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First record of *Stagnicola montenegrinus* Glöer & Pešić, 2009 (Mollusca: Gastropoda: Lymnaeidae) in Bulgaria and its taxonomic relationship to other European lymnaeids based on molecular analysis

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Abstract. *Stagnicola montenegrinus* was found in the floodplain of the river Maritza in Plovdiv. By comparison of the cyt-b sequences (fragment of 329 bp) of one of the specimens with other European *Stagnicola* specimens, this specimen fell in a cluster together with two sequences of *S. montenegrinus* from Skadar Lake, confirming the morphological determination and the first record of this species for Bulgaria. *S. montenegrinus* is a species closely related to *S. corvus*. The species' occurrence is evidently not limited to the Skadar Lake region from where *S. montenegrinus* was originally described.

Key words: *Stagnicola montenegrinus*, Bulgaria, Lymnaeidae, molecular genetics

Introduction

For the freshwater snail fauna of Bulgaria currently, three species of the genus *Stagnicola* Jeffreys, 1830 are known: *S. palustris* (O. F. Müller, 1774), *S. corvus* (Gmelin, 1791), and *S. turricula* (Held, 1836) (Angelov 2000, Hubenov 2007).

When *S. montenegrinus* was described as a new species of this genus, it was only known from three localities in Montenegro: the Skadar Lake, the Crnojevica River and the Humsko Blato near Vitoja (Glöer & Pešić 2009). Up to now no other records became known.

In June 2010 three specimens of genus *Stagnicola* were collected in the floodplain of the river Maritza in Plovdiv (Bulgaria) and sent to the Senckenberg Natural History Collections Dresden, Museum of Zoology (SNSD) for determination and molecular genetics analyses. Anatomical examinations by Peter Glöer determined that these three specimens belong to *S. montenegrinus*. The aim of the study is to prove the determination based on shell and genital characters by molecular techniques.

Material and methods

The specimens of *S. montenegrinus* were found in shallow floods and pools on the north bank of Maritza River in Plovdiv city (Upper Thracian Lowland) near the VHVP-bridge (Fig. 1), N42° 09' 13.5" E24° 43' 34.8", leg. Dilian Georgiev, 09.06.2010.

Figure 1. Habitat of *S. montenegrinus* on the northern banks of Maritza River in Plovdiv city (photo by Stanislava Vassileva).

Dissections and measurements of genital organs and shells were carried out using stereo microscopes (ZEISS and OLYMPUS). Photographs were taken with a digital camera system (OLYMPUS DP10).

For the taxonomy we followed the current European checklists (Falkner et al. 2001, Bank 2011).

For outgroup comparison in the molecular genetic analyses we used Palaearctic specimens of the species *Planorbarius corneus* (Linnaeus 1758), *Aplexa hypnorum* (Linnaeus, 1758). *Lymnaea stagnalis* (Linnaeus, 1758), *Galba truncatula* (O. F. Müller, 1774), *Omphiscola glabra* (O. F. Müller, 1774), *Radix auricularia* (Linnaeus, 1758), *R. balthica* (Linnaeus, 1758), *R. labiata* (Rossmässler, 1835),

Stagnicola palustris, *S. fuscus* (C. Pfeiffer, 1821), and *S. corvus* were used as ingroup. The snails were fixed in 70-80% ethanol. All specimens used in the study are listed in Table 1. They are stored in the Molluscan collection of the Senckenberg Natural History Collections Dresden, Museum of Zoology (SNSD).

Table 1. Material used in the molecular genetic studies. ENA=European Nucleotide Archive.

Code	Collection No. SNSD	Locality	ENA No.	
			cyt-b	ITS-2
Planorbarius corneus (Linnaeus, 1758)				
Planorbarius corneus 1	Moll 52556	Germany, Saxony, Linz, pond Goldgrubenteich, 13°43'09"E 51°19'45"N	FR797880	FR797830
Planorbarius corneus 2	Moll 52557		FR797881	FR797831
Aplexa hypnorum (Linnaeus 1758)				
Aplexa hypnorum 1	Moll S348	Germany, Mecklenburg-Vorpommern, lake, Nebel, 12°42'02"E 53°15'32"N	FR797882	FR797832
Aplexa hypnorum 2	Moll S350		FR797883	FR797833
Galba truncatula (O. F. Müller 1774)				
Galba truncatula 1	Moll 52545	Germany, Saxony, Oelsnitz/Erzgebirge, former pond, 12°42'04"E 50°43'02"N	FR797892	FR797847
Galba truncatula 2	Moll 52546		FR797893	FR797848
Galba truncatula 3	Moll S1130	Bulgaria, Osogovo Mountains, Smolichane Village, karst spring, 22°48'25.2"E 42°07'58.1"N	FR797890	FR797845
Galba truncatula 4	Moll S1131		FR797891	FR797846
Omphiscola glabra (O. F. Müller 1774)				
Omphiscola glabra 1	Moll S303	Germany, Hamburg, Kollau, Mühlenau, 09°55'33"E 53°36'34"N	FR797887	FR797853
Omphiscola glabra 2	Moll S304		FR797888	FR797854
Omphiscola glabra 3	Moll S305		FR797889	FR797855
Lymnaea stagnalis (Linnaeus, 1758)				
Lymnaea stagnalis 1	Moll 53093	Germany, Baden-Württemberg, lake Bodensee, peninsula Mettnau, north side, 09°00'04"E 47°43'52"N	FR797896	FR797836
Lymnaea stagnalis 2	Moll 53094		FR797897	FR797837
Lymnaea stagnalis 3	Moll 53108	Germany, Baden-Württemberg, Konstanz-Egg, ditch Hockgraben, 9°11'34.2"E 47°40'57.3"N	FR797894	FR797834
Lymnaea stagnalis 4	Moll 53109		FR797895	FR797835
Stagnicola palustris (O. F. Müller 1774)				
Stagnicola palustris 1	Moll 48716	Germany, Saxony, wetland west of Burghausen, 12°14'44"E 51°21'33"N	FR797899	FR797841
Stagnicola palustris 2	Moll 53095	Germany, Baden-Württemberg, lake Bodensee, peninsula Mettnau, north side, 09°00'04"E 47°43'52"N	HE577651	HE577631
Stagnicola palustris 3	Moll 53096		HE577652	HE577632
Stagnicola palustris 4	Moll S1345	Germany, Mecklenburg-Vorpommern, lake Grosser Plaetschsee, south bank, 12°19'18"E 53°26'25"N	HE577653	FR797838
Stagnicola fuscus (C. Pfeiffer 1821)				
Stagnicola fuscus 1	Moll 48550	Germany, Saxony, reservoir Lobstädt, north bank, 12°27'27"E 51°07'58"N	HE577654	HE577633
Stagnicola fuscus 2	Moll 51794	Germany, Saxony, marsh wood near Raden, 13°29'57"E 51°22'23"N	HE577655	HE577634
Stagnicola fuscus 3	Moll S2082	Germany, Saxony, nature reserve Alte See Grethen, marsh wood, 12°40'18"E 51°13'42"N	HE577656	HE577635
Stagnicola fuscus 4	Moll S2199	Germany, Baden-Württemberg, nature reserve Erlich, marsh wood, R 3462394 H 5449072	HE577657	HE577636
Stagnicola fuscus 5	Moll S2946	Germany, Thuringia, alder marsh near Appenrode, 10°43'07"E 51°34'27"N	HE577658	HE577637
Stagnicola corvus (Gmelin 1791)				
Stagnicola corvus 1	Moll 49821	Germany, Saxony, Niederspree, pond Großer Tiefzug, 14°53'38"E 51°24'20"N	HE577659	HE577638
Stagnicola corvus 2	Moll 49872	Germany, Saxony, pond Vierteich near Freitelsdorf, 13°41'57"E 51°15'43"N	HE577660	HE577639
Stagnicola corvus 3	Moll 52830	Germany, Saxony, Grethen, ditch on the west side of the pond Kleiner Kirchenteich, 12°39'22"E 51°14'29"N	HE577661	HE577640
Stagnicola corvus 4	Moll 52831		HE577662	HE577641
Stagnicola corvus 5	Moll 52832		HE577663	HE577642
Stagnicola montenegrinus Glöer & Pešić 2009				
Stagnicola montenegrinus 1	Moll 51854	Montenergo: Skutari See, Vranjina 9°07'32.52"E 42°16'37.37"N	HE577664	HE577643
Stagnicola montenegrinus 2	Moll 51855		HE577665	HE577644

Table 1. (continued)

Code	Collection No. SNSD	Locality	ENA No.	
			cyt-b	ITS-2
Stagnicola montenegrinus 3	Moll S2313	Bulgaria: floodplain of the Maritza river in Plovdiv, 24° 43' 34.8"E 42° 09' 13.5"N	HE577666	HE577645
Radix auricularia (Linnaeus 1758)				
Radix auricularia 1	Moll 53070	Germany, Bavaria, Weichering, pond in riverside forest, 11°19'23.6"E 48°43'34.1"N	FR797902	FR797842
Radix auricularia 2	Moll 53071		FR797903	FR797843
Radix auricularia 3	Moll 52857	Russia, Novosibirsk Region, Novosibirsk Reservoir near Kirza River 81° 39.63114"E 54° 14.244″N	HE577667	HE577647
Radix auricularia 4	Moll 52859		HE577668	HE577646
Radix labiata (Rossmässler 1835)				
Radix labiata 1	Moll 51275	Germany, Saxony, pond near Langenberg, 12°51'21"E 50°33'09"N	HE573106	HE573068
Radix labiata 2	Moll 51276		HE573107	HE573069
Radix labiata 3	Moll 51696	Germany, Brandenburg, small lake near Wachow, 12°43'05"E 52°32'05"N	HE577669	HE577648
Radix labiata 4	Moll 51697		HE573108	HE573070
Radix balthica (Linnaeus 1758)				
Radix balthica 1	Moll 51281	Switzerland, canton Basel-Landschaft, Liestal, Orishof, 07°43'03"E 47°28'22"N	HE577670	HE577650
Radix balthica 2	Moll 51283		HE573133	HE573082
Radix balthica 3	Moll 53111	Germany, Baden-Württemberg, Konstanz-Egg, pond near University, 09°11'29"E 47°41'09" N	HE573116	HE573078
Radix balthica 4	Moll 53112		HE573117	HE577649

Molecular techniques

Tissue samples were taken under a microscope from the feet of the snails and fixed in 100% ethanol. The samples were registered in the tissue collection of the SNSD with a new collection number and the collection number of the specimen in the molluscan collection of SNSD and stored at -80°C.

For molecular genetic analyses we obtained sequence data of the complete nuclear ITS-2 marker (280 bp in *A. hypnorum* up to 491 bp in *L. stagnalis*) and a 329 bp fragment of the cyt-b gene as mitochondrial marker.

DNA was extracted using DTAB (dodecyl trimethyl ammonium bromide) buffer (Gustincich et al. 1991). The tissue samples were washed with 100 µl TE buffer and subsequently incubated with 500 µl preheated DTAB for 30 min at 65°C. The incubation was continued after adding 10 µl Proteinase K (50 mg/ml) for 20-24 hours, followed by a short incubation with 10 µl RNase (10 mg/ml) for 30 min at 37°C. Remaining tissue fragments disintegrated after vortexing. For cleaning 550 µl chloroform/isoamyl alcohol (24/1) was used. The samples were vortexed for 20 sec and the phases subsequently separated again at 12 000 g for 3 min. With the upper aqueous phase the procedure was repeated. 100 µl 4M LiCl and 400 µl isopropanol were added to the aqueous phase for precipitation. The samples were cooled at --20°C for 30 min and subsequently the DNA was pelleted by centrifugation at 11 200 g for 20 min at 4°C. The liquid was disposed of and the pellets were dried by inverting the tubes on a paper towel. The pellets were cleaned twice with 200 µl ice-cold 70% ethanol. The DNA pellets were dried 10 min at 50°C and subsequently redissolved in 50 µl of TE buffer.

The PCRs were carried out in a final volume of 20 µl with quantities of DNA from 0.5 to 5.0 µl depending on the concentration estimated by gel electrophoresis, 2 µl 10x PCR buffer (Bioron, incomplete), 1 µl $MgCl_2$ (Bioron, 0.055 µS/cm), 1 µl of each primer (10 pmol/µl), 0.5 µl dNTP (10 mM), 0.2 µl Taq DNA polymerase (DFS-Taq Bioron) and the corresponding volume of sterile H_2O. From the cyt-b gene a region of circa 370 bp was amplified with the primers UCyt*b*151F and UCyt*b*270R (Merritt et al. 1998) and a temperature profile of 94°C for 4 min, 40 cycles of 94°C for 40 s, 48°C for 40 s, 72°C for 1.15 min, followed by an extension at 72°C for 6 min and hold at 8°C, was used. The primers used for ITS-2 were LT1 (Bargues et al. 2001) and ITS2-Rixo (Almeyda-Artigas et al. 2000). The temperature profile used was the following: 94°C for 4 min, 40 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, followed by 7 min at 72°C and 8°C hold.

PCR products were purified with 0.1 µl Exo Sap-It plus 4 µl ddH_2O and incubated for 30 min at 37°C, followed by deactivation for 15 min at 80°C.

The primers used for the cycle sequencing were UCyt*b*151F for cyt-b and LT1 for ITS-2. The quantity of PCR product used for cycle-sequencing ranged from 0.5 to 5.0 µl depending on the concentration estimated by gel electrophoresis. 0.5 µl BigDye T-Mix (ABI, Applied Biosystems), 2.25 µl BigDye buffer (5x), 0.5 µl primer (10pmol), and sterile H_2O were added to a total volume of 10 µl. The following temperature profile was used: 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min and 8°C hold. The products were purified by adding 1 µl 3M NaAc (pH 4.6) and 25 µl EtOH (100%), centrifuging at 13 000 g for 15 min, inverting the tubes on a paper towel and washing with 200 µl 70% EtOH. After removing the EtOH the pellets were dried for 10 min at 50°C. Samples were sequenced on an ABI 3130 xl (Applied Biosystems).

Data analysis

For maximum-likelihood analyses, including bootstrap support, we used raxmlGUI 0.9 beta 2 (RAxML) (Silvestro & Michalak 2010, Stamatakis et al. 2005). The settings were "ML+thorough bootstrap" with 100 (replicate) runs and 1000 (bootstrap) repetitions.

Maximum-parsimony (MP) trees were reconstructed using PAUP (version 4.0b10; Swofford 2002; settings: gapmode=NewState, addseq=closest). For presentation of MP results, one of the best trees was chosen to be able to illustrate branch lengths (one showing the same overall topology as the majority rule consensus tree was chosen).

Genetic distances of the cyt-b were calculated using MEGA version 4 (Tamura et al. 2007).

Results

Habitat and associated freshwater molluscs

S. montenegrinus occurs in pools on the northern banks of Maritza River in Plovdiv city fed by high waters of the main river and a small tributary coming from north. The substrate is sand and mud, water is running very slow or standing depending on the water levels of the river Maritza. Bank vegetation is dominated by *Salix* spp., *Typha* spp., *Phragmites australis* (Cavanilles, 1799) Trinius ex Steudel, 1840, and water plants by *Ceratophyllum demersum* Linnaeus, 1753 and *Elodea canadensis* Michaux, 1803. Other molluscs found in the locality are: *Unio pictorum* (Linnaeus, 1758), *Planorbis planorbis* (Linnaeus, 1758), *Physella acuta* (Draparnaud, 1805), *Radix auricularia* (Linnaeus, 1758), *Valvata piscinalis* (O. F. Müller, 1774), *Lymnaea stagnalis* (Linnaeus, 1758), *Planorbarius corneus* (Linnaeus, 1758), *Galba truncatula* (O. F. Müller, 1774) and *Anisus vortex* (Linnaeus, 1758).

Molecular genetics

Distance analyses. Genetic distances (p-distance) from pair-wise comparisons of cyt-b sequences (fragment of 329 bp) are shown in Table 2. Distances between species of different families (Planorbidae, Physidae and Lymnaeidae) (outgroup comparison) ranged between 31.2% and 25.5%. Differences between different genera within the family Lymnaeidae ranged from 26.4% to 15.1%. Among the four *Stagnicola* species analysed, the values are between 19.4% and 14.7%. The highest difference between *S. montenegrinus* and the other three *Stagnicola* species is the difference to *S. palustris* (19.1%). The difference to *S. corvus* and *S. fuscus* are 17.5% and 14.7% respectively. The values of the three analysed *Radix* species ranged between 17.5% and 13.0%.

Molecular phylogeny. The maximum-parsimony (MP) tree of the cyt-b sequences is illustrated in Fig. 2 (tree length = 532, consistency index = 0.5602, retention index = 0.8769).

It shows low or very low support on basal branches. *Omphiscola glabra* is paraphyletic with respect to the other genera of Lymnaeidae analysed in this paper. *Galba truncatula* is paraphyletic with respect to *Radix auricularia*, *R. labiata*, and *R. balthica*. In contrast the clades of the species have full bootstrap support. Within analysed representatives of the genus *Stagnicola*, *S. montenegrinus* groups sister to *S. fuscus* with low support (66%) whereas *S. palustris* groups sister to *S. corvus* (bootstrap support 71%).

The RAxML tree of the cyt-b sequences (not shown) shows similar results. The basal branches have low or very low support. This poor support is underlined by two polytomies: one between the

Table 2. Evolutionary distances (p-distance) of the cyt-b gene fragment (329 bp) calculated using MEGA version 4 (Tamura et al. 2007).

	P. corneus	*A. hypnorum*	*G. truncatula*	*O. glabra*	*S. corvus*	*S. montenegrinus*	*S. fuscus*	*S. palustris*	*L. stagnalis*	*R. labiata*	*R. balthiac*	*R. auricularia*
Planorbarius corneus	–	–	–	–	–	–	–	–	–	–	–	–
Aplexa hypnorum	0.312	–	–	–	–	–	–	–	–	–	–	–
Galba truncatula	0.294	0.263	–	–	–	–	–	–	–	–	–	–
Omphiscola glabra	0.275	0.255	0.202	–	–	–	–	–	–	–	–	–
Stagnicola corvus	0.306	0.278	0.230	0.214	–	–	–	–	–	–	–	–
Stagnicola montenegrinus	0.308	0.298	0.241	0.237	0.175	–	–	–	–	–	–	–
Stagnicola fuscus	0.305	0.292	0.232	0.214	0.177	0.147	–	–	–	–	–	–
Stagnicola palustris	0.315	0.328	0.259	0.258	0.160	0.191	0.194	–	–	–	–	–
Lymnaea stagnalis	0.284	0.278	0.224	0.218	0.211	0.219	0.187	0.227	–	–	–	–
Radix labiata	0.284	0.259	0.151	0.192	0.224	0.242	0.247	0.264	0.226	–	–	–
Radix balthica	0.308	0.255	0.158	0.207	0.237	0.238	0.216	0.261	0.220	0.130	–	–
Radix auricularia	0.298	0.259	0.161	0.209	0.230	0.263	0.240	0.273	0.249	0.175	0.149	–

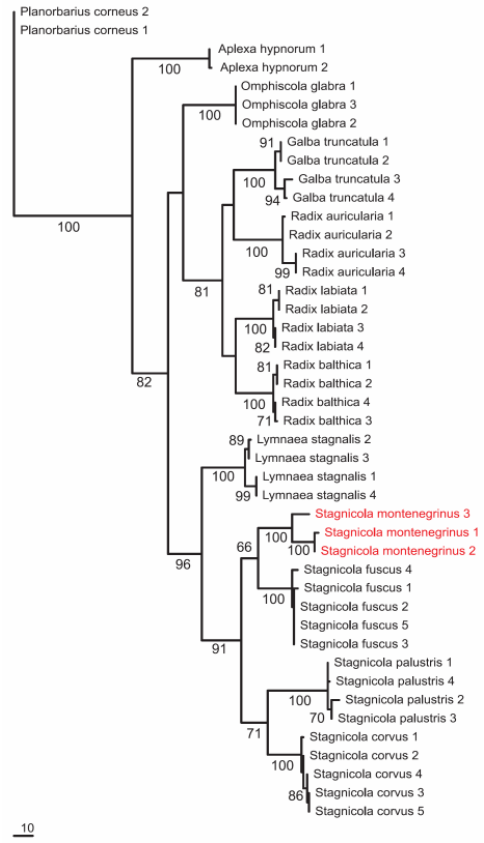

Figure 2. Hypothesis of the phylogenetic relationships of *S. montenegrinus* based on one of the 71 best maximum-parsimony trees of the sequenced fragment of the mitochondrial marker cyt-b (329 bp; tree length = 532, consistency index = 0.5602, retention index = 0.8769). Branch lengths are proportional to the number of substitutions and the overall topology corresponds to that of the strict consensus tree. Bootstrap support values above 50% are reported below nodes.

Figure 3. Hypothesis of the phylogenetic relationships of *S. montenegrinus* based on one of the 9 best maximum-parsimony trees of the nuclear marker ITS-2 (tree length = 1767, consistency index = 0.8087, retention index = 0.9650). Branch lengths are proportional to the number of substitutions and the overall topology corresponds to that of the strict consensus tree. Bootstrap support values above 50% are reported below nodes.

Radix-Galba cluster and the other genera of Lymnaeidae analysed, and the other within the *Radix-Galba* group. *S. montenegrinus* groups sister to *S. fuscus* with low support (67%) too. *S. palustris* is the sister group to *S. corvus* (71%). The clades of all species have high bootstrap support.

The maximum-parsimony (MP) tree of the nuclear marker ITS-2 (tree length = 1767, consistency index = 0.8087, retention index = 0.9650) (Fig. 3) is well-supported within the Lymnaeidae. Most of the basal branches have full bootstrap support. The tree shows the analysed representatives of the genus *Radix* as sister group to the other studied genera of Lymnaeidae. Within the latter *G. truncatula* groups sister to *O. glabra*, *L. stagnalis* and the *Stagnicola* species analysed. *S. palustris* groups sister to *S. fuscus*, *S. corvus* and *S. montenegrinus*. On the other hand, although not well supported (59%), *S. fuscus* groups as the sister group to a cluster consisting of *S. corvus* and *S. montenegrinus* that has full bootstrap support.

The RAxML tree of the nuclear marker ITS-2 (not shown) shows *G. truncatula* as sister group to the other Lymnaeidae analysed. The three *Radix*

species group sister to the genera *Stagnicola*, *Omphiscola*, and *Lymnaea*. In contrast to the MP tree there is no resolution within the genera *Stagnicola*, *Omphiscola*, and *Lymnaea*. *S. montenegrinus* forms a cluster together with *S. corvus* with bootstrap support of 95%. *S. palustris* groups sister to *S. fuscus* with bootstrap support of 66%.

Morphology

Two of the three specimens from the Maritza River floodplain have higher and wider shells (Table 3) than the specimens from Skadar Lake (shell height 10.6-21.5 mm, shell width 4.4-9 mm) (Glöer & Pešić 2009). The aperture is also a little higher than the spire, as defined as a differential characteristic between *S. montenegrinus* and *S. corvus* (Glöer & Pešić 2009). The mantle pigmentation is bluish black and lacks white spots as shown in Glöer & Pešić (2009, p. 55, Fig.2) for *S. montenegrinus* from Skadar Lake. The anatomy is very similar to that of the specimens from Montenegro. The prostate contains 3 folds and the bursa duct is also thickened at the distal end by entering the vagina (Fig. 4).

Table 3. Shell measurements of *S. montenegrinus* from Maritza River in Plovdiv city.

	SNSD Moll S2313	SNSD Moll S2314	SNSD Moll S2315
Shell height	26.7 mm	25.0 mm	21.0 mm
Shell width	10.3 mm	10.3 mm	9.6 mm
Aperture height	14.3 mm	14.1 mm	11.9 mm

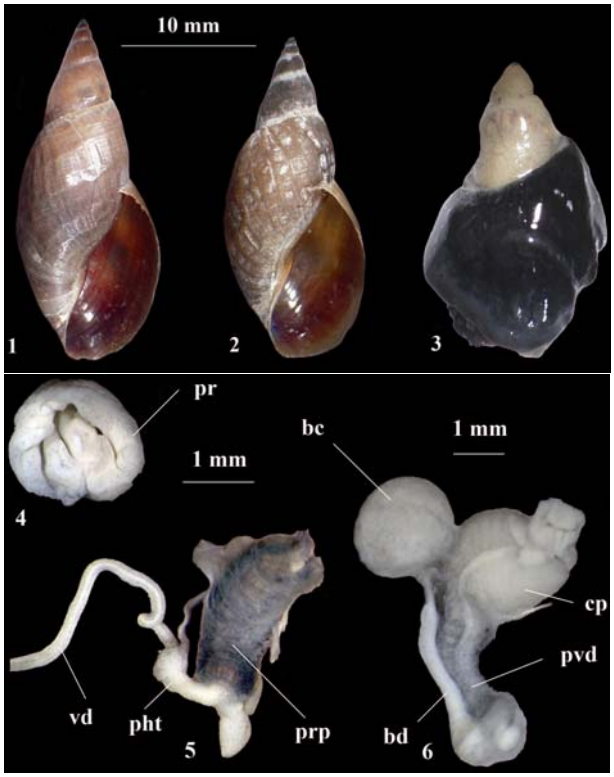

Figure 4. *Stagnicola montenegrinus* from Maritza River floodplain (Bulgaria). – **1**: shell of SNSD Moll S2313; **2**: shell of SNSD Moll S2314; **3**: mantle pigmentation of SNSD Moll S2315; **4**: cross-section through prostate gland SNSD Moll S2313; **5**: male copulatory organ SNSD Moll S2313; **6**: female sex tract. **bc** = bursa copulatrix, **bd** = bursa duct, **cp** = corpus pyriforme, **pht** = phallotheca, **pr** = prostate, = **prp** = praeputium, **pvd** = provaginal duct, **vd** = vas deferens.

Discussion

The molecular distances of 17.5% between *S. montenegrinus* and *S. corvus*, 14.7% between *S. montenegrinus* and *S. fuscus*, and 19.1% between *S. montenegrinus* and *S. palustris* in the cyt-b fragment (about 329 bp) (Table 2) show similar values like the distances between *S. palustris* and *S. fuscus* as well as *S. corvus* and *S. fuscus* (19.4% and 17.7% respectively). The lowest value of 14.7% between *S. montenegrinus* and *S. fuscus* is even higher than the distance between the *Radix* species *R. balthica* and *R. labiata* (13.5%) as well as the distances between *R. balthica* and *R. lagotis* (Schrank, 1803) (9.0%), between *R. ampla* (Hartmann, 1821) and *R. lagotis* (9.2%) (Schniebs et al. 2011) or between *Aenigmomphiscola europaea* Kruglov and Starobogatov, 1981 and *Ae. kazakhstanica* Kruglov and Starobogatov, 1981 (9.0%) (Vinarski et al. 2011). Similar to other authors (e.g. Abramson 2009), we interpret the existing differences between the values of the molecular distances between species clearly distinguishable by morphological characteristics, as a consequence of different evolutionary speeds for the cyt-b gene.

The results of our molecular genetic analyses of the nuclear marker ITS-2 and the mitochondrial marker, the cyt-b fragment (329 bp) (Figs 2,3), are inconsistent and not congruent like in studies of Unionidae using the nuclear ribosomal internal transcribed spacer region in comparison to the mitochondrial genes 16S and COI (Källersjö et al. 2005). Whereas there is a significant difference in gene distances in the cyt-b fragment (about 329 bp) between all *Stagnicola* species analysed (Table 2) and *S. montenegrinus* groups sister to *S. fuscus* and *S. corvus* groups sister to *S. palustris* using this gene (both with only medium support of 66% and 71% respectively), the nuclear marker ITS-2 shows no sequence differences between the three specimens of *S. corvus* from Germany (collection No. SNSD Moll 49821, 49872 and 49872) and the specimens of *S. montenegrinus* from Skadar Lake and Bulgaria (Fig. 3). The result is the same if sequences of *S. turricula* from GenBank as well as own sequences from other Palaearctic *Stagnicola* species are included in the calculation (unpublished data).

Although there is no difference between *S. corvus* and *S. montenegrinus* in nuclear marker ITS-2 sequences we think that the parsimony tree (Fig. 3) reflects the most realistic relationships between the genera *Omphiscola*, *Lymnaea*, and *Stagnicola*, because own studies of the 18S rRNA gene (Vinarski et al. 2011) and analyses of Bargues & Mas-Coma (1997) as well as studies based on 16S, ITS-1 and ITS-2 spacers of other authors (Correa et al. 2010) have shown that these three genera are very closely related. Of the *Stagnicola* species analysed from Bulgaria, *S. palustris* is the only one with one prostate fold and it groups sister to the species with two and more folds in the ITS-2 parsimony tree. *S. fuscus* with two prostate folds groups sister to *S. montenegrinus* and *S. corvus* with three and more folds. So the ITS-2 tree (Fig. 3) appears also to reflect meaningful relations between the analysed species of *Stagnicola*. Anyway the clusters of the ITS-2 (Fig. 3) appear to reflect the subdivision into genera we used following the current European checklists (Falkner et al. 2001, Bank 2011).

We thus have more confidence in this phylogenetic hypothesis based on the nuclear marker, than in the one based on the mitochondrial marker cyt-b.

From anatomical differences important for differentiation of European *Stagnicola* species, as number of prostate folds and diameter of the bursa duct at the distal part, as well as from our molecular genetic analyses we conclude that *S. montenegrinus* is a species closely related to *S. corvus*. Its occurrence is not limited to the Skadar Lake region and needs further investigation.

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