears prominent, paired perioral lappets (= 'lateral lips', 'velum') (Figs 1D, 2A, 4C) on either side of the mouth. The postoral tentacles are inconspicuous (Figs 1B, C, 2A, 4C), represented only by a pair of bulges with only a small distance between them. The foot is oval (slightly longer than wide), sucker-like organ and is surrounded by the mantle cavity. There are four pairs of gills (ctenidia), which are simple papillary appendages (maximum length = 50 µm, approximate diameter = 30 µm). The anus is a simple opening in the centre of the posterior mantle cavity. None of the available specimens shows signs of brooding in the mantle cavity. By transmitted

Figure 1. *Micropilina minuta*. **A–C.** Entire specimen (spm 4) in ethanol. **D.** Decalcified specimen (spm 1) in ethanol prior to embedding. Asterisks mark artefacts, such as sand grains not part of the specimen. **A.** Dorsal view. **B, D.** Ventral view. **C.** View from lateral left. **A, B, D.** Viewed by transmitted light using compound microscope. **C.** Viewed by reflected light using Olympus SZX12 stereo microscope. Abbreviations: ap, apex; fo, foot; il, intestinal loops; ol, organic shell and periostracum layers; me, mantle edge; pe, perioral lappet; po, postoral lappets; so, subradular organ. Scale bar **A–C** = 200 µm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

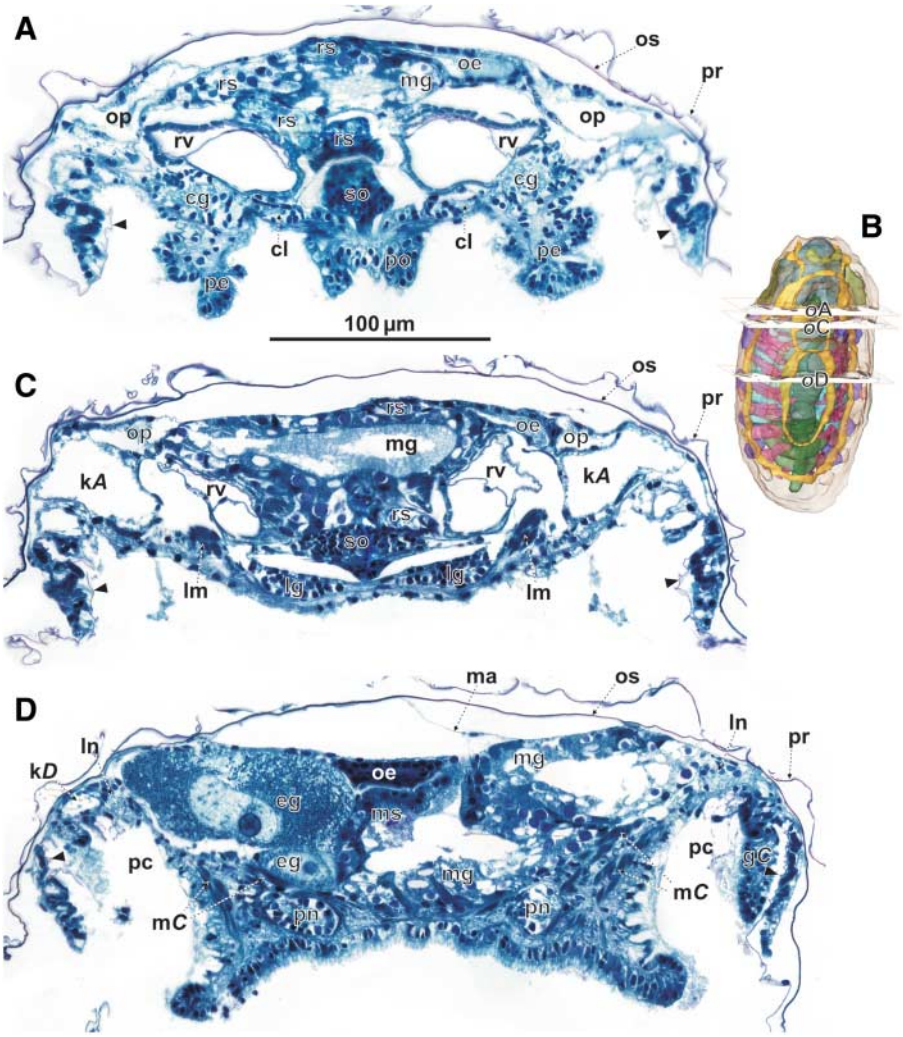


Figure 2. *Micropilina minuta* (spm 1). **A, C, D.** Histological cross-sections. **B.** Transparent surface rendering of same specimen with *orthoslices* (*o*A, *o*C, *o*D) showing section planes of **A, C** and **D.** Arrowheads show free periostracum. Abbreviations: cg, cerebral ganglion; cl, cerebrolabial connective; eg, egg; g*C*, gill of sector *C*; *kA*, kidney of sector *A*; *kD*, kidney of sector *D*; lg, labial ganglion; lm, labial muscle; ln, lateropedal nerve cord; ma, mantle; *mC*, dorsoventral muscle of sector *C*; mg, glandular portion of midgut; ms, stomach portion of midgut; oe, oesophagus; op, oesophageal pouch; os, organic shell matrix; pr, periostracum; pn, pedal nerve cord; pe, perioral lappet; po, postoral lappets; rs, radular sheath; rv, radular vesicle; so, subradular organ. Scale bar **A**, **C**, **D** = 100 µm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

light the subradular organ (Fig. 1B, D) posterior to the mouth opening and the intestinal loops (Fig. 1A, B) in the posterior part of the body are clearly visible.

The pedal sole is uniform, with an epithelium consisting of prismatic, densely ciliated cells with epidermal mucous cells interspersed. Subepithelial glands were not found in any region of the body.

Mantle and gills

The mantle of *Micropilina minuta* shows the typical molluscan features, such as thin dorsal epithelium and so-called tendon cells (significantly higher than surrounding mantle cells and with dense dorsoventrally arranged actin fibres, see e.g. Tompa & Watabe, 1976) at muscle attachment sites. In the fixed specimens, the mantle had partly collapsed, becoming attached to the structures beneath, so that contours of internal organs like intestinal loops and muscle attachment sites are visible (Fig. 4A).

The inner side of the mantle margin is covered with a cuticle-like membrane, extending dorsally almost to the bases of gills (Fig. 2A, C, D). This membrane – herein termed 'free periostracum' (a term similarly applied in bivalves, e.g. Harper, 1997) – represents the extension of the shell periostracum. In fixed specimens there is a space between shell and mantle margin (Fig. 1A, B), but the free periostracum is connected to both bridging this space. In sections the free periostracum is thinner than the shell periostracum (Fig. 2A, C, D), and the former is presumably highly flexible.

Although many structural details of the mantle margin cannot be resolved by light microscopy, a distinct difference from previous descriptions is apparent: there is no deep cleft between an inner and an outer fold (e.g. Schaefer & Haszprunar, 1997b).

The epithelium of the pallial roof is mainly composed of squamous nonciliated supporting cells, while ciliated cells are rarely found. Groups of large mucous cells, the pallial glands, surround the gill bases.

All gills are of about equal size and positioned in the sectors *C* (posteriorly), and *E*–*G* (Figs 4C, 5A, B). On the median (i.e. towards the foot) side the gill epithelium is densely ciliated up to the tips and there are no areas with a squamous epithelium. Internal skeletal elements are absent, while gill retractor muscles are visible reaching almost to the gill tip.

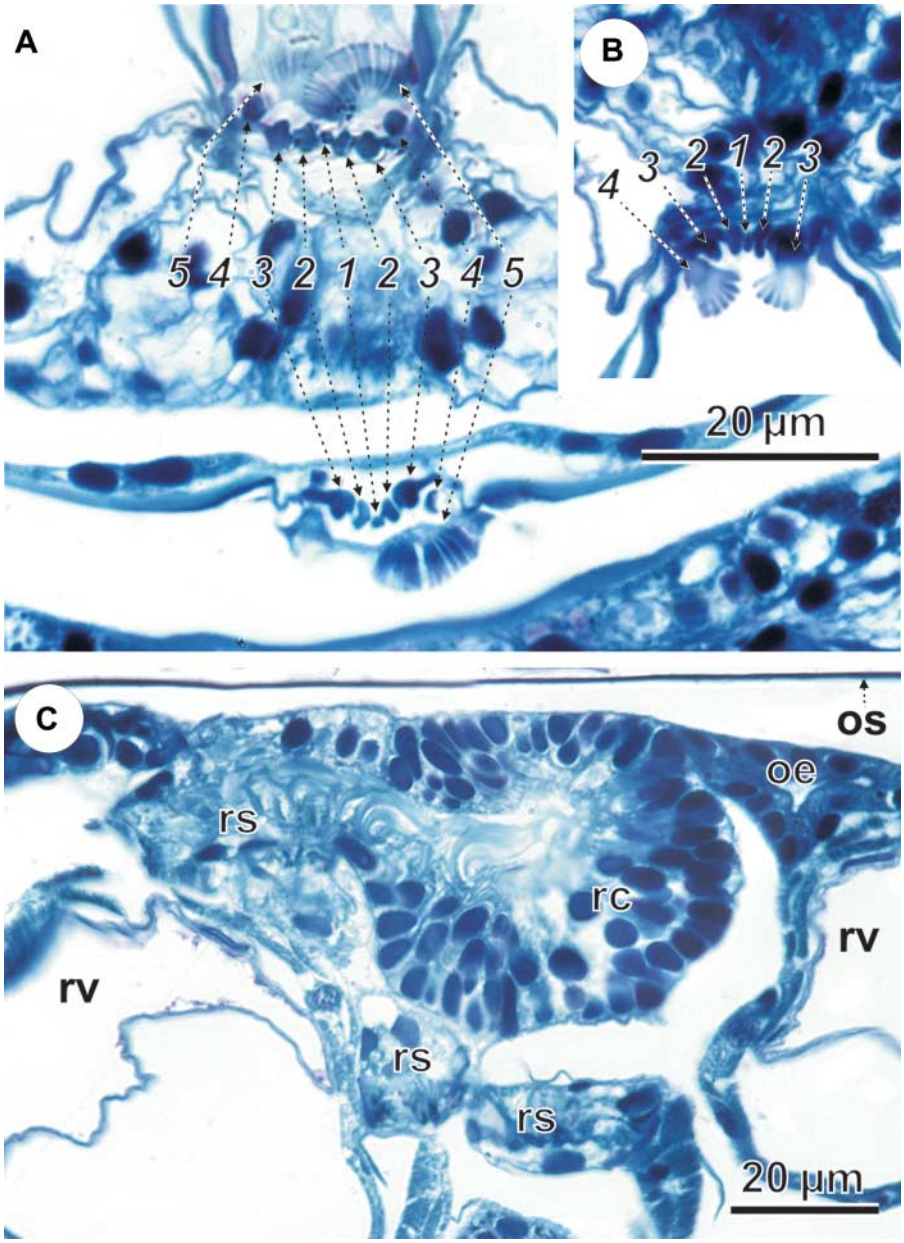


Figure 3. *Micropilina minuta* (spm 3). Cross-sections in radular area. **A, B.** Radula in slightly different positions. **C.** Middle of buccal apparatus. Abbreviations: *1–5*, radular teeth numbered following the terminology of e.g. Warén & Gofas (1996); oe, oesophagus; os, organic shell matrix; rc, radular caecum; rs, radular sheath; rv, radular vesicle. Scale bars **A, B** = 20 µm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

Body muscle systems

There are eight dorsoventral muscle bundles (Figs 4B, F, 5A, B), the shell muscles. Dorsally these form compact bundles inserting on the shell; ventrally they ramify in the foot. The two bundles located most anteriorly (m*A*, m*B*) insert relatively close to each other. Medially to the first muscle (m*A*) another muscle inserts, the labial muscle (Fig. 4F), which extends ventroanteriorly towards the sides of the mouth. As in previous studies of monoplacophoran morphology (e.g. Wingstrand, 1985; Schaefer & Haszprunar, 1997a; Haszprunar & Schaefer, 1997a, b) we do not regard this one as a shell muscle in a strict sense. The distance between bundles increases from anterior to posterior, except that the distance to the last bundle (m*G*–m*H*) is smaller again. In dorsal view the insertion areas of the dorsoventral muscles form a U-shape, as is typical also for most other neopilinoids. In addition there is a pair of prominent oblique muscles inserting far posterior to the anus. Horizontal muscles, as known in *M. arntzi* (Haszprunar & Schaefer, 1997b), are absent.

Circulatory and excretory system

As in *M. arntzi* (Haszprunar & Schaefer, 1997b) we could not detect any trace of a heart or indication of a reduced pericardium. In addition, no blood sinus-like spaces are identifiable. All internal organs are densely packed with little space for haemolymph circulation in between.

Micropilina minuta has four pairs of kidneys (nephridia) in sectors *A*, *D*, *E* and *F* respectively, located outside the shell muscles above the roof of the pallial cavity (Figs 2C, 4B, E,

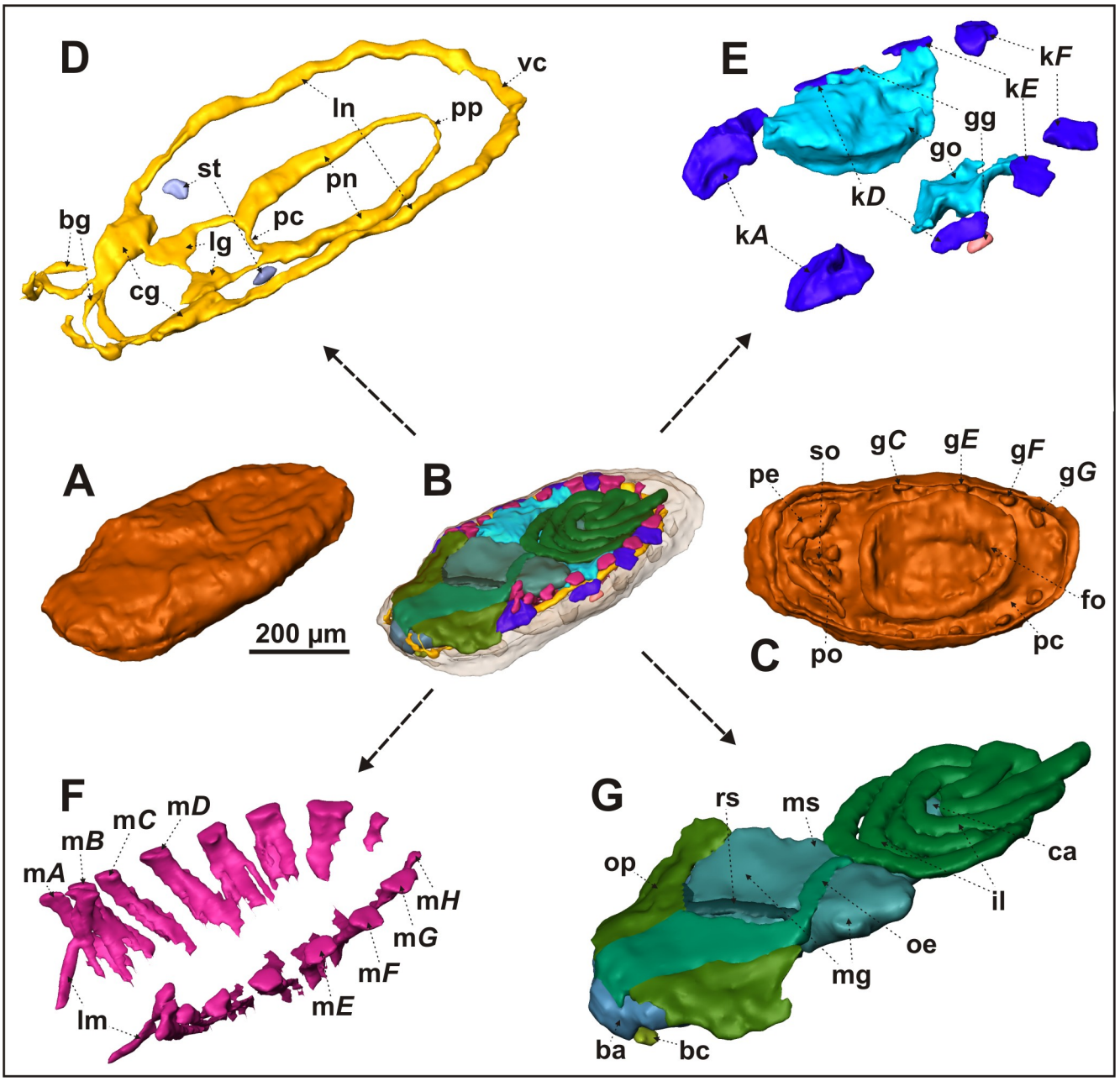


Figure 4. *Micropilina minuta* (spm 1). Exploded view of surface renderings of 3D-processed specimen. **A, B, D–G.** Oblique view from left. **C.** From ventral side. **A, C.** External surface views. **B.** External surface transparent showing all organs at once. **D.** Nervous system and statocysts. **E.** Kidneys and genital system. **F.** Dorsoventral and labial muscles. **G.** Digestive system. Abbreviations: ba, buccal apparatus; bc, buccal glands; bg, buccal ganglion; ca, stomach caecum; cg, cerebral ganglion; fo, foot; g*C*, g*E*, g*F*, g*G*, gills of respective sectors; gg, genital gland; go, gonad; il, intestinal loops; k*A*, k*D*, k*E*, k*F*, kidneys of respective sectors; lg, labial ganglion; lm, labial muscle; ln, lateropedal nerve cord; m*A*–m*H*, dorsoventral muscles of respective sectors; mg, glandular portion of midgut; ms, stomach portion of midgut; oe, oesophagus; op, oesophageal pouch; pc, pedal connective; pe, perioral lappet; pn, pedal nerve cord; po, postoral lappets; pp, posterior pedal connective; rs, radular sheath; so, subradular organ; st, statocyst; vc, visceral commissure. Scale bar **A**–**C** = 200 µm.

This figure appears as 3D model in the online version of *Journal of Molluscan Studies*. Instructions for viewing 3D image in PDF: In *Adobe Reader* (recent version recommended) click the image to activate the 3D mode. A variety of tools allows interactive manipulations ranging from free rotating (Rotate or Spin option activated on 3D bar and drag model with mouse), zooming in and out (Zoom option) or moving the model (Pan option) to viewing only selected portions (use Model Tree—check or uncheck objects) or changing surface visualization (Render Mode, Lighting). In addition to the default view some views (names self-explanatory) are prefabricated and available by clicking on them in the middle of the Model Tree interface.

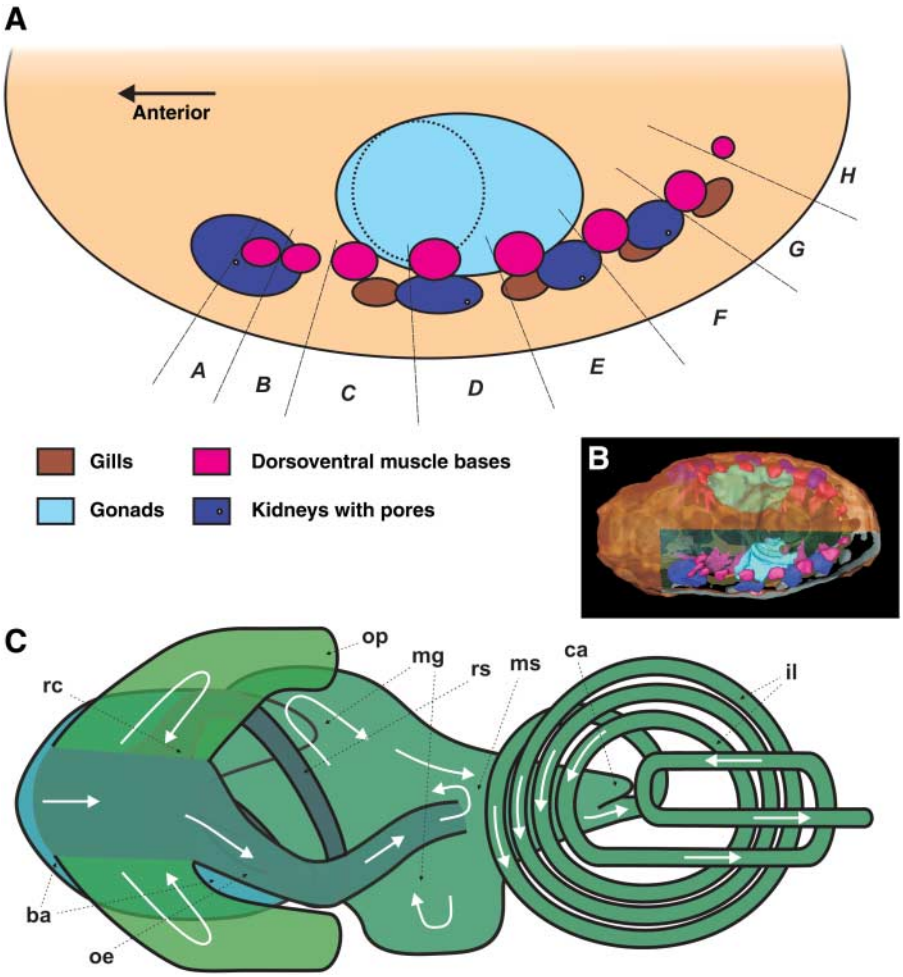


Figure 5. *Micropilina minuta*. **A, C.** Schematic drawings. **B.** Surface rendering with part of the external surface omitted. **A.** Left side; dorsal view. Sector arrangement of gills, kidneys, gonads and dorsoventral muscle bases. **B.** Same view and showing the same structures as **A** with all other structures transparently displayed. **C.** Digestive system. White arrows show inferred food passage. Abbreviations: *A–H*, sectors defined by the anterior edge of the main shell muscle insertion areas; ba, buccal apparatus; ca, stomach caecum; il, intestinal loops; mg, glandular portion of midgut; ms, stomach portion of midgut; oe, oesophagus; op, oesophageal pouch; rc, radular caecum; rs, radular sheath.

5A, B). The kidneys do not show any interconnections. The most anterior kidney (Figs 2C, 4B, E, 5A, B; k*A*) is by far the largest and the only one with two protruding lobes. The latter are due to muscle m*A*, which traverses the kidney. The pore of the kidney is located anterolaterally of the dorsoventral muscle bundle *A*. All remaining kidneys are compact organs. The kidneys in sectors *D* and *E* have the supplementary function of releasing the gametes.

Genital system

The gonads appear as a pair of simple sacs (Figs 2C, 4B, E, 5A, B) occupying a large part of the body cavity and situated laterally near the stomach portion of the midgut. Posteriorly the gonads are covered dorsally by intestinal loops. Along the anteroposterior axis they extend between sectors *B* (on right side) or *C* (on left) as far as *F*.

Each gonad contains several immature oocytes and, if at all, only a single mature egg (Fig. 2D). Mature eggs are of medium size (diameter up to 110 μm), yolk-rich and irregularly shaped. Each egg contains a large nucleus (oval to irregularly shaped, diameter up to 45 μm) with a prominent nucleolus (spherical, diameter up to 11 μm). The degree of egg maturity may differ in the left and right ovary. Certain areas in the lateral and posterior gonad contain material that appears to represent stages of spermiogenesis, resembling in appearance the ones shown in *Laevipilina antarctica* (Healy *et al.*, 1995: fig. 3C). If this interpretation is correct, *M. minuta* is hermaphroditic, but this remains doubtful due to poor tissue fixation. However, another possible suggestion of hermaphroditism is the fact that all investigated specimens carry eggs.

Laterally each gonad is linked to kidneys *D* and *E*, which serve as ducts for releasing the gametes (see above). This dual gononephroduct system might indicate that there are two pairs of gonads, but a clear histological separation between two compartiments could not be discerned. The gononephroducts lack special accessory epithelia and the releasing kidneys are not enlarged (Figs 4E, 5A, B; k*D*, k*F*). The anterior nephro-genital opening is surrounded by the genital gland (Fig. 4E).

Digestive system

The head and buccal apparatus of *M. minuta* are large. Within the buccal cavity the subradular organ (Figs 1B, D, 2A, B, 4C) is represented by an appendage with densely packed cell nuclei. Its anteriorly directed tip can protrude out of the mouth opening, as in the reconstructed specimen.

As far as could be resolved from the section series, *M. minuta* has a typical monoplacophoran radula and prominent radular musculature. Five tooth types of the usual six could be discerned (Fig. 3A, B); tooth number 5 (according to the terminology of Warén & Gofas, 1996) has the usual brush-like shape. The lateral teeth show denticulation. Six denticles were observed for teeth numbers 3 and 4 (Fig. 3B). The radular sheath is very long and forms a loop surrounding the midgut dorsally and reflected forwards to end at a position next its origin. Near the end of the radular sheath, but not terminally, a prominent radular caecum descends; this is positioned anteriorly to and closely attached to the midgut. Developing radular teeth can be seen in this caecum (Fig. 3C). In one specimen (spm 1) the radular vesicles were collapsed (Fig. 2A, C), which could be a result of fixation conditions.

A pair of medially interconnected buccal (salivary) glands sits ventroanteriorly between the buccal apparatus and body wall (Fig. 4G). From the mouth the food passage leads anteriorly and dorsally, passing the radula, and running into the oesophagus. The latter has a pair of large lateral sacs, the oesophageal pouches (Figs 2A, C, 4B, G, 5C). These pouches lie underneath the dorsolateral epithelium and cover the buccal apparatus lateroanteriorly. They extend posteriorly for one-third of the animal's length. The oesophageal lumen shows a ventral and dorsal ridge with ciliation, and these ridges lead laterally into the oesophageal pouches right to their postero-lateral ends. Laterally the pouches show bright homogenous contents (Fig. 2A, C, right [specimen] side).

From the point of separation from the oesophageal pouches, the oesophagus forms a curve to the left and then back to the sagittal plane (Fig. 4B, G). It enters the midgut dorsally at a relatively posterior position. The midgut is by far the most voluminous component of the digestive system. For most of its length it sits atop the ventral body wall (Fig. 2D). The central and anterior parts of the midgut show the histology of digestive glands (Fig. 2D), while the posterior part has a stomach-like wall. Terminally and dorsally there is an inconspicuous internally ciliated extension, the stomach caecum (Figs 4G, 5C).

The intestine emerges ventrally from the midgut. It forms five continuous, horizontal loops, which are situated dorsally in the body (Figs 1A, B, 4B, G, 5C), and lead to the simple anal opening. The diameter of the intestinal loops is uniform within and among individuals, whereas the arrangement of loops varies considerably among specimens (compare Fig. 1A, B and Fig. 4B, G).

Nervous system and sensory organs

In *M. minuta* the main components of the nervous system (Fig. 4D) are a pedal nerve ring, a dorsolateral nerve ring with prominent cerebral ganglia anteriorly (Fig. 2A) and well-separated labial and buccal ganglia. The anterior part of the pedal nerve ring is thicker than the posterior one, where a delicate posterior commissure is present. The labial ganglia (Fig. 2C) show long connectives to the pedal nerve cords and short ones to the cerebral ganglia (Fig. 2A). The latter are relatively voluminous and form distinct anterolateral swellings on the dorsolateral nerve ring. The buccal connectives descend anteriorly from the cerebral ganglia. A few ganglionic swellings lie adjacent to the buccal apparatus; the innermost ones represent the buccal ganglia. It is unclear whether cerebropedal connectives are truly missing or if they could not be resolved because of technical shortcomings.

As is typical for neopilinoids, the unpaired subradular organ (Figs 1B, D, 2A, C; see also Digestive System) is the most prominent sense organ and is located between the labial ganglia. The oval statocysts (Fig. 4D) lie equidistant between cerebral, labial and 'pedal' ganglia adjacent to the body wall. Part of their inner lumen is filled with irregular contents and their dorsal epithelium is thickened, but a tubular extension seems to be absent. There are no traces of eyes or osphradia.

Organ seriality

In *M. minuta* organs that show signs of seriality are the dorsoventral muscle bundles (eight pairs), kidneys (four pairs) and gills (four pairs). As can be judged from Figure 5A, B there is no serial correlation between these three organ complexes. Relative distances differ; dorsoventral muscle bundles are closest to each other, followed by kidneys and gills. Thus, both number and anteroposterior distribution of the respective serial structures differ.

DISCUSSION

General remarks

General accounts of comparative monoplacophoran morphology have been given by Lemche & Wingstrand (1959), Wingstrand (1985), Haszprunar & Schaefer (1997a, b), Haszprunar (2008) and Lindberg (2009). Here we focus on the new results for *Micropilina minuta*.

Mantle margin

A permanent connection between periostracum and mantle margin in the form of a free periostracum, as in *M. minuta*, is probably common in monoplacophorans (e.g. Lemche & Wingstrand, 1959: 16: text description; Warén & Gofas, 1996: figs 2E, 8B, D, 16A, C: remains of the free periostracum visible in SEMs), although it has not received attention in previous studies. As in *M. minuta*, the free periostracum is an extensive membrane that must be flexible in life, because the distance between the shell margin and the contracted mantle edge can be quite large (Fig. 1A, B; Warén & Gofas, 1996: fig. 6B) as also seen in living specimens (Urgorri, García-Álvarez & Luque, 2005: fig. 1B, D). Previous schematic drawings of shell plus mantle edge are more or less misleading in this respect: figure 33 of Lemche & Wingstrand (1959) gives the false impression that mantle and shell edge are directly fixed to each other, whereas figure 14 of Schaefer & Haszprunar (1997b) shows a free periostracum that may be too short. The latter figure may help to understand the difference between *Laevipilina antarctica* with a deep cleft laterally of the inner fold and *M. minuta* without a distinct cleft. The cleft in *L. antarctica* could be due to mantle retraction prior to or at fixation, so that the middle fold became torn towards the outside by the free periostracum, resulting in this cleft underneath. If so, this is an artefact rather than a stable morphological condition. The condition in *L. antarctica*, in which only the very inner (upper) edge of the free periostracum and the middle fold of the mantle edge are fixed to each other, could apply to monoplacophorans in general.

Thus, the tryblidian structure of mantle–shell connection differs substantially from that of other shell-bearing conchiferan taxa. In gastropods (including limpets with a shell shape similar to that of monoplacophorans), scaphopods and cephalopods (of which *Nautilus* alone has an external shell) the highly mobile mantle edge is usually unconnected to the shell edge and touches the latter only during shell growth. In contrast, the thin, large, lamella-shaped mantle folds of bivalves extend to the shell edge to which they are permanently tightly connected by the narrow free periostracum.

Coelomic conditions

The lack of the heart is shared by *M. minuta* and *M. arntzi*. In the latter the heart seems (but see below) to be functionally replaced by a series of ventrally located, horizontal muscles (Haszprunar & Schaefer, 1997b), whereas in *Micropilina minuta* there is no functional equivalent to the heart. This condition might be explained by the miniaturization of these animals and/or by paedomorphic development. In Polyplacophora the pericardium and heart are formed weeks after metamorphosis when the animals have already undergone some growth (e.g. Hammarsten & Runnström, 1926; Salvini-Plawen & Bartolomaeus, 1995; B. Ruthensteiner & G. Haszprunar, unpubl.). It is possible that *M. arntzi* and *M. minuta* never reach these developmental stages. If so, the lack of a heart in both species could be due to paedomorphosis. Alternatively, it could also be interpreted purely functionally, if metabolic transportation systems are not required because of the small size and thus became reduced. However, organization of other organ complexes gives additional evidence for paedomorphosis in these tiny monoplacophorans (see below). Moreover, gastropods and bivalves (except the protobranch *Microgloma*; Sanders & Allen, 1973) within the same size range usually have a heart.

Reproduction

Size and yolk content of the eggs suggest nonplanktotrophic development, but it is improbable that *M. minuta* is a brooding species like *M. arntzi*. There are three reasons: (1) the presence of all stages of oogenesis suggests continuous egg production, yet among four specimens there was not a single one with eggs in the mantle cavity. In contrast, Haszprunar & Schaefer (1997b) found brooded eggs in five of nine adult individuals of *M. arntzi*. (2) *Micropilina arntzi* shows a distinct shift of the insertion areas of muscle pairs *F* inwards to widen the space for the large egg in the mantle cavity, whereas *M. minuta* does not show this condition. (3) In *M. arntzi* the gills are comparatively prominent organs, probably to ventilate brooded eggs or embryos. In *M. minuta* these organs are significantly smaller. The absence of horizontal muscles in *M. minuta* suggests that they might play a role in pushing out the huge eggs in *M. arntzi* rather than replacing the heart function as proposed by Haszprunar & Schaefer (1997b).

On the other hand, *M. minuta* shows, like *M. arntzi*, a very low sperm volume compared with the large testes of probable ectaquatic fertilizers like *Laevipilina antarctica*. This suggests entaquatic fertilization in the mantle cavity of *M. minuta*.

Evolutionary remarks

Micropilina minuta helps to bridge the conceptual gap between the anatomical features of *M. arntzi* and the Neopilinidae. The specific conditions of *M. minuta* also shed light on neopilinoid evolution.

The description of a neopilinoid species with four pairs of gills completes the series of adults with three (*M. arntzi*), four (*M. minuta*), five (e.g. *Neopilina galathaea*, *Laevipilina antarctica*) or six (e.g. *L. hyalina*, *Vema ewingi*) pairs of gills. It has now been demonstrated that in two cases (*Micropilina*, *Laevipilina*) gill numbers are not diagnostic for genera. Accordingly, the validity of *Vema*, which has been separated from *Neopilina* solely based on this character, can be questioned.

Independent seriality of organs *vs* true annelid-like segmentation has been exhaustively discussed in previous studies on monoplacophorans (Lemche & Wingstrand, 1959; Götting, 1980; Wingstrand, 1985; Haszprunar & Schaefer, 1997a, b; Haszprunar, 2008, Lindberg, 2009). As a result it is now widely agreed that monoplacophorans do not show true segmentation. Our results on *M. minuta* which shows obvious incongruencies in seriality patterns of different organ complexes provide additional evidence for this. Distinct differences in relative spacing (Fig. 5A) clearly point towards a nonsegmented condition.

Interpretation of organ seriality in *M. minuta*, in terms of homology within the monoplacophorans (discussed in detail by Haszprunar & Schaefer, 1997b), appears more difficult. When comparing the serial organization of monoplacophorans in general, it seems apparent that the gills are the serial organs that are most affected in number by decreasing body size, as is the case in the two *Micropilina* species (Haszprunar & Schaefer, 1997b; present study). Least affected are the dorsoventral muscle bundles, the number of which remains more or less constant throughout the species investigated. In the range between lie the kidneys that exhibit some decrease in number. Accordingly, the strength of the serial pattern is negatively correlated with body size.

Interpretation of these conditions may follow two different lines: (1) miniaturization in combination with paedomorphosis acted upon relatively large ancestors with a more pronounced serial pattern. (2) The small species represent the plesiomorphic condition, while the large ones are derived. Outgroup comparison, however, is clearly in favour of the first hypothesis, because the heart and kidney organization of the large species resembles those of other molluscan taxa, and a (secondary) reinvention of a heart seems very unlikely.

Organ homologies within monoplacophorans

While the gills in sectors *E*–*G* of *M. minuta* are homologous with the same ones of other species, homology of the anterior one (sector *C*) remains unclear, because the other species do not show gills in this sector. It could correspond to the sector *B* gill of *V. ewingi*, *L. antarctica* and *N. galatheae*. It seems more parsimonious, however, to propose a homology with sector *D* gills of the other species, because then it is not necessary to assume the loss of a previous pair during development. Indeed, because of the posterior position of the gill in sector *C* and the position of gill *E* at the edge of sectors *D* and *E* (Figs 5A, B, 6) it appears possible that there is a slight shift forward from sector *D* as has been found in other organs among monoplacophorans (Haszprunar & Schaefer, 1997a).

The serial arrangement of kidneys in monoplacophorans is not as homogeneous as that of the muscles or gills. As mentioned above, the number of kidneys decreases with body size. The kidney of sector *A* is present in all Neopilinoidea; in most species (*L. antarctica*, *M. arntzi*, *M. minuta*) it is distinctly larger than the other kidneys, as is the case in *M. minuta*. This could be due to function; kidney *A* is not at all involved in reproduction, such as discharge of gametes. It might play a more important role in excretion than the other kidneys. However, the functioning of the monoplacophoran kidneys is unclear, because primary urine in adult molluscs is usually produced by ultrafiltration into the pericardial lumen and transported to the kidney from there. This obviously is not the case for most monoplacophorans kidneys – most obviously for the ones without connections to the pericardium. A remarkable similarity might shed light on this: kidney *A* very much resembles the 'protonephridial kidney' (Baeumler, Haszprunar & Ruthensteiner, submitted) of juvenile polyplacophorans in position and shape. If this resemblance is verified by future fine-structural or ontogenetic examinations, kidney *A* would represent an unambiguous paedomorphic structure. The lack of the most posterior kidney (sector *G*) is shared by *M. arntzi* and *M. minuta*. Again, as for the presence or absence of a circulatory system, this should be regarded as derived due to

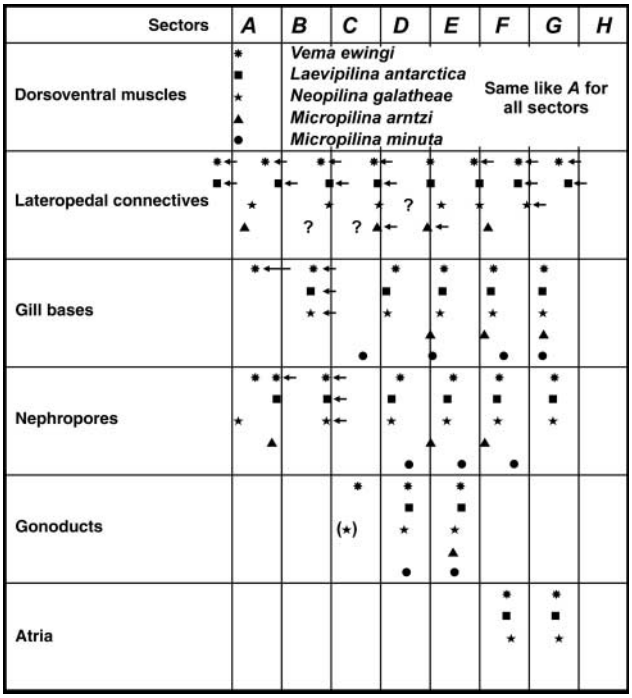


Figure 6. Comparative view of organ seriality in the five species of extant Monoplacophora studied anatomically in detail (adapted from Haszprunar & Schaefer, 1997b).

miniaturization rather than as a plesiomorphic condition. Outgroup comparison with the polyplacophorans (nephropore situated in sector *G*) may also indicate that the lack of a kidney in sector *G* is an apomorphic feature of *Micropilina*. However, contrary to the neopilinoid condition, the polyplacophoran kidneys are located inwards of the shell muscles (Lindberg & Ponder, 1996; Lindberg, 2009), which makes direct comparison difficult. In any case, the conditions in the *Micropilina* species contradict the ideas of Lauterbach (1983) on kidney evolution in monoplacophorans and provide additional evidence that the seriality of gills and kidneys is entirely independent of the annelid condition (contrary to e.g. Götting, 1980).

Taxonomy and systematics

There are certain differences between the shell of the specimen we investigated and the type specimens as described by Warén (1989). The outline of the aperture is more circular and the overall shape more flat, with the apex not overhanging the shell periphery. The shell sculpture is coarser. However, these differences might be due to the smaller size of our shelled specimen (0.815 mm length). Shell sculpture and proportions may change as a function of growth (A. Warén, personal communication). Killeen & Smith (1994) report 1.5 mm as the maximum length for specimens of the entire material from which our specimens came. SEM examination of additional shells should resolve this question. Currently, it seems justified to retain the identification *Micropilina minuta*.

The validity of the genus *Micropilina* is problematic. The genus was erected by Warén (1989) for monoplacophorans with (1) a series of distinct muscle impressions having (2) small size, and (3) a shell sculpture of concentrically arranged small pits. While this probably was a useful concept at the time of its introduction, all of these shell characters have become increasingly obscured by more findings and descriptions of 'micropilinid' monoplacophorans since then. First, while details of the shell interior are unknown for most monoplacophorans at the SEM level, there are species attributed to other genera with distinct muscle impressions (e.g. *Laevipilina cachuchensis*, Urgorri *et al.*, 2005) and *Micropilina* species without muscle scars (e.g. *M. arntzi*, Warén & Hain, 1992). Secondly, there are other taxa in the size range of *Micropilina* species (e.g. *Rokopella segonzaci*, Warén & Bouchet, 2001). Thirdly, there are *Micropilina* species without clearly recognizable concentrically arranged small pits (e.g. *M. arntzi*, Warén & Hain, 1992), whereas species attributed to other genera (e.g. *Veleropilina reticulata* (Seguenza, 1876), information from Warén & Gofas, 1996) have a shell surface with concentrically arranged pits that more closely resembles that of *M. minuta*. Therefore, shell morphology does not support a close relationship between *M. arntzi* and other *Micropilina* species (cf. Marshall, 1990, 2006). Accordingly, a redefinition of the genus *Micropilina* may be required. The same question has been raised for the genus *Laevipilina* by Urgorri *et al.* (2005). We agree with these authors that a thorough general revision of monoplacophorans at the supraspecific level will be useful.

Based on anatomical knowledge of *M. arntzi*, Haszprunar & Schaefer (1997b) erected a new family, Micropilinidae, to highlight the significant differences between this species and all remaining Neopilinoidea (Neopilinidae). The anatomical data on *M. minuta*, the type species of the genus *Micropilina*, permits testing of these supraspecific systematic arrangements.

Aside from the differences in shell morphology, several anatomical characters differ between *M. minuta* and *M. arntzi* (Table 1). These include the number of gills (four *vs* three), kidneys (four *vs* three), oesophageal pouches (one pair *vs* two pairs, small *vs* medium size), gononephroducts (two *vs* one) and the reproductive type (probable nonbrooding *vs* brooding). These differences do not support a particularly close relationship between the two species.

On the other hand *M. minuta* and *M. arntzi* share several features, which are thus diagnostic for the genus and family. Two of these characters may be accepted as synapomorphic among the Neopilinoidea, because this is more parsimonious than assuming independent acquisition: (1) the lack of a heart, (2) lack of sector *G* nephridia. It depends on the proposed line of evolution whether (3) the low number of gills (three or four),

Table 1. Comparison of *Micropilina* species and the Neopilinidae

Taxon character	*Micropilina arntzi*	*Micropilina minuta*	*Neopilina, Laevipilina, Vema*
Postoral lappets	Absent (?)	Present	Present
Gills	Three (sectors *E, F, G*)	Four (sectors *C, E, F, G*)	Five (sectors *C–G*); six (*B–G*)
Kidneys	Three (sectors *A, E, F*)	Four (sectors *A, D, E, F*)	Six (sectors A, C–G); seven (*A–G*)
Gonads	One (sector *D*)	One (or two?) (sectors *D–E*)	Two (sectors D, E), three (*C–E*)
Fertilization	Entaquatic	Entaquatic	Ectaquatic (all?)
Brooding habit	Yes	No	No
Heart	Absent	Absent	Present
Oesophageal pouches	2 pairs, regular size	1 pair, large	2 pairs, large and extended
Intestinal loops	6 irregular	5 circular, horizontal	5 circular, horizontal
Organ density	Space between organs	Organs densely packed	Space between organs
Dorsoventral muscles	*A* + *B* fused, *C–H* separate	*A–H* separate	*A–H* separate

(4) kidneys (three or four) and (5) gonad (one or two) are considered as plesiomorphic or apomorphic characters (see above). It should be noted, however, that two of the potential synapomorphies are losses that might result from the same evolutionary constraint, miniaturization, which obviously is a widespread trend among monoplacophorans. Many of the species described so far – including those from other genera – have sizes in the range of the anatomically investigated *Micropilina* species (e.g. Warén & Gofas, 1996). Consequently, anatomical knowledge of other small monoplacophorans is desirable for unequivocal interpretation of our findings in *Micropilina*.

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