MOLECULES *VS* MORPHOLOGY IN THE TAXONOMY OF THE *RADOMANIOLA/GROSSUANA* GROUP OF BALKAN RISSOOIDEA (MOLLUSCA, CAENOGASTROPODA)

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Abstract *The morphology of the shell, penis, and female reproductive organs, as well as the mitochondrial COI and ribosomal 18S (112 and 38 sequences, respectively) were studied in 40 populations of the Balkan hydrobiids* Radomaniola *and* Grossuana. *In 19 populations of five nominal species/subspecies, shell morphometry based on seven characters did not confirm the distinctness of the taxa. Despite wide variation, we did not find morphological differences between* Radomaniola *and* Grossuana *or between the nominal species assigned to these genera. The COI Bayesian tree proved the monophyly of the group, while the ML tree did not. Both methods revealed three groups: one of* Grossuana *from Serbia, Bulgaria, Romania and NE Greece (disjunct distribution); and two of* Radomaniola *from part of the former Yugoslavia and SE Greece. However, only the* Radomaniola *from the former Yugoslavia was monophyletic. The molecular differentiation was not reflected in morphology, and morphostatic evolution was postulated.*

Key words Grossuana, Orientalina, Radomaniola, *COI, 18S*

INTRODUCTION

The Rissooidea are globally-distributed prosobranch gastropods (Kabat & Hershler, 1993). In most of them the height of the shell scarcely exceeds 5 mm, in many cases not even reaching 1 mm. In the fossil record they appear in the Toarcian, Early Jurassic (Grundel, 1999) and some Recent genera are as old as the Oxfordian, Late Jurassic. The rissooidean family Hydrobiidae is probably not much younger. They inhabit a wide variety of inland aquatic habitats, mostly springs. In Europe, the centre of their distribution is the Balkan Peninsula. The history of the Balkan rissooids began at the time of the Paratethys (Rogl, 1998, 1999; Geary, Magyar & Müller, 2000) and reflects the geological history of the region. The present richness of the fauna is due to multiple orogenies, sea transgressions and other factors. To gain a full understanding of the evolutionary history of this fauna, more phylogeographical and molecular phylogenetic studies are required.

Many rissooid taxa share a tiny, simplified shell without characteristic traits, a female anatomy with a single coil of oviduct and two seminal receptacles, and a penis with a double lobe on the left hand edge. Anatomical studies on rissooid gastropods have not provided sufficient evidence for the resolution of the phylogenetic relationships, or even helped to establish the distinctness of the species within the group. Molecular data are thus necessary to understand the phylogeny of the group.

Radoman (1972) described a new genus *Orientalia* with the type species *O. curta* (Küster 1852). Later, to avoid homonymy, he replaced the name *Orientalia* with *Orientalina* (Radoman 1978), but, in the process, created a new homonym. Szarowska (2006) replaced the latter homonym with *Radomaniola* Szarowska 2006. Radoman (1973) described a new genus *Grossuana*, with the newly described *G. serbica* as type species. Each representative of either of the two genera has a small, ovate-conical shell, a single coil of oviduct and two seminal receptacles, and a double (more or less prominent and bi-lobed) lobe on the left side of the penis. In fact, one may use almost the same characteristics to describe either of the two genera. According to Radoman (1973, 1983), in *Radomaniola* (his "*Orientalina*") the bursa copulatrix is moderately large, irregularly heart-shaped, with a long duct; the rs_1 seminal receptacle is well developed, with a relatively long duct; the rs_2 seminal receptacle is also rather well developed; the penis is prolonged, with a double outgrowth (lobe) on its left side. In *Grossuana* the bursa is pear-shaped, elongate, and proportion-

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ally strongly developed; rs_2 is bigger than rs_1; the penis is long, tapering at its end, and bears a more or less weakly marked outgrowth on its left side. However, all the characters listed above are infra-species variable within the Rissooidea (Falniowski, 1987, 1990; Szarowska, 2006; Szarowska & Falniowski, 2008).

Szarowska *et al.* (2007) described the anatomy and presented the molecular phylogeny of four species of *Grossuana*. Their molecular data (Szarowska *et al.*, 2007) did not confirm the opinion of Reischutz (1988) who had ranked *Radomaniola* (as "*Orientalina*") as a subgenus of *Grossuana*. Bodon, Giusti & Manganelli (1992) extended the range of the genus *Radomaniola* (as "*Orientalina*") to the Apennines in central Italy. Based on the shell morphology, Radoman (1983, 1985) distinguished 13 taxa of species/subspecies rank within the genus *Radomaniola* ("*Orientalina*") and six taxa of the same rank within the genus *Grossuana*. Most of them (12 *Radomaniola* and 5 *Grossuana*) come from the territory of the former Yugoslavia. In contrast to the latter, knowledge of the rissooid fauna of Greece is fragmentary. Schütt (1980) assigned several taxa to the genus *Belgrandiella* A.J. Wagner 1927, thus extending the range of this northwestern Balkan taxon to the south-east of the region. His *Belgrandiella* appears, however, to be a collection of gastropods with tiny shells of a similar outline that are not necessarily closely related.

The aims of the present paper were (1) to establish the reliability of morphological differences between *Radomaniola* and *Grossuana* (in shell morphology and morphometry, also morphology of the penis and female reproductive organs); (2) to use molecular data (COI, 18S), to test the monophyly of the Balkan hydrobiids that share an ovate-conical shell, a bi-lobed outgrowth of the penis, and two seminal receptacles; (3) to prove, with the same molecular and morphological data, the distinctness of selected nominal taxa.

MATERIAL AND METHODS

Material collection and fixation We collected the material in 2001–2008, from 20 localities in Greece, and 20 in Montenegro, Bosnia and Herzegovina, and Serbia (Fig. 1, Table 1). The sampling area covered most of the known range of *Grossuana* and *Radomaniola*. Where possible, we collected snails from type localities (see Discussion); otherwise we sought localities as close to the type localities as possible. Snails were collected with a sieve, or by hand.

For the molecular study snails were washed twice in 80% ethanol, and left to stand in this solution for ca. 12 hours, after which the solution was changed twice in 24 hours. Finally, after a few days, the 80% solution was exchanged for a 96% solution of ethanol and the material was stored at –20°C. Snails for the morphological study were fixed in 10% buffered formalin. Additional materials of *Radomaniola curta germari* (Frauenfeld 1863) for morphometric analysis were collected from the Jadro River at Solin, west of Split, Croatia (43°32′07″N, 16°29′27″E) and the Cetina River at Radmanove Mlinice, north of Omiš, Croatia (43°26′22″N, 16°45′01″E).

Morphological techniques Dissections were made using a Nikon SMZ–U stereoscope microscope with a Nikon drawing apparatus and DS-5 digital camera. Shells were cleaned in an ultrasonic cleaner and photographed using a Nikon DS-5 or Canon EOS 50D digital camera.

Morphometric techniques Seven morphometric parameters of the shell (Szarowska, 2006; Falniowski, Szarowska & Grzmil, 2007) were measured by one person using a Nikon DS-5 digital camera measurement system, in thirty specimens out of each of the 20 samples from 19 localities (locality M4 was sampled twice at a different time). The linear measurements were then logarithmically transformed; for angular measurements the arcsine transformation was applied. We calculated Euclidean distances and computed UPGMA clustering and minimum spanning tree (MST) using NTSYSpc (Rohlf, 1998). The same program was used to compute principal component analysis (PCA), based on the matrix of correlation. The original observations were projected into PC space, with a superimposed minimum spanning tree to detect local distortions in the data.

Molecular techniques Snails were hydrated in TE buffer (3×10 min.) and their DNA extracted using the Sherlock Extracting Kit (A&A Biotechnology); the final product was dissolved in 20 µm of TE buffer. The PCR reaction (Palumbi, 1996) was performed using the following primers: LCOI490 (5′-GGTCAACAAATCATAAAGATATTGG-3′)

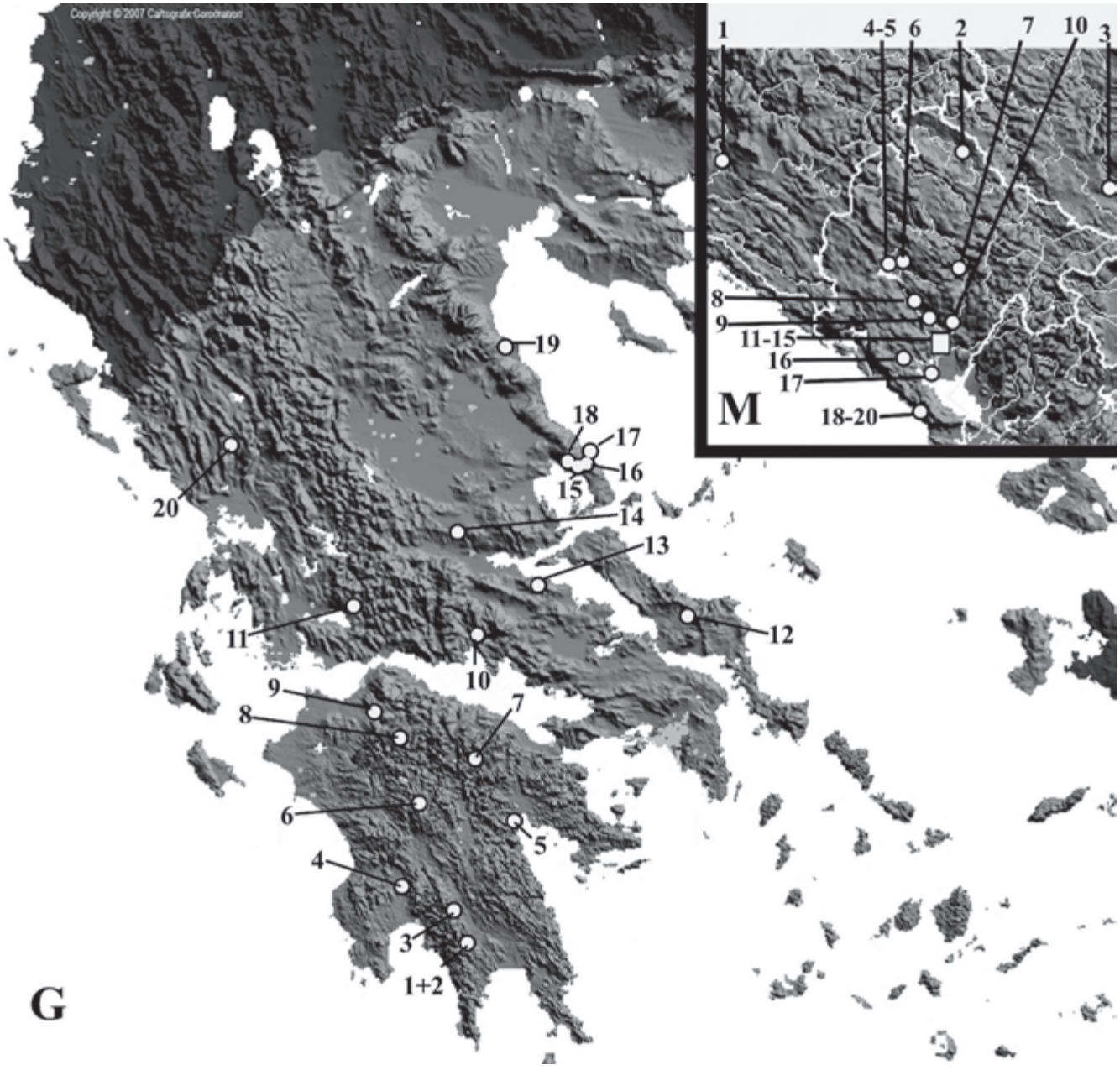


Figure 1 Sampling localities.
Abbreviations: G, Greece; M, Montenegro, Bosnia and Herzegovina, Serbia.

(Folmer *et al.*, 1994) and COR722b (5′-TAAACTT CAGGGTGACCAAAAAATYA-3′) (Wilke & Davis, 2000) for the COI gene and SWAM18SF1 (5′-GAATGGCTCATTAAATCAGTCGAGGTTCC TTAGATGATCCAAATC-3′), and SWAM18SR1 (5′-ATCCTCGTTAAAGGGTTTAAAGTGTACT CATTCCAATTACGGAGC-3′) for the 18S gene (Palumbi, 1996). The PCR conditions were as follows. COI: initial denaturation step of 4 min at 94^{O}C, followed by 35 cycles at 94^{O}C for of 1 min, 55^{O}C for 1 min, 72^{O}C for 2 min, and a final extension of 4 min at 72^{O}C. 18S: initial denaturation step of 4 min. at 94^{O}C followed by 40 cycles of 45 sec. at 94^{O}C, 45 sec. at 51^{O}C, 2 min. at 72^{O}C; after all cycles were completed we performed an additional elongation step of 4 min. at 72^{O}C. The total volume of each PCR reaction mixture was 50 µl. Ten µl of the PCR product was then run on 1% agarose of gel to check the quality the PCR products. The PCR product was purified using Clean-Up columns (A&A Biotechnology) and sequenced in both directions (Hillis *et al.*, 1996) using BigDye Terminator v3.1 (Applied Biosystems), following the manufacturer's

Table 1 Sampled localities in Greece (G) and Montenegro, Serbia and Bosnia and Hercegovina (M); COIn – number of COI sequences, COIh – number of COI haplotypes.

	Locality	Geographic coordinates	COIn	COIh	18S
Greece					
G1	spring S of Koumousta, Taigetos Mts., Peloponnisos	36°56′45″N, 22°24′34″E	6	6	3
G2	spring S of Koumousta, Taigetos Mts., Peloponnisos	36°56′31″N, 22°24′23″E 9	9	5	2
G3	spring at Tripi, N. Taigetos Mts., Peloponnisos	37°05′37″N, 22°20′50″E	9	8	1
G4	springs Piges Pamisou, Peloponnisos	37°10′05″N, 22°01′35″E	1	1	–
G5	spring at Mili (Lérni), Peloponnisos	37°33′07″N, 22°43′03″E	3	2	1
G6	spring between Palaiokhorion and Karkalou,WNW of Tripolis, Peloponnisos	37°37′21″N, 22°02′59″E	6	1	–
G7	spring near Stimfalia Lake, between Bouzion and Kalanoi, Peloponnisos	37°53′38″N, 22°28′27″E 1	1	1	2
G8	spring at Bouboukas, near road to Maneli, Peloponnisos	38°00′53″N, 21°58′31″E	1	1	1
G9	spring E of Katarraktis, Peloponnisos	38°06′05″N, 21°50′00″E	4	2	1
G10	spring Kastalia Pigi, Dhelphi, Parnassos Mts.	38°28′59″N, 22°30′19″E	1	1	1
G11	spring in city centre of Thérmon, NE of Trichonida Lake,	38°34′23″N, 21°39′58″E	3	3	1
G12	spring between Loutsa and Steni Dhirfios, Evvoia Island	38°35′16″N, 23°48′57″E	1	1	1
G13	spring S of Agios Konstantinos, N of Mt. Spartias, Attica	38°45′05″N, 22°51′12″E	1	1	1
G14	spring of Achilles, ESE of Kalamakion, NW of Lamia	38°59′13″N, 22°22′43″E	2	2	3
G15	spring NW of Dhrakia, Oros Pilion, E of Volos	39°23′04″N, 23°00′04″E	1	1	–
G16	spring NW of Dhrakia, Oros Pilion, E of Volos	39°23′36″N, 23°02′33″E	2	2	–
G17	spring E of Anilion, Oros Pilion, E of Volos	39°24′49″N, 23°09′23″E	4	3	2
G18	spring at Makrinitsa/Koukourava, Oros Pilion, E of Volos	39°23′35″N, 22°59′55″E	2	1	–
G19	spring of Athena, Thembi Valley	39°58′26″N, 22°38′17″E	4	3	1
G20	springs of Louros River	39°25′56″N, 20°50′30″E	3	3	1
Montenegro, Bosnia and Hercegovina, Serbia					
M1	Neretva River near Buna, Bosnia and Herzegovina	43°15′08″N, 17°49′25″E	1	1	3
M2	spring of river Breznica, Pljevlja city	43°21′52″N, 19°21′49″E	3	3	–
M3	spring of river Raska, Sopočani Monastery type locality of Grossuana serbica	43°06′57″N, 20°22′15″E	1	1	–
M4	spring Vidrovan in Vidrovan village, Nikšić city	42°50′19″N, 18°54′48″E	1	1	–
M5	spring at Gornji Poliskovic between Gornje Polje, Vidrovan village, Nikšić	42°51′12“N, 18°56′33“E	3	1	–
M6	spring at Niksicko Polje	42°50′02″N, 18°56′43″E	4	4	1
M7	spring close to Moraca Monastery	42°46′01″N, 19°23′26″E	1	1	–
M8	spring at Dobro Polje	42°37′51″N, 19°01′58″E	3	3	1
M9	River Zeta near Prentina Glavica, Danilovgrad town	42°32′45″N, 19°09′10″E	3	2	–
M10	spring at Vranicke-Njive	42°28′05″N, 19°15′30″E	5	4	1
M11	spring Vriješko Vrelo in village Bandići, Podgorica city	42°30′33″N, 19°07′00″E	2	2	–
M12	spring Ribničko Vrelo, Podgorica city	42°26′11″N, 19°17′54″E	3	3	3
M13	spring in village Pričelje, 8 km N of Podgorica centre	42°30′13″N, 19°13′21″E	1	1	–
M14	spring Mareza, Tološi, Podgorica city	42°28′48″N, 19°11′55″E	3	2	1
M15	River Cijevna near Trgaja spring, Podgorica city	42°23′46″N, 19°22′49″E	3	3	2
M16	spring below the road at Lipovik	42°21′10″N, 19°02′09″E	2	2	1
M17	spring on island Vranjina, Skadar Lake area	42°16′21″N, 19°08′49″E	3	3	–
M18	spring Gornje Vrelo in village Dobre Vode, Bar city	42°02′59″N 19°08′55″E	2	2	1
M19	spring Donje Vrelo in village Dobre Vode, Bar city	42°01′59″N 19°08′55″E	3	3	–
M20	spring Škurta near village Dobre Vode, Bar city	42°01′59″N 19°08′55″E	1	1	2
			112	91	38

Table 2. Taxa used for phylogenetic analyses, with their GenBank Accession Numbers and references

Species	18S GB#	CO1 GB#	References
Orientalina callosa (Palucci 1881)	AF367685	AF367649	Wilke *et al*. 2001
Graziana alpestris (Frauenfeld 1863)	AF367673	AF367641	Wilke *et al*. 2001
Pseudamnicola lucensis (Issel 1866)	AF367687	AF367651	Wilke *et al*. 2001
Grossuana codreanui (Grossu 1946)	EF061916	EF061919	Falniowski *et al*.2007
Grossuana serbica Radoman 1973	xxxx	EF061921	Falniowski *et al*.2007
Grossuana delphica (Radoman 1973)	EF061917	EF061922	Falniowski *et al*.2007

protocol and using the primers described above. The sequencing reaction products were purified with ExTerminator Columns (A&A Biotechnology) and the sequences were read using the ABI Prism 3100 Avant Genetic Analyzer .

Data analysis The COI sequences were aligned by eye using BioEdit 5.0.0 (Hall, 1999) and edited with MACCLADE 4.05 (Maddison & Maddison, 2002). For 18S an initial alignment was performed using the CLUSTALX 1.82 (Thompson *et al.*, 1997) and MACCLADE was used to remove variable fragments that could not be unambiguously aligned. To test whether the COI dataset showed a significant level of saturation, we used the entropy-based test of Xia (2000a, Xia *et al*., 2003, Xia & Lemey 2009) as implemented in the software package DAMBE 4.2.13 (Xia 2000b).

We used the ML approach, as implemented in PAUP*4.0b10 (Swofford, 2002), together with MODELTEST (Posada & Crandall, 1998; Posada, 2003) to find the appropriate model of evolution, with the Akaike Information Criterion (Posada & Buckley, 2004). We performed heuristic searches using stepwise addition and treebisection-reconnection (TBR) branch-swapping (Swofford *et al.*, 1996). Confidence in the nodes were assessed by 1000 bootstrap replicates with random addition of taxa (Felsenstein, 1985). Bootstrap values for ML trees were computed using the "fast" heuristic search algorithm and the same model parameters as used for each ML analysis.

For Bayesian inference we used MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). We selected the best model of sequence evolution for each data set using MRMODELTEST 2.2 (Nylander, 2004), applying the Akaike Information Criterion (Posada & Buckley, 2004). The Bayesian inference was performed with the following parameters: 4 chains in two parallel analyses (1 cold, three heated; T=0.15) metropolis-coupled Monte Carlo analysis run twice in parallel for 50,000,000 generations, trees sampled every 1000 generations starting after a burn-in of 30,000 generations. We inferred final consensus trees with Bayesian probabilities. In the phylogeny reconstruction for COI, we used three *Grossuana* and one *Radomaniola* species from GenBank (Table 2) together with outgroup taxa: *Graziana alpestris* and *Pseudamnicola lucensis* (Table 2). The outgroup were chosen to be far enough to find the root of the tree. We also used several other outgroup taxa (*Daphniola louisi* Falniowski & Szarowska 2000, *Hauffenia* sp., *Sadleriana fluminensis* (Küster 1852)) that were closest to *Grossuana* and *Radomaniola* (Szarowska, 2006). To avoid substantial bias in the DNA evolution model inferred, *Daphniola*, *Hauffenia* and *Sadleriana* are not included in the phylogeny presented in this paper.

RESULTS

Morphology The shells of the *Radomaniola/Grossuana* (Figs 2–5) studied from Greece (Figs 2–3), with the exception of G20 (Fig. 3), did not usually exceed 2 mm in height. Some of the shells from Montenegro reached about 3 mm (Figs 4–5). Most of the shells were low-spired and wide (ovate-conical), but in some populations from Montenegro (Fig. 4: M9, Fig. 5: M14 and, especially, M17) the shells were higher-spired (conical). For the shell morphometry study we used five *Radomaniola* taxa from among the 13 ones that were described from the former territory of Yugoslavia: *Radomaniola curta curta* (Küster 1852), *R. curta anagastica* (Radoman 1973), *R. curta germari* (Frauenfeld 1863), *R. curta narentana* (Radoman 1973), and *R. montana* (Radoman 1973). PCA as well as cluster analysis, computed on the males and females from three populations (not presented here), indicated no sexual

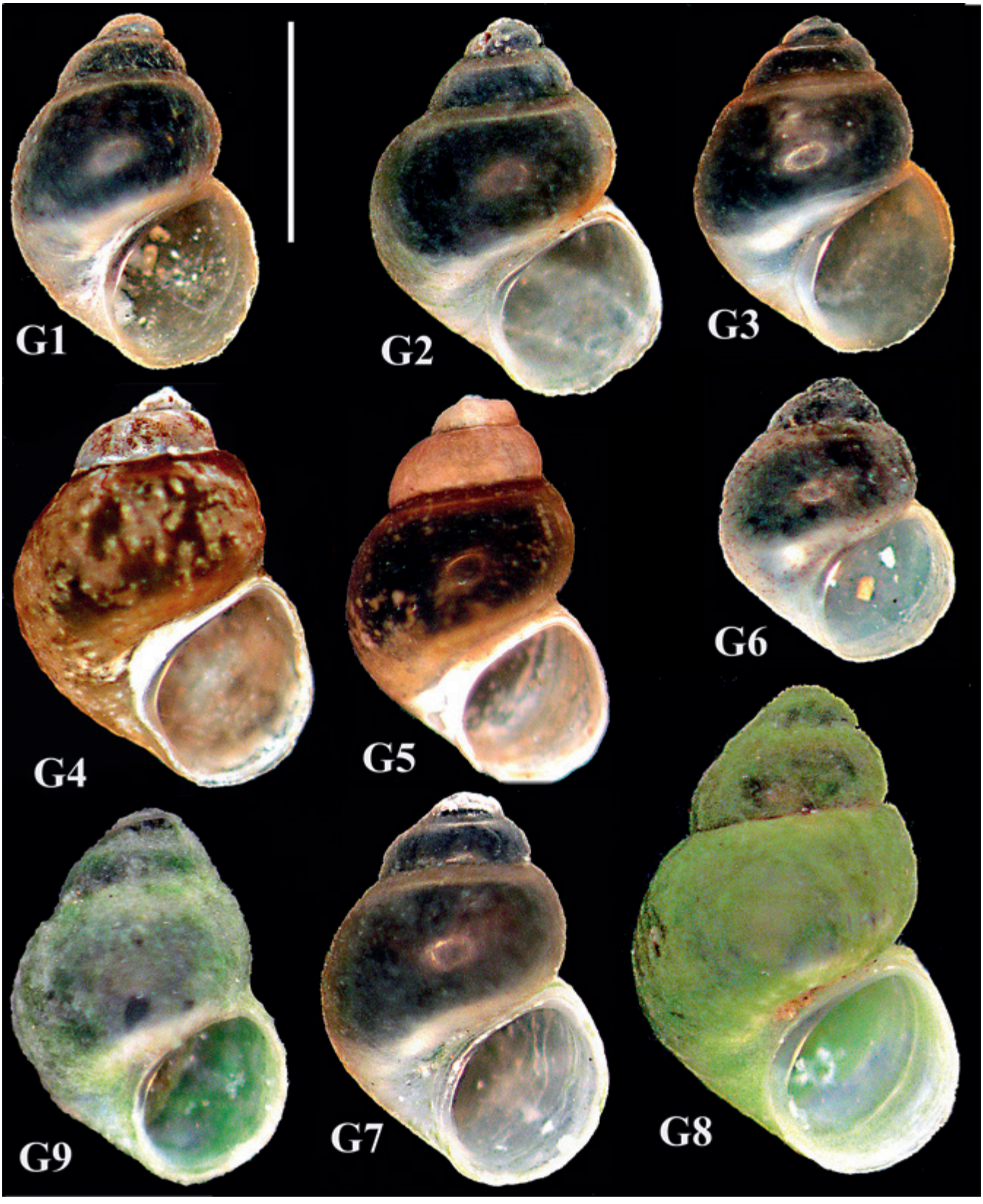


Figure 2 Shells of *Radomaniola* from Greece (localities G1-G9), bar equals 1 mm.

dimorphism. The PCA (Fig. 6) grouped all the eight populations of *R. curta curta* together and connected them by the minimum spanning tree (MST). Likewise, the two populations of *R. curta germari* were close to each other and distant from all the other populations (Fig. 6). In contrast,

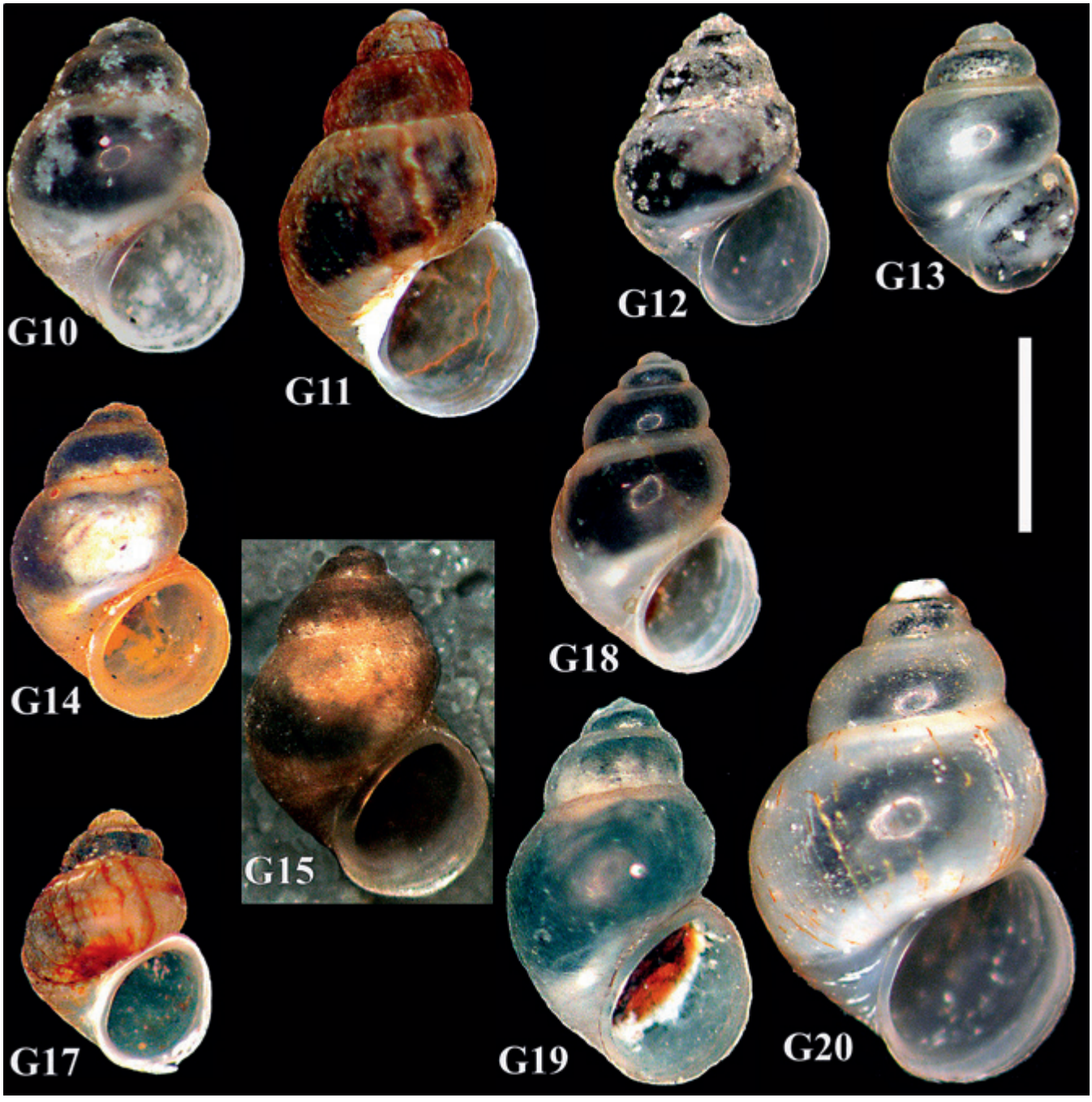


Figure 3 Shells of *Radomaniola* (G11, G13, G14, G15, G18 and G20) and *Grossuana* (G10, G12, G17, G19) from Greece (localities G10-G20), bar equals 1 mm.

R. montana were mixed with *R. curta anagastica*, and *R. curta anagastica* were scattered in two distinct groups. Two samples of *R. curta anagastica*, collected at locality M4 during different seasons, were distinct (Fig. 6). Similar results showed clustering (not presented here). Similarly, the PCA and clustering performed on individuals, weakly confirmed the distinctness of *R. curta curta* and *R. curta germari*, and generally mixed specimens of different nominal taxa.

The penes (Figs 7–8), although variable in size and habitus, were more or less elongated, and all bore a double lobe on the left side. In the Lérni population (G5) the penis was typical of *Radomaniola*, yet its habitus somewhat resembled the one of *Semisalsa* Radoman 1974 (= *Heleobia* Stimpson 1865). As a general rule, the variability of the penis was continuous. There was not a discrete difference between either *Radomaniola* and *Grossuana* or the nominal species/

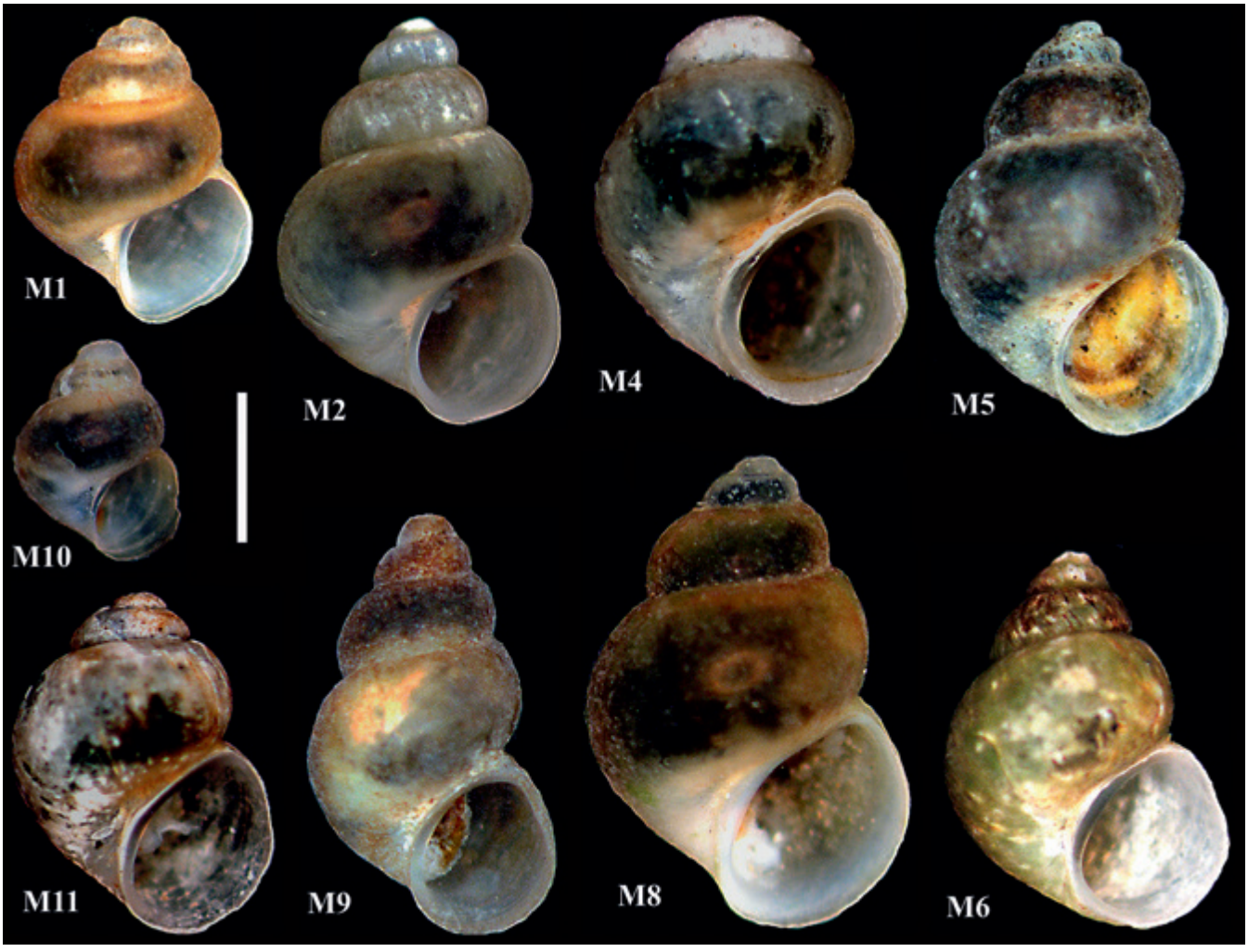


Figure 4 Shells of *Radomaniola* from Neretva River and Montenegro (localities M1-M11), bar equals 1 mm.

subspecies studied. The renal and pallial section of the female reproductive organs (Figs 9–13) had a prominent bursa copulatrix and two seminal receptacles (rs_1 and rs_2). There was a continuous variation of the size and habitus of the bursa and receptacles and of the relative size of the latter ones (rs_1 vs. rs_2). A considerable part of this variation (Figs 9–13) could not unambiguously be ascribed to either *Radomaniola* or *Grossuana*. Similarly, we did not find discrete differences among the nominal taxa studied.

Molecular phylogeny We analyzed 112 COI sequences, 552 bp long (GenBank Accession Numbers: ****), representing 91 haplotypes, and 38 18S sequences, 424 bp long (GenBank Accession Numbers:****) (Table 1). For COI, the DAMBE 4.2.13 entropy-based test resulted in Iss = 0.109; Iss.c.symmetrical = 0.708, d.f. = 500, $p<0.0000$; Iss.c.asymmetrical (more adequate in this case) = 0.386 , d.f. = 500, $p<0.0000$. Thus, this indicates little saturation.

For COI the Akaike Information Criterion (AIC) with MODELTEST found the model TVM+I+Γ, with base frequencies: A =0.3170, C = 0.1974, G = 0.1452, T = 0.3404; substitution rate matrix: [A-C] = 1.1813, [A-G] = 13.0622, [A-T] = 2.6759, [CG] = 0.3250, [C-T] = 13.0622, [G-T] = 1.0000; proportion of invariable sites: (I) = 0.6686, and Γ distribution with the shape parameter = 0.8132. MRMODELTEST selected the model GTR+I+Γ. For the 18S the Akaike Information Criterion (AIC) with MODELTEST found the model K80+I, with equal base frequencies, Ti/tv ratio = 0.9142, proportion of invariable sites: (I) = 0.7842, and equal rates for all sites.

The COI Bayesian tree (Fig. 14) shows the monophyly of the studied group. On the other hand, an ML tree based on the same data set

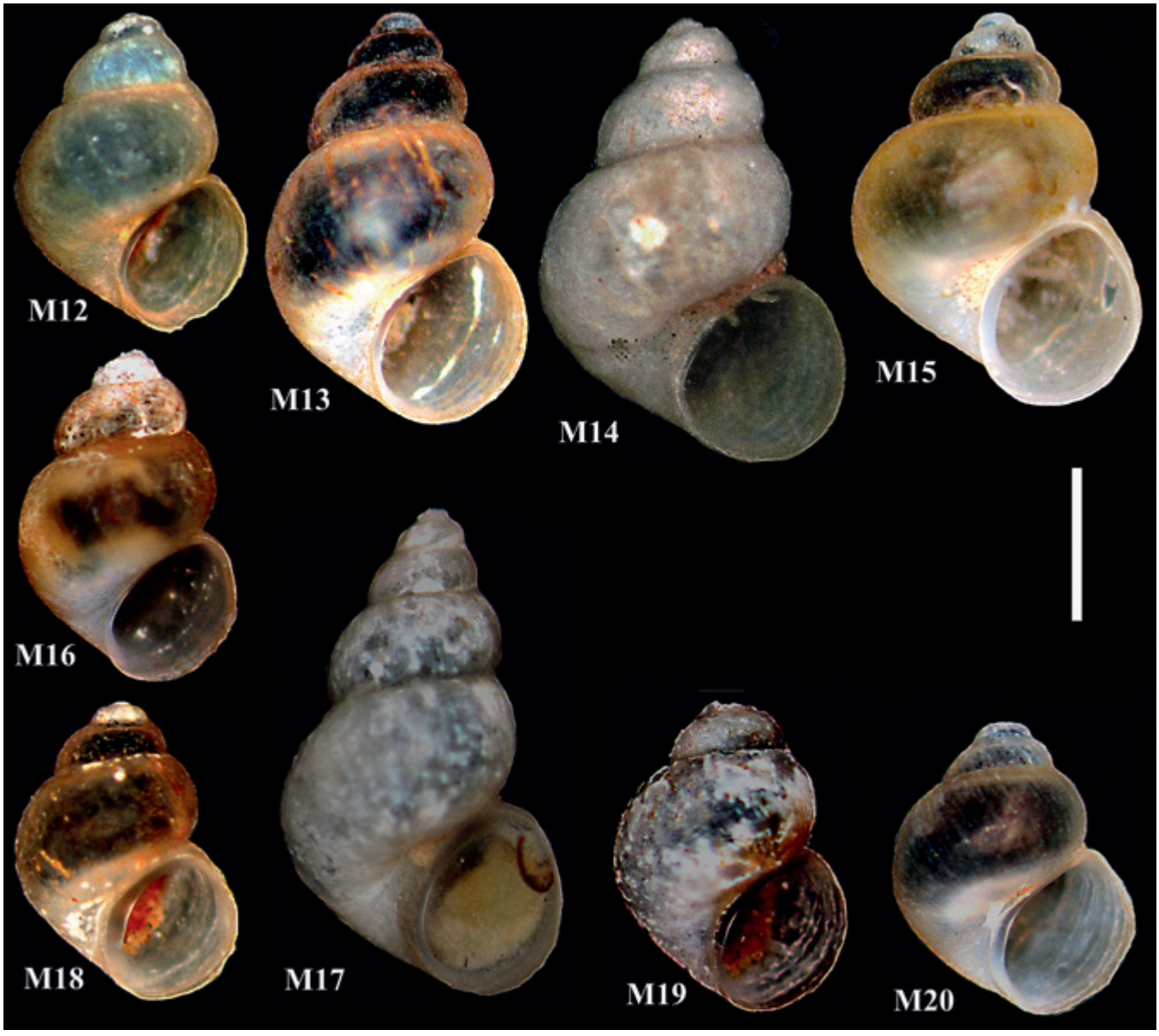


Figure 5 Shells of *Radomaniola* from Montenegro (localities M12-M20), bar equals 1 mm.

(results not shown) placed one of the outgroup species, *Pseudamnicola lucensis*, between the smaller clade including *Grossuana*, and the bigger clade including the remaining taxa (asterisk in Fig. 14). The other outgroup species: *Graziana alpestris*, was placed together with *Grossuana*. At the same place as *Pseudamnicola* (marked with asterisk in Fig. 14) there were found *Daphniola*, *Hauffenia* and *Sadleriana* in both Bayesian and ML trees (not presented), rejecting thus the monophyly of the group.

In both the Bayesian and ML COI trees, the taxa formed three major groups. Those three groups also appeared in our 18S trees, but phylogenetic resolution within them was poor, so the trees are not presented here. All three species of *Grossuana* — *G. codreanui*, *G. serbica* (M3) and *G. delphica* (G10) — formed a clade, and close to this clade, representatives of Greek populations G12, and G17 formed a polytomy, with another group of clades including G16, G19, and part of G14 (haplotype 4R10R). The distinctness of the group formed by all the above taxa was highly supported (Bayesian probability 0.98, ML bootstrap support 0.78). Fig. 14 shows that populations G1-G4 from the Taigetos Mts., Peloponnese (Fig. 1) form a second haplotype-rich group (Bayesian probability 1.00, ML bootstrap 0.66), close to which are population G5 from Lérni (Bayesian probability 1.00, ML bootstrap 0.69), the

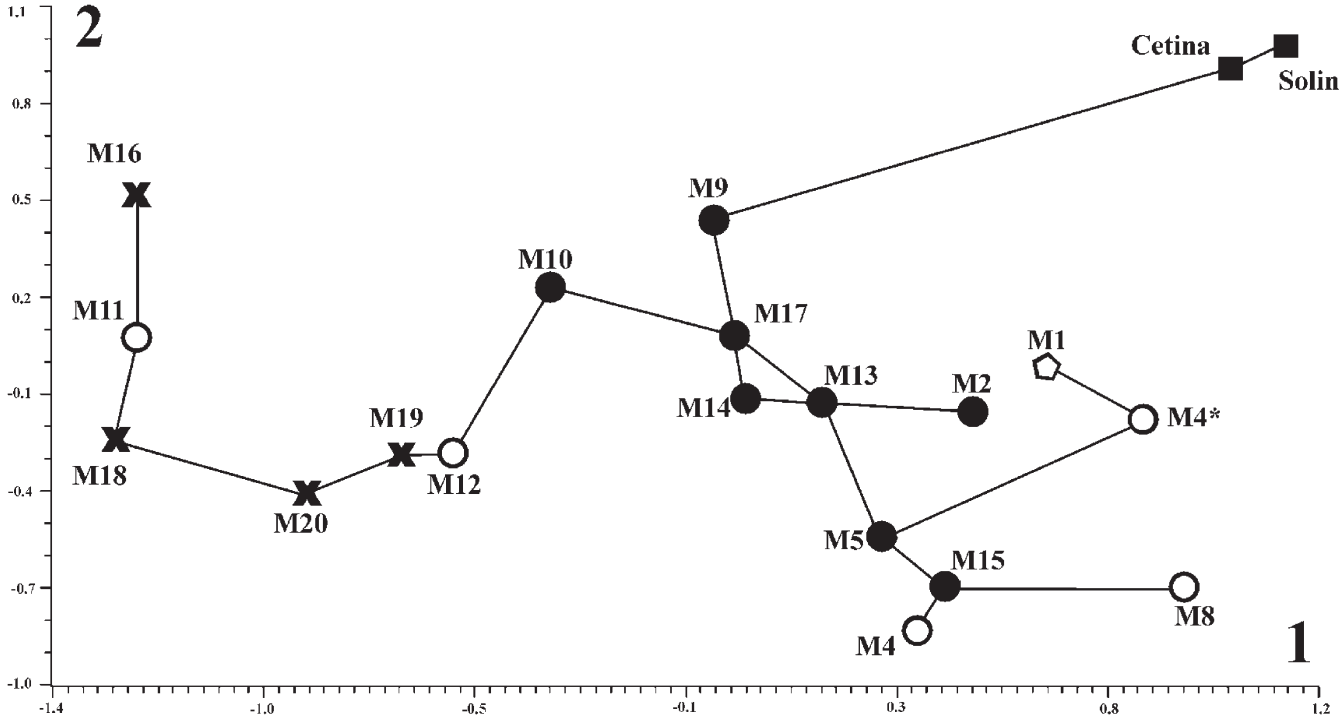


Figure 6 Principal component analysis (PCA) with minimum spanning tree superimposed, computed for the mean values of the seven characters, plotted into PC1 And PC2 space; squares – *Radomaniola curta germari* (Frauenfeld 1863); black circles – *R. curta curta* (Küster 1852), white circles – *R. curta anagastica* (Radoman 1973), crosses – *R. montana* (Radoman 1973), pentagons – *R. curta narentana* (Radoman 1973); M4* and M4 – two samples from locality M4, collected at different time. PC1 explained 51.0904% of variance (37.0408 expected using broken-stick model), PC2 explained 21.6161% (22.7551 expected); PC1 and PC2 cumulatively explained 72.7065% of variance; PC3 (13.0463%, 15.6122 expected) was thus not used for the analysis.

haplotypes from localities G6, G13, G15, G18 and haplotype 4R11R from locality 14.

More than a half of the haplotypes cluster within the third group (Bayesian probability 0.99, ML bootstrap support 0.83) consisting of all the Montenegro populations (M2, M4-M20), the population from the Neretva River (M1), and populations G7-G9, G11, G20 from Greece. *Radomaniola callosa* clusters within this group, as a sister clade of M1 (*Radomaniola curta narentana*). Populations M18-M20 representing *R. montana* are grouped together, but are far from M16 that also represents this nominal taxon. The representatives of *R. curta anagastica* (M8, M11, M12) are not grouped together, as is also the case for *R. curta curta*.

Taxonomic Interpretation and Discussion

In most of the studied populations the shells, which bear rissooid plesiomorphic characters (ovate-conic shape, spire slightly longer than shell width, flat whorl outline, complete aperture: Hershler & Ponder, 1998), closely resemble one another. PCA and clustering on the mean values for 19 populations (and on individual specimens) of the seven morphometric characters for the five *Radomaniola* taxa did not confirm their distinctness. Only *R. curta curta* and *R. curta germari* formed homogeneous groups. This may indicate that we did not select the proper characters to prove the distinctness of the *Radomaniola* taxa. The same set of characters was, however, good enough to confirm species distinctness within *Parabythinella* (Szarowska, 2006) and *Daphniola* (Falniowski *et al*., 2007). The descriptions of all the nominal taxa that we studied were based on the shell alone (Radoman, 1972, 1973, 1983). Our results should, therefore, be sound. The striking difference between the two samples of *R. curta anagastica* from the same locality (M4 and M4*) collected at different seasons is probably connected with seasonal variation in shell morphology (different generations?), which is an important source of variation in a character set that is normally considered as reflecting

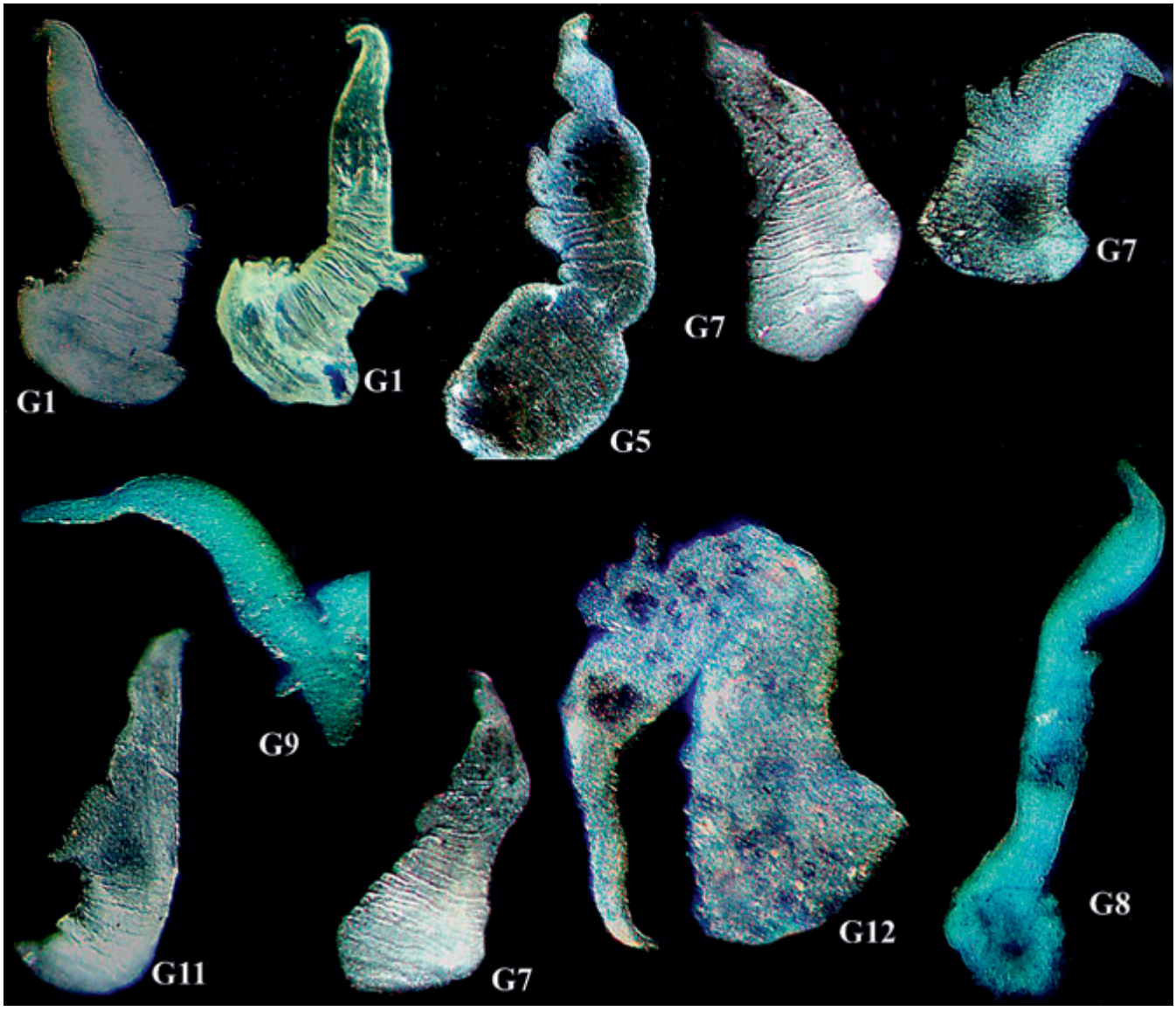


Figure 7 Penes of *Radomaniola* (localities G1, G5, G7, G8, G9, G11) and *Grossuana* (locality G12) from Greece (G1, G8, G12 – ventrally, all others – dorsally).

species distinctness. A similar variation in shell characters, not corresponding to molecular data, was found in *Adriohydrobia* (Wilke & Falniowski, 2001).

We observed a continuous variation in all the penial characters that Radoman used to distinguish between *Radomaniola* and *Grossuana* (Radoman, 1973, 1983): several "intermediate" specimens were not assignable to either the *Radomaniola* or *Grossuana* type according to Radoman's criteria. This raises doubts as to the differentiation of the male genitalia in the two genera. The characteristic penis of the gastropods from Lérni (G5), the habitus of which was somewhat different from that of the other *Radomaniola*/*Grossuana*, might superficially resemble the penis of *Semisalsa*. Lérni is the type locality of *Hydrobia tritonum* Bourguignat 1852, whose shell in Schütt (1980: pl. 9, fig. 6) resembles our specimen of G5 (Fig. 2). Therefore, the *Semisalsa tritonum* of Schütt (1980) is most probably identical with *Radomaniola* from locality G5, which indicates that our specimens from Lérni should be assigned to *Radomaniola tritonum* (Bourguignat 1852) (Bourguignat, 1887). Other than this case, the interspecific variation of the penial characters was continuous.

The renal and pallial section of the female reproductive organs resemble the drawings and descriptions by Radoman (1973, 1983), Szarowska

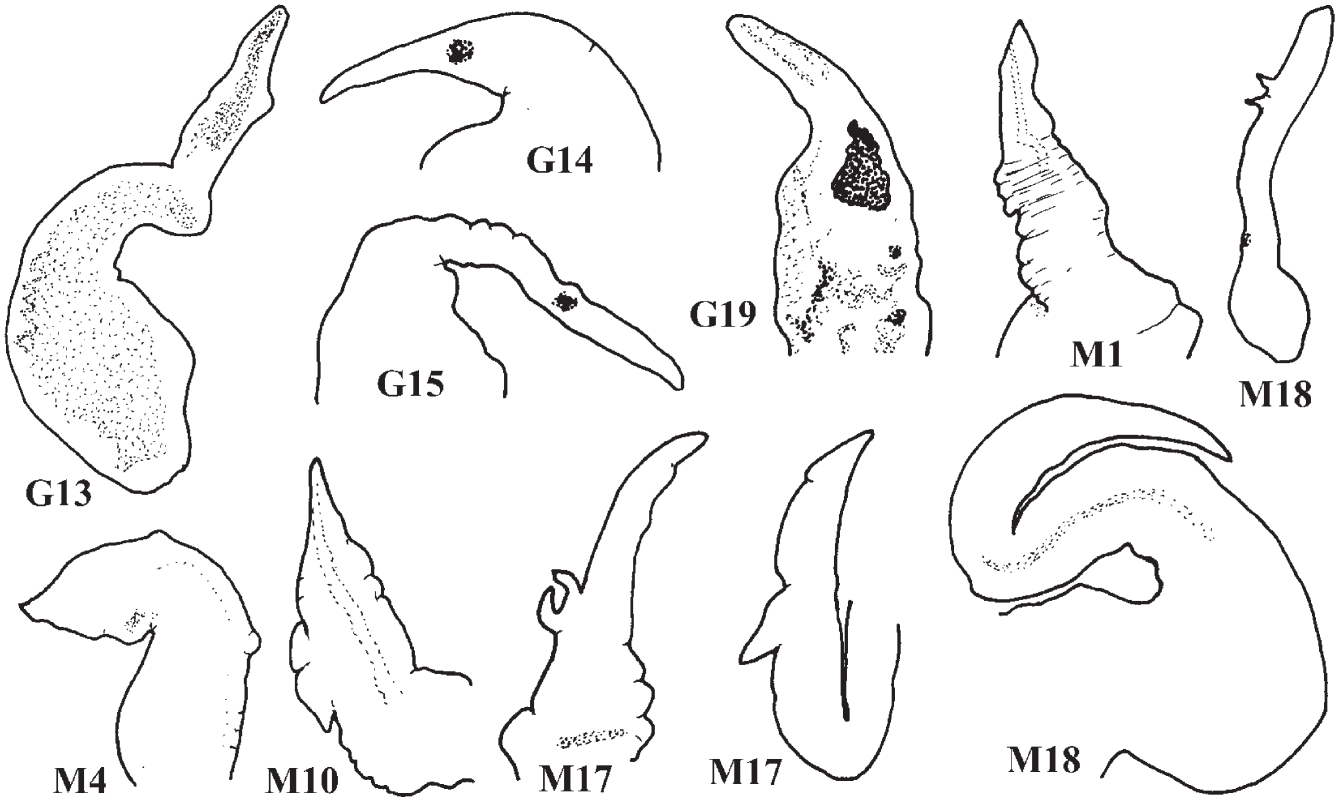


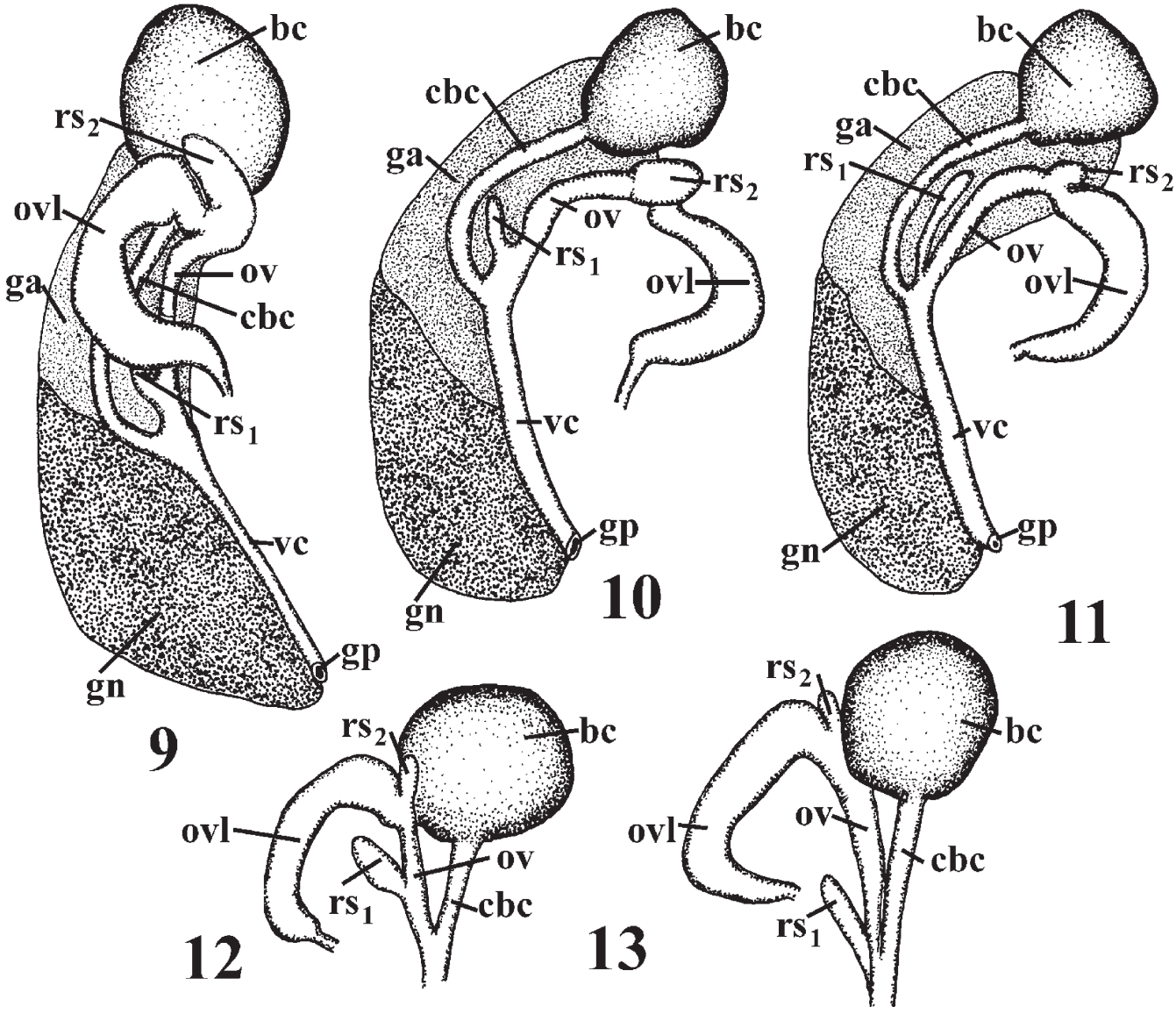
Figure 8 Penes of *Grossuana* (G14, G19) from Greece and *Radomaniola* (all others) from Greece (G), Neretva River (M1) and Montenegro (M) (G14, M4 – ventrally, all others – dorsally).

(2006) and Szarowska *et al*. (2007). However, as in the case of the penis, there is a continuous variation in the shape and size of the bursa copulatrix, as well as in the shape and size proportions of the receptacles. Thus the anatomical distinction between *Radomaniola* and *Grossuana* is unclear, if at all present.

Szarowska (2006) and Szarowska & Falniowski (2008) discussed several examples of morphological characters, the usefulness of which (to phylogeny reconstruction and taxonomy within the Rissooidea) was found to be very limited. Consequently, they proposed an evolutionary interpretation of these observations. Morphostatic evolution, as defined by Davis (1992), is often a result of non-adaptive radiation marked by the rapid proliferation of species without ecological differentiation (Gittenberger, 1991). This results in a flock of species that are not differentiated morphologically (and ecologically). Although traditionally neglected, non-adaptive radiation seems not to be a rare phenomenon in gastropods (Cameron, 1992; Cameron, Cook & Hallows, 1996).

Szarowska (2006) demonstrated that, unexpectedly, the general outline of the penis seems to match the structure of the renal oviduct, and suggested the lock-and-key mechanism regarding the male and female genitalia as a stabilizing factor above species level (Szarowska, 2006; Szarowska & Falniowski, 2008). Such structures as penial lobes and seminal receptacles/bursae copulatrix are thus conservative traits, but some details of their morphology are prone to wide variation. All of this seems to be the case in *Radomaniola* and *Grossuana*. None of the morphological characters of the shell (including morphometry) and soft parts is useful in the species distinctions of *Grossuana* and *Radomaniola*.

Szarowska *et al*. (2007) showed that *Radomaniola* and *Grossuana* do not form a monophyletic clade, since they are not sister clades. The same result shows in our ML COI tree. Thus, it is probable that the group is not monophyletic and that *Radomaniola* and *Grossuana* are distinct genera. However, in our Bayesian tree *Grossuana* is paraphyletic, but this is lost if the other three outgroup species are included in the analysis. The placement of *Pseudamnicola* between *Grossuana* and *Radomaniola* in the ML tree is obviously an artifact, a result of the long-branch attraction.



Figures 9–13 Renal and pallial section of female reproductive organs of *Grossuana* and *Radomaniola*: 9 –10 – *Grossuana serbica*, M3; 11 – *Orientalina curta curta*, M17; 12 – *Orientalina*, G8; 13 – *Orientalina*, G2.
Abbreviations: bc, bursa copulatrix; cbc, canal of bursa copulatrix; ga, albumen gland; gn, capsule gland;, gp, gonoporus; ov, pallial oviduct; ovl, coil of "renal" oviduct; rs, seminal receptacle (1 and 2 after Radoman); vc, ventral channel.

The 18S sequence is commonly used for phylogeny reconstruction within the Rissooidea (e.g. Wilke *et al.*, 2001; Szarowska, 2006; Szarowska *et al.*, 2007). The average rate of change in this molecule is, however, about 1% per 50 million years (Valentine, 2004). Thus its usefulness to phylogeny reconstruction within a group of closely-related species may be very restricted. This concerns our case of *Radomaniola/Grossuana*: the 18S data, confirming the distinctness of the three groups inferred with the COI data, do not provide much more information. Fig. 14 shows the putative species assignment. The group of taxa — consisting of *Grossuana codreanui*, *G. serbica* and *G. delphica*, together with Greek populations G12, G16, G17, G19, and part of G14 — is assigned to the genus *Grossuana* Radoman 1973. Szarowska *et al.* (2007) found representatives of this genus in Greece. The map (Fig. 1) shows that the inferred distribution of *Grossuana* is disjunct. Part of it covers areas of Serbia, Bulgaria and Romania (Radoman, 1985; Szarowska *et al.*, 2007), another part covers northeast Greece. At the northern-most part of the Greek localities studied (the Spring of Achilles: G14),

we found *Grossuana* (4R10R) and a representative of another taxon (4R11R). If, as Radoman suggests (Radoman, 1985), these two genera are vicariant, it is only in the narrow border-zone of their distribution ranges that sympatric populations may be found. It is interesting that, rather close to that locality (in the Volos peninsula: localities G16, G17), in addition to *Grossuana* and *Radomaniola*, we also found two sympatric *Bythinella* species (*Bythinella* G5 and *Bythinella* G6: Falniowski & Szarowska, 2011). The G12 Site (Evvoia) was situated close to the type locality of *Amnicola marginata* Westerlund 1881 (Westerlund, 1881). The G12 shells resembled a shell illustrated in Schütt (1980: in pl. 10, fig. 22). Thus we postulate the assignment of population G12 from Evvoia and *Grossuana* G17 from Volos to *Grossuana marginata* (Westerlund, 1881). Based on shell morphology and data on the distribution of the Greek Rissooidea (Schütt, 1980), we identified *Grossuana* population G19 as *Grossuana hohenackeri* (Küster 1853) (Küster, 1852–1853). Population G16 was probably conspecific with the bearers of haplotype 4R10R, which we found at locality G14 together with another non-*Grossuana* species. Unfortunately, we cannot assign the G16 and G14 *Grossuana* to any species so far known from Greece.

The clade that includes the haplotypes from G1-G4 and G6 (south and central Peloponnese), G15 and G18 (Volos), G13 (north Attica), and haplotype 4R11R from G14, is distant from the clade of *Grossuana* and certainly cannot belong to that genus. This group is probably neither *Grossuana* nor *Radomaniola*. It is somewhat closer to *Radomaniola*, and, with the present knowledge of this group it is assigned to this genus (Fig. 14). The G1-G4 shells resemble the shells that Schütt (1980: pl.10, figs 23–25) presents as *Amnicola seminula* Frauenfeld 1863 (Frauenfeld, 1863), to such an extent that it is appropriate to identify these populations with *Radomaniola seminula* (Frauenfeld, 1863). The shells from locality G6 are, however, also similar to Frauenfeld's illustrations (mentioned above). This Arcadian locality is situated not far from the type locality of *A. seminula*.

As mentioned above, we identified population G5 as *Radomaniola tritonum* (Bourguignat 1852). According to present knowledge, it is, however, not possible to assign other populations from this group to any known species.

The third group consisting of the Montenegro populations, north Peloponnesian populations G7-G9, and populations G11 and G20 from west Greece, represents the genus *Radomaniola* Szarowska 2006. Evidently, the nominal taxa of *Radomaniola* distinguished by Radoman (1973, 1983), i.e. *R. curta curta* (Küster 1852), *R. curta anagastica* (Radoman 1973) and *R. montana* (Radoman 1973), do not form monophyletic taxa in the molecular tree (Fig. 14). Locality M10 is the type locality of *R. curta curta*, and M7 is the type locality of *R. curta anagastica*. The position of these two populations in the tree does not indicate the distinctness of the two nominal taxa.

Thus, neither of our data sets (the molecular data and the shell morphometry data) confirmed the distinctness of those taxa. *Radomaniola curta narentana* (Radoman 1973) appears as a sister species of *R. callosa* (Paulucci 1881) (Bodon, Giusti & Manganelli, 1992) but one population is not sufficient for assessing the distinctness of this nominal taxon. On the other hand, the observed differences suggest a number of distinct species within this clade. M17 is the type locality of *Radomaniola elongata* (Radoman 1973) and we postulate the distinctness of this species (Fig. 14). We identified populations M18-M20 with *R. montana*. Yet, as noted above, not all populations in which identification is based on the shell, are grouped together in the molecular tree. There are a few molecularly-evident groups of a species rank within *Radomaniola* from Montenegro, but without more data (anatomy included) our knowledge is insufficient to make taxonomic decisions.

Schütt (1980) assigned population G11 to *Trichonia kephalovrissonia* Radoman 1973. Again, the present state of knowledge of the group does not permit the identification of all the species. The geological history of the study area is not very helpful in explaining the observed pattern. Modern post-Alpine orogeny European topography of the Mediterranean emerged during the late Tortonian (8MYA) (Kostopoulos, 2009). In the middle-upper Pliocene (3.5–1.8 Myr) there was a strip of freshwater lakes and marshes that stretched along the present shore of the Aegean Sea, and another parallel strip of lakes and marshes that stretched along the eastern margin of the Hellenides (Popov *et al.*, 2004). This may have initiated the three main clades. In the Pleistocene, the unstable fluvio-lacustrine

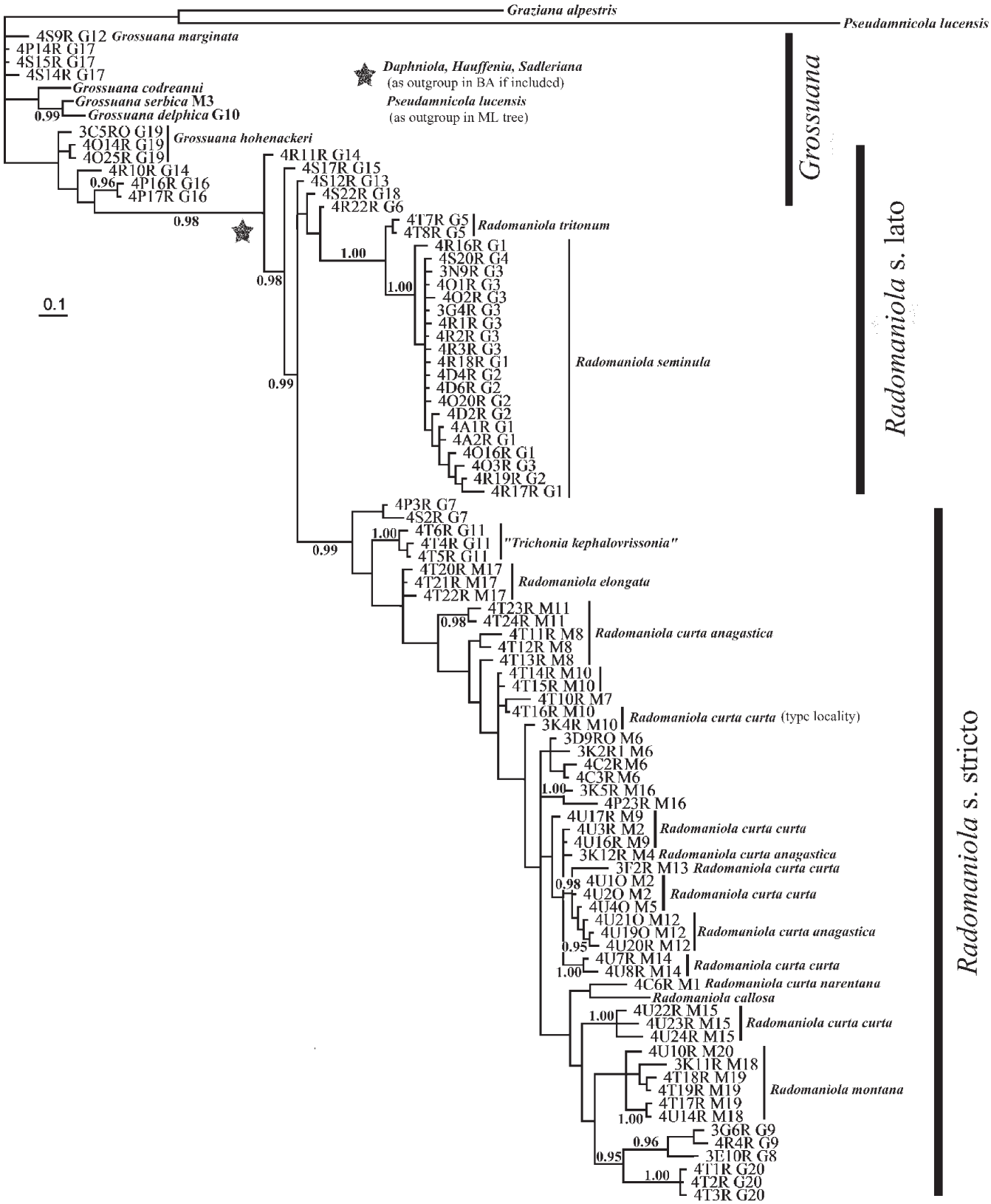


Figure 14 Bayesian tree based on COI sequences, each haplotype name followed by locality symbol, Bayesian probabilities given for each clade if significant (>0.95).

system in SW Bulgaria and northern Greece, with glaciers in Pirin and Rila Mts. (Zagorchev, 2007), probably formed effective temporary barriers for the freshwater gastropods. The sea level during the glacial maxima was up to 120 m lower than today (Hofreiter & Stewart, 2009); thus the Peloponnese and Evvoia were temporarily connected broadly with the mainland. These conditions promoted allopatric speciation. On the other hand, as is the case for *Bythinella*, which co-occurred with *Radomaniola/Grossuana* at many of the studied Greek localities (unpublished data), but also in land snails of the genus *Albinaria* (e.g. Douris, Giokas, Thomaz, Lecanidou & Rodakis, 2007), the general pattern of vicariance has probably been disrupted by subsequent redistribution by passive dispersal, most likely birds (see Wesselingh, Cadee & Renema, 1999 for a review of the literature).

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