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MOLECULAR PHYLOGENY, SYSTEMATICS AND MORPHOLOGICAL CHARACTER EVOLUTION IN THE BALKAN RISSOOIDEA (CAENOGASTROPODA)

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ABSTRACT: Morphological characters in 33 Balkan rissooid genera (*Adriohydrobia*, *Adrioinsulana*, *Alzoniella*, *Anagastina*, *Belgrandiella*, *Bithynia*, *Boleana*, *Bythinella*, *Bythiospeum*, *Daphniola*, *Dianella*, *Emmericia*, *Graecorientalia*, *Graziana*, *Grossuana*, *Hauffenia*, *Heleobia*, *Horatia*, *Hydrobia*, *Islamia*, *Lithoglyphus*, *Litthabitella*, *Marstoniopsis*, *Orientalina*, *Paladilhiopsis*, *Parabythinella*, *Pontobelgrandiella*, *Pseudamnicola*, *Pseudobithynia*, *Pyrgula*, *Sadleriana*, *Trichonia*, *Ventrosia*) are discussed and illustrated based on the literature and, where necessary, on the presented additional data. These include shell macrocharacters, protoconch sculpture, soft part morphology and pigmentation, radulae, stomach, female reproductive organs, male reproductive organs. Based on partial sequences of the ribosomal 18S RNA gene, a molecular phylogeny is presented for all the genera, and based on fragments of CO1 gene in mitochondrial DNA, for all except six genera. Based on the Adams consensus tree the two gene phylogenies are summarised and systematics of the group is proposed. *Adrioinsulana* is considered a junior synonym of *Pseudamnicola*; *Parabythinella* a junior synonym of *Marstoniopsis*; a new name: *Radomaniola* n. gen. is proposed as a replacement name for the preoccupied *Orientalina*. *Litthabitella,* morphologically and molecularly distinct from the hydrobioids, probably belongs to the Assimineidae. *Marstoniopsis* belongs to the Amnicolidae, *Bythinella* to Bythinellidae, *Lithoglyphus* to Lithoglyphidae, *Heleobia* to Cochliopidae, *Bithynia* and *Parabithynia* to Bithyniidae, *Emmericia* to Emmericiidae. *Paladilhiopsis* and *Bythiospeum* belong to the Moitessieriidae, there being no reason for homologising the two genera. All the other genera belong to the monophyletic family Hydrobiidae, within which two subfamilies can be distinguished: Hydrobiinae and Sadlerianinae. The latter includes mostly very closely related genera, which makes splitting of this subfamily into more groups of this rank unjustified. The phylogeny of the molecular characters is mapped on two molecular trees. The caecal appendix on the stomach, reduction of the basal cusps on the rhachis and the so called "spermathecal duct" evolved parallelly, and are thus homoplastic. The network of pores on the protoconch and the flagellum seem to be uniquely derived. The seminal receptacles and lobes on the penis seem to be phylogenetically old, not prone to changes and rather useful in phylogeny reconstruction. The morphologically inferred relationships of Emmericiidae and the systematic position of the two species of *Parabythinella* are discussed in Appendix 2 and Appendix 3, respectively. Destroyed type localities of Balkan rissooids are listed in Appendix 4.

KEY WORDS: Rissoidea, Hydrobiidae, evolution, phylogeny, DNA, CO1, 18S RNA, morphology, Balkans

CONTENTS

INTRODUCTION

The family Hydrobiidae Troschel, 1857, one of the largest families of the superfamily Rissooidea, is a group of small or minute snails that occur in permanent freshwater or (a few taxa) brackish habitats all over the world. Most hydrobiids have smooth shells which lack distinctive characters like denticulations of the aperture, etc. Owing to the miniaturisation of these snails (many hydrobiids are < 1 mm high), their anatomy is also difficult to study, being so simplified that it is impossible to obtain all necessary data by simple dissection. This may explain the confusing state of the higher systematics of the Hydrobiidae and the slow progress in resolving the phylogenetic relationships amongst the higher taxa. From half of the

19th till the end of the 20th c. as many as 16 classifications were proposed for hydrobiid snails. KABAT & HERSHLER (1993) reviewed the proposed classifications and defined the family (in the broader sense), including 725 genera in it, based on anatomical characters. HERSHLER & PONDER (1998) illustrated all the characters and their states that provided the basis to distinguish taxa.

The main problem that arises when one tries to resolve the hydrobiid phylogeny is in evolutionary convergence commonly found in their shell form and anatomy. PONDER (1988), in his first comprehensive cladistic analysis of truncatelloidean (rissooidean) families, defined the hydrobiid clade based on two characters, one of which is a reversal and the other a parallelism (KABAT & HERSHLER 1993, WILKE et al. 2001). FALNIOWSKI & SZAROWSKA (1995a) have shown that different sets of anatomical characters can produce different phylogenies. Neither this nor other cladistic approaches to the morphological data resulted in a well resolved phylogeny (FALNIOWSKI & SZAROWSKA 1995a, BODON et al. 2001). To be fulfilled, this task needs molecular characters. In the last decade the rapid development of molecular techniques yielded increasingly more data (BAKER et al. 1998, HILLIS & WIENS 2000, GIRIBET & DISTEL 2003, MCARTHUR & HARASEWYCH 2003, MEDINA & COLLINS 2003). First attempts to infer higher level relationships involving Hydrobiidae were done by ROSENBERG et al. 1997 (Hydrobiidae vs. Pomatiopsidae and Truncatellidae) using 28S rRNA. Our study of COI and 18S gene fragments from representatives of 40 genera (WILKE et al. 2001) has demonstrated that the family Hydrobiidae (as defined by KABAT & HERSHLER 1993) is polyphyletic and the Cochliopidae are a distinct family. We tentatively assigned the following subfamilies to the Hydrobiidae: Hydrobiinae, Pseudamnicolinae, Nymphophilinae, Islamiinae, and Horatiinae.

For obvious reasons, of all continents, Europe is the one where hydrobiid fauna was studied first. Paradoxically, so many species and genera having been described, where phylogenetic relationships or phylogeography are concerned, the European Hydrobiidae are still far from being satisfactorily studied. This is unlike the case, for instance, of the hydrobiid fauna of Australia, where extensive studies carried out for several decades by PONDER and his co-workers, having yielded numerous excellent papers (e.g. PONDER 1967, 1982, 1983, 1984, 1985, 1992, PONDER & CLARK 1990, PONDER et al. 1989, 1991), improved the current state of knowledge far beyond that of the European fauna.

The centre of distribution of the European Hydrobiidae is the Balkan Peninsula. One of the first monographic papers dealing with the molluscan fauna of the region is that by WAGNER (1927). From among numerous studies covering this region the most worth of mentioning are those of RADOMAN. More than 25 years of his research resulted in more than 30 papers (e.g. RADOMAN 1955, 1965, 1966, 1967a, b, 1973a, b, c, d, 1975, 1976, 1977, 1978) summarized in a monograph (RADOMAN 1983), which, despite all its shortcomings, is still the most exhaustive source of knowledge of the anatomy and distribution of Balkan hydrobiids.

My previous molecular studies on hydrobiids of that region are either limited to the species or genus level (SZAROWSKA & WILKE 2004, SZAROWSKA et al. 2005, 2006, SZAROWSKA et al. in press) or cover only a part of the Balkan hydrobiid genera (WILKE et al. 2001). The aim of this study is to sum up the earlier results and fill up as many of the numerous gaps in the knowledge of phylogenetic relationships among hydrobiid snails and evolution of their characters as possible.

MATERIAL AND METHODS

SPECIMENS AND TAXA

Material for this study was collected in 1999, 2001 (Slovenia, Croatia) and 2003–2005 (Greece, Macedonia, Montenegro, Romania, Bulgaria). For morphological study some material collected in 1985 and 1992 was used (see Table 1). Snails were collected by hand (springs or brooks) or using a light dredge (lakes and rivers). Specimens for the morphological study were initially fixed with either 4% formalin, or Bouin's fluid, and later transferred into 80% ethanol. Specimens for the molecular study were fixed directly with 80% and stored in 96% ethanol.

The rissooidean taxa included in this study are listed in Appendix 1. They comprise nine representatives of the family Hydrobiidae, four of which represent the subfamily Hydrobiinae (*Hydrobia*, *Ventrosia*, *Adriohydrobia*), three Pyrgulinae (*Pyrgula, Dianella, Trachyohridia*), and two Pseudamnicolinae (*Pseudamnicola, Adrioinsulana*). Among the others, 18 genera have, at one point or another, been considered to belong to the Hydrobiidae (see WILKE et al. 2001). These are: *Orientalina, Sadleriana, Anagastina, Grossuana, Graecorientalia, Trichonia, Daphniola, Horatia, Hauffenia, Islamia, Belgrandiella, Graziana, Alzoniella, Boleana, Pontobelgrandiella, Litthabitella, Paladilhiopsis, Bythiospeum.* The other rissooidean taxa included are commonly referred to as "hydrobioids". They comprise representatives of the families Cochliopidae (*Heleobia*), Emmericiidae (*Emmericia*), Bithyniidae (*Bithynia, Pseudobithynia*), Lithoglyphidae (*Lithoglyphus*), "Bythinellidae" (*Bythinella*), Amnicolidae (*Marstoniopsis, Parabythinella*). As outgroup, I used a representative of the family Rissoidae (*Rissoa*). Most

of those taxa were previously studied genetically (e.g. WILKE et al. 2000, 2001, SZAROWSKA & WILKE 2004, SZAROWSKA et al. 2005) (see Table 1, page 128).

MORPHOLOGICAL AND ANATOMICAL WORK

Dissections were done using a NIKON SMZ-U stereomicroscope with a NIKON drawing apparatus, and a NIKON COOLPIX 4500 digital camera. The protoconchs and radulae were examined using a JEOL JSM–5410 scanning electron microscope (SEM), applying the techniques described by FALNIOWSKI (1990a). For histology, Bouin-fixed and/or formalin-fixed specimens were embedded in paraplast (Sigma, Saint Louis), serially sectioned (10 µm), and stained with haematoxylin and eosin. Every third section was photographed with a COHU 3715 camera, coupled with a frame-grabber and a PC equipped with the MultiScanBase v. 11.06 software. The files were then used for three-dimensional reconstructions performed with SURFDRIVER (MOODY & LOZANOFF 1999).

All the materials, as well as histological sections, are deposited at the Zoological Museum of Jagiellonian University in Kraków.

MOLECULAR WORK

DNA sequencing and alignment

Ethanol-fixed snails were washed twice with ice-cold water, than DNA was isolated according to the methods of SPOLSKY et al. (1996) and DAVIS et al. (1998), with modifications. Isolated DNA was used as a template in the PCR reaction with the following primers: LCO1490 (5'-GGTCAACAAATCAT AAAGATATTGG-3') COR722b (5'-TAAACTTCA GGGTGACCAAAAAATYA-3') to amplify the gene of mitochondrial cytochrome oxidase subunit 1 (CO1; FOLMER et al. 1994, DAVIS et al. 1998) and SWAM18SF1 (5'-GAATGGCTCATTAAATCAGTCGA GGTTCCTTAGATGATCCAAATC-3'), SWAM18SR1 (5'-ATCCTCGTTAAAGGGTTTAAAGTGTACTCATT CCAATTACGGAGC-3') to amplify the nuclear gene of small ribosomal subunit RNA (18S; PALUMBI, 1996). The PCR conditions were as follows: 1. For CO1: 4 min. at 94°C followed by 35 cycles of 1 min. at 94°C, 1 min. at 55°C 2 min. at 72°C, after all cycles an additional elongation step of 4 min. at 72°C was performed; 2. For 18S: 4 min. at 94°C followed by 40 cycles of 45 sec. at 94°C, 45 sec. at 51°C, 2 min. at 72°C, after all cycles an additional elongation step of 4 min. at 72°C was performed. The total volume of each PCR reaction mixture was 50 µl, out of which 10 µl was analysed to determine the quality of PCR products by electrophoresis in a 1% agarose gel. After amplification the PCR product was purified using the Clean-Up columns (A&A Biotechnology) according to the manuals. The purified PCR product was sequenced (HILLIS et al. 1996) using BigDye Terminator v3.1 (Applied Biosystems) according to the manuals and with the primers described above. The sequencing reaction products were purified using ExTerminator Columns (A&A Biotechnology) according to the manuals, and the sequences were read using the ABI Prim sequencer. For each taxon not less than three specimens for 18S, and four for CO1 were sequenced.

Unfortunately, the materials of some taxa were either old or not fixed well enough, or the CO1 primers were not appropriate (*Emmericia*), thus it was impossible to sequence CO1 for seven of the genera considered in the paper. The new sequences I obtained for 13 species were combined with sequences from earlier papers of mine and/or my co-workers (WILKE & DAVIS 2000, WILKE et al. 2000, 2001, WILKE & FALNIOWSKI 2001, SZAROWSKA & WILKE 2004, SZAROWSKA et al. 2005). For the GenBank Accession Numbers, see Table 1 in Appendix 1.

Molecular data analysis

The sequences were initially edited with BIOEDIT 7.0.5.3 (HALL 1999), and aligned by eye with the same program. In the CO1 sequences neither insertions nor deletions were found, thus all the sequences were cropped to the same length (638 bp) and used for further analysis.

In order to test whether the CO1 dataset shows a significant level of saturation, the test implemented in the software package DAMBE 4.2.13 (XIA 2000, SALEMI 2003) was used. It revealed a significant degree of saturation in the third position of the sequences. However, to avoid a substantial loss of information in the case of closely related species, I did not exclude this position from the dataset and used it for the analysis. To estimate the effects of saturation, the phylogenetic inference was performed also on the sequences translated to amino acids (see below). The 18S sequences were initially aligned manually with the help of the knowledge of the secondary structure information from the SSU rRNA database (WUYTS et al. 2004). Later the iterative alignment with ClustalX (THOMPSON et al. 1997) and editing with MacClade 4.05 (MADDISON & MADDISON 2002) were performed. All regions with deletions were excluded in the majority of the sequences. The final alignment of the 18S sequences was certainly not unambiguous, but this may only have biased the deeper parts of the phylogeny. Finally the 436 bp long sequences were used.

There is a common opinion that parsimony is assumption-free, and, on the other hand, that maximum likelihood that applies a model as close to the real mode of evolution as possible performs much better. None of the two opinions is true (FALNIOWSKI, 2003). Parsimony assumes the simplest mode of evolution that minimizes all the evolutionary changes. Maximum likelihood is not sensitive to some violation of its as-

sumptions (SWOFFORD et al. 1996), but may show a tendency towards finding wrong reconstructions, especially where one deals with many taxa and short sequences (NEI et al. 1998, NEI & KUMAR 2000). After all, in the maximum likelihood theory as a whole one cannot find a parameter connected with the tree topology. The only thing one can do is to believe that the tree with the "truest" branch lengths is, at the same time, the one with the best topology (YANG et al. 1995, NEI 1987, 1996). There is also a strong evidence that the more complex the model of evolution, the higher the variance of the resulting reconstructions. Our understanding of the DNA evolution is not yet sufficient, thus all the models are far from realistic. Thus, it may happen that the simplest models will result in phylogeny reconstructions which approach the real historical processes the most closely (GAUT & LEWIS 1995, YANG 1997, TAKAHASHI & NEI 2000).

Hence I decided to use maximum parsimony, with all the characters (positions) treated in the same way for the non-coding 18S, with PAUP*4.0b10 (SWOFFORD 2002), as the first step, applying the minimum assumptions, parameters to be estimated. The same program was used, for each gene, to calculate parameters for the 56 models of evolution included in PAUP, and 24 included in MRBAYES 3.1 (HUELSENBECK & RONQUIST 2001, RONQUIST & HUELSENBECK 2003). Later the program MODELTEST 3.7 (POSADA & CRANDALL 1998) was applied to choose the models best fitting the datasets. For 18S the maximum likelihood model applied for the heuristic search (TBR) for the maximum likelihood tree with PAUP was the one of TAMURA & NEI (1993), with invariable sites and Γ distribution, assuming the equal base frequencies, rate matrix: 1.000, 1.744, 1.000, 1.000, 4.862, 1.000, proportion of invariable sites 0.707, and Γ distribution shape parameter 0.519. The SYM + I + Γ model was applied for the same 18S data for the Bayesian inference with the package MRBAYES 3.1, with 2,000,000 generations.

For the CO1 data the model TVM + I + Γ, with the base frequencies: A=0.333, C=0.133, G=0.120, T=0.414, rate matrix: 0.698, 8.159, 0.293, 1.605, 8.159, 1.000, proportion of invariable sites 0.511, and the Γ distribution shape parameter: 0.570, was found with the MODELTEST, and used for inferring the maximum likelihood tree with PAUP, applying heuristic search (TBR). With the same model TVM + I + Γ, the Bayesian tree was inferred with MRBAYES. For the coding CO1 sequences the translation to the protein was performed with BIOEDIT. Later, the maximum parsimony tree was inferred with PAUP, all the amino acids assumed to be unordered reversible characters. This too simplifying assumption was used as a safer solution, which, unlike assumptions not justified enough, would not increase the variance of the results in the case of 27 sequences and only 18 parsimony-informative characters.

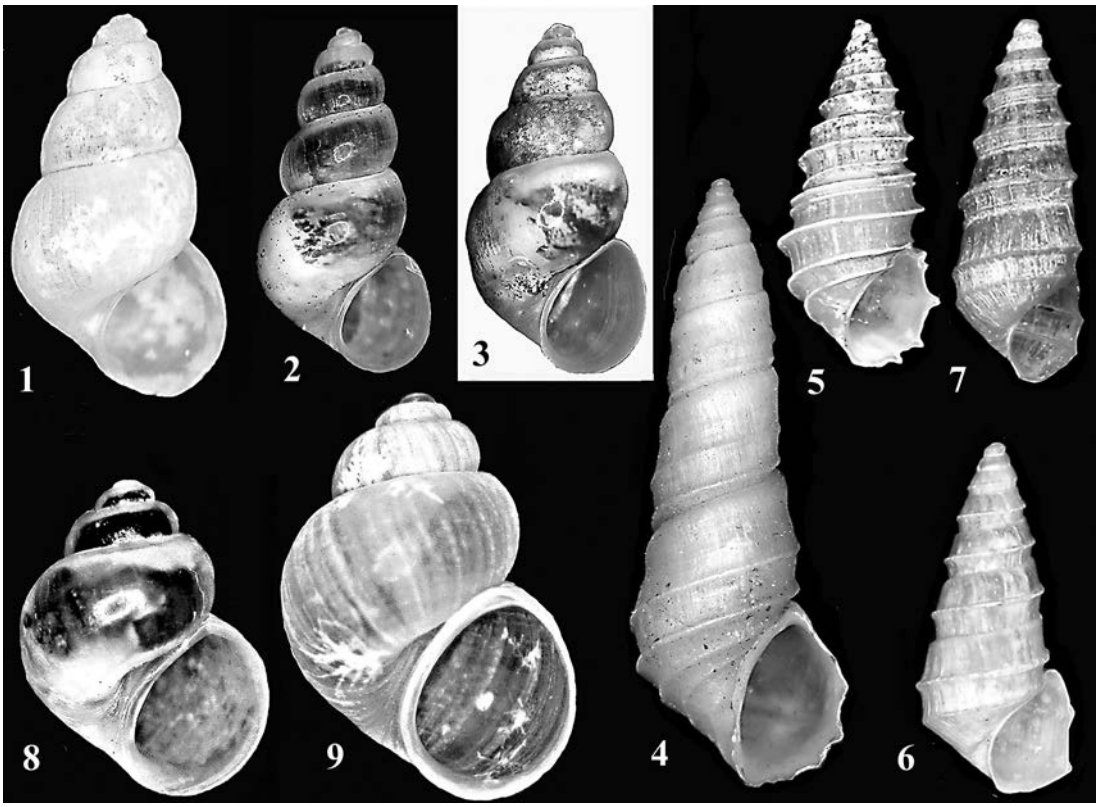
Finally, I had to choose between a separate analysis of the two genes, and total evidence analysis. BULL et al. (1993) and CHIPPINDALE & WIENS (1994) have demonstrated that total evidence analysis fails to result in a good reconstruction when one set of data consists of rapidly evolving characters (like CO1 in our case) and the other includes slowly evolving characters (like 18S in our case). Thus I decided to avoid total evidence analysis of the data. It must be stressed, that phylogeny reconstruction, even if reliable (which can never be known for sure) reflects the phylogeny of a gene, which is not exactly the same for different genes, and not necessarily the same as the phylogeny of the species (e.g. AVISE 2000). To summarize the results of the molecular phylogeny reconstructions the strict and Adams consensus trees were computed for the taxa sequenced for both genes. Later, the taxa lacking in the CO1 tree were added based on their position in the 18S tree. Finally, the two trees were constructed manually with MACCLADE, using a data matrix consisting of morphological data, to reconstruct the phylogeny of the morphological characters with MACCLADE.

RESULTS

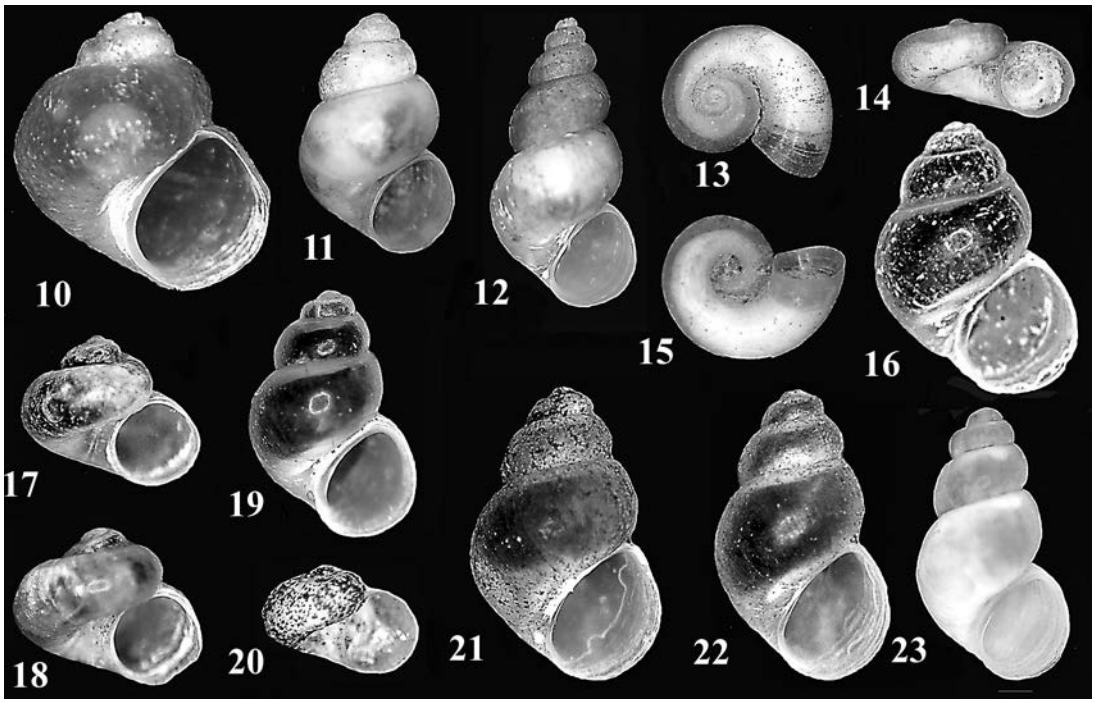
MORPHOLOGICAL CHARACTERS OF THE STUDIED GENERA

Teleoconch

The studied gastropods (Figs 1–46) are tiny, the height of their shells varies from about 1 mm (*Hauffenia*: Figs 13–15, *Daphniola*: Figs 17–18, *Islamia*: Fig. 20, *Graziana*: Fig. 27) to 12 mm (*Bithynia*: Fig. 37); in most of them the shell is between 1.5 and 4 mm high. None of the representatives of the group that inhabit the studied area, is planispiral- or nearly planispiral-shelled. Some of them have a valvatiform shell (Figs 13–15, 17–18, 20), the umbilicus being wide (*Daphniola:* Figs 17–18, *Islamia:* Fig. 20) or very wide (*Hauffenia:* Fig. 15). A neritiform shell with a broad columellar lip is an autapomorphy of *Lithoglyphus* (Figs 34–35). A trochiform shell is characteristic of *Sadleriana* (Fig. 10), but also of some representatives of *Pseudamnicola* (Fig. 8) and *Pseudobithynia* (Fig. 39). All the other shells range (as distinguished by HERSHLER & PONDER 1998) from ovate-conical (*Pseudamnicola*: Fig. 9, *Orientalina*: Fig. 11, *Grossuana*: Fig. 16, *Graecorientalia*: Fig. 19, *Trichonia kephalovrissonia*: Figs 21–22, *Belgrandiella*: Figs 24–25, *Graziana*: Fig. 27, *Pontobelgrandiella*: Figs 28–30, *Boleana*: Fig. 26, *Emmericia*: Fig. 36, *Bithynia*: Fig. 37, *Bythinella*: Fig. 40, *Parabythinella*: Fig. 41, *Litthabitella:* Figs 45–46), through conical (*Adriohydrobia*: Fig. 1,



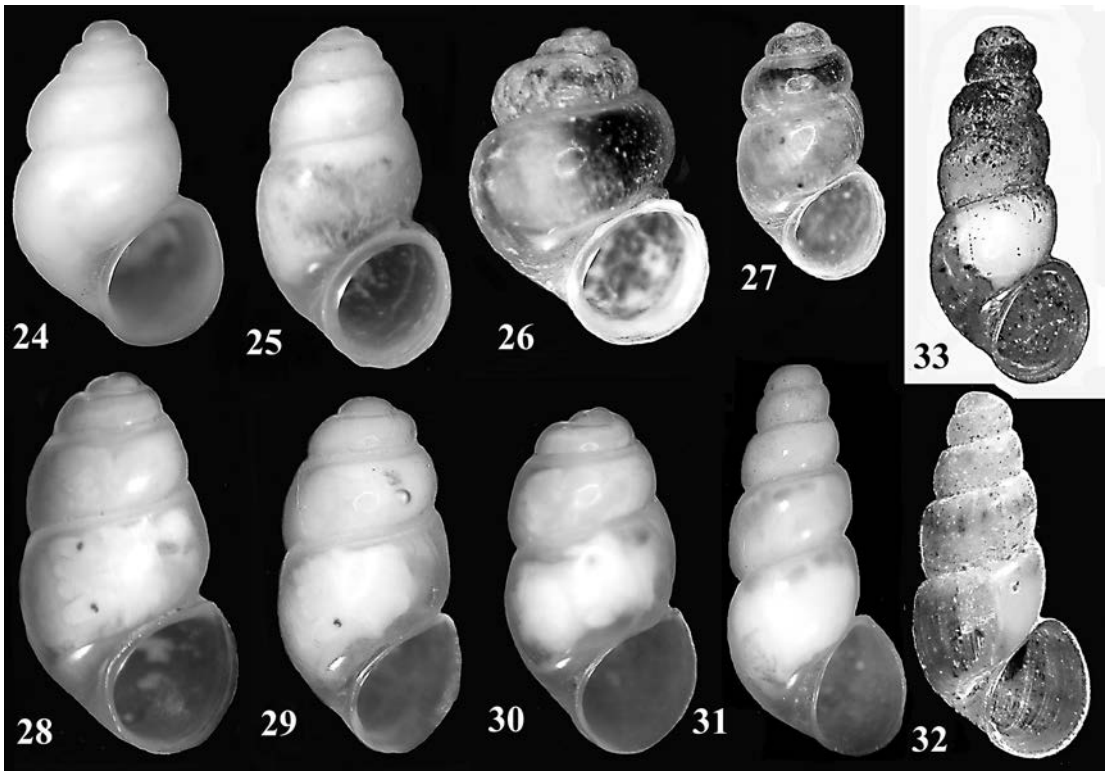
Figs 1–9. Shells of the Hydrobiidae: 1 – *Adriohydrobia gagatinella*, Cetina River Estuary, Croatia; 2–3 – *Ventrosia*: 2 – Itea, Greece, 3 – Euboia, Greece; 4–5 – *Dianella thiesseana*: 4 – Trichonida Lake, 5 – Lysimachia Lake; 6–7 – *Pyrgula annulata*: 6 – Garda Lake, Italy, 7 – Neretva River, Croatia; 8 – *Adrioinsulana conovula*, Zubovici, Pag Island, Croatia; 9 – *Pseudamnicola negropontina*, Marmaris, Euboia Island, Greece; size proportions not constrained between the species



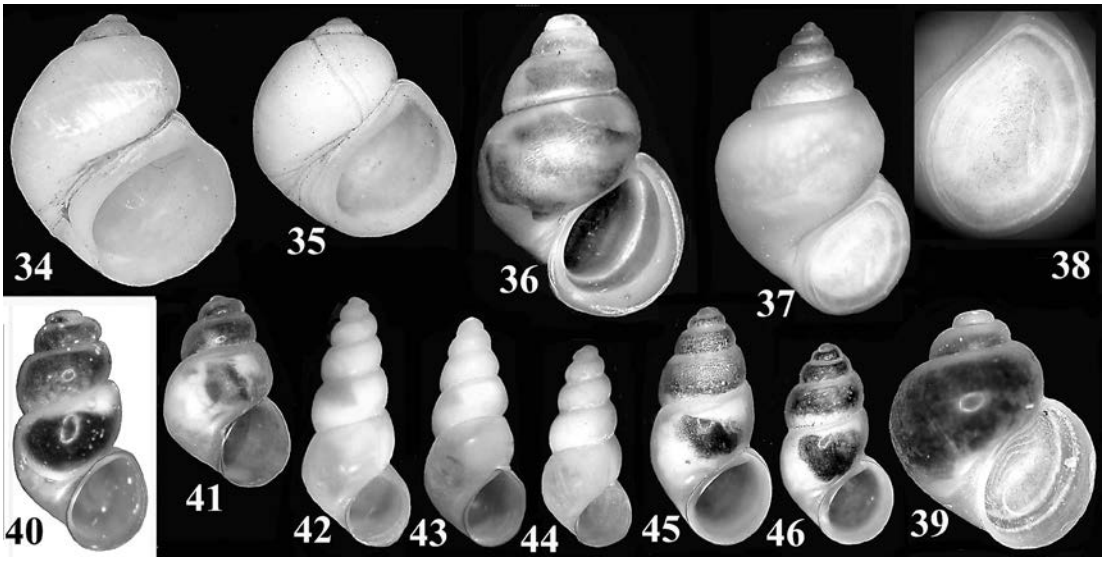
Figs 10–23. Shells of the Hydrobiidae: 10 – *Sadleriana fluminensis*, Močilnik, Slovenia; 11 – *Orientalina curta curta*, Nikšicko Polje, Montenegro; 12 – *Anagastina scutarica*, Scutari Lake, Montenegro; 13–15 – *Hauffenia* sp., Slovakia; 16 – *Grossuana codreanui*, spring at Terchighiol Lake, Romania; 17–18 – *Daphniola graeca*, Daphne spring, Greece; 19 – *Graecorientalia vrissiana*, Makrinitsa/Koukourava, Greece; 20 – *Islamia zermanica*, spring close to the Zrmanje River, Croatia; 21–22 – *Trichonia kephalovrissonia*, Termos, Greece; 23 – *Trichonia trichonica*, Trichonida Lake, Greece; size proportions not constrained among species

Ventrosia: Figs. 2–3, *Anagastina*: Fig. 12, *Trichonia trichonica*: Fig. 23), to turriform (*Daphniola*: Figs 4–5, *Pyrgula*: Figs 6–7, *Paladilhiopsis*: Figs 31–33, and *Heleobia*: Figs 42–44).

Whorl translation, as defined by RAUP (1966), in the majority of the studied shells is isometric, thus their shell outline is (almost) straight. There are, however, some cases where a gradual increase in whorl translation produces a convex shell outline, like in *Belgrandiella* (Figs 24–25), *Graziana* (Fig. 27), *Pontobelgrandiella* (Figs 28–30), *Bythinella* (Fig. 40), (less marked) *Litthabitella* (Figs 45–46). Whorl con-



Figs 24–33. Shells of the Belgrandiellinae and Moitessieriidae: 24–25 – *Belgrandiella kusceri*, Rakek, Slovenia; 26 – *Boleana umbilicata*, Močilnik, Slovenia; 27 – *Graziana lacheineri*, Bele Vode, Slovenia; 28–30 – *Pontobelgrandiella nitida*, Jasenovo, Bulgaria; 31–33 – *Paladilhiopsis carpathica*, Vadu Crisul Cave, Romania; size proportions not constrained among species



Figs 34–46. Shells of the Rissooidea: 34–35 – *Lithoglyphus naticoides*, Danube River near Calafat, Romania; 36 – *Emmericia expansilabris*, Ombla, Croatia; 37 – *Bithynia tentaculata*, Mala Neretva River, Croatia; 38 – operculum of *Bithynia*; 39 – "*Pseudobithynia graeca*", Piges Pamisou, Greece; 40 – *Bythinella austriaca*, Ojców, Poland; 41 – *Parabythinella graeca*, Vegorritida Lake, Greece; 42–44 – *Heleobia dobrogica*, Movile Cave, Romania: 42–43 – female, 44 – male; 45–46 – *Litthabitella chilodia*, W of Sotonici, Montenegro; size proportions not constrained among species

vexity is little differentiated among clades: apart from intraspecific variation, the whorls are flat in *Dianella* (Figs 4–5) and *Pyrgula* (Figs 6–7), and slightly to moderately convex in all the other genera (Figs 1–3 and 8–46).

In all the shells considered the peristome is continuous. Relative to the remainder of the apertural plane the outer lip may be simple (in most of the studied genera), reflected or fluted (*Belgrandiella, Graziana, Paladilhiopsis, Pontobelgrandiella, Bithynia, Pseudobithynia)* or thickened behind the outer lip like in *Emmericia* (the outer lip is both fluted and thickened). In lateral profile, the outer lip may be simple (in most of the studied cases), adapically sinuated (*Belgrandiella, Graziana, Paladilhiopsis, Boleana, Trichonia, Heleobia*), or abapically sinuated (*Horatia*).

The spiral sculpture in the studied genera is either absent (most cases) or composed of keels (*Dianella*: Figs 4–5, *Pyrgula:* Figs 6–7). The axial sculpture is in the form of either growth lines (most cases) or rounded ribs (*Dianella)*. In *Lithoglyphus* (Figs 34–35) the shell is thick-walled, in the other genera it is thin or very thin-walled.

The umbilicus may be absent (*Lithoglyphus*), narrow (most cases) or wide (*Hauffenia, Islamia, Horatia, Daphniola).*

Operculum

In *Bithynia* and *Pseudobithynia* (Figs 37–39) the operculum is calcified and thick, concentric, the nucleus central. In most of the other genera considered it is thin, paucispiral, ovate, the nucleus submarginal. In *Hauffenia, Islamia, Daphniola* and *Horatia* the operculum is circular with a central nucleus. In *Hauffenia* there is a spiral thickening of the inner surface of the nucleus; in the other genera the nucleus is not thickened.

Protoconch

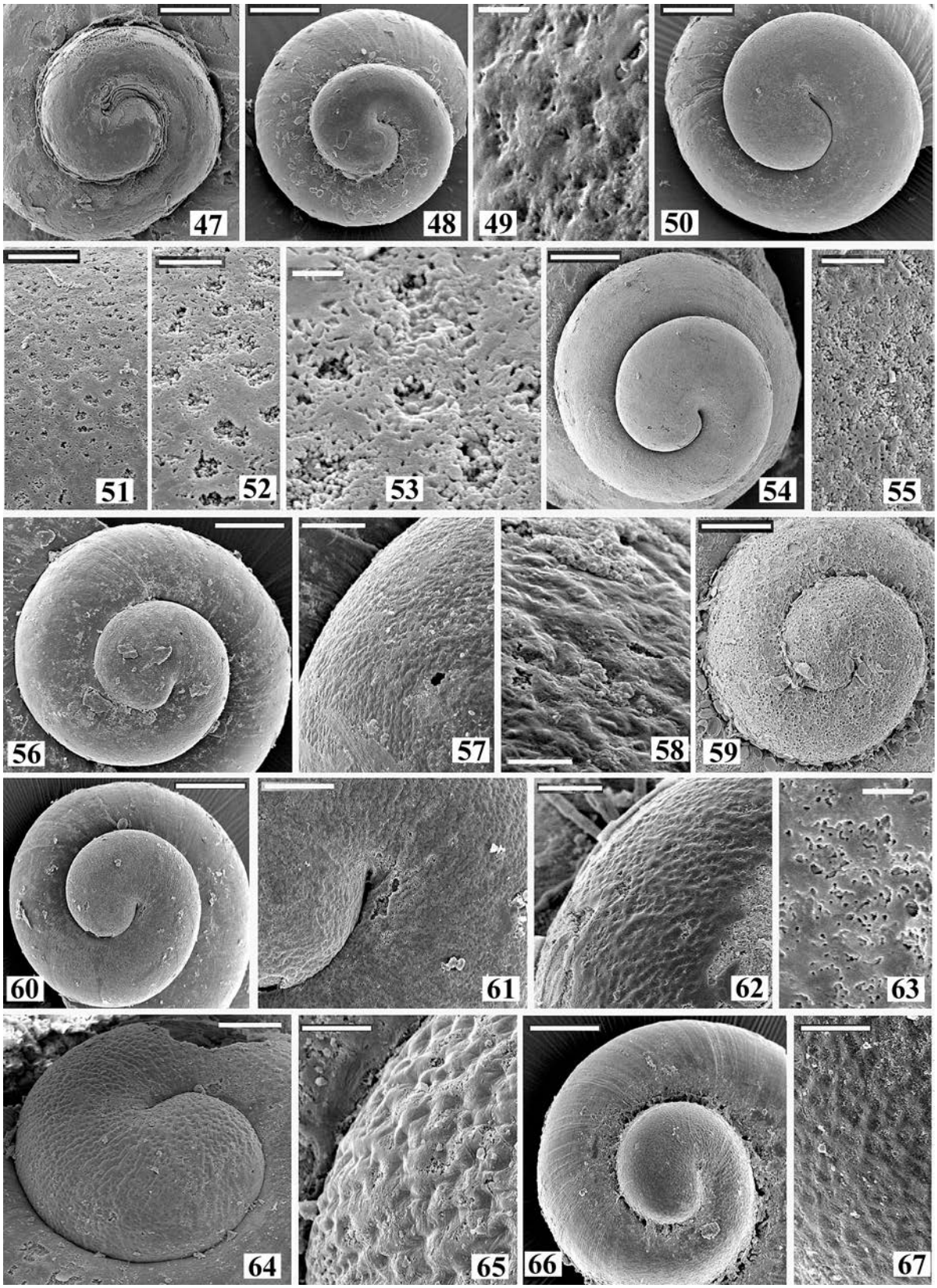
In the present paper only the protoconchs of the genera not considered in the literature are described and illustrated (Figs 47–119).

The border between the proto- and teleoconch is easily discernible in *Ventrosia* (Fig. 48), *Pyrgula* (Fig. 50), *Dianella* (Fig. 54), *Orientalina* (Fig. 56), *Grossuana* (Fig. 68), *Boleana* (Fig. 77), and *Litthabitella* (Figs 105–106), in each case there are somewhat more than 1½ whorls forming the protoconch. In *Paladilhiopsis* (Fig. 94) the protoconch is relatively broad and massive, first (at apex) broadening abruptly, then slowly. The protoconch habitus in all the other studied species is similar (Figs 47–48, 50, 54, 56, 59–60, 66, 68, 70, 72, 75, 77, 80, 82, 86–88, 90–91, 97, 99, 102–103, 105–106, 110, 115–116). In most of the genera its initial part is broad (*Adriohydrobia:* Fig. 47, *Ventrosia:* Fig. 48, *Pyrgula:* Fig. 50, *Orientalina*: Fig. 56, *Grossuana*: Fig. 68, *Daphniola*: Fig. 70, *Graziana*: Fig. 72, *Belgrandiella*: Fig. 75, *Boleana*: Fig. 77, *Pontobelgrandiella*: Fig. 80, *Graecorientalia*: Fig. 82, *Islamia*: Fig. 91, *Heleobia*: Figs 97, 99 and 102, *Bythinella*: Fig. 103, *Pseudobithynia*: Fig. 110 and *Lithoglyphus*: Figs 115–116); in some others it is narrow (*Dianella:* Fig. 54, *Sadleriana:* Fig. 59, *Anagastina*: Fig. 60 and *Litthabitella*: Figs 105–106). However, dependent on species, in some genera it may either be broad or narrow (*Hauffenia:* Figs 64 vs. 66, respectively; *Trichonia*: Figs 86–87 vs. 88 and 90, respectively). Thus the protoconch habitus may characterise a species, but rather not a genus.

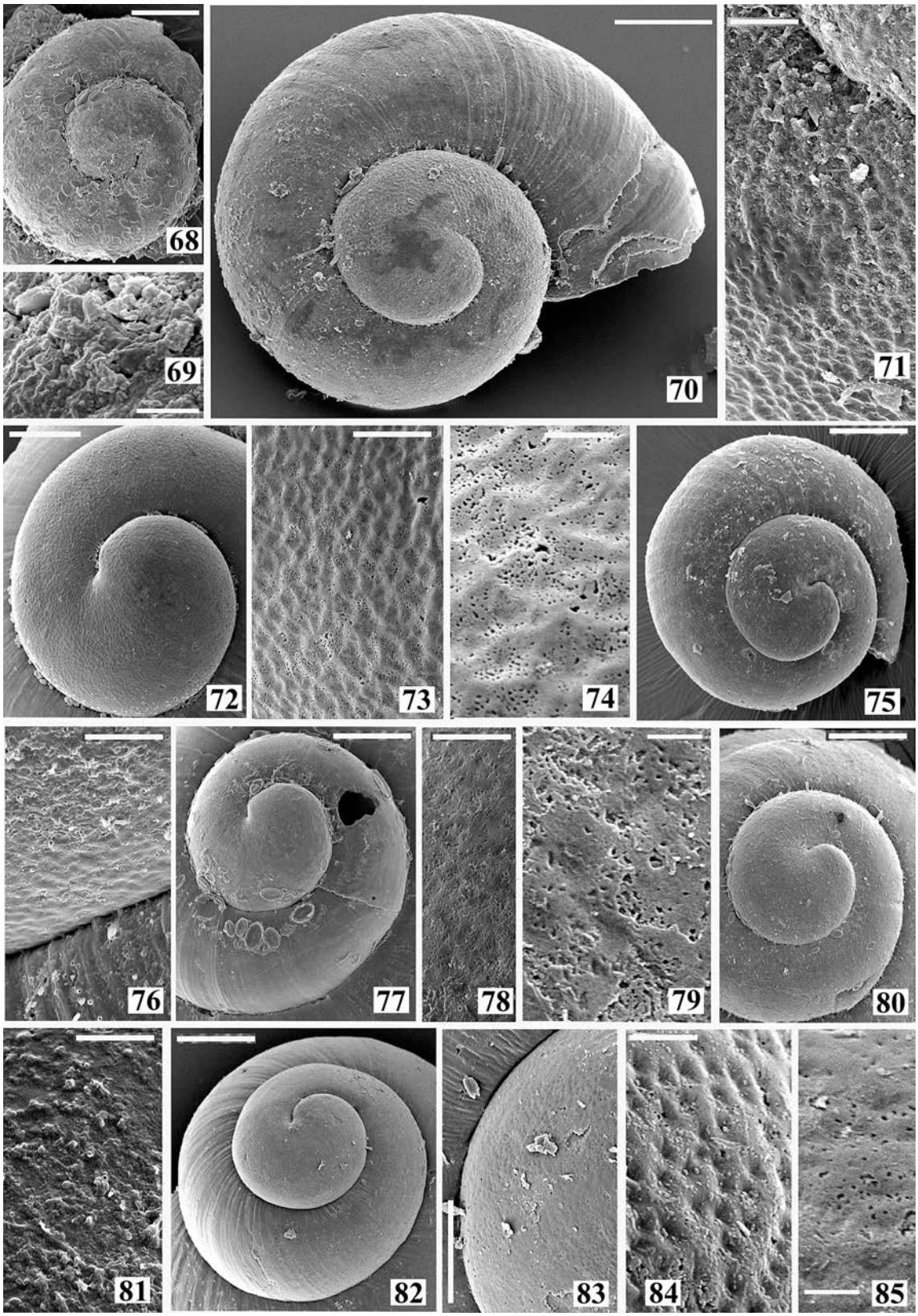
The protoconch of *Bythinella* has a delicate spiral sculpture, which in some species, like *B. charpentieri* (Fig. 103), is almost indiscernible. A similar spiral sculpture is found in *Parabythinella* (Appendix 3, Fig. 21). In *Pseudobithynia* there is a pattern of rather flat and irregular spiral threads (Fig. 111). Small patches of it could only be found closer to the suture. In *Lithoglyphus,* a very fine and hardly discernible spiral sculpture in the form of spiral grooves could only be seen in an embryo (Fig. 117). It seems that the periostracum is so delicate that soon after hatching the sculpture is destroyed: it was only in patches that it could be found, even in a very young snail (Figs 118–119).

In *Daphniola exigua* a net-like pattern of dense depressions, their shape irregular, covers all the protoconch and initial part of the teleoconch (Figs 70–71). A very similar pattern of protoconch sculpture is found in *Hauffenia* (Figs 64–67), *Graziana* (Figs 72–73), *Belgrandiella* (Figs 75–76), *Boleana* (Figs 77–78: very weakly marked), *Graecorientalia* (Figs 83–84), *Islamia* (Figs 91–92: weakly marked), and *Trichonia* (Figs 86–90: very weakly marked). A similar pattern, with relatively lower and narrower ridges surrounding the depressions, is found in *Heleobia* (Figs 98–100). A smooth protoconch is characteristic of *Sadleriana* (Fig. 59). In the other taxa there are only delicate irregularities of the protoconch surface (*Orientalina*: Figs 56–58, *Anagastina*: Figs 60–62, *Grossuana*: Fig. 69, *Pontobelgrandiella*: Fig. 80, *Paladilhiopsis*: Fig. 93, *Litthabitella*: Figs 105–109).

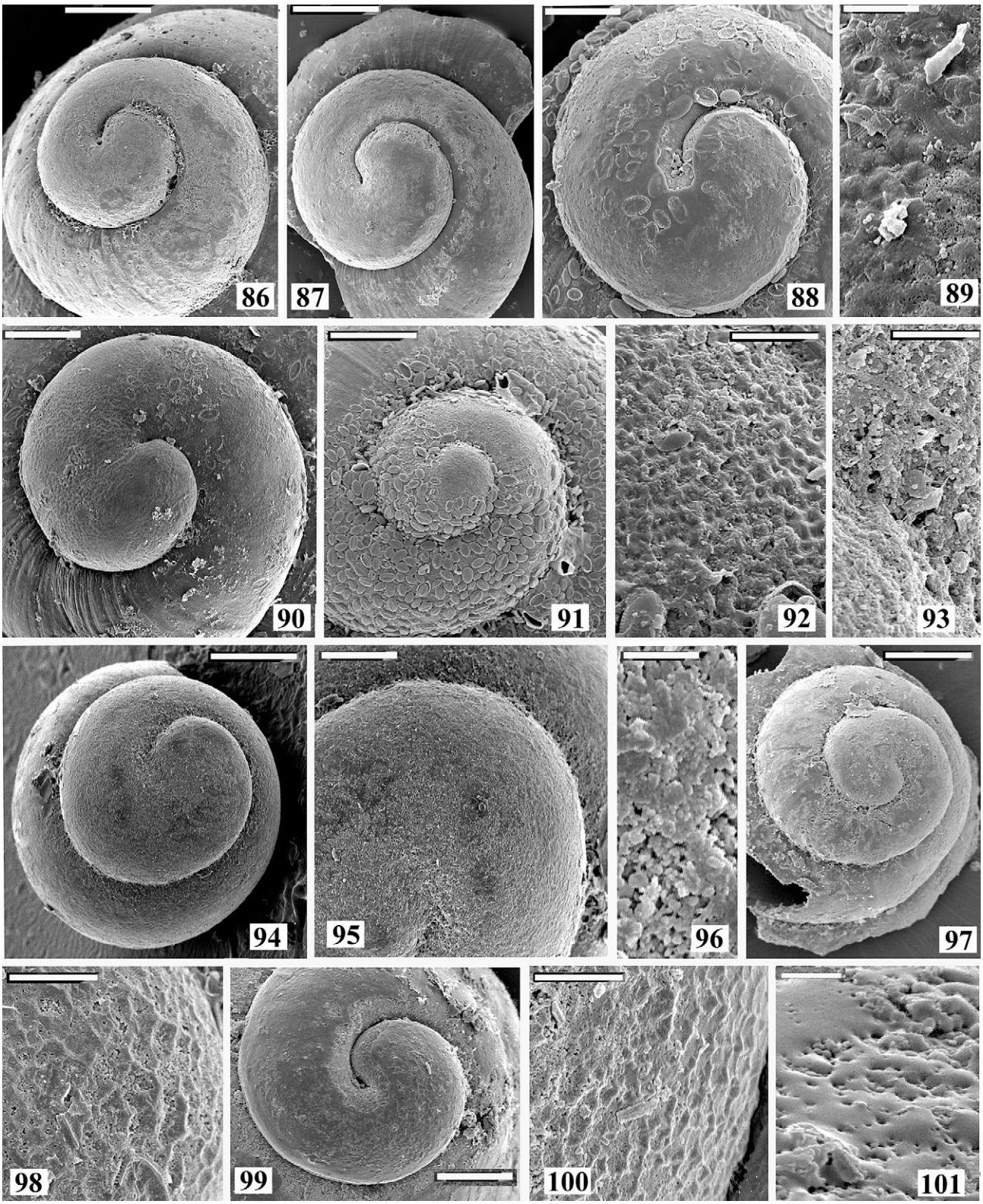
Extremely fine pores, scattered irregularly and not densely, are found in *Pyrgula* (Figs. 51–53) and *Dianella* (Fig. 55). The same type of pores is found in *Parabythinella* (Fig. 22 in Appendix 3 in the present paper), but similar pores occur in *Heleobia* (Figs 96, 101). A characteristic network of pores, densely arranged and lying entirely within the periostracum, is found in *Bythinella* (Fig. 104), *Pseudobithynia* (Figs 112–114), and *Emmericia* (Figs 16–17 in Appendix 2 in the present paper). Coarse cavities occur on the protoconch surface of *Pyrgula* (Figs 51–53); the cavities are numerous, some of them fused, in *Pyrgula* they have sharp edges. Another pattern of sculpture, composed of small pores of various size and outline, their edges sharp, density irregular, was found in *Anagastina* (Fig. 54), *Graziana* (Fig. 74), *Belgrandiella* (Fig. 76), *Boleana* (Fig. 79), and *Graecorientalia* (Fig. 85).



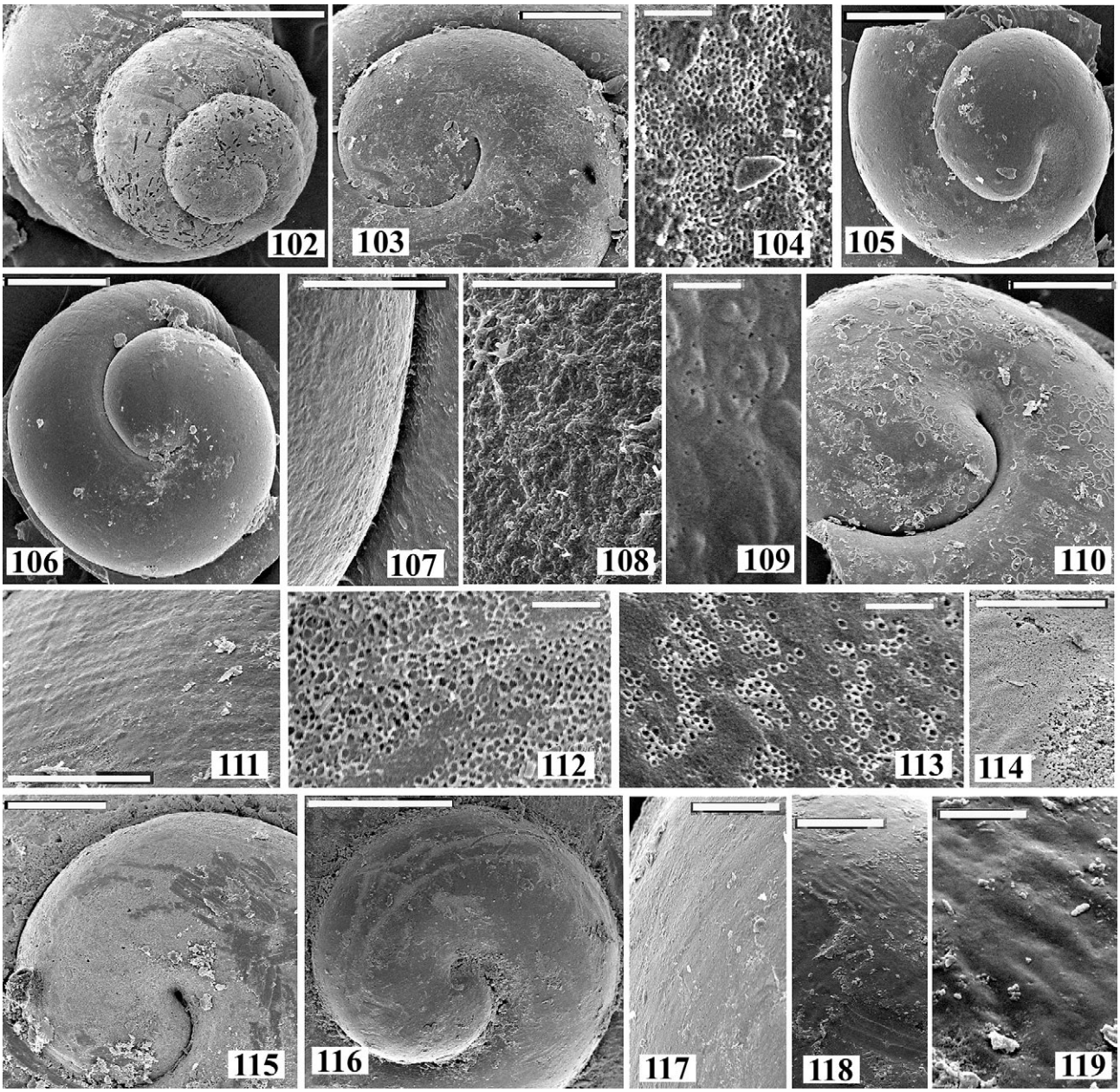
Figs 47–67. Protoconchs: 47 – *Adriohydrobia gagatinella*, habitus, 48–49 – *Ventrosia spalantiana*: 48 – habitus, 49 – surface; 50–53 – *Pyrgula annulata:* 50 – habitus; 51–53 – surface; 54–55 – *Dianella thiesseana:* 54 – habitus, 55 – surface; 56–58 – *Orientalina curta curta*: 56 – habitus, 57–58 – surface; 59 – *Sadleriana fluminensis*, habitus; 60–63 – *Anagastina scutarica*: 60 – habitus, 61–63 – surface; 64–65 – *Hauffenia* sp.: 64 – habitus, 65 – surface; 66–67 – *H. michleri*: 66 – habitus, 67 – surface. Scale bars: 100 μm in 59; 50 μm in 47, 48, 50, 54, 56, 60 and 66; 25 μm in 64; 20 μm in 61; 10 μm in 51, 57 and 62; 5 μm in 65 and 67; 2.5 μm in 52 and 55; 2 μm in 58; 1 μm in 49, 53 and 63



Figs 68–85. Protoconchs: 68–69 – *Grossuana codreanui*: 68 – habitus, 69 – surface; 70–71 – *Daphniola exigua*: 70 – habitus of proto and teleoconch, 71 – surface; 72–74 – *Graziana lacheineri*: 72 – habitus, 73–74 – surface; 75–76 – *Belgrandiella croatica*: 75 – habitus, 76 – proto and teleoconch surface; 77–79 – *Boleana umbilicata*: 77 – habitus, 78–79 – surface; 80–81 – *Pontobelgrandiella nitida*: 80 – habitus, 81 – surface; 82–85 – *Graecorientalia vrissiana*: 82 – surface, 83 – proto and teleoconch surface, 84–85 – protoconch surface. Scale bars: 100 μm in 70; 50 μm in 68, 75, 77, 80 and 82; 25 μm in 72; 20 μm in 71 and 83; 5 μm in 73, 76, 78 and 81; 2 μm in 69, 74 and 84; 1 μm in 79 and 85



Figs 86–101. Protoconchs: 86–87: *Trichonia kephalovrissonia*, habitus; 88–90 – *T. trichonica*: 88 and 90 – habitus, 89 – surface; 91–92 – *Islamia zermanica*: 91 – habitus, 92 – surface; 93–95 – *Paladilhiopsis carpathica*: 93 – surface, 94 – habitus, 95 – proto- and teleoconch surface; 96–98 – *Heleobia dalmatica*: 96 and 98 – surface, 97 – habitus; 99–101 – *Heleobia* sp.: 99 – habitus, 100–101 – surface. Scale bars: 50 μm in 86, 87, 91, 94, and 97; 25 μm in 88, 90, 95 and 99; 5 μm in 92, 93, 98 and 100; 2 μm in 89; 1 μm in 96 and 101



Figs 102–119. Protoconchs: 102 – *Heleobia dalmatica;* 103–104 – *Bythinella charpentieri:* 103 – habitus, 104 – surface; 105–109 – *Litthabitella chilodia*: 105–106 – habitus, 107–109 – surface; 110–113 – *Pseudobithynia*: 110 – habitus, 111–113 – surface, 112–113 – higher magnification reveals characteristic net of pores; 114–119 – *Lithoglyphus naticoides*: 114, 117–119 – surface, 115–116 – habitus, 117 – surface of embryonic shell. Scale bars: 100 μm in 102; 50 μm in 103, 105, 106, 110, 115 and 116; 20 μm in 117; 10 μm in 107, 108, 111 and 118; 2 μm in 109 and 119; 1 μm in 104 and 112–114

Soft parts external morphology

Within the studied gastropods soft parts external morphology shows either no differences or differences at a species level. Many of them, like the presence/absence of the eyes or black pigment, are mostly correlated with the habitat: in the subterranean forms (like *Paladilhiopsis* or *Heleobia dobrogica*) there are neither eyes nor pigment. The ctenidium proportions and lamellae number are widely variable, also within a genus. In *Bithynia* and *Pseudobithynia* there is a typical ciliated food groove running on the right side of the mantle cavity. In *Litthabitella* the osphradium is broadly ovate (Fig. 183), unlike in the other taxa studied.

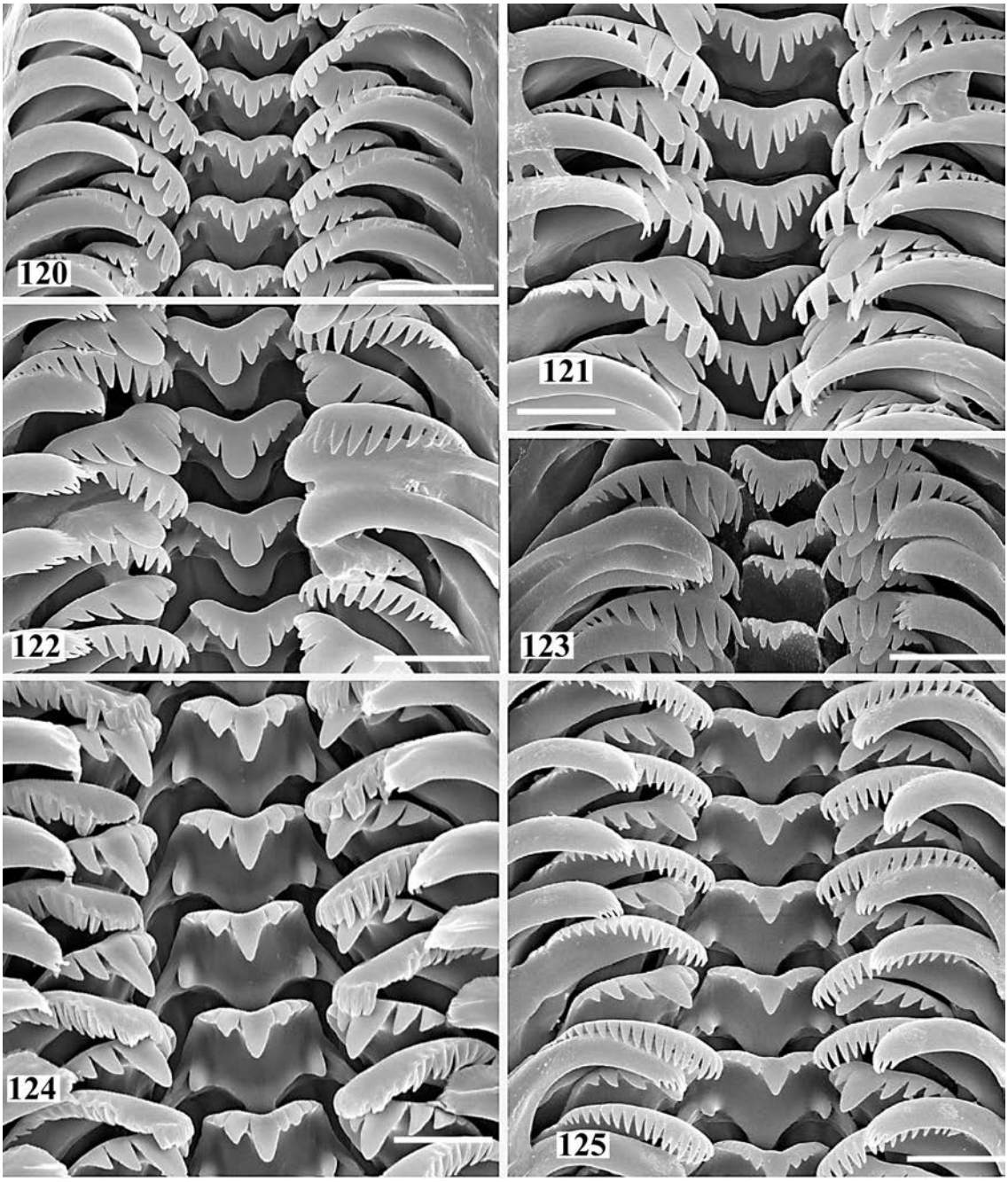
Radula

In the present paper only the radulae not considered elsewhere are illustrated (Figs 120–152). In *Ventrosia* (Figs. 120–121) there are two basal cusps on each side of the rhachis (Fig. 120) and four-five cusps on each side of the median cusp of the rhachis (Figs 120–121). The median cusp is only slightly longer

than the adjacent cusps, all the cusps relatively long and narrow. On the lateral tooth there are three-four cusps on each side of the biggest cusp, all the cusps long and narrow like those of the rhachis. On the inner marginal tooth there are about 15 cusps, the distal seven of which are long and narrow, while the proximal eight are much smaller. In *Adriohydrobia* (Fig. 122) there is only one basal cusp on each side of the rhachis and four on each side of the median cusp, all the cusps stouter than in *Ventrosia*. The cusps of the lateral and marginal teeth, like those in *Ventrosia,* are long and narrow. On the lateral tooth there are three cusps on each side of the biggest one. On the inner marginal tooth, like in *Ventrosia*, there are long and prominent cusps in the distal part of the tooth and much smaller cusps in the proximal part, there being no cusps of intermediate size.

As a "pyrgulid" representative, the radula of *Trachyohridia filocincta* from the Ohrid Lake is shown in the present paper (Fig. 123). The radula resembles the ones of *Ventrosia* and *Adriohydrobia*, but its central tooth is without basal cusps.

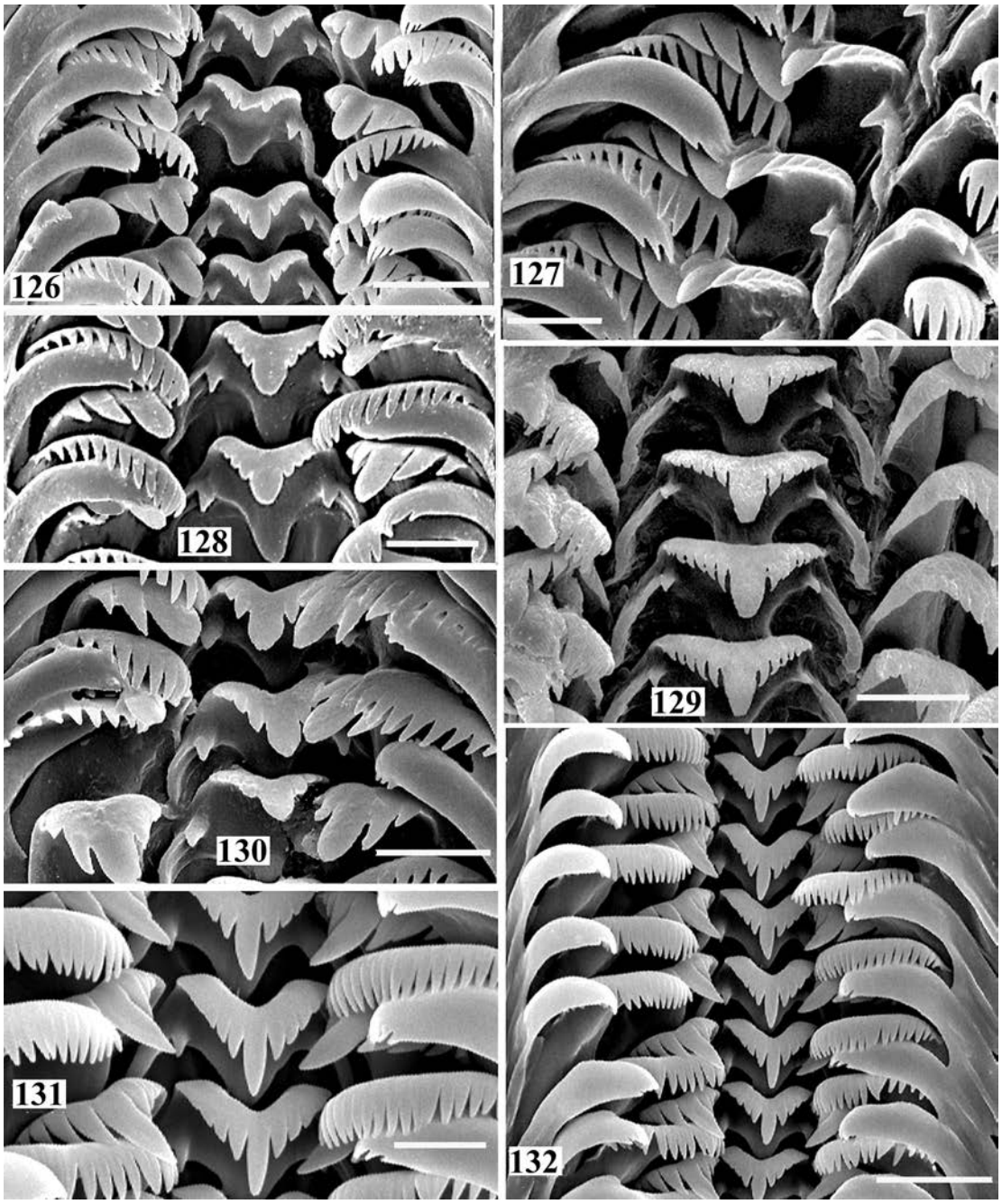
On the rhachis of *Adrioinsulana* (Fig. 124) there is one basal cusp on each side of the tooth, and three cusps on each side of the median cusp, the latter prominent, approximately triangular in shape and



Figs 120–125. Radulae: 120–121 – *Ventrosia spalatiana*, 122 – *Adriohydrobia gagatinella*, 123 – *Trachyohridia filocincta*, 124 – *Adrioinsulana conovula*, 125 – *Pseudamnicola negropontina*. Scale bars: 10 μm in 120, 122 and 123; 5 μm in 121, 124 and 125

more than twice as long as the adjacent cusps. The lateral tooth fulfils the formula: 2–1–2(3) (the third cusp on its outer side rudimentary). In *Pseudamnicola* (Fig. 125) the radula is almost the same as in *Adrioinsulana*, but the rhachis has five, instead of three, cusps on each side of the median cusp, this being less prominent than in *Adrioinsulana*. On the inner marginal tooth there are about 16 cusps, their length diminishing slowly and gradually from the distal to the proximal part of the tooth.

In the radula of *Orientalina* (Figs 126–127) there is one prominent basal cusp on each side of the rhachis and five cusps on each side of the median cusp. The latter is obtuse, twice as long as the adjacent cusps. The lateral tooth formula is 2–1–2(3); the biggest cusp is broad and massive. There are only 12 cusps on the inner marginal tooth, their length diminishing gradually starting from the distal ones that are long and slender. The radula of *Anagastina* (Fig. 128) resembles the one of *Orientalina*. On the rhachis there are 4(5) cusps on each side of the prominent median one and one pair of prominent basal cusps. The lateral tooth has two cusps on each side of the biggest one. There are 14 cusps on the inner marginal tooth. The central tooth of *Sadleriana* (Fig. 129) has one small basal cusp on each side; the median cusp is



Figs 126–132. Radulae: 126–127 – *Orientalina curta germari*, 128 – *Anagastina scutarica*, 129 – *Sadleriana fluminensis*, 130 – *Trichonia trichonica*, 131–132 – *Grossuana codreanui*. Scale bars: 10 μm in 126 and 129; 5 μm in 127, 128, 130 and 132; 2 μm in 131

broad and twice as long as the adjacent ones. The latter, seven on each side, are long, narrow and sharp; towards the margins they slowly diminish in size. The radula of *Trichonia* (Fig. 130) resembles the ones of *Anagastina* (Fig. 128) and *Orientalina* (Fig. 126). On the rhachis there is one rather big basal cusp on each side of the tooth and four cusps on each side of the massive median cusp. The lateral tooth has two cusps on each side of the broad and massive biggest cusp. There are ten cusps on the inner marginal tooth.

In the radula of *Grossuana* (Figs 131–132) there is a deep sinus along the anterior side of the central tooth. The basal cusps, one on each side, are rather big and sharp. The median cusp is narrow and slender, more than twice as long as the adjacent ones. On its each side there are four cusps, the margins of the cutting edge of the rhachis cuspless. The lateral tooth, its formula: 3–1–4, has a narrow, triangular, point-tipped long cusp. The inner marginal tooth bears 20 long and slender cusps, their size gradually decreasing. In *Daphniola* (Figs 133–134) the sinus on the central tooth is weakly marked. There is one prominent basal cusp on each side of the rhachis. The median cusp, less than twice as long and about twice as broad as the adjacent cusps, is relatively not as prominent as in the radulae described above. There are 3(4) cusps on both its sides (Fig. 133). Of the lateral tooth (Fig. 134), its formula: 2–1–6, the biggest cusp is only slightly bigger than the adjacent ones. The inner marginal tooth bears 22 long and slender cusps.

The central tooth of *Graecorientalia* (Fig. 135) has a deep and narrow sinus on the proximal side of the cutting plate, a pair of fine basal cusps and a narrow and fine median cusp, which is twice as long as the adjacent cusps, five on each side. The lateral tooth, its formula: 3–1–4, has a slender and fine biggest cusp. There are about 20 cusps on the inner marginal tooth. In *Belgrandiella* (Fig. 136) all cusps except the basal ones (one pair) are similar in shape and proportions to those of *Graecorientalia*. The latter cusps are big and massive, much bigger than in *Graecorientalia*. There are five cusps on both sides of the median cusp in the rhachis. The lateral tooth formula is 4–1–5; there are 24 or more cusps on the inner marginal tooth. In the radula of *Boleana* (Figs 137–138) all the cusps are long and narrow. In the central tooth there are two pairs of the basal cusps, one pair long and conspicuous, one pair vestigial. The sinus at the anterior side of the cutting plate is well marked, but not as deep as in *Graecorientalia* (Fig. 135). The median cusp on the rhachis is narrow and more than twice as long as the adjacent cusps (Fig. 138), there being five of them on each side of the median cusp. The lateral tooth formula is 4–1–4(5). There are 24–26 cusps on the inner marginal tooth.

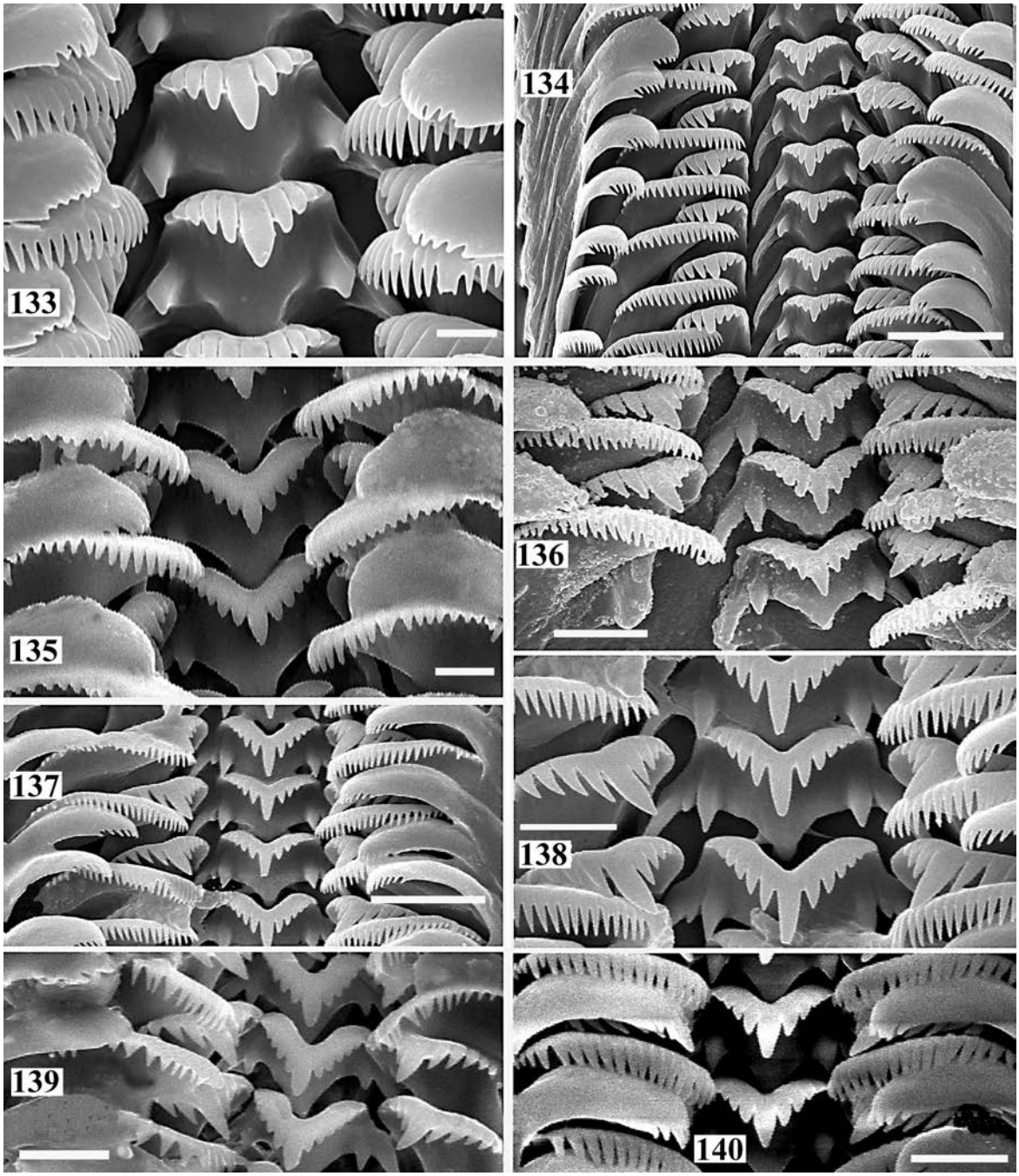
The radula of *Graziana* (Fig. 139) resembles the one of *Graecorientalia* (Fig. 125). The rhachis has a deep and narrow sinus at the anterior side of the cutting plate, one pair of basal cusps, and four (sometimes five) cusps on each side of the median cusp, which is broader than in *Graecorientalia*. The lateral tooth formula being 3–1–5, the biggest cusp is long and triangular in shape. There are about 18 cusps on the inner marginal tooth. In *Pontobelgrandiella* (Fig. 140) the sinus at the central tooth is weakly marked; there is one pair of prominent basal cusps. The median cusp is more than twice longer than the adjacent ones, which are triangular in shape, four on its each side. The lateral tooth formula is 3–1–5. The inner marginal tooth bears 22 cusps, the proximal of which are vestigial and the other increasing abruptly in length.

In the radulae of *Hauffenia* (Fig. 141), *Islamia* (Fig. 142), *Heleobia* (Figs 143–144) and *Paladilhiopsis* (Figs 145–146) the rhachis has a narrow and slender median cusp, on both sides of which there are prominent and slender cusps, due to which the cutting edge has a triangular outline. In the radula of *Hauffenia* (Fig. 141) there are two basal cusps on the rhachis; the median cusp is only slightly longer than the adjacent cusps, five of which are situated on each side of it. The lateral tooth fulfils the formula: 4–1–5, all its cusps, the biggest included, long, sharp and slender, like the median cusp on the rhachis. In the inner marginal tooth there are about 22 long and narrow cusps. In *Islamia* (Fig. 142) there is one pair of small basal cusps on the rhachis. Like in *Hauffenia* (Fig. 141), the median cusp on the central tooth is not much longer than the adjacent cusps, there being four of them on each side. The lateral tooth resembles that of *Hauffenia*. There are 22 cusps on the inner marginal tooth (Fig. 142).

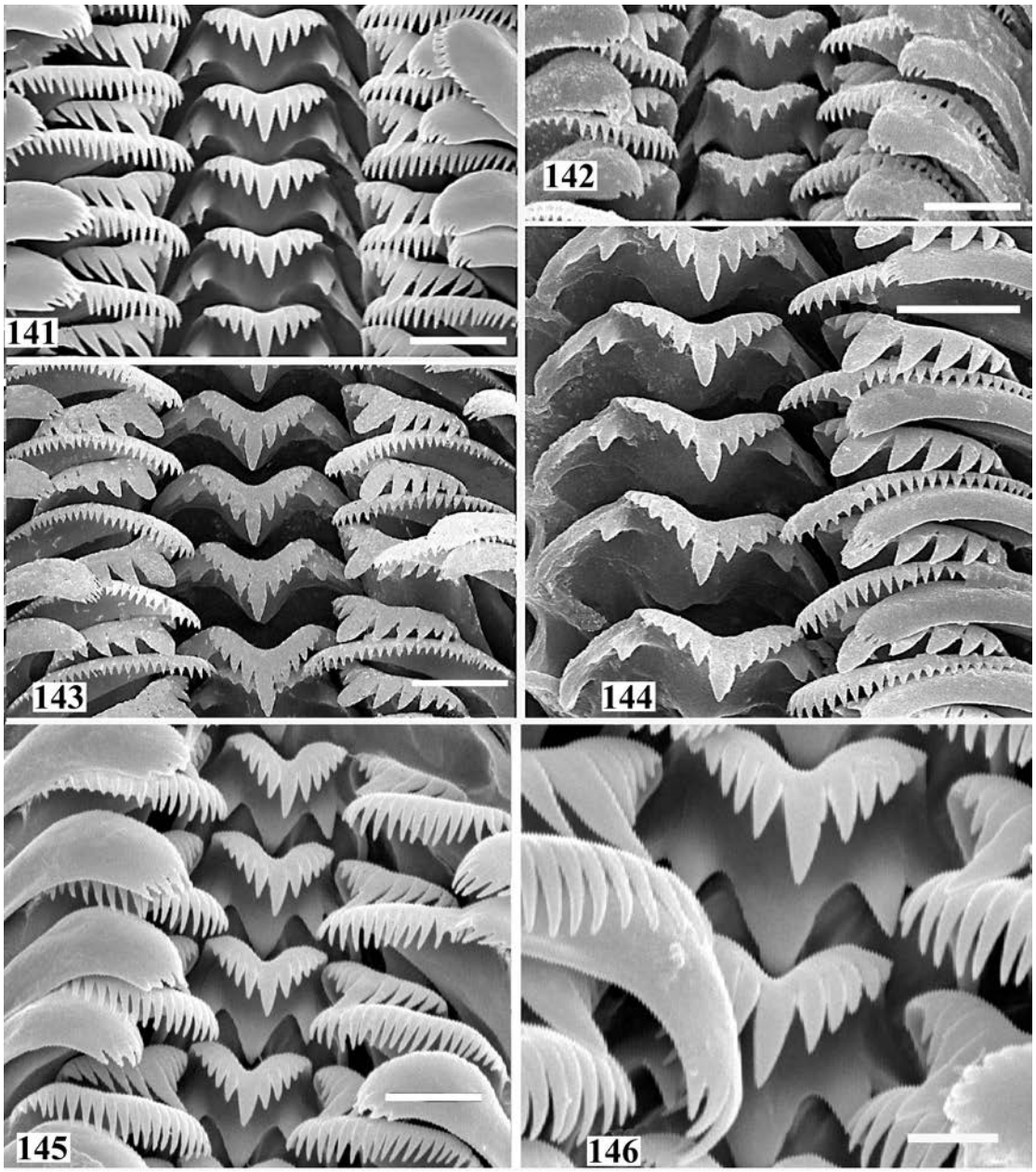
In the radula of *Heleobia* (Figs 143–144) there is a broad sinus at the proximal side of the cutting plate of the central tooth and a pair of rather small basal cusps; the median cusp is about twice as long as the adjacent six-seven on its both sides. In the lateral tooth the biggest cusp is broader than in *Hauffenia* or *Islamia,* rounded at the tip and not much longer than the adjacent cusps. The formula of the lateral tooth is: 3–1–5(6). On the inner marginal tooth there are 29–30 short cusps. The central tooth of *Paladilhiopsis* (Figs 145–146) resembles the one of *Heleobia*, but the basal cusps are bigger, and the median cusp proportionally shorter. On each side of the median cusp there are six (or, less frequently, five) cusps. The biggest cusp of the lateral tooth, unlike in *Heleobia* (Figs 143–144), like in *Hauffenia* (Fig. 141) and *Islamia* (Fig. 142) is long and narrow. The formula of the lateral tooth is: 5–1–5. The inner marginal tooth (Fig. 145) bears 19–20 long cusps.

In the other hydrobioids described above the bases of the basal cusps are parallel to the cutting edge of the tooth. In *Pseudobithynia* (Figs 149–150) the basal cusps lie along the lateral margins of the tooth base. In *Pseudobithynia* there are five basal cusps. The me-

dian cusp in *Pseudobithynia* is rather broad and about twice as long as the adjacent cusps (Figs 147–150), three-four of which lie on each side of it. The lateral tooth (Figs 147–148 and 150) fulfils the formula: 3–1–3; its biggest cusp is similarly broad but only slightly longer than the adjacent cusps, all the cusps prominent and triangular in shape. In the inner marginal tooth (Figs 147–148) there are 16–17 moderately long, triangular cusps. In the radula of *Litthabitella* (Figs 151–152) the central tooth has a deep sinus at the proximal part of the cutting plate and one pair of big and prominent basal cusps. The median cusp is narrow and slender, more than twice as long as the adjacent cusps, which it resembles in shape. On each side of the median cusp, laterally, there are four-five cusps; the cutting edge straight, with no cusps (Fig. 151). The biggest cusp in the lateral tooth is triangular, twice as long and broad as the adjacent cusps. The formula of the tooth is: (5)4–1–5. There are 26–28 long and slender cusps on the inner marginal tooth.



Figs 133–140. Radulae: 133 – *Daphniola graeca*, 134 – *D. exigua*, 135 – *Graecorientalia vrissiana*, 136 – *Belgrandiella croatica*, 137–138 – *Boleana umbilicata*, 139 – *Graziana lacheineri*, 140 – *Pontobelgrandiella nitida*. Scale bars: 5 μm in 134 and 137; 2 μm in 136 and 138–140; 1 μm in 133 and 135



Figs 141–146. Radulae: 141 – *Hauffenia* sp., 142 – *Islamia zermanica*, 143–144 – *Heleobia dalmatica*, 145–146 – *Paladilhiopsis carpathica*. Scale bars: 5 μm in 143 and 144; 2 μm in 141, 142 and 145; 1 μm in 146

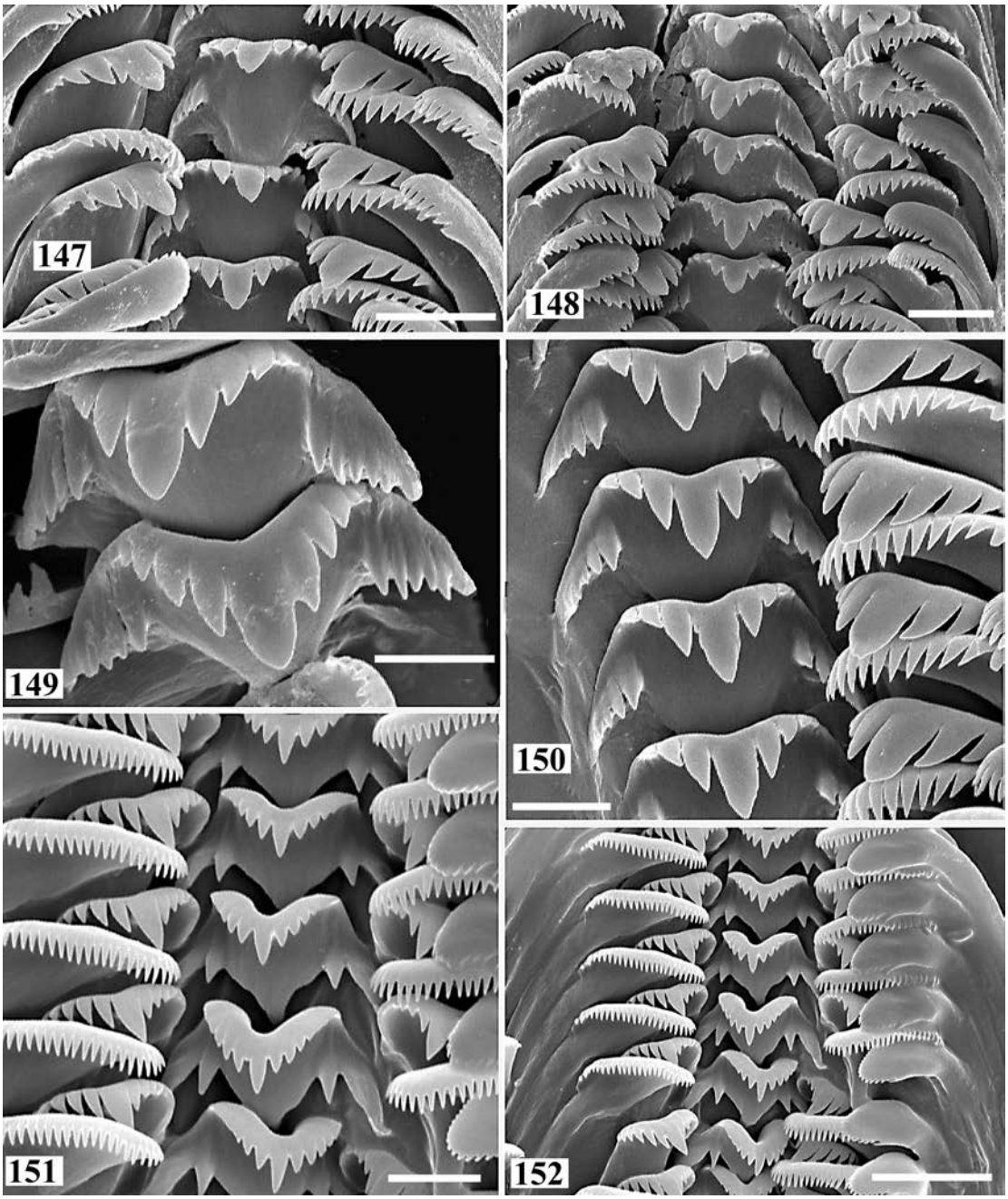
Stomach

In all the studied genera, the stomach (Figs 153–157) has a style sac, the oesophagus reaching the stomach on the same side as the intestine, and the opening of the digestive gland close to the oesophagus. In the majority of them, like *Bythinella* (Fig. 155) there is no caecum of any kind at the pyloric end of the stomach. A caecal appendix, the reminiscence of the spiral caecum of the less advanced prosobranchs (FRETTER & GRAHAM 1962) is found at the pyloric end of the stomach in *Hydrobia* (Fig. 153), *Dianella/Pyrgula* (Fig. 154), *Bithynia* (Fig. 156), and *Heleobia* (Fig. 157). The structure is more or less developed and does not look the same in all genera. The caeca of *Hydrobia* and *Dianella/Pyrgula* (Figs 153–154) are alike, the rudimentary caecum of *Heleobia* (Fig. 157) being different from them. Yet another type of caecum, which resembles neither *Heleobia* nor *Dianella/Pyrgula,* is found in *Bithynia* (Fig. 156).

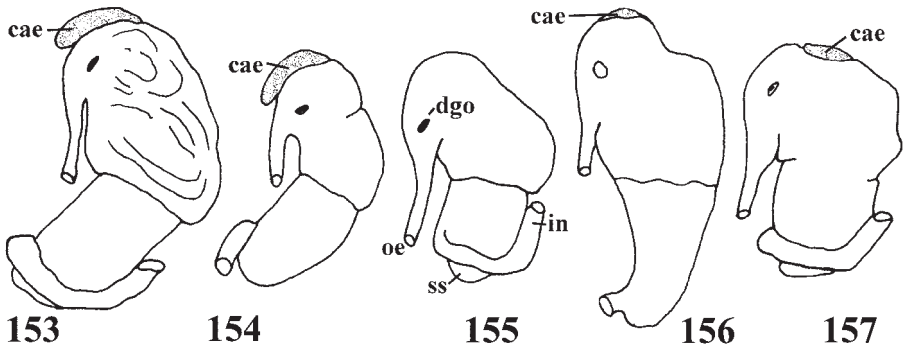
Female reproductive organs

In the present study, the descriptions and drawings are restricted to some corrections and observations not published elsewhere, and to diagrammatic representation of the characters whose evolution will be discussed later.

The structure of the ventral channel has been examined by means of histological techniques. In *Ventrosia* (Figs 158–160) the channel, heavily ciliated (Fig. 160) is separated from the lumen of the capsule



Figs 147–152. Radulae: 147–150 – *Pseudobithynia*, 151–152 – *Litthabitella chilodia*. Scale bars: 10 μm in 147 and 148; 5 μm in 149, 150 and 152; 2 μm in 151



Figs 153–157. Stomachs of Rissooidea: 153 – *Hydrobia*, 154 – *Dianella*, 155 – *Bythinella*, 156 – *Bithynia*, 157 – *Heleobia*: cae – caecum, dgo – opening of digestive gland, in – intestine, oe – oesophagus, ss – style sac

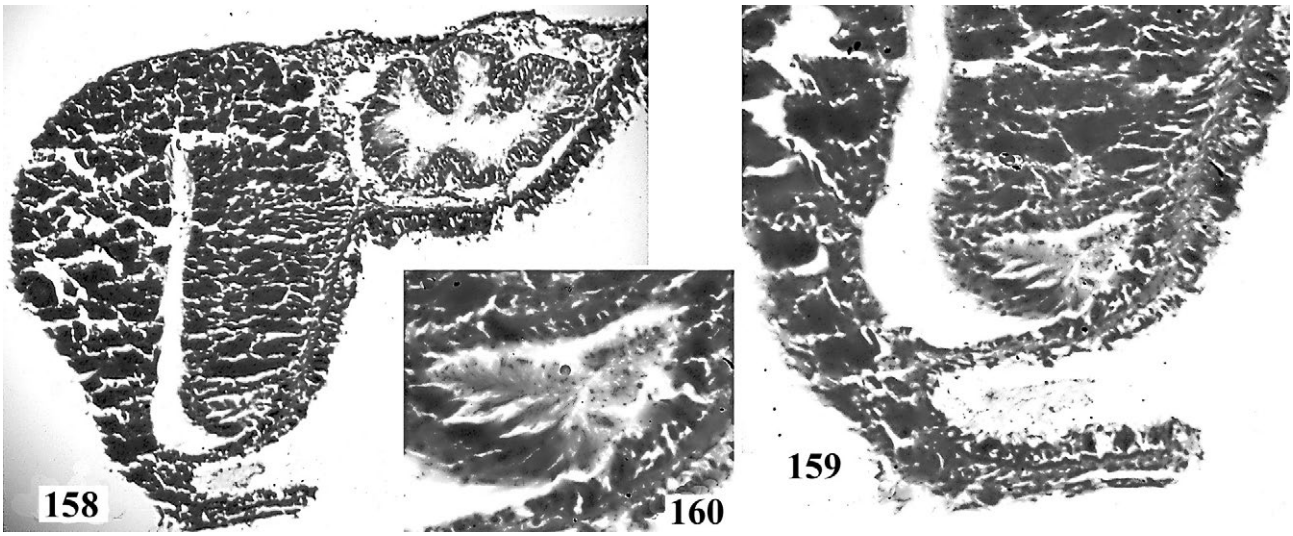
gland duct by a rather narrow fold (Fig. 159). In *Lithoglyphus* (Figs 161–169), like in the other rissooids that have a ventral channel, the oviduct becomes a closed tube (Fig. 166) that runs along the albumen gland, proximal (posterior) to the capsule gland, and after a short distance outbranches the duct of the bursa copulatrix (Figs 168–169). In this genus the fold that separates the ventral channel (Figs 161–166) is somewhat broader and bent (Fig. 166). The ventral channel is situated not ventrally, like in *Hydrobia*, but somewhat laterally (Figs 161–164).

In *Bithynia* (Figs 170–174) the pallial oviduct is a massive tube whose lateral walls are heavily thickened by sub-epithelial glands (Figs 170, 173), while the dorsal and ventral walls are thin. Despite its characteristic tubular habitus, the pallial oviduct organisation is not much different from the one found in e.g. *Ventrosia* or *Lithoglyphus*. The dorsal wall is surrounded by glands (Figs 170, 172, 173); the ventral wall in its section lying within the proximal (posteriormost) part of the albumen gland is glandular (Fig. 170), while the section that lies within the distal part of the albumen gland and the capsule gland (Figs 172–173) consists of a few muscle fibres. Formed in this way, the thin-walled ventral channel of *Bithynia* looks thus somewhat different than in *Hydrobia* or *Lithoglyphus*. The other unique structures of *Bithynia* and *Pseudobithynia* are: the seminal receptacle that, situated hydrobioid-typical, is unusually large (Fig. 171), and the vast bursa copulatrix (Fig. 174) that lies anteriorly (distally).

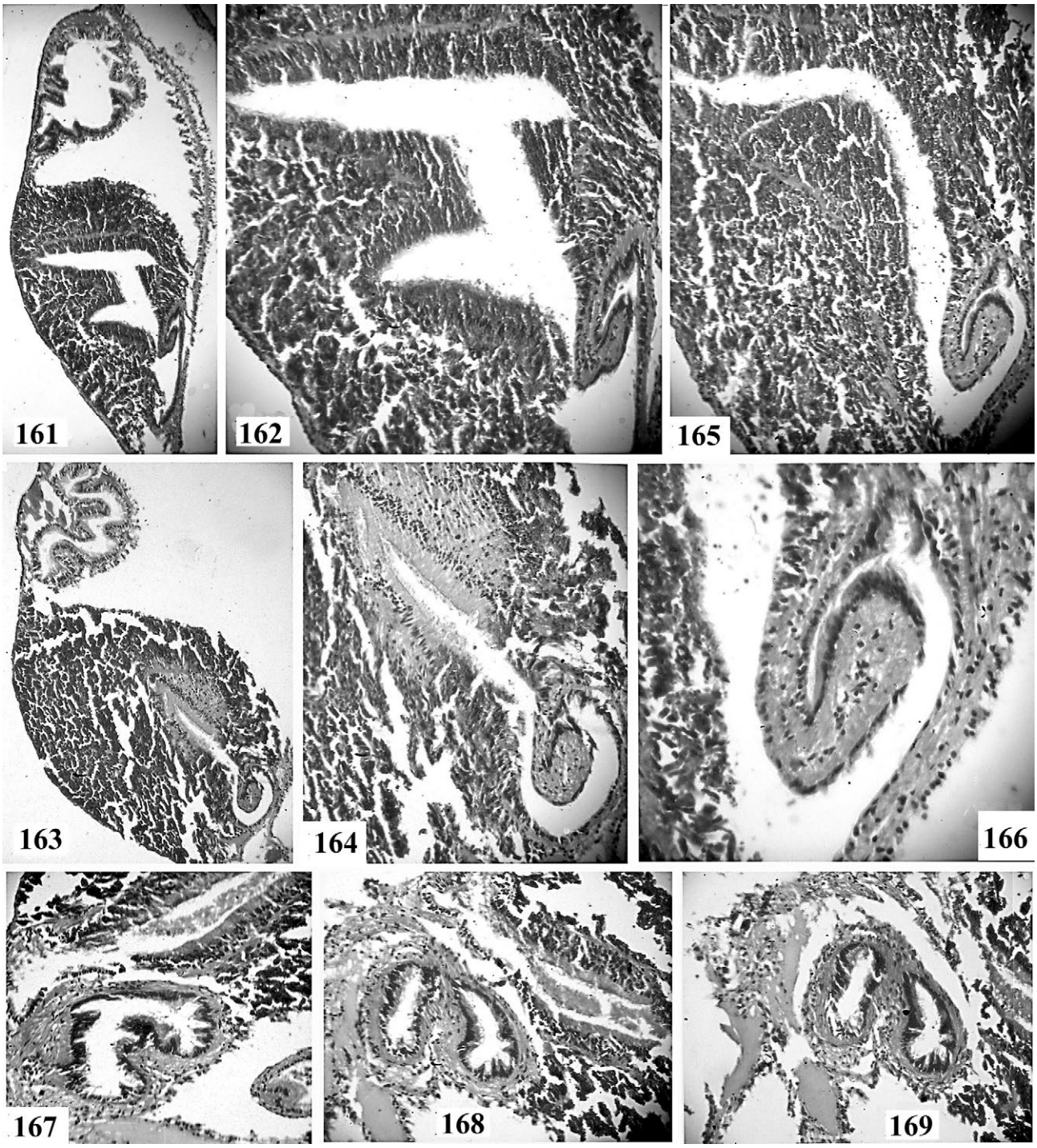
In *Bythinella*, semi-thin serial sections made perpendicular to the pallial oviduct along all the length of the capsule gland (Figs 175a-i, 197) show a very broad fold separating a broad and flat ventral channel which contacts with the lumen of the capsule gland along its whole length. Hence, though somewhat atypical, it is a "true" ventral channel, definitely not a duct.

The copulatory duct found in *Parabythinella* (Figs 176–177, 179–180 and 198) is separated from the lumen of the capsule gland along most of its length (Figs 176, 198) except a short anteriormost (distalmost) part where it joins the lumen of the gland (Fig. 177). Thus, there is only one gonoporus that opens in this region (Figs 179–180, 198) and serves two purposes: copulation and egg-lying. An identical duct is found in *Marstoniopsis*. From among the studied Balkan rissooideans a copulatory duct can also be found in *Heleobia* (Figs 181–182). It, however, does not resemble the one of *Parabythinella*. In *Heleobia* the females are diaulic: the gonoporus that occupies the terminal position at the capsule gland (the typical position of the gonoporus within the other rissooidea considered) serves only the purpose of egg deposition, there being another gonoporus situated at the posterior wall of the mantle cavity, which is for copulation only (Fig. 181). The duct terminates with a big, bulbous bursa copulatrix, and this, copulatory part of the organs is connected with the oviduct (thus all the other parts of the reproductive system) only at the terminal part of the renal oviduct, by a narrow bifurcation of the duct of the seminal receptacle (Fig. 182). In topography, the copulatory duct of *Heleobia* corresponds to the duct of the bursa copulatrix of other hydrobioids.

The studied specimens of *Paladilhiopsis carpathica* from Romania are most probably the first collected alive, all the literature data concerning the shell only. Thus the female reproductive organs of the species are illustrated herein (Fig. 178). The pallial complex of the glands is relatively short, folded, and externally the capsule gland cannot be easily distinguished from the albumen one. The gonoporus is situated at the pointed, distal end of the capsule gland; there is a typical ventral channel running along the gland. The most striking character of the organs is the extremely



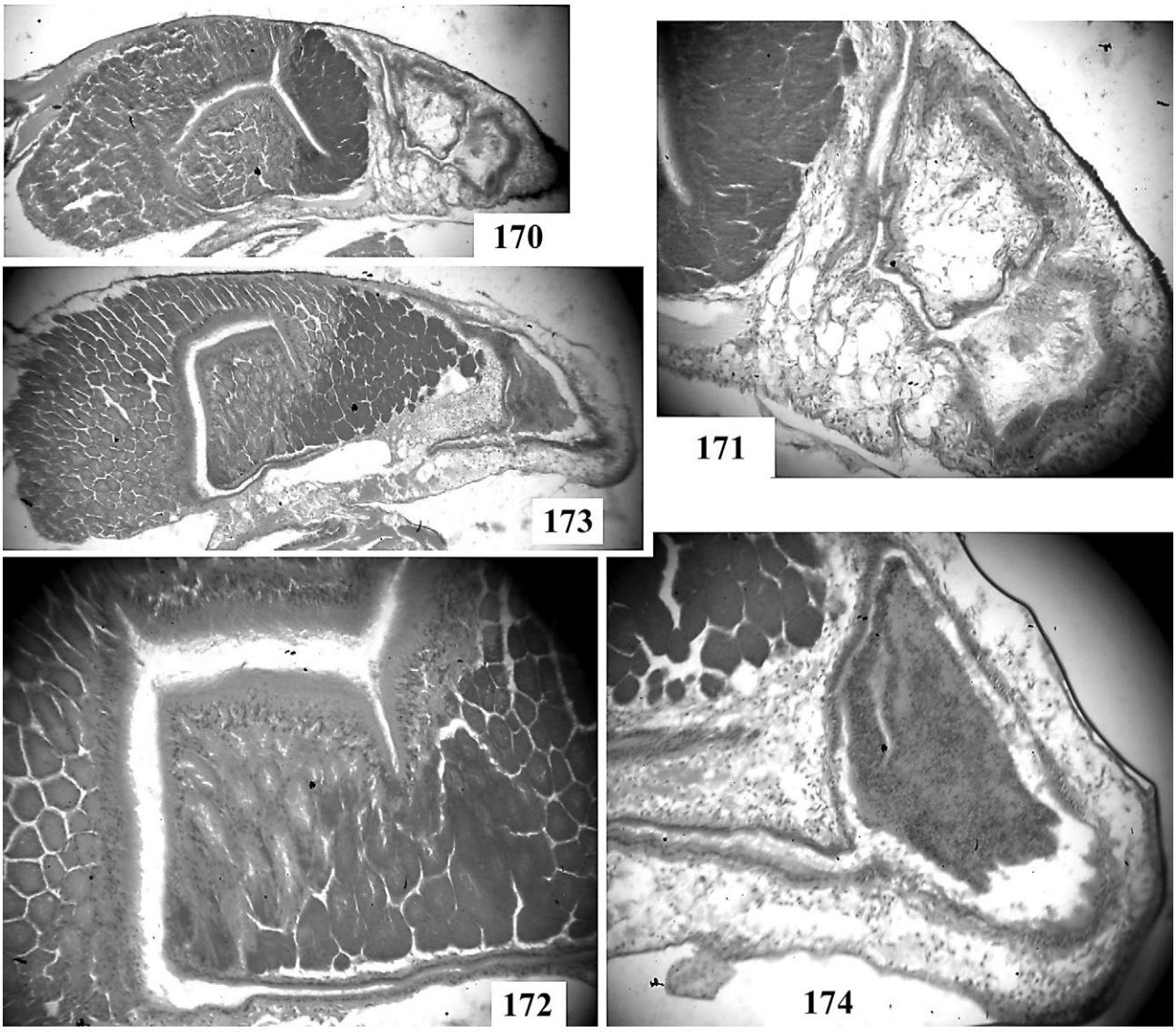
Figs 158–160. Perpendicular cross sections of female reproductive organs of *Ventrosia spalatiana*: 158 – section of capsule gland and kidney, 159 – fragment of 158, showing ventral channel, 160 – fragment of 159 showing details of ventral channel



Figs 161–169. Perpendicular cross sections of female reproductive organs of *Lithoglyphus naticoides*: 161–166 – sections of capsule gland: 161 – gross morphology of gland and kidney, 162 – fragment of 161 showing lumen of gland and ventral channel, 163 – gross morphology at another part, 164 – fragment of 163 showing details of gland and ventral channel, 165 – lumen of gland and ventral channel at another part of gland, 166 – fragment of 165 showing details of ventral channel; 167–169 – separation of oviduct from bursal duct

big, bulky bursa copulatrix. The bursa, approaching the length of nearly a half of the gland complex, lies behind the posterior end of the complex. The bursal duct, beginning at the ventral side of the posterior part of the bursa, runs along the bursal wall and joins the oviduct close to the posterior end of the albumen gland. The receptacle is pin-shaped, its small bulb lying at the end of a long duct, the diameter of which is similar to the diameter of the bursal duct. The renal oviduct forms a thick, U-shaped loop.

The female reproductive organs of *Parabythinella graeca* (Figs 179–180) look the same as in *Marstoniopsis*. As described above, they have a typical copulatory duct of the Amnicolidae. Examining the organs in



Figs 170–174. Perpendicular cross sections of female reproductive organs of *Bithynia tentaculata*: 170–171 – section at posterior (proximal) part: 170 – capsule gland together with seminal receptacle, 171 – fragment of 170 showing seminal receptacle with spermatozoa submerged with their heads within epithelium of receptacle, 172 – capsule gland showing its lumen and ventral channel; 173–174 – section at anterior (distal) part: 173 – gross morphology showing capsule gland and bursa copulatrix, 174 – fragment of 173 showing bursa copulatrix

Parabythinella (Figs 179–180) I found a big seminal receptacle (with oriented sperm). The same shape and dimensions of the receptacle, coupled with the lack of the bursa, I found in *Marstoniopsis* as well. In both taxa the complex of the accessory glands of the pallial oviduct is long and narrow, kidney-shaped. The renal oviduct is in the form of a small, slightly thickened loop. Having outbranched the duct of the receptacle, the oviduct divides into the copulatory duct and the branch that enters the lumen of the albumen gland. The big receptacle is probably used as a place for temporary sperm storage, thus may function as a bursa as well (I found also some unoriented sperm inside).

In *Heleobia dobrogica* as well as in *H. dalmatica* the females are much bigger and more common than males which comprise only a few percent of the population. It is possible to determine sex because in the females the zone of the pallial oviduct complex of glands is seen through the walls of the shell (Figs 42–43). In the fixed material it is bright reddish-yellowish (Fig. 181: the part intensively shaded black). The female reproductive organs in *H. dobrogica* (Figs 181–182) are diaulic, with a short copulatory duct opening at the posterior wall of the mantle cavity, a moderately big and bulbous bursa copulatrix, and a moderately big receptacle with a very long duct. The renal oviduct is rather thick, coiled to form a spire (that is more than one loop). The organs look in every detail the same as the ones, also examined, of *H. dalmatica*.

The pallial oviduct gland complex of *Litthabitella* is relatively short, lying far posteriorly, thus the gonoporus that lies at the terminal part of the capsule

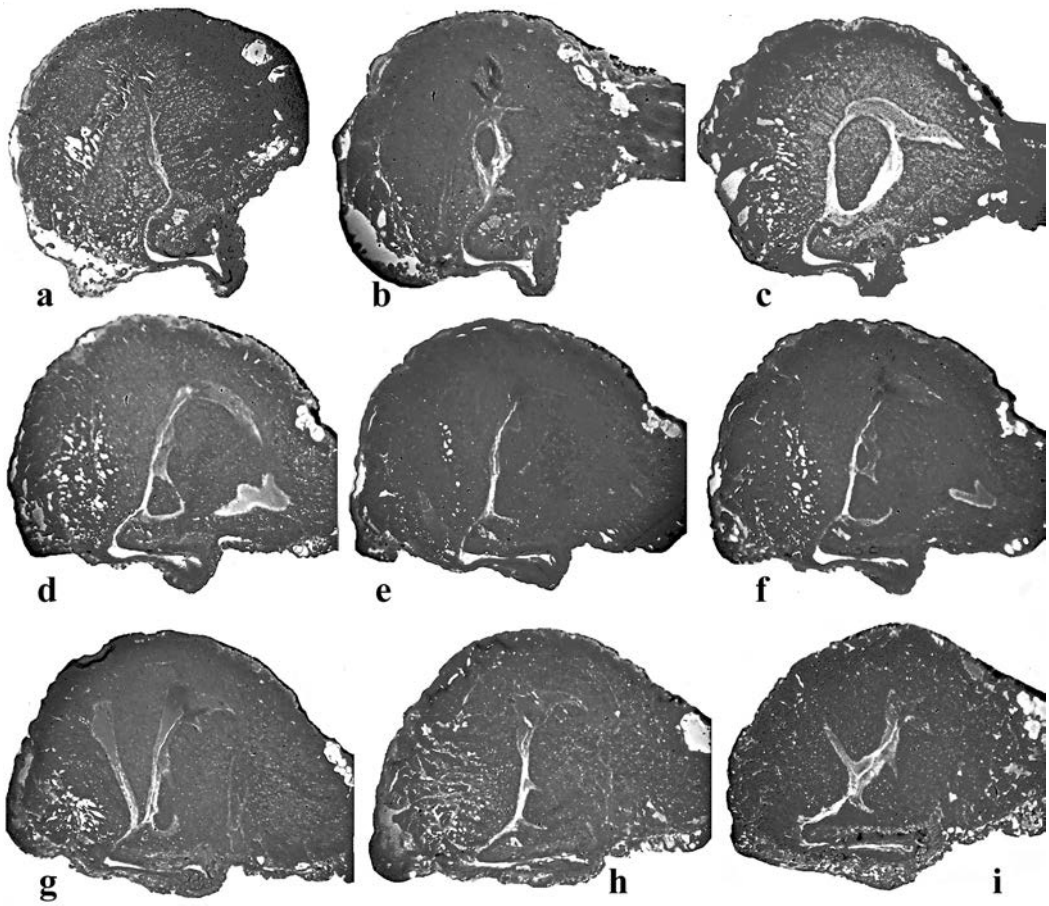
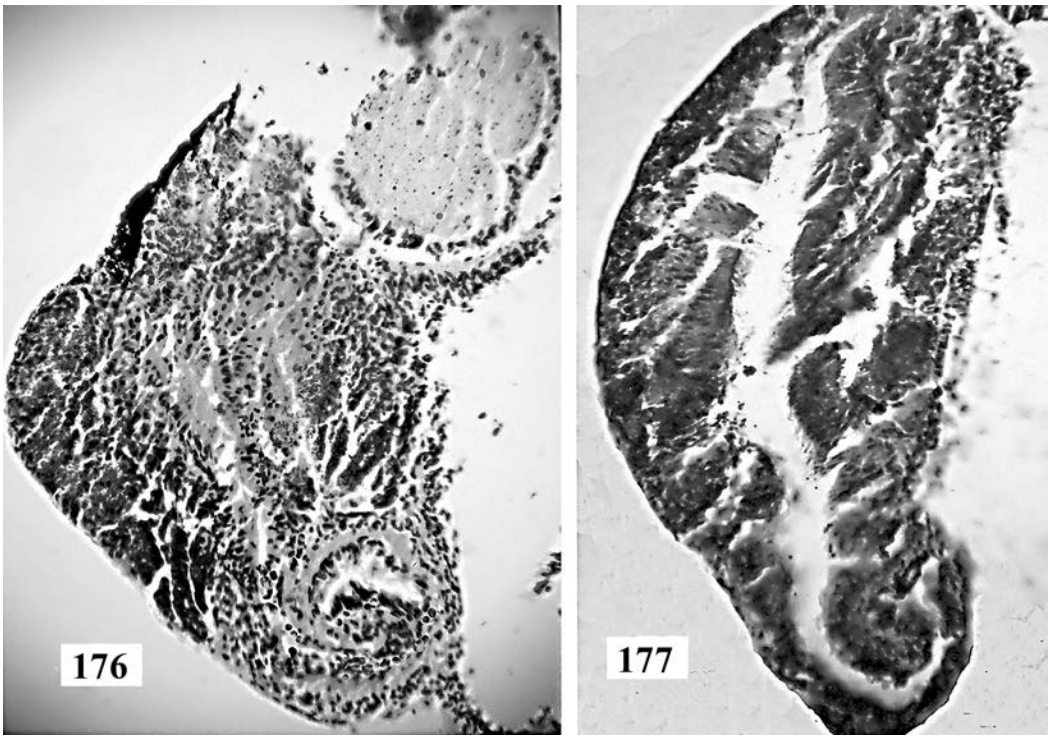


Fig. 175. Perpendicular cross sections of capsule gland in *Bythinella austriaca*, showing ventral channel separated by wide folds along all the gland



Figs 176–177. Perpendicular cross sections of capsule gland of female reproductive organs of *Parabythinella graeca*: 176 – proximal part with separate "spermathecal" duct, 177 – close to gonoporus with "spermathecal" duct connected with lumen of capsule gland

gland is situated deep inside the mantle cavity, far behind the anus (Fig. 183). Carefully examined, the female reproductive organs of *Litthabitella,* though they may mimic typical organs of the hydrobioids, reveal their distinctness. The ventral channel runs not on the ventral side of the capsule gland, but somewhat laterally (Figs 184, 186). The bursa copulatrix is situated like in a hydrobioid and there are two seminal receptacles: one near the junction of the oviduct and the duct of the bursa and the other at the end of the thickened part of the renal oviduct. The receptacles may or may not be homologues of the rs_1 and rs_2 of the hydrobioids (see below). The renal oviduct (Fig. 185) forms neither a spire nor a horseshoe-like loop but, unique among the studied gastropods, it is circular instead (Figs 184–185).

The female reproductive organs of all the taxa included in the present paper are schematically represented in Figs 187–199, which is sufficient to analyse the evolution of particular characters. All the characters in all the taxa are described in detail either in the literature cited above or in this paper. All data have been carefully checked. In all the genera there is a complex of accessory glands of the pallial oviduct: an albumen gland posteriorly, and a capsule gland anteriorly, a thickened muscular renal oviduct, a bursa copulatrix (may be lacking) and either one or two seminal receptacles. A receptacle can be in either rs_1 or rs_2 position (terminology after RADOMAN 1983); rs_1 means close to the junction of the oviduct and bursa copulatrix, while rs_2 means at the distal end of the thickened renal oviduct (Figs 187–199). In the major-

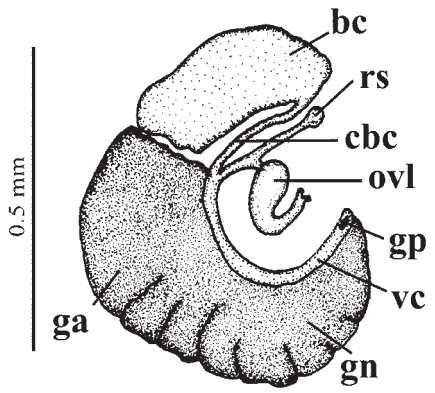
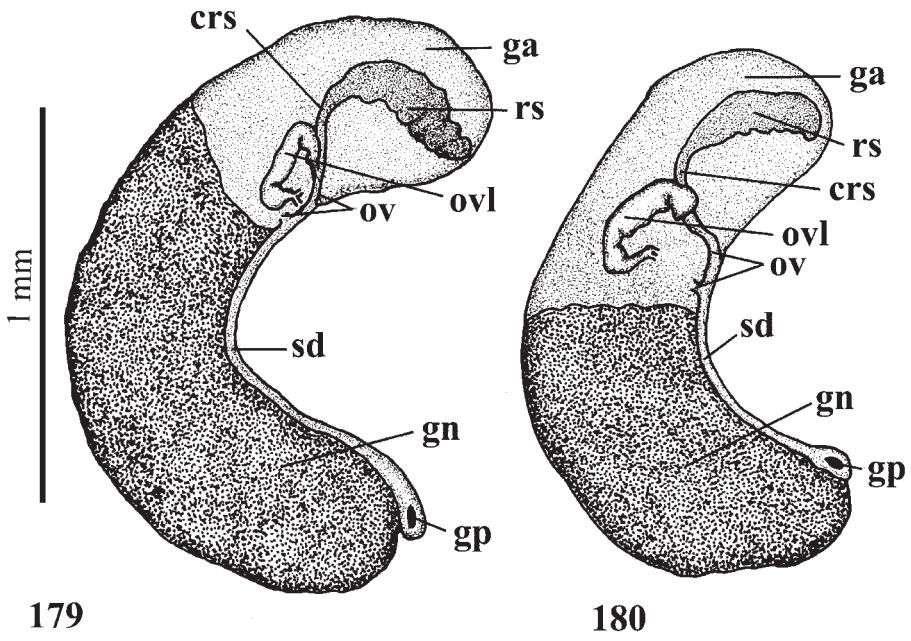


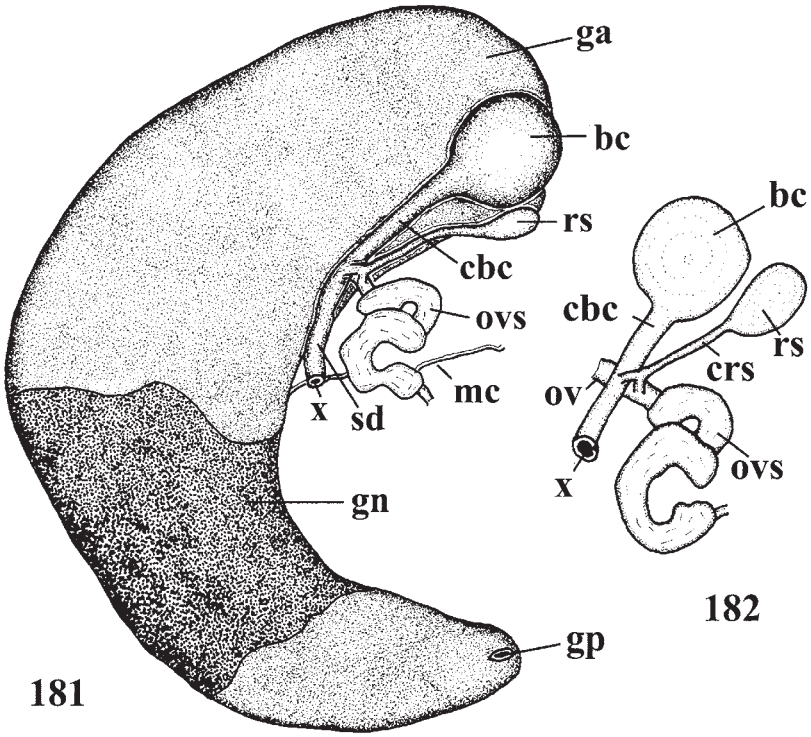
Fig. 178. Female reproductive organs of *Paladilhiopsis carpathica* (bc – bursa copulatrix, cbc – bursal duct , ga – albumen gland, gn – capsule gland, gp – gonoporus, ovl – loop of (renal) oviduct, rs – seminal receptacle (in position of rs_1), vc – ventral channel)

ity of the genera there is a ventral channel (Figs 187–197), which may have broad folds (Figs 196–197), whereas in some genera there is a copulatory duct instead (Figs 198–199).

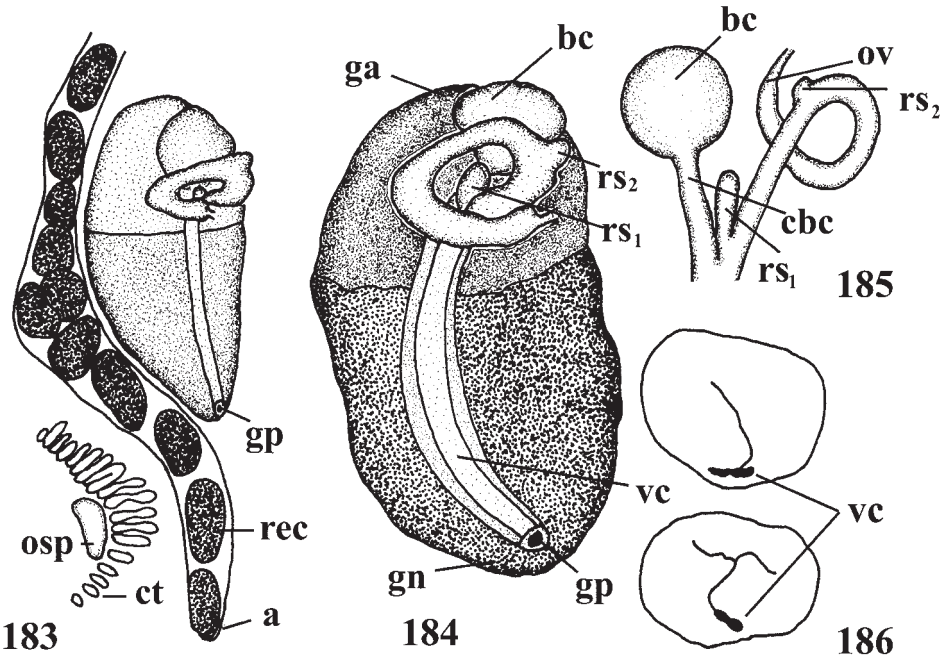
In *Hydrobia*, *Ventrosia*, *Adriohydrobia*, and *Pseudamnicola* (Fig. 187) there is a bursa copulatrix, one seminal receptacle (rs_1) and a coiled renal oviduct in the form of a black-pigmented spire. In *Pyrgula* and *Dianella* (Fig. 188) there is one receptacle (rs_2) situated at the end of a thickened, coiled renal oviduct in the form of a pigmentless spire. In *Belgrandiella*, *Graziana*, *Boleana*, *Pontobelgrandiella*, and *Hauffenia* (Fig. 189), besides the bursa copulatrix, there is one seminal receptacle (rs_1) and a single,



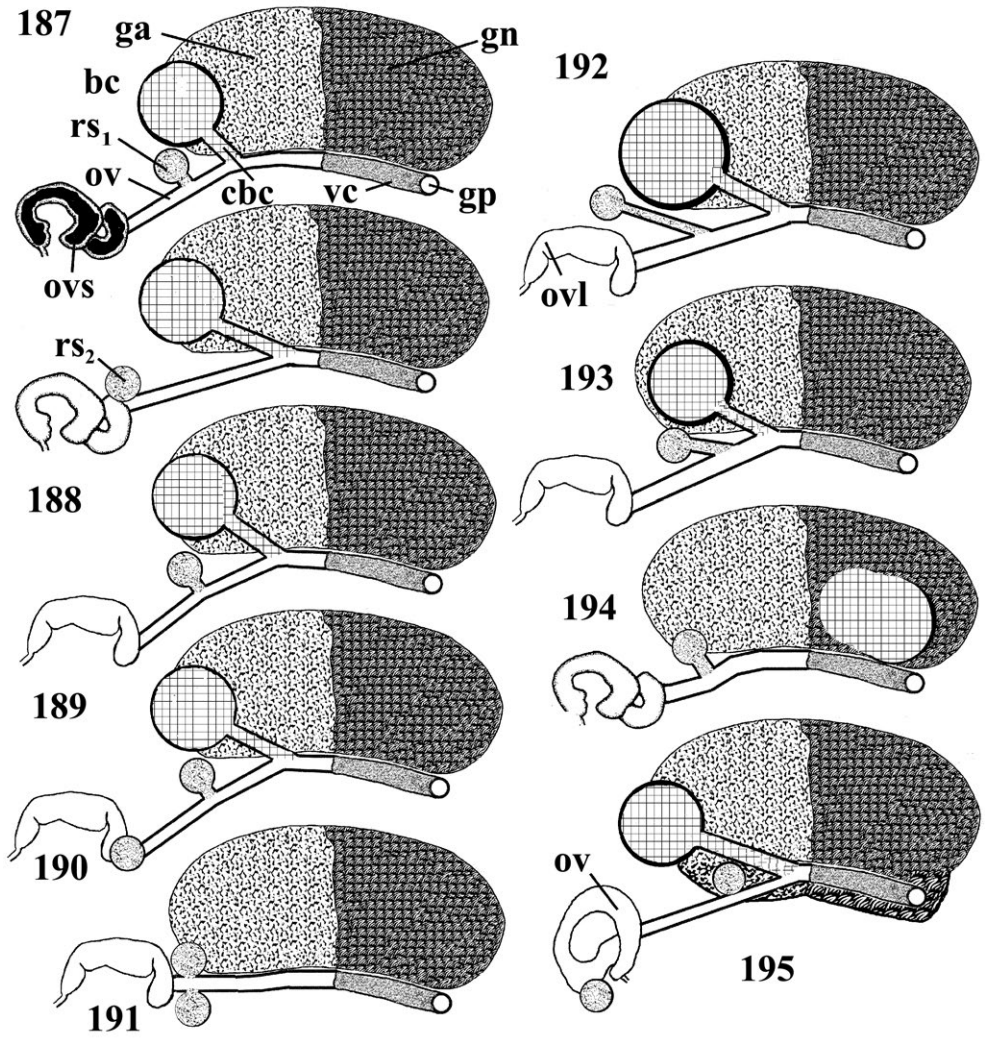
Figs 179–180. Female reproductive organs of *Parabythinella graeca* (crs – duct of seminal receptacle, ga – albumen gland, gn – capsule gland, gp – gonoporus, ov – oviduct, ovl – loop of (renal) oviduct, rs – seminal receptacle (in position of rs_1), sd – "spermathecal" duct)



Figs 181–182. Female reproductive organs of *Heleobia* (bc – bursa copulatrix, cbc – bursal duct , crs – duct of seminal receptacle: note its bifurcation forming junction between "spermathecal" duct and bursa with oviduct not joined at any other point, ga – albumen gland, gn – capsule gland, gp – gonoporus, mc – posterior end of mantle cavity, ov – oviduct, ovs – spire of (renal) oviduct, rs – seminal receptacle, sd – "spermathecal" duct, x – second (copulatory) gonoporus)



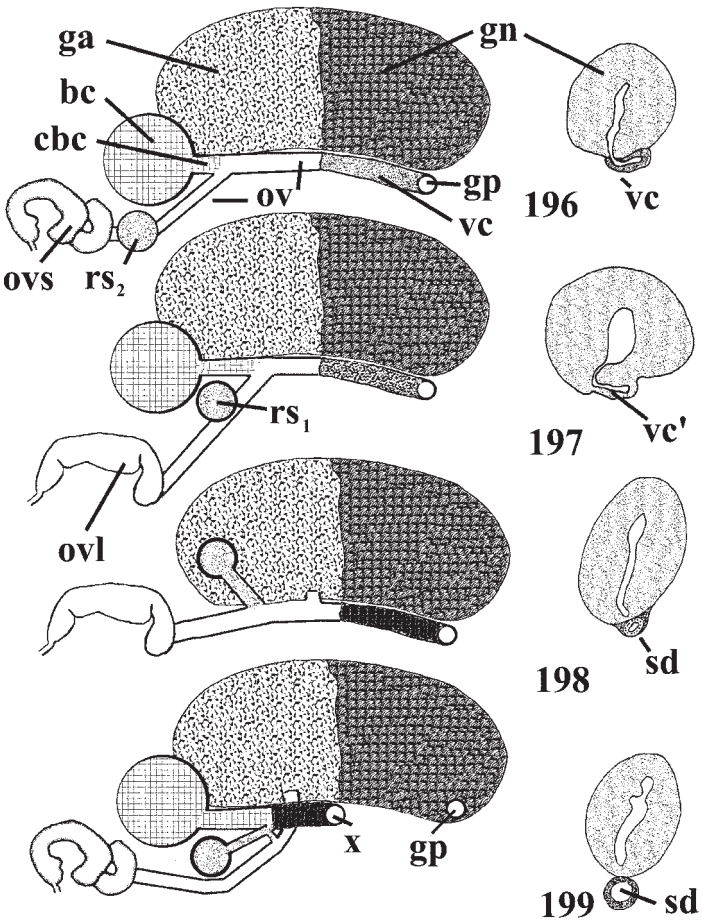
Figs 183–186. Female reproductive organs of *Litthabitella chilodia*: 183 – female organs with rectum, anus, ctenidium and osphradium; 184 – pallial section of the organs; 185 – complex of bursa copulatrix, receptacles and oviduct; 186 – perpendicular sections through the capsule gland (a – anus, bc – bursa copulatrix, cbc – bursal duct , ct – ctenidium, ga – albumen gland, gn – capsule gland, gp – gonoporus, osp – osphradium, ov – oviduct, rec – rectum, rs_1 – seminal receptacle close to junction of bursal duct and albumen gland, rs_2 – seminal receptacle adjoining renal oviduct, vc – ventral channel, vc' – ventral channel separated with broad folds, imitating spermathecal duct)



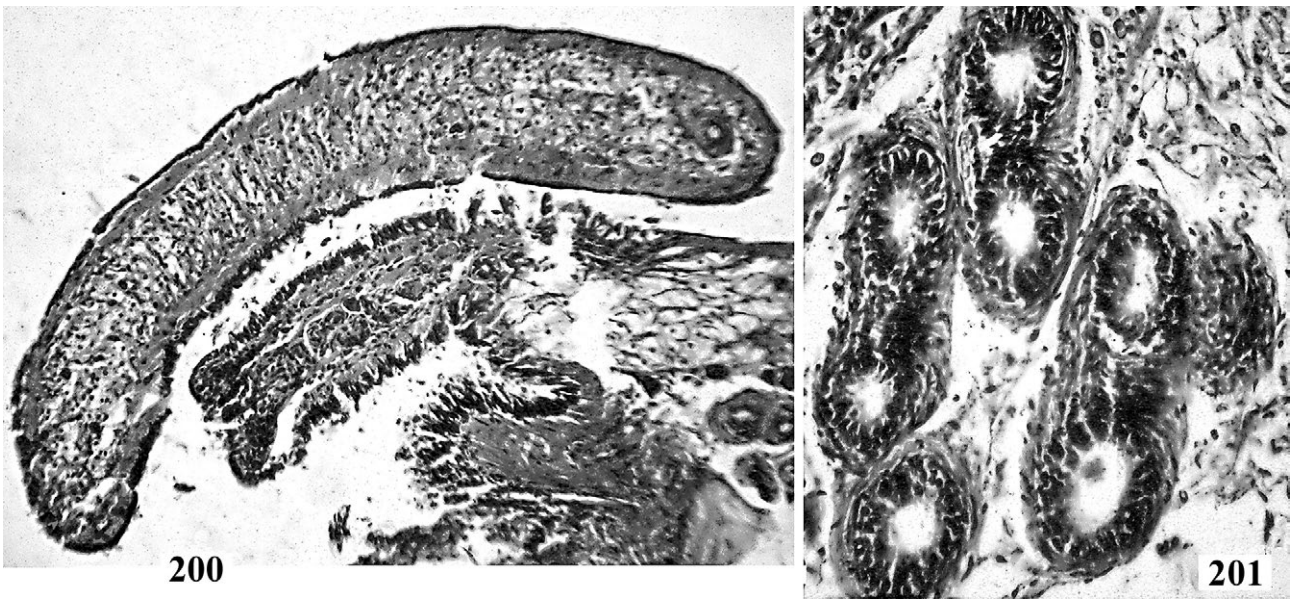
Figs 187–195. Schematic representation of female reproductive organs: 187 – *Hydrobia*, *Ventrosia*, *Adriohydrobia*, *Pseudamnicola*; 188 – *Pyrgula*, *Dianella*; 189 – *Belgrandiella*, *Graziana*, *Boleana*, *Pontobelgrandiella*, *Hauffenia*; 190 – *Orientalina*, *Sadleriana*, *Grossuana*, *Anagastina*, *Trichonia*, *Graecorientalia*, *Horatia*, *Daphniola*, *Alzoniella*; 191 – *Islamia*, 192 – *Paladilhiopsis*, 193 – *Lithoglyphus*, 194 – *Bithynia*, *Pseudobithynia*, 195 – *Litthabitella* (bc – bursa copulatrix, cbc – bursal duct , ga – albumen gland, gn – capsule gland, gp – gonoporus, ov – oviduct, ovl – loop of (renal) oviduct, ovs – spire of (renal) oviduct, rs_1 – seminal receptacle close to junction of bursal duct and albumen gland, rs_2 – seminal receptacle adjoining renal oviduct, vc – ventral channel)

horseshoe-shaped, flat loop of coiled oviduct. In *Orientalina*, *Sadleriana*, *Grossuana*, *Anagastina*, *Trichonia*, *Graecorientalia*, *Horatia*, *Daphniola*, and *Alzoniella* (Fig. 190) there is a bursa, two receptacles (rs_1 and rs_2) and a single, horseshoe-shaped, flat loop of coiled oviduct. The same coiling pattern (a single loop) of renal oviduct, and two receptacles can be found in *Islamia* (Fig. 191). However, situated at the same section of the oviduct, one receptacle in front of the other (Fig. 191) and there being no bursa, the receptacles cannot be homologized with either rs_1 or rs_2, (the region of rs_1 is distinguished as the region of the outlet of the bursa to the oviduct).

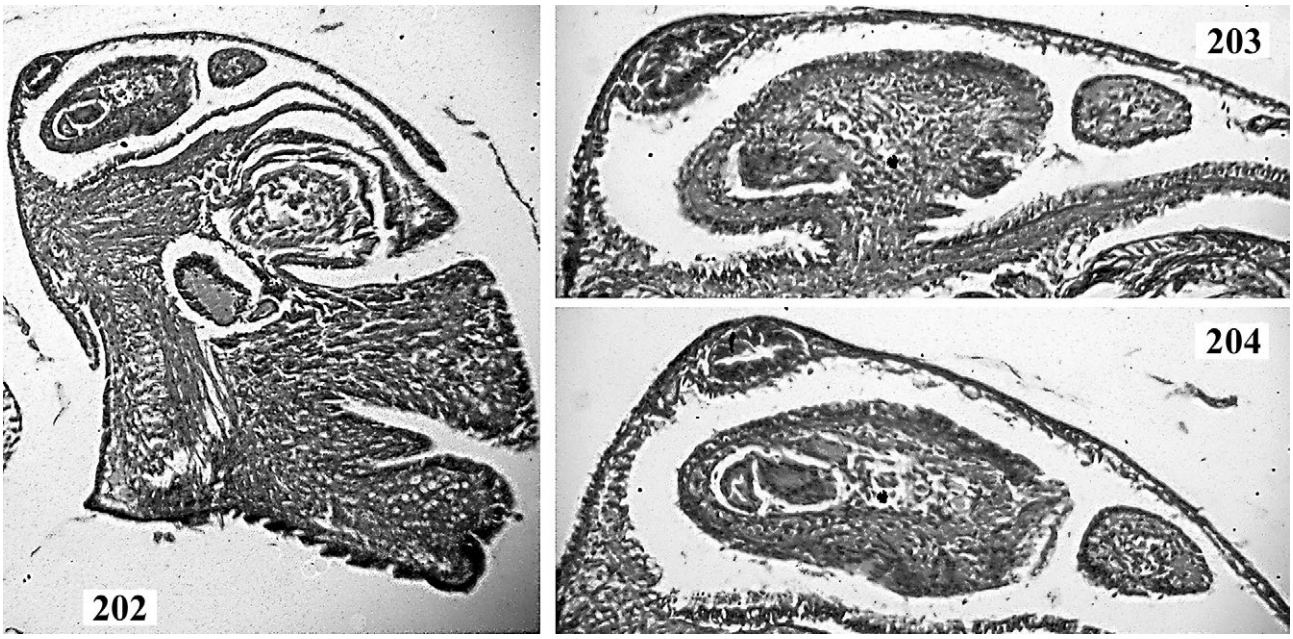
In *Paladilhiopsis* (Fig. 192) the bursa copulatrix is enormously big (compare Fig. 178), there is one pin-shaped receptacle (rs_1) with a very long duct, the renal oviduct forming a single, horseshoe-shaped, flat loop. The same coiling patter (single loop) of the renal oviduct, and one seminal receptacle in the position of rs_1 are characteristic of *Lithoglyphus* (Fig. 193), but the bursa copulatrix of this genus is embedded in the tissue of the albumen gland. In *Bithynia* and *Pseudobithynia* (Fig. 194) there is no posteriorly-located bursa copulatrix in the typical hydrobioid form, situated at the proximal end of the albumen gland. Instead, there is a vast, thin-walled, anterior bursa which spreads along the distal part of the capsule gland. Surrounded by the tissue of the albumen gland, there is one seminal receptacle which may be a homologue of rs_1 (this, however, is not easy to determine without a posterior bursa), and a renal oviduct in the form of a spire. In the aforementioned *Littha-*



Figs 196–199. Schematic representation of female reproductive organs: 196 – *Emmericia*; 197 – *Bythinella*; 198 – *Parabythinella*, *Marstoniopsis*; 199 – *Heleobia* (bc – bursa copulatrix, cbc – bursal duct , ga – albumen gland, gn – capsule gland, gp – gonoporus, ov – oviduct, ovl – loop of (renal) oviduct, ovs – spire of (renal) oviduct, rs_1 – seminal receptacle close to the junction of bursal duct and albumen gland, rs_2 – seminal receptacle adjoining renal oviduct, sd – "spermathecal" duct, x – second (copulatory) gonoporus, vc – ventral channel, vc' – ventral channel separated with broad folds, imitating spermathecal duct)



Figs 200–201. Male reproductive organs of *Parabythinella graeca*: 200 – section of penis, 201 – perpendicular section of flagellum

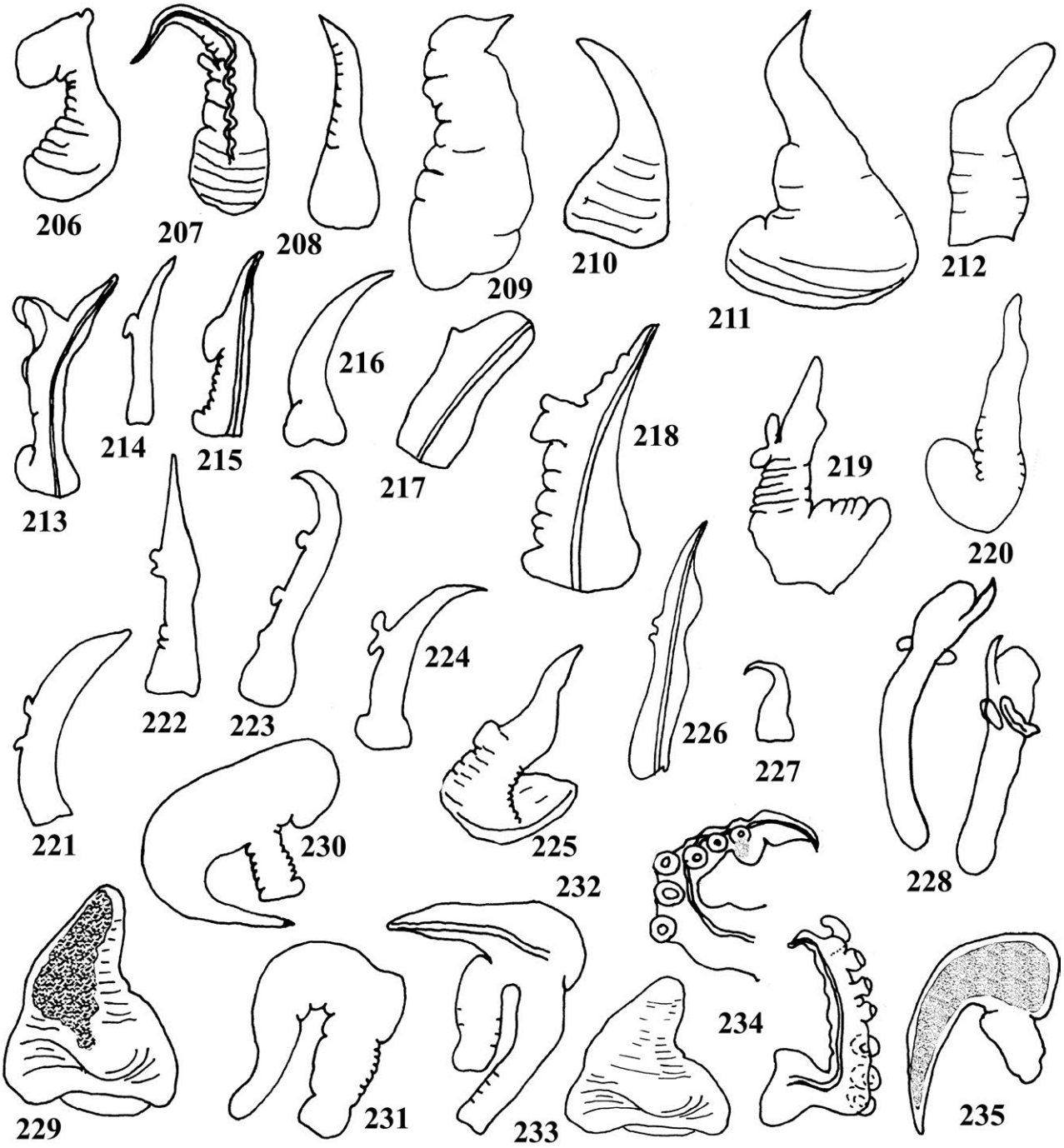


Figs 202–204. Sections of male of *Bythinella austriaca*: 202 – section of snail showing foot, buccal mass and mantle cavity with penis inside it; 203–204 – sections of penis

bitella (Fig. 195) the female reproductive organs have the appearance of the ones found in the other hydrobioids, the ventral channel runing in a different manner, the renal oviduct forming neither a spire nor horseshoe-shaped loop but a circular coil, the homology of the two receptacles and rs_1 and/or rs_2 uncertain.

In *Emmericia* (Fig. 196) there is a ventral channel separated from the lumen of the capsule gland by a relatively broad fold. The bursa copulatrix is in the typical, posterior position (Fig. 196), close to the proximal part of the albumen gland, there is one seminal receptacle (rs_2), and a spire of the thickened renal oviduct. In *Bythinella* (Fig. 197) the fold that separates the ventral channel from the lumen of the capsule gland is very broad; there is a bursa, one receptacle in the position of rs_1, and a horseshoe-shaped, flat loop of the thickened renal oviduct. In *Parabythinella* (Fig. 198) there is a copulatory duct running along the capsule gland to a common opening (gonoporus) of the duct and the lumen of the capsule gland; there is no bursa copulatrix, instead of which there is one very big receptacle with a duct; the thickened renal oviduct forms a horseshoe-shaped, flat loop. In *Heleobia* (Fig. 199) there also is a copulatory duct, but it terminates at the posterior wall of the mantle cavity, thus the female is diaulic. The copulatory duct terminates with a big bursa copulatrix and is

Fig. 205. Semi-thin sections of flagellum in *Bythinella austriaca*



Figs 206–235. Penes of Rissooidea: 206 – *Hydrobia*, 207 – *Ventrosia*, 208 – *Adriohydrobia*, 209 – *Pseudamnicola*, 210 – *Pyrgula*, 211 – *Dianella*, 212 – *Alzoniella*, 213 – *Islamia*, 214 – *Graziana*, 215 – *Belgrandiella*, 216 – *Boleana*, 217 – *Hauffenia*, 218– *Orientalina*, 219 – *Grossuana*, 220 – *Sadleriana*, 221 – *Anagastina*, 222 – *Horatia*, 223 – *Pontobelgrandiella*, 224 – *Graecorientalia*, 225 – *Daphniola*, 226 – *Trichonia*, 227 – *Paladilhiopsis*, 228 – *Litthabitella*, 229 – *Lithoglyphus*, 230 – *Bithynia*, 231 – *Bythinella*, 232 – *Emmericia*, 233 – *Pseudobithynia*, 234 – *Heleobia*, 235 – *Parabythinella, Marstoniopsis*

connected with the oviduct by a bifurcation of the duct of the seminal receptacle (whose homology thus cannot be determined, though apparently it is not an rs_2). The thickened renal oviduct is coiled to form more than one loop.

Male reproductive organs

The flagellum (penial gland) is a long tube with a blind terminus within the haemocoel of the head, thickened in this region and arranged in a series of tight coils positioned dorsal to the oesophagus, and

outletting by the second (left) arm of the penis (Figs 200, 202–204) or the second and third arms (*Emmericia* – see Appendix 2 in the present paper: Figs 25–34). The structure looks similar in all the gastropods in which it has been found. The morphological identity of a structure in different taxa, especially if the structure is not simple, supports the assumption of homology of the structure. For this reason I have examined the histological structure of the penial gland. In *Bithynia*, *Parabythinella* (Fig. 201), *Marstoniopsis*, *Bythinella* (Fig. 205) and *Emmericia* (Fig. 29 in Appendix 2 in this volume) serial sections reveal exactly the same structure. The tube is coated with a thin layer of circular muscles that surround a series of columnar glandular cells that have basal nuclei and discharge into a narrow, central lumen, not cilliated (Figs 201, 205, also Appendix 2 in this volume: Fig. 29).

The penes (except the flagella in the bi- and triarmed verges) are heavily ciliated. Their habitus and gross morphology (Figs 206–235) are much differentiated among the studied gastropods. A simple penis with neither a second arm nor an outgrowth is found in *Adriohydrobia* (Fig. 208), *Pseudamnicola* (Fig. 209), *Pyrgula* (Fig. 210), *Alzoniella* (Fig. 212), *Boleana* (Fig. 216), *Sadleriana* (Fig. 220), *Paladilhiopsis* (Fig. 227), *Lithoglyphus* (Fig. 229), and *Pseudobithynia* (Fig. 233). In some of the taxa listed above the simple penis, when in resting position, is folded in two (see Fig. 210, but this arrangement is found in many others, like *Pseudamnicola*, *Pyrgula*, *Dianella*). The penis of *Pseudobithynia* (Fig. 233) is worth mentioning. In this genus, there being no trace of a second arm or a tubular gland in the penis, the other conchological, ultrastructural, opercular, radular, anatomical characters considered (the characteristic female reproductive organs included) agree in every detail with the corresponding characters of *Bithynia*.

In the other genera there may be a small accessory arm (not containing a flagellum) and/or outgrowths. Such penes are found in *Hydrobia* (Fig. 206), *Ventrosia* (Fig. 207), *Dianella* (Fig. 211), *Graziana* (Fig. 214), *Belgrandiella* (Fig. 215), *Hauffenia* (Fig. 217), *Orientalina* (Fig. 218), *Grossuana* (Fig. 219), *Anagastina* (Fig. 221), *Horatia* (Fig. 222), *Pontobelgrandiella* (Fig. 223), *Graecorientalia* (Fig. 224), *Daphniola* (Fig. 225) and *Trichonia* (Fig. 226).

The penis of *Islamia* (Fig. 213) with two-three distinct arms is very characteristic, the same concerns the very big penis of *Litthabitella* (Fig. 228), whose three outgrowths are not found elsewhere.

The penis of *Heleobia* (Fig. 234) bears seven-nine outgrowths in the form of circular suckers. The morphology of the outgrowths strongly suggests that they may act as real suckers. They probably help to position the penis inside the female's mantle cavity, the gonoporus used for copulation in *Heleobia* being situated as deep as the posterior wall of the mantle cavity. Finally, in *Bithynia* (Fig. 230), *Bythinella* (Fig. 231), and *Parabythinella/Marstoniopsis* (Fig. 235), there are penes, the second arm of which contains the terminal part of the tubular gland (flagellum), all the penes looking alike. In *Emmericia* (Fig. 232) the penis is different, there being a third arm (for details see Appendix 2 in this paper).

MOLECULARLY INFERRED PHYLOGENIES

The maximum-parsimony heuristic search based on 18S data, assuming each base substitution equally probable and not compensating for hidden substitution, thus obviously underestimating the number of substitutions and prone to homoplasies (STRIMMER & HAESELER 2003), resulted in 24,550 trees, 215 steps long, with CI=0.512, RC=0.362. Out of 436 bp, 354 were constant, 22 variable but evolutionary parsimony-uninformative, and 60 parsimony-informative. The majority-rule consensus tree (Fig. 236), despite the oversimplified model given above, has a topology that is not much different from the one inferred with the more sophisticated techniques (compare Fig. 236 with 237 and 238). Close to the outgroup there are: *Lithoglyphus*, *Emmericia*, *Paladilhiopsis* and *Bythiospeum* (the latter two close to each other, although, strictly, not in the same clade), and *Heleobia*. The amnicolids form one clade included in the clade of *Bythinella*, *Bithynia*, and *Pseudobithynia* (the latter close to *Bithynia*). *Litthabitella* is placed between all the above non-hydrobiid (in the strict sense) taxa and the hydrobiids. The Hydrobiidae sensu stricto (*Hydrobia*, *Ventrosia*, *Adriohydrobia*, *Pyrgula*, *Dianella*, *Pseudamnicola*, and *Adrioinsulana*) are nearly monophyletic. *Pontobelgrandialla* and *Alzoniella* are not close to *Belgrandiella*, the latter forming a clade with *Boleana* and *Trichonia*, close to *Graecorientalia*, *Anagastina* and *Daphniola*.

In the maximum-likelihood tree based on 18S data (Fig. 237) the placement of *Lithoglyphus*, *Emmericia*, *Paladilhiopsis*, *Bythiospeum*, and *Heleobia* is the same as in the maximum-parsimony tree. The amnicolids are in one clade, close to *Bythinella*, *Bithynia*, and *Pseudobithynia*, the latter two genera being close to each other but not in the same clade. The position of *Litthabitella* is the same as in the maximum-parsimony tree, the same concerning *Pontobelgrandiella* and *Islamia*. The Hydrobiidae sensu stricto (*Hydrobia*, *Ventrosia*, *Adriohydrobia*, *Pyrgula*, *Dianella*, *Pseudamnicola*, *Adrioinsulana*) are monophyletic without *Adriohydrobia*, with which they are paraphyletic. All the other taxa form one clade, which suggests monophyly of the rest of the hydrobiids (excepting *Pontobelgrandiella* and *Islamia*). *Boleana*, *Trichonia* and *Belgrandiella* are in one clade, which contains *Graziana* besides. *Graecorientalia* and *Belgrandiella* showing the higher amounts of anagenesis, all the other branches are short.

Table 1. Morphological characters and their states: 1 – shell outline (0: valvatiform, 1: trochiform, 2: neritiform, 3: ovate-conical, 4: conical, 5: turriform); 2 – outer lip relative to rest of apertural plane (0: simple, 1: reflected, 2: with varex behind outer lip); 3 – shape of adapical and abapical portions of outer lip (0: simple, 1: adapically sinuous, 2: abapically sinuous); 4 – umbilicus (0: absent, 1: narrow, 2: wide); 5- protoconch sculpture: (0: absent, 1: reticulate, 2: spiral); 6 – arrangement of pores on protoconch (0: absent, 1: irregular, 2: honeycombe-like net); 7 – number of pairs of basal cusps on central tooth (0: one/two pairs, 1: two pairs, 2: more than two pairs, 3: absent); 8 – position of basal cusps (0: arise from lateral wing, 1: arise from tooth face, 2: absent); 9 – posterior caecum (0: present, 1: rudimentary or absent); 10 – pigmentation of coiled renal oviduct (0: absent, 1: present); 11 – coiling pattern of renal oviduct (0: single loop, 1: two or more loops (spire)); 12 – pallial oviduct organisation (0: *Hydrobia*-like, 1: *Bythinella*-like, 2: amnicolid-like, 3: *Heleobia*-like); 13 – number and position of seminal receptacles (0: one distal (rs_1), 1: one proximal (rs_2), 2: two receptacles (one distal, one proximal)); 14 – position of bursa copulatrix (0: posterior, 1: absent, 2: anterior); 15 – flagellum (0: absent, 1: simple, 2: bifurcate); 16 – simple lobes on left edge of penis (0: absent, 1: apical, 2: lateral) 17 – sucker-like glands (0: absent, 1: present)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Hydrobia	4	0	0	1	2	1	0	0	0	1	1	0	0	0	0	0	0
Ventrosia	4	0	0	1	1	1	0	0	0	1	1	0	0	0	0	0	0
Adriohydrobia	4	0	0	1	2	1	0	0	0	1	1	0	0	0	0	0	0
Pyrgula	5	0	1	0	2	1	3	2	0	0	1	0	1	0	0	0	0
Dianella	5	0	1	1	2	1	3	2	0	0	1	0	1	0	0	0	0
Pseudamnicola	3	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0
Adrioinsulana	3	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0
Orientalina	3	0	0	1	1	0	0	0	1	0	0	0	2	0	0	2	0
Sadleriana	1	0	0	1	0	0	0	0	1	0	0	0	2	0	0	0	0
Anagastina	4	0	0	1	1	1	0	0	1	0	0	0	2	0	0	2	0
Grossuana	3	0	0	1	1	0	0	0	1	0	0	0	2	0	0	2	0
Trichonia	3	0	1	1	1	0	0	0	1	0	0	0	2	0	0	2	0
Daphniola	0	0	0	2	1	0	0	0	1	0	0	0	2	0	0	2	0
Horatia	0	0	2	2	1	0	0	0	1	0	0	0	2	0	0	2	0
Graecorientalia	3	0	0	1	1	1	0	0	1	0	0	0	2	0	0	2	0
Belgrandiella	3	1	1	1	1	1	0	0	1	0	0	0	0	0	0	2	0
Boleana	3	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0
Alzoniella	3	0	0	1	1	?	0	0	1	0	0	0	2	0	0	0	0
Graziana	3	1	1	1	1	1	0	0	1	0	0	0	0	0	0	2	0
Pontobelgrandiella	3	1	0	0	1	0	0	0	1	0	0	0	0	0	0	2	0
Hauffenia	0	0	0	2	1	0	0	0	1	0	0	0	0	0	0	2	0
Islamia	0	0	0	2	1	0	0	0	1	0	0	0	2	1	0	1	0
Emmericia	3	2	0	1	2	2	3	2	1	0	1	0	1	0	2	0	0
Lithoglyphus	2	1	1	0	2	0	2	1	1	0	0	0	0	0	0	0	0
Bythinella	3	0	0	1	2	2	2	0	1	0	0	1	0	0	1	0	0
Marstoniopsis	3	0	0	1	2	1	2	0	1	0	0	2	0	1	1	0	0
Parabythinella	3	0	0	1	2	1	1	0	1	0	0	2	0	1	1	0	0
Bithynia	3	1	0	1	2	2	2	1	0	0	1	0	0	2	1	0	0
Pseudobithynia	3	1	0	1	2	2	2	1	0	0	1	0	0	2	0	0	0
Paladilhiopsis	5	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0
Bythiospeum	5	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0
Heleobia	5	0	1	1	1	1	0	0	0	0	1	3	0	0	0	0	1
Litthabitella	3	1	1	1	1	0	0	0	1	0	0	0	2	0	0	1	0

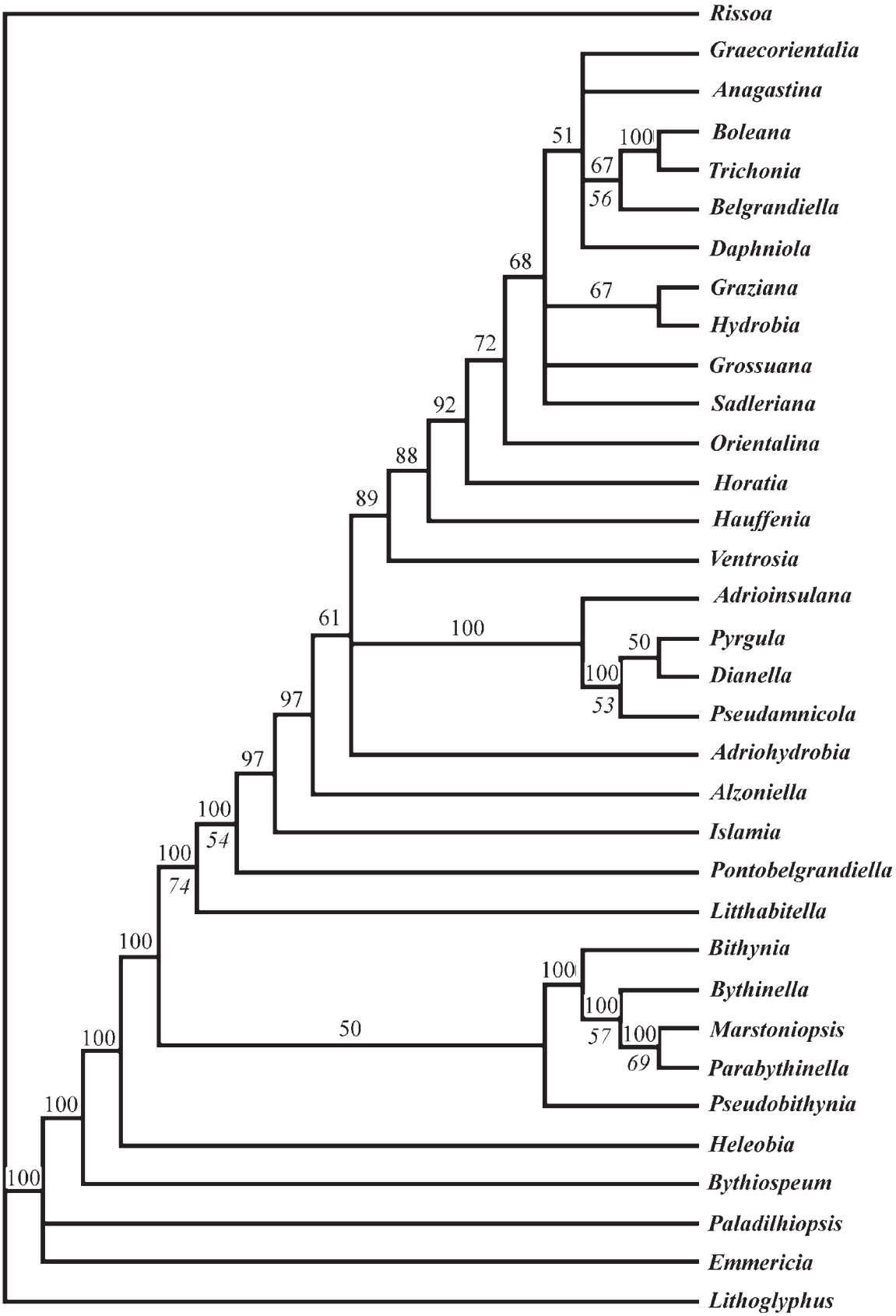


Fig. 236. Majority–rule consensus tree based on 18S RNA gene sequences (436 bp, 354 constant, 22 variable parsimony-uninformative, 60 parsimony–informative), heuristic search, computed of 24,550 maximum parsimony trees 215 steps long, whose CI=0.512, RC=0.362. In normal font given percent of trees with a given branch, in italics bootstrap support (1,000 replicates) for the branches whose support was higher than 50%

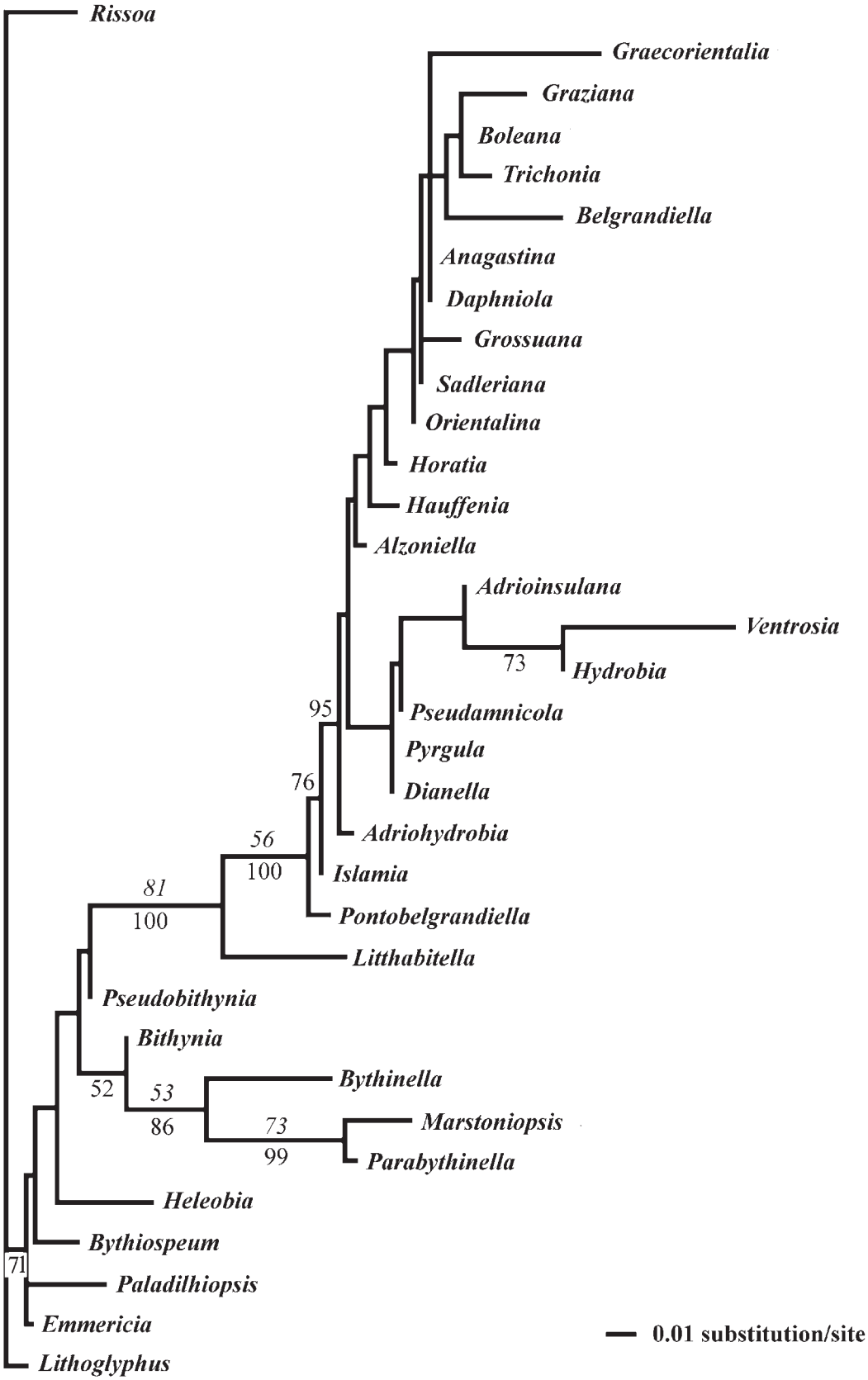


Fig. 237. Maximum likelihood phylogram based on 18S RNA gene sequences, model applied: Tamura & Nei + I + Γ; equal frequencies; rate matrix: 1.000, 1.744, 1.000, 1.000, 4.862, 1.000; proportion of invariable sites: 0.707; Γ distribution shape parameter: 0.519. Heuristic search (TBR), score of tree: 1669.662. In italics bootstrap support (1,000 replicates) for the branches whose support was higher than 50%, in normal font given Bayesian probabilities for the branches common with the tree presented in Fig. 238

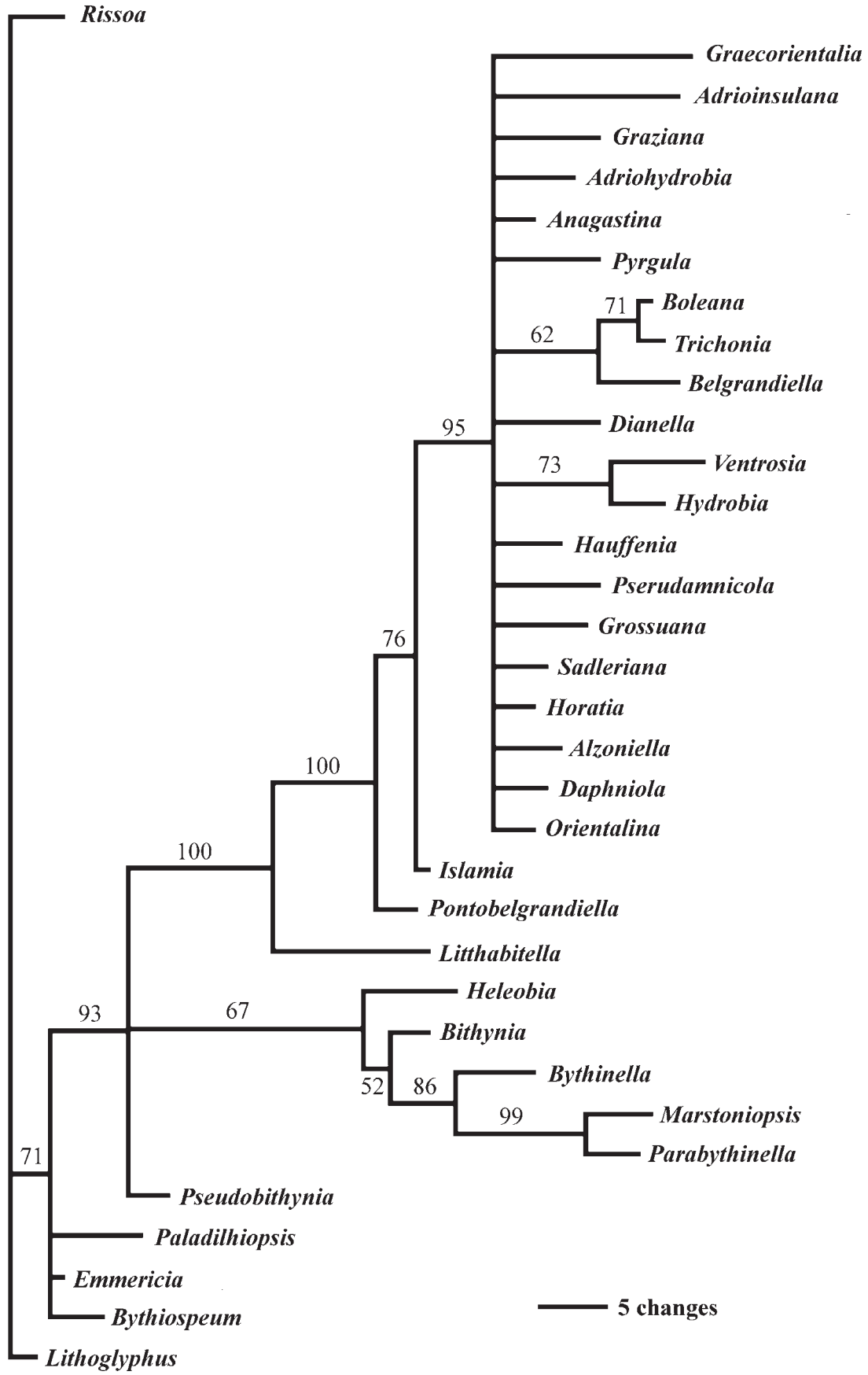


Fig. 238. Bayesian maximum likelihood phylogram based on 18S RNA gene sequences, equal frequencies, model SYM + I + Γ, rate matrix estimated by MrBayes. 2,000,000 generations. Bayesian probabilities given for each clade

The Bayesian maximum likelihood consensus phylogram based on the 18S data (Fig. 238) is resolved less than the maximum likelihood PAUP-computed one (Fig. 237) but shows the same pattern. Like in the latter, *Litthabitella* is here placed between the hydrobioids and the other taxa, *Lithoglyphus*, *Emmericia*, *Paladilhiopsis* and *Bythiospeum* are close to the root. *Paladilhiopsis* and *Bythiospeum* are also close to each other but not forming a monophyletic clade. The amnicolids, *Bythinella* and *Bithynia* form a clade, which besides them includes *Heleobia*. Interestingly, *Pseudobithynia* is far from *Bithynia*. *Pontobelgrandiella* and *Islamia* are again placed not outside the other hydrobiids. The latter form one unresolved polytomy, with only two clades: one of *Hydrobia* and *Ventrosia* and the other of *Boleana*, *Trichonia* and *Belgrandiella*. The model assumed being basically the same as in the maximum likelihood PAUP tree, the parameters estimated by MRBAYES are somewhat different from the ones applied to PAUP.

Unfortunately, for CO1, not all of the taxa considered for 18S were included, thus the positions of some taxa, like *Belgrandiella*, *Pontobelgrandiella*, *Litthabitella*, or *Emmericia* are checked with another data set. The maximum likelihood tree computed for the CO1 data with PAUP (Fig. 239) shows *Heleobia* close to the outgroup. *Bithynia* and *Bythinella* form a monophyletic clade, although the amounts of anagenesis along both branches are high. Together with *Bythinella*, *Marstoniopsis*, *Lithoglyphus* and *Bythiospeum*, the former two taxa form one clade. The Hydrobiidae s. stricto (*Hydrobia*, *Ventrosia*, *Adriohydrobia*, *Pyrgula*, *Dianella*, *Pseudamnicola*, *Adrioinsulana*) form one clade: *Hydrobia* with *Ventrosia*, *Dianella* with *Pyrgula*, *Adriohydrobia*, *Adrioinsulana* with *Pseudamnicola*. All the other hydrobiids form a clade, in which *Orientalina*, *Horatia* and *Anagastina* form one monophyletic group, the other group including the remaining taxa. *Alzoniella* and *Islamia* form a clade with high amounts of anagenesis along the branches, the branch terminated with *Sadleriana* being of a similar length. Here, like previously, there also is a clade consisting of *Grossuana*, *Trichonia*, *Daphniola* and *Boleana*.

To avoid the effect of saturation of the 3rd positions in the CO1 sequences a maximum-parsimony tree was computed based on the CO1 data translated to amino acids. The equal probability of each change of the amino acid (all characters unordered, reversible) was assumed. This obviously oversimplifying assumption was used to avoid a too high variance of the results, there being no reasonable, indubitable way of weighting such changes. Out of the 212 characters, 171 were constant, 23 variable but evolutionary-parsimony-uninformative, and only 18 parsimony-informative. The heuristic search resulted in three almost identical trees, 65 steps long, with CI=0.754, RC=0.526. The strict consensus tree (Fig. 240) confirms the monophyly of the Hydrobiidae s. stricto (*Hydrobia*, *Ventrosia*, *Adriohydrobia*, *Pyrgula*, *Dianella*, *Pseudamnicola*, *Adrioinsulana*). Like in the previous trees, there also is a monophyletic group of *Boleana*, *Grossuana*, *Daphniola* and *Trichonia*. *Bithynia*, *Bythiospeum, Bythinella* and *Pseudobithynia* form a clade of an "unusual" topology and placed deep within the hydrobioids. *Orientalina*, *Anagastina* and *Horatia* form another clade. *Heleobia*, *Marstoniopsis* and *Lithoglyphus* are close to the outgroup. The tree topology is obviously affected by the low number of parsimony-informative characters, and too many evolutionary events hidden by the translation to amino acids.

The strict consensus tree computed for the two (18S and CO1) maximum-likelihood trees resulted in one polytomy, except one clade (*Hydrobia*, *Ventrosia*). Thus the Adams consensus tree (Fig. 241) was computed for all the taxa, the CO1 sequences of which were known. Adams consensus tree is a useful approach where two topologies look alike, but the strict or even majority-rule consensus tree shows just polytomies. It is especially useful where the trees, having some parts in common, include some "wandering taxa", the positions of which are so much different that it is impossible to find any common elements by the usually applied consensus techniques (KITCHING et al. 1998). Such is the case in the present study, and this explains why the Adams consensus tree was computed. However, one must bear in mind that this kind of consensus tree shows some "clades" which, not to be found in any of the basic tree, are not the real clades. Thus one has to be careful when interpreting such a tree – it need not reflect evolution. However, no better way to summarize the information gained from the molecular data is found in the present case. Next, the taxa on which there are no CO1 data, are added to the Adams consensus tree based on their position on the 18S trees (Fig. 242). The resulting tree summarizes all the molecularly-based information to be used in the systematics of the studied taxa.

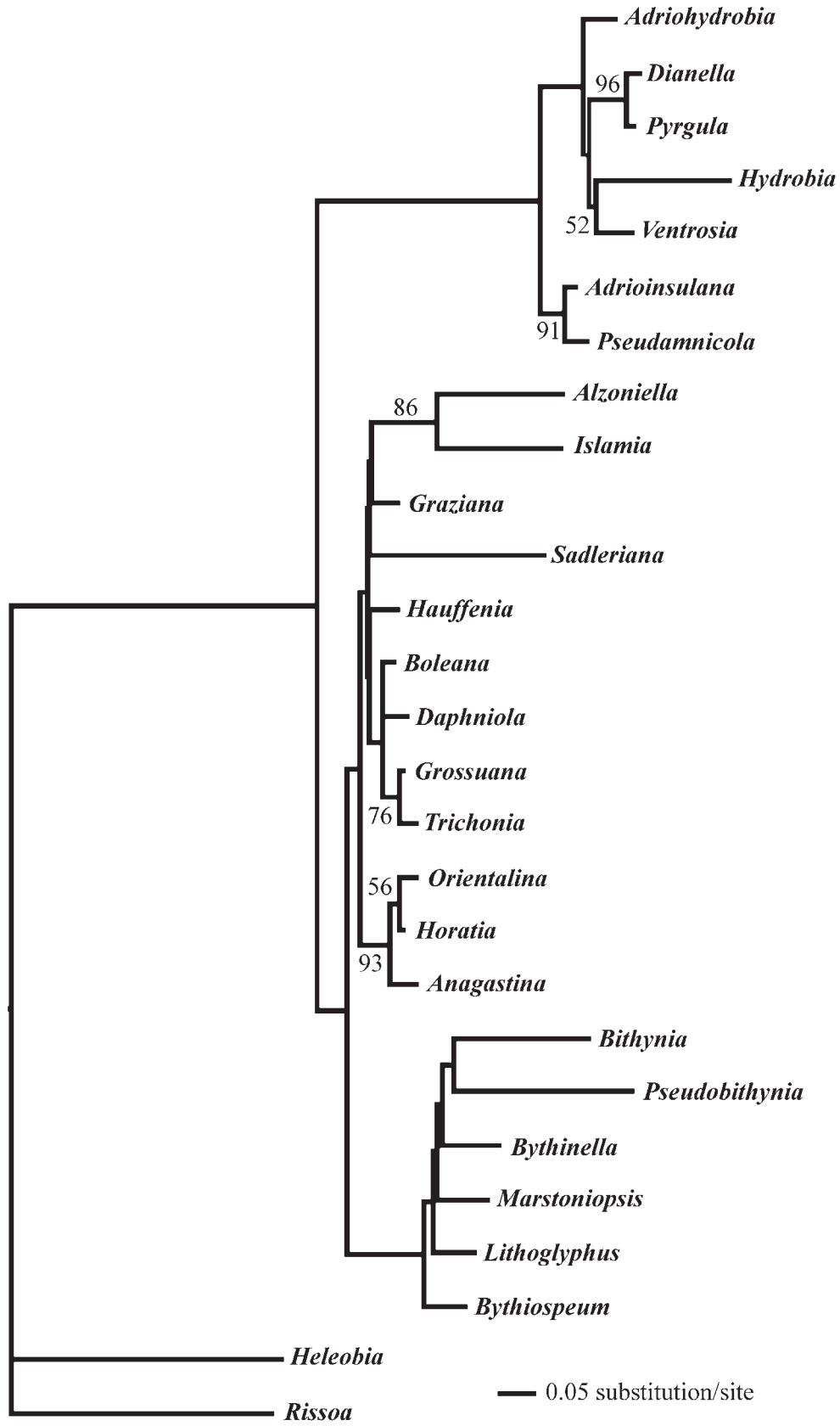


Fig. 239. Maximum likelihood phylogram based on CO1 gene sequences, model applied: TVM + I + Γ, frequencies: A=0.333, C=0.133, G=0.120, T=0.414; rate matrix: 0.698, 8.159, 0.293, 1.605, 8.159, 1.000; proportion of invariable sites: 0.511; Γ distribution shape parameter: 0.570. Heuristic search (TBR), score of tree: 7336.787. Bootstrap supports (1,000 replicates) given for the branches whose support was higher than 50%

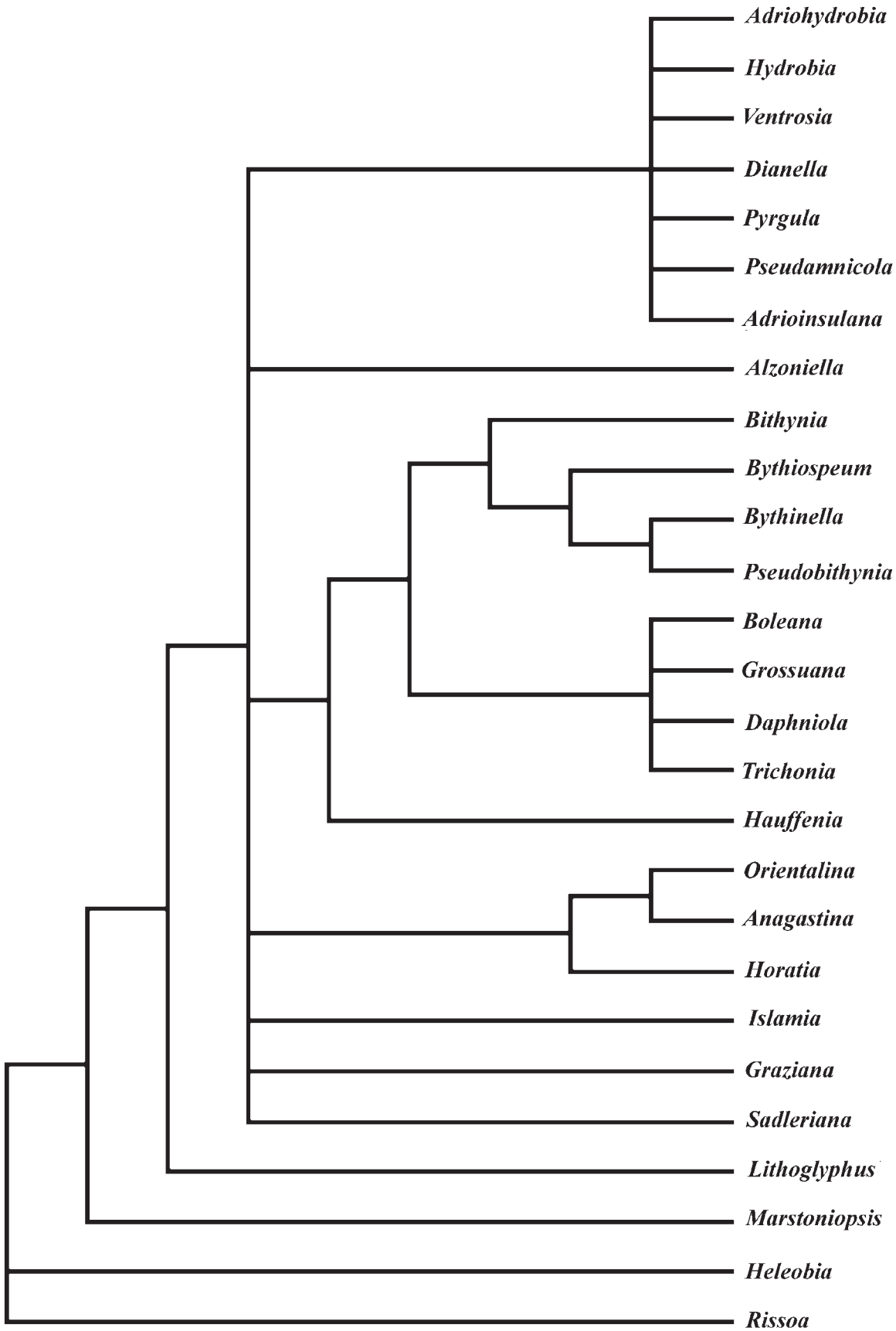


Fig. 240. Strict consensus tree based on CO1 gene sequences translated to amino acids, computed on three maximum parsimony trees, 65 steps long, CI=0.754, RC=0.526. 212 characters, of which 171 constant, 23 variable parsimony–uninformative, 18 parsimony–informative; bootstrap support (1,000 replicates) not exceeding 60% for any branch

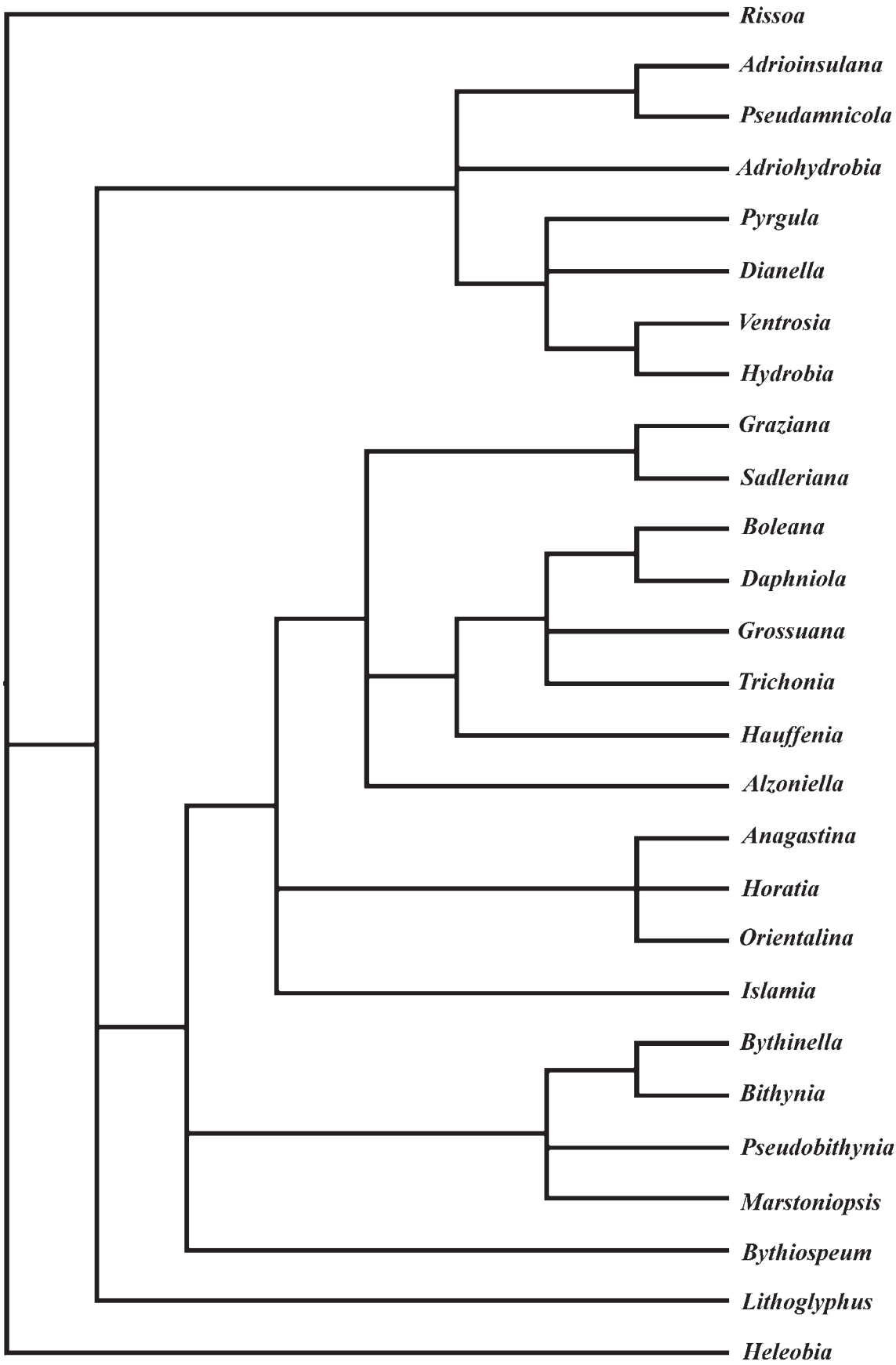


Fig. 241. Adams consensus tree computed for trees from Figs. 237 and 239 (based on 18S and CO1 gene sequences), for the taxa present in CO1 tree

DISCUSSION

MORPHOLOGY

Teleoconch

The teleoconch outer surface and internal structure in several of the studied genera are described in FALNIOWSKI (1989a, b, 1990a, b), FALNIOWSKI & SZAROWSKA (1995a, b, c) and FALNIOWSKI et al. (1996a). Some of these characters seemed to be useful in taxonomy. However, the results of cladistic analysis done by FALNIOWSKI & SZAROWSKA (1995a) are not necessarily in agreement with the present-day knowledge of the molecularly-based phylogeny of the group (e.g. WILKE et al. 2001). As their research was not continued, there are no data about the majority of the taxa considered in the present paper.

Protoconch

The protoconch habitus and macro- and microsculpture in some of the studied species are described in FALNIOWSKI (1989a, b, 1990a, b), FALNIOWSKI & SZAROWSKA (1995a, b, c, 2000), SZAROWSKA (1996) and FALNIOWSKI et al. (1996a). In some genera, like *Hauffenia* (Figs 64, 66) and *Trichonia* (Figs 86–88, 90) the protoconch habitus is not genus- but species-specific. Similar remarks concern other taxa considered in the present study (FALNIOWSKI 1989a, FALNIOWSKI & SZAROWSKA 1995a, SZAROWSKA et al. 2006, in press).

In most of the taxa considered but not illustrated here the protoconch is without a spiral sculpture. In *Hydrobia* (PONDER 1988) and *Bythinella* (FALNIOWSKI 1990a, SZAROWSKA 1996, SZAROWSKA & WILKE 2004) there is a delicate spiral sculpture. In some *Bythinella* species, like *B. charpentieri* (Fig. 103), it is almost indiscernible (FALNIOWSKI 1990a). The protoconch sculpture of *Daphniola exigua* (Figs 70–71) is the same as the pattern found in *D. louisi* Falniowski et Szarowska, 2000 (FALNIOWSKI & SZAROWSKA 2000). This sculpture pattern resembles those of *"Hauffenia kerschneri"* (HAASE 1990), *Kerkia brezicensis* (BODON & CIANFANELLI 1996) and *"Alzoniella" manganellii* (BODON et al. 1997). The protoconch surface picture that can be seen under higher magnifications is only known in few hydrobioids (FALNIOWSKI & SZAROWSKA 1995a). Extremely fine pores, scattered irregularly and not densely, have been found in *Marstoniopsis* (FALNIOWSKI 1990b), and *Dianella* (FALNIOWSKI & SZAROWSKA 1995b). A characteristic network of pores, densely arranged and lying entirely within the periostracum, has been described in *Bythinella* (FALNIOWSKI 1990a, SZAROWSKA 1996) and *Bithynia* (FALNIOWSKI 1989a, b). Coarse cavities have been found on the protoconch surface of *Pseudamnicola* (FALNIOWSKI & SZAROWSKA 1995c), *Pyrgula* (Figs 51–53), *Dianella* (FALNIOWSKI & SZAROWSKA 1995b) and *Rissoa* (FALNIOWSKI et al. 1993); the cavities are scarce and with sharp edges in *Pseudamnicola*; numerous, with sharp edges, some of them fused, in *Pyrgula* and *Dianella*; numerous with rounded edges in *Rissoa*.

Radula

The radulae of many of the taxa the present paper deals with are described and illustrated in FALNIOWSKI (1989a, b, 1990a), FALNIOWSKI & SZAROWSKA (2000), SZAROWSKA & WILKE (2004), SZAROWSKA et al. (2005, 2006 and in press). The species assigned to the "Pyrgulidae" have no basal cusps on the central tooth (e.g. THIELE 1929–1935, GIUSTI & PEZZOLI 1980, RADOMAN 1983), and basal cusps were found in neither *Pyrgula* and *Dianella* (SZAROWSKA et al. 2005) nor *Trachyohridia filocincta* (Fig. 123).

The radula of *Pseudobithynia* (Figs 137–140) looks almost the same as the one of *Bithynia* (e.g. FALNIOWSKI 1990a). In both radulae the arrangement of the basal cusps lying along the lateral margins of the tooth base is similar to that found in *Lithoglyphus* (FALNIOWSKI 1990a). Another similarity is the high number of basal cusps (4–5) that can be found in *Bithynia, Pseudobithynia* and *Lithoglyphus*. In all the other genera there were one-two (more often one) basal cusp on each side of the rhachis. Three pairs of basal cusps can be found in some specimens of *Potamopyrgus antipodarum* (Gray, 1843). It occurs in the Balkans but as a non-native species, has not been considered in the present study.

It is evident that the variability ranges of all the radular characters described above overlap among the genera. Neither "Pyrgulidae" nor *Emmericia* have basal cusps on the rhachis. In all the other genera there are always basal cusps on the central tooth, and no "rudimentary" basal cusp can be found. In some cases one or two cusps can be found in the same species; one cusp characterises *Parabythinella* and two are found in *Marstoniopsis* (Appendix 3 in the present study). The position of the basal cusps in *Bithynia, Pseudobithynia* and *Lithoglyphus* is different from that in the others; in the three genera the number of basal cusps is the same (4–5). Numerous and prominent cusps on the cutting edge of the rhachis are characteristic of the representatives of the Cochliopidae, Moitessieriidae and Belgrandiellinae.

Female reproductive organs

The female reproductive organs of the genera considered in the present paper have been described and illustrated by many authors (BREGENZER 1916, ROBSON 1922, ANKEL 1923, KRULL 1935, REGTEREN ALTENA 1936, KRAUSE 1948, LILLY 1953, FRETTER & GRAHAM 1962, DAVIS 1966, RADOMAN 1966, 1967a, b, 1968, 1970, 1972, 1973a, b, c, 1978, 1983, BOLE 1970, 1982, BOETERS 1971, 1973, 1998, GIUSTI & PEZZOLI 1980, HERSHLER & DAVIS 1980, FALNIOWSKI 1983,

1987, 1989a, b, 1990a, BANK & BUTOT 1984, HAASE 1990, 1994, 1995, BODON & CIANFANELLI 1996, BODON et al. 1997, 1999, 2001, FALNIOWSKI & SZAROWSKA 2000, HAASE et al. 2000, RIEDEL et al. 2001, WILKE et al. 2001, GLÖER 2002, SZAROWSKA & WILKE 2004, SZAROWSKA et al. 2005, 2006, in press, ARCONADA & RAMOS 2006, GLÖER & PEŠIČ 2006).

The way the penis and sperm penetrate the female reproductive organs has for many years (DAVIS 1967) been considered to reflect the main lines of evolution, and thus to be important in the taxonomy of the rissooid gastropods. In the majority of the Rissooidea, like the majority od the Caenogastropoda, e.g. Littorinidae (JOHANSSON 1939, FRETTER & GRAHAM 1962), there is a fold that runs along the ventral side of the capsule gland and forms the so called ventral channel (HERSHLER & PONDER 1998). JOHANSSON (1948) observed that in *Hydrobia* the pallial oviduct commences as an open gutter in the ontogeny, later to form the ventral channel. The ontogeny of the structure in the other taxa remains unknown, but the ventral channel seems to be homologous and plesiomorphic. The structure of the ventral channel of *Ventrosia* (Figs 158–160) is typical of *Hydrobia* and related genera, like *Peringia*, or *Adriohydrobia*, and does not differ from the one described by DAVIS (1966) and HERSHLER & DAVIS (1980). In *Lithoglyphus* (Figs 161–169), it resembles the one described for *Potamolithus*, a south American representative of the family Lithoglyphidae (DAVIS & PONS DA SILVA 1984).

In *Bithynia* and *Pseudobithynia* the anterior, vast bursa copulatrix (Fig. 174) resembles some Rissoidae (JOHANSSON 1939, FRETTER & GRAHAM 1962, FALNIOWSKI 1988) rather than hydrobioids. The structure of the pallial oviduct in *Bithynia* is described in LILLY (1953).

In some representatives of the Rissooidea the tract that harbours the penis and transports sperm during copulation is truly separated from the lumen of the capsule gland. DAVIS (1967) introduced the term "spermathecal duct" for the structure, the presence of which he considered to be crucial in discriminating the main evolutionary pathways within the Rissooidea. The term is, in fact, not correct. Spermatheca is a sperm-accumulating structure in insects and some other arthropods. In molluscs the analogous structure is known as bursa copulatrix. The bursa harbours spermatozoa, together with prostatic fluid, for a short time during and after copulation. Inside the bursa the spermatozoa are unoriented and not fed (FRETTER & GRAHAM 1962). For a longer storage spermatozoa, without fluid, are kept in the seminal receptacle. Spermatozoa are there arranged with their heads (in Neritopsina the flagella) embedded in the epithelium of the receptacle, which feeds the spermatozoa, thus making it possible to keep them a in good condition for several months. Some structures, like the bursa of *Hydrobia* (FRETTER & GRAHAM 1962) and also probably the big receptacle of *Marstoniopsis* and *Parabythinella,* may combine both functions. Considering the above, instead of "spermathecal duct" the proper term should rather be "copulatory duct". It is believed that this kind of a duct evolved from the ventral channel (HERSHLER & PONDER 1998), although its real homology may be different.

The presence/absence in *Bythinella* of the so called spermathecal duct (see above), a character that would confirm or contradict the hypothetical close relationship of the genus with the North American Amnicolidae (and the assignment of *Bythinella* to this family), was controversial. Originally the female anatomy of *Bythinella* was presented as resembling that of *Hydrobia,* without a spermathecal duct. Based on a simple section of a female, GIUSTI (unpublished data) supposed that what he found in *Bythinella* was a spermathecal duct. Serial sections of the capsule gland of *Bythinella* (SZAROWSKA & WILKE 2004) revealed that instead of a spermathecal duct, it has a ventral channel separated by very broad folds, which may have mimicked a closed duct. A similar broad fold, although not as broad as in *Bythinella*, is found in *Emmericia* (Fig. 196; Appendix 2: Fig. 43), and in the North American genus *Fontingens* (HERSHLER et al. 1990).

The female reproductive organs of *Parabythinella graeca* (Figs 179–180) look the same as in *Marstoniopsis*. According to KRULL (1935) and BOETERS (1973, 1998) in *Marstoniopsis* there is an enormously big seminal receptacle and no bursa copulatrix. Similarly, RADOMAN (1983) has noted the lack of the bursa in *Parabythinella*. On the other hand, GIUSTI & PEZZOLI (1980) and FALNIOWSKI (1987) portrayed the female reproductive organs of *Marstoniopsis* as having a "normal" bursa and a long but very thin, tube-like receptacle. Examining the organs in *Parabythinella* (Figs 179–180) I found a big seminal receptacle (with oriented sperm), its shape resembling more or less the shape of the "bursa" illustrated by FALNIOWSKI (1987). The homology of the receptaculum being not clear, I have assumed, after RADOMAN (1983) and BODON et al. (2001), that it is in rs_2 position.

In *Heleobia dobrogica* as well as in *H. dalmatica* the males are rare: they comprise only a few percent of the population. Like in *H. stagnorum* (BANK & BUTOT 1984) they are much smaller than the females. A similar zone found in *H. dalmatica* is described by RADOMAN (1973c). The female reproductive organs of *Heleobia dobrogica* (Figs 181–182) look in every detail the same as the ones, also examined, of *H. dalmatica,* and very similar to the ones described by BANK & BUTOT (1984) for *H. stagnorum* (Gmelin, 1791). RADOMAN (1973c) and BERNASCONI (1991) have not properly described the details of the female reproductive organs of *H. dalmatica* and *H. dobrogica*, respectively. The copulatory duct of *Heleobia* corresponds in topography to the duct of the bursa copulatrix of other hydrobioids.

The female reproductive organs of *Paladilhiopsis carpathica* closely resemble the ones described and illustrated by BOLE (1970) for *P. robiciana* (Clessin, 1882), and by RADOMAN (1983) for *P. grobbeni* (Kuščer, 1928).

Litthabitella chilodia was either assigned, as a subgenus, to the genus *Belgrandiella* (SCHÜTT 1980) or regarded as belonging to the genus *Litthabitell*a assigned to the Orientalinidae/Orientaliinae (RADOMAN 1983) or Hydrobiidae (BODON et al. 1999). In all the aforementioned papers the female reproductive organs of *Litthabitella* are portrayed like ones typical of the Hydrobiidae in a broad sense. In all the other gastropods considered in the present paper the oviduct, before joining the bursa and receptacle (one or two), forms either a spire (helix) composed of a couple of flat loops, or a flat loop in the form of a wide horseshoe. This section of the oviduct is muscular, thus thickened, and usually known as the renal oviduct. Its homology, however, is uncertain (FRETTER & GRAHAM 1962). In *Litthabitella* (Fig. 185) the renal oviduct forms neither a spire nor a horseshoe-like loop but, unique among the studied gastropods, it is circular instead (Figs 184–185).

Male reproductive organs

In the taxa considered the male reproductive organs are all alike: in any taxon the testis, seminal vesicles and prostate do not provide a discriminant character. On the other hand, the penis may be genus-characteristic, some of the taxa considered (*Bithynia*, *Bythinella*, *Parabythinella*, *Marstoniopsis*, *Emmericia)* having a flagellum (tubular penial gland). FRETTER & GRAHAM (1962) speculate that the secretion of the flagellum may be poured onto the wall of the female's mantle cavity and anchor the penis in position during copulation. During the penis erection the arm that contains the flagellum diverges from the one including the vas deferens, thus it seems doubtful that the flagellum tip is able to penetrate the female ducts. The secretion of the flagellum may attract a female or help to penetrate the female opening during the succeeding copulation. Sometimes a portion of the flagellum can be seen turned outside the tip of the left arm (FALNIOWSKI 1990a). At any rate, the function of the flagellum remains enigmatic. It must be noted that in the literature the term flagellum may be restricted to the second arm of the penis only, while the long tube lying in the haemocoel of the head is called tubular accessory prostate gland (e.g. LILLY 1953). The function of the structure remains unknown (see above) so that it can hardly be classified as a prostate. Hence, I have not adopted the aforementioned terminology.

In *Bithynia*, *Parabythinella* (Fig. 201), *Marstoniopsis*, *Bythinella* (Fig. 205) and *Emmericia* (Fig. 29 in Appendix 2 in this volume) the flagellum has the same structure. This has also been found in the North American genera *Amnicola* (HERSHLER & THOMPSON 1988) and *Fontingens* (HERSHLER et al. 1990).

In the case of a penis without a flagellum it may be not easy to tell an arm from an outgrowth. For instance, *Ventrosia* is usually defined in terms of having a second arm (MUUS 1967); an apparent arm is found in *Hydrobia*, *Belgrandiella*, *Orientalina,* or *Grossuana*, but in many cases there is an appendage (maybe glandular) which is either an arm or outgrowth. The outgrowths are often bifurcate, or there may be two separate outgrowths. Their function is unclear, but some of them may produce mucus or other secretion which may be useful in the process of copulation. Another function may be to keep the penis in the proper position and/or well attached during copulation.

PROPOSED SYSTEMATICS FOR THE GROUP

As one can see in the tree (Fig. 242) the phylogeny is still not well resolved (and, as pointed above, some "clades" are not necessarily real). This is due to the still poor taxon sampling (however, the present-day state of the habitats in the Balkans considered: see Appendix 4, many of the lacking taxa may never be collected without an extensive, long-time field work) and the lack of the CO1 data for some genera. Anyway, the available data enable some obvious taxonomic decisions to be made.

The family status of the Cochliopidae (represented in Europe by the single genus *Heleobia*) is once more confirmed. *Lithoglyphus* represents the distinct family Lithoglyphidae. *Litthabitella*, based solely on the 18S gene, is situated between the hydrobioids and the other families, with high bootstrap supports, and 100% Bayesian probabilities on the two internal branches that are connected to its terminal branch. However, its morphological pecularities described in the present paper, considered together with the information on its CO1 sequences (WILKE, personal communication) *Litthabitella* is very distant from the other genera and should probably be assigned to the Assimineidae. *Emmericia* is molecularly distinct, which justifies assigning it to the separate family Emmericidae. Without a sequence of its CO1 gene, the position of *Emmericia* remains uncertain. Thus the morphological data remain decisive (see Appendix 2), placing it close to *Bithynia*, so confirming the monophyly of the flagellum-bearing rissooideans (see below).

Paladilhiopsis and *Bythiospeum* represent Moitessieriidae, although the generic status of both remains valid. On the other hand, *Parabythinella* is nothing more than a junior synonym of *Marstoniopsis* (see Appendix 3) and belongs to the Amnicolidae unlike *Bythinella*, in the case of which either the molecules or morphology show that it constitutes a distinct family (Bythinellidae). All the morphological data confirm the close relationships of *Bithynia* and *Pseudobithynia*. The molecular data are somewhat equivocal, but the

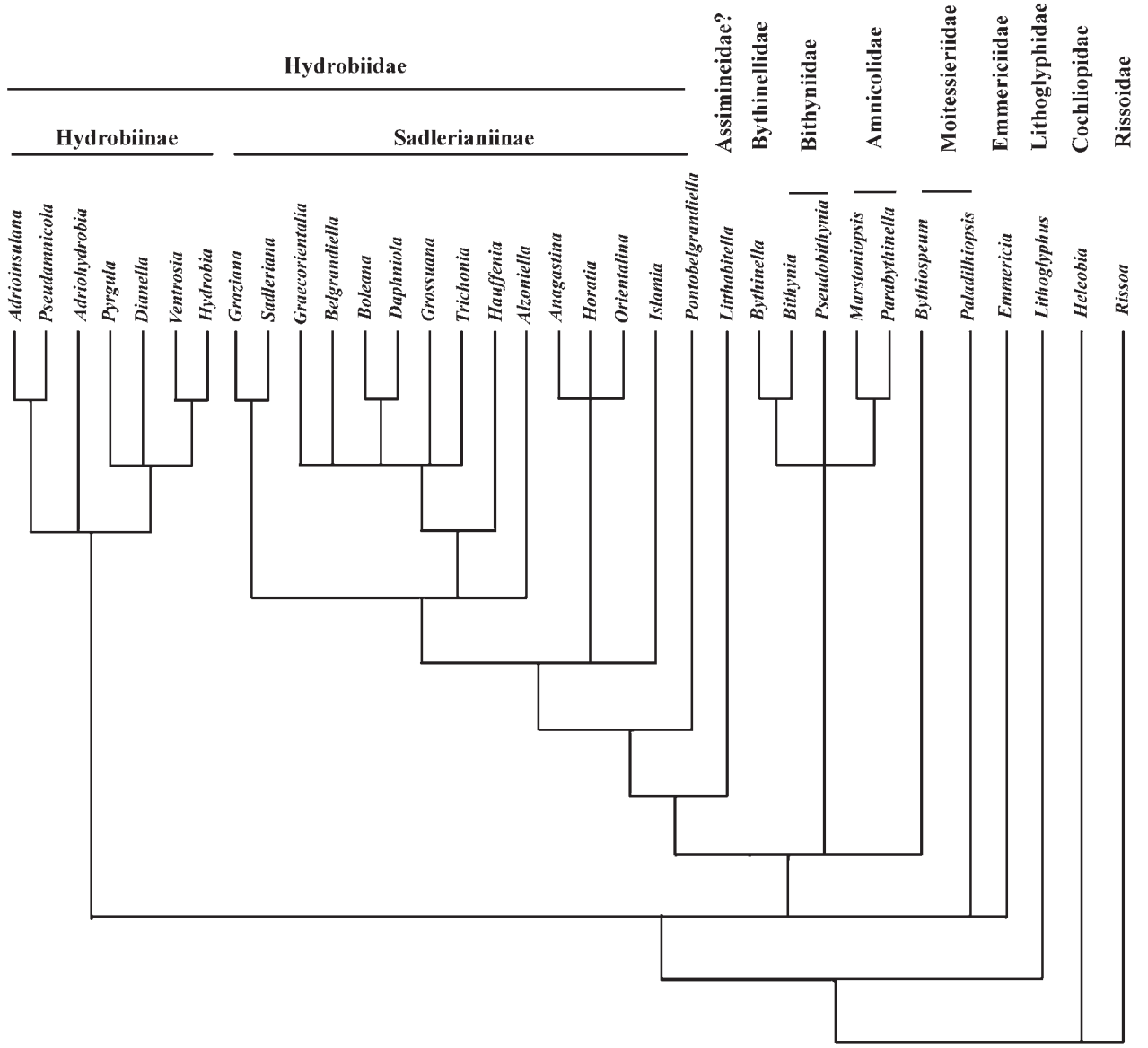


Fig. 242. Adams consensus tree from Fig. 241, with additional taxa included in positions based on 18S RNA gene sequences only, with proposed classification of the studied genera

two genera most probably belong to the same family Bithyniidae. All the other genera are assigned to one family: Hydrobiidae, with two subfamilies. Perhaps each of them could be given a family rank. The Hydrobiinae are monophyletic, including the so called "pyrgulids" (even a subfamily rank cannot be assigned to those snails: see SZAROWSKA et al. 2005). *Adrioinsulana* is no more than a junior synonym of *Pseudamnicola*: see SZAROWSKA et al. 2006). The other clade of the Hydrobiidae is formed by a group of closely related species (short branches on the phylograms), whose relationships are differently reconstructed using different data sets. Thus it is the safest not to propose any more detailed classification, and to include all of them in one subfamily: Sadlerianinae (with the type genus *Sadleriana* Clessin, 1890). The name *Orientalina* must be replaced, as a junior homonym. The proposed system is the following:

Hydrobiidae Troschel, 1857

Hydrobiinae Troschel, 1857
- *Hydrobia* Hartmann, 1821
- *Ventrosia* Radoman, 1977
- *Adriohydrobia* Radoman, 1973
- *Dianella* Gude, 1913
- *Pyrgula* Cristofori et Jan, 1832
- *Pseudamnicola* Paulucci, 1878 (with *Adrioinsulana* Radoman, 1978, as a junior synonym)

Sadlerianidae Radoman, 1973
- *Sadleriana* Clesin, 1890
- *Alzoniella* Giusti et Bodon, 1984
- *Anagastina* Radoman, 1978
- *Belgrandiella* Wagner, 1927
- *Boleana* Radoman, 1973
- *Daphniola* Radoman, 1973
- *Graecorientalia* Radoman, 1973

Graziana Radoman, 1975
Grossuana Radoman, 1973
Hauffenia Pollonera, 1898
Horatia Bourguignat, 1887
Islamia Radoman, 1973
Pontobelgrandiella Radoman, 1978
Radomaniola n. gen. (replacement name for *Orientalina*, type species: *Paludina curta* Küster, 1852)
Trichonia Radoman, 1973

Moitessieriidae Bourguignat, 1863
Bythiospeum Bourguignat, 1882
Paladilhiopsis Pavlovic, 1913

Lithoglyphidae Tryon, 1866
Lithoglyphus Hartmann, 1821

Emmericidae Brusina, 1870
Emmericia Brusina, 1870

Bithyniidae Gray, 1857
Bithynia Leach, 1818
Pseudobithynia Glöer et Pešić, 2006

Amnicolidae Martens, 1858
Marstoniopsis van Regteren Altena, 1936 (with *Parabythinella* Radoman, 1973 as a junior synonym)

Bythinellidae Germain, 1930
Bythinella Moquin-Tandon, 1855

Cochliopidae Tryon, 1866
Heleobia Stimpson, 1865 (BANK & BUTOT (1984) do not agree with DAVIS et al. (1982) who synonymise *Semisalsa* Radoman, 1984 with *Heleobia* inhabiting Lake Titicaca; the problem remains open)

Assimineidae H. et A. Adams, 1856
Litthabitella Boeters, 1970

Rissoidae Gray, 1847
Rissoa Fréminville, 1814 (outgroup)

EVOLUTION OF MORPHOLOGICAL CHARACTERS

As argued above, the phylogenies inferred with the two genes differ so much, that they should be analysed separately. Adams consensus tree does not reflect phylogeny in some of its parts and cannot be used for reconstructing character evolution. Thus I decided to reconstruct the evolution of the morphological characters described above and listed in Table 1 separately, for each (18S and CO1) of the maximum-likelihood molecular trees (Figs 243–244). The user-defined trees with the topology inferred with the molecular data were used together with the data matrix shown in Table 1, to reconstruct the character evolution with MACCLADE.

In the 18S tree (Fig. 243) the shell habitus is synapomorphic in some of the clades (turriform within the pyrgulids: *Pyrgula* and *Dianella*; turriform for the close, though not forming a clade, *Heleobia*, *Bythiospeum* and *Paladilhiopsis*; neritiform as an autapomorphy of the Lithoglyphidae; conical within the clade *Hydrobia* and *Ventrosia*; ovate-conical in the clades *Bithynia*, *Bythinella*, Amnicolidae, and *Boleana*, *Graziana*, *Trichonia*, *Belgrandiella*). The character shows, however, a high amount of homoplasy. This, coupled with a high variation within many genera, is the reason why the usefulness of the character is very restricted at the genus level. One of the most evident cases of the complete uselessness of the character within the hydrobioids is *"Sadleriana" pannonica* (SZAROWSKA & WILKE 2004). Similar remarks concern the other three shell "macrocharacters". The protoconch sculpture is either reticulate or spiral, the latter being a synapomorphy of the clade consisting of the Amnicolidae, *Bythinella* and *Bithynia*, but is also found in *Pseudobithynia* (very close, thus not definitely excluded from the clade), *Emmericia* and *Lithoglyphus*. The characteristic network of pores is found in *Bithynia*, *Pseudobithynia* (see above), *Bythinella*, also in *Emmericia* (see Appendix 2), but not in the Amnicolidae (a secondary loss?). The network occurs in *Peringia* (FISH & FISH 1977, FALNIOWSKI et al. 1996), but is not found in the Hydrobiidae considered in the present paper. The irregular, very small pores were found in amnicolids as well as "pyrgulids".

Central teeth without basal cusps are found not only in "pyrgulids": *Pyrgula* and *Dianella* (a crucial character documenting their presumed, long postulated distinctness, e.g. GIUSTI & PEZZOLI 1980, RADOMAN 1983, PONDER & WARÉN 1988), but also in *Emmericia*. More than two pairs of basal cusps occur in *Lithoglyphus*, and the clade consisting of the Amnicolidae (in *"Parabythinella"* not found so far), *Bythinella*, and the Bithyniidae. The cusps arise from the lateral sides of the central tooth in the Bithyniidae and, parallelly, in *Lithoglyphus*. A caecal appendix on the pyloric end of the stomach is found in the Hydrobiinae (also in *Adrioinsulana*), *Bithynia* and *Pseudobithynia*. As stressed above, the caecum, though probably a reminiscence of the spiral caecum found in many more primitive prosobranchs (FRETTER & GRAHAM 1962), looks different in each of the groups and can hardly be homologous. It may be no more than an outgrowth, and in some hydrobioids it may or may not occur even within the same genus. The black pigmentation of the renal oviduct is clearly a synapomorphy of the clade consisting of *Hydrobia*, *Ventrosia*, *Adrioinsulana/Pseudamnicola*, and occurs in *Adriohydrobia*, but not within the "pyrgulid" clade (a secondary loss?).

More than one loop of the coiled oviduct characterises not only the Hydrobiinae, but also Bithyniidae, *Heleobia* and *Emmericia*. A single loop occurs in all the Sadlerianinae, Amnicolidae, Moitessieriidae and *Lithoglyphus*. Perhaps the single loop is primitive, but the state of more loops also arose very early in the phylogeny. A primitive character state, ventral channel, is present in all but three clades. In *Bythinella* it has a special form of broad folds. Copulatory ducts are present in the Cochliopidae and Amnicolidae, and they cannot be homologous (as supposed above, considering their

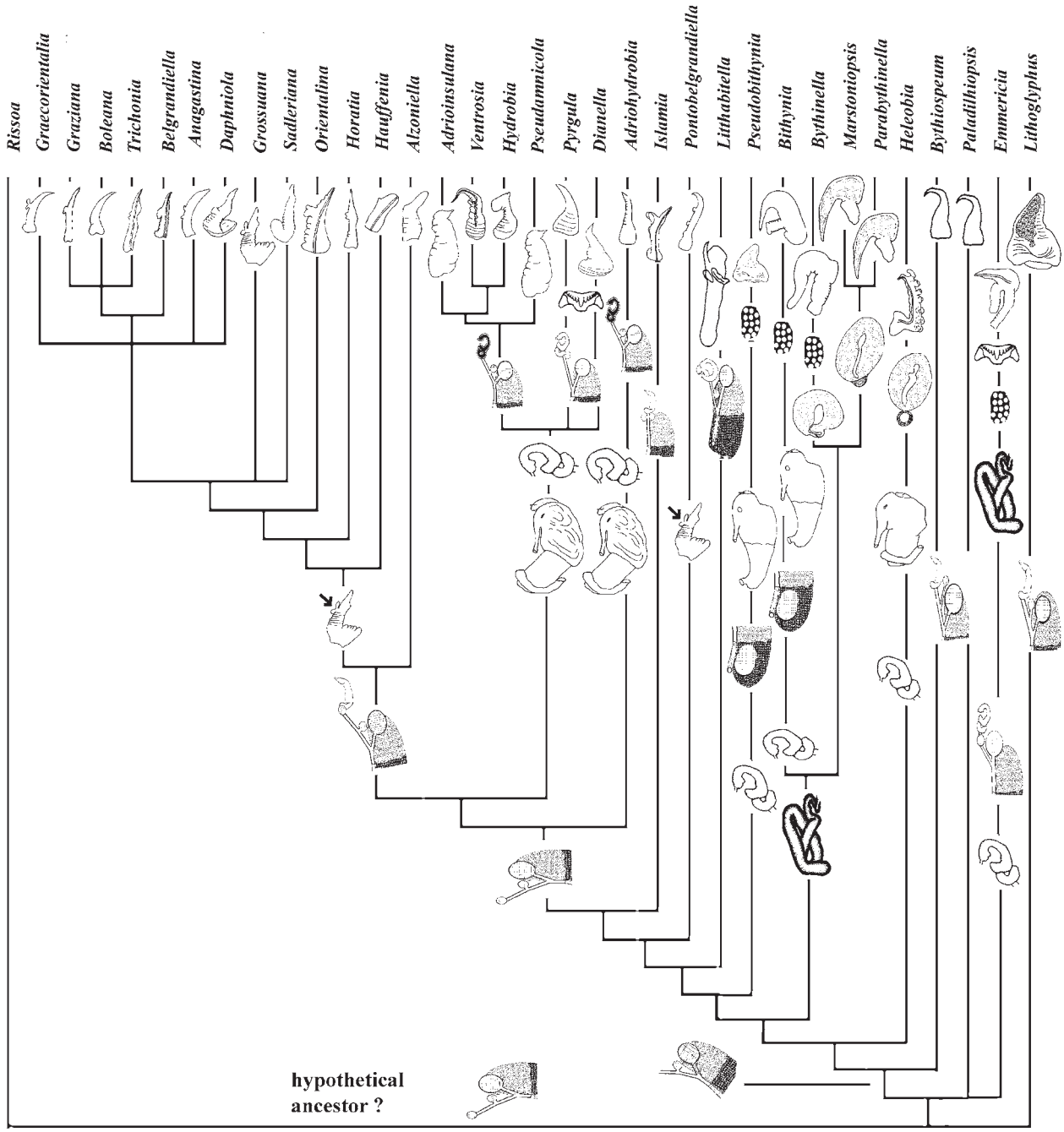


Fig. 243. Evolution of morphological characters reconstructed on 18S tree given in Fig. 237

different anatomy). The number and position of the seminal receptacles is shown in Fig. 243. Probably there were two receptacles in the ancestor, but rs_2 was lost in some lineages very early. Two receptacles characterise the biggest clade of all but two (*Islamia* and *Pontobelgrandiella*) genera of the Sadlerianinae; two receptacles were present in the ancestor of all the Hydrobiinae: later the rs_1 was lost in the "pyrgulid" clade, and the rs_2 was lost in the other Hydrobiinae. There is an rs_2 receptacle in *Emmericia*, while rs_1 receptacles in Lithoglyphidae, Moitessieriidae, Cochliopidae, Bithyniidae, and Bythinellidae. The single, big receptacle of the considered Amnicolidae, being ductless and not accompanied with a bursa, can hardly be homologised; similar remarks concern *Islamia* which has two receptacles and no bursa.

An anterior bursa copulatrix is present in the Bithyniidae, despite their possible non-monophyly suggested by the tree (see above). Unique within the group, it perhaps can be homologised with such bursae found in some rissoids (JOHANSSON 1939, FRETTER & GRAHAM 1962, FALNIOWSKI 1988). Any

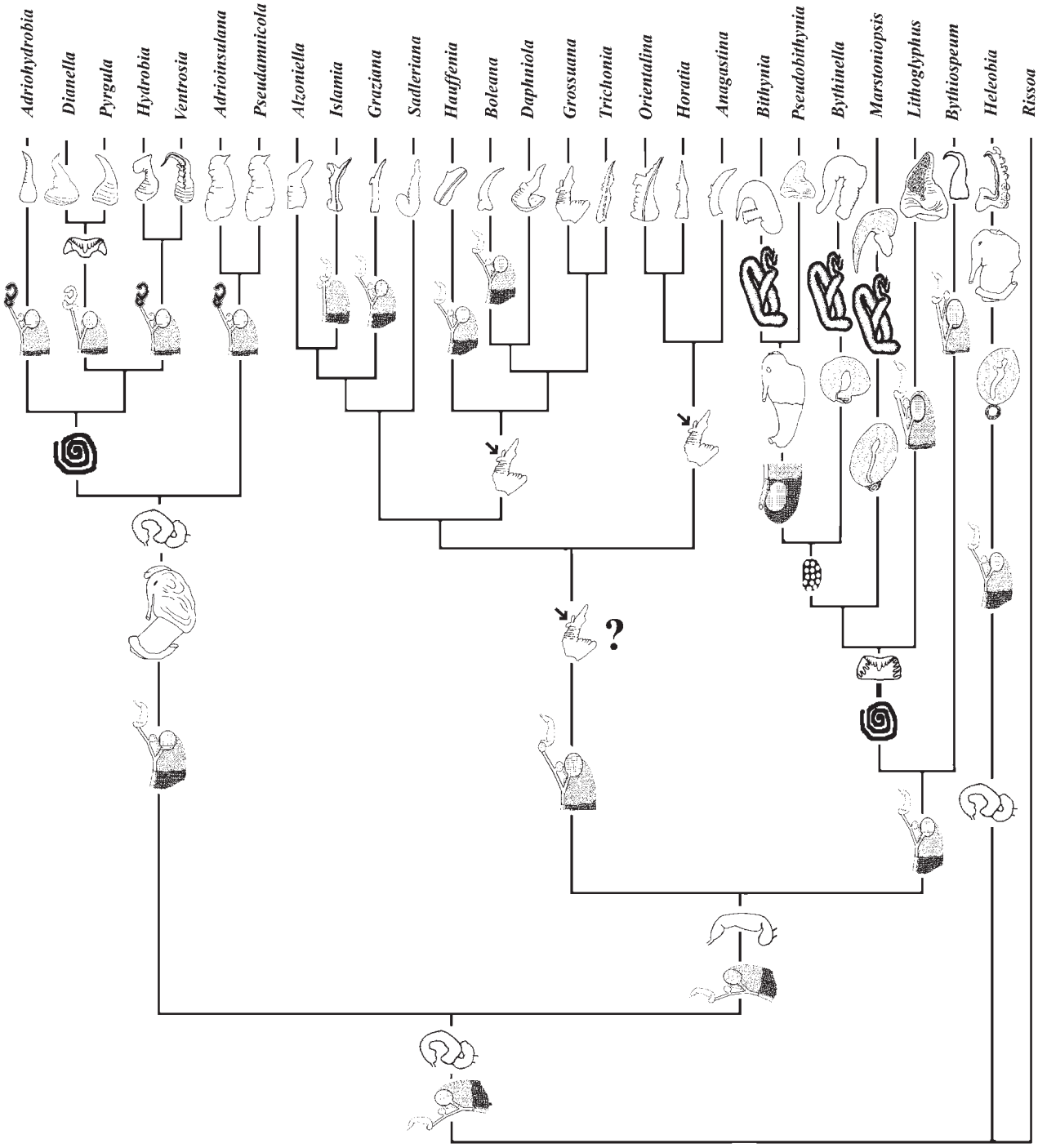


Fig. 244. Evolution of morphological characters reconstructed on CO1 tree given in Fig. 239

kind of bursa copulatrix is absent in either the amnicolids or *Islamia*; this lack must be secondary. On the other hand, there are autapomorphies concerning the "normal" posterior bursa, like the extremely big one of the Moitessieriidae, or the lithoglyphid bursa embedded in the tissue of the albuminoid gland.

The flagellum is a synapomorphy of the clade consisting of the Amnicolidae, Bythinellidae and *Bithynia*. It is also found in *Emmericia*. The position of the latter remains enigmatic (see above and Appendix 2), and it can be closer to the flagellum-bearing clade. On the other hand, the absence of both flagellum and second arm of the penis in *Pseudobithynia* may indicate that the structure was similarly lost in genera like *Heleobia*, *Lithoglyphus*, *Paladilhiopsis* and *Bythiospeum*. Thus its homology remains probable. The sucker-like glands on the penis of *Heleobia* are autapomorphic. On the other hand, the presence of the simple lobes on the left edge of the penis, a seemingly labile character, characterises *Pontobelgrandiella* and the big clade consisting of 12 genera (thus all but one) of the Sadlerianinae. In fact, the strange penis of the re-

maining *Islamia* can perhaps be homologised with the former type of penis. Thus the lobes seem evolutionarily old and stable; it has to be noted that their occurrence seems to be correlated with the presence of two receptacles (see Fig. 243).

For the reconstruction shown in Fig. 244 for the CO1 gene, similar remarks can be formulated. None of the shell "macrocharacters" (1–4) shows any evolutionarily sound pattern. Here the Bithyniidae are monophyletic and share their unique anterior bursa as a synapomorphy (though it may have appeared as a reversal to the state of the remote ancestors). With their closest group, Bythinellidae, they share the unique network of pores and flagellum, the latter present in the Amnicolidae as well. Thus both the network and the flagellum seem to be synapomorphies. The above group shares with *Lithoglyphus* the spiral pattern of protoconch sculpture (the latter occurs also in the clade formed by the Hydrobiinae, except *Pseudamnicola/Adrioinsulana*) and the rhachis with more than two pairs of basal cusps (the arrangement of which differs from that found in *Bythinella* and the Amnicolidae). Once more, the caecal appendix appears parallelly in each case, the same concerning the copulatory duct. The "pyrgulids" are secondarily devoid of basal cusps on the rhachis and a black pigment on the coiled oviduct, the latter being a synapomorphy of the Hydrobiinae. Perhaps the coiling pattern of the renal oviduct with more than one loop is a primitive state, but a single loop appeared also very early and there must have been reversals in the phylogeny of the character (see Fig. 244). Probably one seminal receptacle rs_1 is a primitive character state, but rs_2 must have arised early: the common ancestor of the Hydrobiinae possesed both, or else the receptacle of the "pyrgulids" and rs_2 are not homologous. The ancestor of the clade including all Sadlerianinae (thus monophyletic) had two receptacles, and a simple lobe on the left edge of the penis. Only one receptacle, rs_1, must have had the common ancestor of the Moitessieriidae, Lithoglyphidae, Amnicolidae, Bythinellidae, and Bithyniidae. Thus either one receptacle, rs_1, is primitive and rs_2 arose not earlier than in the ancestor of the sadlerianid clade (and, parallel, in the ancestor of the Hydrobiinae, or later in the "pyrgulids" whose rs_2 was next reduced), or two receptacles are a primitive state, rs_2 being reduced in many, while rs_1 in a few, clades.

As it can be seen, several characters, traditionally regarded as "good" ones, of key importance to the rissooid taxonomy, evolved parallelly. This concerns the caecal appendix, copulatory duct, or the basal cusps of the rhachis. On the other hand, more than two pairs of basal cusps and the characteristic network of pores are real synapomorphies. It seems that the unique flagellum is really uniquely derived then homologous, it is lost in some lineages secondarily. The latter seems the more probable that in *Pseudobithynia* the whole anatomy except the absence of flagellum is the same as in *Bithynia*. Finally, the presence of simple lobes on the left edge of the penis seems to be correlated with the presence of two receptacles, both characters reflecting a mode of copulation which is much more conservative than supposed. The sperm can be stored also within the renal oviduct, thus one can imagine that somewhere in the region there may appear some diverticulum (one or more) to enable more sperm to be stored, and that such outgrowth may be easily gained or lost. Despite the above speculations, the reconstruction of the evolution suggests the evolutionary stability of the receptacles. It must be stressed as well that there is either rs_1, or rs_2, or both, but not a receptacle in any intermediate position. Thus the receptacles, penial lobes, and mode of copulation correlated with those structures must have been stable throughout the evolution.

In the present study the reconstruction of the evolution of the morphological characters is limited to the rissooids whose geographical range is rather restricted. In such cases morphological characters work better (SZAROWSKA et al. 2005). Rissooidea are known since the Toarcian, Early Jurassic (GRÜNDEL 1999), some Recent genera having originated as early as the Oxfordian, Late Jurassic. Hydrobioids are probably not much younger. Therefore there has been much time for evolution within the group. All rissoids are small or even minute, thus one of the most important factors that have affected their morphology is miniaturization. All rissoids had to cope with the freshwater conditions, which means to evolve the osmoregulatory system, copulatory organs and the structures that are responsible for formation of the egg capsules. All the above must have left a rather restricted morphospace, which makes parallelisms and convergences common. Furthermore, it is evident that reversals were common within the group. Apparently, almost all their character states appeared very early in the phylogeny. Later, many character states were lost, and then some were regained. This concerns even complex structures like the bifurcate penis with a flagellum or the caecal appendix. The observed macroevolutionary processes confirm the supreme importance of the regulatory genes, which can activate or inactivate the expression of the genotypic information accumulated long time ago.

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APPENDIX 1

TAXA AND LOCALITY INFORMATION

Rissoa labiosa (Montagu, 1803) (outgroup), canal of Neretva River at Ploče, Croatia
Hydrobia acuta (Draparnaud, 1805), Hérault, Etang du Prévost, France, leg. C. CASAGRANDA
Ventrosia spalatiana (Radoman, 1973), Pontana spring, Croatia
Ventrosia sp. Itea Greece, South Euboa Island, Greece
Adriohydrobia gagatinella (Küster 1852), Cetina River Estuary, Croatia
Pyrgula annulata (Linnaeus, 1767), Garda Lake, Italy (1992); Neretva River, Croatia
Dianella thiesseana (Kobelt, 1878), Trichonida Lake, Greece
Trachyohridia filocincta Poliński, 1939, Ohrid Lake, Macedonia, leg. T. WILKE
Pseudamnicola negropontina (Küster, 1853), S. Euboa Island, Greece
Adrioinsulana conovula (Frauenfeld, 1863), Pag Island, Croatia
Orientalina curta curta (Küster, 1852), Nikšicko Polje, Montenegro
Orientalina curta germari (Frauenfeld, 1863), Cetina River, Croatia
Sadleriana fluminensis (Küster, 1852), Močilnik spring, Slovenia
Anagastina zetaevalis (Radoman, 1973), spring at Zeta River by the bridge at Vranicke njive, Montenegro
Anagastina scutarica (Radoman, 1973), Skutari Lake, Montenegro, leg. V. PEŠIČ
Grossuana serbica Radoman, 1973, spring of Raška River by the monastery of Sopoćani, Serbia
Grossuana codreanui (Grossu, 1946), spring SW of Techirghiol Lake, Romania
Trichonia kephalovrissonia Radoman, 1973, Termos,Greece
Trichonia trichonica Radoman, 1973, Trichonida Lake, Greece (1985)
Daphniola graeca Radoman, 1973, Dafne Spring, Greece
Daphniola exigua (Schmidt, 1856), Agia Paraskevi, Greece
Daphniola louisi Falniowski et Szarowska, 2000, Kessariani, Athens, Greece
Horatia klecakiana Bourguignat, 1887, spring of Vrana River, Croatia
Graecorientalia vrissiana Radoman, 1966, Makrinitsa/Koukourava, Greece
Belgrandiella kusceri (Wagner, 1914), Rak brook by Rakovski Škocjan, Rakek, Slovenia
Belgrandiella croatica (Hirc, 1881), NW Croatia
Boleana umbilicata (Kuščer, 1932), Močilnik spring, Slovenia
Alzoniella finalina Giusti et Bodon, 1984, Liguria, Savona, Molino, spring at the Porra River, leg. M. BODON, S. CIANFANELLI
Alzoniella slovenica (Ložek et Brtek, 1964), leg. M. HORSAK
Graziana lacheineri (Küster, 1852), spring at Bele Vode, Slovenia
Paladilhiopsis carpatica Soos, 1940, Vadu Crisul Cave, Romania, leg. A. BENEDEK
Bythiospeum sp., Source de la Nizon, France, leg. M. BODON, H. GIRARDI, B. BOMBA
Pontobelgrandiella nitida (Angelov, 1972), spring at Jasenovo, Bulgaria, leg. M. HORSAK
Hauffenia michleri Kuščer, 1932, Močilnik spring, Slovenia
Hauffenia sp., Pätřočnica spring, Gemerska Hôrka, Slovakia, leg. J. GREGO
Islamia zermanica Radoman, 1973, Slime, Croatia
Emmericia patula (Brumati, 1838), Cetina River, Croatia
Emmericia expansilabris Bourguignat, 1880, spring of Dubrovačka Rijeka, Croatia
Lithoglyphus naticoides (Pfeiffer, 1828), Narew River at Drozdowo, Poland
Bythinella austriaca (Frauenfeld, 1856), Młynnik spring, Ojców, Poland
Bythinella robiciana (Clessin, 1890), Potoče by Preddvor Slovenia, leg. T. WILKE
Bythinella pannonica (Frauenfeld, 1865), Hrhov, Slovakia
Bythinella charpentieri Roth, 1855, Delfi, Greece
Marstoniopsis insubrica (Küster, 1853), Warnow River at Rostock Germany, leg. M. L. ZETTLER
Parabythinella macedonica (Hadžišče, 1958), Prespa Lake, Macedonia, leg T. WILKE
Parabythinella graeca Radoman, 1978, Vegorritida Lake, Greece
Bithynia tentaculata (Linnaeus, 1758), Cetina River, Croatia
"Pseudobithynia graeca", Piges Pamisou (Springs of Pamisou), Greece
Litthabitella chilodia (Westerlund, 1886), W of Sotonici, Montenegro
Heleobia dalmatica (Radoman, 1973), Pirovac spring, Croatia
Heleobia dobrogica (Grossu, 1986), Movile Cave, Romania, leg. I. SIRBU
Heleobia sp., Peloponnissos, Greece, leg. A. FALNIOWSKI

Table 1. Gene Bank Accession Numbers of the sequences used for phylogeny inference; numbers of sequences not published earlier marked with asterisks

Species	18S	CO1	Species	18S	CO1
Adriohydrobia gagatinella	AF367657	AF317881	*Horatia klecakiana*	AF367669	AF367637
Adrioinsulana conovula	AF367656	AF367628	*Hydrobia acuta*	AF367680	AF278812
Alzoniella finalina	AF367686	AF367650	*Islamia piristoma*	AF367671	AF367639
Anagastina zaetavalis	————	EF070616*	*Lithoglyphus naticoides*	AF367674	AF367642
Anagastina scutarica	EF070622*	————	*Litthabitella chilodia*	EF070629*	————
Belgrandiella kusceri	EF070632*	————	*Marstoniopsis insubrica*	AF367676	AY027813
Bithynia tentaculata	AF367675	AF367643	*Orientalina callosa*	AF367685	AF367649
Boleana umbilicata	EF070623*	EF070615*	*Paladilhiopsis carpathica*	EF070631*	————
Bythinella austriaca	AF212917	EF070617*	*Parabythinella graeca*	EF070627*	————
Bythiospeum sp.	AF367664	AF367634	*Pontobelgrandiella nitida*	EF070621*	————
Daphniola graeca	EF070624*	EF070618*	*Pseudamnicola negropontina*	————	EF061915*
Dianella thiesseana	AY676125	AY676127	*Pseudamnicola lucensis*	AF367687	————
Emmericia expansilabris	EF070625*	————	*Pseudobithynia* sp.	EF070628*	EF070620*
Graecorientalia vrissiana	EF070626*	————	*Pyrgula annulata*	AY676124	AY341258
Graziana alpestris	AF367673	AF367641	*Rissoa labiosa*	AY676126	AY676128
Grossuana codreanui	EF061916*	EF061919*	*Sadleriana fluminensis*	AF367683	AY273996
Hauffenia tellinii	AF367672	————	*Trichonia kephalovrissonia*	EF070630*	EF070619*
Hauffenia sp.	————	EF070614*	*Ventrosia maritima*	————	AY616140
Heleobia dalmatica	AF367661	AF367631	*Ventrosia ventrosa*	AF367681	————

APPENDIX 2

FLAGELLUM-BEARING HYDROBIOIDS. 1. *EMMERICIA* (GASTROPODA: PROSOBRANCHIA: RISSOOIDEA): FROM MORPHOLOGY TO MOLECULES AND BACK TO MORPHOLOGY

INTRODUCTION

The phylogenetic relationships of the genus *Emmericia* Brusina, 1870 are, like all the phylogeny of the Rissooidea, far from being understood (e.g. KABAT & HERSHLER 1993, FALNIOWSKI & SZAROWSKA 1995a, WILKE et al. 2001). The range of the genus spans along the Adriatic coast from North-East Italy to the south of Croatia. Besides, isolated localities are known from France and Germany (BRUSINA 1870, BOURGUIGNAT 1880, BOETERS & HEUSS 1985, MOUTHON 1986, KABAT & HERSHLER 1993, GLÖER 2002). The representatives of the genus inhabit rivers and springs (GIUSTI & PEZZOLI 1980, RADOMAN 1983, BOETERS 1998, GLÖER 2002). *Emmericia patula* (Brumati, 1838) is known from Monfalcone in Italy to the Neretva River in Croatia, occurring not higher than about 70 m a.s.l. RADOMAN (1967b, 1968, 1970, 1983) lists three more Croatian species. One of them occurs in the Neretva River, the other two are locally found in springs. THIELE (1929–1935) describes the radula, RADOMAN (1967a, 1968, 1973a, 1983), GIUSTI & PEZZOLI (1980) and BOETERS (1998) describe the radula, verge and female reproductive organs of *E. patula*. BRUSINA (1870) established the monogeneric subfamily Emmericiinae, belonging to the family Rissoidae. THIELE (1929–1935) placed Emmericieae, with *Emmericia* as the only genus, in the Hydrobiidae, subfamily Hydrobiinae, not far from Lithoglypheae, Benedictieae, and Amnicoleae. RADOMAN (1967a, 1968, 1970) considered *Emmericia* to be the most similar to *Lithoglyphus* but, at the same time, to belong to a distinct family Emmericiidae (vs. Lithoglyphidae). GIUSTI & PEZZOLI (1980) placed Emmericiidae together with the Pyrgulidae in a superfamily Pyrguloidea, separated from Hydrobioidea. PONDER & WARÉN (1988) included Emmericiinae in the Hydrobiidae, placing the former close to the Baicaliinae, Benedictiinae and Tateinae, and far from the Lithoglyphinae and Amnicolinae.

Nuclear 18S RNA and mitochondrial 16S RNA genes were sequenced in *Emmericia expansilabris* from the type locality. Unfortunately, despite several efforts, it proved impossible to obtain a PCR product of CO1. The phylogenetic analysis showed that *Emmericia* represents a good and distinct clade, of rather a family level. However, different alignment strategies and different sets of taxa considered resulted each time in different sister-group relationships. It seems simply impossible to align the sequences in unequivocal way, and the sequences are not sufficient to resolve the polytomy including this genus. The lack of data on CO1 seems crucial: 18S is apparently not a molecule the mutation rate of which is adequate to the level of universality that is sufficient in this case. Despite all the limitations, *Emmericia* belongs certainly neither to the Hydrobiidae, nor to Pomatiopsidae, Cochliopidae, or Tateidae. It clusters in a big group which includes *Bythinella*, Lithoglyphidae, Amnicolidae, and Bithyniidae. It is impossible to assess which of them is the closest relative of *Emmericia*.

All the above considered, apart from checking and supplementing the morphological data, the aim of the study was to make a morphology-based analysis of phylogenetic relationships of *Emmericia* and answer the following questions:

1) Is the morphology-inferred position of *Emmericia* compatible with the molecularly inferred one?
2) What, according to morphological data, is the sister taxon of *Emmericia*?
3) Which, if any, of the morphological characters are universal, that is apparently homoplasy-free throughout all the "hydrobioid" group?

MATERIAL AND METHODS

SPECIMENS AND TAXA

Specimens of *Emmericia patula* (Brumati, 1838) were collected in September, 1999 and 2001 at two localities on each occasion:

1) Jadran river at the bridge at Solin (43°32.120'N, 16°29.447'E), macrophytes and gravel, about 0.5 m deep;
2) Cetina river at Radmanove Mlinice (43°26.367'N, 16°45.019'E), macrophytes, sand and gravel, about 1 m deep.

Topotypical specimens of *E. expansilabris* Bourguignat, 1880 were collected in September, 2001 at Izvor Rijeke (spring of the Rijeka Dubrovacka/Ombla river (42°40.652'N, 18°08.088'E), a huge spring with a waterfall below a big water intake; snails were taken from among dense weeds, from a pool at the water intake, about 2 m deep.

TAXA USED FOR COMPARISON

In order to assess the phylogenetic relationships of *Emmericia*, 15 other representatives of the Rissooidea were included (see Table 1): four of the Hydrobiinae (*Hydrobia, Peringia, Ventrosia*, *Adriohydrobia*); one of the Pseudamnicolinae (*Pseudamnicola*); two other taxa of the Hydrobiidae (*Horatia*, *Sadleriana*); six of other rissooidean families that were, at one point or another, considered to belong to the Hydrobiidae (see WILKE et al. 2001). The latter taxa are commonly referred to as "hydrobioids". They comprise the families Cochliopidae (*Heleobops*), Bithyniidae (*Bithynia*), "Bythinellidae" (*Bythinella*), Amnicolidae (*Amnicola*), Pomatiopsidae (*Gammatricula*) and Lithoglyphidae (*Lithoglyphus*). As outgroup, we used representatives of the family Rissoidae (*Setia*) and Truncatellidae (*Truncatella*).

MORPHOLOGICAL TECHNIQUES

The techniques are the same as described in Material and Methods of the main part.

PHYLOGENETIC ANALYSIS

Phylogenetic inference based on the morphological data was performed with PAUP*4.0 (Swofford 2002), applying branch-and-bound technique, and the character evolution traced with MACCLADE 4.05 (MADDISON & MADDISON 2002), on a MACINTOSH POWERPC G4.

The analysis is primarily based on the characters listed by HERSHLER & PONDER (1998). Unfortunately, many of those characters can only be examined in fresh material. As most previous anatomical studies are based on preserved specimens, very little comparative information is available in the literature. The microsculpture of the protoconch surface, though characteristic, is excluded from the character list as unknown in many of the studied taxa. The concentrated nervous system is also omitted, as it represents rather not a synapomorphy but a grade of evolution. The characters whose states were polymorphic and/or unknown in any taxa are not included, as well as all the morphometric characters that are useless at that level of universality. The remaining eleven characters, used for subsequent analyses, are listed in Table 1. As discussed by POE & WIENS (2000), exclusion of characters because of their variability, polymorphism, unknown states in some taxa, etc., is problematic and may impair the resolution of phylogeny. (It must be noted, however, that none of the algorithms used in phylogenetic inference so far will treat unknown and polymorphic characters in a way that is

Table 1. Morphological characters and their states (after DAVIS 1967, RADOMAN 1973a, b, c, 1983, DAVIS et al. 1982, FALNIOWSKI 1987, HAASE 1994, HERSHLER & PONDER 1998, BODON et al. 2001, WILKE et al. 2001, SZAROWSKA & WILKE 2004): 1 – shell habitus (0: valvatiform, 1: trochiform, 2: neritiform, 3: ovate-conical, 4: conical, 5: turriform); 2 – basal cusps on rhachidian tooth (0: absent, 1: present); 3 – stomach (0: without caecal appendix, 1: with caecal appendix, 2: not hydrobioid bauplan); 4 – coiled oviduct (0: absent, 1: spiral, 2: loop); 5 – pigment on coiled oviduct (0: absent, 1: present); 6 – rs_1 (0: absent, 1: present); 7 – rs_2 (0: absent, 1: present); 8 – sperm passes through (0: ventral chanel, 1: ventral chanel separated by wide folds, 2: sperm duct type A, 3: sperm duct type B, 4: sperm duct type C, 5: neither channel nor duct); 9 – flagellum (0: absent, 1: present); 10 – glandular lobe on penis (0: absent, 1: present); 11 – ganglionic thickening on tentacle nerve (0 – absent, 1 – present), 12 – central nervous system (0: not *Hydrobia*-like, 1: *Hydrobia*-like)

	1	2	3	4	5	6	7	8	9	10	11	12
Emmericia	3	0	0	1	0	0	1	0	1	0	0	0
Bythinella	3	1	0	2	0	1	0	1	1	0	1	0
Lithoglyphus	2	1	0	2	0	0	1	0	0	0	0	0
Amnicola	1	1	0	2	0	1	0	2	1	0	0	0
Heleobops	3	1	0	1	0	1	0	3	0	1	1	0
Gammatricula	4	1	0	2	0	0	1	2	0	1	1	0
Truncatella	5	0	2	0	0	0	0	4	0	0	0	0
Setia	4	0	2	0	0	0	0	5	0	0	0	0
Bithynia	3	1	2	1	0	0	0	0	1	0	0	0
Hydrobia	4	1	1	1	1	1	0	0	0	0	1	1
Pseudamnicola	2	1	1	2	1	1	0	0	0	0	1	1
Peringia	4	1	1	1	1	1	0	0	0	0	1	1
Ventrosia	4	1	1	1	1	1	0	0	0	1	1	1
Adriohydrobia	4	1	1	1	1	1	0	0	0	0	1	1
Horatia	1	1	0	2	0	1	1	0	0	1	1	0
Sadleriana	1	1	0	2	0	1	1	0	0	0	1	0

Table 2. The same morphological characters as in Table 1, binary-coded (0 – absent, 1 – present): 1 – shell valvatiform, 2 – shell trochiform, 3 – shell neritiform, 4 – shell ovate-conical, 5 – shell conical, 6 – shell turriform, 7 – basal cusps on rhachidian tooth, 8 – hydrobioid bauplan of stomach, 9 – stomach with caecal appendix, 10 – coiled oviduct, 11 – spiral (vs. loop) of oviduct, 12 – pigment on coiled oviduct, 13 – rs_1, 14 – rs_2, 15 – ventral chanel, 16 – ventral chanel separated by wide folds, 17 – sperm duct type A, 18 – sperm duct type B, 19 – sperm duct type C, 20 – flagellum, 21 – glandular lobe on penis, 22 – central nervous system *Hydrobia*-like

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Emmericia	0	0	0	1	0	0	0	1	0	1	1	0	0	1	1	0	0	0	0	1	0	0
Bythinella	0	0	0	1	0	0	1	1	0	1	0	0	1	0	0	1	0	0	0	1	0	0
Lithoglyphus	0	0	1	0	0	0	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0
Amnicola	0	1	0	0	0	0	1	1	0	1	0	0	1	0	0	0	1	0	0	1	0	0
Heleobops	0	0	0	1	0	0	1	1	0	1	1	0	1	0	0	0	0	1	0	0	1	0
Gammatricula	0	0	0	0	1	0	1	1	0	1	0	0	0	1	0	0	1	0	0	0	1	0
Truncatella	0	0	0	0	0	1	0	0	?	0	?	?	0	0	0	0	0	0	1	0	0	0
Setia	0	0	0	0	1	0	0	0	?	0	?	?	0	0	?	?	?	?	?	0	0	0
Bithynia	0	0	0	1	0	0	1	0	?	1	1	0	0	0	1	0	0	0	0	1	0	0
Hydrobia	0	0	0	0	1	0	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	1
Pseudamnicola	0	0	1	0	0	0	1	1	1	1	0	1	1	0	1	0	0	0	0	0	0	1
Peringia	0	0	0	0	1	0	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	1
Ventrosia	0	0	0	0	1	0	1	1	1	1	1	1	1	0	1	0	0	0	0	0	1	1
Adriohydrobia	0	0	0	0	1	0	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	1
Horatia	0	1	0	0	0	0	1	1	0	1	0	0	1	1	1	0	0	0	0	0	1	0
Sadleriana	0	1	0	0	0	0	1	1	0	1	0	0	1	1	1	0	0	0	0	0	0	0

always acceptable). The present study follows a more traditional way of character selection. All autapomorphies, as cladistically uninformative, are excluded from the data set, too. It should be noted that the anatomical characters used here for cladistic analyses are the characters the European hydrobioid taxonomy is primarily based on. The central tooth of the radula, central nervous system, and the caecal appendix on the stomach are mainly applied to higher grouping; the details of the female reproductive organs and (treated as less useful) male reproductive and copulatory organs are used to distinguish the genera.

Very little is still known about character evolution within the hydrobioid snails, so some assumptions as to the transformation series are preliminary. HERSHLER & PONDER (1998) list the characters that may be useful in phylogenetic analysis in the hydrobioid snails, and suggest a scheme for coding those characters' states. However, BODON et al. (2001), who applied the coding scheme to their analysis, have received a poorly resolved phylogeny with some clades formed by certainly not closely related taxa.

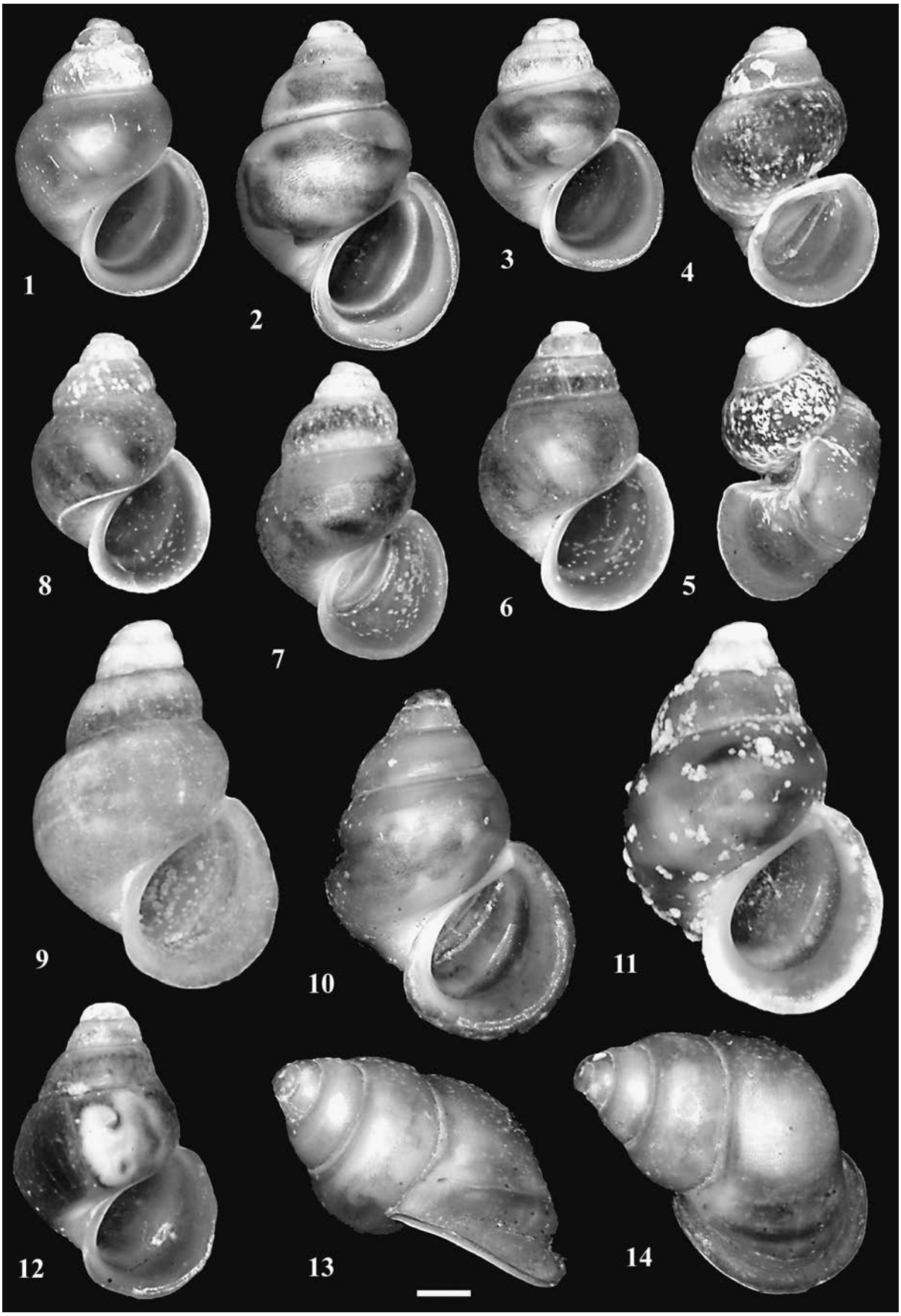
The characters listed in Table 1 were coded as either multistate (characters 1, 3, 4, 8,), or binary (remaining characters). SZAROWSKA et al. (2005) proposed some transitional series and weighting scheme for those characters, but in this analysis all characters were treated as unordered and reversible, and equally weighted.

The long debate of the recent 20 years, concerning the pros and cons of multistate versus binary coding of characters (PIMENTEL & RIGGINS 1987, MEIER 1994, PLEIJEL 1995, WILKINSON 1995, LEE & BRYANT 1999, STRONG & LIPSCOMB 1999, SEITZ et al. 2000, FALNIOWSKI 2003, ROE & HOEH 2003) has not brought about a consensus. Thus, the data from Table 1 were recoded as binary, reversible (Table 2).

RESULTS

MORPHOLOGY

The shells of *E. expansilabris* (Figs 1–5) were similar to the ones of *E. patula* (Figs 6–14). The shell size variation range of the former species was contained in that of the latter, none of *E. expansilabris* having the shells as big as the biggest of *E. patula* (Figs 9–11). The protoconch of *E. patula* (Fig. 15) had a narrow nucleus, slowly and regularly growing whorl width, and was not distinctly demarcated from the teleoconch. Its surface was always covered with diatoms and calcareous deposits and corroded, so that it was impossible to examine its microsculpture. In *E. expansilabris* (Figs 16–19) the habitus was the same as in the preceding species (Figs 18–19). In specimens extracted from egg capsules deposited on the shells of the representatives of the species the surface showed, under higher magnifications (10,000 ×), a characteristic network of pores lying entirely in the periostracum, part of them open (Figs 16–17).

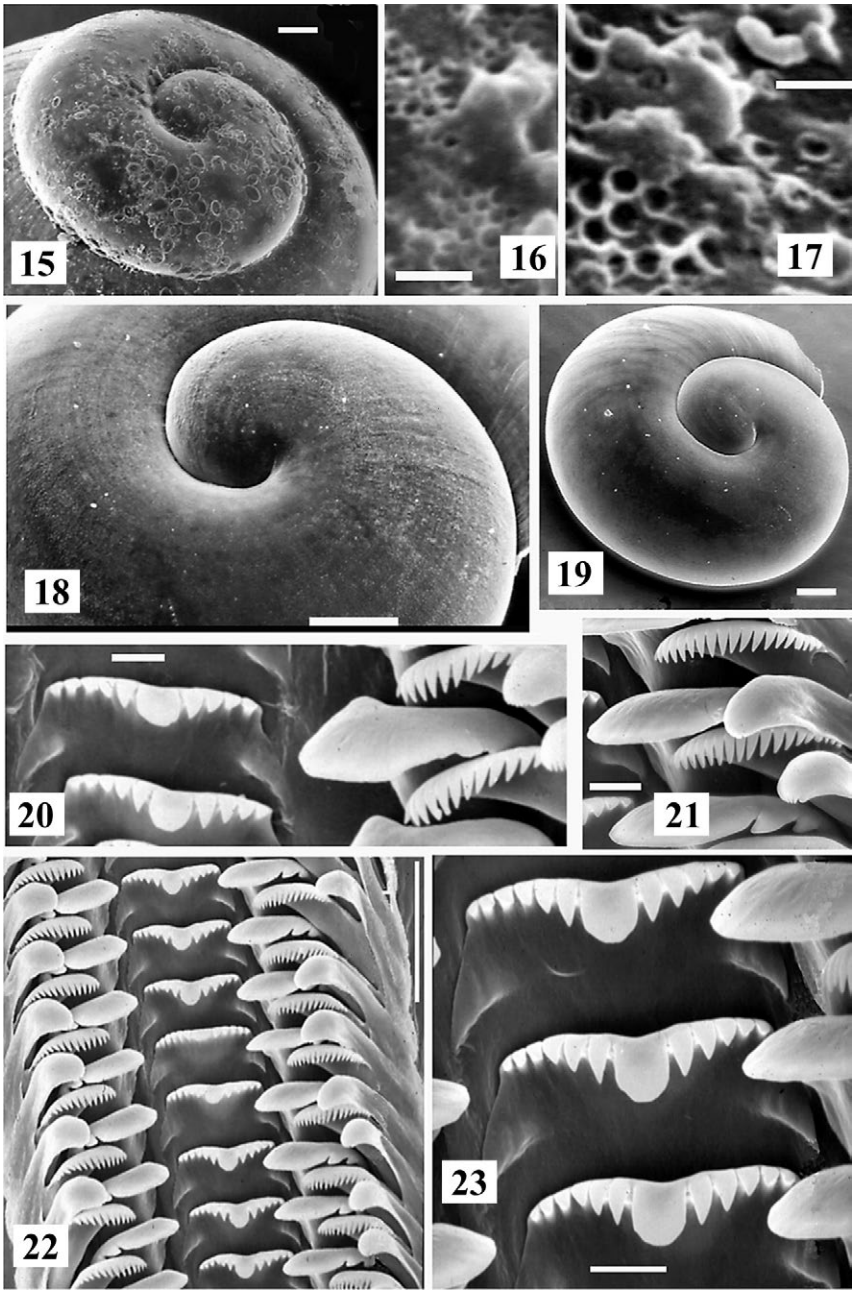


Figs 1–14: Shells of *Emmericia*: 1–5, *E. expansilabris*, Ombla; 6–14 – *E. patula*: 6–9 – river at Solin, 10–14, Cetina river at Radmanove Mlinice, scale bar 1 mm

The typically taenioglossate radula of *E. expansilabris* (Figs 20–23) had a central tooth with no basal cusps (Figs 20, 20–23) and 4 – 6 cusps on both sides of the central cusp. All the cusps on the rhachis were moderately big and sharp, often asymmetrically distributed on the tooth. The lateral tooth (Figs 20–22) had a large, lobe-shaped cutting edge and a pair of outer accessory cusps. The inner marginal tooth (Figs 20–22) had 18–20 long, elliptical, sharp-pointed cusps. The outer marginal tooth (Figs 20–22) bore a couple of very small and irregular cusps along its distal edge. The radulae of *E. patula* did not differ from the ones of *E.expansilabris*.

The central nervous system was highly concentrated, with shortened pleuro-supraintestinal and pleuro-subintestinal connectives, and without a ganglionic thickening on the tentacle nerve.

The head of both species (Fig. 24) has all the snout except its distal part pigmented black, the same as the tentacles at their distal half; the neck bearing grains of black pigment regularly spotted. The pigmentation pattern was little variable. The verge is triple-armed

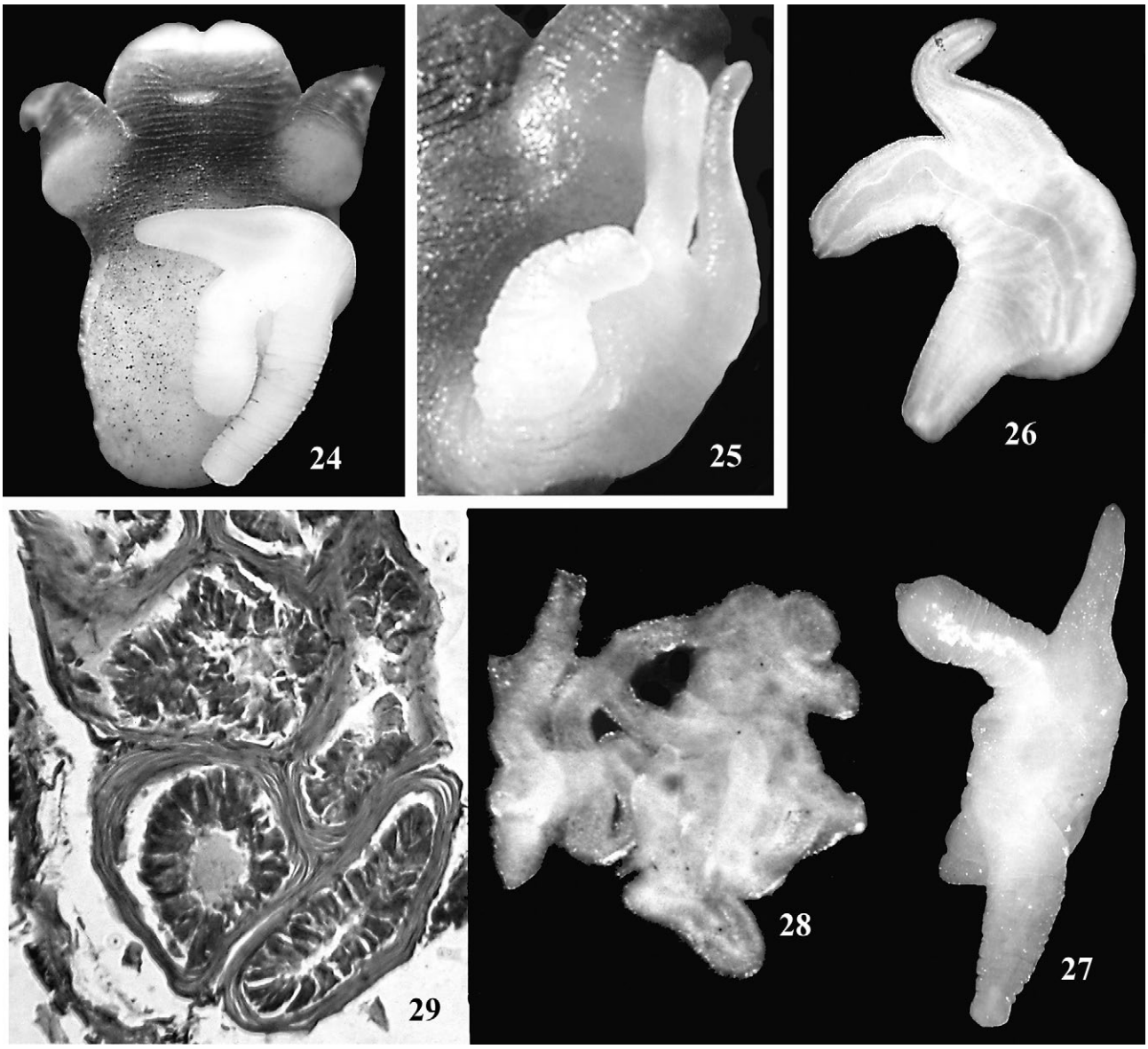


Figs 15–23. SEM photographs of protoconchs and radulae of *Emmericia*: 15–19: protoconchs: 15 – *E. patula*, habitus (scale bar = 100 μm), 16–19 – *E. expansilabris*: 16–17 – surface with net of pores (scale bar = 1 μm), 18–19 – habitus of shell of embryo extracted from egg capsule (scale bars = 100 μm in 18 and 100 μm in 19); 20–23 – radulae of *E. expansilabris*: 20 – central, lateral and inner marginal teeth (scale bar = 10 μm), 21 – lateral and marginal teeth (scale bar = 10 μm), 22 – several transverse rows (scale bar = 50 μm), 23 – marginal teeth (scale bar = 10 μm)

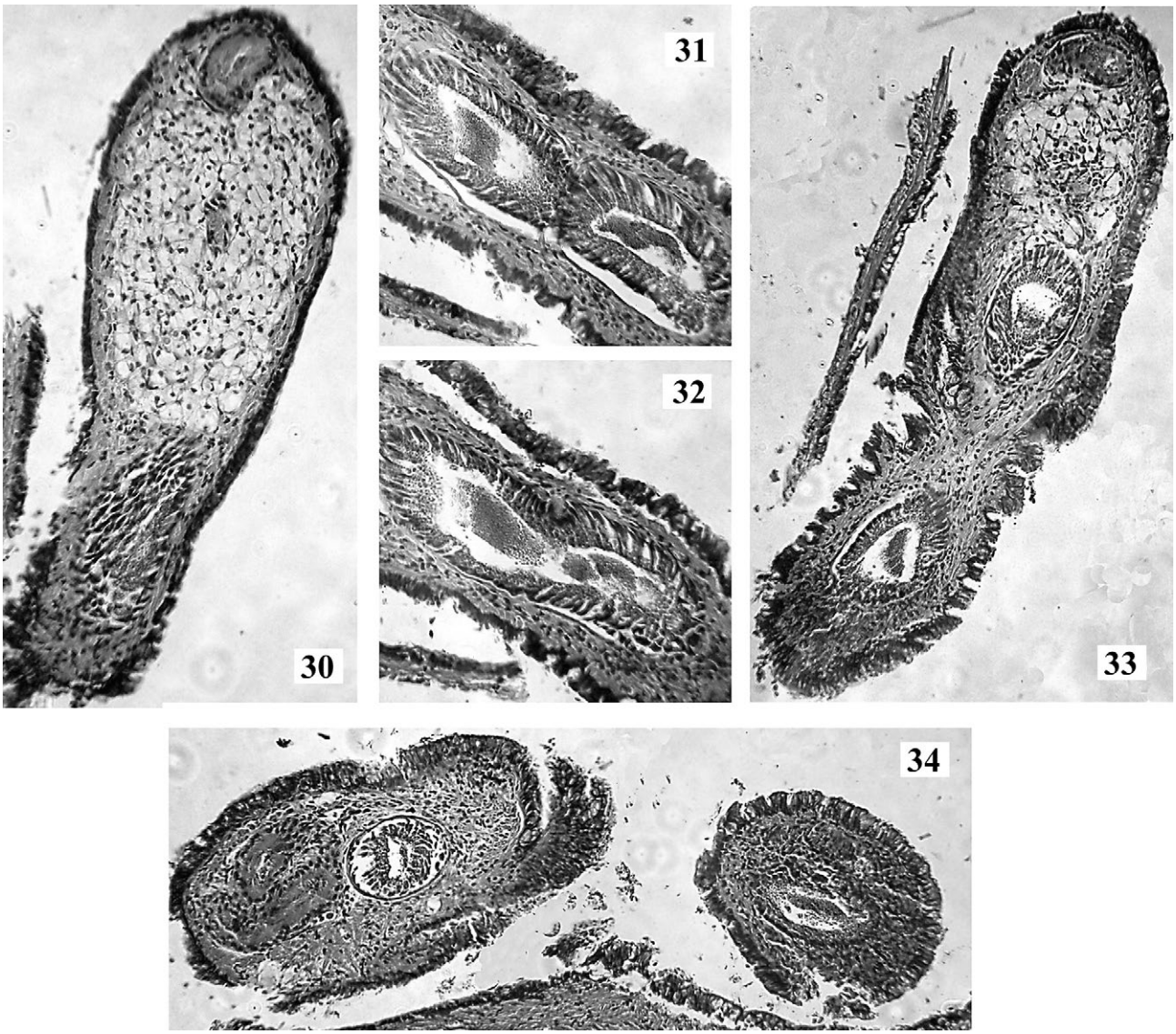
(Figs 24–27), the central and left arms have canals inside (Figs 26–27). The left arm, containing the terminal part of the vas deferens is narrow, slender and sharply pointed, the other two arms broader and blunt. The habitus of the verge in *E. expansilabris* (Figs 24, 26) and *E. patula* (Figs 25, 27) was variable (mostly due to fixation-caused contraction), the variablility ranges of the two taxa overlapped.

The big and long flagellum (Fig. 28), contained in the haemocel of the head, has the form of a tube of muscle fibres (Fig. 29), the glandular tissue and connective tissue arranged radially around the lumen of the tube. Serial histological sections of the verge (Figs 30–34), cut perpendicular to the axes of its arms, showed "cartilaginous" structures reinforcing the right arm, and a relatively narrow vas deferens running along this arm. In both central and left arm canals of the same structure were found (Figs 33–34). Closer to the base of the penis, the two canals merge (Figs 31–32) to form one canal (Fig. 30). This is the terminal part of the flagellum, which bifurcates into the central and left arm canals of the penis. No trace of any gland situated entirely in the verge was found.

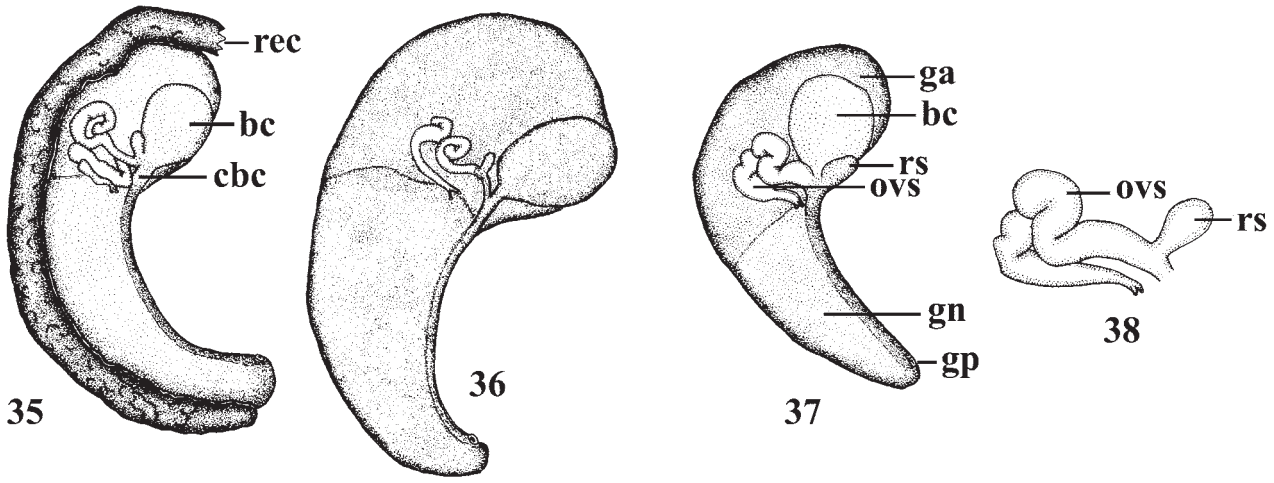
The female reproductive organs are shown in Figs 35–38. The capsule gland is relatively narrower than the albumen gland (Figs 35–37). The coiled "renal" (homology still not quite clear) oviduct forms an unpigmented spire (Fig. 38). There is one, medium-sized seminal receptacle, in rs_2 position (Fig. 38). In *E. patula* (Fig. 35) the spire of oviduct is moderately thick, the bursa copulatrix is ovoid, rather small, with a short duct. Both the bursa and receptacle are embedded in the tissue of the albumen gland (Fig. 35). In *E. expansilabris* (Figs 36–38) the female



Figs 24–29. Head, penes and flagellum of *Emmericia*: 24 – *E. expansilabris*, head and penis; 25 and 27 – *E. patula*, penis; 26 – *E. expansilabris*, penis in dark field, showing duct of flagellum running through central arm; 28–29 – *E. expansilabris*, flagellum: 28 – external view, 29 – serial section



Figs 30–34. *E. expansilabris*, serial sections of penis, showing vas deferens, cartilagineous support along vas deferens, and branching of duct of flagellum



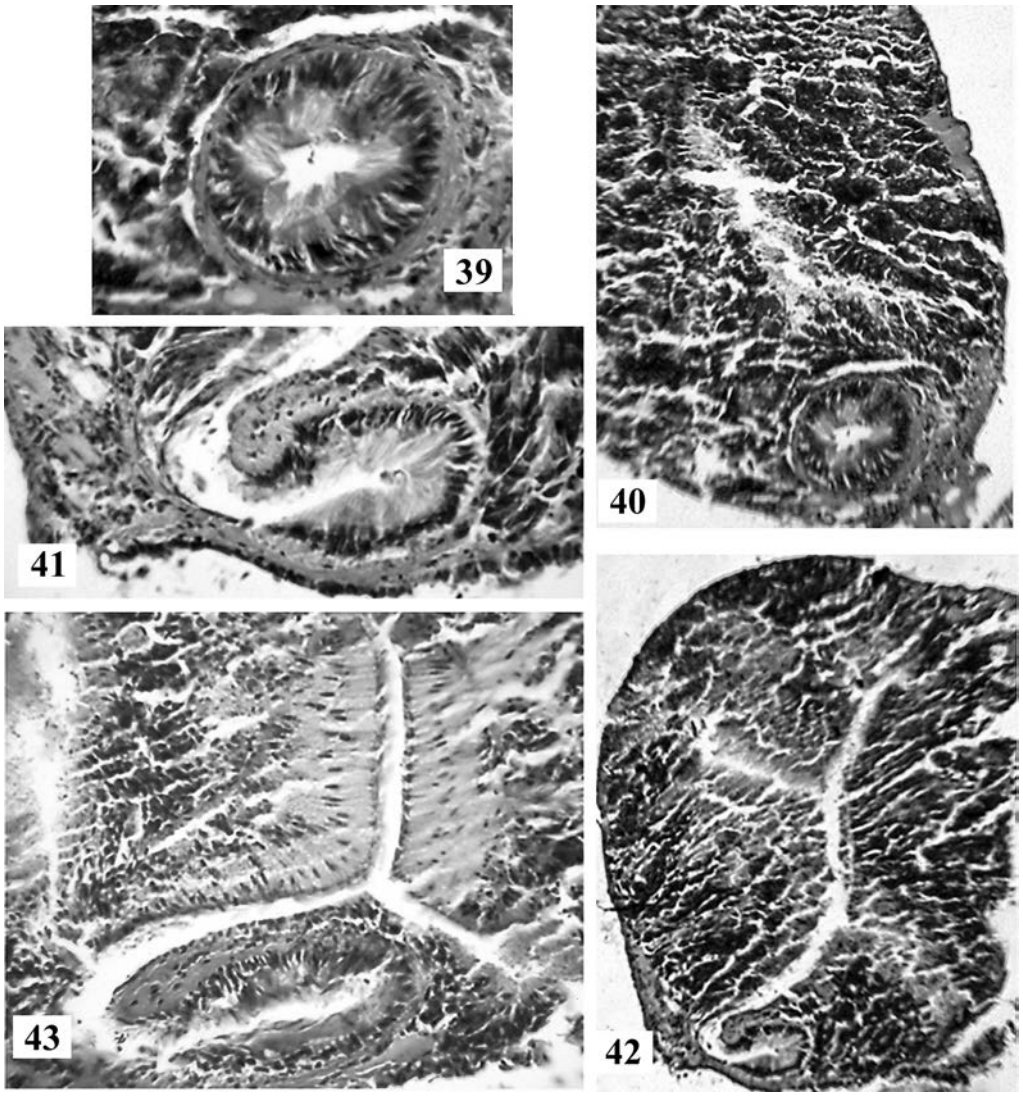
Figs 35–38. Renal and pallial section of female reproductive organs in *Emmericia*: 35 – *E. patula*, 36–38 – *E. expansilabris* (bc – bursa copulatrix, cbc – canal of bursa copulatrix, ga – albumen gland, gn – capsule gland, gp – gonoporus, ovs – coiled "renal" oviduct, rec – rectum, rs – seminal receptacle)

reproductive organs are either the same as in *E. patula* (Fig. 36) or the bursa is relatively bigger and the spire of oviduct thicker (Fig. 37). Serial sections of the pallial accessory gland complex of the female reproductive organs are shown in Figs 39–43. The duct of the bursa (Fig. 39) runs through the tissue of the albumen gland (40), the lumen of the gland is short and straight in outline. In the capsule gland (Figs 41–43) the lumen of the gland is more spacious and x-shaped (Fig. 42), and a typical, ciliated ventral channel is found (Figs 41, 43).

INFERRED PHYLOGENIES

The branch-and-bound technique applied to the characters listed in Table 1, resulted in 35 MPR trees, with CI=0.6286, RC=0.4683. Majority-rule consensus tree (Fig. 44) shows a polytomy grouping all the genera of Hydrobiinae s. stricto (*Hydrobia*, *Adriohydrobia*, *Peringia* and *Ventrosia*) together with the clade grouping all the other genera. The branch separating all four genera from the other taxa was supported by 61% of the bootstrap trees. 88% support was computed for the branch separating the above genera and *Pseududamnicola* from the other taxa. All the non-hydrobioid genera (*Heleobops*, *Gammatricula*, *Truncatella* and *Setia*) were grouped together. A polytomy grouped a branch terminated with *Lithoglyphus*, another "hydrobiid" branch including *Horatia* and *Sadleriana*, and a branch including *Bythinella*, *Amnicola*, *Bithynia* and *Emmericia*. In all the trees *Emmericia* formed a clade with *Bithynia*, bootstrap support of the clade was 59%.

Branch-and-bound applied to the binary-coded characters (Table 2) gave 12 MPR trees, 40 steps long, CI=0.5750, RC=0.4148. Majority-rule consensus tree (Fig. 45) was very similar to the one based on the multistate characters. The difference was only in grouping *Heleobops* and *Gammatricula* in one clade, and the trichotomy of *Amnicola*, *Bythinella* and the



Figs 39–43. *E. expansilabris*, transverse serial sections through female reproductive organs: 39–40 – albumen gland with duct of bursa (39) running through it; 41–43 – capsule gland and ventral channel (41 and 43)

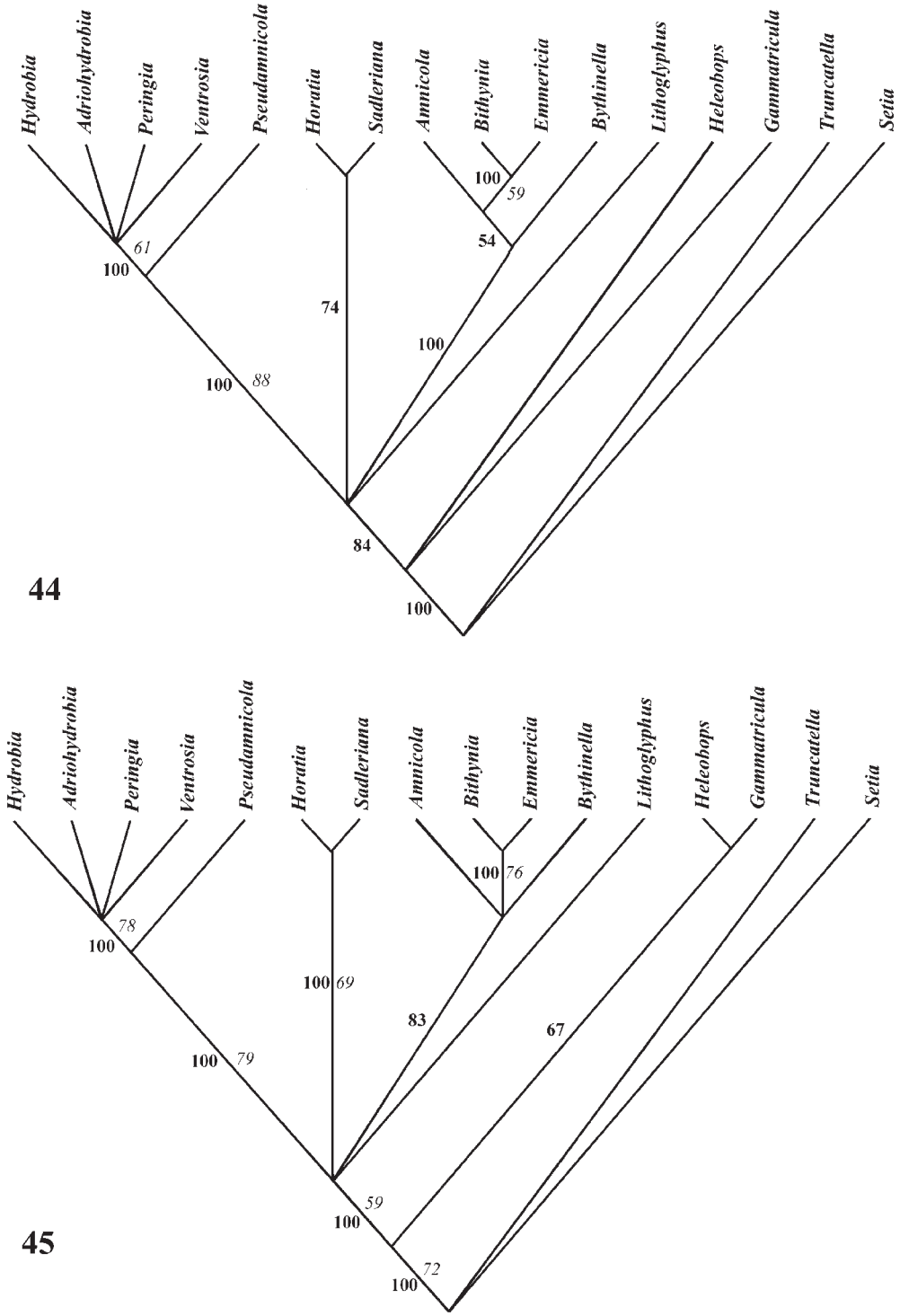


Fig. 44–45. Majority–rule consensus trees: 44 – summarizing 35 MPR trees, based on the multistate characters, all unordered, unweighted, CI=0.6286, RC=0.4683; 45 – tree summarizing 12 MPR trees, based on binary characters, 40 steps, CI=0.5750, RC=0.4148; in bold given percent of basic trees supporting given branch, in italics bootstrap support, 1,000 replicates

Bithynia/Emmericia clade. However, the bootstrap supports were different, and usually higher: 76 for the *Bithynia/Emmmericia* clade, 78 for the four genera of the Hydrobiinae s. stricto, 79 for the Hydrobiinae + *Pseudamnicola*, etc.

In all the reconstructions, the flagellum was a synapomorphy of the clade composed of the amnicolids (*Amnicola*), *Bythinella*, bithyniids, and *Emmericia*. The caecal appendix on the stomach was a synapomorphy of the Hydrobiinae but it occurred also in cochliopid *Heleobops*.

DISCUSSION

MORPHOLOGY

The shells of the studied species look like the ones described and illustrated by RADOMAN (1967a, 1983). The characteristic network of pores found on the surface of the protoconch is identical with the pores found on the protoconchs of *Bithynia* and *Bythinella* (FALNIOWSKI 1990a, FALNIOWSKI & SZAROWSKA 1995a) and may be a synapomorphy.

To my knowledge the radulae in *Emmericia* have not been illustrated in much detail so far. Their rhachis without basal cusps, they are similar to the ones described and illustrated by RADOMAN (1973a, 1983) and GIUSTI & PEZZOLI (1980), The highly concentrated central nervous system is described by RADOMAN (1973a, 1983) and GIUSTI & PEZZOLI (1980). As noted above, this grade of evolution may have been independently reached in the *Emmericia, Lithoglyphus* and *Emmericia* lineages.

The habitus of the verge is as given by RADOMAN (1973a, 1983) and GIUSTI & PEZZOLI (1980). Unlike in their description, however, there is no trace of a gland lying entirely within the verge, its duct opening on the tip of the central arm; instead, the flagellum duct branches into the central and left arms. The female reproductive organs resemble the ones figured by RADOMAN (1973a, 1983) and GIUSTI & PEZZOLI (1980), although the bursa copulatrix, seminal receptacle and the coiled oviduct are proportionally smaller when compared with the accessory gland complex; the shape of the bursa is different from the one figured by BOETERS (1998).

SPECIES DISTINCTNESS OF THE STUDIED TAXA

All the distinction between the studied species of *Emmericia* found in the literature (RADOMAN 1967a, 1983) concerns minor differences in shell characters and distribution. The shells of all the nominal species of *Emmericia* are alike. In the studied species the variability ranges overlap each other, although in general *E. expansilabris* is somewhat smaller. Despite careful examination, we found differences in neither the radulae, nor soft part external morphology, pigmentation, and anatomy. It seems that there is only one species, *E. patula*, whose dwarfish ecotype is *E. expansilabris*. The latter are probably dwarfed by the shortage of food and high densities in the spring.

PHYLOGENY AND CHARACTER EVOLUTION

Despite all the difference among the two morphology-based consensus trees presented above, as well as between any one of them and the molecularly-based trees, in all the trees the position of *Emmericia* is conformable. In all the morphology-based trees its sister taxon is *Bithynia*. In fact, this sister- group relationship would be even more evident, if the unique network of pores of the periostracum was considered.

FRETTER & GRAHAM (1962) speculated that the secretion of the flagellum may be poured onto the wall of the female's mantle cavity and anchor the penis in position during the copulation. During the penis erection the arm containing the flagellum diverges from the one including the vas deferens, thus it seems doubtful if the flagellum tip penetrates female ducts. The secretion of the flagellum may attract female or help penetrating female orifice during succeeding copulation. Sometimes a portion of the flagellum can be seen turned outside the tip of the left arm (FALNIOWSKI 1990). Anyway, the function of the flagellum remains enigmatic. Whatever its function may be, the organ seems unique, and wherever it is found, its histological structure looks the same (*Bithynia, Bythinella, Amnicola, Marstoniopsis, Parabythinella, Emmericia*). This suggests homology, confirmed by the inferred phylogeny. However, the character mapping on the molecular trees (WILKE et al. 2001) suggests that the flagellum might have been secondarily lost in some of the lineages.

Apart from the flagellum, not a single morphological character considered in the present paper can be safely applied to the phylogeny reconstruction of higher hydrobioid taxa. In particular, problems arise where one compares family-level taxa from different continents. It is evident that the hydrobioid evolution has for a very long time been running independently in several territories. The Rissooidea are known since the Toarcian, Early Jurassic (GRÜNDEL 1999), some Recent rissoidean genera having originated as early as in the Oxfordian, Late Jurassic. The hydrobioids are probably not much younger. Morphological characters will work better if one considers hydrobioid genera of some more restricted geographical range. They will work better, as well, if one considers restricted monophyletic groups, but are not sufficient at such a high level of universality as the Hydrobioidea as a whole.

APPENDIX 3

FLAGELLUM-BEARING HYDROBIOIDS. 2. *PARABYTHINELLA* (GASTROPODA: PROSOBRANCHIA: RISSOOIDEA): A CONTRIBUTION TO THE TAXONOMY AND PHYLOGEOGRAPHY OF THE EUROPEAN AMNICOLIDAE

INTRODUCTION

Parabythinella is a group of tiny hydrobioid inhabitants of Balkan lakes. The taxon includes: *P. macedonica* (Hadžišče, 1958) found in lakes Prespa and Mala Prespa, and *P. graeca* Radoman, 1978 found in lake Vegorritida (RADOMAN 1983, 1985). Based on historical geomorphological data, RADOMAN (1985) classified lake Vegorritida in the Aegean-Anatolian lake group, which is separated by the emerging Hellenides from the Adriatic-Ionian group including lakes Garda, Bacina, Skutari, Ohrid, Trichonida and Amvrakia. Prespa and Mala Prespa are included in a distinct Prespa-system. The latter, somewhat intermediate, system, is not well defined geomorphologically. Some of the above lakes are regarded as Tertiary; RADOMAN (1985) emphasizes the high levels of endemism of those "Tethys-derivatives". His concept of the distinctness of the "intermediate" Prespa-system of lakes is not, in fact, supported by historical geology data. It is derived from the fact that those lakes are inhabited by both pyrgulid representatives of the Adriatic-Ionian group and *Parabythinella* known from lake Vegorritida of the Aegean-Anatolian lake group. *Parabythinella* is thus a crucial taxon for the verification of Radoman's speculations about the history of the hydrobioid malacofauna; as well as all the Balkan lakes system. He emphasizes the Tertiary (or even older) origin of the hydrobioid genera (including *Parabythinella*) and obviously overestimates the stability of the occurrence of a gastropod species which according to him may neither come from nor migrate to a new lake. The latter assumption is hardly realistic.

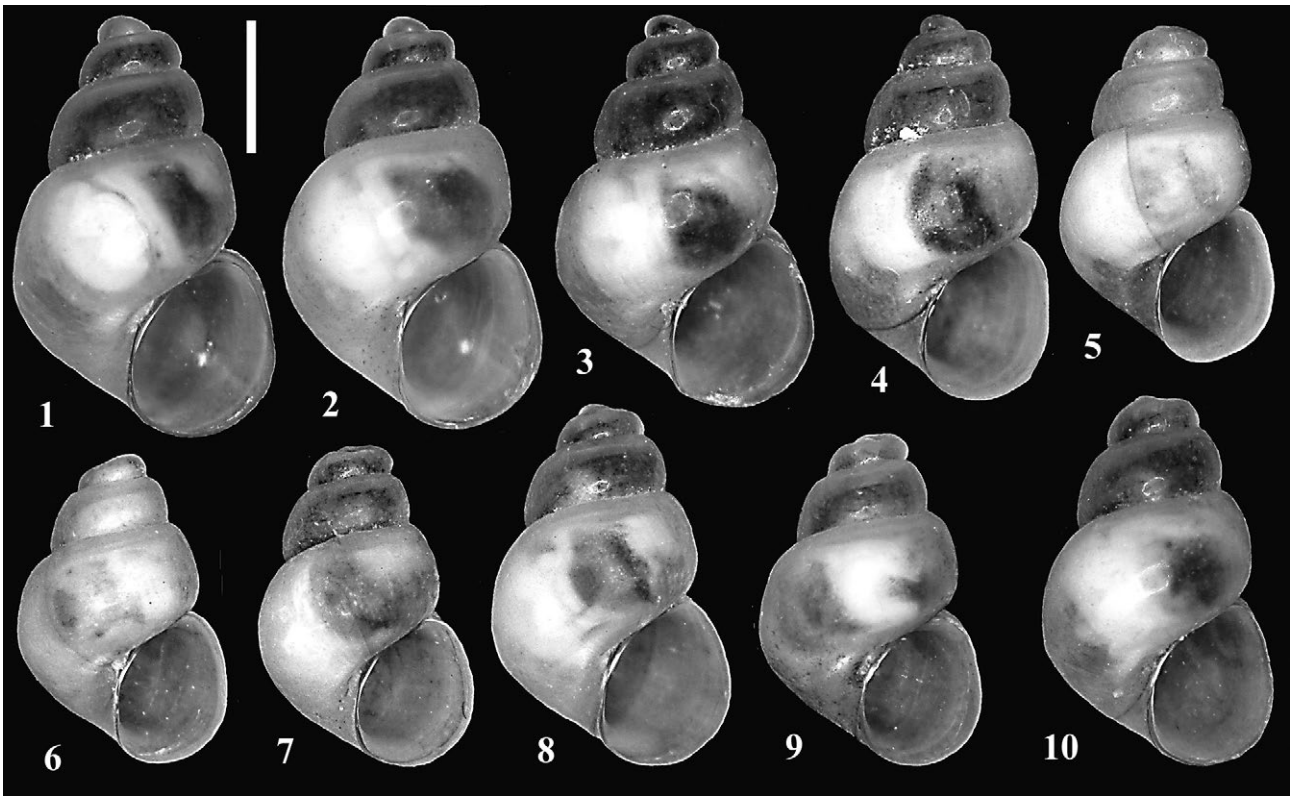
The present study is aimed at: (1) relationships within the genus, (2) the position of the genus within the hydrobioid snails, (3) estimation of the time and possible scenario of divergence of the genus in the context of the origin and history of the Balkan lakes system.

MATERIAL AND METHODS

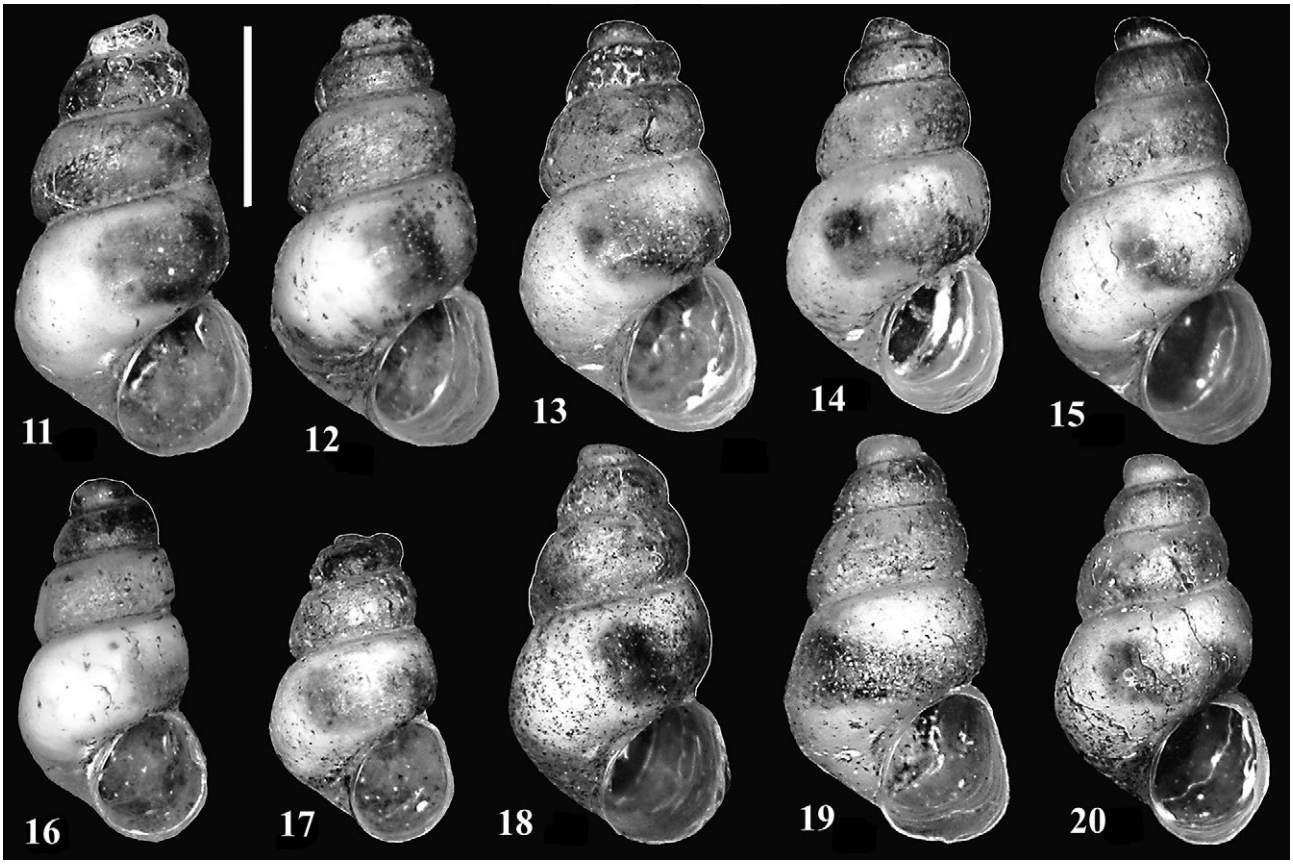
In 2003, numerous specimens of *Parabythinella graeca* were collected at the type locality of the species, in lake Vegorritida. Most of them were fixed in 4% formalin, and later kept in 80% ethanol, some specimens were fixed in 80% ethanol for the molecular work. A few specimens of *P. macedonica* collected in lake Prespa were fixed in 80% ethanol. The techniques applied to the morphological and molecular study were the same as described in the main text. To compare the shells, seven morphometric parameters (Fig. 44) were measured, with a COHU 3715 camera, coupled with a frame-grabber and a PC equipped with the MultiScanBase v. 11.06 software. The linear measurements were logarithmically transformed, for the angular ones the arcsine transformation was applied. Euclidean distances were calculated, and UPGMA clustering and minimum spanning tree were computed with NTSYSpc (ROHLF 1998). PCA was computed basing on the matrix of correlation, and the original observations were projected into PC space, with superimposed minimum spanning tree to detect local distortions in the data.

RESULTS

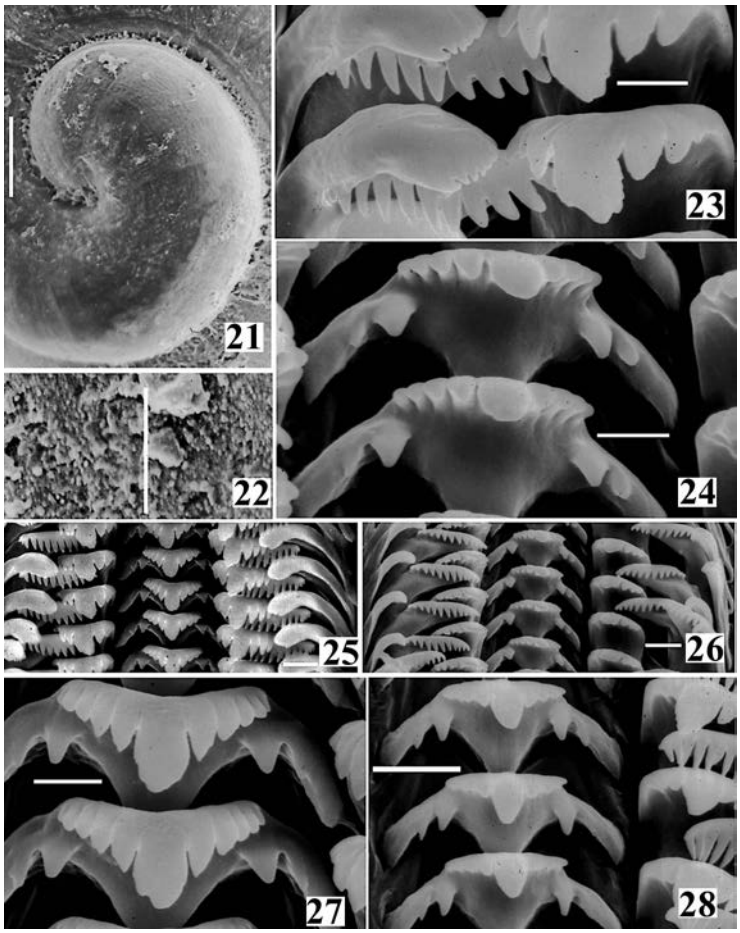
Parabythinella graeca (Figs 1–10) shells differ from the ones of *P. macedonica* (Figs 11–20). The UPGMA clustering (Fig. 45) has classified the measured specimens in two clusters, each consisting of the specimens of one of the nominal taxa, the variability range of *P. macedonica* being somewhat broader. The first two PC explained cumulatively 84.083% of the total variance (56.963 and 27.200, respectively). The third PC explained only 11.916%, which is less than expected under the broken-stick model (15.612%). Hence, the analysis is limited to the first two PCs. PCA also shows the distinctness of the two taxa (Fig. 46), this, however, occurs along the PC1 axis only. *P. macedonica* being smaller, all or almost all the differences are connected with size differences.



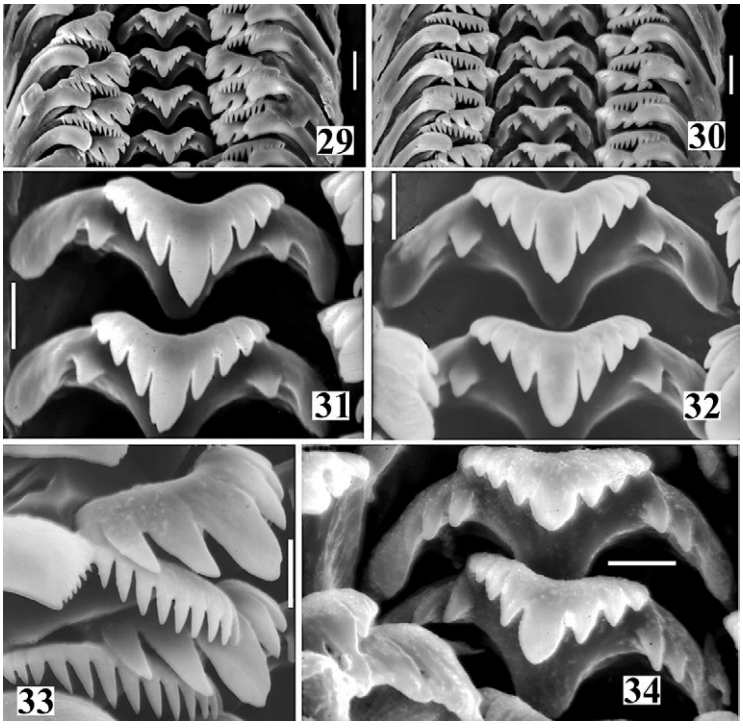
Figs 1–10. Shells of *Parabythinella graeca* from lake Vegorritida; scale bar 0.5 mm



Figs 11–20. Shells od *Parabythinella macedonica* from lake Prespa; scale bar 0.5 mm

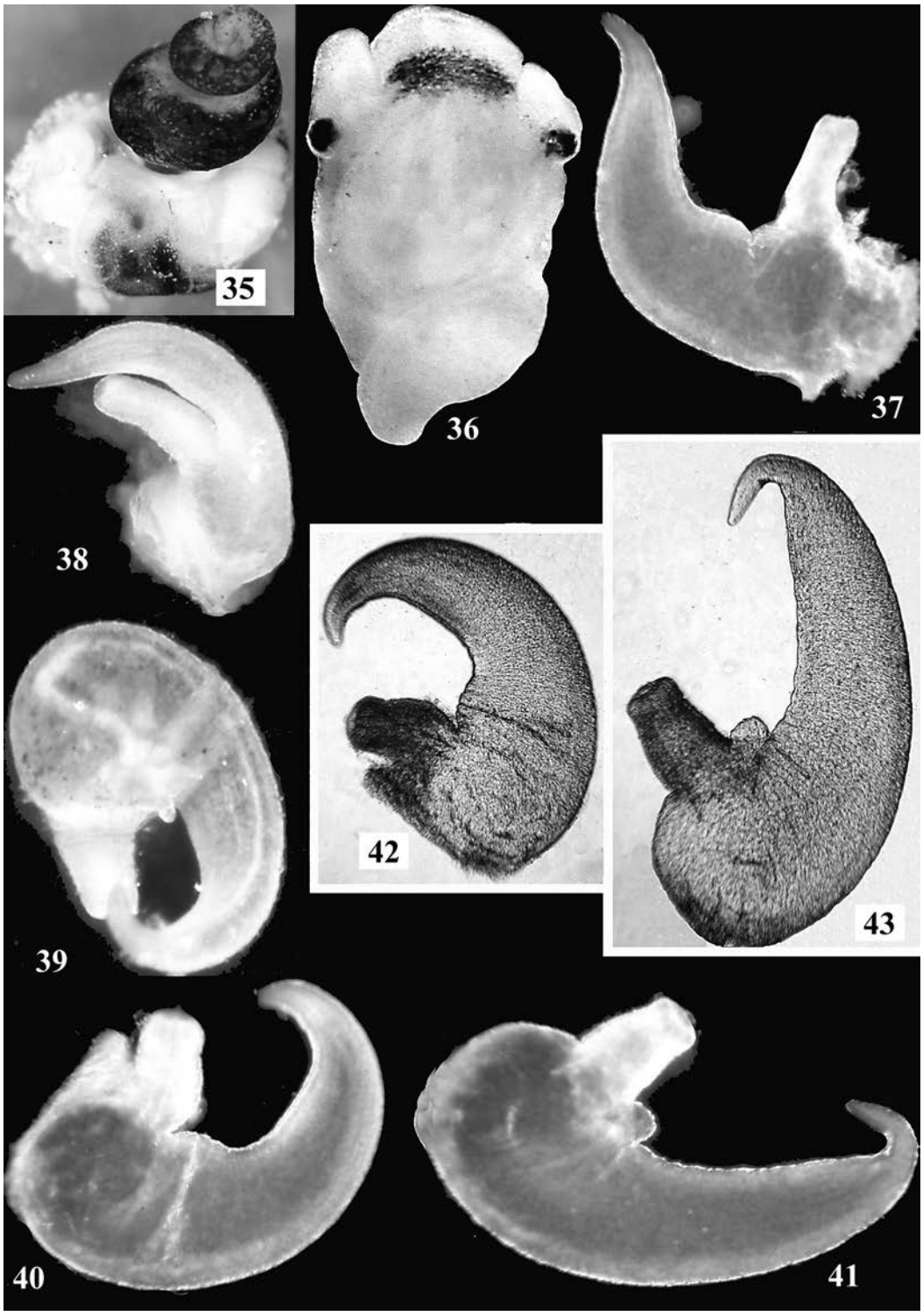


Figs 21–28. *Parabythinella graeca*: 21 – protoconch surface, scale bar 100 μm; 22 – higher magnification of protoconch surface with traces of small pores, scale bar 5 μm; 23–28 – radulae: 23 – lateral and marginal teeth, 24 – central teeth, 25–26 – transverse rows, 27–28 – central teeth; scale bars 5 μm in 23, 24 and 27, and 10 μm in 25, 26 and 28

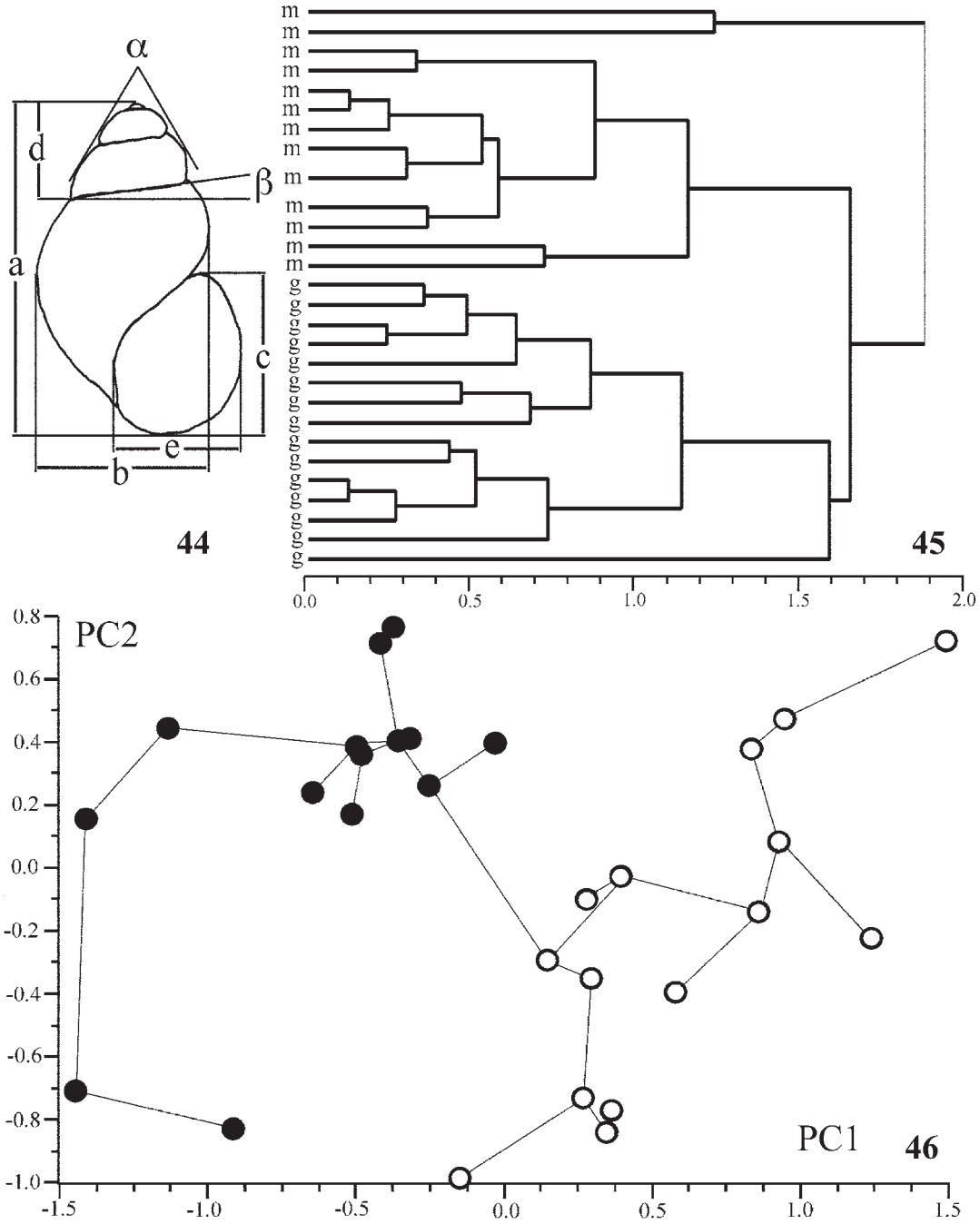


Figs 29–34. Radulae: 29–33 – *Parabythinella macedonica*: 29–30 – transverse rows, 31–32 – central teeth, 33 – lateral and marginal teeth; 34 – *Marstoniopsis insubrica*, Sarąg Lake, Masurian Lakes, central teeth; scale bar 10 μm in 29 and 30, and 5 μm in 31–34

The protoconch of *P. graeca* has a characteristic, spiral sculpture (Fig. 21) and, seen under higher magnifications, traces of a network of pores (Fig. 22). The radulae of *P. graeca* (Figs 23–28) look the same as those of *P. macedonica* (Figs 29–33), and are very similar to the ones of *Marstoniopsis insubrica* (Küster, 1853) (Fig. 34). The pigmentation of the soft parts in *P. graeca* is characteristic: a black pigment entirely covers the visceral sac, this coupled with a somewhat lighter band of pigment running across the head (Figs 35–36). The penis (Figs 36–43), with its broad and massive right arm and a much smaller left arm, looks like the one of *Marstoniopsis*. The penes of *Parabythinella graeca* and *P. macedonica* are identical. The typi-



Figs 35–43. *Parabythinella graeca*: 35 – pigmentation of head and visceral sac; 36 – head of male, with visible penis, big eyes and pigmentation of snout; 37–43 – penis habitus (37 and 39: ventral side, others: dorsal side)



Figs 44–46. Shell biometry of *Parabythinella*: 44 – measurements of shell: a – shell height, b – body whorl breadth, c – aperture height, d – spire height, e – aperture breadth, α – apex angle, β – angle between body whorl suture and horizontal surface; 45 – UPGMA clustering computed on Euclidean distances calculated on the measurements listed above: g – *Parabythinella graeca*, m – *P. macedonica*; 46 – specimens of *P. macedonica* (black circles) and *P. graeca* (white circles) with superimposed minimum spanning tree, projected into space of first two PCs (PC1, PC2)

cally amnicolid female reproductive organs (with a spermathecal duct and a very large seminal receptacle, without a bursa copulatrix: see the main text) do not differ in either gross anatomy or histology from the one described for *Marstoniopsis*.

DISCUSSION

The above data suggest that the two taxa of *Parabythinella* represent one species which is congeneric with *Marstoniopsis*, and belongs to the family Amnicolidae. The unexpectedly close relationship between *Parabythinella* and *Marstoniopsis* needs some explanation. Although the molecular clock rate is not constant either throughout the animal kingdom or the mollusca (AVISE 2000), it has fortunately been calibrated at the hydrobioid snails (WILKE 2003, 2004). Given the calculated divergence (K2P) between *Parabythinella* and *Marstoniopsis* averaging 1.51% ±0.44%, the estimated time of divergence would be no less than 600,000, and no more than 1,000,000 years. Thus we have to check the geological history of the Central Europe and the Balkans.

At the Eocene/Oligocene boundary the intercontinental Paratethys Sea arose in the western part of the earlier Tethys Ocean (RÖGL 1998, 1999), formed by aggregate basins ranging from the valley of the river Rhone in Western Europe to Central Asia. The West Paratethys relates to the region of the Rhone, the Central Parathetys to the Pannonian or Middle Danube lowland, the East Paratethys to the Black and the Caspian Seas. The Lower Sarmatian Sea existed in the region of the Central and Eastern Paratethys, about 15 million years ago, at the end of the Miocene. It ranged from the Pannonian lowland to the Aral sea, possibly extending further into Central Asia. Although its salinity was only about 20‰, it was inhabited by marine fauna. Later, 11.5 million years ago, the Pannonian brackish lake became isolated. An endemic fauna, living at salinity 12–15‰, was formed in that lake (GEARY et al. 2000). The Pannonian Lake connected the Recent South Balkans with the Recent Middle Danube lowland, by a wide arch expanding to the east. Unfortunately, about four million years ago the Pannonian Lake disappeared, so we cannot correlate it with the much later estimated time of divergence between the North European *Marstoniopsis* and the Balkan *Parabythinella*. All these events are much later than what RADOMAN (1985) speculated about the Tethys as the primary source of the Balkan freshwater malacofauna. Like in many cases mentioned by STANLEY (1998), evolution was less time-consuming than traditionally believed.

FALNIOWSKI & WILKE (2001) listed three possible scenarios of how the recent habitat range of *Marstoniopsis* may have been reached: (a) the species originated south of the Alps and then spread throughout the northern part of Central Europe, perhaps after the last glaciation, either (b) it survived the glaciations in refuges in both Northern Europe and northern Italy, or (c) it survived the Pleistocene glaciations in refugia on the northern side of the Alps with a subsequent dispersal to southern Switzerland and northern Italy on the one hand, and to the North of Europe on the other. The distance between the Recent North European and Swiss/Italian populations of *Marstoniopsis* used to be shorter than it is now (1,220 km). FÜKÖH (2000), for example, reports on the periodic occurrence of *Marstoniopsis scholtzi* (Schmidt) in Holocene sediments, in the Boreal biozone. The species occurs neither in the recent fauna of Hungary, nor in the older Quaternary (Pleistocene) faunas. Given the present data on the high genetic similarity between *Parabythinella* and *Marstoniopsis*, the estimated time and hypothetical place of their divergence, the Swiss and Italian populations of *Marstoniopsis* seem to be secondary, despite their Recent shortest distance from the populations of *Parabythinella*. It must be added that lake Garda, of glacial origin, is no more than 22,000 years old, except for the obviously older, tectonic depression forming the northern part of the lake. However, covered by ice, it was hardly suitable as a refugium during glaciation.

To conclude, *Parabythinella graeca* and *P. macedonica* are most probably no more than two subspecies of *P. macedonica*. Both belong to the genus *Marstoniopsis* van Regteren Altena, 1936. The genus is very little varied molecularly. Among the hydrobioid snails, differences like those observed between *M. insubrica* and *Parabythinella graeca* can be found within a species. Thus the two species, the North European *Marstoniopsis insubrica* and the Balkan *M. macedonica,* must have got separated not earlier than in the Holocene. Perhaps, as FÜKÖH's (2000) data suggest the genus was once scattered throughout the area where it does not occur recently. Given the long existence of the Tertiary lake Prespa, lake Vegorritida being probably not much younger, one can assume that those lakes, not affected by glaciations, are the place of origin/centre of distribution of the genus in Europe. On the other hand, the inconspicuous differences between the *Parabythinella* populations of Prespa and Vegotrritida suggest that it is not long since that the two lakes have been inhabited by the amnicolids.

APPENDIX 4

DESTROYED LOCALITIES OF BALKAN HYDROBIIDS[1]

Croatia:

1. Zrmanja River (freshwater part), Croatia, the type locality of: *Dalmatinella fluviatilis* Radoman, 1973, *Belgrandiella zermanica* Radoman, 1973, *Islamia zermanica* Radoman, 1973, *Lithoglyphulus tedanicus* Schlickum et Schütt, 1971; the river having been dammed in its upper course was apparently changed into a system of stagnant pools (like that at Kaštel Zegarski) interspersed with a dry river bed (like that at Ervenik); furthermore, since the beginning of the nineties of the 20th century the towns and villages in Krajina have been not inhabited and any spot in the area was hardly accessible.
2. Spring Glogi, Podugrinac at Bribir, the type locality of *Vinodolia fiumana* Radoman, 1973, a locality of *Graziana lacheineri adriolitoralis* Radoman, 1975; in the years 1999, 2001, 2002, 2004 there was no water in the spring.

Montenegro:

3. Velje oko, Gluhi Do village, the type locality of *Anagastina gluhodolica* (Radoman, 1973), a locality of *Antibaria notata* (Frauenfeld, 1865); there was a big water intake and no water on the surface.
4. Spring at Lipovik near Rijeka Crnojevića, the type locality of *Anagastina matjasici*; the biggest spring at Lipovik was used by the local people as a source of water; there being no permanent water intake, only several "provisional" pumps installed on the spring, it was not quite destroyed, yet no snails could be found there; in another, smaller spring at Lipovik, which was much overgrown and would not have been accessible without help of the local people, I found only *Orientalina* sp.
5. Popovo selo (Vodice) in Buljarica near Petrovac na moru, the type locality of *A. notata*; there was nothing left of the natural spring; there was only tap water taken from the spring, available at the monastery.
6. Other springs along the Adriatic coast which RADOMAN (1983) cites as localities of *A. notata*: Bostanj by Pelinovo, Smokovijenac near Sveti Stefan, Golubovici and Dobra voda at Buljarica, Studenac at Braići above the Budva town; at the latter there was a huge water intake; the whole area close to the seaside was changed to a holiday and health resort built with numerous hotels, etc. and I did not find any of the above localities.
7. Spring Smokovijenac at Tomići, the drainage area of Skutari lake, a locality of *A.notata* and *Orientalina montana* Radoman 1973; the very small village seemed to be almost uninhabited, there being a few old, empty houses; the spring was overgrown with dense bushes, only a thin trickle of its water was accessible, there being no hydrobiids besides *Litthabitella chilodia* (Westerlund, 1886) and *O. montana*.
8. Spirov izvor, Podmeret below Braćeni, the type locality of *Bracenica spiridoni* Radoman, 1973; the spring did not exist and the locals did not even know its name.

Greece:[2]

9. Spring at Perama, springs at the north bank of the lake Pamvotis at Janina, once the type localities of: *Paladilhia* (*Paladilhiopsis*) *janinensis* (Schütt, 1962), *Horatia* (*Neohoratia*) *epirana* Schütt, 1962, *Semisalsa steindacheri* (Westerlund, 1902), *Orientalina curta albanica* Radoman, dried out at the beginning of the eighties of the 20th century.
10. Spring at Kefalovriso, the type locality of *Trichonia kephalovrissonia* Radoman, 1973; nothing was left of the spring as water intake had been built on it.
11. The holy spring at Vravrona (the ancient Brauron), near Athens, east of the city, one of the very few "terra typica" localities of *Pseudamnicola macrostoma* (Küster, 1853) [referred to as *Pseudamnicola* cf. *moussoni* (Calcara) in FALNIOWSKI & SZAROWSKA 1995] left; dried out by digging a deep and broad drainage ditch at the place.
12. Spring at Kessariani, Athens, the type locality of *Daphniola louisi* Falniowski et Szarowska, 2000; no snails were found in the small, artificial pool beneath the spring where they were once abudant (FALNIOWSKI & SZAROWSKA 2000), there were fish in the pool and only very few specimens in the spring whose water was no more suitable for drinking; it is doubtful that the topotypical population of the species would persist for long.
13. Spring at Myli (the ancient Lerna), the Peloponnese, the type locality of *Semisalsa tritonum* (Bourguignat, 1852); due to the water intake built on the spring, the snails, though still numerous, were endangered; if more water were taken from the spring, the snails would easily disappear; the species (whatever it is, not a *Semisalsa*) recorded

[1] The list includes only the localities I visited in 1999–2004, so most probably it is incomplete.

[2] Most of the destroyed hydrobiid localities in Greece are described in SZAROWSKA & FALNIOWSKI (2004 and in press).

from only two localities could not be found at the other one, at Kefalari.

14. Spring 5 km SW of Githion, the type locality of *Horatia (Neohoratia) hadei* Gittenberger, 1982; a water intake was built on the spring and the snails, if they still existed, were not accessible.
15. Spring Kamena Vourla, the type locality of *Grossuana serbica vurliana* Radoman, 1966 and one of the few localities of *Semisalsa achaja achaja* (Clessin, 1879) given by SCHÜTT (1980); if at all present, the snails were inaccessible, as no water of the thermal spring, taken by pipes and pumps to supply the huge health resort at the place Agios Konstantinos, reached the surface.
16. Spring at Vrysia, the type locality of *Graecorientalia vrissiana* (Radoman, 1966); due to the water intake built on the spring there is neither water nor snails on the surface.
17. Spring at Velestino. According to SCHÜTT (1980) once a locality of *Belgrandiella (Turcorientalia) hohenackeri hohenackeri* (Küster, 1853) and *Semisalsa achaja sorella* (Westerlund, 1879); the huge spring dried out in the nineties of the 20th century due to deep wells drilled to supply the nearby cotton fields with water (information obtained from a local).
18. Vegorritida Lake, the type locality of *Parabythinella graeca* Radoman, 1978 and *Graecoanatolica vegorriticola* (Schütt, 1962); the conditions in the lake deteriorated due to the water pollution and drop in the water level; of the two species I could only find live *P. graeca*, of *G. vegorriticola* I found empty shells in 2003, while two years later WILKE and ALBRECHT (personal communication) could not find even those.
19. Trichonida Lake, the type locality of *Trichonia trichonica, Islamia graeca*, a locality of *Dianella thiesseana*; in 2003, compared to 1985 (FALNIOWSKI personal communication) this once oligotrophic lake was apparently eutrophicated, the water level a couple of metres lower; I found only *D. thiesseana*.