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## 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. lancevevei* 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. lancevevei* 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 lancevevei*

**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. lancevevei* 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. lancevevei* 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 lancevevei*

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. lancevevei* 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. lancevevei* 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. lancevevei* 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. lancevevei* 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. lancevevei* 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. lancevevei*. 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 lancevevei* Locard, 1884

Original combination: *Bythinella lancevevei* 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. lancevevei* 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 lancevevei* was considered as a synonym of *B. viridis* by Germain (1931), but re-established by Falkner et al. (2002). *B. lancevevei* 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).

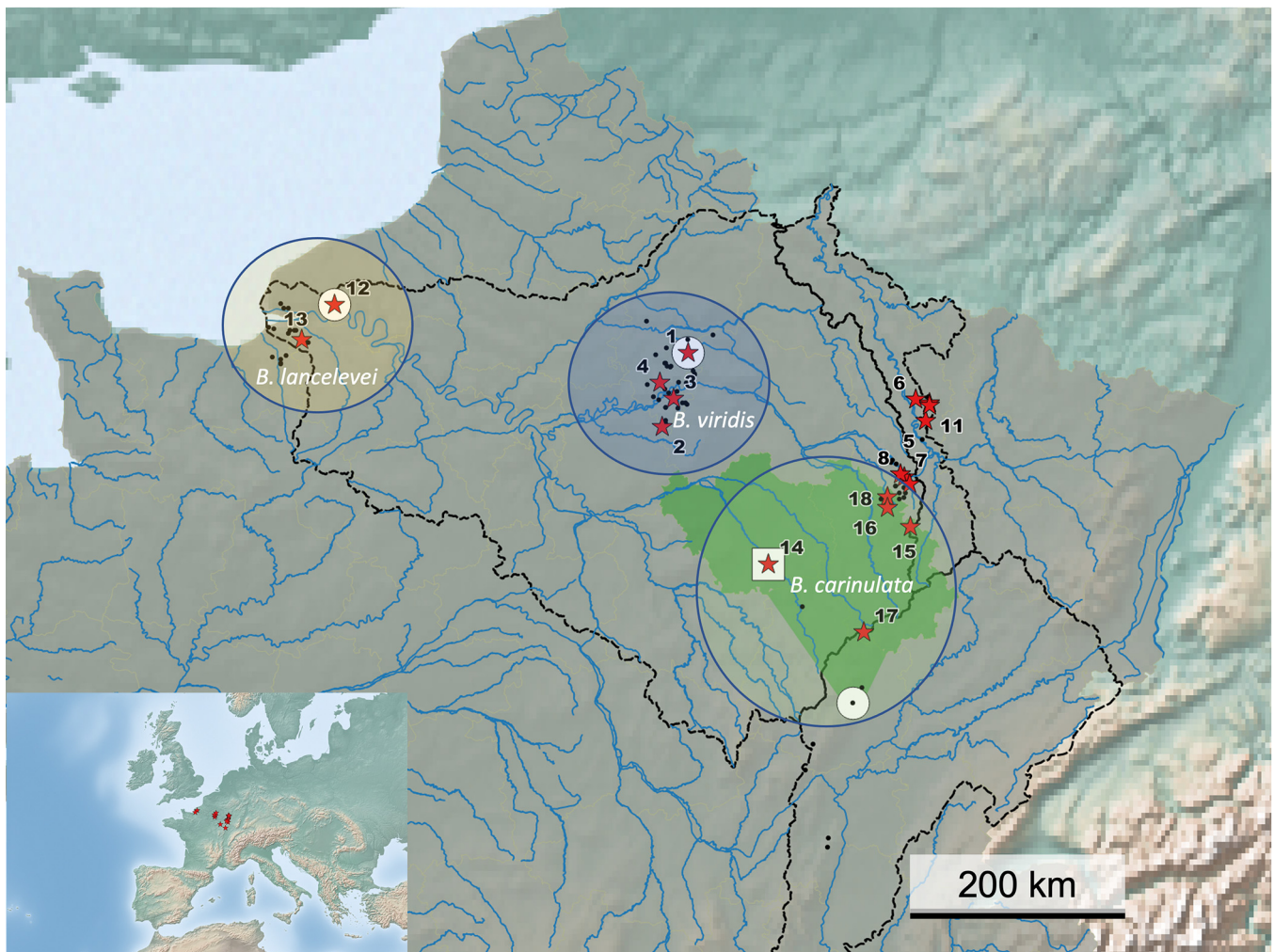
The type locality of *Bythinella carinulata* (“fontaine de Larrey, près Dijon”) should be the actual Fontaine d'Ouches, which springs north of the Larrey district, in the city of Dijon. No *Bythinella* live in this spring anymore (E. Farat, com. pers.). According to Boeters (1998), the populations from 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	ITS	
<i>Bythinella viridis</i>	Chery Chartreuse	1	49.2592° N	3.6333° E	3169	FJ029102	FJ028871		Benke <i>et al.</i> , 2009
					Moll5959/399-1	EF016165	DQ318901	Bichain <i>et al.</i> , 2007b	
	Montdauphin	2	48.8559° N	3.4172° E	Moll5959/Che-1			DQ318902	Bichain <i>et al.</i> , 2007a
					Moll5959/Che-3				Bichain <i>et al.</i> , 2007a
					B17836	KP765282			This study
					B17837	KP765283			This study
					B17813	KP765280			This study
					B17803	KP765277			This study
	Epoux-Bezu	4	49.0960° N	3.3931° E	B17798	KP765276			This study
					B17808	KP765278			This study
	Saint-Mihiel	5	48.8870° N	5.6092° E	B17808	KP765278			This study
					B17812	KP765279			This study
	Troyon	6	49.0047° N	5.5295° E	B2085	KP765264	KP765288	KP765257	This study
					B2086	KP765265	KP765289		This study
	Saint-Joire	7	48.5803° N	5.4341° E	B2087	KP765266	KP765290	KP765258	This study
					B2089	KP765267	KP765291		This study
	Treveray	8	48.5937° N	5.3994° E	B2095	KP765296			This study
					B2096	KP765272	KP765297	KP765255	This study
Houdelaincourt	9	48.5423° N	5.4787° E	B2097	KP765273	KP765298		This study	
				B2098	KP765274	KP765299		This study	
Lamorville	10	48.9844° N	5.6386° E	B2099	KP765275	KP765300		This study	
				B2090	KP765268	KP765292		This study	
Lamorville	11	48.9707° N	5.6392° E	B2091	KP765269	KP765293		This study	
				B2092	KP765270	KP765294	KP765261	This study	
Villequier, Bois du Nord, La Roquette	12	49.5197° N	0.6880° E	B2094	KP765271	KP765295		This study	
				2926	OL310181	OL322510	OL322501	This study	
Fort-Movile, Source-Vannier	13	49.3286° N	0.4133° E	2927	OL310182	OL322511	OL322502	This study	
				2928	OL310183	OL322512	OL322503	This study	
July-sur-Sarce, Fontaine du Gris	14	48.0978° N	4.3003° E	2929	OL310184	OL322513	OL322504	This study	
				3794	FJ029064	FJ028871		Benke <i>et al.</i> , 2009	
Lafauche	15	48.3032° N	5.4800° E	3795	FJ029065	FJ028872		Benke <i>et al.</i> , 2009	
				3796	FJ029066			Benke <i>et al.</i> , 2009	
Thonnance-les-Moulins	16	48.4088° N	5.2931° E	B17818	KP765281			This study	
				B17843	KP765284			This study	
Vais-des-Tilles	17	47.7171° N	5.0910° E	B17844	KP765285			This study	
				2949	FJ029099			Benke <i>et al.</i> , 2009	
Pancey	18	48.4676° N	5.2915° E	2950	FJ029100			Benke <i>et al.</i> , 2009	
				2951	FJ029101	FJ028892		Benke <i>et al.</i> , 2009	
<i>Bythinella lanceleveii</i>	Lafauche	15	48.3032° N	5.4800° E	2930	OL310185	FJ028884		This study
					2931	OL310186	OL322514	OL322505	This study
<i>Bythinella carminulata</i>	Thonnance-les-Moulins	16	48.4088° N	5.2931° E	2924	OL310179	OL322508	OL322499	This study
					2925	OL310180	OL322509	OL322500	This study
Pancey	18	48.4676° N	5.2915° E	2932	OL310187	OL322516	OL322506	This study	
				2933	OL310188	OL322517	OL322507	This study	
<i>Bythinella lanceleveii</i>	Pancey	18	48.4676° N	5.2915° E	B2083	KP765262	KP765286	KP765260	This study
					B2084	KP765263	KP765287		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. lancelevei*, 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



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. lancevevei* 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. lancevevei* is a junior synonym of *B. viridis*. There is no morphological argument to jeopardize the genetic data. *B. lancevevei* 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. lancevevei* 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. lancevevei*) 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. lancevevei* 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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