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PSEUDAMNICOLA PAULUCCI, 1878 (GASTROPODA: HYDROBIIDAE) IN THE BALKANS

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ABSTRACT: The shell, protoconch, radula, head, penis, and female reproductive organs are described and illustrated for Greek *Pseudamnicola macrostoma* (Küster) from two localities in Attica, *P. negropontina* (Clessin) from Evvia Island, and *Adrioinsulana conovula* (Frauenfeld) from Pag Island in Croatia. The two populations of *P. macrostoma* were sampled in 1985, and both were later destroyed. *P. negropontina* was sampled again in 2003, and the material was used for extraction of DNA. Four sequences of mitochondrial CO1 gene were obtained. The parsimony-based molecular phylogeny (with eight sequences from the GenBank, including *P. lucensis* (Issel) and *Adrioinsulana conovula*) showed that the Greek *P. negropontina* actually belongs to the genus *Pseudamnicola*, and that the molecular differences between *P. negropontina* and Italian *P. lucensis* are twice greater than between the latter and *A. conovula*, thus distinguishing the genus *Adrioinsulana* is not justified: all the species belong to *Pseudamnicola*.

KEY WORDS: freshwater snails, Hydrobiidae, *Pseudamnicola*, phylogeny

INTRODUCTION

The genus *Pseudamnicola* Paulucci, 1878, with its type species *Paludina macrostoma* Küster, 1853 (KABAT & HERSHLER 1993), once harboured dozens of taxa, unified by small, ovate cone-shaped umbilicate shells with a fairly low spire and large body whorl, and by the radula (THIELE 1929) whose description can be applied to many rissooid gastropods. The species assigned to this genus were known from the British Isles, Netherlands, and Spain, through France, Italy and the Balkans, to Romania. However, after checking the anatomy, especially female reproductive organs, it became evident that such "*Pseudamnicola*" was a collection of several evolutionary lineages, not necessarily close to each other (RADOMAN 1973, 1983, GIUSTI & PEZZOLI 1980), now assigned to such genera as *Mercuria*, *Sadleriana*, *Orientalina*, *Grossuana*, *Polinskiola*, *Ohridohauffenia*, *Ohrigocea*, *Dolapia*, *Graecorientalia*, *Belgrandia*, *Lyhnidia*, *Adriohydrobia*, etc. (WAGNER 1927, RADOMAN 1983, GLÖER 2002). Shell characters are often positively misleading within the Rissooidea (e.g. SZAROWSKA & WILKE 2004). BOETERS (1971) described the anatomy of *Pseudamnicola lucensis* (Issel, 1866) from its type locality in Italy. RADOMAN (1972) described the anatomy of *P. conovula* (Frauenfeld, 1863) from Pag Island in Croatia; later he described a new monotypic genus *Adrioinsulana* for that species (RADOMAN 1978). GIUSTI & PEZZOLI (1980) distinguished three species of *Pseudamnicola* in Italy: *P. lucensis*, *P. moussoni* (Calcara, 1841) and *P. conovula*, and illustrated their reproductive organs.

Within the literature on the Greek malacofauna (see BUTOT & WELTER-SCHULTES 1994) there are some reports on the occurrence of *Pseudamnicola*, most of them not based on anatomy. SCHÜTT (1980) distinguishes in Greece seven taxa of *Pseudamnicola* (six species and one subspecies), four of them from the islands, two species and one subspecies in continental Greece and Evvoia Island. However, all of them (two new for the science) are distinguished based on the shell alone, anatomy is illustrated (not described)

for two species only, and the drawings are poor. Moreover, in the case of *Pseudamnicola* the author follows the same rule as with his "*Belgrandiella*", "*Semisalsa*" or *Bythinella* – cuts the territory into separate pieces, each one of them harbouring one "species" of the "genus". FALNIOWSKI & SZAROWSKA (1995a, b) described the shell surface and internal structure in *Pseudamnicola* cf. *moussoni* from Vravrona.

The aim of the paper is to describe the shells, radulae and anatomy of some Greek *Pseudamnicola*, and to compare it with the Italian species. We also wanted to check if the Greek species belong really to this genus, basing on molecular data – mitochondrial CO1 gene. Finally, we reconsidered the distinctness of the genus *Adrioinsulana*.

MATERIAL AND METHODS

1) In March 1985 several hundred specimens of *Pseudamnicola macrostoma* (Küster, 1853) were collected in Vravrona (the ancient Brauron), Attica, from the small stream flowing to the sea from the holy spring, at the historical place (FALNIOWSKI & SZAROWSKA 1995a). The material was fixed in 4% formalin and stored in 70% ethanol. Visiting the place in 2003 we found it totally destroyed – the spring had recently been dried out by digging a deep and broad drainage ditch at the place – making collection of some new material for the molecular work impossible.
2) In May 1985 several hundred specimens of *P. macrostoma* were collected in Kato Souli, from a deep ditch close to the small airport. The snails were fixed in 4% formalin and stored in 70% ethanol. Visiting the same place in 2003 we found no ditch, only some brackish, small water bodies, and all the area covered with garbage – apart from some *Ventrosia* there were no snails there. Despite visiting several places known from the literature, we did not find any *P. macrostoma* for molecular work.
3) In May 1985, and in September 2003 several specimens of *P. negropontina* (Clessin, 1878) were collected in Marmaris at Evvoia Island, from an artificial pond, forming the water intake at a spring. For morphological study, the other *P. negropontina* were fixed with 4% formalin and after 24 hours transferred to 80% ethanol for storage. For molecular study several specimens were fixed with 80% ethanol.
4) In September 1999 a few dozen specimens of *Adrioinsulana conovula* were collected from the outer side of the concrete block surrounding the spring at Zubovici, on Pag Island. The material was fixed in 80% ethanol.

Dissections were done using a NIKON SMZ-U stereomicroscope with a NIKON drawing apparatus, and a NIKON COOLPIX 4500 digital camera. The radulae were examined using a JEOL JSM-5410 scanning electron microscope (SEM), applying the techniques described by FALNIOWSKI (1990).

Ethanol-fixed snails were washed three times with ice-cold water, than DNA was isolated according to the method described by SPOLSKY (SPOLSKY et al. 1996) and DAVIS (DAVIS et al. 1998) with modifications. Isolated DNA was used as a template in PCR reaction with primers: LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') COR722b (5'-TAAACTTCAGGGTGACCAAAAAATYA-3') to amplify the gene of mitochondrial cytochrome oxidase subunit 1 (CO1; FOLMER et al. 1994, DAVIS et al. 1998). The PCR conditions were: 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. The PCR was made in 50 µl volume, 10 µl was analysed in 1% agarose gel. After amplification the PCR product was purified using the Clean-Up columns (A&A Biotechnology) according to the manuals. Purified PCR product was sequenced using the BigDye Terminator v3.1 (Applied Biosystems) according to the manuals and the above described primers. The reaction product was purified using the ExTerminator Columns (A&A Biotechnology) according to the manuals, and sequences were read at the ABI Prism sequencer.

To infer the phylogeny, a set of sequences from the GenBank (Table 1) was used. The sequences were aligned by hand using BioEdit 5.0.0 (HALL 1999), and further edited with MacClade 4.05 (MADDISON & MADDISON 2002). 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. However, 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 any violation of its assumptions (SWOFFORD et al. 1996), but often shows 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, there simply is no parameter connected with a tree topology in all the maximum likelihood theory: nothing but to believe that the tree with the most "true" 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 complicated the model of evolution, the higher the variance of the resulting recon-

Table 1. Species included in phylogenetic analysis based on the mitochondrial CO1 gene, with GenBank accession numbers (GB access), references and localities

Species	GB access	References	Locality
Adriohydrobia gagatinella (Küster, 1852)	AF317881	WILKE & FALNIOWSKI (2001)	Krka River near Skradin, Croatia
Adrioinsulana conovula (Frauenfeld, 1863)	AF367628	WILKE et al. (2001)	spring at Zubovici, Pag Island, Croatia
Bythinella compressa (Frauenfeld, 1857)	AF367653	WILKE et al. (2001)	Altengronau, Hessen, Germany
Dianella thiesseana (Kobelt, 1878)	AY676127	SZAROWSKA et al. (2005)	Lake Trichonida at Loutres Mirtias, Greece
Hydrobia acuta (Draparnaud, 1805)	AF278808	WILKE et al. (2000)	Etang du Prévost, Hérault, France
Peringia ulvae (Pennant, 1777)	AF118302	WILKE & DAVIS (2000)	"Levin navolok lagoon", White Sea, Russia
Pseudamnicola lucensis (Issel, 1866)	AF367651	WILKE et al. (2001)	Bagni Caldi, Bagni di Lucca, Lucca, Tuscany, Italy
Ventrosia ventrosa (Montagu, 1803)	AF118335	WILKE & DAVIS (2000)	Snettisham lagoon, The Wash, Norfolk, Great Britain

structions. 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 are the closest to the real historical processes (GAUT & LEWIS 1995, YANG 1997, TAKAHASHI & NEI 2000). Hence we decided to use parsimony, with all the characters (positions) treated in the same way. Phylogenetic inference was performed with PAUP*4.0b10 (SWOFFORD 2002), on an APPLE POWER MACINTOSH G4 computer.

RESULTS

The shells of *Pseudamnicola macrostoma* from Kato Souli (Figs 1–9) were much bigger, and their variability range wider than in any other *Pseudamnicola* or *Adrioinsulana* studied. The spire was relatively higher and more massive, and some of the shells displayed moderately marked scalarity (Figs 5, 9). Dissection showed that nearly all the snails were heavily infected with the trematode larvae, with the reproductive organs nearly destroyed; in males the penes were much reduced. The shells of *P. macrostoma* from Vravrona (Figs 10, 11) were much smaller, with relatively higher body whorls, slightly variable. The snails were either not attacked by the trematodes or – rarely – with a few parasites within the visceral sac.

The shells of *P. negropontina* from Marmaris (Figs 12–18) were bigger than the shells of *P. macrostoma* from Vravrona, but smaller than the ones from Kato Souli. Their variability was also wider than that observed in Vravrona, but narrower than in Kato Souli. It has to be noted that the variability ranges of the two taxa overlap. Most *P. macrostoma* shells have relatively higher but also more slender spires. The specimens attacked by the trematodes were more numerous than in Vravrona, but much less numerous than in Kato Souli. Shells of *Adrioinsulana conovula* from Zubovici (Figs 19–24) were thin-walled, relatively small, with a big body whorl, slightly variable. In general, they were similar to the shells of *Pseudamnicola macrostoma* from Vravrona (Figs 10–11).

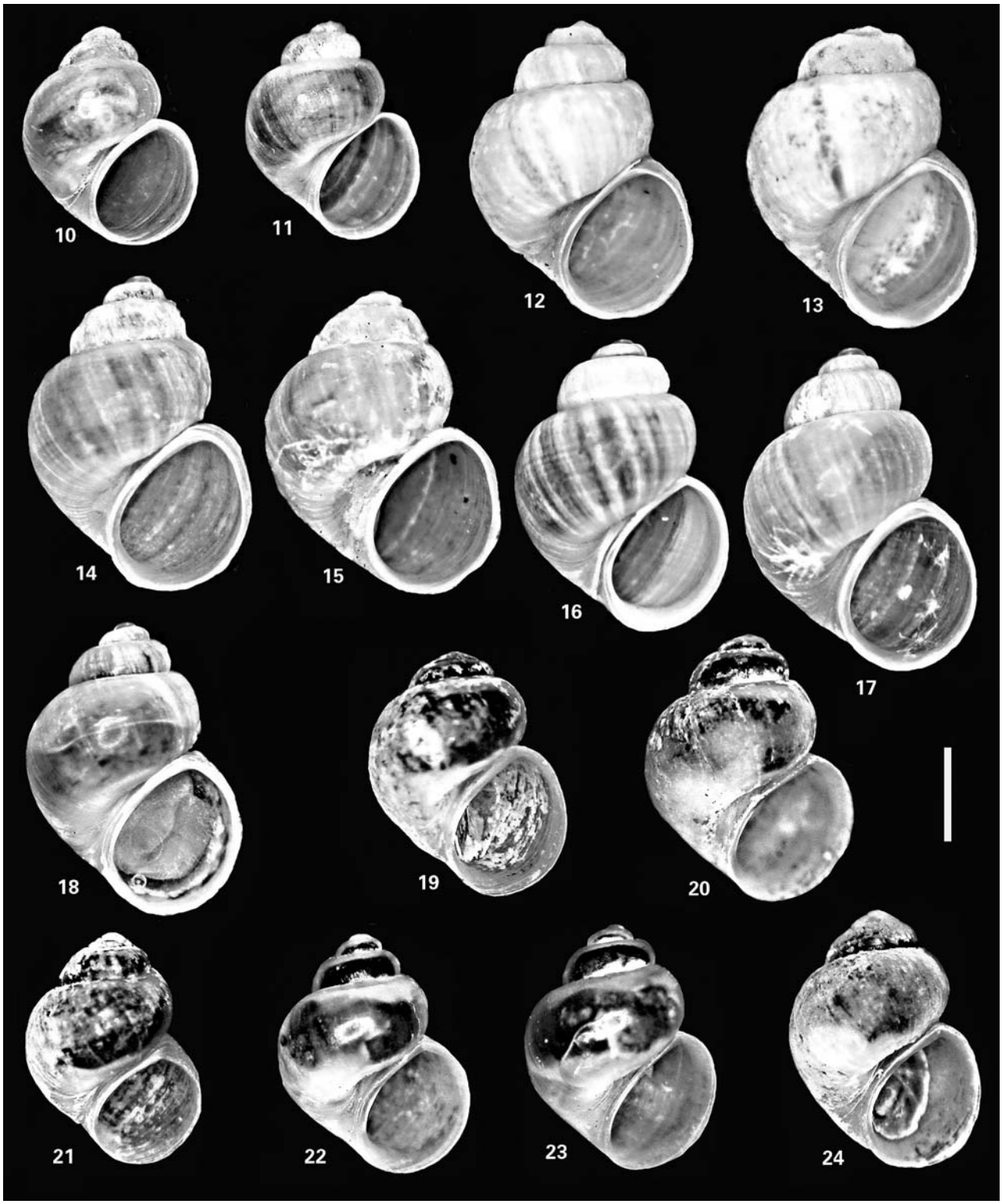
The protoconchs of the Greek *Pseudamnicola* were usually heavily corroded. In those uncorroded (Figs 25–30) there was no macrosculpture (Figs 25, 27, 29), in *P. macrostoma* (Figs 25, 27) the protoconch consisted of about 2 1/8 whorls, growing slowly and regularly after narrow first half of the whorl. There was more (Fig. 27) or less (Fig. 25) well marked border between the proto- and teleoconch. In *P. negropontina* the first half of the protoconch was broader (Fig. 29). The protoconch surface visible under higher magnifications (Figs 26, 28, 30) was composed of irregular depressions (Fig. 26), usually more or less covered by the sediment despite cleaning (Figs 28, 30). There were no differences between *P. macrostoma* (Figs 26, 28) and *P. negropontina* (Fig. 30).

The radulae (Figs 31–38) were characterized by the rhachis with one pair of basal cusps in each of the studied species. In *P. macrostoma* (Figs 31–34) the central cusp fulfilled the formula:

$$\frac{(6)5-1-5(6)}{1-1}$$

Figs. 1–9. Shells of *Pseudamnicola macrostoma*, Kato Souli; scale bar 1 mm

In Kato Souli (Figs 31–32) on the rhachis there were up to six cusps on both sides of the central one, the sixth cusp not fully developed. The central cusp was long, more or less slender. The lateral tooth fulfilled the formula: 3–1–2, with the biggest cusp prominent and broad, inner marginal tooth with about 15 long and slender cusps, and the outer marginal tooth with about 15 thin cusps. In Vravrona (Figs 33–34) there were no more than five cusps on each side of the central tooth (Fig. 34), the lateral tooth fulfilled the formula: 2–1–4, on the inner marginal tooth

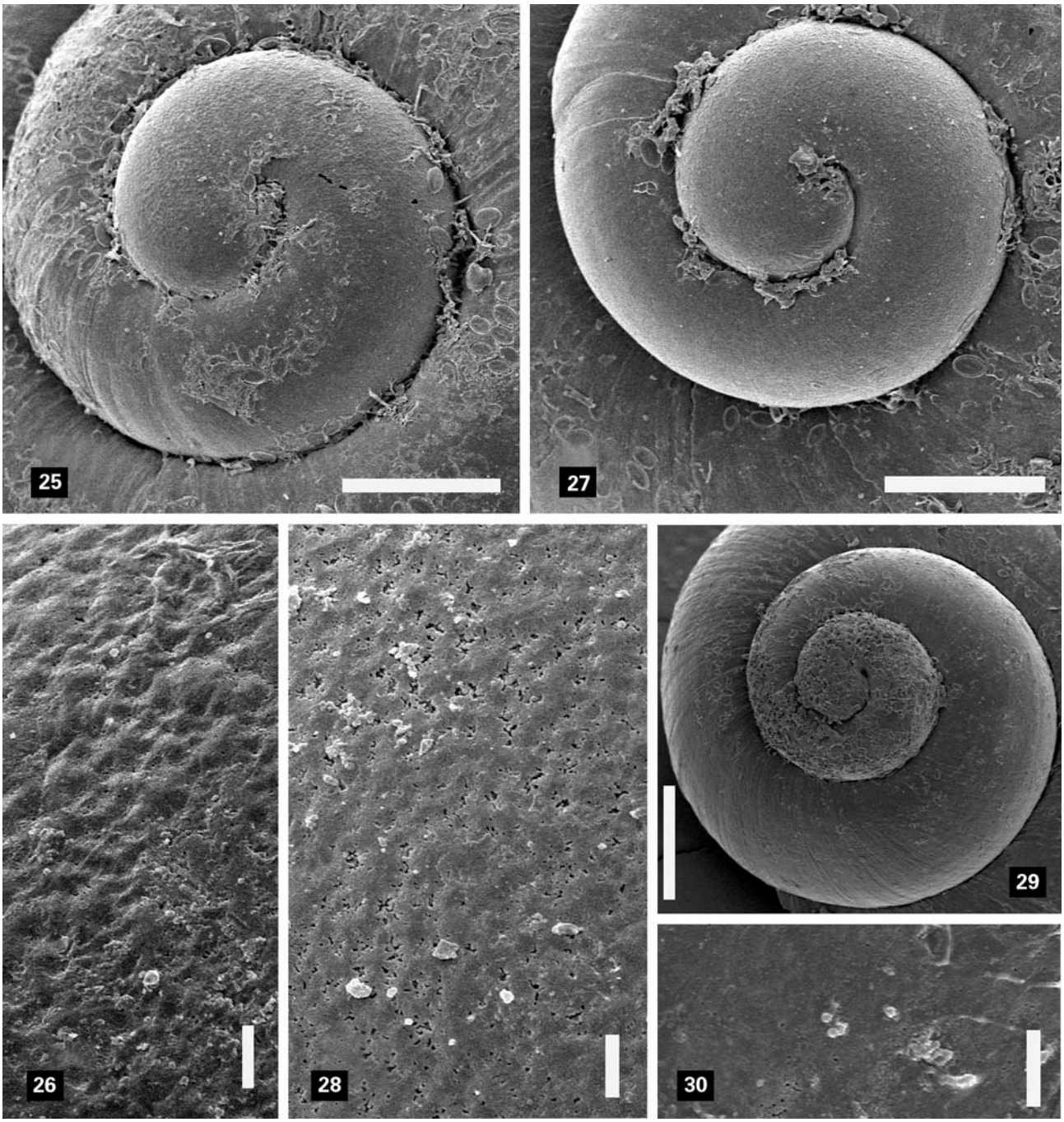


Figs. 10–24. Shells: 10–11 – *Pseudamnicola macrostoma*, Vravrona; 12–18 – *Pseudamnicola negropontina*, Marmaris, Evvoia Island, 19–24 – *Adrioinsulana conovula*, Zubovici, Pag Island; scale bar 1 mm

there were 17–19 cusps, and 15 cusps on the outer marginal tooth.

In *P. negropontina* (Figs 35–36) there were only four cusps on both sides of the rhachis, and the central cusp was in the form of a long triangle. The lateral tooth followed the formula: 3–1–2, and there were about 15 cusps both on the inner and outer marginal tooth. In *A. conovula* (Figs 37–38) there were three cusps (sometimes with a very slightly developed fourth one) on both sides of the central cusp. The lateral tooth fulfilled the formula: 2–1–2, and there were about 12 cusps on both inner and outer marginal tooth.

As already noted above, in Kato Souli the snails were heavily parasitized, thus we do not present their soft parts, affected by the parasites. In *P. macrostoma* from Vravrona (Figs 39–42) the snouts and tentacles were often intensively brown-pigmented (Fig. 41), but sometimes the pigmentation was very delicate (Fig. 42). The penes (Figs 39–42) were simple, triangular, with many folds. In *P. negropontina* (Figs 43–45) the brown or black pigmentation of the snout and tenta-

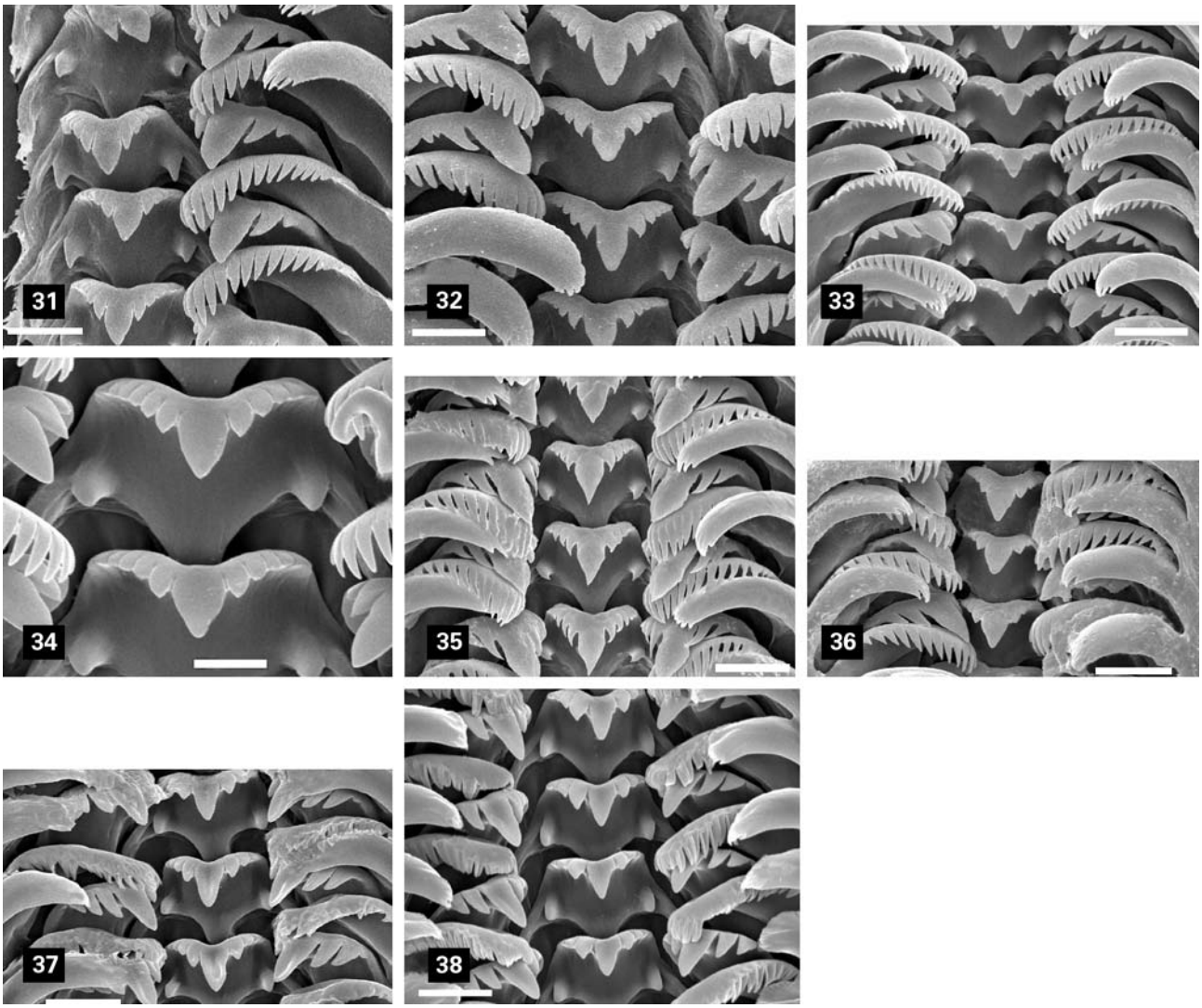


Figs. 25–30. Protoconchs of *Pseudamnicola*: 25–28 – *P. macrostoma*, Kato Souli (25 and 27 – habitus, 26 and 28 – fragments of 25 and 26, respectively); 29–30 – *P. negropontina*, Marmaris (29 – habitus, 30 – fragment of 29); scale bar 50 μm in 25 and 27, 100 μm in 29, and 2 μm in 26, 28 and 30

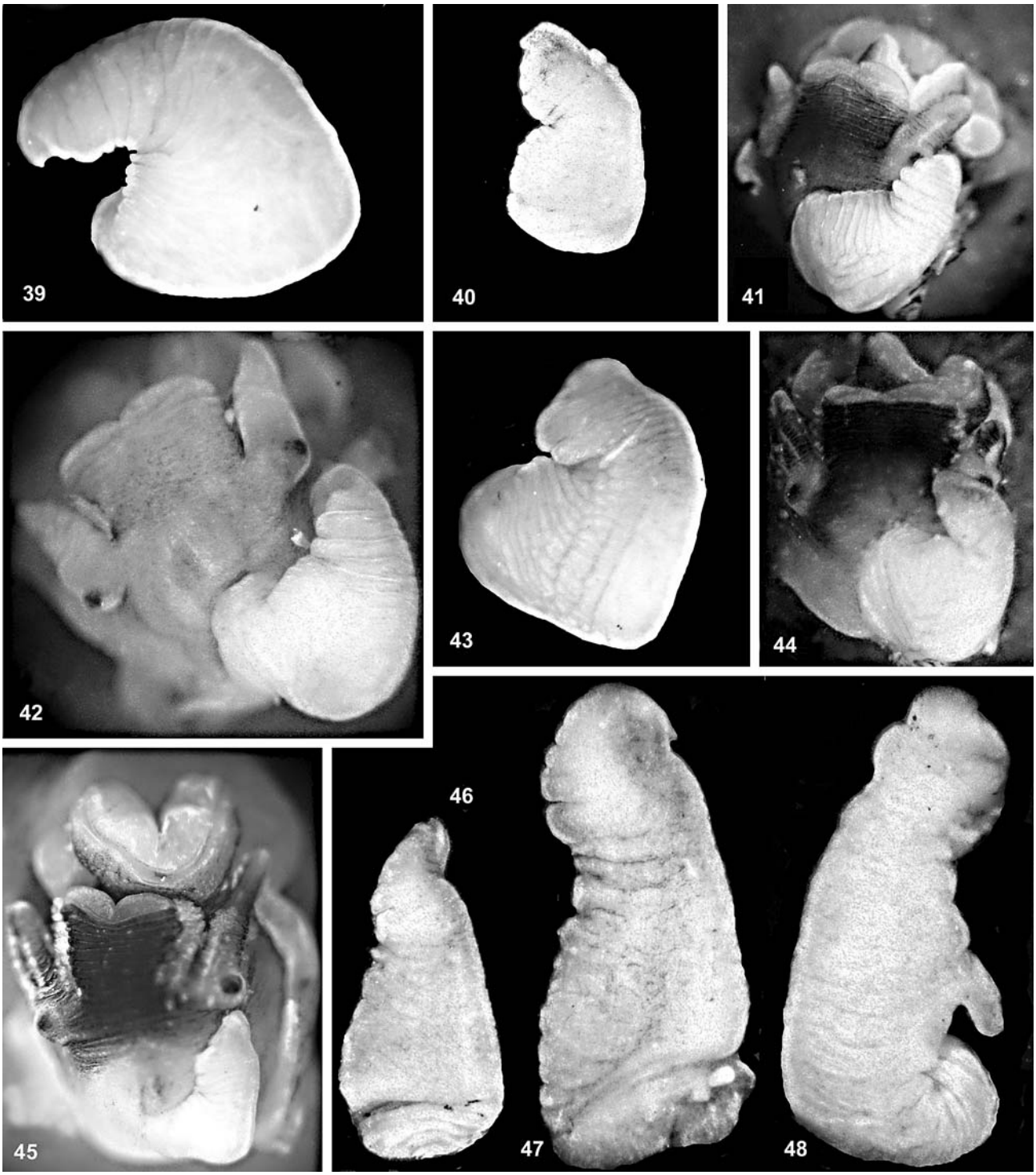
cles was always intensive (Figs 44–45). The penes (Figs 43–45) as in *P. macrostoma*, had no characteristic features. The penes of *A. conovula* (Figs 46–48) were similar, also simple and triangular, but much more elongated than in the other two species.

The female reproductive organs (Figs 49–50) were characterised by the massive, intensively black pigmented spire of coiled "renal" oviduct, the moderately big and approximately sphaerical/oval bursa copulatrix with the long duct, one seminal receptacle (in the position of rs_1 as defined by RADOMAN), ventral channel, and the accessory gland complex (albumen gland + capsule gland) with three zones of different colour discernible. The organs of *Pseudamnicola macrostoma* (Fig. 49) from Vravrona were smaller than the ones of *P. negropontina* from Marmaris (Fig. 50), and the shape of the bursa was different in the two species, but no more differences were observed. The female reproductive organs of *Adrioinsulana conovula* were characterised by the very long tube-shaped seminal receptacle and moderately big, sac-shaped bursa; the organs were as illustrated in RADOMAN (1972, 1983) and GIUSTI & PEZZOLI (1980).

CO1 sequences were obtained for four specimes of *Pseudamnicola negropontina* from Marmaris, two of them used in phylogeny reconstruction. There were three polymorphic positions, 99% identity between the sequences. Together with eight sequences from the GenBank (Table 1) we obtained the matrix of 643 characters, 408 of them constant, 56 parsimony-uninformative, and 179 parsimony-informative. The exhaustive search resulted in one tree (Fig. 51), with length 503, CI=0.658, RC=0.359. *Pseudamnicola negropontina* formed the clade with *P. lucensis*, and with *Adrioinsulana conovula*. Bootstrap support (10,000 replicates) as high as 99% supported the clade grouping *Pseudamnicola* and *Adrioinsulana*. It is also evident that the molecular difference between *Pseudamnicola lucensis* and *Adrioinsulana conovula* is half that between the two species of *Pseudamnicola* (Fig. 51).



Figs. 31–38: Radulae: 31–34 – *Pseudamnicola macrostoma*: 31–32 – Kato Souli, 33–34 – Vravrona; 35–36 – *Pseudamnicola negropontina*, Marmaris, Evvoia Island; 37–38 – *Adrioinsulana conovula*, Zubovivi, Pag Island; scale bar 2 μm in 34 and 4 μm in other figures

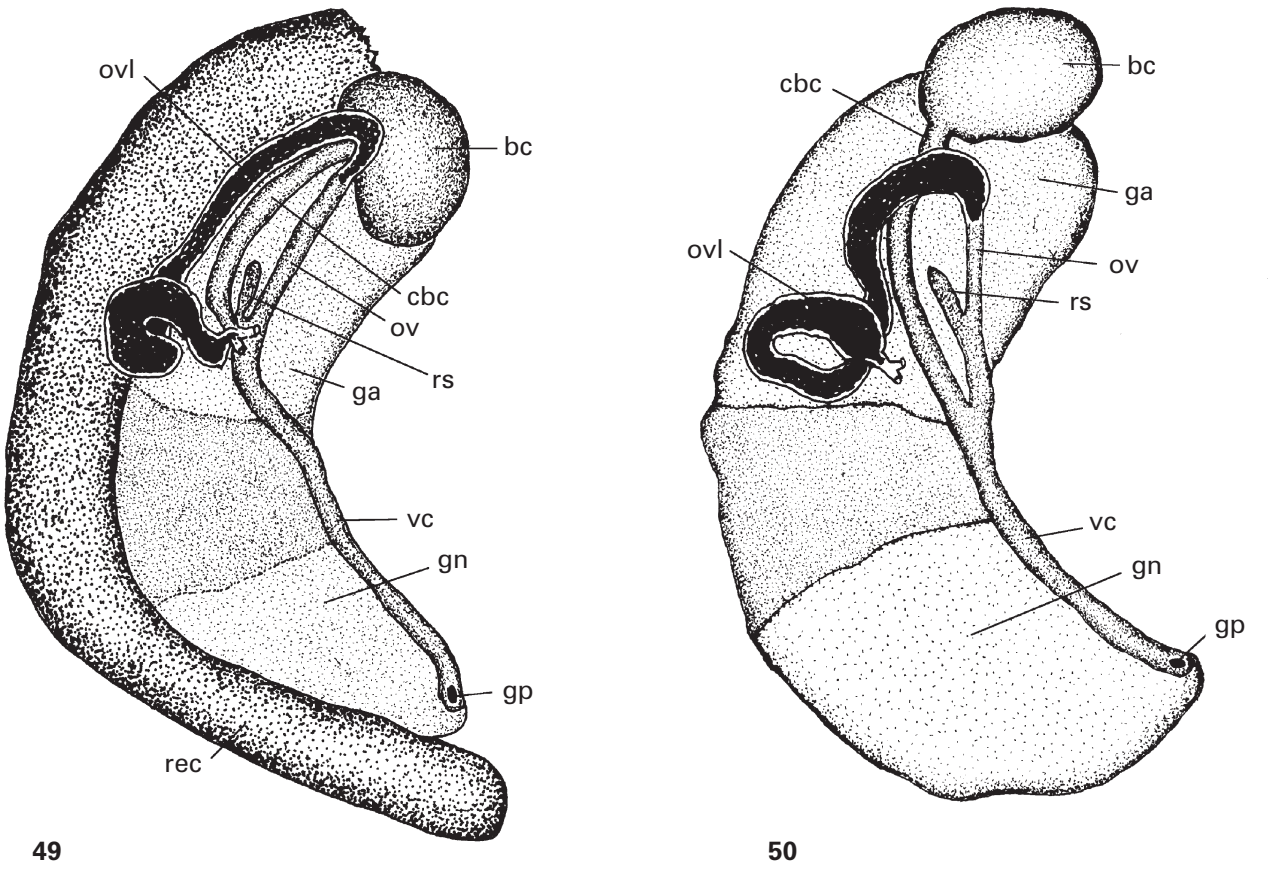


Figs. 39–48. Heads and penes: 39–42 – *Pseudamnicola macrostoma*, Vravrona (39–40 – penis, 41–42 – head with penis); 43–45 – *Pseudamnicola negropontina*, Marmaris (43 – penis, 44–45 – head with penis); 46–48 – *Adrioinsulana conovula*, penis

DISCUSSION

Both the size and abnormalities of the shells in Kato Souli were typical of gigantism, caused by the parasitic trematodes (FRETTER & GRAHAM 1962, MUUS 1967, FALNIOWSKI 1987). In Kato Souli *P. macrostoma* formed an enormously dense population, inhabiting the ditch along hundreds of metres, thus making the snails ideal hosts for those parasites.

As clearly visible in the photographs, the shell variability ranges overlap. In fact, the differences are less than slightly marked. SCHÜTT (1980) presents the



Figs. 49–50. Female reproductive organs: 49 – *Pseudamnicola macrostoma*, Vravrona; 50 – *Pseudamnicola negropontina*, Marmaris (bc – bursa copulatrix, cbc – canal of bursa copulatrix, ga – albumen gland, gn – capsule gland, gp – gonoporus, ov – pallial oviduct, ovl – loop of coiled "renal" oviduct, rec – rectum, rs – seminal receptacle, vc – ventral channel)

photograps of the shells of *P. macrostoma* and *P. negropontina*, which are even less different one from the other. As noted in the Introduction, SCHÜTT distinguishes the species and subspecies considering their geographic range solely, which seems more than doubtful. However, the problem must wait for some further, molecularly-based study, with some freshly collected material of *P. macrostoma*, not available now. SCHÜTT (1980), considering shell characters only, treats *P. negropontina* as a subspecies of *P. macrostoma*. However, in our opinion the differences between the two taxa, although slight, are not less marked than those between the other *Pseudamnicola* species illustrated and described by SCHÜTT (1980). Thus, we consider *P. negropontina* a distinct species, at least as long as there are no molecular data on *P. macrostoma*. The similarity of the shells of *P. macrostoma* from Vravrona and *Adrioinsulana conovula* – the two taxa whose distinctness is evident molecularly – supports our skepticism considering shell-based taxonomy in *Pseudamnicola*.

The difference in the shape and breadth of the first whorl of the protoconch may reflect some differences in life history, thus confirming species distinctness of the taxon (e.g. FALNIOWSKI 1990). The radular teeth are always with one pair of the basal cusps on the rhachis, as noted by THIELE (1929). Somewhat unusually, the cusp numbers on the central and lateral teeth are different in each of the three studied taxa, confirming their distinctness.

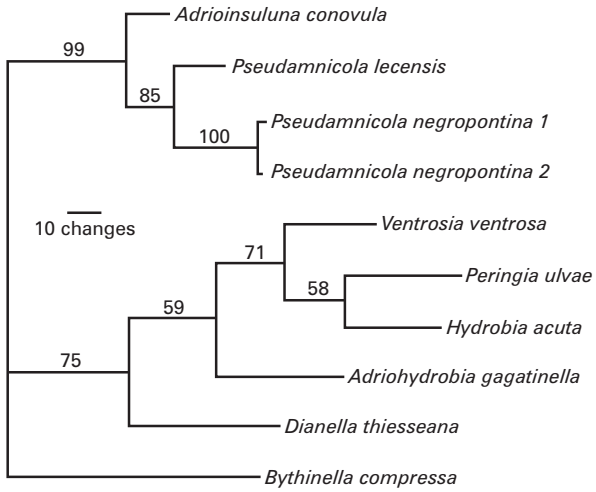


Fig. 51. Phylogenetic position of the studied psudamnicolid species: MPR in form of phyllogram, 503 steps long, CI=0.658, RC=0.359; bootstrap supports (10,000 replicates) given

The penes are simple, thus hardly useful in taxonomy at the species level. They resemble the ones described and illustrated by BOETERS (1971), RADOMAN (1972, 1983), and GIUSTI & PEZZOLI (1980). The female reproductive organs of all the three taxa studied were characteristic of *Pseudamnicola*, as defined by BOETERS (1971), RADOMAN (1972, 1983) and GIUSTI & PEZZOLI (1980). As noted in the Results, the female organs of *Adrioinsulana conovula* were identical with the ones illustrated by RADOMAN (1972) and GIUSTI & PEZZOLI (1980). The organs of *Pseudamnicola macrostoma* are illustrated by SCHÜTT (1980), but the illustration is so bad that it could be assigned to any *Pseudamnicola*, or even any representative of the Hydrobiinae s. stricto. Apart from *Adrioinsulana conovula*, the anatomy of not one of the species considered in the present paper was described or illustrated in the literature. There are only descriptions and illustrations of *Pseudamnicola lucensis* (BOETERS 1971, GIUSTI & PEZZOLI 1980, RADOMAN 1983) and *P. moussoni* (GIUSTI & PEZZOLI 1980). The comparison of the reproductive organs of all the five species shows slightly marked differences, expressed in such characters as the shape and dimensions of the bursa copulatrix, and of the seminal receptacle. Those characters are very labile within the Rissooidea, prone also to ontogenetic and physiological variation. It has to be noted, as well, that the differences in the female reproductive organs between *P. macrostoma* and *P. negropontina* seem not smaller than the ones between *P. lucensis* and *P. moussoni*, and that, say, *P. lucensis* does not differ more from *P. negropontina* than the latter from *P. macrostoma*. To conclude, the anatomy of the female reproductive organs neither confirms nor rejects their species distinctness, which seems true for all the species of *Pseudamnicola* and *Adrioinsulana* studied so far.

As already stated in the Results, the variability ranges of the shells also overlap between the species. On the other hand, the molecular differences – as expressed in the CO1 mtDNA sequences – undoubtedly confirm the species distinctness of *Pseudamnicola negropontina* and *P. moussoni*. It seems that in *Pseudamnicola* we can observe a morphostatic radiation, similar to the one typical of the Hydrobiinae s. stricto (WILKE & DAVIS 2000, WILKE et al. 2000). This means that neither speciation nor later phyletic evolution modifies morphology more than slightly, despite the growing molecular differences reflected in high genetic distances between completely reproductively isolated clades. Unfortunately, we have to wait for some fresh material to get sequences of *P. macrostoma*. The phylogenetic analysis demonstrated also that the Greek *P. negropontina* actually belongs to the genus *Pseudamnicola*, and that there is no basis for distinguishing the genus *Adrioinsulana* Radoman, 1978: also *A. conovula* belongs to *Pseudamnicola*.

Pseudamnicola has thus a disjunct distribution: it inhabits all the Appenninian Italy together with Sicilly, Sardegna and Elba on one side of the Adriatic Sea, and on the other side of it all the continental Greece together with islands, like Crete, Evvoia, etc. It does not inhabit the Balkans north of Greece, with an exception of some islands inhabited by *P. conovula*. Such geographic range of the genus may be easily explained, considering the geological history of the region, with numerous episodes of orogenies, sea transgressions, etc. (RÖGL 1998, 1999, GEARY et al. 2000).

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