Additional molecular data on the protected springsnail species *Bythinella viridis* (Poiret, 1801) (Gastropoda: Bythinellidae) suggest synonymy of related taxa

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Abstract – The taxonomic status of the springsnails *B. viridis*, *B. carinulata* and *B. lanceleveii* remains unclear despite the molecular evidence data provided by Benke *et al.* (2009). Based on extensive sampling and the analysis of COI, 16S, 28S and ITS genes, we investigate analyze the genetic variability of *Bythinella* populations sampled within the area of occurrence of the three nominal taxa. Topotypic populations of *B. lanceleveii* and *B. viridis* cannot could not be distinguished. Some of the populations included in the putative area of distribution range of *B. carinulata* form distinct supported clades, but these distinct clades are not monophyletic and the overall genetic divergence is less than the 3% barcoding gap for species of the genus the barcoding gap of c.a. 3% for *Bythinella* species. Therefore, we propose to synonymize these three nominal species under the name *B. viridis* (Poiret, 1801). Our results have important conservation implications, as they significantly expand the range of the protected species *B. viridis*. This species should be considered in impact studies in a large northeastern quarter of France.

Keywords: Taxonomy / molecular phylogeny / species delimitation / *Bythinella carinulata* / *Bythinella lanceleveii*

Résumé – Des données moléculaires supplémentaires sur l'espèce protégée *Bythinella viridis* (Poiret, 1801) (Gastropoda: Bythinellidae) suggèrent la synonymie des taxons apparentés. Le statut taxonomique des Bythinelles *B. viridis*, *B. carinulata* et *B. lanceleveii* reste mal défini en dépit des données moléculaires fournies par Benke *et al.* (2009). Sur la base d'un échantillonnage extensif et de l'analyse des gènes COI, 16S, 28S et ITS, nous analysons la variabilité génétique des populations de Bythinelles échantillonnées au sein de la zone d'occurrence des trois taxons nominaux. Les populations topotypiques de *B. lanceleveii* et *B. viridis* n'ont pas pu être distinguées. Certaines des populations incluses dans l'aire de répartition supposée de *B. carinulata* forment des clades distincts et soutenus, mais ces clades ne sont pas monophylétiques et la divergence génétique globale est inférieure au barcoding gap de 3% pour les espèces du genre *Bythinella*. Par conséquent, nous proposons de synonymiser ces taxons sous le nom *B. viridis* (Poiret, 1801). Nos résultats ont des implications importantes en termes de conservation, puisqu'ils élargissent considérablement l'aire de distribution de l'espèce protégée *B. viridis*. Cette espèce devrait être prise en compte dans les études d'impact dans un grand quart nord-est de la France.

Mots clés : Taxonomie / phylogénie moléculaire / délimitation d'espèces / *Bythinella carinulata* / *Bythinella lanceleveii*

The springsnail *Bythinella viridis* (Poiret, 1801) is endemic to France and protected by French law. According to available data, this species is restricted to a few springs in the Oise and Marne River catchments (Falkner *et al.*, 2002). *Bythinella carinulata* (Drouët, 1867), also protected by French law was described from a large area around the city of Dijon, including

the Aube and Haute-Marne departments, *i.e.*, south of the distribution area of *B. viridis*. *B. lanceleveii* Locard, 1884 was described from specimens collected in the lower Seine, a few tens of kilometers away from the sea, about 200 km west of the distribution area of *B. viridis*.

Bythinella carinulata (Drouët, 1867) is revalidated by Boeters (1998) from material collected in the upper Seine drainage. *B. lanceleveii* is morphologically very close, if not identical, to *B. viridis*, but Falkner *et al.* (2002) consider it as a

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distinct species, based on the fact that it occurs 200 km away from the nearest localities of *B. viridis*. For the establishment of the IUCN red list, Bichain and Prié (2010) considered *B. lancevelei* as a synonym of *B. viridis* based on the molecular results of Bichain (2007a,b). Benke et al. (2009) published a review of the phylogeography of *Bythinella* species throughout Europe based on molecular data and their results suggest *B. viridis* and *B. lancevelei* should be synonymized, but the authors do not discuss the taxonomic implications of their results. In the absence of a taxonomic conclusion by Benke et al. (2009), Gargominy et al. (2011) maintained *B. lancevelei* and *B. carinulata* as distinct species in the French checklist. More recently, Pouchard and Bichain (2013) and Pouchard (2018) have published new localities for *B. lancevelei*. Finally, the two electronic taxonomic registers TAXREF (French register, Gargominy et al., 2020) and MolluscaBase (global register of molluscs, MolluscaBase 2021) follow the validity of that species.

In this note we give additional molecular data from 15 localities from north-east France and address the question of the taxonomic status of the currently valid species in the *Bythinella viridis* group.

Family Bythinellidae Locard, 1893

Genus *Bythinella* Moquin-Tandon, 1856

Bythinella viridis (Poiret, 1801)

Original combination: *Bulimus viridis* Poiret, 1801: 45–46.

Type locality: « Le ruisseau qui tombe en cascade de la montagne en bas de laquelle est situé le moulin de Veau, proche de Chartreuve [nowadays Chery-Chartreuve, in the Aisne department] »

Distribution: according to Boeters (1998) and Falkner et al. (2002), restricted to a few springs in the Oise and Marne Rivers catchments. The type locality of *B. viridis* is unambiguous and still hosts the species (locality 1 in Tab. 1). COI sequences from topotypes are provided by Benke et al. (2009) and Bichain et al. (2007b); ITS sequence fragments come from Bichain et al. (2007a). *Bythinella viridis* is protected by French law and categorized as ENB1ab(iii,iv)+2ab(iii,iv) by the IUCN red list (Bichain and Prié, 2010).

Bythinella lancevelei Locard, 1884

Original combination: *Bythinella lancevelei* Locard, 1884.

Type locality: Villequier (Seine-Maritime), « (...)sous les pierres submergées du ruisseau du chemin du Nord, qui coule vers la Seine ».

Distribution: according to Falkner et al. (2002), known only from the type locality, but Pouchard and Bichain (2013) and Pouchard (2018) give a few more locations in the Eure and Seine-Maritime departments. Specimens of *B. lancevelei* collected from the spring of La Roquette in Villequier are here considered as topotypes. COI, 16S and 28S genes fragments were amplified by Benke et al. (2009). Additional COI gene fragments from three specimens sampled in Fort-Movil, about 30 km south-west of the type locality, were included in the analysis (Fig. 1, Tab. 1).

Bythinella lancevelei was considered as a synonym of *B. viridis* by Germain (1931), but re-established by Falkner et al. (2002). *B. lancevelei* is considered synonym of *B. viridis* by the IUCN red list (Bichain and Prié, 2010).

Bythinella carinulata (Drouët, 1867)

Original combination: *Hydrobia carinulata* Drouët, 1867: 90–91.

Type locality: « Habite les sources au pied de la Côte (calcaire jurassique), sur les pierres : fontaine de Larrey, près Dijon (type) ! source de la Norges ! la Douix, à Chatillon-sur-Seine !... (...) Je l'ai recueilli dans l'Aube et la Haute Marne dans les mêmes circonstances ».

B. carinulata has five recognized subjective synonyms.

- *Turbo griseus* Vallot, 1801. Vallot (1801) does not give any precise type locality, but the original publication deals with the Côte d'Or department). The name *Turbo griseus* was considered *nomen oblitum* by Boeters (1974) and Falkner et al. (2002) considered that the strict application of the priority was not advisable in this case.
- *Paludinella turgidula* Paladilhe, 1869. The type locality is Billy-les-Chanceaux (source de la Seine) and Paladilhe (1869) adds « dans le département de l'Aube, aux environs de Bar-sur-Seine et des Riceys ».
- *Paludinella scalarina* Paladilhe, 1876. Châtillon-sur-Seine (Côte d'Or) and Boutigny (Seine-et-Marne).
- *Bythinella burgundina* Locard, 1893. Locard (1893) attributes this name to Beaudouin (1890) [sic], probably referring to Beaudouin (1888). This nominal species is known from pits in Châtillon-sur-Seine (Côte d'Or).
- *Bythinella viridiformis* R. Bernasconi, 1989. The type locality is Barbirey-sur-Ouche (Côte-d'Or). Bernasconi (1989) gives a large distribution including the Plateau de Langres and the départements of the Côte-d'Or, Haute-Marne, Haute-Saône and Vosges.

Boeters (1998) re-established the name *Bythinella carinulata* and considered *Paludinella turgidula* Paladilhe, 1869, from Ricey and Bar-sur-Seine (Aube department) as a junior synonym. According to Falkner et al. (2002), the specimens from Barbirey-sur-Ouche (Côte-d'Or department) studied by Bernasconi (1989) would also belong to *B. carinulata*, although Bernasconi himself eventually considered them as synonyms of *B. viridis* (Bernasconi com. pers. in Falkner et al., 2002). *B. carinulata viridiformis* is considered by Fauna Europaea as synonym of *B. carinulata*, but is not considered in MolluscaBase.

Distribution: Drouët (1867) gives a wide range for this species, encompassing the drainages of the Seine and the Rhône Rivers (Fig. 1).

The type locality of *Bythinella carinulata* (“fontaine de Larrey, près Dijon”) should be the actual Fontaine d'Ouches, which springs north of the Larrey district, in the city of Dijon. No *Bythinella* live in this spring anymore (E. Farat, com. pers.). According to Boeters (1998), the populations from Bar-sur-Seine are conspecific with *B. carinulata*. Benke et al. (2009) used specimens collected in July-sur-Sarce, which lies about five kilometers away from Bar-sur-Seine, and considered them as *B. carinulata*. This later locality can be used as ergonymotope (*sensus* Frétey et al., 2018) for this species. We used the COI, 16S and 28S genes fragments amplified by Benke et al. (2009) for *B. carinulata*. *Bythinella carinulata* is protected by French law and categorized as EN B1ab(iii)+2ab(iii) by the IUCN red list (Prié, 2010).

1 Material and methods

Morphological characters and anatomy are not relevant for species delimitation in the *Bythinella* genus

Table 1. specimens and localities.

Nominal species	Locality	Locality #	Lat N.	long E	Specimen #	GenBank #			References
						COI	16S	28S	
<i>Bythinella viridis</i>	Chery Chartreuse	1	49.2592° N	3.6333° E	3169 Moll5959/399-1 Moll5959/Che-1 Moll5959/Che-3	FJ029102 EF016165	FJ028871		Benke <i>et al.</i> , 2009 Bichain <i>et al.</i> , 2007b Bichain <i>et al.</i> , 2007a Bichain <i>et al.</i> , 2007a This study This study This study This study This study This study This study This study This study This study
	Montdauphin	2	48.8559° N	3.4172° E	B17836	KP765282			
	Saint-Eugene	3	49.0111° N	3.5115° E	B17837	KP765283			
	Epoux-Bezu	4	49.0960° N	3.3931° E	B17813	KP765280			
	Saint-Mihiel	5	48.8870° N	5.6092° E	B17798	KP765277			
					B17808	KP765276			
	Troyon	6	49.0047° N	5.5295° E	B17812	KP765278			
					B2085	KP765279			
					B2086	KP765264	KP765288	KP765257	
		Saint-Joire	7	48.5803° N	5.4341° E	B2087	KP765265	KP765289	
<i>Bythinella sp.</i>					B2087	KP765266	KP765290	KP765258	
					B2089	KP765267	KP765291		
					B2095	KP765267	KP765296		
					B2096	KP765272	KP765297	KP765255	
	Treveray	8	48.5937° N	5.3994° E	B2097	KP765273	KP765298		
					B2098	KP765274	KP765299		
					B2099	KP765275	KP765300		
					B2090	KP765268	KP765292		
					B2091	KP765269	KP765293		
	Houdelaincourt	9	48.5423° N	5.4787° E	B2092	KP765270	KP765294	KP765254	KP765261
<i>Bythinella lanceleviei</i>					B2094	KP765271	KP765295		
	Lamorville	10	48.9844° N	5.6386° E	2926	OL310181	OL322510		OL322501
					2927	OL310182	OL322511		OL322502
	Lamorville	11	48.9707° N	5.6392° E	2928	OL310183	OL322512		OL322503
					2929	OL310184	OL322513		OL322504
	Villequier, Bois du Nord, La Roquette	12	49.5197° N	0.6880° E	3794	FJ029064	FJ028871		
					3795	FJ029065	FJ028872		
					3796	FJ029066		FJ028892	
	Fort-Moville, Source-Vannier	13	49.3286° N	0.4133° E	B17818	KP765281			
					B17843	KP765284			
<i>Bythinella carimulata</i>					B17844	KP765285			
					2949	FJ029099			
	July-sur-Sarce, Fontaine du Gris	14	48.0978° N	4.3003° E	2950	FJ029100			
					2951	FJ029101		FJ028896	
	Lafauche	15	48.3032° N	5.4800° E	2930	OL310185	FJ028884		
					2931	OL310186	OL322514		
<i>Bythinella</i>	Thonnance-les-Moulins	16	48.4088° N	5.2931° E	2924	OL310179	OL322515	OL322505	This study
					2925	OL310180	OL322508	OL322499	This study
	Vals-des-Tilles	17	47.7171° N	5.0910° E	2932	OL310187	OL322509	OL322500	This study
					2933	OL310188	OL322516	OL322506	This study
<i>Bythinella</i>	Pancey	18	48.4676° N	5.2915° E	B2083	KP765262	KP765286	KP765256	This study
					B2084	KP765263	KP765287	KP765260	This study

Fig. 1. Known *Bythinella* records in the area (black dots), sampled sites (red stars) and putative distribution of the three nominal species. Sampling sites numbers refer to Table 1. Circles: type localities; square: ergonymotope for *B. carinulata*. The green polygon refers to the distribution of *B. carinulata* according to Drouët (1867). Black dotted lines represent the major watershed limits (Seine, Meuse and Rhône drainages). Scale bar: 100 km.

(Bichain *et al.*, 2007a, b). We thus relied on the genetic distances between specimens to delimit species. Following a barcoding approach, we used the pairwise distribution of the genetic distances calculated with the Barcode gene COI to identify a gap in the distribution, referred to as the “barcoding gap” (Puillandre *et al.*, 2012), between intra and interspecific distances. A total of 1074 COI sequences of *Bythinella* species downloaded from GenBank (Dataset 1) were used to estimate the barcoding gap (supplementary material 1). MEGA 10.05 (Tamura *et al.*, 2011) was used to calculate the p-distances (following Srivathsana and Meier, 2012) between each pair of specimens. The distribution of the genetic distances was then calculated using an excel sheet (Supplementary material 1). The COI barcoding gap for the *Bythinella* genus was found to be around 3%. This result is congruent with the species level divergence observed in other *Bythinella* taxonomic papers (Bichain *et al.*, 2007a, b; Prié and Bichain, 2009).

We analyzed a total of 48 specimens from 18 localities (Tab. 1). Extraction and amplification were performed using

standard protocols (ex. Bichain *et al.*, 2007b). Total genomic DNA was extracted using the Qiagen DNA Mini Kit and following the manufacturer’s protocol. Universal primers were used for the amplification of 16S (Palumbi *et al.*, 1991) and 28S (Park and O’Foighil, 2000). For ITS and COI, we used the primers designed by Bichain *et al.* (2007a, b).

Sequences were automatically aligned using CLUSTALW multiple alignments implemented in BIOEDIT 7.2.5 (Hall, 1999). The accuracy of automatic alignments was confirmed by eye. Only a few gaps, unambiguously aligned, were inferred for the 28S, ITS and 16S genes: they were conserved for the analyses.

We obtained 34 sequences for the COI gene (Tab. 1), resulting after alignment in a 557 bp fragment. Seven additional sequences downloaded from GenBank were included in the dataset (two sequences for both *B. viridis* and *B. lanceolevei*, three sequences for *B. carinulata*). A total of 25 sequences were obtained for the 16S gene, and four additional sequences were included in the dataset (two for *B. viridis*, one

Fig. 2. Phylogenetic tree based on Bayesian inference, using the COI gene. Only posterior probabilities > 0.8 are shown. Numbers refer to the sampled localities (Fig. 1, Tab. 1). Scale bar (0.3) indicate the minimum inter-specific genetic divergence for the COI gene (barcoding gap). Top left: distribution of the genetic divergence within the genus *Bythinella* illustrating the barcoding gap.

for *B. carinulata* and one for *B. lanceleveii*), resulting after alignment in a 502 bp fragment. Three sequences were obtained for the 28S gene, and two additional sequences are available from GenBank (one for *B. lanceleveii* and one for *B. carinulata*). After alignment, 28S gene sequences resulted in a 799 bp fragment. Fourteen sequences were obtained for the ITS gene, and two additional sequences (*B. viridis*) were downloaded from GenBank. After alignment, ITS gene sequences resulted in a 267 bp fragment.

For phylogenetic analyses of the COI gene fragment, *B. badensis* Boeters, 1981 and *B. cebennensis* (Dupuy, 1849) were chosen as outgroups, following the results of Benke *et al.* (2009). Bayesian analysis were performed running two parallel analyses in MRBAYES V 3.1.2 (Huelsenbeck *et al.*, 2001), each consisting of two Markov chains of 5 000 000 generations each, sampled every 1 000 generations. We defined three partitions (one for each codon position). Convergence of the analysis was checked using TRACER V 1.7.1 (Rambaut *et al.*, 2018); all ESS values were > 200 (default burnin). A consensus tree was then calculated after omitting the first

10% trees as burn-in. Chains had converged after 3.000.000 generations.

2 Results

COI gene phylogeny of the genus *Bythinella* (based on Dataset 1) confirmed the monophyly of all studied populations (results not shown). Within this clade, corresponding to clade V of Benke *et al.* (2009), *B. viridis* and *B. lanceleveii* topotypes could not be distinguished. Some populations of the putative *B. carinulata* formed distinct supported clade, but these were not monophyletic (Fig. 2). These results confirm those of Benke *et al.* (2009). The 16S gene was little variable (overall p -distance=0.01). The topotypes of *B. viridis* and *B. lanceleveii* shared the same haplotype for this gene. Regarding the nuclear genes, all the specimen studied shared the same haplotype for the 28S fragment. The ITS gene fragment was a bit more variable (overall p -distance=0.07). Topotype specimens of *B. viridis* had an insertion (AGA) in

position 99 and a G in position 238. For all gene fragments, nominal species and newly sequenced specimens could not be distinguished.

Average genetic distance for COI gene was 0.0093. Maximum distance observed was 0.0250 between specimens of *B. carinulata* from July-sur-Sarce (ergonymotope, locality 14) and specimens presumably of the same species from Vals-des-Tilles (locality 17, the closest locality to the type locality in Dijon). The genetic distance between *B. viridis*, *B. lancelevei* and *B. carinulata* was below the barcoding gap threshold, suggesting that all the specimens analyzed belong to the same species.

3 Discussion

B. viridis is the oldest name available. In our opinion, it makes no doubt that *B. lancelevei* is a junior synonym of *B. viridis*. There is no morphological argument to jeopardize the genetic data. *B. lancelevei* was considered a distinct species by Falkner *et al.* (2002) only based on one argument: the geographic distance from *B. viridis*. However, (i) the known populations of *B. lancelevei* are in the same drainage as the known populations of *B. viridis* (Seine River drainage) and (ii) some localities may have been overlooked in between. The apparent isolation between the populations of the lower Seine (*B. lancelevei*) and the populations from the upper Seine drainage (*B. viridis*) could result from human impact or other recent geographic isolation, but is very likely a collection bias. Regarding *B. carinulata*, although all the specimens studied formed a distinct clade in COI and 16S, genetic divergence was very low, suggesting it should also be synonymized with *B. viridis*.

In the absence of contradictory evidence, we propose to synonymize *B. viridis*, *B. lancelevei* and *B. carinulata* under the name *Bythinella viridis* (Poiret, 1891). As *B. viridis* is protected in France, conservation measures and consideration in impact studies should apply throughout its entire range, *i.e.*, from the lower Seine west (Fig. 1, 13) to the Meuse drainage east (Fig. 1, 11) and down to the upper Rhône south (Fig. 1, 17).

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