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SPECIES DISTINCTNESS OF *LITTHABITELLA* BOETERS, 1970 (CAENOGASTROPODA: TRUNCATELLOIDEA) FROM THE IONIAN ISLANDS

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ABSTRACT: The shell, protoconch, operculum and radula of *Litthabitella chilodia* (Westerlund, 1886) from two localities in Croatia and two in Montenegro are presented, as well as the shell of "*L. chilodia ionica* (Schütt, 1980)" from Levkada island. Principal Component Analysis (PCA) of the shell showed distinct differences between the Levkada population and the other four localities, but also – although with some overlap – between the populations from Croatia and Montenegro. *18SrRNA* nuclear gene sequences confirmed that *Litthabitella* did not belong to the Hydrobiidae. Histone *H3* nuclear gene sequences confirmed distinctness of the Levkada population, and thus, combined with the shell morphometrics, it confirmed the species-level distinctness of *Litthabitella* from the Ionian islands.

KEY WORDS: shell morphology, morphometrics, histone H3, 18SrRNA, DNA, taxonomy, phylogeny, transadriatic and transionian distribution

INTRODUCTION

The genus *Litthabitella* Boeters, 1970 lives in freshwater springs in the coastal regions of the Balkan Peninsula (BOETERS 1970), from Greece to Slovenia (RADOMAN 1983, 1985, KABAT & HERSHLER 1993), and in southern Italy (BODON et al. 1995, GIUSTI et al. 2010), as well as in the Ionian Islands: Kerkyra, Korfu and Levkada (GIUSTI et al. 2010). It inhabits springs, mostly located close to the sea, but occasionally also subterranean waters (BOLE & VELKOVRH 1986). Its type species: *L. chilodia* (Westerlund, 1886), described from the spring Zwebina in Pridvorje, about 20 km SE of Dubrovnik, Croatia (RADOMAN 1983), is thus a transadriatic-transionian taxon. SCHÜTT (1980) described *L. chilodia ionica* (as *Belgrandiella*) from Korfu, tributary to the Mesongi river (Greece). He characterised the subspecies as having a smaller, more slender and more thin-walled shell than *L. chilodia chilodia*. GIUSTI et al. (2010) examined the type material of *L. chilodia ionica* (Schütt, 1980) and found that some specimens represented *L. chilodia*, and others were most probably some *Belgrandia*, since its anatomy remained unknown. The holotype was a *Belgrandia*; according to the ICZN, the name "*ionica*" should be applied to *Belgrandia* Bourguignat, 1869. Thus, now the name *ionica* is used as *Belgrandia ionica* (Schütt, 1980), which is known from Korfu and from Albania (FEHÉR & PÉTER 2009, RADEA 2011). In fact, the shells illustrated by SCHÜTT (1980) represent *Belgrandia* (fig. 14) and *Litthabitella* (fig. 15). On the other hand, the drawing of the female reproductive organs (fig. 2B) may represent both genera, but the penis (fig. 2A) is characteristic of *Litthabitella*. Another species of *Litthabitella*: *L. elliptica* (Paladilhe, 1874) is known from France and Spain (ARCONADA & PRIÉ 2010). THOMPSON (1979) placed *Litthabitella* in the Nymphophilinae, SZAROWSKA (2006) included it putatively in Assimineidae.

The aim of the present paper was to examine the anatomy of *L. chilodia*, to check the taxonomic status of *Litthabitella* from Levkada using molecular markers, and to unravel the phylogenetic relationships of *Litthabitella*.

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MATERIAL AND METHODS

Specimens of *Litthabitella* came from five localities (Fig. 1) in Croatia, Montenegro and Greece (Table 1). The snails were collected from the sediment using a metal sieve.

Individuals for molecular analyses were washed in 80% ethanol and left to stand in it for ca. 12 hours. Afterwards, the ethanol was changed twice during 24 hours and, after a few days, 80% ethanol was replaced with 96% ethanol. The samples were then stored at –20°C prior to DNA extraction.

The snails were dissected using a NIKON SMZ18 stereo-microscope with dark field and phase contrast; their shells and penes were photographed with CANON EOS 50D digital camera. The protoconchs and radulae were examined using a JEOL JSM-5410 scanning electron microscope, applying the techniques described by FALNIOWSKI (1990).

A NIKON DS-5 digital camera measurement system was used to measure seven shell parameters (SZAROWSKA 2006, FALNIOWSKI et al. 2012a). The linear measurements were then logarithmically transformed. For angular measurements, the arcsine transformation was applied. Euclidean distances were calculated and the minimum spanning tree was computed (MST) using NTSYSpc (ROHLF 1998). The same software was used for Principal Component Analysis (PCA), based on the correlation matrix (FALNIOWSKI 2003). The original observations were projected into PC space, with a superimposed MST (performed for clarity, not presented in the figures) to detect local distortions in the data. Such usage of the PCA in a descriptive approach makes it possible to detect morphologically distinct groups without any a priori classification.

DNA was extracted from foot tissue of each snail. The tissue was hydrated in TE buffer (3 × 10 min.). Total genomic DNA was extracted with the SHERLOCK extracting kit (A&A Biotechnology), and the final product was dissolved in 20 μl TE buffer.

The PCR reaction was performed with the following primers: H3F 5'-ATGGCTCGTACC AAGCAGACVGC-3' and H3R 5'-ATATCCTTRGGC ATRATRGTGAC-3'; COLGAN et al. 1998) for histone *H3* and SWAM18SF1 5'-GAATGGCTCATTAAA TCAGTCGAGGTTCCTTAGATGATCCAAATC-3' and SWAM 18SR1 5'-ATCCTCGTTAAAGGGTTT

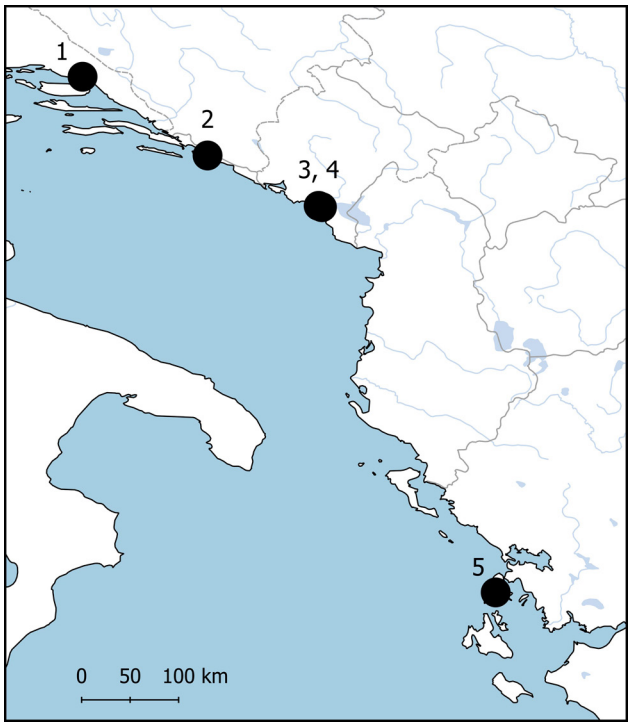


Fig. 1. Localities of the studied populations of *Litthabitella*, numbers as in Table 1

AAAGTGTACTCATTCCAATTACGGAGC-3' (ATTWOOD et al. 2003) for *18SrRNA* nuclear genes. The PCR conditions were as follows: for *H3* – initial denaturation step of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 50°C, 1 min at 72°C, and after all cycles were completed, an additional elongation step of 4 min at 72°C; for *18SrRNA* – initial denaturation step of 4 min at 94°C, followed by 40 cycles of 45 s at 94°C, 45 s at 51°C, 2 min at 72°C and, after all cycles were completed, an additional elongation step of 4 min at 72°C. To check the quality of the PCR products 10 μl of the product was run on a 1% agarose gel. Sequencing methods are described in SZAROWSKA et al. (2014). The PCR products were purified using Clean-Up columns (A&A Biotechnology) and the purified products were amplified in both directions using BigDye Terminator v3.1 (Applied Biosystems), following the manufacturer's protocol and with the primers described above. The sequencing reaction products were purified using ExTerminator Columns

Table 1. Localities of the studied populations of *Litthabitella*

Id	Taxon	Localities	Coordinates	
1	*Litthabitella chilodia*	Croatia, Cetina Valley, Kostanje, spring with water intake, 133 m a.s.l.	43°26'28"N	16°49'22"E
2	*Litthabitella chilodia*	Croatia, Trsteno, spring below the main water intake, ~50 m a.s.l.	42°42'47"N	17°58'51"E
3	*Litthabitella chilodia*	Montenegro, W of Scutari lake, Tomići, small spring 280 m a.s.l.	42°14'49"N	19°00'35"E
4	*Litthabitella chilodia*	Montenegro, W of Scutari lake, W of Sotonići, small spring 183 m a.s.l.	42°14'12"N	19°02'38"E
5	*"Litthabitella ionica"*	Greece, Levkada Island, Sivros, Piges Kerasias springs 260 m a.s.l.	38°40'15"N	20°39'01"E

(A&A Biotechnology); DNA sequences then underwent electrophoresis on an ABI Prism sequencer.

The sequences were initially aligned in the MUSCLE (EDGAR 2004) program in MEGA 6 (TAMURA et al. 2013) and then checked in Bioedit 7.1.3.0 (HALL 1999). Saturation test of XIA et al. (2003) was performed with DAMBE (XIA 2013). The *H3* sequence of *Ecrobia maritima* (Milashewitsch, 1916) was used as outgroup.

Maximum likelihood (ML) approach was conducted in RAxML v8.0.24 (STAMATAKIS 2014). One thousand searches were initiated with starting trees obtained through randomised stepwise addition maximum parsimony method. The tree with the highest likelihood score was considered to be the best representation of the phylogeny. Bootstrap support was calculated with 1,000 replicates and summarised on the best ML tree. RAxML analyses were performed using free computational resources of the CIPRES Science Gateway (MILLER et al. 2010). The Bayesian analyses were run with MrBayes ver. 3.2.3 (RONQUIST et al. 2012) with default priors. Two simultaneous analyses were performed, each lasting 40,000,000 generations with one cold chain and three heated chains, starting from random trees and sampling trees every 1,000 generations. The first 25% of trees were discarded as burnin. The analyses were summarised on a 50% majority-rule tree.

RESULTS

The shells of *Litthabitella chilodia chilodia* (Figs 2–11) are slightly variable, the specimens from Croatia: Kostanje (Figs 2–4) and Trsteno (Figs 5–6) are more tumid than the ones from Montenegro: Tomići (Figs 7–9) and Sotonići (Figs 10–11). The difference, with a slight overlap of the variation ranges, is also reflected by the PCA. The shells of *L. chilodia ionica* (Figs 12–13) are somewhat smaller and more slender; the PCA clearly shows their distinctness (Fig. 14).

The protoconch of *L. chilodia* from Sotonići (Figs 15–17) is formed by 1¾ whorls, growing slowly and regularly, with a broad apex and no macrosculpture (Figs 15–18). The protoconch microsculpture (Fig. 19) is in the form of delicate irregularities of the surface. The border between the proto- and teleoconch is well marked. The operculum (Fig. 20) is elongate-ellipsoidal, spiral, paucispiral, with submarginal nucleus, its growth lines (Figs 21–22) are flat but well marked.

The radula (Figs 23–26) has the central tooth formula:

$$\frac{4-1-4}{2-2}$$

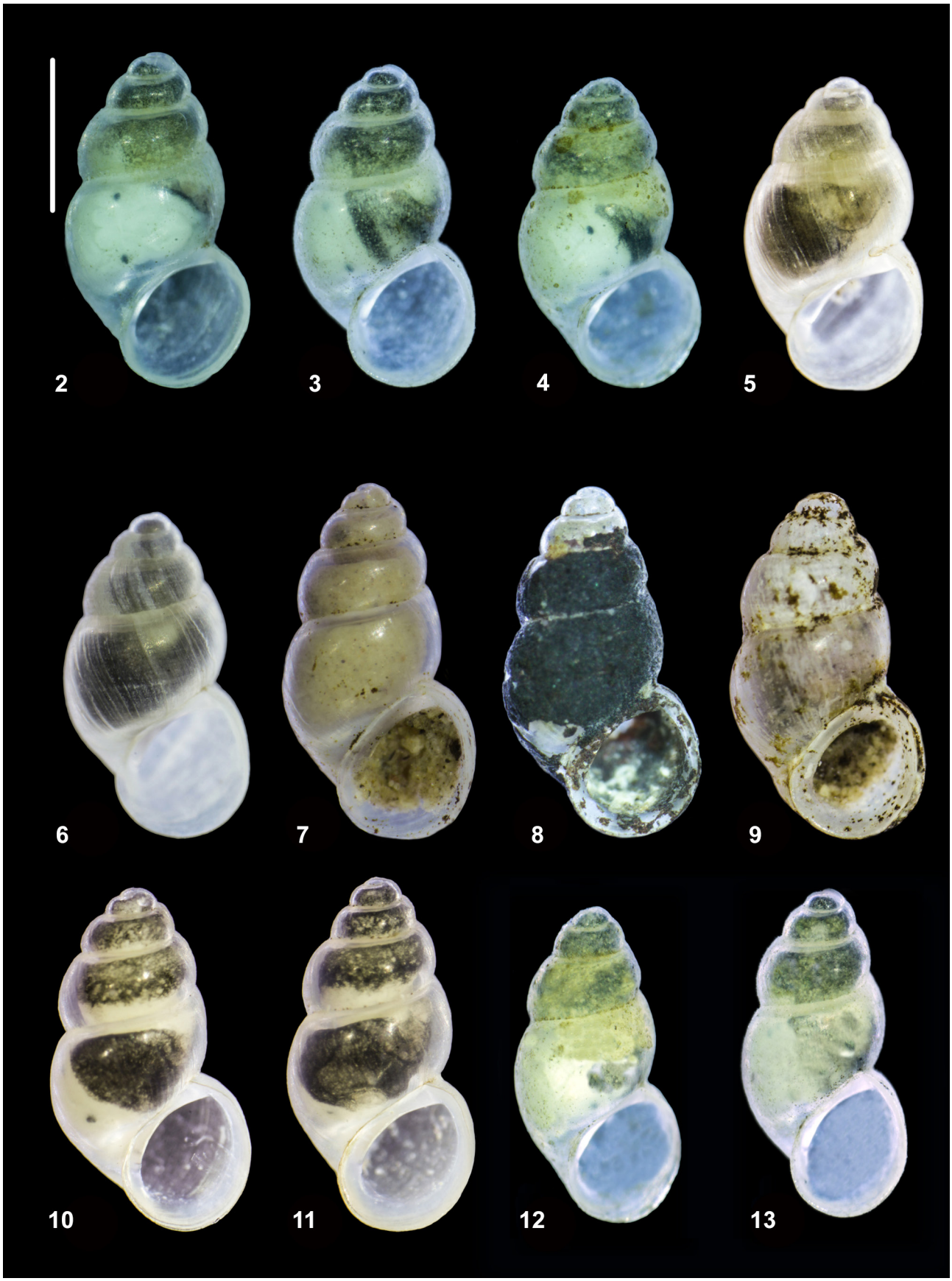
The central cusp is no more than twice longer than the adjacent cusps, all the cusps are rather blunt. There are two pairs of basal cusps (Figs 23–25). Their arrangement is noteworthy: instead of lying one by one along a line more or less parallel to the plate of the tooth, like in nearly all the hydrobioids, or to the lateral margins like in the Bithyniidae, in *Litthabitella* they are arranged one behind the other (Figs 24–25). The lateral tooth (Figs 23–24 and 26) fulfills the formula: 4–1–4, with the cusps similar as on the rhachis. There are about 24 long and sharp cusps on the inner marginal tooth (Figs 24 and 26), and about 8 on the outer marginal tooth.

The penis (Figs 27–28) has a broad and massive base, and terminates with a vast lobe and a narrow, stylet-like terminal part including the vas deferens; ventrally there are two big folds on the lobe (Fig. 28). The female reproductive organs (not shown) are identical to those presented by BOLE (1971), BOETERS (1974) and SZAROWSKA (2006). A few specimens of *L. chilodia* from Levkada ("*ionica*") were used for molecular analysis, thus it was impossible to check their soft parts anatomy and morphology.

The shell measurements (Table 2) and, especially, the PCA of the shell (Fig. 14) clearly separate the Levkada specimens from the other four populations (*L. chilodia chilodia*). They also show – although less well marked – differences between the populations of *L. chilodia chilodia* from Montenegro vs. Croatia; those are visible mostly in the proportions, but also in the shell size.

In total, we obtained 13 sequences of *H3* (283 bp; GenBank Accession numbers KY215955–KY215967) and six sequences of *18SrRNA* (401 bp; GenBank Accession numbers KY215947–KY215952). The saturation tests of XIA et al. (2003) for *H3* revealed no saturation. In all analyses, the topologies of the resulting phylograms were identical in both the maximum likelihood and Bayesian inference.

According to the *H3* analyses, *Litthabitella* was divided into four main clades (Fig. 29). The most distinct was *Litthabitella* from Levkada, with p-distances to other *Litthabitella* clades ranging from 0.016 to 0.021 (Table 3). Such high divergence, characteristic of the species level, confirmed that *Litthabitella* from Levkada was a distinct species. At the same time, p-distances between three *L. chilodia chilodia* clades were 0.006. The sequences from Trsteno in Croatia and Tomići from Montenegro (about 100 km apart) were identical. Small differences were obtained for the sequences from Sotonići which was closest to Tomići (separated by only 3 km). The most dis-



Figs 2–13. Shells of *Litthabitella*: 2–11 – *L. chilodia*: 2–4 – Kostanje (locality 1), 5–6 – Trsteno (locality 2), 7–9 – Tomići (locality 3), 10–11 – Sotonići (locality 4); 12–13 – "*L. ionica*", Levkada Island; bar equals 1 mm

Table 2. Shell morphometrics of *Litthabitella*: localities as in Table 1, measurements as in Fig. 14

Locality	a	b	c	d	e	α	β
1 – Kostanje							
M	2.22	1.09	0.96	0.82	0.88	98.1	18.6
SD	0.13	0.05	0.05	0.06	0.04	1.59	1.65
Max	2.38	2.13	1.02	0.89	0.94	102	21
Min	1.94	0.99	0.87	0.7	0.79	96	16
2 – Trsteno							
M	2.2	1.11	1	0.7	0.88	94.4	15.5
SD	0.07	0.04	0.05	0.05	0.08	1.35	1.08
Max	2.32	1.17	1.06	0.76	0.98	97	18
Min	2.13	1.05	0.92	0.62	0.73	93	14
3 – Tomići							
M	2.42	1.11	1.03	0.98	0.92	96.1	22.6
SD	0.18	0.06	0.06	0.1	0.05	2.17	1.75
Max	2.85	1.27	1.16	1.15	0.98	100	25
Min	2.18	1.05	0.95	0.79	0.84	94	20
4 – Sotonići							
M	2.54	1.62	1.05	1.04	0.95	92.8	19.5
SD	0.13	0.05	0.03	0.08	0.05	3.06	1.04
Max	2.86	1.25	1.1	1.24	1.03	96	21
Min	2.4	1.09	1.01	0.96	0.89	85	18
5 – Levkada I.							
M	2.33	1.27	1.11	0.62	0.96	121.7	12.7
SD	0.1	0.04	0.01	0.13	0.29	2.08	1.15
Max	2.4	1.31	1.12	0.71	0.98	124	14
Min	2.21	1.23	1.1	0.47	0.93	120	12

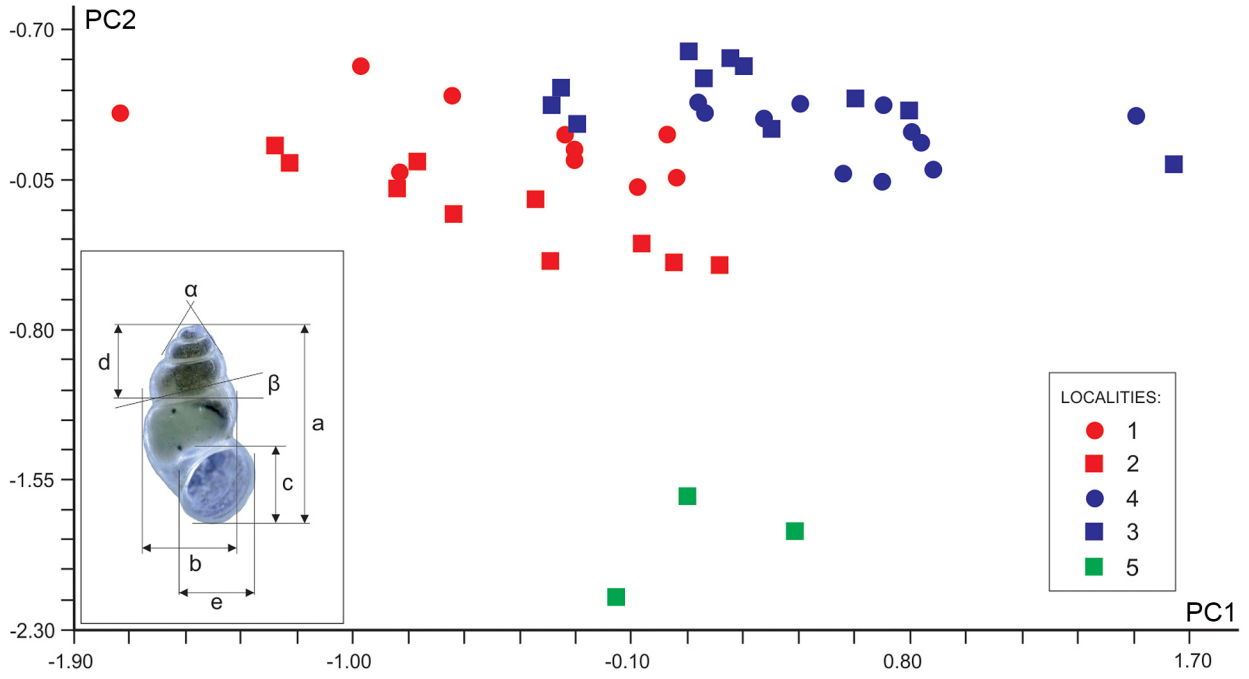
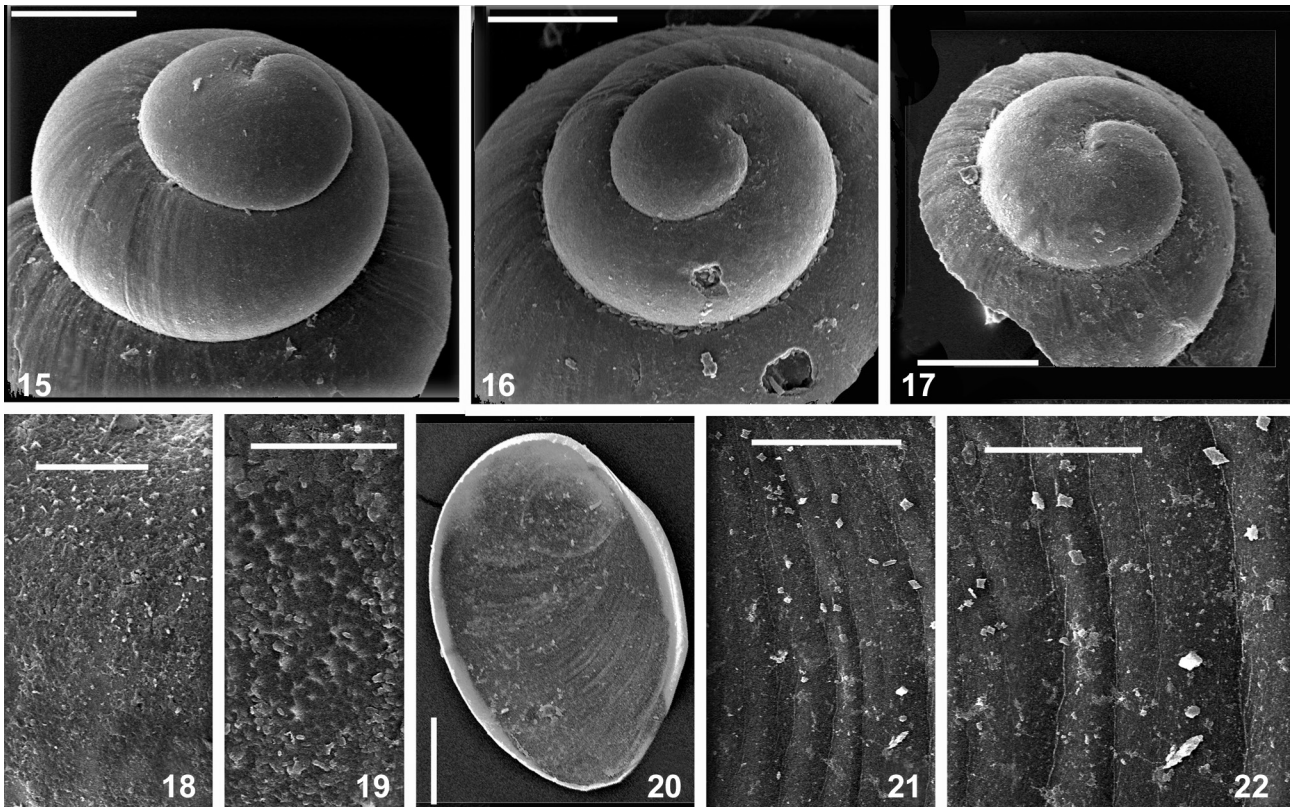
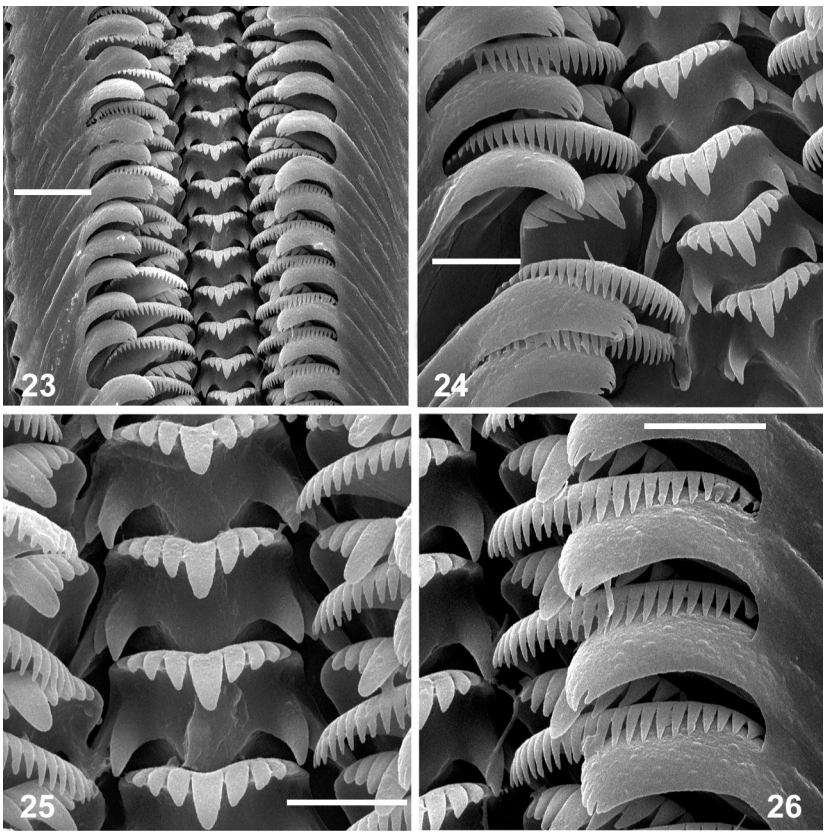


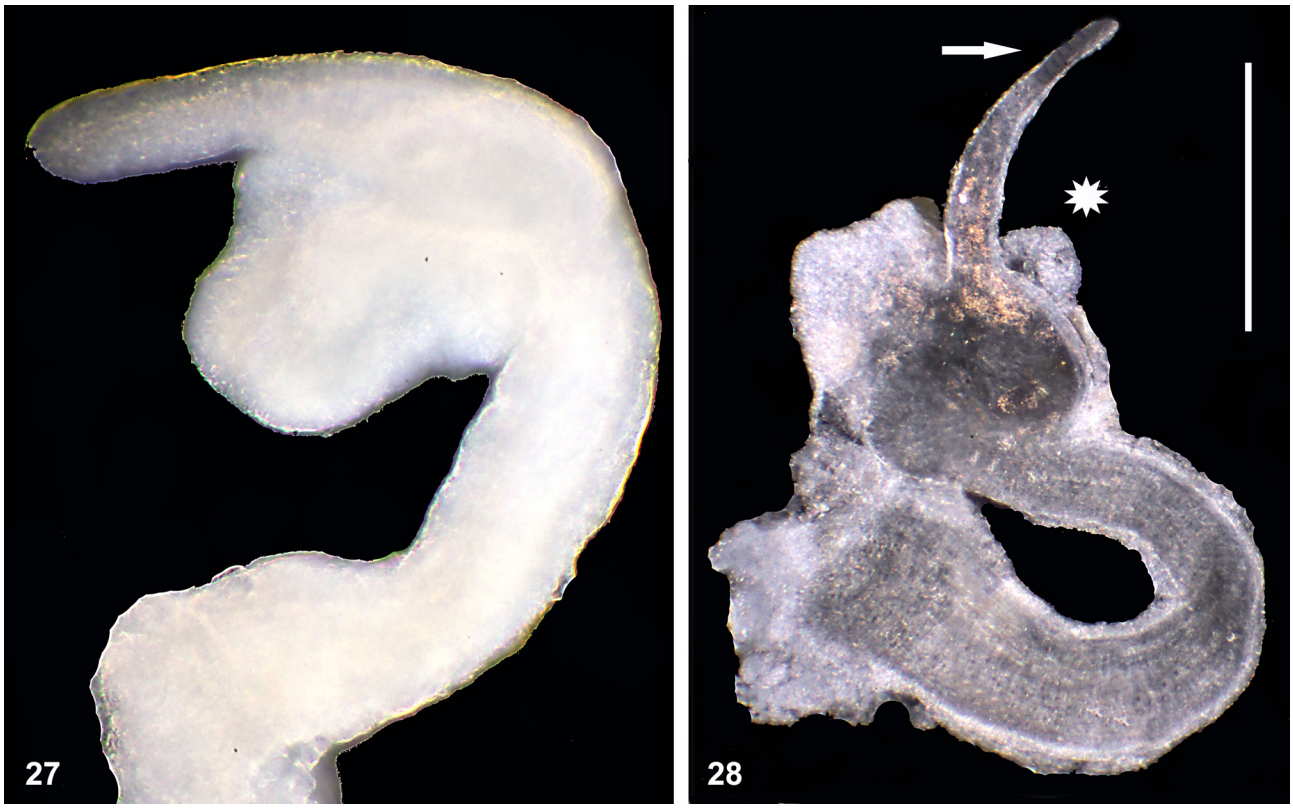
Fig. 14. Shell morphometrics (PCA) of *Litthabitella* for five studied populations. Localities as in Tables 1 & 2. Shell measurements shown: a – shell height, b – body whorl breadth, c – aperture height, d – spire height, e – aperture breadth, α – apex angle, β – angle between body whorl suture and horizontal surface. PC1 explains 60.43% and PC2 explains 19.66%, PC1 and PC2 cumulatively 80.09% of the total variance



Figs 15–22. Protoconch and operculum of *Litthabitella chilodia* from Trsteno (locality 2): 15–17 – protoconch habitus, bar equals 50 μm; 18–19 – protoconch surface, bar equals 10 μm for 18 and 5 μm for 19; 20 – operculum from inner side, bar equals 50 μm; 21–22 – operculum surface, bar equals 20 μm for 21 and 10 μm for 22



Figs 23–26. Radulae of *Litthabitella chilodia* Trsteno (locality 2): 23 – general view, bar equals 4 μm; 24 – half of transverse row, 25 – central tooth, 26 – lateral and marginal teeth; 24–26 – bar equals 2 μm



Figs 27–28. Penes of *Litthabitella chilodia* from Trsteno (locality 2), bar equals 250 μm; arrow indicates terminal part containing vas deferens, asterisk indicates one of the two folds

Table 3. p-distances between *Litthabitella* localities for *H3*. Localities as in Table 1

	Trsteno – 2	Tomići – 3	Sotonići – 4	Kostanje – 1
Tomići – 3	0.000			
Sotonići – 4	0.006	0.006		
Kostanje – 1	0.006	0.006	0.006	
Levkada – 5	0.021	0.021	0.021	0.016

tinct clade of *L. chilodia chilodia* was formed by the sequences from Kostanje, the outermost localities, 125 km from Trsteno and 225 from Tomići/Sotonići. The geographical distance between *L. chilodia ionica* and the nearest localities of *L. chilodia chilodia* is long (about 420 km).

All the *Litthabitella* sequences of *18SrRNA* were identical for all five *Litthabitella H3* subclades. They formed a clearly distinct clade, closest to the lineages of Sadlerianinae and Hydrobiinae (Fig. 30), although outside the Hydrobiidae. The *18SrRNA* p-distance between *Litthabitella* clade and Sadlerianinae/Hydrobiinae was 0.031.

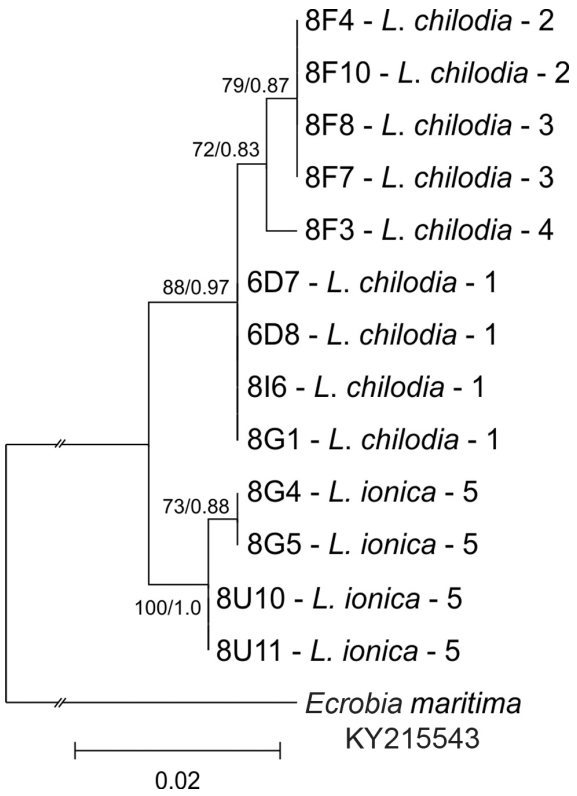


Fig. 29. Maximum-likelihood phylogram for histon *H3* gene. ML tree was rooted by using *Ecrobia maritima*. Bootstrap support (> 70%) and Bayesian posterior probabilities (> 0.8) shown

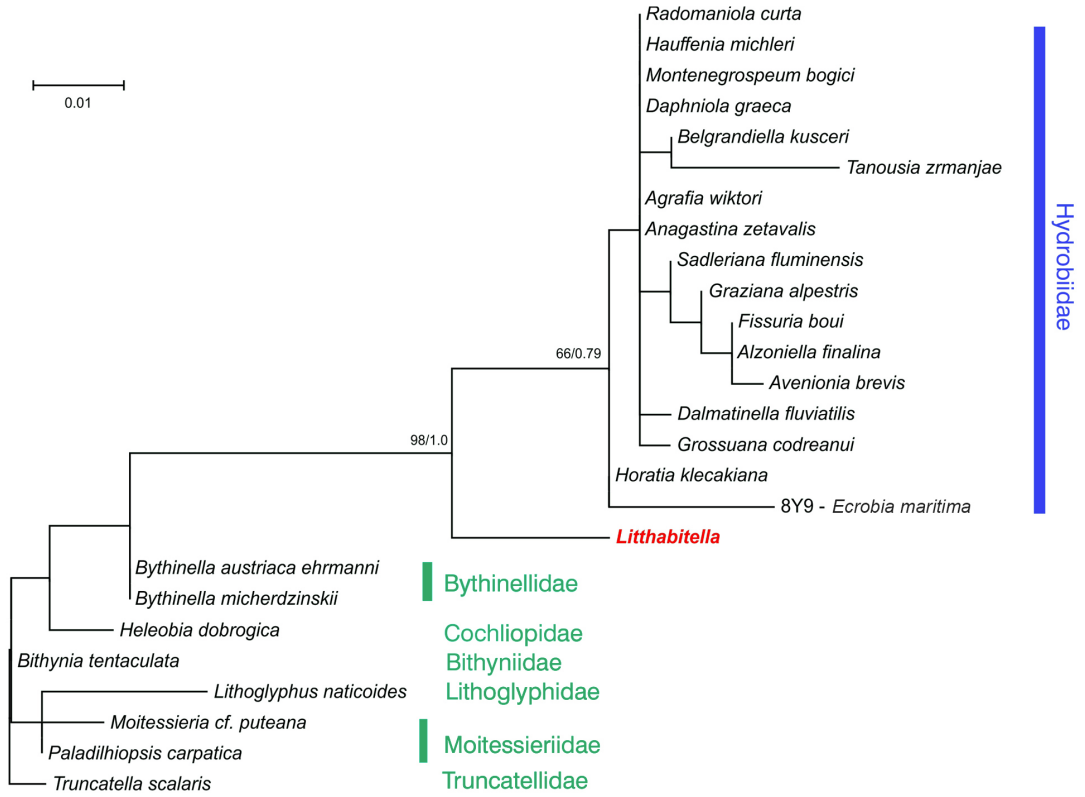


Fig. 30. Maximum-likelihood phylogram for *18SrRNA* gene. Bootstrap supports shown when > 60%

Table 4. GenBank numbers and references to *18SrRNA* sequences

Species	18S rRNA GB#	References
Agrafia wiktori (Szarowska et Falniowski, 2011)	JF906758	Szarowska & Falniowski (2011)
Alzoniella finalina (Giusti et Bodon, 1984)	AF367686	Wilke et al. (2001)
Anagastina zetavalis (Radoman, 1973)	EF070622	Szarowska (2006)
Avenionia brevis berenguieri (Bourguignat, 1882)	AF367670	Wilke et al. (2001)
Belgrandiella kusceri (Wagner, 1914)	JX970574	Wilke et al. (2013)
Bithynia tentaculata (Linnaeus, 1758)	AF367675	Wilke et al. (2001)
Bythinella austriaca ehrmanni (Pax, 1938)	JQ639798	Falniowski et al. (2012b)
Bythinella micherdzinskii (Falniowski, 1980)	JQ639793	Falniowski et al. (2012b)
Dalmatinella fluviatilis (Radoman, 1973)	KC344539	Falniowski & Szarowska (2013)
Daphniola graeca (Radoman, 1973)	EF070624	Szarowska (2006)
Fissuria boui (Boeters, 1981)	AF367690	Wilke et al. (2001)
Graziana alpestris (Frauenfeld, 1863)	AF367673	Wilke et al. (2001)
Grossuana codreanui (Grossu, 1946)	EF061916	Szarowska et al. (2007)
Hauffenia michleri (Kuščer, 1932)	KT236155	Falniowski & Szarowska (2015)
Heleobia dobrogica (Grossu et Negrea, 1989)	EU938133	Falniowski et al. (2008)
Horatia klecakiana (Bourguignat, 1887)	KJ159127	Szarowska & Falniowski (2014)
Lithoglyphus naticoides (C. Pfeiffer, 1828)	AF367674	Wilke et al. (2001)
Moitessieria cf. *puteana* (Coutagne, 1883)	AF367665	Wilke et al. (2001)
Montenegrospeum bogici (Pešić et Glöer, 2012)	KM875509	Falniowski et al. (2014)
Paladilhiopsis carpatica (Soós, 1940)	EF070631	Szarowska (2006)
Radomaniola curta (Küster, 1852)	KC011722	Falniowski et al. (2012b)
Sadleriana fluminensis (Küster, 1853)	AF367683	Wilke et al. (2001)
Truncatella scalaris (Michaud, 1830)	JX970596	Wilke et al. (2013)
Ecrobia maritima (Milaschewitsch, 1916)	KY215953	this study
Litthabitella chilodia (Westerlund, 1886)	KY215947–KY215951	this study
"*Litthabitella ionica*" from Levkada	KY215952	this study

DISCUSSION

Considering the results presented above, namely distinctness in histone 3 locus, coupled with the shell distinctness apparent in the PCA, *Litthabitella* from Levkada should be regarded as a distinct species. The phylogenetic position of *Litthabitella* inferred from *18SrRNA* is very similar to the one presented by SZAROWSKA (2006), despite the differences in the set of taxa included. *Litthabitella* is far from the Nymphophilinae(dae), and does not belong to the Hydrobiidae.

The protoconch habitus and sculpture are similar to those presented by BODON et al. (1999) and SZAROWSKA (2006). FALNIOWSKI (1989) reported differences in the basal cusps arrangement in the "Hydrobioidea" and Bithyniidae: in the former the basal cusps formed a line approximately parallel to the cutting edge of the tooth (terminology after HERSHLER & PONDER 1998), in the latter they were approximately parallel to its lateral margin. In both groups the cusps were arranged in a line parallel to the basal tongue. SZAROWSKA (2006) presented the radula with one pair of basal cusps, SCHÜTT (1980) with three pairs. BODON et al. (1999) presented some blunt and dirty teeth, hardly interpretable. In my radulae of *Litthabitella* there were always two pairs of basal cusps, but situated one behind the other, which had not been seen in any truncatelloid so far. The female reproductive organs, as well as the penes, were identical with those presented by BODON et al. (1999) and SZAROWSKA (2006).

The PCA confirmed the distinctness of the shells of *Litthabitella chilodia* and *Litthabitella* from Levkada. Similarly, histone *H3* confirmed the distinctness of the two taxa. Unfortunately, there were no *COI* sequences, since we failed to amplify the product for this locus, perhaps due to the rather long time from collection of the material. Anyway, the molecular data confirmed the species-level distinctness of *Litthabitella* from Levkada. As stated above, the name *ionica* must not be used for this taxon which should be described as new for the science.

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