

VIVIPARUS MAMILLATUS (KÜSTER, 1852), AND PARTIAL CONGRUENCE BETWEEN THE MORPHOLOGY-, ALLOZYME- AND DNA-BASED PHYLOGENY IN EUROPEAN VIVIPARIDAE (CAENOGASTROPODA: ARCHITAENIOGLOSSA)

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ABSTRACT: Shells and three DNA loci of *Viviparus mamillatus* (Küster, 1852), *V. contectus* (Millet, 1813), *V. acerosus* Bourguignat 1862 and *V. viviparus* (Linnaeus, 1758) were analysed. Despite slight morphological differences between the nominal species, and the near-absence of differences in nuclear 18S rRNA (18S) and histone 3 (H3) loci, mitochondrial cytochrome oxidase subunit I (COI) confirmed species distinctness of all but *V. mamillatus*. The latter should be synonymised with *V. contectus*. The comparison of COI-based phylogeny with the earlier, allozyme- and morphology-based, phylogenies suggests that *V. contectus* and *V. viviparus* are the most distantly related, in the DNA tree *V. acerosus* is closer to *V. contectus*, while in the allozyme and morphology-based tree – to *V. viviparus*.

KEY WORDS: species distinctness, shell, shell sculpture, radula, soft parts, mtDNA, COI

INTRODUCTION

Viviparidae are big, dioecious, ovoviviparous freshwater gastropods, whose history – and invasion of the freshwater habitats – dates back to the Palaeozoic, more precisely to the Devonian (FRETTER & GRAHAM 1962, HASZPRUNAR 1988). In Europe the genus *Viviparus* Montfort, 1810 is represented by widely distributed and rather ubiquitous *V. contectus* (Millet, 1813), somewhat less widely distributed *V. viviparus* (Linnaeus, 1758) and *V. ace-*

rosus Bourguignat, 1862. *V. ater* (De Cristofori et Jan, 1832) is known only from northern Italy and a few localities in Switzerland. *V. janinensis* (Mousson, 1859) has been recorded only from Lake Pamvotis in western Greece, but its geographical distribution (spanning ca. 130 km) as well as its shell morphology suggest its identity with *V. hellenicus* (Clessin, 1879) from Lake Trichonida in SW continental Greece (FALNIOWSKI et al. 1996a, 1998); the latter



species often synonymised with *V. ater* (e.g. WELTER-SCHULTES 2012). It has to be noted that all the species distinction in the European Viviparidae is based on the shell alone.

KÜSTER (1852: pl. 2, figs 1–5) described *Paludina mamillata*: “a species showing transitional features between [*V. contectus*] and [*V. viviparus*]” from “Montenegro, in a creek that flows into Lake Scutari, frequent”. Later it was recorded from Croatia to North Albania (Lake Shkodra: DHORA 2002, GLÖER & PEŠIĆ 2007). Its shell is usually olive brown, prominently striated, sometimes with malleate pattern,

apex blunt, 5–6 convex whorls; juvenile shells with three colour bands (KÜSTER 1852). In May 2018, we collected a few specimens of *V. mamillatus* in Montenegro. The aim of this paper was to test its distinctness and phylogenetic relationships within *Viviparus* applying DNA markers. Some time ago the phylogeny of the other European viviparid species was reconstructed on the basis of morphological and allozymic characters (FALNIOWSKI et al. 1993a, 1996a, b, c, 1997, 1998). Thus there was an opportunity to compare morphology-, allozyme- and DNA-based phylogenies.

MATERIAL AND METHODS

The snails were collected from six localities (Table 1, Fig. 1), and fixed in 80% analytically pure ethanol. The shells were photographed with a CANON EOS 50D digital camera attached to NIKON SMZ-18 stereoscopic microscope with the dark field. The shells of young *V. mamillatus* were cleaned in ultrasonic cleaner and examined using a HITACHI S-4700 scanning electron microscope, applying the techniques described by FALNIOWSKI (1990).

DNA was extracted from foot tissue. The tissue was hydrated in TE buffer (3 × 10 min.); then total genomic DNA was extracted with the SHERLOCK extracting kit (A&A Biotechnology) and the final product was dissolved in 20 µl TE buffer. The extracted DNA was stored at –80°C at the Department of Malacology, Institute of Zoology and Biomedical Research, Jagiellonian University in Kraków. Details of PCR conditions, primers used and sequencing were

Table 1. Sampling localities with geographical coordinates (bold) and references used in the present study

Id	Site	Coordinates	COI GB numbers	References
<i>Viviparus acerosus</i> (Bourguignat, 1862)				
1	Czech Republic, Švihov dam water reservoir	49°37'25"N 15°14'52"E	MK517425-MK517427	present study
<i>Viviparus ater</i> (De Cristofori & Jan, 1832)				
	Switzerland, Lake Zurich, Goldbach	–	FJ405882	SENGUPTA et al. 2009
<i>Viviparus chui</i> Yen, 1937				
	–	–	NC_035733	WANG et al. 2017
<i>Viviparus contectus</i> (Millet, 1813)				
2	Czech Republic, Libický luh NNR, canal in floodplain forest	50°06'06"N 15°11'07"E	MK517420-MK517422	present study
	Denmark, Haellebaek Lakes	–	EU274557	JØRGENSEN et al. 2008
	Denmark, Haellebaek Lakes	–	FJ405835	SENGUPTA et al. 2009
<i>Viviparus georgianus</i> (I. Lea, 1834)				
	USA	–	KY781182	DAVID et al. 2017
	–	–	AF120634	GIRIBET & WHEELER 2002
<i>Viviparus mamillatus</i> (Küster, 1852)				
3	Montenegro, M4, Mareza	42°28'26"N 19°10'47"E	MK517423	present study
4	Montenegro, M8, Crno Oko	42°29'05"N 19°09'16"E	–	present study
5	Montenegro, M19, Vranjina	42°16'54"N 19°08'19"E	MK517424	present study
	Macedonia, Lake Ohrid	–	KY574011	VAN BOCXLAER et al. 2018
<i>Viviparus viviparus</i> (Linnaeus, 1758)				
6	Czech Republic, Velký Osek, Labe River	50°06'09"N 15°09'28"E	MK517417-MK517419	present study
	Germany, Odra River	–	KY574013	VAN BOCXLAER et al. 2018
<i>Viviparus</i> sp.				
	Greece, Lake Pamvotis	–	KY574012	VAN BOCXLAER et al. 2018
<i>Bellamya angularis</i> (Müller, 1774)				
	–	–	KF535563	DING et al. 2013

given in SZAROWSKA et al. (2016). Sequences were initially aligned in the MUSCLE (EDGAR 2004) programme in MEGA 6 (TAMURA et al. 2013) and then checked in Bioedit 7.1.3.0 (HALL 1999). The saturation test (XIA 2000, XIA et al. 2003) was performed using DAMBE (XIA 2013). In the phylogenetic analysis additional sequences from GenBank were used (Table 1). The data were analysed using approaches based on Bayesian inference and maximum likelihood (ML). We applied the GTR model, whose parameters were estimated by the RAXML (STAMATAKIS 2014).

The Bayesian analyses were run using MrBayes v. 3.2.3 (RONQUIST et al. 2012) with the default priors. Two simultaneous analyses were performed, each of which lasted 10,000,000 generations, with one cold chain and three heated chains, starting from random trees and sampling the trees every 1,000 generations. The first 25% of the trees were discarded as burn-in. The analyses were summarised as a 50% majority-rule tree. The ML approach was applied with RAXML v. 8.0.24 (STAMATAKIS 2014). One thousand searches were initiated with starting trees obtained through the randomised stepwise addition maximum parsimony method. The tree with the highest likelihood score was considered as the best representation of the phylogeny. Bootstrap support was calculated with 1,000 replicates and summarised on the best ML tree. RAXML analyses were performed using the free computational resource CIPRES Science Gateway (MILLER et al. 2010).

RESULTS

The shells of *V. contectus* (Figs 2–4), *V. mamillatus* (Figs 5–8), *V. acerosus* (Figs 9–11) and *V. viviparus* (Figs 12–14) show interspecies differences described in the literature (e.g. FALNIOWSKI 1989). The shells of the sequenced *V. mamillatus* (Figs 5–8) resemble the ones of *V. contectus* (Figs 2–4). All the sequenced *V. mamillatus* were juvenile, thus their shells do not resemble those typical of this species. This concerns also the specimens from Vranina (locality M19, Figs 5–6), at Lake Skadar, thus representing the topotypes. We have not found any adult specimens in 2018. The adult ones were collected earlier, but fixed not well enough to get COI sequences. Their shells (Figs 15–18) resembled the ones drawn by KÜSTER (1852), reproduced in Figs 19–21. It should be noted that our juvenile sequenced specimens resembled the very young *V. mamillatus* drawn by KÜSTER (1852: fig. 4). The protoconchs of *V. mamillatus* (Figs 22–23), as well as the sculpture of its first teleoconch whorls (Figs 24–29), with prominent bristles, were similar to the ones found in *V. contectus* (FALNIOWSKI et al. 1996b).

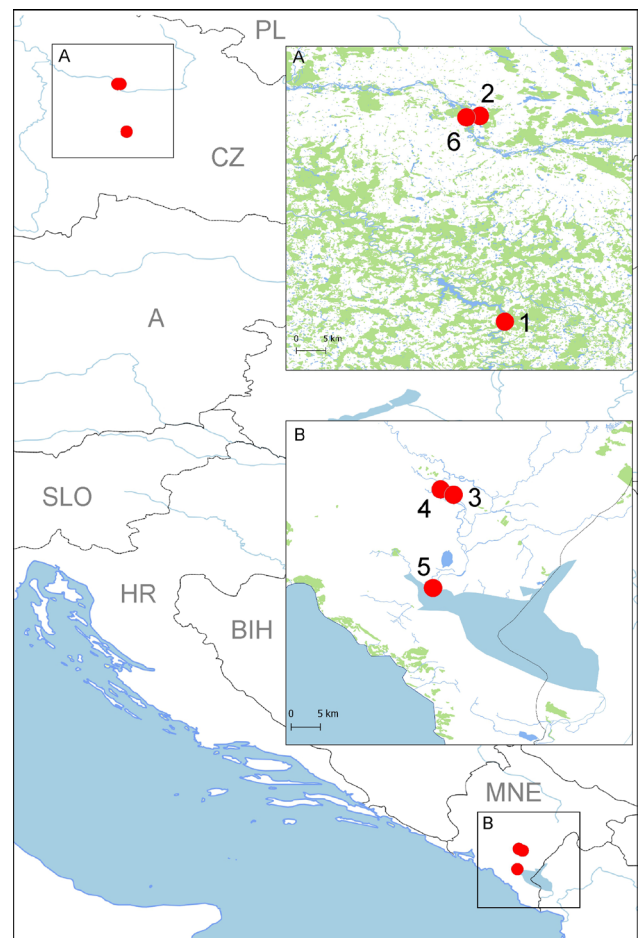
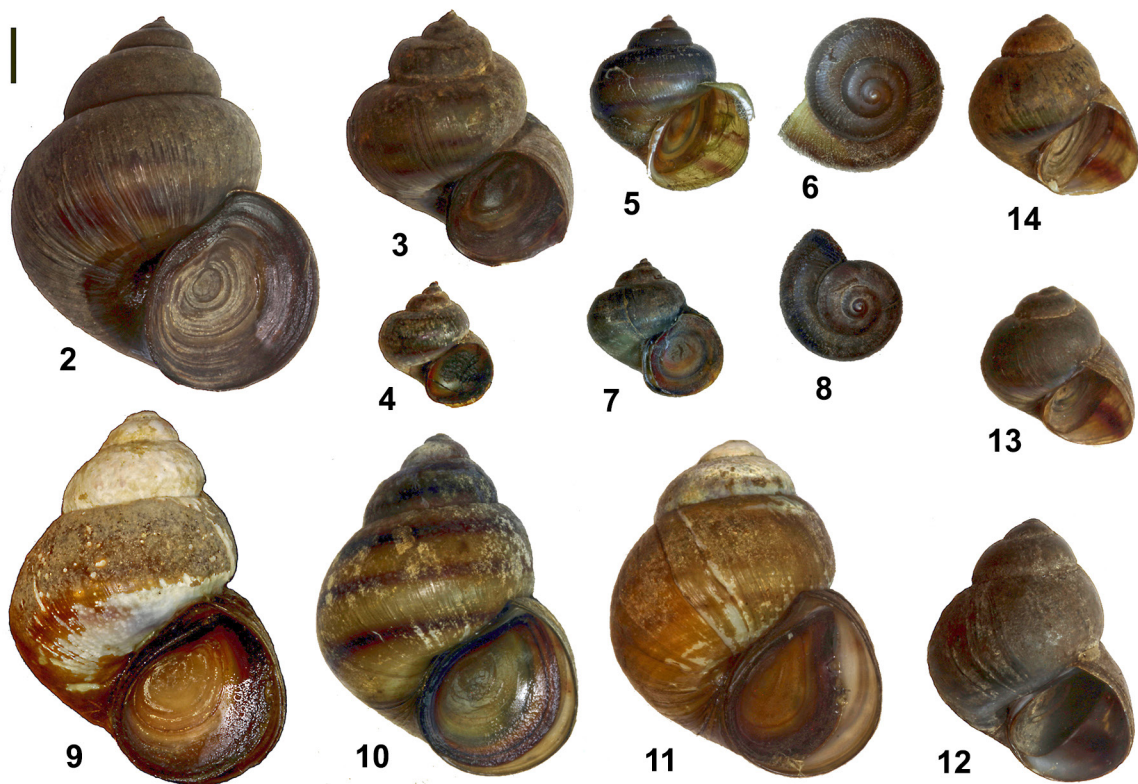


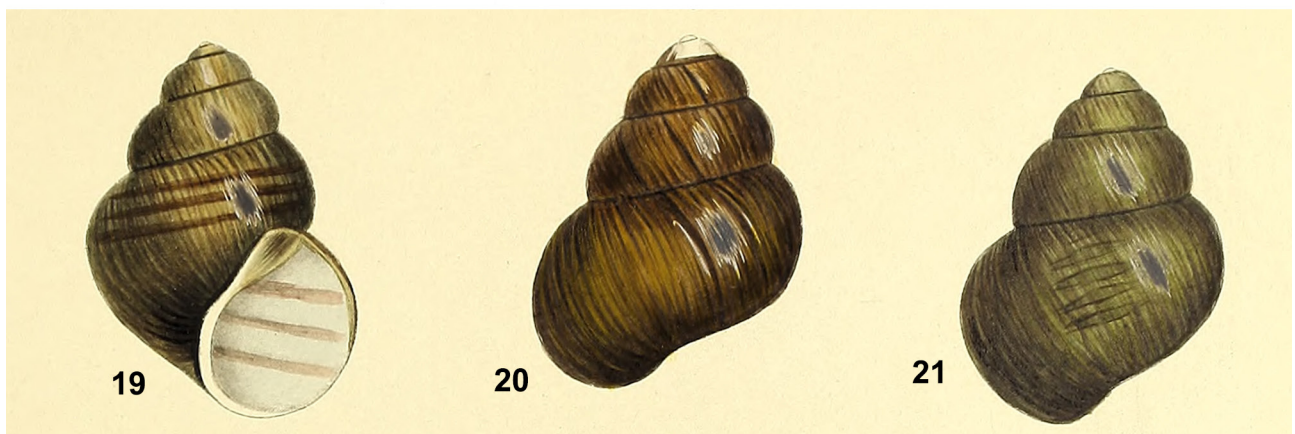
Fig. 1. Localities of the studied Viviparidae (numbers as in Table 1)

In total we analysed three molecular markers: cytochrome oxidase subunit I (COI), histone 3 (H3) and 18S rRNA (18S). The sequences of nuclear 18S rRNA (345 bp, GenBank Accession numbers MK506907–MK506920) and histone H3 (283 bp, GenBank Accession numbers MK517428–MK517441) genes were nearly identical in all the studied species. For COI locus (552 bp, GenBank Accession numbers see Table 1) the tests of XIA et al. (2003) revealed no saturation. The topology of the trees obtained from BI and ML analyses was identical.

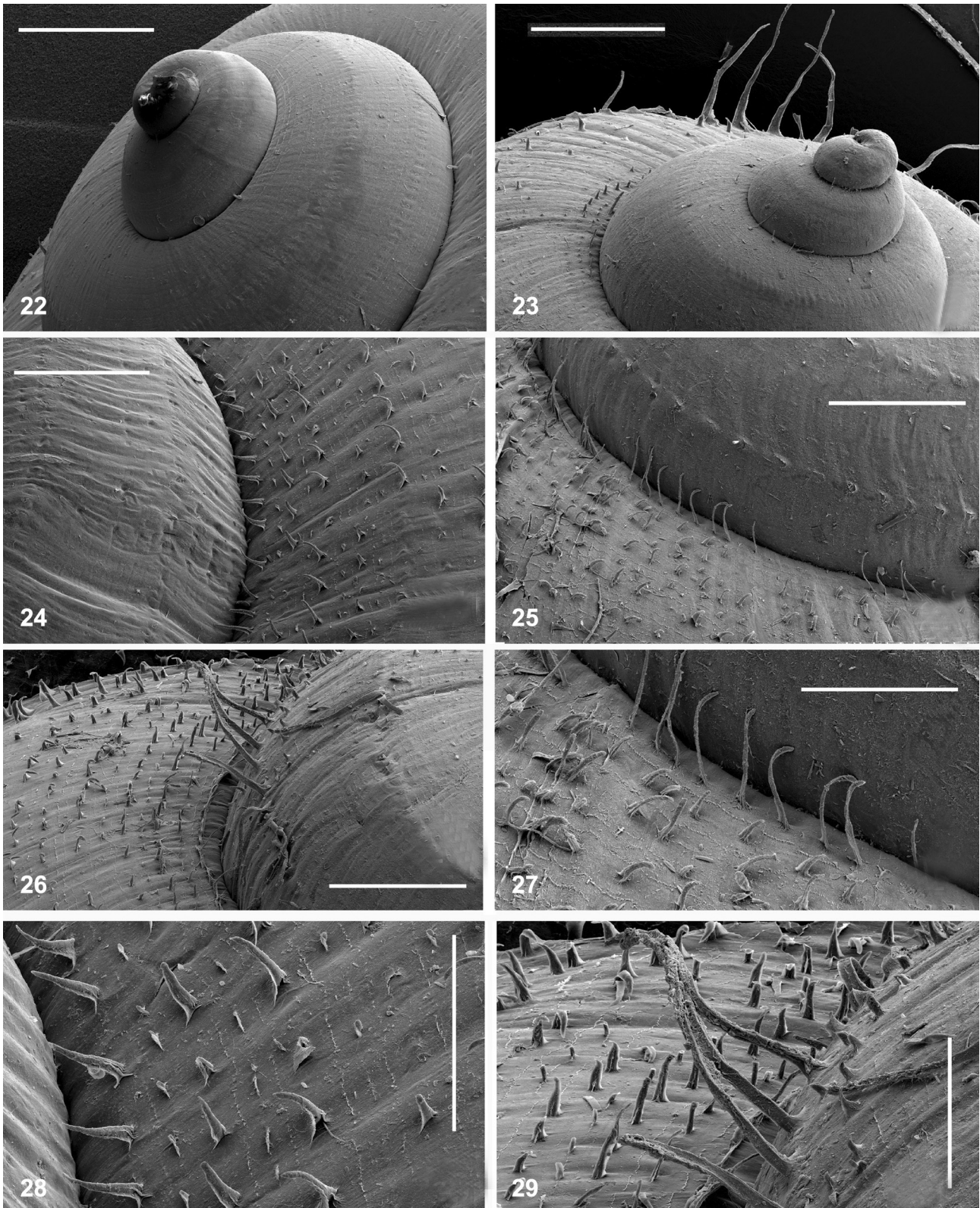
The cytochrome oxidase subunit I (COI) tree (Fig. 30) confirmed distinctness of all the studied species, defined by their shell character states, with an exception of *V. mamillatus*. The p-distances between the species of *Viviparus* are given in Table 2. The sequences of our specimens from Montenegro were nearly identical with *Viviparus* cf. *contectus* from Lake Ohrid found in GenBank, and very close to the ones of our *V. contectus*, as well as *V. contectus* from Denmark taken from GenBank. The p-distance between *V. mamillatus* and *V. contectus* was 0.026



Figs 2–14. Shells of sequenced *Viviparus*: 2–4 – *V. contectus*; 5–8 – *V. mamillatus*: 5–6 – M19, 7–8 – M4; 9–11 – *V. acerosus*; 12–14 – *V. viviparus*; scale bar 5 mm



Figs 15–21. Shells of *V. mamillatus*: 15–18 – unsequenced adult specimens from Lake Skadar; 19–21 – original drawings of KÜSTER (1852: pl. 2, figs 1–3); scale bar 10 mm



Figs 22–29. SEM photograph of shells of *V. mamillatus*: 22–23 – protoconch, bar equals 1 mm; 24–29 – details of teleoconch sculpture; scale bar 1 mm in 24–26, and 0.5 mm in 27–29

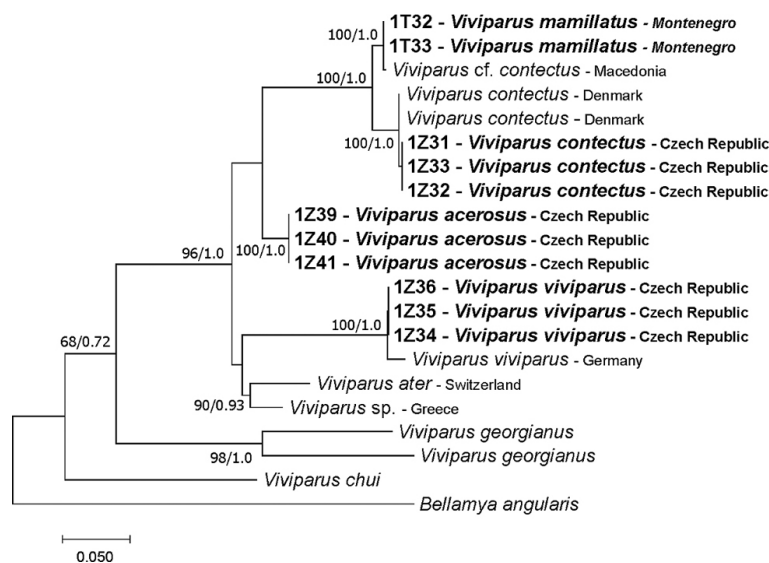


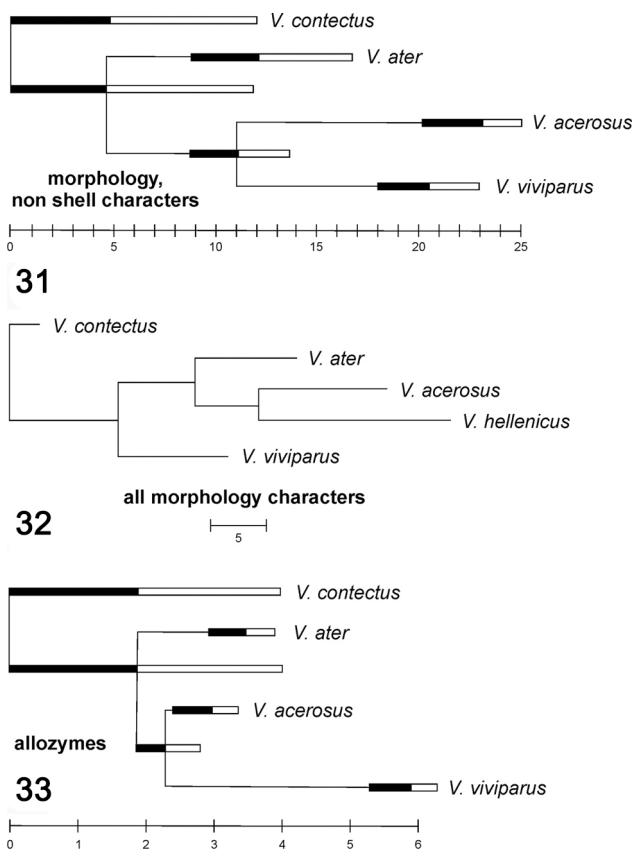
Fig. 30. Cytochrome oxidase subunit I (COI) maximum likelihood tree; bootstrap supports and Bayesian probabilities given

(Table 2). “*Viviparus* sp.” from Lake Pamvotis – most probably representing *V. janinensis* – clustered with *V. ater*, with the p-distance of 0.056.

The morphology-based trees (Figs 31–32) resemble the one based on allozymes (Fig. 33). The comparison of our COI-based phylogeny (Fig. 30) with the allozyme-based one (Fig. 33) confirms the most distant relationships between *V. contectus* and *V. viviparus*, although in the DNA tree *V. acerosus* is closer to *V. contectus*, while in the allozyme tree – to *V. viviparus*.

Table 2. The p-distances for COI locus, between the main <i>Viviparus</i> species						
	1	2	3	4	5	6
1 <i>V. mamillatus</i>						
2 <i>V. contectus</i>	0.026					
3 <i>V. acerosus</i>	0.078	0.084				
4 <i>V. viviparus</i>	0.117	0.115	0.115			
5 <i>V. ater</i>	0.108	0.107	0.075	0.098		
6 <i>V. sp. Greece</i>	0.103	0.097	0.060	0.091	0.056	

Figs 31–33. Inferred non-DNA phylogenies in *Viviparus*: 31 – based on morphology, non-conchological characters (after FALNIOWSKI et al. 1996a), 32 – maximum parsimony tree considering all the morphological characters listed in FALNIOWSKI et al. (1996b, c, 1997), 33 – based on allozymes (after FALNIOWSKI et al. 1996a)



DISCUSSION

Considering the lack of evident morphological differences between *V. mamillatus* and *V. contectus*, combined with the slight differences in COI, *V. mamillatus* should be regarded as conspecific with *V. contectus*, and thus *V. mamillatus* (Küster, 1852) becomes a jun-

ior synonym of *V. contectus* (Millet, 1813). The range of the latter species should be thus expanded to include Montenegro, Albania and Macedonia (lakes Scutari and Ohrid). Our results certainly do not confirm the suggestion of WELTER-SCHULTES (2012)

that *V. mamillatus* may be conspecific with *V. janinensis* from Lake Pamvotis. On the other hand, accepting the probable hypothesis that *V. janinensis* is conspecific with *V. hellenicus*, its close relationship with *V. ater* confirms the suggestion of SCHÜTT (1962) and WELTER-SCHULTES (2012) that *V. hellenicus* is a subspecies of *V. ater*. Considering the priority rule, its name should then be *V. ater janinensis*. It should be noted, however, that neither FALNIOWSKI et al. (1997), nor GLÖER & GEORGIEV (2014) confirm such relationships, based on the shell, radula, and soft parts morphology.

Although morphology need not reflect speciation (FALNIOWSKI 1987, 1992, LARSON 1989, GIUSTI & MANGANELLI 1992), it reflects it in the case of our *Viviparus*. The incongruence of morphological and molecular evolution is not rare (CHEVERUD 1988, FALNIOWSKI et al. 1993b, DAVIS 1994, OMLAND 1994). HAASE (1994) found two species of *Graziana*, morphologically and anatomically different enough

to justify their specific distinctness, to be genetically identical at all 27 loci. Our study (unpublished) on the Polish *Bythinella*: *B. austriaca* (Frauenfeld, 1857) and *B. zyvionteki* Falniowski, 1987, found no differences in four DNA loci, despite clear interspecific differences in morphology, especially penis.

In the case of our Viviparidae the molecules and morphology are congruent in species distinction. The DNA-based phylogeny also confirms the distant relationship between *V. contectus* and *V. viviparus*, despite the fact that the two species sometimes hybridize (FALNIOWSKI et al. 1993a). On the other hand, considering all the data, the phylogenetic relationships of *V. ater* and *V. acerosus* remain unclear.

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