THE PHYLOGENY OF THE ACTEONOIDEA (GASTROPODA): MOLECULAR SYSTEMATICS AND FIRST DETAILED MORPHOLOGICAL STUDY OF *RICTAXIS PUNCTOCAELATUS* (CARPENTER, 1864)

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ABSTRACT

The Acteonoidea are a taxon of about 150 species with uncertain systematic affinity. They have been variously regarded as basal Opisthobranchia, as Architectibranchia, or placed basal to Opisthobranchia together with other heterobranch clades with uncertain interrelationships. We performed molecular phylogenetic analyses employing four gene markers (nuclear 18S rDNA and 28S rDNA; mitochondrial 16S rDNA and COI) to reassess the phylogenetic position of Acteonoidea and provide the first comprehensive study on interrelationships of the constituent families. Our analyses reveal a sister-group relationship of the Acteonoidea to the 'lower' heterobranch taxon Rissoelloidea, implying a basal placement outside the Opisthobranchia. However, the Acteonoidea/Rissoelloidea clade is sister group to the morphologically derived opisthobranch clade Nudipleura, implying an opisthobranch origin. Regarding the interrelationships of the Acteonoidea, the current division into three families is challenged by recovery of only two clades. The genus *Bullina* (sole genus in Bullinidae) resolves as a member of the Aplustridae, which is thus rendered paraphyletic. The Acteonidae are paraphyletic due to the strongly supported position of *Rictaxis punctocaelatus* basal to the Aplustridae. The first detailed investigation of the morphology and histology of *R. punctocaelatus* revealed similarities with both Acteonidae and Aplustridae, implying an intermediate position. Owing to the placement of *R. punctocaelatus* basal to the Aplustridae, the shared acteonid characters are therefore considered plesiomorphic for the whole Acteonoidea and are probably the features of the last common ancestor of the clade.

INTRODUCTION

The Acteonoidea d'Orbigny, 1843 are a clade of marine gastropods displaying a mixture of basal and derived characters. The opisthobranch organization of most of their organ systems contrasts with the well-developed shells, especially prominent in the family Acteonidae (Simone, 2006). Thus their systematic position has been a matter of debate for decades, even raising doubts about their monophyletic origin (Mikkelsen, 2002; Wägele & Klussmann-Kolb, 2005), although similarity of the reproductive systems, mantle cavity and the streptoneurous nervous system support a close relationship among acteonid taxa (Rudman, 1972a).

Based on morphological considerations the Acteonoidea are supposed to be either basal Opisthobranchia belonging to the Cephalaspidea (Gosliner, 1981, 1994; Burn & Thompson, 1998; Dayrat & Tillier, 2002) or are excluded from the Opisthobranchia (Mikkelsen, 1996, 2002; Wägele & Klussmann-Kolb, 2005). Molecular analyses reveal a rather derived position within the Opisthobranchia (Grande *et al.*, 2004a, b; Vonnemann *et al.*, 2005; Klussmann-Kolb *et al.*, 2008) or also support a basal origin (Dayrat *et al.*, 2001; Dinapoli & Klussmann-Kolb, 2010) within Heterobranchia.

In 1985 Haszprunar proposed the new clade Architectibranchia, comprising the Acteonoidea, Ringiculoidea and Diaphanoidea. Paraphyly of this clade is suggested by the cladistic analysis of Mikkelsen (1996), whereas that by Dayrat & Tillier (2002) supports polyphyly of Architectibranchia. As a result of a molecular study, Malaquias *et al.* (2009) exclude the Diaphanidae from the Architectibranchia clade (Acteonidae and Aplustridae) and label the other architectibranch families (Bullinidae, Ringiculidae and Notodiaphanidae) as *incertae sedis*. Thus the systematic position of the Acteonoidea remains unresolved.

The Acteonoidea are currently divided into three families: Acteonidae, Aplustridae and Bullinidae (Rudman, 1972a; Burn & Thompson, 1998; Bouchet & Rocroi, 2005). (The Aplustridae are often referred to as Hydatinidae, because *Aplustrum* is a synonym of *Hydatina*; however, in their review of gastropod classification, Bouchet & Rocroi (2005) retain the older name Aplustridae.) The acteonoidean families have been said to 'demonstrate an evolutionary sequence' (Burn & Thompson, 1998: 943) from the infaunal Acteonidae with 'primitive' characters, through the intermediate Bullinidae to the epifaunal and more specialized Aplustridae (Rudman, 1972a; Burn & Thompson, 1998).

The Acteonidae with about 110 described species (often based only on shell morphology) are the most species-rich family. The valid genera are debated; *Acteon*, *Japonacteon*, *Maxacteon*, *Pupa* and *Rictaxis* are widely accepted (and except *Rictaxis* morphologically well studied), whereas *Pseudacteon* is regarded as a junior synonym of *Rictaxis* and *Punctacteon* as a *nomen nudum* (Rudman, 1971a). Several other genera have been erected based on shell morphology and radular structure. The genus *Crenilabium* was erected for a Tertiary fossil (Cossmann, 1889) and species descriptions (mainly based on shells) have been given by several authors (Marcus, 1974; Bouchet, 1975;

Smriglio & Mariottini, 1996; Simone, 2006). *Mysouffa* was proposed by Marcus (1974) to replace *Tomlinula*, which is still used by other authors (Bouchet, 1975; Bouchet, Le Renard & Gofas, 2001). Further possibly acteonid genera are *Neactaeonina* (Powell, 1960; Marcus, 1974; Burn & Thompson, 1998), *Inopinodon* (Bouchet, 1975; Burn & Thompson, 1998; Bouchet *et al*., 2001), *Liocarenus* (Smriglio & Mariottini, 1996; Bouchet *et al*., 2001) and *Callostracon* (Smriglio & Mariottini, 1996; Bouchet *et al*., 2001). However, validity of these genera as well as of many described species (especially of the genus *Acteon*) is arguable until studies on their morphology and anatomy are provided. Smriglio & Mariottini (1996) report two cases of misidentified pulmonate shells that were dredged at great depths due to resedimentation processes and erroneously classified as acteonids. Additionally, Burn & Thompson (1998) report that *Leucotina* (long regarded as an acteonid, e.g. Marcus, 1974) is a member of the Pyramidelloidea; they also indicate that *Obrussena*, *Ovulactaeon* and perhaps several other genera are unlikely members of the Acteonidae. Therefore, we focus our attention on the above mentioned widely accepted and well-studied genera.

The Aplustridae comprise *Micromelo* and *Hydatina*, as well as the dubious, minute, deep-water *Parvaplustrum tenerum* which has been assigned to the Aplustridae (Powell, 1960; Burn & Thompson, 1998). The Bullinidae contain only the circumtropical and subtropical genus *Bullina*.

Phylogenetic investigations on the relationships of the acteonoidean families have been fragmentary until now, because all studies have suffered from limited taxon sampling. So far, no study has focused on the interrelationships of acteonoidean families; they have mainly been included in phylogenetic studies of higher clades like Euthyneura (Dayrat *et al*., 2001; Grande *et al*., 2004b; Klussmann-Kolb *et al*., 2008), Opisthobranchia (Grande *et al*., 2004a; Vonnemann *et al*., 2005; Wägele & Klussmann-Kolb, 2005) or Cephalaspidea (Mikkelsen, 1996; Malaquias *et al*., 2009). Hitherto the only study to include members of all described families revealed two main clades, the Acteonidae (*Acteon* and *Pupa*) and the Aplustridae/Bullinidae with *Bullina* as the basal offshoot and a sister-relationship of *Micromelo* and *Hydatina* (Wägele & Klussmann-Kolb, 2005), supporting the current hypotheses on family systematics.

Diverse and extensive studies on the morphology of most of the investigated acteonoidean genera are available (*Acteon*: Fretter & Graham, 1954; Johansson, 1954; Rudman, 1972d; Yonow, 1992; *Pupa*: Rudman, 1971a, 1972c, d; *Japonacteon*: Taki, 1956; *Micromelo*: Marcus & Marcus, 1967; Rudman, 1972a; *Hydatina*: Marcus & Marcus, 1967; Rudman, 1972b; *Bullina*: Rudman, 1971b, 1972a). However, little is known of *Rictaxis punctocaelatus* except for its radula (Thiele, 1925; Habe, 1956; Marcus, 1972) and scattered information on other characters (Marcus, 1972; Gosliner, 1981).

The present investigation has three aims. We will give the first detailed description of the morphology and fine structure of *Rictaxis punctocaelatus*. We will test current phylogenetic hypotheses for the Acteonoidea by a molecular systematic analysis based on the most comprehensive taxon set yet studied. Finally, morphological and molecular results will be compared.

MATERIAL AND METHODS

Histology and scanning electron microscopy

Specimens of *Rictaxis punctocaelatus* preserved in formalin were decalcified and embedded in hydroxyethyl methacrylate resin. Serial sections (2 μm) were stained with toluidine blue and examined with a light microscope (Leica DM LB2). Photographs were taken with a digital camera (Leica DC 300F) using the software IM 50.

Jaws and radula were dissected from one specimen of *Rictaxis punctocaelatus*, sputter coated with gold (and examined with a Hitachi S4500 SEM). Photographs were taken with DISS – Digital Image Scanning System (Point Electronic, Halle, Germany).

Taxon sampling

The present study includes nine specimens of Acteonoidea covering all three extant families: four genera of Acteonidae, two out of three genera of Aplustridae (without the dubious *Parvaplustrum*) and *Bullina* (Bullinidae). Five further Heterobranchia were included (belonging to Orbitestellidae, Cimidae, Rissoelloidea and Valvatoidea) together with representatives of major euthyneuran subgroups with a focus on Opisthobranchia (particularly clades that have been postulated as sister groups of or closely related to Acteonoidea, i.e. Cephalaspidea, Nudipleura). The caenogastropod *Littorina littorea* was used as outgroup taxon, yielding a total of 36 taxa.

Specimens were preserved in 80–100% ethanol. Published sequences from GenBank were utilized for several species. The origin of all taxa and accession numbers of sequences are summarized in Table 1.

DNA extraction, PCR and sequencing

The DNeasy Tissue Kit (Qiagen) was used to extract genomic DNA from muscle tissue following the animal tissues/spin-column protocol.

Two nuclear (complete 18S rDNA and partial 28S rDNA) and two mitochondrial gene fragments (partial 16S rDNA and COI) were amplified. All fragments were sequenced in both directions. See Supplementary Data 1 for details on primers and PCR protocols.

PCR products were purified from an agarose gel using the QIAquick Gel Extraction Kit (Qiagen). Cycle sequencing was conducted with the CEQ DTCS Quick Start Kit. The final sequences were obtained using a CEQ 2000 Beckmann Coulter capillary sequencer.

Sequence alignment

MUSCLE 3.6 (Edgar, 2004) was used under the default parameters for alignment of sequences. BioEdit v.7.0.9 (Hall, 1999) was employed to manually exclude inserts and hypervariable base positions from the alignments of 18S rDNA, 28S rDNA and 16S rDNA prior to phylogenetic reconstruction. Third codon positions of the COI sequences were removed from the alignment due to substantial substitution saturation. Details about alignment length and excluded positions are given in Supplementary Data 2.

Statistical tests

The significance of incongruence in a combined data set was evaluated by the incongruence length difference (ILD) test as described by Farris *et al*. (1995). This test is implemented in PAUP v.4.0b10 (Swofford, 2002) as the partition homogeneity test and was used to test if data sets of the single gene markers can be concatenated and analysed as a single data set. One hundred replicates of a heuristic search under the maximum parsimony criterion were performed.

The test by Xia *et al*. (2003) as implemented in the software package DAMBE (Xia & Xie, 2001) was used to determine the degree of substitution saturation.

Table 1. Taxon sampling.

Taxon	Family/subfamily	Locality	GenBank accession numbers			
			18S	28S	16S	COI
CAENOGASTROPODA						
Littorina littorea (Linnaeus, 1758)	Littorinidae	Atlantic	X91970	AJ488672	DQ093481	DQ093525
'LOWER HETEROBRANCHIA'						
Orbitestella sp.	Orbitestellidae	New Zealand	EF489352	EF489377	EF489333	EF489397
Cima sp.	Cimidae	Australia, NSW	FJ917206	FJ917228	FJ917260	FJ917279
Cornirostra pellucida (Laseron, 1954)	Cornirostridae	Australia, NSW	FJ917215	FJ917225	FJ917249	FJ917282
Rissoella rissoaformis (Powell, 1939)	Rissoellidae	New Zealand	FJ917214	FJ917226	FJ917252	FJ917271
Rissoella elongatospira (Ponder, 1966)	Rissoellidae	New Zealand	FJ917203	FJ917232	–	FJ917270
Acteon tornatilis (Linnaeus, 1758)	Acteonidae	France, Atlantic Ocean	GQ845182* GQ845183*	GQ845177*	GQ845190*	GQ845172*
Japonacteon nipponensis (Yamakawa, 1911)	Acteonidae	Japan	GQ845184*	GQ845178*	GQ845191*	–
Pupa solidula (Linnaeus, 1758)	Acteonidae	Australia, QLD	AY427516	AY427481	EF489319	DQ238006
Pupa nitidula (Lamarck, 1822)	Acteonidae	Australia, QLD	GQ845185*	GQ845179*	GQ845192*	GQ845173*
Rictaxis punctocaelatus (Carpenter, 1864)	Acteonidae	USA, California	GQ845186*	EF489370	GQ845193*	EF489393
Hydatina physis (Linnaeus, 1758)	Aplustridae	Australia, NSW	AY427515	AY427480	EF489320	GQ845174*
Micromelo undatus1 (Bruguiere, 1792)	Aplustridae	Samoa	GQ845187*	GQ845180*	GQ845194*	GQ845175*
Micromelo undatus2 (Bruguiere, 1792)	Aplustridae	Guam	GQ845188*	GQ845181*	GQ845195*	GQ845176*
Bullina lineata (Gray, 1825)	Bullinidae	Australia, NSW	GQ845189*	–	GQ845196*	AY296847
OPISTHOBRANCHIA						
CEPHALASPIDEA						
Bulla striata (Bruguiere, 1792)	Bullidae	Portugal	DQ923472	DQ986683	DQ986632	DQ986566
Toledonia globosa (Hedley, 1916)	Diaphanidae	Scotia Arc, Atlantic	EF489350	EF489375	EF489327	EF489395
Scaphander lignarius (Linnaeus, 1758)	Scaphandridae	Portugal	EF489348	EF489372	EF489324	DQ974663
Philine aperta (Linnaeus, 1767)	Philinidae	Spain	DQ093438	DQ279988	DQ093482	AY345016
APLYSIOMORPHA						
Akera bullata (Müller, 1776)	Akeridae	Denmark, Kattegat	AY427502	AY427466	AF156127	AF156143
Aplysia californica (Cooper, 1863)	Aplysiidae	USA, Pacific	AY039804	AY026366	AF192295	AF077759
PTEROPODA						
Cavolinia uncinnata (Rang, 1829)	Cavoliniidae	USA, Atlantic	DQ237964	DQ237983	–	DQ237997
Spongiobranchaea australis (dapos;Orbigny, 1834)	Pneumodermatidae	Scotia Arc, Atlantic	DQ237969	DQ237988	–	DQ238002
UMBRACULIDA						
Umbraculum umbraculum (Lightfoot, 1786)	Umbraculidae	Atlantic	AY165753	AY427457	EF489322	DQ256200
Tylodina perversa (Gmelin, 1791)	Tylodinidae	Spain	AY427496	AY427458	FJ917424	AF249809
NUDIPLEURA						
NUDIBRANCHIA						
Bathydoris clavigera (Thiele, 1912)	Bathydorididae	Antarctica	AY165754	AY427444	AF249222	AF249808
Hypselodoris infucata (Rüppell & Leuckart, 1830)	Chromodorididae	Australia, NSW	FJ917442	FJ917467	FJ917426	FJ917484
Hoplodoris nodulosa (Angas, 1864)	Discodorididae	Australia, NSW	FJ917443	FJ917469	FJ917428	FJ917486
PLEUROBRANCHOMORPHA						
Tomthompsonia antarctica (Thiele, 1912)	Pleurobranchidae	Antarctica	AY427492	AY427452	EF489330	DQ237992
Pleurobranchus peroni (Cuvier, 1804)	Pleurobranchidae	Australia, NSW	AY427494	AY427455	EF489331	DQ237993
Pleurobranchaea meckeli (De Blainville, 1826)	Pleurobranchidae	Spain	FJ917449	FJ917481	FJ917439	FJ917499
PULMONATA						
SIPHONARIOIDEA						
Siphonaria capensis (Quoy & Gaimard, 1833)	Siphonariidae	South Africa	EF489335	EF489354	EF489301	EF489379
HYGROPHILA						
Chilina sp.	Chilinidae	Chile	EF489338	EF489357	EF489305	EF489382
EUPULMONATA						
Otina ovata (Brown, 1827)	Otinidae	France	EF489344	EF489363	EF489310	EF489389

Continued

Table 1. *Continued*

Taxon	Family/subfamily	Locality	GenBank accession numbers			
			18S	28S	16S	COI
SYSTELLOMMATOPHORA						
Onchidella floridana (Dall, 1885)	Onchidiidae	Bermuda	AY427521	AY427486	EF489317	EF489392
STYLOMMATOPHORA						
Arion silvaticus (Lohmander, 1937)	Arionidae	USA	AY145365	AY145392	EU541969	AF513018

Taxon names and classification according to Bouchet & Rocroi (2005). –, missing sequences.

*Sequences generated in current study.

Alternative tree topologies enforcing monophyly of the Acteonidae and Aplustridae, respectively, were tested using the approximately unbiased (AU) test (Shimodaira, 2002). The likelihood at each nucleotide position was calculated for both alternative topologies as well as for the unconstrained topology using PAUP v.4.0b10 (Swofford, 2002). The obtained likelihoods were used to compute *P*-values in CONSEL version 0.1 (Shimodaira & Hasegawa, 2001).

The software k2WuLi (Wu & Li, 1985) was employed to conduct a relative rate test in order to investigate rate heterogeneity in the sequences.

Phylogenetic analyses

Determination of the best fitting model of sequence evolution for all five gene partitions (single codon positions of COI treated separately) was performed prior to phylogenetic analyses by MrModeltest v.2.2 (Nylander, 2004) based on the Akaike information criterion. Details about the models are provided in Supplementary Data 2.

Bayesian inference analysis was performed using MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001) with separate models of evolution for each of the five gene partitions. Two separate runs of four chains (one cold, three heated) of a Metropolis coupled Markov chain Monte Carlo algorithm operated for 1,000,000 generations. The first 1,000 trees were ignored as burn-in for construction of the 50% majority rule consensus tree. Posterior probabilities were calculated for each node, a value of 0.95 or higher being considered as good statistical support.

RAxML v.7.0.3 (Stamatakis, 2006) was employed for maximum likelihood analyses. This program uses a GTR model-based approach under the gamma model of rate heterogeneity. Model parameters are estimated by RAxML for all data partitions. Preliminary analyses to estimate the best settings for the final analyses yielded the best likelihood values for an initial rearrangement setting of 10; the default setting proved best for other parameters. Two hundred best-known likelihood trees were computed based on this setting. One thousand bootstrap replicates were performed and the results were plotted onto the best-known likelihood tree. Bootstrap values above 75 were considered as having good statistical support.

In order to assess the results of these phylogenetic analyses we additionally conducted a split-decomposition analysis on the concatenated alignment using SplitsTree v.4.9.1 (Huson, 1998; Huson & Bryant, 2006). The neighbour-net graph was based on uncorrected p-distances. Split graphs show networks of phylogenetic relationships and are thus able to reveal conflicts in the data set.

Character tracing

Family-specific characters were identified from the literature (Rudman, 1972a; Burn & Thompson 1998) and coded for all Acteonoidea as well as three outgroup taxa (details in Supplementary Data 3). Reconstruction of character evolution was based on the 50% majority rule consensus tree of the Bayesian inference analysis. Character states were mapped onto the phylogeny using a parsimony approach implemented in MacClade v.4.0 (Maddison & Maddison, 2000).

RESULTS

Morphology of Rictaxis punctocaelatus

Rictaxis punctocaelatus possesses a strongly calcified shell (up to about 20 mm length) with a large body whorl composing about two-thirds of the shell length (Fig. 1A). The shell is highly coiled with a well-elevated spire. It is white with black stripes in the living animal, which fade to brown in preserved specimens; seven to five stripes per whorl are visible with a decreasing number towards the apex. The sculpture is spiral and consists of punctate grooves (Fig. 1B).

The body of the animal is translucent white, its thin broad foot extending laterally beyond and posteriorly up to the end of the shell. The animal can withdraw completely into the shell, but an operculum is absent.

The cephalic shield is vertically divided by a shallow groove into left and right halves, each consisting of an anterior and posterior lobe (Fig. 1A). The black eyes are visible through the cephalic shield although they are deeply embedded in the tissue.

The mantle cavity opens anteriorly; a lower and an upper raphe are present adjacent to large secretory cells forming the hypobranchial gland (Fig. 1C). Mucus producing repugnatorial glands are present at the mantle edge (Fig. 1D). There are two groups of five and eight black stripes, respectively, present at the mantle rim. The anterior half of the large plicate gill hangs freely from the mantle roof; the posterior half is attached to the kidney on one side. The laminae are strongly convoluted (Fig. 1E), some secondary folding is visible. The gill is attached to a large vessel running around the kidney to join the pericardium. This vessel is composed of a single cell layer.

Digestive system: The mouth opens into an oral tube composed of ciliated columnar epithelial cells anteriorly and lined with a cuticle in its posterior region. It is succeeded by the buccal bulb, which is surrounded by strong muscle layers. The muscle fibres project in all directions forming a muscular network, whereas the epithelial cells are columnar and covered by a cuticular layer. The buccal bulb contains the jaws at its anterior end and the radula posteriorly. The jaws consist of tightly packed denticulate elements (of about 50 μm) that are composed of one large lateral cusp and three to five small denticles per element (Fig. 2A). The multiseriate radula lacks a central tooth. The radula formula of the *c.* 30 rows is: 5.0.5 (Fig. 2B). Each tooth consists of a basal plate and a triangular

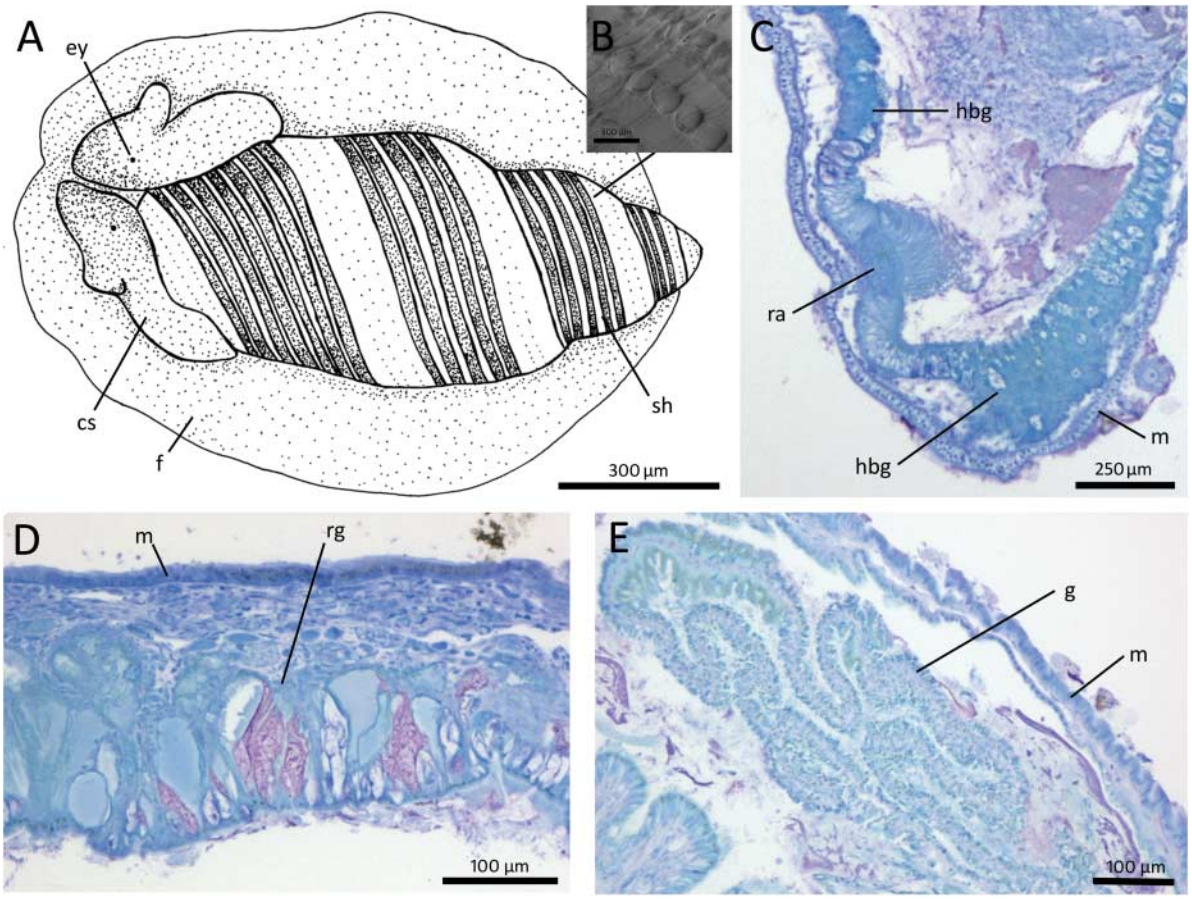

Figure 1. *Rictaxis punctocaelatus*. **A.** Living animal. **B.** Scanning electron microscopy (SEM) of sculpture of shell showing punctate grooves. **C.** Mantle cavity with raphe (ra) and hypobranchial gland (hbg). **D.** Repugnatorial glands (rg) in mantle edge. **E.** Gill (g). Other abbreviations: cs, cephalic shield; ey, eye; f, foot; m, mantle; sh, shell. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

to spine-like cusp (Fig. 2C). The innermost teeth are triangular, bearing approximately nine denticles. The teeth increase in length outwards (first and second *c*. 110 µm; third *c*. 135 µm; fourth *c*. 165 µm) and the denticle number is slightly increased towards the outer rows to about 12 in the fourth tooth. The outermost teeth are extremely long (*c*. 270 µm), forming a spine with numerous lateral denticles. The radula is supported on a large muscular odontophore, which is situated at the postero-ventral end of the buccal bulb below the oesophageal opening.

The oesophagus (Fig. 3A) initially forms a capacious crop surrounded by a greatly folded and granular epithelial wall that produces an extensive secretion (Fig. 3B). Following the crop the oesophagus narrows to form a duct, its epithelium still being highly convoluted. Oesophagus and intestine open into the large stomach in the same region and partly run parallel to each other (Fig. 3C). The stomach is embedded in the digestive gland and its epithelium consists of cuticularized cuboidal cells. The lining of the following intestine is thrown into longitudinal folds and consists of ciliated columnar cells. The large digestive gland occupies most of the upper whorls of the shell and is partly enveloped by the ovotestis (Fig. 5A).

A pair of large salivary glands (Fig. 3D) surrounds the digestive tract and open into the posterior part of the buccal bulb. Their proximal part is composed of cuboidal cells, and the distal part of ciliated columnar cells. All cells contain many diverse vesicles staining light blue to purple, possibly producing different kinds of secretion. An oral gland could not be detected.

Nervous system: The central nervous system (Fig. 4A) of *Rictaxis punctocaelatus* is streptoneurous. The cerebro-pedal nerve ring encircles the oral tube anteriorly to the jaws. The fused cerebro-pleural ganglia are connected by a short and thick commissure. Thick connectives lead to the pedal ganglia situated one below each cerebro-pleural ganglion. One elliptical statocyst with many spherical statoconia, each with a diameter of *c*. 10–15 µm (Fig. 4B) is attached to each pedal ganglion.

The large supraoesophageal ganglion is located above the muscular buccal bulb. It is situated close to the right cerebro-pleural ganglion separated by a short connective. Posteriorly the visceral loop widens to form a small accessory ganglion located above the buccal bulb.

The left cerebro-pleural ganglion is connected via a thin connective to a small accessory ganglion located below the buccal bulb. Posteriorly the large sub-oesophageal ganglion is found at a similar level beneath the muscular buccal bulb. The visceral ganglion is located below the genital gland mass deep in the pallial cavity. Sub- and supraoesophageal ganglia are connected to the visceral ganglion via thin connectives.

Each ganglion is surrounded by a thin layer of connective tissue and contains nerve cells of different sizes which are concentrated in the periphery of the ganglion (Fig. 4B).

The optic nerve projects from each cerebro-pleural ganglion to innervate the eye (Fig. 4C). Each eye is formed by a spherical lens (diameter: *c*. 100 µm) and a layer of black pigment (Fig. 4C). A Hancock's organ is present laterally on each side of the head shield in the groove between the head shield and

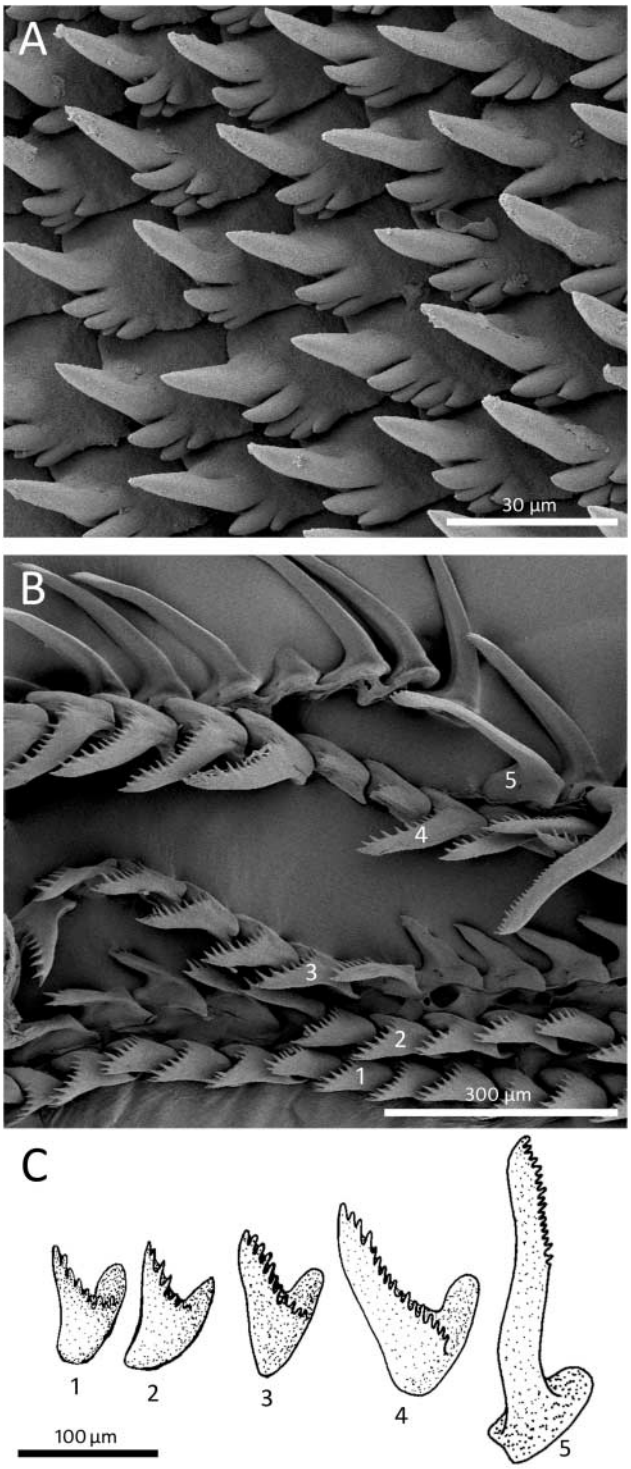

Figure 2. SEM of *Rictaxis punctocaelatus*. **A.** Jaw elements. **B.** One half of radula. **C.** Lateral radula teeth. Abbreviations: 1, first lateral tooth; 2, second lateral tooth; 3, third lateral tooth; 4, fourth lateral tooth; 5, fifth lateral tooth.

the foot. It is characterized by folded epithelia with dense nervous structures underneath.

Reproductive system: *Rictaxis punctocaelatus* has an androdiaulic reproductive system. The external penis is adjacent to the entrance to the mantle cavity. The large ovotestis sits over the digestive gland and is closely attached to it (Fig. 5A); it projects deeply into the shell, occupying the upper whorls. Inside the ovotestis spermatozoa are located in central acini, whereas ovules are located at the periphery (Fig. 5B).

The genital gland mass consists of an albumen gland, an elaborated mucous gland and a membrane gland (Fig. 5C). The mucous gland surrounds both albumen and membrane gland.

Only a single sperm-containing structure could be detected, and this possibly functions as a bursa copulatrix because its content is partly digested. A receptaculum seminis is absent.

The ampullar region of the spermoviduct is situated at the posterior end of the body, surrounded by a thin double-layered epidermis (Fig. 5D). The stored spermatozoa are not orientated.

Pericardium and circulatory system: The pericardium is situated deep in the mantle cavity adjacent to the kidney. The atrium consists of a thin muscle layer, whereas the ventricle is composed of thick muscular cells. A pericardial gland was not detected.

Excretory system: The large kidney is bordered by the gill on one side and the heart on the other. It is composed of delicate tissue consisting of highly vacuolized cells and uniformly blue-staining cells with a visible nucleus. The epithelium is strongly convoluted. It is surrounded by a dense cell layer consisting of nonstaining columnar cells with a central nucleus.

Phylogenetic analyses of molecular data

Statistical tests: The ILD test revealed that the combination of the four gene partitions improves the phylogenetic signal with a *P*-value of 0.01. Thus concatenation of the single genes is reasonable.

Substitution saturation analysis yielded little saturation in the 16S alignment and substantial saturation in the third codon positions of COI. The latter were therefore removed from further analyses.

The relative rate test showed that evolutionary rates differ among the investigated taxa and genetic markers. The highest *z*-scores (between 5.0 and 6.0) indicated that major differences were found between the 18S rDNA sequences of the nudipleuran taxa and *Micromelo undatus1* as well as between the 28S sequences of *Cornirostra pellucida* and *Pleurobranchaea meckeli*. 16S rDNA and COI sequences generally yielded lower *z*-scores with the highest being for *Orbitestella* sp. in the 16S rDNA sequences (about 3.0). In the concatenated alignment *z*-scores were maximal (between 5.0 and 7.5) for *Cornirostra pellucida*, *Rissoella elongatospira*, *Pleurobranchaea meckeli* and *Bathydoris clavigera*.

Phylogenetic analyses: Results of the different phylogenetic analyses were mostly congruent and significant statistical support values were obtained for most nodes. The resulting phylogram of the Bayesian analysis along with posterior probabilities as well as bootstrap support values from the maximum likelihood analysis is shown in Figure 6.

The monophyly of the Acteonoidea has maximal support. The Rissoelloidea are recovered as the sister group of the Acteonoidea and together these are sister to the opisthobranch Nudipleura (support from posterior probability only).

The traditional division of the Acteonoidea into three families was not supported. Instead we found a well-supported division into two clades. The traditional Acteonidae are paraphyletic due to the position of *Rictaxis punctocaelatus* which falls outside the well-supported clade of the other Acteonidae; instead *R. punctocaelatus* is found at the base of the second clade

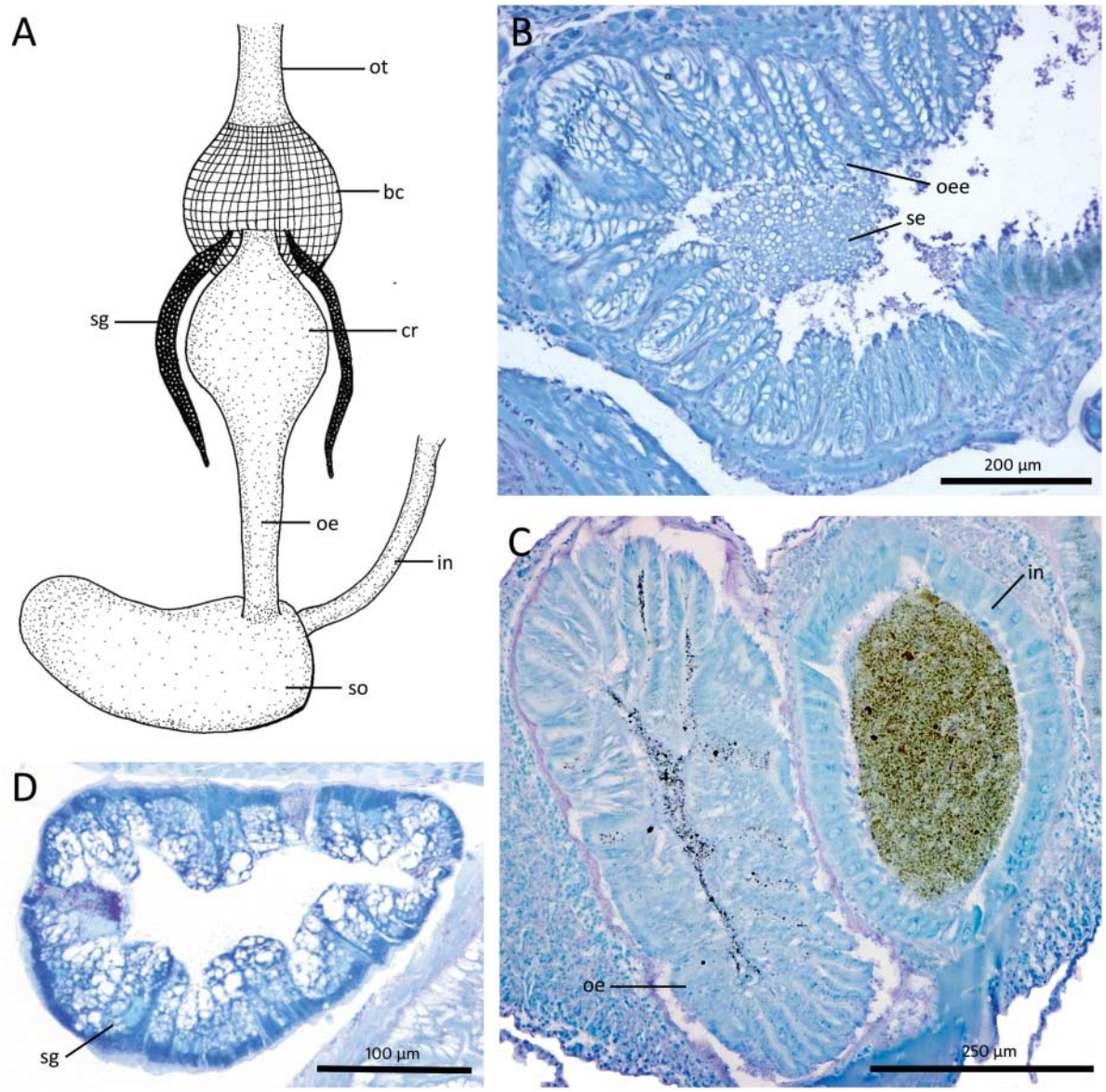

Figure 3. Digestive system of *Rictaxis punctocaelatus*. **A.** Diagram of digestive system. **B.** Transverse section of oesophagus in region of crop. **C.** Transverse section of distal oesophagus (oe) and proximal intestine (in). **D.** Transverse section of salivary gland (sg). Other abbreviations: bc, buccal bulb; cr, crop; oee, oesophagus epithelium; ot, oral tube; se, secretion; so, stomach. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

which also includes the Aplustridae and Bullinidae. The Aplustridae are paraphyletic due to the inclusion of *Bullina lineata* in this clade. According to our results the Bullinidae with its single genus *Bullina* cannot form a separate family of rank equivalent to the other two. In order to test these unexpected results we performed an AU test to reassess the monophyly of the Acteonidae and Aplustridae. The AU test yielded a *P*-value of 0.953 for the unconstrained topology, implying paraphyly of Acteonidae and Aplustridae. On the contrary, enforced monophyly of the Acteonidae and Aplustridae yielded *P*-values of 0.0001 and 0.050, respectively. Both values are within or below the significance value of 0.050, so that monophyly of these families is rejected based on our data set.

The split network analysis (Fig. 7) confirms excellent split support for the monophyly of the Acteonoidea. However, there was no support for a sister-group relationship with Rissoelloidea or for a closer relationship with Nudipleura. In fact, no possible sister group receives any support; the Acteonoidea are clearly separated from all other clades in the network analysis. The division of Acteonoidea into two main clades (Acteonidae without *Rictaxis*; and Aplustridae plus *Bullina* and *Rictaxis*) is confirmed and receives split support.

Character tracing

Character-tracing analyses of family-level characters revealed that the last common ancestor of the Acteonoidea probably had an operculum, a small foot, an anterior mantle cavity opening and a short oral tube, whereas an oesophageal crop and an oral gland were missing (for detailed results of character tracing see Supplementary Data 4). Regarding structure of the shell, presence or absence of a central radular tooth and the position of the supraoesophageal ganglion in relation to the right cerebro-pleural ganglion the plesiomorphic conditions remain unclear. *Rictaxis punctocaelatus* shares apomorphies with the Aplustridae [absence of operculum (2), presence of

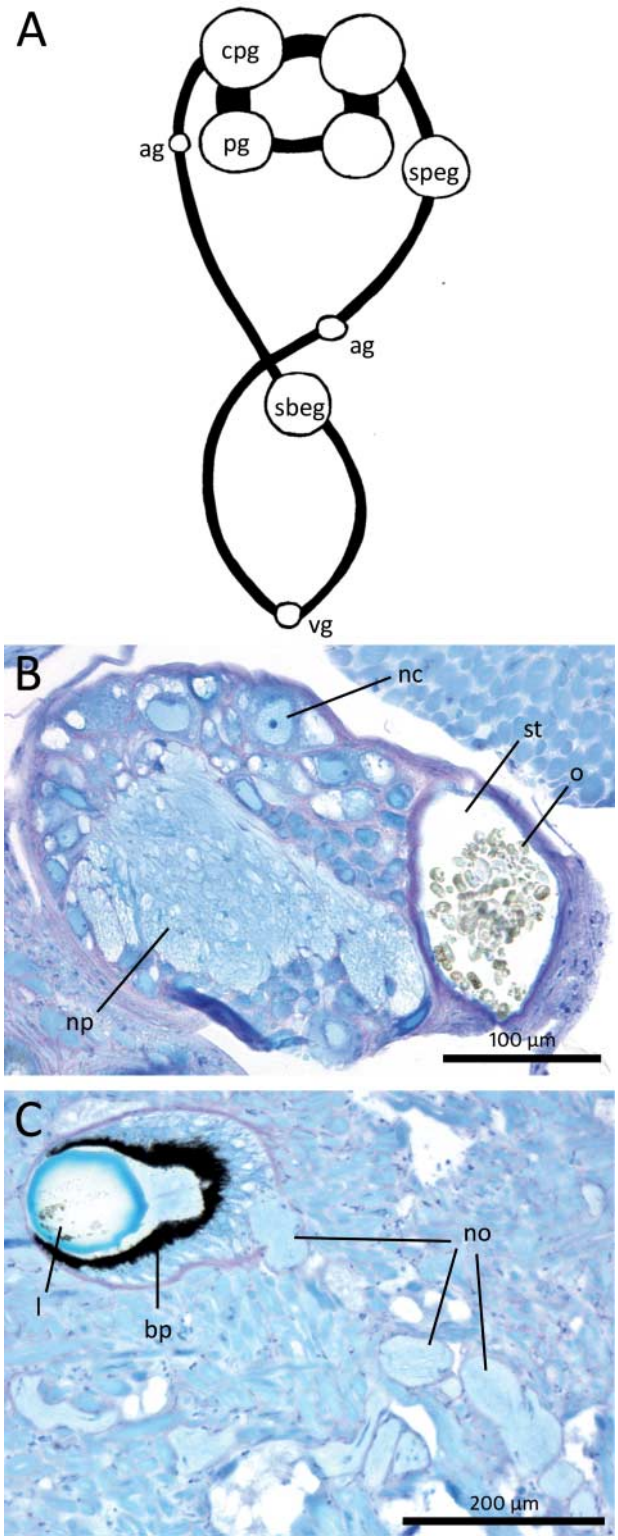

Figure 4. Nervous system of *Rictaxis punctocaelatus*. **A.** Diagram of central nervous system reconstructed from serial sections. **B.** Pedal ganglion with statocyst (st). **C.** Eye and optic nerve (no). Other abbreviations: ag, accessory ganglion; bp, black pigment; cpg, cerebro-pleural ganglion; l, lens; nc, nerve cell; np, neuropil; o, statoconia; pg, pedal ganglion; sbeg, sub-oesophageal ganglion; speg, supraoesophageal ganglion; vg, visceral ganglion. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

oesophageal crop (7) and position of supraoesophageal ganglion (9)], plesiomorphic conditions with the Acteonidae [anterior mantle cavity opening (4) and absence of oral gland (8)], and reveals other character states that are shared with Acteonidae but differ in the outgroup taxa [shell elongate (1) and central radula tooth absent (6)] or exhibits an intermediate state [large foot (3) and distinct oral tube (5)] (Table 2, Fig. 6).

DISCUSSION

Morphology and anatomy of Rictaxis punctocaelatus

The external morphology of *R. punctocaelatus* exhibiting a strongly calcified shell with an elevated spire, a translucent body with a moderately large foot and an anterior mantle cavity opening, resembles those of other genera of the Acteonidae (Rudman, 1971a, 1972c; Marcus, 1972; Gosliner, 1981). However, *R. punctocaelatus* is the only species without an operculum, which is otherwise only missing in the Aplustridae and one as yet undescribed species of *Bullina* (Rudman, 1972b; Gosliner, 1981; Burn & Thompson, 1998; Rudman, 2000). The presence and structure of the raphe and hypobranchial gland in the mantle cavity, as well as of repugnatorial glands at the mantle edge, correspond to these structures in other Acteonoidea (Rudman, 1972a, b, c; Wägele & Klussmann-Kolb, 2005). The black stripes at the mantle edge correspond to those in the pattern of the shell (Marcus, 1972). The relative locations of gill, kidney and pericardium are as in other Acteonoidea, whereas the rather complex structure of the gill is more similar to the Aplustridae than to the plesiomorphic features of Acteonidae (Rudman, 1972a, b, c). A pericardial gland (present in *Pupa*, *Maxacteon*, *Bullina*, *Micromelo* and *Hydatina*; Rudman, 1972b) could not be detected.

Regarding the digestive system, *R. punctocaelatus* exhibits features corresponding to the Acteonidae, whereas some structures resemble those in the Aplustridae and still others show intermediate states. The latter is true for the oral tube, which is reported to be absent or very short in the Acteonidae (Rudman, 1972a), whereas it is long in the Aplustridae (Rudman, 1972b). The Bullinidae exhibit varying configurations; *Bullina lineata* has a long oral tube whereas *B. roseana* has a relatively short one (Rudman, 1972a). *Rictaxis* possesses a distinct oral tube; however, it is not as long and prominent as in the Aplustridae.

The radula of *R. punctocaelatus* has been described previously by Habe (1956) and Marcus (1972), and our study confirms their reports. Radula formula and tooth structure of *Rictaxis* agree with other Acteonidae (Rudman, 1971a) besides the anomalous *Acteon* (Marcus, 1972; Gosliner, 1981; Yonow, 1992) and differ from those of Aplustridae (Rudman, 1972a, b) and Bullinidae (Rudman, 1971b). However, the radula structure is variable in species of *Hydatina* (Marcus & Marcus, 1967) and a central tooth is sometimes lacking (Rudman, 1972b), suggesting that loss may have occurred more than once.

The composition of the buccal bulb is conserved among Acteonoidea (Rudman, 1972a, b, d) and *Rictaxis* shows the usual structure. However, the extensive musculature of its buccal bulb is remarkable and indicates a possible function as a simple suction pump (Rudman, 1972d) for these vermivorous opisthobranchs (Marcus, 1972).

The oesophagus of *Rictaxis* enlarges to form a capacious crop, which has also been described in the same position for the Aplustridae; in contrast the crop is found more distally in *Bullina* and is absent in the Acteonidae. However, the strongly convoluted glandular epithelium in the crop of *Rictaxis* does

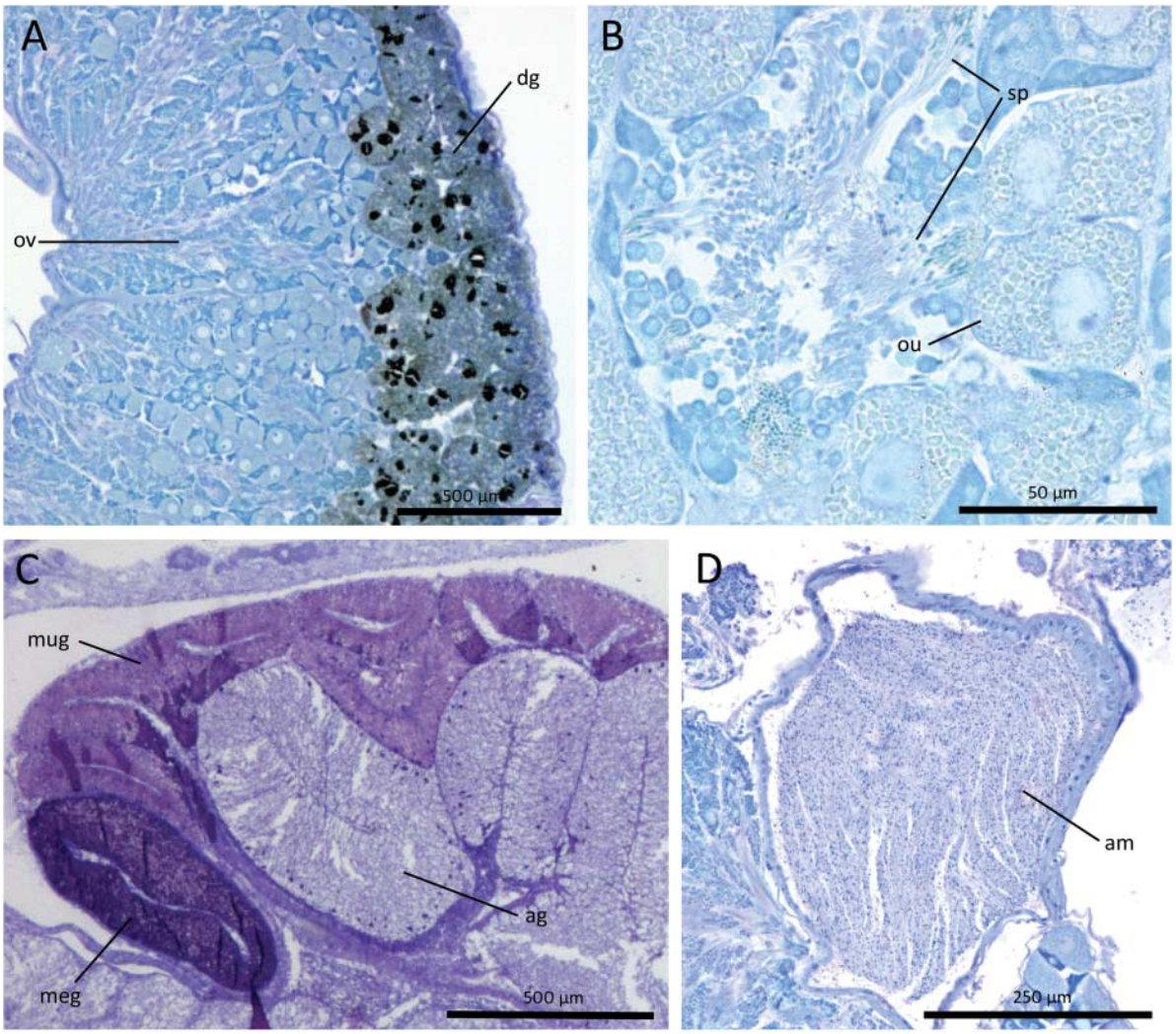

Figure 5. Reproductive system of *Rictaxis punctocaelatus*. **A.** Ovotestis (ov) and digestive gland (dg). **B.** Detail of ovotestis in detail showing position of ovules (ou) and spermatozoa (sp). **C.** Genital glands – albumen gland (ag), mucus gland (mug) and membrane gland (meg). **D.** Ampulla (am). This figure appears in colour in the online version of *Journal of Molluscan Studies*.

not resemble the thin, cuticular, nonciliated lining of Aplustridae (Rudman, 1972b).

A further diagnostic feature of Aplustridae and Bullinidae is the presence of a special unpaired gland in the digestive system, the so-called oral gland which is absent in all Acteonidae and could not be detected in *Rictaxis* in the present study.

The presence, structure and location of paired salivary glands, stomach and intestine is likewise conserved among Acteonoidea (Rudman, 1972a, b, d) and *Rictaxis* exhibits the common pattern.

The general composition of the streptoneurous central nervous system of *R. punctocaelatus*, comprising a pair of fused cerebro-pleural ganglia that are closely associated with pedal ganglia, a supra- and sub-oesophageal ganglion as well as a visceral ganglion, is typical for the Acteonoidea (Burn & Thompson, 1998). Two additional accessory ganglia were detected which corresponds to descriptions of *Pupa* and *Maxacteon* (Rudman, 1972c), whereas only one accessory ganglion has been reported for the Aplustridae and *Bullina* (Rudman, 1972a, b). The main difference between the central nervous system of Acteonidae and Aplustridae is the position of the supraoesophageal ganglion, which is found very close or even fused to the right cerebro-pleural ganglion in Aplustridae (Rudman, 1972a, b), whereas these ganglia are separated by a long connective in Acteonidae (Rudman, 1972c) and *Bullina* (Rudman, 1972a). The supraoesophageal ganglion of *R. punctocaelatus* is found close to the cerebro-pleural ganglion, although they are not fused, resembling the pattern in *Micromelo*.

The configuration of the reproductive system is conserved among Acteonoidea, without distinct features separating the families. *Rictaxis* shows the typical features of this androdiaulic reproductive system; its one peculiarity is the possession of a single auxiliary reproductive storage organ (not receptaculum seminis and bursa copulatrix as in other Acteonoidea), which probably functions as a bursa. This feature has been previously described by Gosliner (1981) and is shared by *Rictaxis* and *Acteon* (Fretter & Graham, 1954; Johansson, 1954; Gosliner 1981). Mikkelsen (1996) also noted that in *Acteon* this single allosperm storage sac includes separated areas for orientated sperm storage (the expanded duct functioning as a receptaculum) and for sperm disposal (the pouch itself functioning as a bursa). However, we were not able to identify a region resembling the expanded duct with oriented sperm storage for *R. punctocaelatus*.

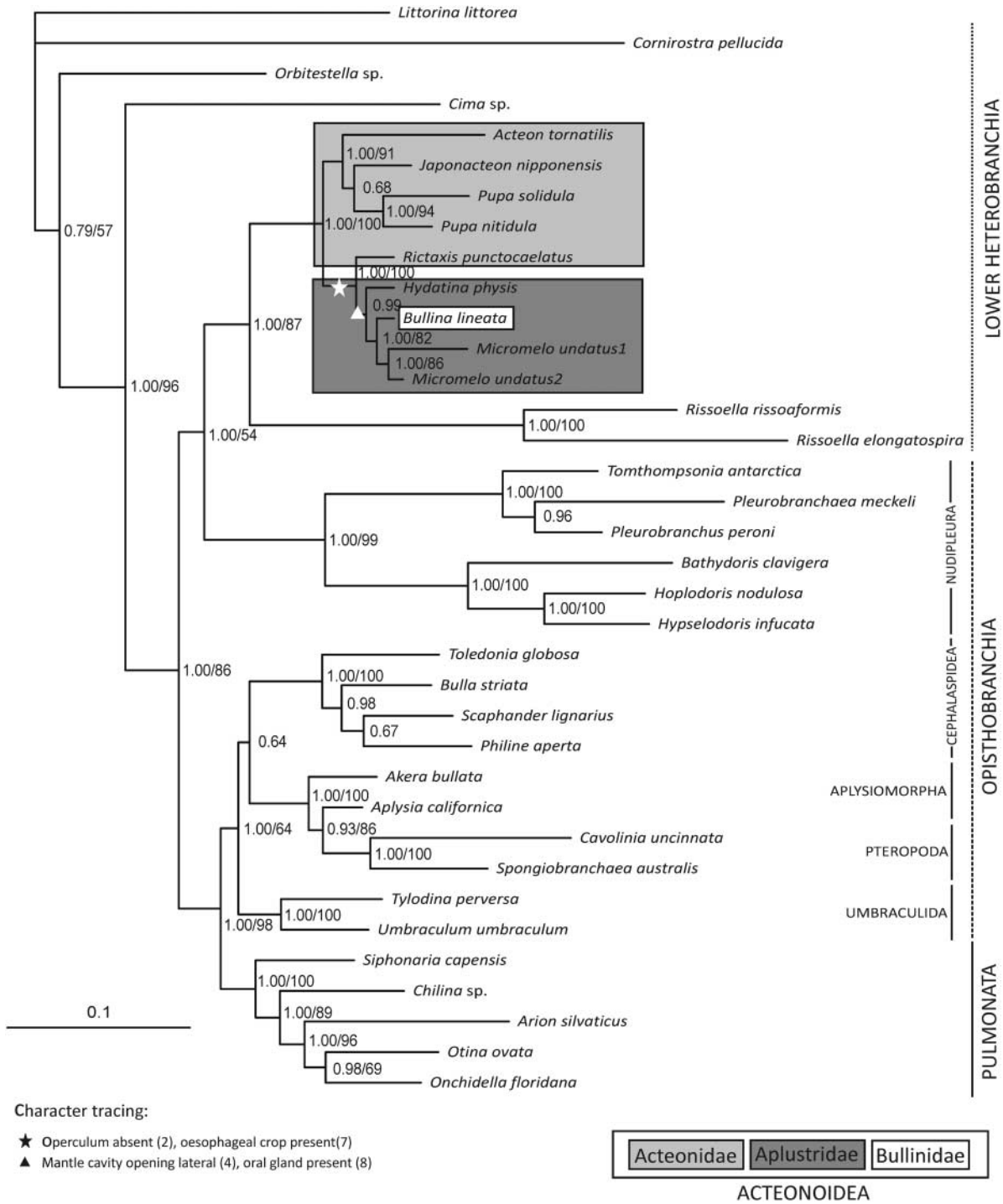

Figure 6. Bayesian inference phylogram based on a concatenated alignment of 18S rDNA, 28S rDNA, 16S rDNA and the first two codon positions of COI (50% majority rule consensus tree). Support values are posterior probabilities (Bayesian analysis) and bootstrap values (Maximum Likelihood analysis, as percentage). Only support values above 0.5 and 50, respectively, are given. Current systematic placements are indicated on the right side and the two clades of the Acteonoidea are marked by shaded boxes.

PHYLOGENY OF THE ACTEONOIDEA

Although the monophyly of the Acteonoidea has been doubted (Mikkelsen, 1996, 2002), our analyses strongly support this, in agreement with other molecular (Klussmann-Kolb *et al.*, 2008; Malaquias *et al.*, 2009) and morphological (Dayrat & Tillier, 2002; Wägele & Klussmann-Kolb, 2005) studies.

The systematic position of this group has also been debated, but unfortunately our results do not conclusively resolve this question. Our study suggests a position outside and basal to the Opisthobranchia (here polyphyletic), because we recovered a sister-group relationship with the 'lower heterobranch' clade Rissoelloidea. This sister-group relationship has also been found in a study of heterobranch phylogeny using the same markers but a different sampling of taxa (Dinapoli & Klussmann-Kolb, 2010). However, we found no split support for the sister-group relationships in our network analyses and

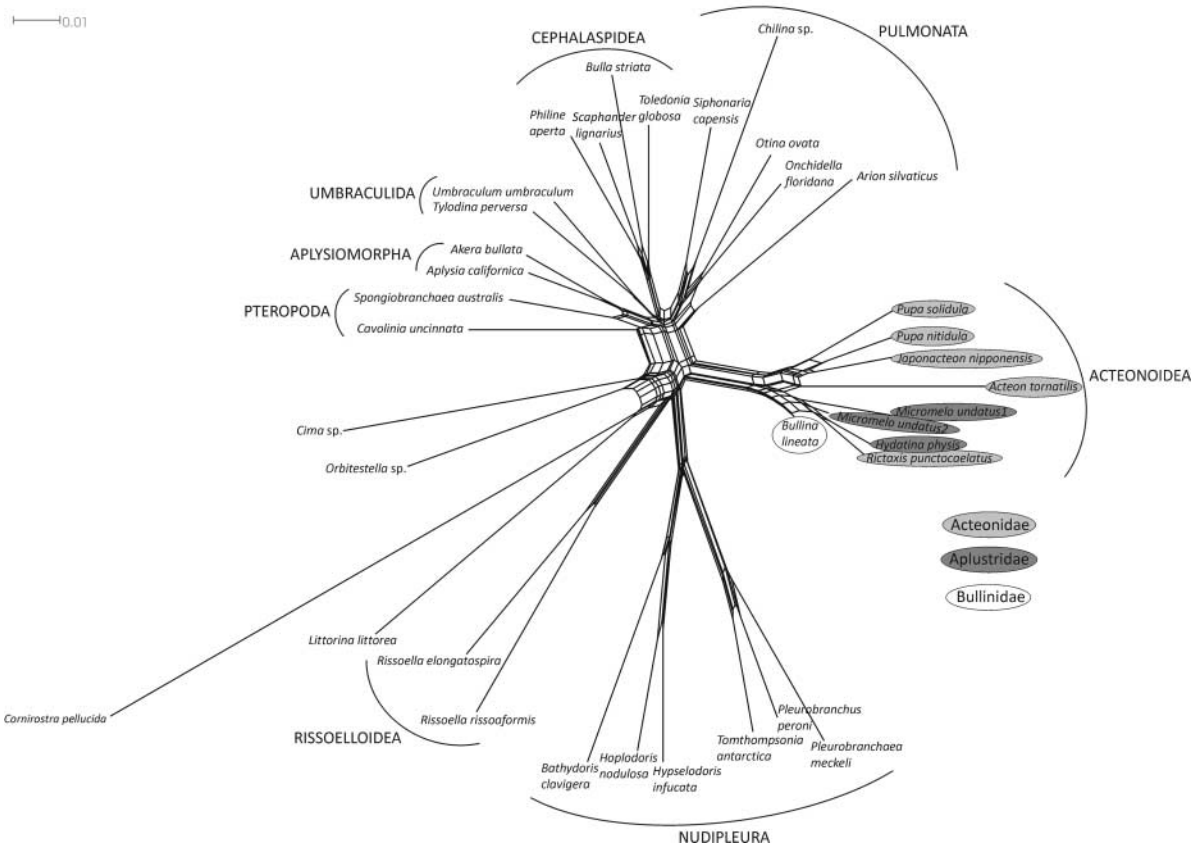

Figure 7. Neighbour-net graph of the split decomposition analysis. Systematic placement of taxa indicated at the periphery.

we found high evolutionary rates for *Rissoella elongatospira*, resulting in long branches for the Rissoelloidea in the phylogram. In the only morphology-based cladistic analysis including species of Rissoelloidea and Acteonoidea, no close relationship of these clades could be detected (Dayrat & Tillier, 2002). Additional analyses employing different markers or ESTs are required, as well as studies on the morphology of the tiny Rissoelloidea to search for possible synapomorphies are necessary to test this sister-group relationship.

According to our results the Acteonoidea/Rissoelloidea clade is sister to the Nudipleura, confirming earlier molecular analyses (Grande *et al.*, 2004a, b; Vonnemann *et al.*, 2005; Klussmann-Kolb *et al.*, 2008). However, this relationship is only supported in the Bayesian analysis (with maximum support). It is also recovered in the best likelihood tree, but receives only weak bootstrap support (54%). Furthermore there was no split support in the network analysis. Therefore, although there is some support for a sister-group relationship with Nudipleura, consistent with similarities in their reproductive system (Ghiselin, 1965), this possible relationship has to be regarded with caution and needs further investigation.

Our study is the first to focus on the phylogenetic relationships among the acteonoidean families. Our results do not support the morphology-based division into three families (Rudman, 1972a; Burn & Thompson, 1998; Bouchet & Rocroi, 2005). Instead, we have found a division into two clades, which do not exactly match any of the described families. This is because *Bullina lineata* (Bullinidae) clusters within the Aplustridae, rendering the latter paraphyletic. However, before establishment of the family Bullinidae (Rudman, 1972a) the genus *Bullina* was placed in the Aplustridae (e.g. Iredale & McMichael, 1962). Rudman (1972a) noted that together the Bullinidae and Aplustridae can be separated from the Acteonidae by the presence of a central radular tooth, an oesophageal crop, an unpaired oral gland and an oral tube. However, the Bullinidae differ from the Aplustridae in the possession of an operculum and a relatively open nervous system. Therefore, the Aplustridae might have arisen from bullinid-like ancestors (Rudman, 1972a), as shown by a morphology-based cladistic analysis (Wägele & Klussmann-Kolb, 2005). Nevertheless, our results do not recover *Bullina* in a basal position in the Aplustridae and suggest that the proposal of a new family for the single genus *Bullina* (mainly based on the aforementioned two morphological characters) was perhaps premature.

On the other hand *Rictaxis punctocaelatus* clusters at the base of the Aplustridae/Bullinidae clade and not within the other Acteonidae. This systematic position was recovered in all of our analyses with maximum statistical support values, as well as considerable split support. Furthermore, the only other study including *Rictaxis* and other Acteonoidea also reveals a sister-group relationship with an aplustrid taxon and not as expected with the acteonid taxon included (Klussmann-Kolb *et al.*, 2008). Thus, as currently defined the Acteonidae are paraphyletic and the placement of *Rictaxis* in the family has to be doubted. Up to now, *R. punctocaelatus* has always been assigned to the Acteonidae (Habe, 1956; Gosliner, 1981; Klussmann-Kolb *et al.*, 2008) although detailed analyses on its morphology have been lacking. The shape of its solid shell and unlobed foot which resemble other Acteonidae rather than Aplustridae seemed to provide enough justification. According to Rudman (1972a), the main differences between the Acteonidae and Aplustridae are found in external features (shape and structure of the shell; presence of operculum; shape and colour of foot; position of mantle cavity opening), the digestive system (presence of oral tube, oral glands and central

Table 2. Distribution of selected (family-specific) morphological characters among acteonoidean families and *Rictaxis punctocaelatus*.

Taxon/character		Acteonidae	Aplustridae	Bullinidae	*Rictaxis punctocaelatus*	References
(1) Shell	Elongate and strongly calcified	×			×	Burn & Thompson (1998)
	Globose and thin		×	×		Burn & Thompson (1998)
(2) Operculum	Present	×		×		Burn & Thompson (1998)
	Absent		×		×	Burn & Thompson (1998)
(3) Foot	Small without lobes	×				Rudman (1972a)
	Large without lobes			×	×	Rudman (1971b)
	Large and lobed		×			Burn & Thompson (1998)
(4) Mantle cavity opening	Anterior	×			×	Gosliner (1981)
	Lateral		×	×		Rudman (1972a)
(5) Oral tube	Short or absent	×				Rudman (1972a)
	Distinct			×	×	Rudman (1972a)
	Extensively long		×			Rudman (1972a)
(6) Radula - central tooth	Present		×	×		Rudman (1971b, 1972a, b)
	Absent	×	×		×	Habe (1956), Marcus & Marcus (1967), Rudman (1972b, d), Yonow (1992)
(7) Oesophageal crop	Present		×	×	×	Rudman (1972a, b)
	Absent	×				Rudman (1972d)
(8) Oral gland	Present		×	×		Rudman (1972a)
	Absent	×			×	Rudman (1972d)
(9) Supraoesophageal ganglion and right cerebro-pleural ganglion	Fused		×			Rudman (1972b)
	Short connective		×		×	Rudman (1972a)
	Long connective	×		×		Rudman (1972a, c)

radular tooth) and the central nervous system (position of supraoesophageal ganglion).

Our morphological investigations of *R. punctocaelatus* revealed that although the external congruencies with the Acteonidae are striking there is some evidence for a closer relationship with the Aplustridae (Table 2, Fig. 6). Four out of nine family-specific characters are shared by Acteonidae and *R. punctocaelatus*, whereas three are shared between *R. punctocaelatus* and the Aplustridae and Bullinidae. The external similarities in shell, foot and mantle structure might potentially be related to the habitat of these species. The Acteonidae and *Rictaxis* live infaunally, whereas the Aplustridae and *Bullina* live epifaunally (Burn & Thompson, 1998). A solid shell and a rather small foot as found in *Rictaxis* are more suitable for this mode of living than a large, globose shell and an elaborately lobed foot. These shared characters might be an adaption to infaunal burrowing, which is a possible plesiomorphic feature of Acteonoidea. The last common ancestor of the Acteonoidea arguably had a solid shell, a small foot and lived infaunally, while the epifaunal habit was established later, and present in the last common ancestor of the Aplustridae/Bullinidae.

Regarding the digestive system, *Rictaxis* lacks one crucial feature of the Aplustridae/Bullinidae, the unpaired oral gland. The exact function of this gland is not known, but because it is located at the functional mouth when the animal everts the oral tube to feed, Rudman (1972b) speculated that the secretion immobilizes the prey. All Acteonidae as well as *Rissoella* and *Orbitestella* lack this gland, implying that its absence is the plesiomorphic condition. Furthermore, the radula of *Rictaxis* resembles that of the Acteonidae (except for *Acteon*) and differs from the Aplustridae in the absence of a central tooth and the structure of the outer teeth. In contrast, the presence of an oral tube and an oesophageal crop in *Rictaxis* resemble the Aplustridae, although the oral tube is not as long and the epithelium of the crop is different in *Rictaxis*. Thus, these features are somewhat intermediate between those of Acteonidae and Aplustridae.

Finally, the central nervous system with the supraoesophageal ganglion closely associated with the cerebro-pleural ganglion in *Rictaxis* is more similar to the configuration in Aplustridae (Rudman, 1972a, b) and does not resemble that of Acteonidae (Rudman, 1972c).

Rictaxis punctocaelatus is the type species of *Rictaxis*, but this genus also includes at least three other described species (*R. punctostriatus* (Adams, 1840), *R. albus* (Sowerby, 1873), *R. painei* (Dall, 1903)). These additional species have so far been described based only on shell morphology and radular structure. In fact the radulae of *R. punctocaelatus* and *R. punctostriatus* differ regarding the longest tooth (fifth in *R. punctocaelatus* and fourth in *R. punctostriatus*; Marcus, 1972). The monophyly of the genus *Rictaxis* remains to be tested by additional anatomical and molecular data.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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