








A taxonomic position of Armenian endemic freshwater snails of the genus *Shadinia* Akramowski, 1976 (Caenogastropoda: Hydrobiidae): combining morphological and molecular evidence

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ABSTRACT

According to analyses of anatomical data and nucleotide sequences of three genes (16S, 18S rDNA and CO 1 mtDNA), the Armenian endemic genus *Shadinia* Akramowski, 1976 should be considered as a separate subfamily, Shadiniinae of Hydrobiidae Stimpson, 1865. A combination of genetic resemblance and similarity in conchology, morphology of radula, male and female anatomy (except some details) assures us of the close relationship of *Shadinia* and the genera *Persipyrgula* and *Intermaria* recently described from riverine waters of Iran. We provisionally assign these two genera to the new subfamily, which also includes the genus *Nicolaia*, established in 2015 for small hydrobiids from the Kasach River (Armenia). The latter genus has not yet been studied genetically, but morphological evidence leads us to consider it as compatible with *Persipyrgula* and *Intermaria*. All three genera differ significantly from the genus *Shadinia* in only one respect: females of these snails have one seminal receptacle, while those of *Shadinia* have two. The subfamily Shadiniinae is a sister group to Pyrgulinae; the former differs morphologically from the latter in possessing a pigmented nervous system and odontophore, presence of basal cusps on the central tooth of the radula and a black pigmented coiled oviduct without the widening that characterises most of the Pyrgulinae.

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Introduction

Hydrobioid snails of the genus *Shadinia* Akramowski, 1976 are rarely found in the lakes, streams and rivers of the South Caucasus; they are endemic inhabitants of the Ararat depression (Akramowski 1976). At present only three nominal species of *Shadinia* are known. The type species was originally described as *Pyrgula terpoghassiani* Shadin, 1952, most likely because the holotype (see below) has a well-developed spiral keel, a characteristic feature of *Pyrgula*. Its keel-less morphotype *Sh. terpoghassiani* morpha *ecarinata* Akramowski, 1976 from Hrazdan River was recently considered (Glöer et al. 2015) as the distinct species *Sh. bjniensis* Bößneck, Walther & Neiber, 2015. The third member of the *Shadinia* genus was described as *Hydrobia akramowskii* Shadin, 1952 from springs in North Armenia. Finally, Glöer et al. (2015) established a new monotypic genus *Nicolaia* Glöer, Bößneck, Walther & Neiber, 2015 for hydrobiid *Shadinia*-like snails from Kasach River (Armenia).

Originally, Shadin (1952) treated the snails known to him from the group under discussion as belonging to Hydrobiidae; later the genus *Shadinia* was assigned

(Akramowski 1976) to the family Lithoglyphidae Tryon, 1866 and this position was unchanged for a considerable time (Kantor et al. 2010). Recently, based on sequences of COI mtDNA gene fragments Delicado et al. (2016) demonstrated that the genus *Shadinia* forms a monophyletic group with two newly-described genera *Persipyrgula* Delicado, Pešić & Glöer, 2016 and *Intermaria* Delicado, Pešić & Glöer, 2016 from Iran. The authors treated this group as closely related to Pyrgulinae though representing a separate hydrobiid lineage positioned between Pseudamnicolinae and Pyrgulinae. However, no specified taxonomic rank has been provided for this cluster of genera including *Shadinia*.

Independently of these authors, we examined the protoconch sculpture, radular teeth morphology and anatomy of *Sh. terpoghassiani* (the smooth morphotype) from the type locality and also performed a phylogenetic analysis using the mitochondrial cytochrome c oxidase subunit I (COI), the mitochondrial large ribosome subunit rRNA (16S) and the nuclear small ribosome subunit rRNA (18S). Our results complement data obtained by Glöer et al. (2015) and Delicado

et al. (2016) and provide new insight into the taxonomic position of the genus *Shadinia*.

Based on molecular-phylogenetic analysis (sequences of the COI and 18S genes) it was demonstrated that the family Hydrobiidae (as defined by Kabat and Hershler 1993) can be considered as monophyletic with the exclusion of Cochliopidae (Wilke et al. 2001). Additionally, on the basis of sequence data from COI, 16S and 18S, Ponder et al. (2008) revealed several well-supported family-level clades within Rissooidea including a broadly-based taxon Hydrobiidae. Later, Wilke et al. (2013), using COI, LSU rRNA and SSU rRNA sequence data, showed that Rissooidea are grouped into 13 family-level clades, including Hydrobiidae; these authors provided morphological/anatomical diagnoses for each family. As well, Wilke et al. (2013) subdivided the family Hydrobiidae into eight or nine subfamilies, among them the closely related taxa Hydrobiinae and Pyrgulinae.

Here we consider a critical analysis of all available data on *Shadinia*, as well as related taxa. The present investigation focuses on a combination of morphological and

molecular evidence with these aims: 1) to confirm the relationships of the genus *Shadinia*; and 2) to determine the family-group rank for this genus.

Materials and methods

More than 50 snails of *Shadinia* were sampled in Armenia (South Caucasus) by hand collecting, mostly from stones or macrophytes in the littoral zone of Lake Aiger-Lich (Marz Armavir, Aknalich settlement east of Metzamor (40°8'41.28" N, 44°10'13.73" E) by Dmitry Palatov 31 July 2014 (Figure 1). The materials used for the present study are stored in the Institute of Zoology NAS of Ukraine (Kiev, Ukraine) and in Limnological Institute SD RAS (Irkutsk, Russia).

Additionally, we studied the type series of species of *Shadinia* housed in the Zoological Institute of RAS (St Petersburg, Russia), including the holotype and seven paratypes of *Pyrgula terpoghassiani* and 10 syntypes of *Hydrobia akramowskii* (Figure 2).

We fixed the specimens in 80% ethanol for 24 h with a subsequent re-fixation in 70% ethanol for permanent



Figure 1. Geographic position (A) and view for sampling of *Shadinia* site from Lake Aiger-Lich, Armenia (B).

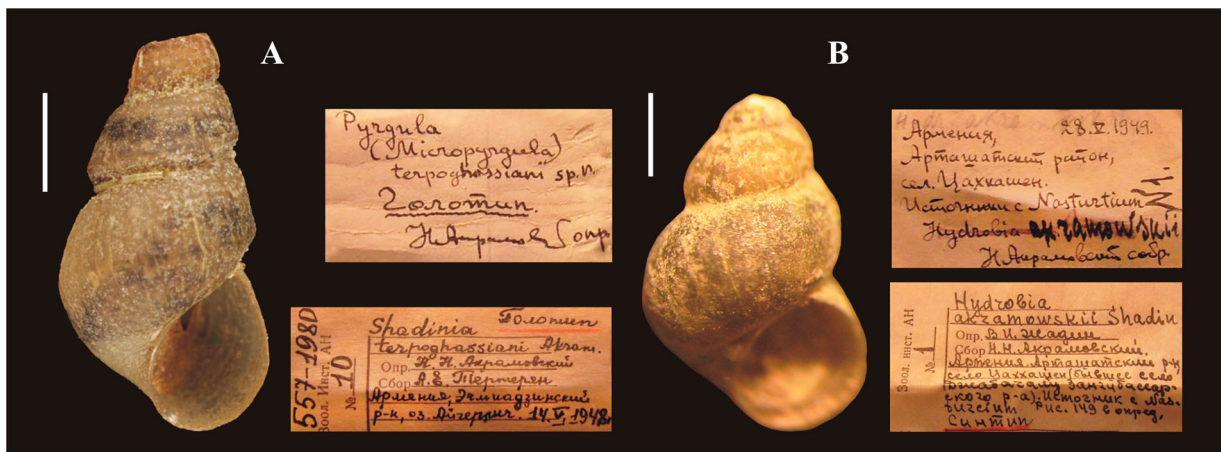


Figure 2. Shells of *Shadinia* species. **A**, *Pyrgula terpoghassiani* Shadin, 1952, holotype, height 4.5 mm; **B**, *Hydrobia akramowskii* Shadin, 1952, syntype #1, height 4.0 mm. Original labels of Nicolai Akramowski and labels of Zoological Institute RAS (St Petersburg, Russia) are reproduced. The shell of the *Pyrgula terpoghassiani* holotype bears a well-developed spiral keel, a characteristic feature of *Pyrgula*. Scale bar 1 mm.

Table 1. Primers used in the present study.

Gene	Primer	References
COI	LCO1490 (f) 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' HCO2198 (r) 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'	Folmer et al. (1994)
16S	ARL (f) 5'-CGC CTG TTT ATC AAA AAC AT-3' BRH (r) 5'-CCG GTC TGA ACT CAG ATC ACG T-3'	Palumbi (1996)
18S	SWAM18S (f) 5'-GAA TGG CTC ATT AAA TCA GTC GAG GTT CCT TAG ATG ATC CAA ATC-3' SWAM18S (r) 5'-ATC CTC GTT AAA GGG TTT AAA GTG TAC TC ATT CCA ATT ACG GAG C-3'	Palumbi (1996)

storage. We photographed the shells of the snails before dissection. Altogether 24 specimens were used to study the anatomy and radula of *Shadinia*. Serial histological sections were prepared for four females, collected from Lake Ayger-Lich, of which the seminal receptacle (rs_1) was hardly visible under the stereomicroscope; gonoducts were embedded in paraffin, sectioned at 5–7 μ m, and stained with haematoxylin (Lillie 1965). Six snails were used to study the operculum and protoconch morphology and for the molecular analysis. In order to identify the snails, we referred to the gastropod collections, including types of *Shadinia*, kept in the Zoological Institute RAS (St Petersburg, Russia).

We examined the protoconchs, radulae and opercula using a scanning electron microscope (SEM) CamScanS2. Prior to the investigations, the protoconch, radula (extracted from buccal mass) and operculum were rinsed in chlorine bleach, washed with distilled water and alcohol, dried, mounted onto SEM stubs and sputter-coated with gold. The morphological terminology used came from reviews of hydrobioid gastropods (Radoman 1983; Riedel 1993; Hershler and Ponder 1998; Szarowska 2006).

We extracted genomic DNA from muscle tissue using a modified Sokolov (2000) method. Gene fragments of mitochondrial cytochrome c oxidase subunit I (COI), mitochondrial large ribosome subunit rRNA (16S) and nuclear small ribosome subunit rRNA (18S)

were then amplified in polymerase chain reaction (PCR) using the primer sets listed in Table 1. Sequencing was carried out in an ABI 3130 automated sequencer. All sequences were deposited into GenBank (see Accession Numbers in Table 2).

Nucleotide sequences were aligned separately with MAFFT v6.2 (e-ins-i algorithm). COI was manually aligned in SeaView 4.5.4 (Gouy et al. 2010), after which the sequences were appended for each operational taxonomic unit (OTU). The alignment differed, although this did not cause any visible differences in the trees. The resulting combined alignment was 1426 bp long and contained the following partitions: COI (559 bp), 18S (422 bp) and 16S (445 bp). Mean pairwise, inter-specific *p*-distances between 16S and 18S sequences were calculated using MEGA 6 (Tamura et al. 2013). For phylogenetic reconstruction we used Bayes inference (BI), implemented in MrBayes v3.2.2 (Ronquist and Huelsenbeck 2003). The dataset consisted of 16 species (three genes) and 20 species (18S) retrieved from GenBank and *Shadinia* sequences produced for this study (Table 2). To estimate the posterior probabilities of the phylogenetic tree, we used 15,000,000 generations of Metropolis-coupled Markov chain Monte Carlo simulation (two runs with four chains). We used the jModelTest 2.1 (Posada 2008; Darriba et al. 2012) to determine the substitution models for the three genes separately. In every case the best-fit model for phylogenetic analysis was GTR

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

Species name	18S GB#	COI GB#	16S GB#	References
<i>Adriohydrobia gagatinella</i> (Kuster, 1852)	AF367567	AF317881	AF478400	Wilke and Falniowski (2001)
<i>Anagastina zetaevallis</i> (Radoman, 1973)	EF070622			Szarowska (2006)
<i>Bithynia tentaculata</i> (Linnaeus, 1758)	JX970566	AF445334	AF445344	Wilke et al. (2013); Hausdorf et al. (unp.)
<i>Bythinella molcsanyi</i> H. Wagner, 1941	JQ639804	FJ545062		Falniowski et al. (2012); Benke et al. (2011)
<i>Bythinella schmidtii</i> (Kuster, 1852)	JQ639802	EF379349	FJ028880	Falniowski et al. (2012); Wilke (unp.); Benke et al. (2009)
<i>Cecina manchurica</i> A. Adams, 1861	AB611744	AB611747	AB611746	Kameda and Kato (2011)
<i>Dianella thiesseana</i> (Kobelt, 1878)	AY676125	AY676127	EF379305	Szarowska et al. (2005); Wilke et al. (2007)
<i>Graziana alpestris</i> (Frauenfeld, 1863)	AF367673	AF367641	AY676123	Wilke et al. (2001); Wilke (unp.)
<i>Horatia klecakiana</i> Bourguignat, 1887	KJ159127	KJ159128	AY222656	Szarowska and Falniowski (2014); Szarowska and Wilke (2004)
<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF367680	AF278808	AY222659	Wilke and Davis (2000); Szarowska and Wilke (2004)
<i>Islamia piristoma</i> Bodon et Cianfanelli, 2002	AF367671			Wilke et al. (2001)
<i>Lithoglyphus naticoides</i> (C. Pfeiffer, 1828)	AF367674	AF367642	AF445341	Wilke et al. (2001); Hausdorf et al. (unp.)
<i>Oncamelania minima</i> Bartsch, 1936	AB611792	AB611791	AB611794	Kameda and Kato (2011)
<i>Pseudamnicola lucensis</i> (Issel, 1866)	AF367687	AF367651	AF478394	Wilke et al. (2001); Wilke (2003)
<i>Pyrgula annulata</i> (Linnaeus, 1767)	AY676124	AY341258	AY676122	Szarowska et al. (2005); Wilke (unp.)
<i>Rissoa labiosa</i> (Montagu, 1803)	AY676126	AY676128	AY676117	Szarowska et al. (2005); Wilke (unp.)
<i>Sadleriana fluminensis</i> (Kuster, 1852)	AF367683	AY273996	AY222657	Wilke et al. (2001); Szarowska and Wilke (2004)
<i>Shadinia terpoghasiani</i> morphotype <i>ecarinata</i> Akramowski, 1976	KT934419	KT934418	KT934417	Present study
<i>Spurwinkia salsa</i> (Pilsbry, 1905)	AF367663	AF354765	EU573991	Wilke et al. (2001); Wilke and Ponder (unp.)
<i>Trichonia kephalovrissonia</i> Radoman, 1973	EF070630			Szarowska (unp.)
<i>Ventrosia ventrosa</i> (Montagu, 1803)	AF367681	AF118335	AF478402	Wilke and Davis (2000); Wilke (2003)

+I+G. Thus their parameters were unlinked while inferring the topology with MrBayes. We constructed a majority-rule (50%) consensus tree following 25% burn-in of all sampled trees, to allow likelihood values to reach stationary equilibrium. Maximum likelihood (ML) analysis was performed in PHYML v3.0 (Guindon and Gascuel 2003). Bootstrap support values were estimated from 1000 replicates. A reasonable amount of variation and the relatively small number of OTUs made a high resolution possible.

Results

The samples of *Shadinia* we studied from Lake Aygerlich (Figure 3A–B) differ from the typical form of *Sh. terpoghassiani* by the lack of a peripheral spiral keel on the shell (compare with Figure 2A). They correspond to the keel-less form designated by Akramowski (1976: 97–98) as *Sh. terpoghassiani* morpha *ecarinata* and recently named by Glöer et al. (2015) as *Sh. bjniensis*.

Morphology

The shell is ovate-conic in shape, and 3–6 mm in length. The raised, domelike protoconch consists of 1.1–1.2 whorls; it is about 3.2–4.2 mm in height and has a maximum diameter of 0.40–0.46 mm. The initial cap-like onset of the embryonic shell is 0.13–0.17 mm wide. The wrinkled or granulated (= fine densely-reticulated) ornamentation covers the entire surface of the embryonic shell. This sculpture covers the protoconch

surface more or less evenly, although there are some delicate spiral rows of granules on the surface near the teleoconch boundary (Figure 3B–C). The transition from the protoconch to the teleoconch is well marked by a thickened notch, and by an appearance of weak axial and spiral lines that become more prominent and turn into fine reticulate ornamentation in the teleoconch whorls.

The operculum of the studied snails (Figure 3D–E) is flat, thin, transparent, a little smaller than the aperture; ovate, paucispiral with about 10 axial growth lines and a subcentral nucleus. The muscle attachment area edges are undifferentiated.

The radula of *Shadinia* (Figure 4) is typically taenioglossate, the length of the radular ribbon is around 640 µm and bears 45 transverse rows, seven of which are rudimentary. There are two basal cusps on each side of the central tooth and three to four cusps on each side of the median cusp. The latter is obtuse-lanceolate, twice as long as the adjacent cusps. The lateral tooth formula is 2–1–2(3); the biggest cusp is broad and massive. On the inner marginal tooth there are about 16 cusps, their length diminishing gradually from the long and slender distal cusps. The outer marginal tooth bears 12 long and slender cusps, their size gradually decreasing from the central part. The odontophore is pigmented grey.

The body black, mantle edge usually grey, gill with 18–22 triangular leaflets; nervous ganglia with dark pigmentation, osphradium ovate, not pigmented, long, lies in the middle part of the gill.

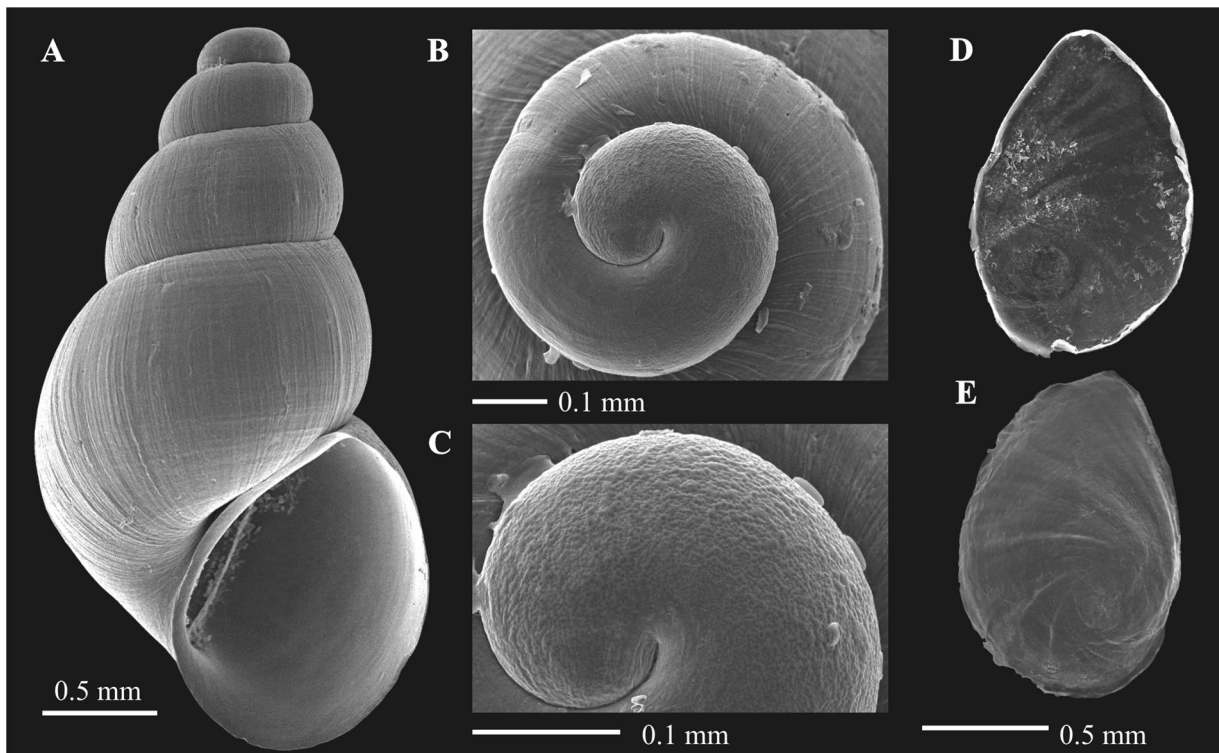


Figure 3. Scanning electron microscope images of the shell and operculum of *Shadinia* sp. **A**, Apertural view of the shell; **B–C**, protoconch of the same specimen at higher magnification, border between protoconch and teleoconch and characteristic fine dense-reticulated ornamentation on the embryonic shell are visible; **D**, operculum external side; **E**, operculum internal side.

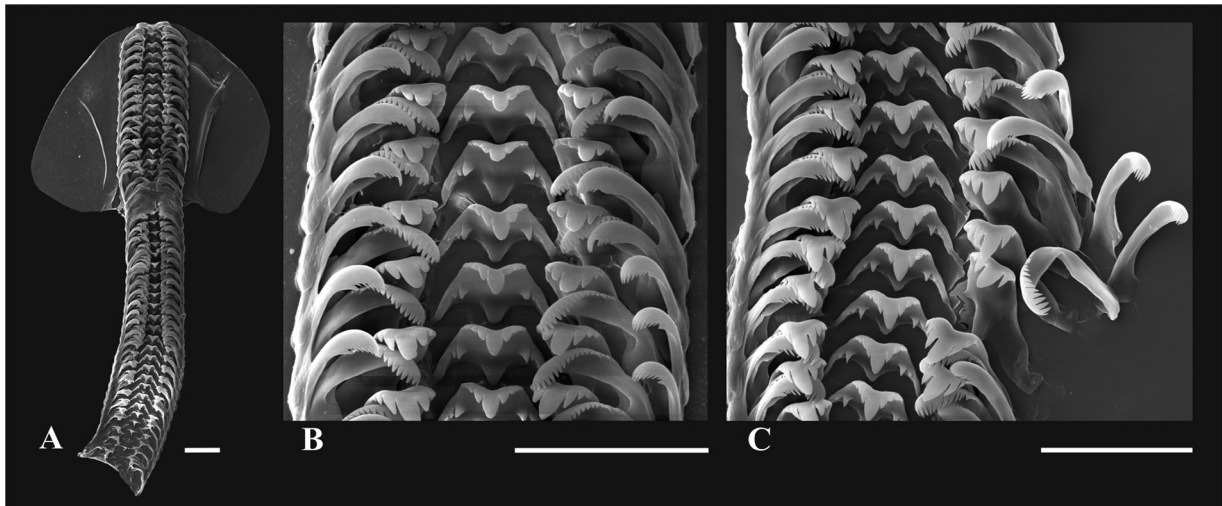


Figure 4. Radula of *Shadinia* sp. **A**, General view of radular ribbon with teeth in natural position; **B**, enlarged set of transverse rows of the same radula; **C**, details of the same radula; central tooth with two pairs of clearly visible sub-basal cusps, minute details of lateral, inner and outer marginal teeth can be seen. Scale bar = 0.05 mm.

Female reproductive system (Figures 5–6) with black-pigmented coiled renal oviduct, forming one loop and bearing claw-shaped black-pigmented seminal receptacle (rs_2), seminal receptacle (rs_1) small, covered by connective tissue and usually not visible under the stereomicroscope, but detected in histological slides (Figure 6B), it contains oriented sperm, its duct close to the junction of bursa copulatrix duct and albumen gland. The bursa copulatrix is ovate or pyriform, and covers the middle part of albumen gland, not reaching its distal end. The albumen gland is shorter than the capsule gland on the ventral side but longer on the dorsal side (Figure 5C). The ventral channel is separated from the lumen of the capsule gland with slightly thickened folds, and contains oriented sperm (Figure 6A), which suggests it functions as a ‘spermathecal duct’.

The prostate gland is bean-shaped, and the penis is simple, non-pigmented, broad-triangular, tapered or moderately elongated, with a grey-pigmented tip which is sometimes slightly hooked. The slightly flexuous vas deferens runs along the right side of the penis.

Molecular analysis

Phylogenetic reconstruction was based on combined data of 559 bp of the COI, 422 bp of the 18S and 445 bp of the 16S genes.

All six specimens of *Shadinia* were shown to have identical COI nucleotide sequences, therefore nucleotide sequences of two rDNA fragments (18S and 16S) were obtained from three specimens only. These sequences were identical as well. In addition, specimens studied were identical at COI nucleotide

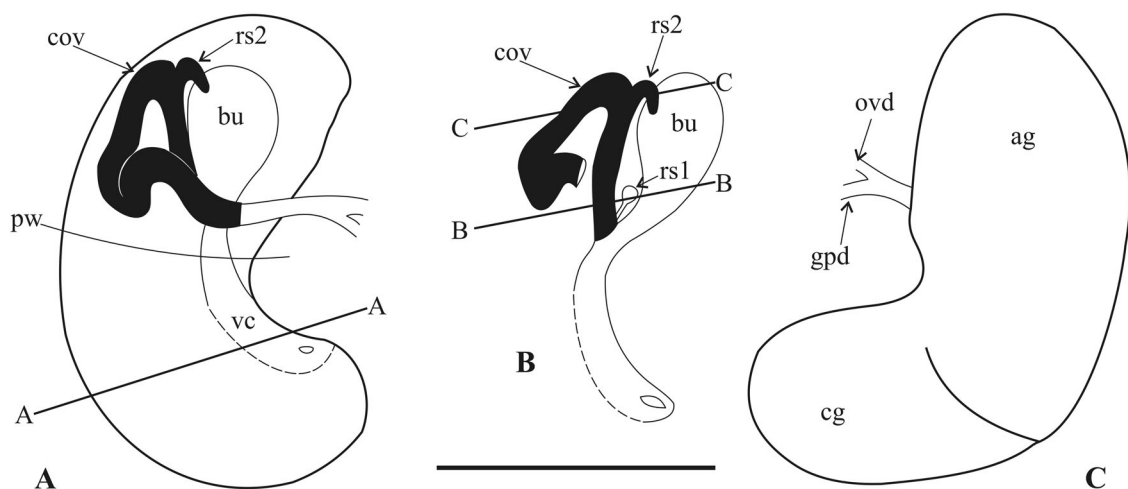


Figure 5. Distal female genitalia of *Sh. terpoghassiani* (morphotype *ecarinata*). **A**, Ventral view; **B**, view of enlarged part of coiled part of oviduct and associated structures; **C**, dorsal view. Abbreviations: ag—albumen gland; bu—bursa copulatrix; cg—capsule gland; gpd—gonopericardial duct; cov—coiled oviduct; ovd—oviduct; pw—pallial wall; rs_1 —seminal receptacle 1; rs_2 —seminal receptacle 2; vc—ventral channel of capsule gland. Histological sections in positions A-A, B-B and C-C shown in Figure 6. Scale bar = 1 mm.

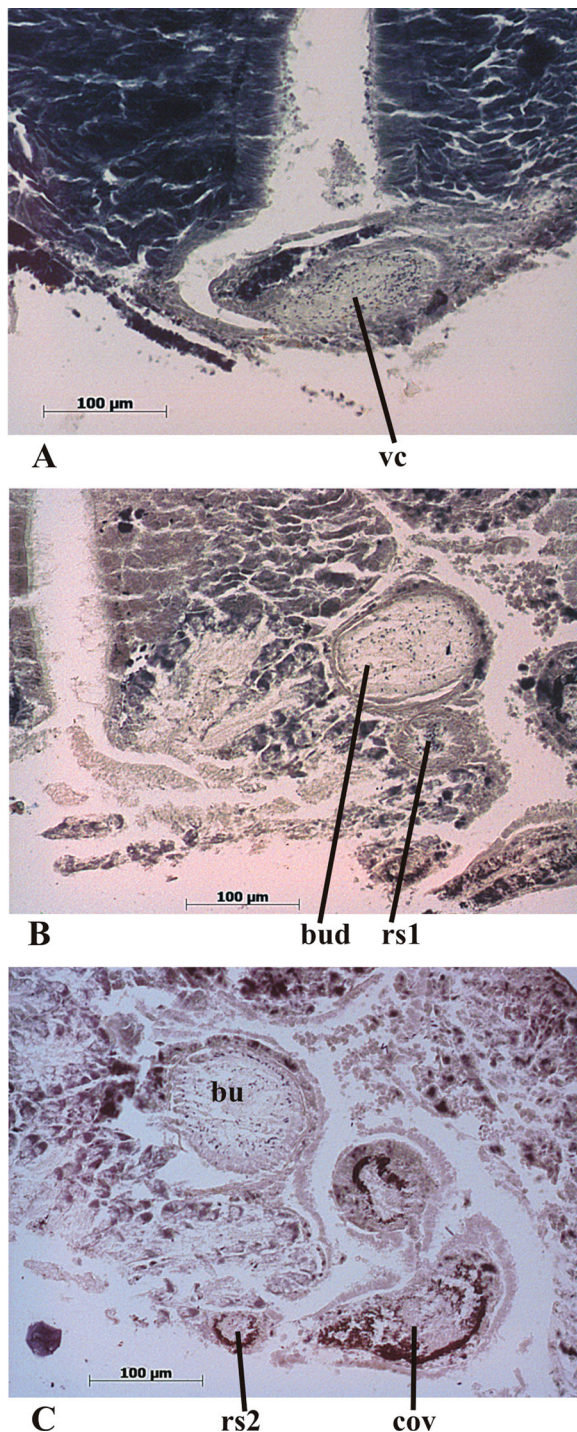


Figure 6. Histological sections of female genital system of *Sh. terpoghassiani* (morphotype *ecarinata*). Corresponding positions of sections are shown in Figure 5. **A**, Section across capsule gland and ventral channel; **B**, section across rs_1 and bursal duct; **C**, section across black-pigmented oviduct with rs_2 and bursa. Abbreviations: bud—bursal duct, other abbreviations as in Figure 5.

sequences with *Sh. bjniensis* collected in Hrazdan River, Yerevan Province, Armenia (Delicado et al. 2016).

The topology of the phylogenetic tree based on 18S sequences (Figure 7A) differs significantly from the tree based on combined data for three genes (Figure 7B). The lineage comprising *Shadinia* on the 18S tree is separate from the hydrobiid taxa, and situated in the unresolved part of the tree. The polytomy could be a result

of the short length of analysed sequences (422 bp). Uncorrected p -distances for the 18S sequences are relatively high ($> 4\%$) between *Shadinia*, *Hydrobia* Hartmann, 1821 (Hydrobiinae), *Dianella* Gude, 1913 and *Pyrgula* Cristofori & Jan, 1832 (Pyrgulinae) in comparison with p -distances ($< 1.5\%$) between *Shadinia* and *Bithynia* Leach, 1818 (Bithyniidae) or *Lithoglyphus* Hartmann, 1821 (Lithoglyphidae) (Table 3). To clarify genetic relationships of *Shadinia* we used phylogeny inferred from the combined dataset (18S+COI+16S), where sequence length, reasonable amount of variation and relatively small number of OTUs made a high resolution. The Bayesian tree based on the combined dataset recovers the phylogeny at family and subfamily levels. This corresponds well to relationships presented by Wilke et al. (2013). On this tree *Shadinia* appears as well supported both by ML (bootstrap value 96) and BI (BPP 0.98) and is the sister group to Pyrgulinae, while Hydrobiinae is the closest relative to these lineages. Pairwise p -distances for 16S gene indicate low genetic divergence between *Shadinia* and *Hydrobia* (4.6%), *Dianella* (4.8%), *Pyrgula* (5.3%), and correspond to COI mutational distances demonstrated by Delicado et al. (2016).

Thus, our phylogenetic analysis, in combination with conchological and anatomical characteristics, allows us to consider the genus *Shadinia* as a distinct lineage of Hydrobiidae representing a separate taxon deserving subfamily rank.

Despite the absence of nucleotide sequences of the 18S as well as 16S genes for the genera *Persipyrgula* and *Intermaria*, we provisionally include them into the same subfamily based on the analysis of COI gene sequences (Delicado et al. 2016). Here we also provisionally allocate the genus *Nicolaia* because of conchological, radular and anatomical characters of the latter genus which correspond well to those of *Persipyrgula* and *Intermaria*. Diagnosis for the new subfamily is compiled with consideration of all available new data as well as that provided by Glöer et al. (2015) and Delicado et al. (2016).

Family Hydrobiidae Stimpson, 1865

Shadiniinae Anistratenko, Peretolchina, Sitnikova & Palatov n. subfam.

Type genus *Shadinia* Akramowski, 1976

Diagnosis

Shell small (up to 6 mm high), ovate to conical, smooth or with spiral keel in periphery of whorls. Operculum oval, paucispiral with subcentral nucleus. Protoconch raised, reticulate, sculptured with irregular granules or wrinkled. Nervous ganglions and odontophores black pigmented. Osphradium lying in the middle part of ctenidium; triangular gill leaflets up to 22. Central radular tooth bears two basal cusps on each side. Oviduct coiled, black pigmented with slightly thickened and

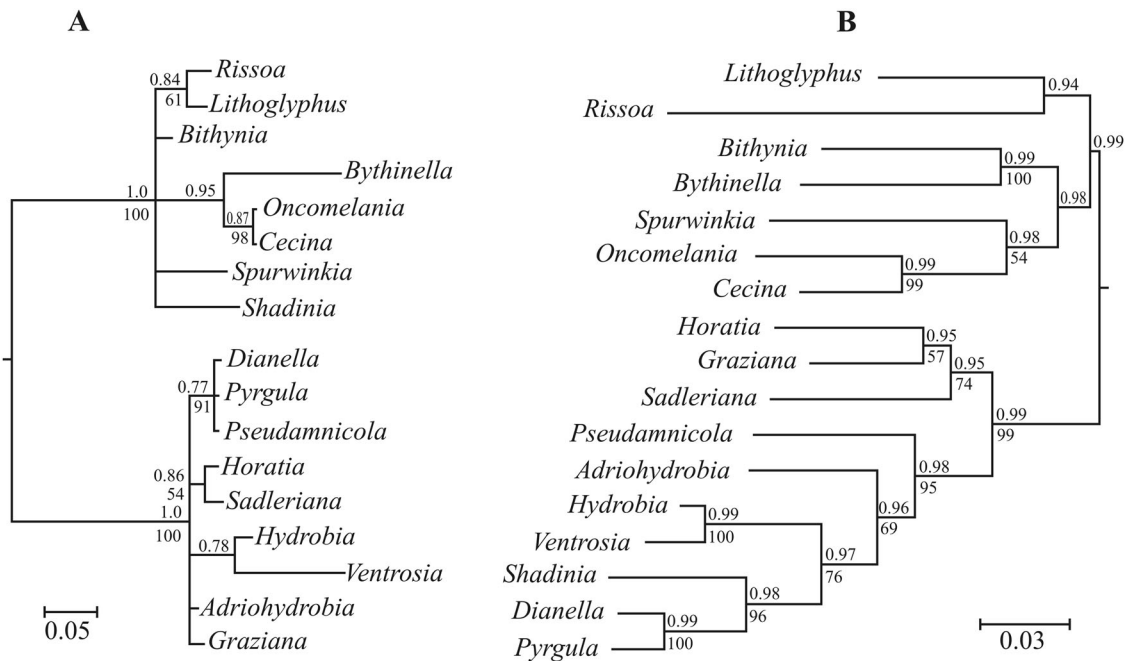


Figure 7. Bayesian tree for 'hydrobioid' species based on the 18S rDNA sequences (**A**) and 18S rDNA + COI and 16S mtDNA concatenated gene fragments (**B**). Posterior probabilities >0.5 are given above branches, bootstrap supports >50% are given below branches.

'corrugated' wall in middle part, or with seminal receptacle (rs_2); small seminal receptacle (rs_1) close to the junction of bursal duct and albumen gland; reservoir of bursa copulatrix does not reach the distal end of albumen gland; bursal duct almost equal to bursa length; ventral channel functions as spermathecal duct. Penis simple or with small distal lobe, spiral vas deferens along right side of penis.

Differential diagnosis

The new subfamily differs from the most closely related subfamily Pyrgulinae by the presence of basal cusps on the central teeth of the radula, and the black pigmented coiled oviduct without a 'pouch' or 'crest' (i.e. the widened part of the loop that is characteristic of

some Pyrgulinae); also Pyrgulinae do not possess a true seminal receptacle (Radoman 1983; Szarowska et al. 2005). Contrary to Shadiniinae, the subfamily Turricaspiinae is characterised by the lack of basal cusps on the central tooth of the radula, though a bursa copulatrix and a seminal receptacle rs_1 are both present (see Anistratenko 2008). Some Turricaspiinae (e.g. genus *Euxinipyrgula* Sitnikova & Starobogatov, 1999) differ from Shadiniinae in having a non-pigmented nervous system and odontophore, and an osphradium opposite the posterior part of the ctenidium that bears more leaflets (about 30–32; unpubl. data) than Shadiniinae (18–22). The ventral channel of the female gonoduct of Shadiniinae contains oriented sperm and possibly acts as a spermathecal duct in a

Table 3. Pairwise p -distances (%) between 18S (below) and 16S (above) sequences of different species of the 'hydrobioid' gastropods.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. <i>Dianella</i> Gude, 1913	–	9.2	5.3	2.0	7.1	10.7	7.9	8.9	10.4	17.0	17.8	17.0	16.8	11.5	26.0	16.3	4.8
2. <i>Horatia</i> Bourguignat, 1887	0.8	–	8.1	9.4	8.1	12.5	10.4	6.4	8.1	15.5	16.5	17.0	16.0	8.9	25.2	14.2	7.4
3. <i>Hydrobia</i> Hartmann, 1821	0.8	0.5	–	6.1	3.6	9.7	5.6	8.7	10.7	16.5	18.3	18.3	17.8	10.9	26.2	16.0	4.6
4. <i>Pyrgula</i> Cristofori & Jan, 1832	0.0	0.8	0.8	–	7.9	11.2	8.9	9.2	10.7	17.8	18.6	17.8	17.6	12.0	26.7	17.0	5.3
5. <i>Ventrosia</i> Radoman, 1977	2.1	1.8	1.3	2.1	–	11.2	6.6	9.2	11.5	16.0	16.8	17.8	18.6	11.5	27.0	15.8	6.6
6. <i>Pseudamnicola</i> Paulucci, 1878	0.0	0.8	0.8	0.0	2.1	–	6.9	9.2	9.2	16.3	16.8	18.1	17.6	14.8	30.0	14.0	9.9
7. <i>Adriohydrobia</i> Radoman, 1973	0.3	0.5	0.5	0.3	1.8	0.3	–	6.9	8.1	14.8	15.8	16.5	16.5	14.0	29.3	14.5	7.6
8. <i>Graziana</i> Radoman, 1975	0.5	0.8	0.8	0.5	2.1	0.5	0.3	–	5.1	13.0	14.0	14.5	13.2	10.4	26.5	10.9	7.4
9. <i>Sadleriana</i> Clessin, 1890	0.8	0.5	1.0	0.8	2.3	0.8	0.5	0.8	–	14.8	15.3	15.3	14.8	11.5	27.2	13.0	8.9
10. <i>Bithynia</i> Leach, 1818	3.6	3.9	3.9	3.6	4.4	3.6	3.9	3.6	4.4	–	15.0	15.5	15.5	14.8	29.0	11.2	17.0
11. <i>Spurwinkia</i> Davis & Mazurkiewicz, 1982	4.7	5.4	5.4	4.7	6.0	4.7	4.9	4.7	5.4	1.8	–	14.5	13.0	17.0	27.7	14.5	18.3
12. <i>Oncomelania</i> Gredler, 1881	4.7	4.9	4.9	4.7	5.4	4.7	4.9	4.7	5.4	1.0	2.3	–	5.1	15.0	26.5	14.5	16.8
13. <i>Cecina</i> Adams, 1861	4.7	4.9	4.9	4.7	5.4	4.7	4.9	4.7	5.4	1.0	2.3	0.0	–	14.0	27.0	13.0	16.8
14. <i>Lithoglyphus</i> Hartmann, 1821	4.1	4.9	4.9	4.1	5.7	4.1	4.4	4.1	4.9	1.3	1.6	1.8	1.8	–	24.4	11.5	10.7
15. <i>Rissoa</i> Fréminville, 1814	4.1	4.9	4.9	4.1	5.4	4.1	4.4	4.1	4.9	1.0	1.6	1.6	1.6	0.5	–	25.2	24.7
16. <i>Bythinella</i> Moquin-Tandon, 1855	4.9	4.7	4.7	4.9	5.2	4.9	4.7	4.4	5.2	1.6	1.8	1.0	1.0	2.3	2.1	–	15.3
17. <i>Shadinia</i> Akramowski, 1976	4.1	4.9	4.9	4.1	5.4	4.1	4.4	4.1	4.4	1.0	1.3	2.1	2.1	1.3	1.3	2.1	–

The lowest genetic distances between *Shadinia* sp. and other molluscs are in bold.

way that does not occur in Pyrgulinae or Turricaspiinae. Both the Hydrobiinae and Caspiinae have the same radular features as Shadiniinae but *Shadinia* differs in the presence of a bursa copulatrix and two seminal receptacles (rs_1 and rs_2) in the female reproductive system. Although they have two pairs of basal cusps on the central tooth of the radula, members of Caspiinae lack both a bursa copulatrix and a seminal receptacle while Hydrobiinae have a bursa, but lack a second seminal (rs_2). Bithyniidae have similar central radular teeth but differ from Shadiniinae by their unusually large single seminal receptacle (which may be a homologue of rs_1) and the bursa copulatrix lies anteriorly (distally). The members of the family Lithoglyphidae differ from Shadiniinae by the presence of more (3–4) basal cusps on the central teeth of the radula, and having only one seminal receptacle at the position of rs_1 .

Discussion

It should be stressed again that individuals of *Shadinia* studied in the present investigation have identical COI nucleotide sequences to *Sh. bjniensis* and do not differ from *Sh. terpoghassiani* collected in the type locality of that species (Delicado et al. 2016), where our samples were also gathered. However, the snails we studied differ anatomically from both species investigated by Delicado et al. (2016) in shape and position of rs_2 and the shape of the penis. Therefore we do not identify our *Shadinia* as *Sh. bjniensis* and tentatively keep the name *Sh. terpoghassiani* morphotype *ecarinata* given by Akramowski (1976). More detailed analysis of intraspecific variability and species-level taxonomy of *Shadinia* will be provided in a paper in preparation.

The subfamilies Shadiniinae, Pyrgulinae (*Pyrgula* Cristofori & Jan, 1832) and Turricaspiinae (*Falsipyrgula* Radoman, 1973, *Turricaspiia* B. Dybowski & Grochmalicki, 1915 and *Euxinipyrgula*) have a similar (granulated) protoconch microsculpture (Riedel et al. 2001; Anistratenko 2008; Szarowska 2006), but such protoconch microsculpture is also known in some other hydrobiid taxa including *Ventrosia* Radoman, 1977 (Hydrobiinae) and *Anagastina* Radoman, 1978 (Sadlerianinae) (Bodon et al. 2001; Szarowska 2006; Szarowska and Falniowski 2014). Weak striae observed on the initial part of the protoconch of *Shadinia* are also characteristic of *Pyrgula* (Riedel et al. 2001).

Most of the lithoglyphids and hydrobiids, including Pyrgulinae and Turricaspiinae, have similar opercular morphology, except for *Horatia* Bourguignat, 1887 (Belgrandeillinae), *Islamia* Radoman, 1973 (Islamiinae) and *Sadleriana* Clessin, 1890 (Sadlerianinae), which have a circular operculum with a central nucleus (Hershler and Ponder 1998; Bodon et al. 2001; Szarowska 2006).

The radula of *Shadinia* is typically taenioglossate and similar to those of the majority of truncatelloidean taxa and nearly all Hydrobiidae (e.g. Hershler and Ponder

1998) excluding Pyrgulinae and Turricaspiinae which lack basal cusps on their central teeth (Anistratenko 2008, 2013).

A black-pigmented coiled oviduct is present not only in Shadiniinae, but also in *Hydrobia*, *Ventrosia*, *Adriohydrobia* Radoman, 1973 (Hydrobiinae) and *Pseudamnicola* Paulucci, 1878 (Pseudamnicolinae), but in these genera the pigmentation does not extend beyond the 'loop' (e.g. Szarowska 2006: fig. 187), while the coiled oviduct in Shadiniinae is pigmented up to the junction of the bursal duct. As well, all the genera mentioned above possess a single seminal receptacle (rs_1). Among Pyrgulinae the pigmented coiled oviduct is described by Radoman (1983) for *Ohridopyrgula macedonica* (Brusina, 1896) only, but the oviduct is voluminous and glandular in this species and it has no distinct seminal receptacles.

Two seminal receptacles (rs_1 and rs_2) are present in *Shadinia* and six hydrobiid genera (*Anagastina*, *Horatia*, *Islamia*, *Trichonia* Radoman, 1973, *Sadleriana* and *Lithabitella* Boeters, 1970) of two different subfamilies. However, in those genera the bursa copulatrix extends up to the posterior end of the albumen gland (e.g. Szarowska 2006: fig. 188), while in *Shadinia* it does not reach the end of albumen gland. Histological sections revealed oriented sperm inside the ventral channel of *Shadinia*, which shows that the new subfamily differs from Pyrgulinae and other listed subfamilies of Hydrobiidae. The penis shape varies within Hydrobiidae; the absence or presence of penis lobes (besides glands) are also variable characteristics within the family. Such variation probably accounts for the lobe present in some Shadiniinae (Delicado et al. 2016; unpubl. data) and in Pyrgulinae (e.g. Szarowska 2006).

Sadlerianinae (namely *Anagastina*, *Trichonia*) have the same shell shape, almost identical radular morphology and very similar structure of the male and female genital systems to those seen in Shadiniinae, but the molecular analysis does not indicate a close relationship of these groups.

The evidence of the molecular data appears to support the merging of *Shadinia*, *Persipyrgula*, *Intermaria* and *Nicolaia* into the same subfamily, despite *Shadinia* possessing two seminal receptacles, while the other three genera possess only one.

The modern range of the subfamily Shadiniinae is restricted to South Caucasus (Armenia, Azerbaijan) and water bodies of northwest Iran (Shadin 1952; Akramowski 1976; Glöer et al. 2015; Delicado et al. 2016).

Direct relatives of the genus *Shadinia* are as yet unknown in either the Caspian Sea, or from the Azov-Black Sea Basin or from Balkan or Anatolian regions. However, there are some common traits in the history of Lake Ohrid and Lake Aiger-Lich allowing us to estimate the age of this taxon (Akramowski 1952). The fauna of Lake Ohrid contains elements that are the remnants of Pliocene brackish and freshwater

Pontian fauna. The Ararat depression, where Lake Aiger-Lich, is also of Pliocene age because it was formed when the Aragats Mountains and Gegam Ridge uplifted (Akramowski 1952). The ancient and relic nature of *Shadinia* agrees indirectly with the fossil finding of *Pyrgula shadini* Akramowski, 1956 and *Hydrobia sieversi* Boettger, 1881—both conchologically similar to *Sh. terpoghassiani*—from the Pliocene–Pleistocene deposits of Armenia (Akramowski 1956). Some authors consider this region to be a refuge for certain invertebrates derived from the Ancient Caspian Sea (Birstein 1932), so it appears that the genus *Shadinia* may belong to one of the relic and endemic branches of the Ponto-Caspian fauna, which survived in the continental refugia of Caucasus and Asia Minor. However, the clarification of the relationships of *Shadinia* requires further investigations using morphological, molecular and phylogeographical approaches.

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