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A barcode pitfall in Palaearctic *Stagnicola* specimens (Mollusca: Lymnaeidae): Incongruence of mitochondrial genes, a nuclear marker and morphology

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Abstract. Molecular genetic analyses of the nuclear ITS-2 spacer and the two mitochondrial markers COI and a cyt-b fragment (329 bp) of the Palaearctic freshwater snail species *Stagnicola corvus* (Gmelin, 1791), *S. montenegrinus* Glöer & Pešić 2009, *S. fuscus* (C. Pfeiffer, 1821), and *S. palustris* (O. F. Müller, 1774) are inconsistent, revealing a high percentage of hybrid specimens. The results lead to different conclusions depending on the phylogenetic relationships of the species analysed. Determined as *S. montenegrinus*, these specimens can now be interpreted as hybrids between *S. corvus* and another *Stagnicola* species, possibly *S. palustris* or *S. fuscus*. Hybrids between *S. fuscus* (role of females, i.e. inheriting mitochondria) and *S. palustris* (role of males) can only be distinguished from "genuine" *S. palustris* by the mitochondrial markers COI and cyt-b, but not by morphology. The study shows that the mitochondrial COI and cyt-b genes are not nearly as suitable for phylogenetic analyses within *Stagnicola* as is the nuclear marker ITS-2.

Key words: molecular genetics, morphology, *Stagnicola*, hybridization.

Introduction

The mitochondrial markers cytochrome c oxidase subunit I (COI) and to a lesser extent cytochrome b (cyt-b) have been frequently used for species delimitation in the case of molluscs that are difficult to distinguish by morphological methods (e.g. de Aranzamendi et al. 2009, von Proschwitz et al. 2009; Köhler & Johnson 2012, Weigand et al. 2012), in studies on phylogentic relationships (e.g. Wethington & Lydeard 2007, Remigio & Hebert 2003, Plazzi et al. 2011), or for analyses of the spatial pattern of intraspecific mitochondrial diversity (e.g. Fehér et al. 2013, Pfenninger et al. 2014), and climate niche modelling (e.g. Cordellier & Pfenninger 2009, Patel et al. 2014).

However, recent studies pointed out discordant patterns between mtDNA and nuclear markers (e.g. Toews & Brelsford 2012) and the unreliability of mitochondrial genes in phylogenetic studies or for species discovery (e.g. Dasmahapatra et al. 2010, Sauer & Hausdorf 2012, Schniebs et al. 2012), such conclusions being often based on their characteristic to be more likely to introgress than nuclear DNA (Ballard & Whitlock 2004). It has been known since over 10 years that mtDNA alone is not enough to resolve many of the questions asked of it, including species delineation and finding phylogeographic patterns (Ballard & Whitlock 2004).

During molecular studies of Palaearctic species of the freshwater family Lymnaeidae, the authors are increasingly confronted with incongruent results of mtDNA (COI, cyt-b) and nuclear markers (ITS1, ITS2) (Schniebs et al. 2011, 2012, Vinarski et al. in prep.). When reexamining the determination based on shell and genital characters of the first record of *Stagnicola montenegrinus* in Bulgaria using molecular techniques (Schniebs et al. 2012), the authors established the inconsistency of the results of genetic analyses of ITS-2 and cyt-b for the specimens analysed. *S. montenegrinus* was characterised as a species closely related to *S. corvus*. New genetic analyses of another specimen of *S. montenegrinus* and specimens of *S. palustris* allow a new interpretation.

Material and methods

We used samples of four Palaearctic species of the genus *Stagnicola*: *S. corvus*, *S. montenegrinus*, *S. fuscus*, and *S. palustris*.

All specimens used for molecular and morphological examination in this study are listed in Table 1., and are stored in the Molluscan collection of the Senckenberg Natural History Collections Dresden, Museum of Zoology (SNSD).

For outgroup comparison, in the molecular genetic analyses we used specimens of the freshwater snail

Table 1. Material used for the molecular genetic and morphological analyses. ENA=European Nucleotide Archive.

Code	Collection No. SNSD	Locality	ENA No.		
			COI	cyt-b	ITS-2
Aplexa hypnorum (Linnaeus, 1758)					
Aplexa hypnorum 1	Moll S348	Germany, Mecklenburg-Vorpommern, lake Nebel, N 53°15'32" E 12°42'02"	FR797859	FR797882	FR797832
Aplexa hypnorum 2	Moll S350	Germany, Mecklenburg-Vorpommern, lake Nebel, N 53°15'32" E 12°42'02"	FR797860	FR797883	FR797833
Galba truncatula (O. F. Müller, 1774)					
Galba truncatula 1	Moll 52544	Germany, Saxony, Oelsnitz/Erzgebirge, former pond, N 50°43'02" E 12°42'04"	FR797875	–	–
Galba truncatula 2	Moll 52545	Germany, Saxony, Oelsnitz/Erzgebirge, former pond, N 50°43'02" E 12°42'04"	–	FR797892	FR797847
Galba truncatula 3	Moll 52546	Germany, Saxony, Oelsnitz/Erzgebirge, former pond, N 50°43'02" E 12°42'04"	HG932215	FR797893	FR797848
Galba truncatula 4	Moll S1130	Bulgaria, Osogovo Mountains, Smolichane Village, karst spring, N 42°07'58.1" E 22°48'25.2"	FR797873	FR797890	FR797845
Galba truncatula 5	Moll S1131	Bulgaria, Osogovo Mountains, Smolichane Village, karst spring, N 42°07'58.1" E 22°48'25.2"	–	FR797891	FR797846
Galba truncatula 6	Moll S302	Iran: Fars Provinz, Maregoon waterfall, N 30°29′30“ E 51°53′40“	HG932216	–	–
Lymnaea stagnalis (Linnaeus, 1758)					
Lymnaea stagnalis 1	Moll 53108	Germany, Baden-Württemberg, Konstanz- Egg, ditch Hockgraben, N 47°40'57.3" E 9°11'34.2"	FR797865	FR797894	FR797834
Lymnaea stagnalis 2	Moll 53109	Germany, Baden-Württemberg, Konstanz- Egg, ditch Hockgraben, N 47°40'57.3" E 9°11'34.2"	FR797866	FR797895	FR797835
Lymnaea stagnalis 3	Moll S1321	Germany: Mecklenburg-Western Pomerania, Lake Plauer See. N 53°25′54“ E 12°18′34“	HG932253	HG932144	HG931960
Lymnaea stagnalis 4	Moll S2311	Bulgaria: Plovdiv, floodplain of the Mariza River, N 42° 09' 13.5'' E 24° 43' 34.8''	HG932255	HG932147	HG931965
Lymnaea taurica (Clessin, 1880)					
Lymnaea taurica 1	Moll S1678	Russia, Omsk Region, Isilkul District, a small nonpermanent steppe pool, N 54°54.7288′ E 71°10.8252′	HG932240	HE613165	HE613316
Lymnaea taurica 2	Moll S2922	Russia, Altai Region, pond in the floodplain of the Kulunda River near Shimolino, N 52°59.506' E 80°59.506'	HG932241	HE613171	HE613318
Lymnaea taurica 3	Moll S3245	Ukraine, Odessa Region, floodplain of the Kogilnik River near Tatarbunar, N 45°49′32.3" E 29°37′43.8"	HG932242	HE613173	HE613320
Stagnicola montenegrinus Glöer & Pešić 2009					
Stagnicola montenegrinus 1	Moll 51854	Montenergo: Skutari See, Vranjina, N 42°16'37.37" E 19°07'32.52"	–	HE577664	HE577643
Stagnicola montenegrinus 2	Moll 51855	Montenergo: Skutari See, Vranjina, N 42°16'37.37" E 19°07'32.52"	LN515536	HE577665	HE577644
Stagnicola montenegrinus 3	Moll S2313	Bulgaria: Plovdiv, floodplain of the Mariza River, N 42° 09' 13.5'' E 24° 43' 34.8''	LN515537	HE577666	HE577645
Stagnicola montenegrinus 4	Moll S2314	Bulgaria: Plovdiv, floodplain of the Mariza River, N 42° 09' 13.5'' E 24° 43' 34.8''	–	LN515578	LN515550
Stagnicola corvus (Gmelin, 1791)					
Stagnicola corvus 1	Moll 49821	Germany, Saxony, Niederspree, pond Großer Tiefzug, N 51°24'20" E 14°53'38"	HG932236	HE577659	HE577638
Stagnicola corvus 2	Moll 49872	Germany, Saxony, pond Vierteich near Freitelsdorf, N 51°15'43" E 13°41'57"	LN515538	HE577660	HE577639
Stagnicola corvus 3	Moll 52682	Germany, Baden- Württemberg, lake Illmensee, N 47 51'43.07" E 9 22'40.60"	–	LN515579	LN515551
Stagnicola corvus 4	Moll 52683	Germany, Baden- Württemberg, lake Illmensee, N 47 51'43.07" E 9 22'40.60"	–	LN515580	LN515552
Stagnicola corvus 5	Moll 52830	Germany, Saxony, Grethen, ditch on the west side of the pond Kleiner Kirchenteich, N 51°14'29" E 12°39'22"	HG932237	HE577661	HE577640
Stagnicola corvus 6	Moll 52831	Germany, Saxony, Grethen, ditch on the west side of the pond Kleiner Kirchenteich, N 51°14'29" E 12°39'22"	HG932238	HE577662	HE577641
Stagnicola corvus 7	Moll S2147	Germany, Mecklenburg- Western Pomerania, lake Koelpinsee, N 53° 30.833' E 12° 36.7'	–	LN515581	LN515553
Stagnicola corvus 8	Moll S2304	Germany, Saxony, ditch in deciduous wood near Papitz, N 51°23'02" E 12°14'29"	–	LN515582	LN515554
Stagnicola corvus 9	Moll S3596	Germany, Saxony, pond near Deuben, N 51°21'50" E 12°41′05"	–	LN515583	LN515555

Continued on the next page

Table 1. (continued)

Stagnicola fuscus (C. Pfeiffer, 1821)					
Stagnicola fuscus 1	Moll 48550	Germany, Saxony, reservoir Lobstädt, north bank, N 51°07'58" E 12°27'27"	LN515539	HE577654	HE577633
Stagnicola fuscus 2	Moll 51794	Germany, Saxony, marsh wood near Raden, N 51°22'23" E 13°29'57"	–	HE577655	HE577634
Stagnicola fuscus 3	Moll 53076	Germany, Bavaria, Weichering, pond in riverside forest N 48°43’34.1”, E 11°19’23.6”	HG932234	HG932133	HG931948
Stagnicola fuscus 4	Moll S2082	Germany, Saxony, nature reserve Alte See Grethen, marsh wood, N 51°13'42" E 12°40'18"	–	HE577656	HE577635
Stagnicola fuscus 5	Moll S2199	Germany, Baden- Württemberg, nature reserve Erlich, marsh wood, R 3462394 H 5449072	–	HE577657	HE577636
Stagnicola fuscus 6	Moll S2946	Germany, Thuringia, alder marsh near Appenrode, N 51°34'27" E 10°43'07"	LN515540	HE577658	HE577637
Stagnicola palustris (O. F. Müller, 1774)					
Stagnicola palustris 1	Moll 52882	Russia, Tjumen Region, Polar Urals, mountain range Ra-Iz, pond at 141th kilometer of railway Seida-Labytnangi	–	LN515584	LN515556
Stagnicola palustris 2	Moll 53095	Germany, Baden- Württemberg, lake Bodensee, peninsula Mettnau, north side, N 47°43'52" E 09°00'04"	FR797871	HE577651	HE577631
Stagnicola palustris 3	Moll 53096	Germany, Baden- Württemberg, lake Bodensee, peninsula Mettnau, north side, N 47°43'52" E 09°00'04"	FR797872	HE577652	HE577632
Stagnicola palustris 4	Moll S375	Germany, Saxony, Dresden, pond Zschoner Mühlteich, R 5404540 H 5659042	LN515541	LN515585	LN515557
Stagnicola palustris 5	Moll S381	Sweden, Södermanlands Län, Askö Island, near Biostation in 0.2 m depth, 17.631°E 58.827°N	–	FR797900	LN515558
Stagnicola palustris 6	Moll S1345	Mecklenburg-Western Pomerania, lake Grosser Plaetschsee, south bank, N 53°26'25" E 12°19'18"	LN515542	HE577653	FR797838
Stagnicola palustris 7	Moll S2148	Mecklenburg-Western Pomerania, lake Koelpinsee, N 53° 30.833' E 12° 36.7'	–	LN515586	LN515559
Stagnicola palustris 8	Moll S2149	Mecklenburg-Western Pomerania, lake Koelpinsee, N 53° 30.833' E 12° 36.7'	LN515543	LN515587	LN515560
Stagnicola palustris 9	Moll S2284	Germany, Saxony, former clay-pits near Papitz, R 4517551 H 5693965	–	LN515588	LN515561
Stagnicola palustris 10	Moll S2299	Germany, Saxony, former clay-pits near Papitz, R 4517551 H 5693965	–	LN515589	LN515562
Stagnicola palustris 11	Moll S2550	Germany, Saxony, pond Dammühlenteich near Schönfeld, R 5408973 H 5683830	–	LN515590	LN515563
Stagnicola palustris 12	Moll S2554	Germany, Saxony, pond Dammühlenteich near Schönfeld, R 5408973 H 5683830	–	LN515591	LN515564
Stagnicola palustris 13	Moll S2555	Germany, Saxony, pond Dammühlenteich near Schönfeld, R 5408973 H 5683830	–	LN515592	LN515565
Stagnicola palustris 14	Moll S3494	Germany, Saxony, old ox- bow of river Röder between Rödern and Oberrödern, R 5409383 H 5678432	–	LN515593	LN515566
Stagnicola palustris 15	Moll S3495	Germany, Saxony, old ox- bow of river Röder between Rödern and Oberrödern, R 5409383 H 5678432	LN515544	LN515594	LN515567
Stagnicola palustris 16	Moll S3496	Germany, Saxony, old ox- bow of river Röder between Rödern and Oberrödern, R 5409383 H 5678432	–	LN515595	LN515568
Stagnicola palustris 17	Moll S3498	Germany, Saxony, old ox- bow of river Röder between Rödern and Oberrödern, R 5409383 H 5678432	–	LN515596	LN515569
Stagnicola palustris 18	Moll S3500	Germany, Saxony, old ox- bow of river Röder between Rödern and Oberrödern, R 5409383 H 5678432	LN515545	LN515597	LN515570
Stagnicola palustris 19	Moll S3717	Germany, Saxony, Dresden, small pond, N 51°00'22.49" O 13°42'24.51"	LN515546	LN515598	LN515571
Stagnicola palustris 20	Moll S5141	Russia, Tjumen Region, Jamal peninsula, River Shchuchya, N 67°23' E 67°36'	LN515547	LN515599	LN515572
Stagnicola palustris 21	Moll S6910	Russia, Republic of Karelia, Tiksheozero Lake near Chupa settlement, bay № 1, N 66˚16.5 E 33˚ 01.17	–	LN515600	LN515573
Stagnicola palustris 22	Moll S6911	Russia, Republic of Karelia, Tiksheozero Lake near Chupa settlement, bay № 1, N 66˚16.5 E 33˚ 01.17	–	LN515601	LN515574
Stagnicola palustris 23	Moll S6912	Russia, Republic of Karelia, Tiksheozero Lake near Chupa settlement, bay № 1, N 66˚16.5 E 33˚ 01.17	–	LN515602	LN515575
Stagnicola palustris 24	Moll S6916	Russia, Moscow City, a pond in Nekrasovka District, N 55°41'32.52" E 37°57'0.68"	LN515548	LN515603	LN515576
Stagnicola palustris 25	Moll S6917	Russia, Moscow City, a pond in Nekrasovka District, N 55°41'32.52" E 37°57'0.68"	LN515549	LN515604	LN515577

species *Aplexa hypnorum* (Linnaeus, 1758, family Physidae), including also sequences of the lymnaeid mollusc species *Galba truncatula* (O. F. Müller, 1774), *Lymnaea stagnalis* (Linnaeus, 1758), and *L. taurica* (Clessin, 1880), for comparison.

Morphology

The snails were fixed in 70-80% ethanol. The shell morphology and anatomy of the specimens studied were recorded. The dissections and measurements of the genital organs and shells were carried out using stereo microscopes (Zeiss and Olympus). The photographs were taken with digital camera systems (Leica R8 and Nikon DS-Fi2).

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

Molecular techniques

Tissue samples taken from the foot were fixed in 100% ethanol. They were registered in the tissue collection of the SNSD with a new collection number as well as the collection number of the specimen in the mollusc collection of SNSD, and stored at –80°C.

For molecular genetic analyses we obtained sequence data of the nuclear ITS-2 spacer (280 bp in *A. hypnorum*, up to 498 bp in *L. taurica*), as well as a 329 bp fragment of the cyt-b gene and a 626 bp fragment of the COI gene as mitochondrial markers.

For primers and protocols of DNA extraction, Polymerase Chain Reaction (PCR), purification of PCR products and DNA sequencing see Vinarski et al. (2011).

Sequence alignments. Alignments were assembled using the sequence alignment editor BioEdit (Hall, 1999). The ITS-2 alignment was obtained using the Clustal algorithm of MEGA version 4 (Tamura et al. 2007) and improved by eye. MEGA 4 was also used to check the mitochondrial sequences for stop codons.

Phylogenetic analyses of sequences. For maximum-likelihood analyses, including bootstrap support, we used RAxML (raxmlGUI 0.9 beta 2, Silvestro & Michalak 2010, Stamatakis et al. 2005). The settings were "ML+thorough bootstrap" with 100 (replicate) runs and 1000 (bootstrap) repetitions. Phylogenetic analyses under the maximum parsimony (MP) criterion for ITS-2 spacer and cyt-b fragment were carried out using equal weighting (EW) implemented with TNT version 1.1 (Willi Hennig Society Edition, August 2011, Goloboff et al. 2008). The maximum number of trees held was set to 10,000. Tree searches were conducted using all four "New Technology" search options: sectorial searches, ratchet, tree drifting and tree fusing. For the ratchet, the number of replicates was set to 200, and the number of cycles of tree drifting to 50. All other search parameters were left at their default settings. Analyses were terminated once the most parsimonious cladogram(s) (MPCs) had been found 1000 times. The maximum-parsimony (MP) tree of COI was reconstructed using PAUP (version 4.0b10, Swofford 2002; settings: gapmode = NewState, addseq = closest, the maximal number of trees with the initial default setting of maxtree = 100 did not have to be increased, since the number of best trees remained below 100; number of bootstrap replicates = 10000). For presentation of the MP results for COI one of the 14 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).

All DNA-sequences have been placed in the European Nucleotide Archive (ENA, see http://www.ebi.ac.uk/ena/).

Results

Morphology

Shell measurements of the *Stagnicola* specimens analysed with preserved first whorls and aperture are given in Table 2.

The shell height of the *S. corvus* specimens (n=7) ranged between 13.5 and 23.6 mm, the shell width between 6.7 and 10.1 mm, the aperture height between 4.45 and 12.7 mm, and the average number of whorls varied between 4.25 and 6.0. In *S. montenegrinus* specimens (n=4) the shell height varied in the range of 15.9–26.7 mm, the shell width in the range 7.4–10.3 mm, the aperture height in the range 10.0–14.3 mm, and the average number of whorls varied between 5.0 and 6.25. The height of the shells of the three *S. fuscus* specimens with preserved first whorls and aperture ranged between 10.8 and 15.3 mm, the width between 5.1 and 8.1 mm, the aperture height between 6.0 and 8.4 mm. On average, all three shells had 5.0 whorls. The shell height of the *S. palustris* specimens with preserved first whorls and aperture (n=21) ranged between 10.25 and 21.5 mm, the width between 5.3 and 8.9 mm, the aperture height between 5.4 and 10.3 mm, and the average number of whorls between 4.5 and 6.0.

The number of prostate folds of most of the 37 *Stagnicola* specimens analysed is given in Table 3. In some cases examination was not possible because specimens were juvenile or organs were damaged while removing the soft body from the shell.

When visibility permitted accurate counting, at least three main folds and in most cases a few additional smaller secondary folds were discerned at the prostate of the analysed *S. corvus* specimens (n=8) (Fig. 1) The four *S. montenegrinus* specimens had at least four main and secondary folds (Fig. 1). The prostate of two *S. fuscus* specimens (specimens 2 and 4) had two folds inside (Fig. 1). For the other two specimens examined, the number of folds on the prostrate was not well visible. The prostate of the majority of *S. palustris* specimens (n=21) had one fold inside (Fig. 1). In specimen

Table 2. Shell measurements of the *Stagnicola* specimens analysed (only specimens with preserved first whorls) and data from literature.

Species	Specimen/Reference	shell height in mm	shell width in mm	aperture height in mm	aperture width in mm	number of whorls
S. corvus	*S. corvus* 2	23.60	10.10	12.70	7.70	4.25
	S. corvus 3	22.00	9.60	10.75	6.40	6.00
	S. corvus 4	13.50	6.70	4.45	4.50	5.00
	S. corvus 5	18.10	8.40	9.35	6.35	5.00
	S. corvus 6	17.70	8.50	9.20	6.35	5.00
	S. corvus 8	18.50	8.60	11.30	6.50	5.00
	S. corvus 9	20.70	8.30	10.30	7.00	6.00
	Steńko-Krodkiewska 1994	10.50 to 35.50	4.50 to 16.20	--	--	--
	Gittenberger et al.1998	up to 45.00	up to 19.50	--	--	up to 8.00
	Jackiewicz 1998	up to 45.00	up to 16.00	--	--	6.00 to 7.00
	Glöer 2002	13.00 to 34.00	6.00 to 16.50	--	--	--
	Stadnichenko 2004	up to 33.00	up to 13.60	--	--	6.00 to 8.00
	Kruglov 2005	31.66±0.76	13.08±0.29	15.38±0.26	7.29±0.17	7.00 to 8.00
S. montenegrinus	*S. montenegrinus* 1	15.90	7.40	10.00	5.70	5.00
	S. montenegrinus 2	20.90	9.40	11.70	7.30	6.00
	S. montenegrinus 3	26.70	10.30	14.30	8.30	6.25
	S. montenegrinus 4	25.00	10.30	14.10	8.60	6.00
	Glöer & Pešić 2009	10.60 to 21.50	4.40 to 9.00	--	--	5.50 to 6.00
	Schniebs et al. 2012	21.00 to 26.70	9.60 and 10.30	--	--	--
S. fuscus	*S. fuscus* 1	15.30	7.70	8.20	4.90	5.00
	S. fuscus 3	15.70	8.10	8.40	5.70	5.00
	S. fuscus 5	10.80	5.10	6.00	4.20	5.00
	Jackiewicz 1998	up to 25.00	up to 11.50	--	--	up to 6.00
	Glöer 2002	10.00 to 25.00	5.50 to 11.50	--	--	6.00 to 8.00
	Kruglov 2005	21.00	6.80	10.20	--	6.00
S. palustris	*S. palustris* 1	16.50	8.00	7.60	4.80	6.00
	S. palustris 2	21.50	8.80	10.00	6.50	6.00
	S. palustris 3	16.40	7.90	8.50	6.00	5.00
	S. palustris 4	19.20	8.60	10.30	6.10	6.00
	S. palustris 6	17.50	8.90	9.60	6.85	5.50
	S. palustris 7	12.70	5.90	6.60	3.70	5.00
	S. palustris 8	11.90	5.30	5.60	3.80	6.00
	S. palustris 9	13.80	6.50	6.60	4.40	5.00
	S. palustris 10	13.90	6.30	6.30	4.60	6.00
	S. palustris 11	13.60	6.40	6.50	4.40	5
	S. palustris 12	16.90	8.80	9.20	5.90	5.5
	S. palustris 13	11.70	6.80	6.40	5.00	5
	S. palustris 14	16.30	7.20	8.70	6.10	4.5
	S. palustris 16	19.60	8.10	8.20	6.50	5.5
	S. palustris 17	14.60	6.35	7.50	4.40	5
	S. palustris 19	10.25	5.30	5.40	4.00	4.75
	S. palustris 20	13.40	6.00	6.00	3.60	5.5
	S. palustris 21	11.60	6.35	7.00	5.00	5
	S. palustris 22	11.10	6.10	8.00	4.00	5
	S. palustris 23	12.30	6.20	7.30	4.85	5
	S. palustris 25	12.50	6.25	7.60	4.70	5
	Gittenberger et al. 1998	up to 20.00	up to 8.50	--	--	6.00
	Jackiewicz 1998	up to 30.00	up to 15.00	--	--	6.00 to 7.00
	Glöer 2002	10.00 to 17.50	6.0 to 8.0	--	--	6.00
	Stadnichenko 2004	up to 25.60	up to 11.70	--	--	6.00 to 7.00
	Kruglov 2005	25.00	11.50	11.2	--	6.25
	Khokhutkin et al. 2009	up to 28.00	--	--	--	7.00 to 7.50

Table 3. Measurements of the male genitalia and number of prostate folds of the *Stagnicola* specimens analysed and data from literature.

Species	Specimen/Reference	length of the praeputium in mm	length of the penial sheath in mm	ratio of the length of the praeputium to the length of the penial sheath	number of prostate folds
S. corvus	*S. corvus* 1	3.5	2.0	1.75:1	?
	S. corvus 2	3.0	1.5	2:1	>5
	S. corvus 3	3.5	1.0	3.5:1	8
	S. corvus 4	2.0	1.0	2:1	>3
	S. corvus 5	2.6	1.2	2.16:1	8?
	S. corvus 6	3.5	2.5	1.4:1	6?
	S. corvus 7	3.3	1.0	3.3:1	?
	S. corvus 8	2.3	1.5	1.53:1	9
	Falniowski 1980	–	–	3:1	numerous
	van der Velde & van Kessel 1984	–	–	1.77-3.44	
	Jackiewicz 1988	–	–	–	numerous
	Jackiewicz & Gerber 1990	–	–	–	numerous
	Gittenberger et al. 1998	–	–	2:1	numerous
	Jackiewicz 1998	–	–	3:1	numerous
	Glöer 2002	–	–	3:1	numerous
	Stadnichenko 2004	–	–	3.5:1	numerous
	Kruglov 2005	–	–	3.5:1	5-10
S. montenegrinus	*S. montenegrinus* 1	3.0	0.8	3.75:1	4
	S. montenegrinus 2	3.0	1.2	2.5:1	6?
	S. montenegrinus 3	2.7	1.5	1.8:1	6?
	S. montenegrinus 4	4.0	1.5	2.66:1	5?
	Glöer & Pešić 2009	–	–	3.5:1–4.2:1	3-4
	Soes 2014	–	–	–	3-4
S. fuscus	*S. fuscus* 1	3.5	1.5	2.33:1	?
	S. fuscus 2	2.5	1.0	2.5:1	2
	S. fuscus 3	3.0	1.0	3:1	?
	S. fuscus 4	2.5	1.0	2.5:1	2
	Jackiewicz 1988	–	–	3:1	2
	Jackiewicz & Gerber 1990	–	–	2:1	2
	Gittenberger et al. 1998	–	–	–	2
	Jackiewicz 1998	–	–	3:1	2
	Glöer 2002	–	–	3:1	2
	Kruglov 2005	3.6	5.00	1:1.4	1
S. palustris	*S. palustris* 1	2.8	3.3	1:1.8	1
	S. palustris 2	4.0	4.5	1:1.25	1
	S. palustris 3	2.2	3.5	1:1.59	1
	S. palustris 4	3.5	4.0	1:1.14	1
	S. palustris 5	2.5	3.0	1:1.2	1
	S. palustris 6	2.5	5.0	1:2	1
	S. palustris 9	2.5	3.0	1:1.2	1
	S. palustris 11	3.0	3.5	1:1.16	1
	S. palustris 12	2.5	4.0	1:1.6	1
	S. palustris 14	3.5	4.5	1:1.28	1
	S. palustris 15	4.0	4.0	1:1	1
	S. palustris 16	4.0	4.0	1:1	1
	S. palustris 17	2.5	4.5	1:1.8	1
	S. palustris 18	5.0	4.0	1.25:1	1
	S. palustris 19	2.0	3.0	1:1.5	1
	S. palustris 20	2.7	3.5	1:1.29	1
	S. palustris 21	2.2	2.5	1:1.13	1
	S. palustris 22	2.0	2.5	1:1.25	1
	S. palustris 23	2.0	3.0	1:1.5	?
	S. palustris 24	2.0	3.0	1:1.5	1

Continued on the next page

Table 3. (continued)

Species	*Specimen/Reference*	length of the praeputium in mm	length of the penial sheath in mm	ratio of the length of the praeputium to the length of the penial sheath	number of prostate folds
	S. palustris 25	2.8	4.0	1:1.42	1
	van der Velde & van Kessel 1984	–	–	0.49:1.00	–
	Kruglov & Starobogatov, 1986	–	–	0.70-0.80	1
	Gittenberger et al. 1998	–	–	50-100%	1
	Jackiewicz 1998	–	–	1:1	1
	Glöer 2002	–	–	1:1	1
	Stadnichenko 2004	–	–	1.25-1.40:1.00	1
	Kruglov 2005	4.0	5.0	1:1.25	1
	Andreeva et al. 2010	–		0.60-0.82	–

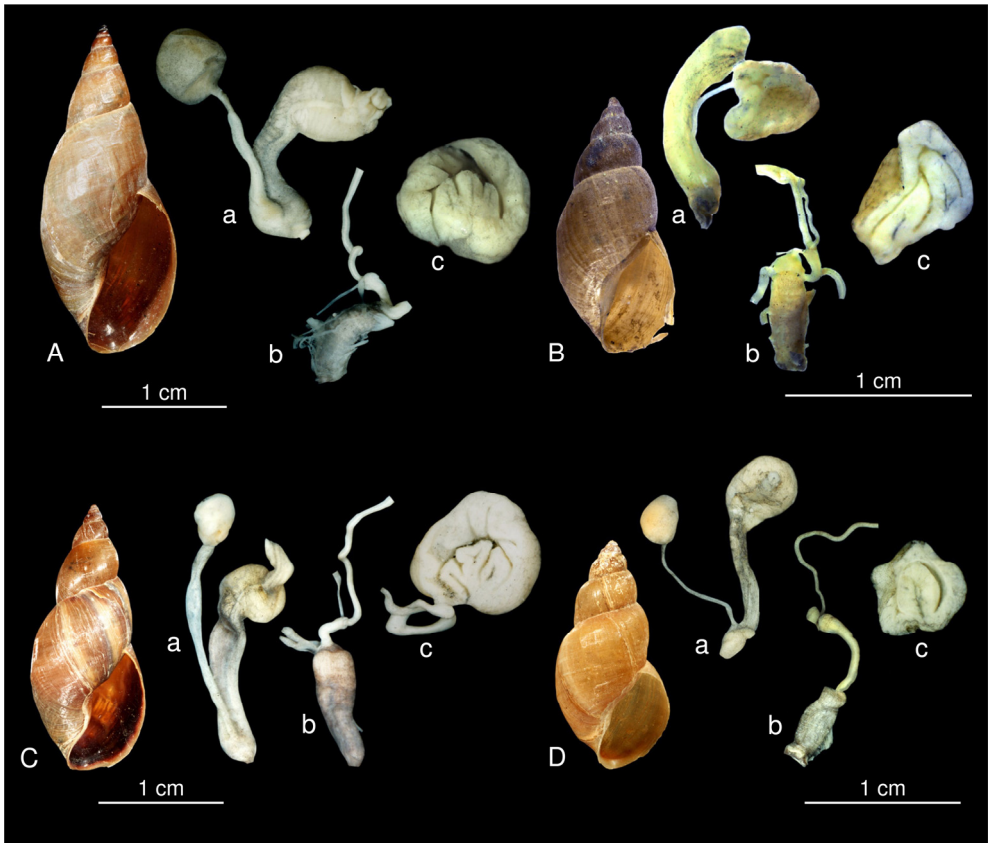

Figure 1. A) *Stagnicola montenegrinus* (Bulgaria: floodplain of the Maritza river in Plovdiv); B) *Stagnicola fuscus* (Germany, Saxony, nature reserve Alte See Grethen, marsh wood); C) *Stagnicola corvus* (Germany, Baden-Württemberg, lake Illmensee); D) *Stagnicola palustris* (Russia, Tjumen Region, Polar Urals, mountain range Ra-Iz, pond at 141th kilometer of railway Seida-Labytnangi). a) bursa copulatrix and bursa duct entering the vagina; b) male genitalia; c) cross section through prostate gland.

no. 23 it was not clearly visible whether the prostate had one or two folds.

The measurements of praeputium and penial sheath of 37 *Stagnicola* specimens are reported in Table 3. The ratio of the length of the praeputium to that of the penial sheath varied between 1.4–3.5 in the analysed *S. corvus* specimens (n=8). For the analysed *S. montenegrinus* specimens (n=4) this

character varied between 1.80–3.75. In the four *S. fuscus* specimens analysed, the ratio of praeputium length to that of the penial sheath varied between 2.33–3.00. In the *S. palustris* specimens 1, 2, 3, 5, 6, and 20 that group as a sister to the *S. corvus* specimens and to one *S. montenegrinus* specimen in the cyt-b trees, the ratio of the praeputium length to that of the penial sheath varied between 0.50 and 0.88, whereas in the *S. palustris* specimens, that group together with the *S. fuscus* specimens (n=15), the ratio varied from 0.55 to 1.25.

The duct of bursa copulatrix is thickened at the distal end by entering the vagina in all specimens of *S. corvus* and *S. montenegrinus*, whereas in the *S. fuscus* and *S. palustris* specimens, the spermathecal duct is narrow and very long (Fig. 1) as reported in literature (Jackiewicz & Gerber 1990, Gittenberger et al. 1998, Jackiewicz 1998, Glöer 2002).

Molecular genetics

Unfortunately, fragments of the COI gene could not be obtained from all specimens analysed. For that reason the COI trees (RAxML and MP) show a different composition of individuals than the trees of the cyt-b gene fragment and the ITS-2 spacer.

The maximum-parsimony (MP) tree of COI (strict consensus tree, SCT, of 14 trees retained; tree length= 415, CI=0.67, RI=0.89; Fig. 2) shows full, high or low support for basal branches. The species-clades received full support in most cases. *S. corvus* specimen group (with full support) as a sister clade to that of only four of the *S. palustris* specimens analysed. The other seven *S. palustris* specimens (marked red) group together with the *S. fuscus* specimens (full bootstrap support). This mixed cluster of *S. palustris* and *S. fuscus* groups as a sister to the specimens of *S. montenegrinus* (marked sky-blue) with low support (85%). The clade formed by *S. montenegrinus* and the mixed cluster of *S. palustris* and *S. fuscus* groups as a sister to the clade formed by *S. corvus* and the other four *S. palustris* specimens with low support (86%).

In the RAxML tree of the COI gene (not shown) the basal branches and species clades have lower support in most cases. The topology of the clades is concurrent to that in the MP SCT of COI (Fig. 2).

In the MP tree of the cyt-b fragment (one best tree; tree length=432, CI=0.6134, RI=0.9053; Fig. 3), the basal branches are highly or very lowly supported. The species-clades themselves show full support with the exception of two clades: The specimens of *S. corvus* analysed group together with one specimen of *S. montenegrinus* (marked sky-blue and from which no COI could be gained) with bootstrap support of 86% and 19 specimens

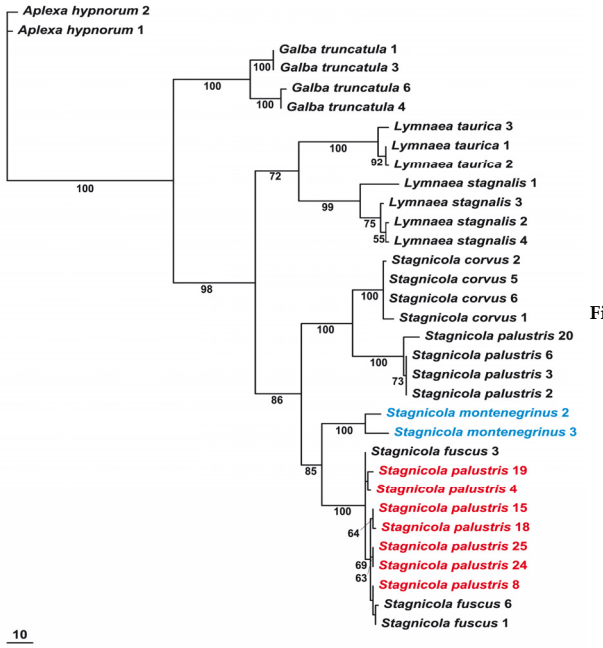

Figure 2. Hypothesis of the phylogenetic relationships of *Stagnicola*: one of the 14 best maximum-parsimony trees of the sequenced fragment of the mitochondrial marker COI (626 bp; tree length= 415, CI=0.67, RI=0.89). Branch lengths are proportional to the number of substitutions and the overall topology corresponds to that of the strict consensus tree. Bootstrap support values above 50% are reported below nodes. Hybrids between *S. palustris* and *S. fuscus* are marked red. Specimens of *S. montenegrinus* are marked blue.

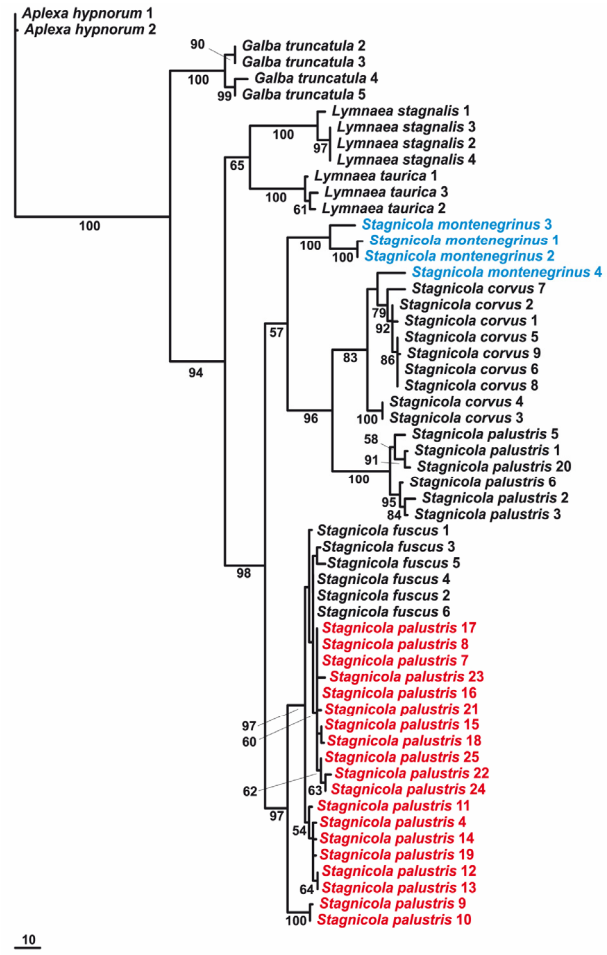

Figure 3. Hypothesis of the phylogenetic relationships of *Stagnicola*: Best maximum-parsimony tree of the fragment sequenced of the mitochondrial marker cyt-b (329 bp). Branch lengths are proportional to the number of substitutions. Bootstrap support values above 50% are reported below nodes. Hybrids between *S. palustris* and *S. fuscus* are marked red. Specimens of *S. montenegrinus* are marked blue.

of *S. palustris* (marked red) group together with the six specimens of *S. fuscus*, with high support (97%).

Six specimens of *S. palustris* group as a sister to the mixed cluster of *S. corvus* and *S. montenegrinus*. Together they group as a sister with very low support (57%) to the other three specimens of *S. montenegrinus*.

The RAxML tree of the cyt-b fragment (not shown) is very similar in topology to the MP tree but the basal branches and species clades have lower support in most cases.

The maximum-parsimony (MP) tree of ITS-2 spacer (SCT of four best trees; CI=0.86, RI=0.96; not shown) shows high or fully supported basal branches (between 91% and 100% bootstrap support). The species-clades are fully supported with the exception of the clade of *S. palustris* (low support of 67%). There are two main differences to the mitochondrial trees:

1. All four specimens of *S. montenegrinus* group together with the specimens of *S. corvus* with full bootstrap support. 2. The specimens of *S. palustris* do not fall into two separate clusters. The specimens of *S. fuscus* group as sister to all specimens of *S. palustris*. Together they group as sister to the clade of *S. corvus*-*S. montenegrinus*.

In the RAxML tree of the ITS-2 spacer (Fig. 4) the basal branches are supported by bootstrap values >80%. The species-clades show full or high support in most cases. The topology of the tree is similar to that of the MP tree.

Discussion

Morphology

The shell measurements of the analysed *Stagnicola*

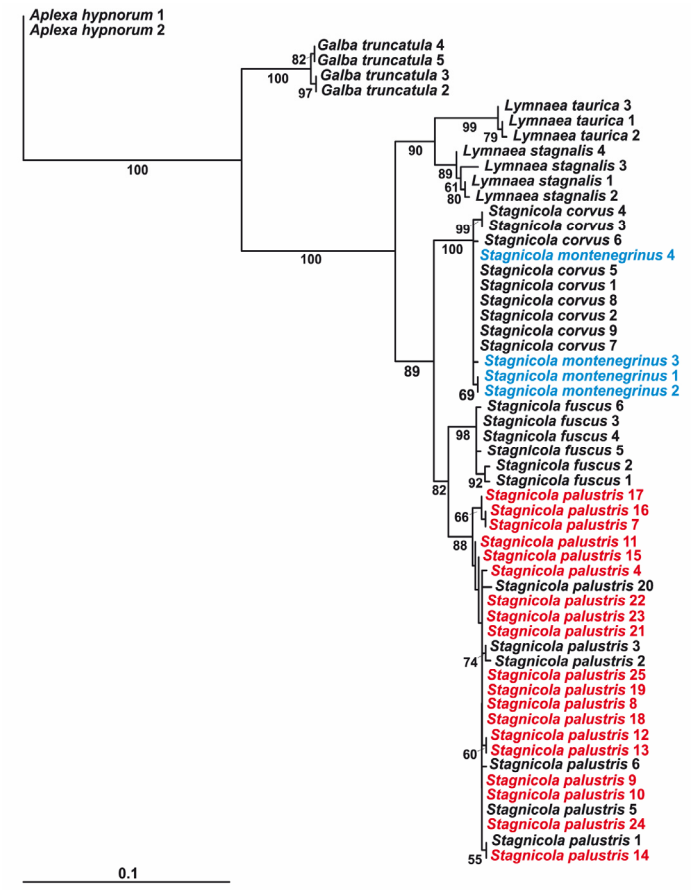

Figure 4. Hypothesis of the phylogenetic relationships of *Stagnicola*: the RAxML tree of the nuclear marker ITS-2 (280 - 498 bp). Branch lengths are proportional to the number of substitutions and the overall topology corresponds to that of the strict consensus tree. Bootstrap support values above 50% are reported below nodes. Hybrids between *S. palustris* and *S. fuscus* are marked red. Specimens of *S. montenegrinus* are marked blue.

specimens correspond to data from literature (Tab. 2). In case of *S. corvus* we can add to the diagnosis given by Glöer & Pešić (2009) that the aperture of the shell could be higher than the spire (specimen 2), just as in *S. montenegrinus*. While large *S. corvus* can reach a height up to 45 mm (Tab. 2), *S. montenegrinus* reaches a height of about 27 mm (Tab. 2). *S. palustris* and *S. fuscus* are smaller. In addition, *S. montenegrinus* has a characteristic spire with flat whorls and suture, as *L. stagnalis* (Fig.1).

The shells of the *Stagnicola* species analysed can vary considerably in form and number of whorls (Tab. 2, Fig.1). Therefore, some anatomical characters, such as the number of prostate folds, the external diameter of the distal end of the duct of the bursa copulatrix, where it enters the vagina, and the ratio of the praeputium length to the penial sheath length are very important in species' determination.

The prostate of the *S. corvus* and *S. montenegrinus* specimens have numerous folds inside, which is consistent with the data from literature (Tab. 3, Fig. 1). *S. corvus* and *S. montenegrinus* are the only known European *Stagnicola* species with more than two prostate folds and a duct of bursa copulatrix thickened at the distal end (Fig. 1) (Kruglov & Starobogatov 1984, van der Velde & van Kessel 1984, Gittenberger et al. 1998, Jackiewicz 1998, Glöer 2002, Kruglov 2005, Glöer & Pešić 2009, Schniebs et al. 2012; Soes 2014). Conversely, *S. fuscus* and *S. palustris* have two or one prostate folds (Tab. 3, Fig. 1) and a narrow and very long spermathecal duct (Jackiewicz & Gerber 1990 [*Lymnaea vulnerata* Küster, 1862], Gittenberger et al. 1998, Jackiewicz, 1998 [*L. vulnerata*], Glöer 2002). Kruglov (2005) mentioned only one fold for *S. fuscus* (Table 3). But *S. fuscus* sensu Kruglov is not identical with *L. vulnerata* sensu Jackiewicz, 1988 (non Jackiewicz, 1962 = *S. turricula*) and *S. fuscus* sensu Glöer, because measurements of the male genitalia given by Kruglov (2005) match those of *S. palustris* (Table 3). This problem of the true taxo-

nomic identity of this species needs further investigation.

In *S. montenegrinus* the praeputium is darker than in *S. corvus* and it is curved at the entry point of the vas deferens. Furthermore, in *S. montenegrinus* its shape is cylindrical, whereas the praeputium of *S. corvus* has a more conical form.

S. corvus, *S. montenegrinus*, and *S. fuscus* are distinguished from *S. palustris* by the fact that the praeputium is considerably longer than the penial sheath (Fig. 1), which is consistent with the data from literature (Tab. 3). Between the three species mentioned first, no differences in this characteristic could be seen in this dataset. In the *S. palustris* specimens 1, 2, 3, 5, 6 and 20 we found however a lower variability in the ratio of the praeputium length to that of the penial sheath (0.50–0.88) than in the *S. palustris* specimens 4, 7–19 and 21–25 that group together with the *S. fuscus* specimens (0.55–1.25). However, this is a relatively small difference that does not allow an anatomical differentiation between "genuine" *S. palustris* and *S. palustris*-like hybrids between *S. fuscus* and *S. palustris* found in our analyses. Besides, the use of this parameter (praeputium : penial sheath ratio) for reliable species delimitation in Lymnaeidae has recently been questioned (Vinarski, 2011, Schniebs et al. 2011).

Molecular phylogeny

The results of our molecular genetic analyses of the nuclear ITS-2 spacer and the two mitochondrial markers COI and cyt-b fragment (329 bp) are inconsistent. The three specimens of *S. montenegrinus* (specimens 1, 2, 3) analysed already in 2012 (Schniebs et al. 2012) show a predictable grouping in the phylogenetic trees: In the nuclear ITS-2 trees they form a cluster together with the *S. corvus* specimens analysed with full bootstrap support (Fig. 4). In the trees of the cyt-b fragment (Fig. 3) and COI (Fig. 2) they form their own cluster with full or nearly full bootstrap support that groups sister to *Stagnicola* specimens of other species. Based on the cyt-b fragment (Fig. 3), the *S. montenegrinus* specimens 1, 2, 3 group together in a fully supported clade (without specimen 4). Its sister group relationship to a mixed cluster consisting of six *S. palustris* specimens and all *S. corvus* specimens plus the *S. montenegrinus* specimen 4 has very low bootstrap support (57), indicating that it should rather be interpreted as a polytomy. This result is in contrast with the phylogenetic trees gained from the *Stagnicola* specimens analysed in 2012 (Schniebs et al. 2012), in which the three *S. montenegrinus* specimens formed a sister group to *S. fuscus*. Based on a smaller number of specimens, the COI tree (Fig. 2) also shows a different result: with medium bootstrap support (85) the two *S. montenegrinus* specimens 2, 3 group as a sister to a mixed cluster of *S. fuscus* plus *S. palustris* specimens. The two mitochondrial genes thus give different information about the phylogenetic relationship of *S. montenegrinus*. Both hypotheses are in contrast with morphology: *S. corvus* and *S. montenegrinus* have more than two prostate folds and the bursa copulatrix duct thickened at the distal end (entering the vagina). They thus have a special position with respect to the other European *Stagnicola* species. In Russian taxonomy the special position of *S. corvus* is expressed by positioning this species into a separate subgenus *Corvusiana* Servain, 1881, whereas *S. palustris* and *S. fuscus* are placed in the subgenus *Stagnicola* Leach, 1830 (e.g. Kruglov 2005). In our phylogenetic trees of nuclear marker ITS-2 (Fig. 4), the special position of *S. corvus* is reflected adequately. In this tree (and the nuclear ITS-2 MP tree, not shown) the *S. corvus* specimens form a monophylum with the *S. montenegrinus* specimens. With strong support (89), this clade groups as a sister to all other *Stagnicola* specimens analysed. This relationship is not found in the mitochondrial trees (see above). Similarly to the conclusions in our earlier study (Schniebs *et al*., 2012), we are thus of the opinion that none of the two mitochondrial trees (cyt-b, COI) reflects the relationships between the analysed *Stagnicola* species. Likely explanations for the incongruence between the tree topologies of the two mitochondrial markers are incomplete taxon sampling, an insufficient number of individuals analysed due to the high level of hybridisation observed and the inadequate length of the cyt-fragment. In addition, we cannot rule out that the differences in the overall topology of the two mt-trees could be the result of using two different MP-programs (PAUP and TNT). Nevertheless, it is unlikely that well supported clusters would consist of different individuals: the *S. montenegrinus* specimen 4 would not cluster with specimens 1–3 if a different MP-algorithm was used to analyse the cyt-b data.

Very interesting is the phylogenetic position of a new specimen of *S. montenegrinus* (specimen 4) analysed, from which, unfortunately, we obtained sequences only from the ITS-2 and cyt-b fragments. Based on the nuclear marker ITS-2 (Fig. 4), all *S. montenegrinus* specimens group in a clade together with *S. corvus* (without resolution, see

above), whereas *S. montenegrinus* specimens 1–3 form a separate clade based on cyt-b. In the cyt-b tree the new specimen 4 clusters together with the other *S. corvus* specimens analysed (Fig. 3). This allows a new interpretation of the inconsistency of nuclear and mitochondrial markers. In 2012 we concluded that *S. montenegrinus* is a species closely related to *S. corvus* (Schniebs et al. 2012). With respect to the new results we can interpret the specimens with the morphological disposition described as *S. montenegrinus* as hybrid specimens between *S. corvus* and another *Stagnicola* species.

The mitochondrial sequences of the *S. montenegrinus* specimens 1, 2, 3 and 4 contained no stop codons typical for pseudogenes. Nevertheless, we cannot definitively exclude the argument that we sequenced pseudogenes in the case of these three specimens.

Cases in which species with nearly identical nuclear sequences show a high differentiation of mitochondrial lineages as result of hybridisation are known from many other animal groups, ex. from butterflies (Dasmahapatra et al. 2010), crocodiles (Franke et al. 2013) or lizards (Renoult et al. 2009).

In our opinion we found a compelling case of hybridisation in *S. palustris* (specimens 4, 7, 8, 9–19, 21, 22, 23, 24, and 25). Specimens with the morphological disposition of *S. palustris* show different positions in nuclear and mitochondrial trees. In the RAxML as well as in the MP tree of the nuclear marker ITS-2 these specimens form a cluster with a bootstrap support of 88% (Fig. 4) and 67% (not shown) respectively, which corresponds to their morphology. In the trees of the mitochondrial markers, the specimens form a cluster together with the *S. fuscus* specimens: fully supported in the COI MP tree, nearly fully supported in the cyt-b MP tree, with 80% and 77% bootstrap support in the COI RAxML and in the cyt-b RAxML trees respectively. Ballard & Whitlock (2004) concluded that introgression does not necessarily leave a signal in the nuclear genome. Our nineteen *S. palustris* specimens clustering together with the *S. fuscus* specimens in the mitochondrial trees appear to be a typical case of such a past event. Surprisingly, in fact, is the high percentage of hybrids among the *Stagnicola* specimens. Perhaps adaptive advantages could also play a role here, as it was observed in water frogs of the genus *Rana* where *R. ridibunda* Pallas, 1771 larvae with introgressed mitochondrial DNA of *R. lessonae* Camerano, 1882 were postulated to be less sensitive to oxygen deficiency than normal *R. ridibunda* larvae (Plötner et al. 2008). Boratynski et al. (2014) suggest that the evolution of mtDNA in a rodent species affected by mtDNA introgression may have functional, ecological and adaptive significance.

The usual theory for animals is that in case of an introgression that took place relatively recently, specimens with the „wrong" mitochondrial genome should be found mainly in the area of sympatry of two species, but with increasing time elapsing since the event took place, such haplotypes can be found further away from the area of sympatry (Abramson 2009). In our opinion, introgression events in hermaphroditic freshwater snails are theoretically possible in every waterbody. Single specimens of species new for the waterbody could be introduced by zoochory and be confronted with specimens of only another (related) species. Freshwater gastropods are transported mainly by aquatic birds or stock fishes (Reise & Glöer 2004) into other waterbodies, probably even over long distances. In experiments, Wullschleger et al. (2002) could show for *Radix balthica* and *R. labiata* that "snails from the sympatric location avoided mating with the opposite species, while allopatric snails showed less discrimination against the opposite species" (Wullschleger et al. 2002: p. 247). Some of the *Stagnicola* freshwater snail specimens analysed in this study appear to be typical examples of introgression of mtDNA from one species into another (Ballard & Whitlock, 2004), so that we could assume similar reproducing behaviour in *Stagnicola* species as in *R. balthica* and *R. labiata*. As we know from monitoring programmes in some federal states of Germany (e.g. Mecklenburg-Western Pomerania, Saxony), *S. fuscus* appears to be a widespread but rare species (Zettler 2006; <www.weichtiere-sachsen.de>). The transport of single specimens of this species by aquatic birds, stock fishes or flood events into waterbodies inhabited by *S. palustris* could be a possible opportunity for hybridisation. Indisputably, *S. corvus* is one of the parent species of *S. montenegrinus*. Not native to the area, *S. corvus* was reported from the southern side of the Skadar Lake (on the border between Albania and Montenegro) in 1996 (Dhora & Welter-Schultes 1996) and from the northern side of the same waterbody in 2008 (Glöer & Pešić 2008). Whether *S. palustris* could be the second, and rarer, parent species is doubtful. This species has neither been found in Lake Skadar (Glöer &

Pešić 2008) nor in the entire Balkan region. It is questionable whether reports of the occurrence of *S. palustris* in Lake Skadar (Wohlberedt 1909; Dhora & Welter-Schultes 1996) were based on anatomical determination. Concerning the distribution of *S. palustris* and *S. fuscus* we cannot trust older literature in which species identification is based only on the shells. If we do so, we get a wrong picture (for an example see Welter-Schultes 2012). *S. palustris* occurs from Britain and the North German lowlands to Siberia. In the south of the German lowlands there are only some disjoint populations known (e.g. Lake Constance) (Glöer 2002). *S. fuscus* is distributed southwards of the North German lowlands, into Southern Europe. It occurs in Croatia (Beran et al. 2013), although it was not found in Montenegro (V. Pešić, oral pers. comm.), Bulgaria (Georgiev 2014) or Macedonia (Bank 2011). *S. palustris* has not been recorded from Bulgaria by anatomical studies and considering only the shells, it could be confused with *S. turricula* (= *S. vulnerata* sensu Jackiewicz, 1962).

We cannot exclude that *Lymnaea* (*Corvusiana*) *gueretiniana* Servain, 1881 (locus typicus Lake Balaton, Hungary) mentioned (amongst others) by Kruglov & Starobogatov (1984), Stadnichenko (2004), and Kruglov (2005) from the South European part of Russia and from Ukraine, is identical with *S. montenegrinus*. This assumption has to be verified in future. We therefore hypothesise that the hybridisation event between *S. palustris* and *S. corvus* has occurred many years ago or in other regions than the Balkans, for example in South-European Russia or in Ukraine. Probably, all that we can see now is only a „footprint" of an earlier hybridisation.

Temporary waterbodies in floodplains that become isolated when the level of the river is low and disappear by flooding could theoretically be suitable places for hybridisation events. If different genital organ morphology and complex mating behaviour represent only incomplete barriers to interspecific sperm exchange in land slugs *Arion rufus* (Linnaeus, 1758) and *A. lusitanicus* auct. non J. Mabille, 1868 (Dreijers et al. 2013) all the more sperm exchange is possible between *Stagnicola* species, considering the relatively simple mating behaviour in representatives of Lymnaeidae. Numerous cases of interspecific and even intersubgeneric hybridisation in aquatic pulmonate snails are reviewed by Beriozkina & Starobogatov (1988). An astonishing example is a report of hybridization between *Lymnaea stagnalis* (Linnaeus, 1758) and *Radix auricularia* (Linnaeus, 1758) (Chaster 1900). However, we regard this report as very doubtful.

Other evidence of hybridization in land snails was found in the genus *Euhadra* in Japan (Shimizu & Ueshima 2000), and Stankowski & Johnson (2014) found incongruence between morphological and molecular phylogenetic variation in species of the genus *Rhagada* in the Dampier Archipelago, W. Australia.

An intermediate morphological position of *S. montenegrinus*, as well as its possible origin as a product of hybridisation between *S. corvus* and *S. palustris* or *S. fuscus*, allows one to resolve the question about the taxonomic rank of the (sub-)genus *Corvusiana*. There are compelling conchological, anatomical and karyological differences between *Corvusiana* and *Stagnicola* s. str. (summarised in Table 4) that brought some authors (Kruglov & Starobogatov 1984, Kruglov 2005, Vinarski, 2013) to raise *Corvusiana* to the rank of a genus. Our results show that successful hybridisation between representatives of *Corvusiana* and *Stagnicola* s. str. is possible, therefore we cannot corroborate the former as a separate genus, following Dubois' (1988: 77) recommendation that all species "able to give birth to viable adult hybrids, be these fertile or not" should be placed in the same genus. But nevertheless, the morphological, caryological and genetic differences between the *S. corvus* group and *Stagnicola* s. str. suffice for keeping *Corvusiana* as a subgenus.

Unfortunately, with only four specimens analysed using molecular methods, our data are too scarce to elucidate the species status of *S. montenegrinus* unequivocally. The problem needs further study. However, the very possibility of reticulate speciation in Lymnaeidae (not known previously) is intriguing and may partially explain the high level of morphological variation in the pond snails that caused the extreme taxonomic inflation in the family in the past.

Our analyses expose the existence of a relatively high percentage of hybrid specimens in Palaearctic *Stagnicola*.

In contrast to our conclusions in an earlier molecular genetic study of *S. montenegrinus* (Schniebs et al. 2012), we can now consider that specimens showing the morphology described for *S. montenegrinus* are probably hybrids of *S. corvus* with another *Stagnicola* species, possibly *S. palustris* or *S. fuscus*.

At the present time, neither *S. palustris* nor *S.*

Table 4. Morphological and caryological comparison between species of *Corvusiana* and *Stagnicola* s. str.

Character	*Corvusiana*	*Stagnicola* s. str.	Source
Shell height	Up to 45 mm	Up to 30 mm	See Table 2
Praeputium: penial sheath ratio	> 2.00	< 1.50	Kruglov 2005
Number of prostate folds	5–10	1	Kruglov 2005
NF*	60	72	Garbar et al. 2004

*NF (nombre fundamental) – total number of chromosome arms.

fuscus occur in those regions where *S. montenegrinus* is found, we thus interprete *S. montenegrinus* as a result of ancient hybridisation or hybridisation due to zoochory-mediated long-distance dispersal. Thus, the taxonomy of *Stagnicola montenegrinus* can only be unravelled by an integrative approach combining morphology, anatomy, biogeography, and genetics, similar to the study of the caenogastropod freshwater snail genus *Bythinella* (Haase et al. 2007), and needs further investigation.

In case of the hybrids between *S. palustris* and *S. fuscus* analysed from Germany and Russia, there was no considerable morphological signal found, and their status as a separate species of hybrid origin is unwarranted. Specimens with the typical morphology of *S. palustris* have mitochondrial COI and cyt-b sequences so similar to *S. fuscus* that they cluster together and allow no genetic differentiation by these two genes. Therefore, molecular genetic analyses of the mitochondrial COI and cyt-b genes lead to results inconsistent with those of morphological examination and molecular genetic analyses of the nuclear ITS-2 spacer. Palaearctic *Stagnicola* snails are another example for the occurrence of gene flow between species.

Conclusions

With the new results presented here, we show that the mitochondrial COI and cyt-b genes are not suitable for phylogenetic analyses and for barcoding within *Stagnicola* and possibly also for other Lymnaeidae. As already established in our earlier molecular genetic studies on *Stagnicola* (Schniebs et al. 2012), we hold the view that the phylogenetic hypotheses based on nuclear markers are much more reliable than the hypotheses based on the mitochondrial markers COI and cyt-b.

Our results present another example of possible obstacles related to DNA barcoding procedures that are rather popular in current zoology, including malacological studies (Mikkelsen et al. 2007, Davison et al. 2009, Weigand et al. 2011). It is strongly recommended not to base taxonomic changes on mtDNA alone (Ballard & Whitlock 2004, Nygren 2013). Careless usage of the single-molecule approach and formal statistical algorithms of species delimitation may enhance taxonomic inflation (Stålstedt et al. 2013). We believe that only multidimensional approaches (including the study of morphological traits, mtDNA, nuclear DNA, zoogeographic data) can lead malacologists to reliable species delineation in aquatic pulmonates.

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