Phylogenetic relationships and description of a new stygobite species of *Bythinella* (Mollusca, Gastropoda, Caenogastropoda, Amnicolidae) from southern France

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ABSTRACT

A population of the aquatic gastropod *Bythinella* that does not conform to any described hypogean or epigean species of this genus was discovered in a cave stream in southern France (Folatière cave). Members of this population lacked body pigments and eyespots, which suggests adaptation to a subterranean environment. Mitochondrial COI sequences revealed two lineages within this population, separated by a genetic divergence higher than the inter-specific threshold commonly admitted for hydrobioid species. In contrast, a phylogeny based on the nuclear ITS1 gene clustered the sequenced hypogean specimens in a single clade. Moreover multivariate analyses performed on shell parameters did not enable discrimination between hypogean specimens attributed to each of these two mitochondrial lineages. The high COI variability within the same population and conflict between mtDNA and nDNA trees could be explained by a scenario of introgression by hybridization. Consequently, based on nuclear gene phylogeny, shell and anatomical characters, and ecological evidences, we hypothesize that the easily recognizable hypogean morph of the Folatière cave can be attributed to a distinct and new species – here described and named *B. navacellensis* n. sp. Our extensive sampling suggests that *B. navacellensis* n. sp. is distributed within a small delimited and cohesive area located around southern Larzac, covering *c.* 300 km^2. Considering its distribution, restricted to a few subterranean aquifers only, *B. navacellensis* n. sp. can be categorised as a vulnerable species, VU D2.

KEY WORDS
Mollusca,
Gastropoda,
Bythinella,
DNA-taxonomy,
France,
introgression by hybridization,
stygobite,
subterranean habitat,
IUCN red list,
new species.

RÉSUMÉ

Relations phylogénétiques et description d'une nouvelle espèce stygobie du genre Bythinella *(Mollusca, Gastropoda, Caenogastropoda, Amnicolidae) du sud de la France.*

Une population de gastéropodes aquatiques du genre *Bythinella*, ne correspondant à aucune espèce décrite, a été découverte dans une rivière souterraine dans le sud de la France (la grotte de la Folatière). Les individus de cette population sont dépigmentés et anophtalmes, suggérant une adaptation à l'environnement souterrain. Les séquences du gène mitochondrial COI ont révélé deux lignées au sein de cette population, avec une divergence génétique supérieure à la moyenne de variabilité interspécifique communément admise pour les hydrobies. Toutefois, une phylogénie sur la base du gène nucléaire ITS1 regroupe tous les individus séquencés dans le même clade. De plus, les analyses multivariées réalisées sur les paramètres morphologiques de la coquille n'ont pas permis de discriminer ces deux lignées mitochondriales. La forte variabilité du gène COI au sein de la même population et le conflit entre les arbres de l'ADN mitochondrial et nucléaire pourraient s'expliquer par un scénario d'introgression par hybridation. En conséquence, en nous appuyant sur la phylogénie du gène nucléaire, les caractères anatomiques et morphologiques de la coquille et les particularités écologiques, nous posons l'hypothèse que ce morphe facilement reconnaissable peut être attribué à une espèce nouvelle – décrite ci-après et nommée *Bythinella navacellensis* n. sp. Notre échantillonnage intensif suggère que *B. navacellensis* n. sp. est limitée à une petite zone cohésive des causses du Larzac méridional et de Blandas, couvrant environ 300 km². En raison de sa distribution restreinte à quelques aquifères souterrains, *B. navacellensis* n. sp. peut être catégorisée comme vulnérable VU D2.

MOTS CLÉS
Mollusca,
Gastropoda,
Bythinella,
taxonomie moléculaire,
France,
introgression par hybridation,
stygobie,
milieu souterrain,
liste rouge UICN,
espèce nouvelle.

INTRODUCTION

The genus *Bythinella* Moquin-Tandon, 1856 consists of small hydrobioid springsnails widespread from northern Spain to eastern Turkey. This group is considered as one of the most diverse amongst European hydrobioids, with 91 species and subspecies (Boeters 2006, 2008; Glöer 2006; Glöer & Pesic 2006; Haase *et al.* 2007; Boeters & Falkner 2008; Fauna Europaea Web Service 2009), and France, with its 49 alleged valid taxa, is its main centre of species richness.

Bythinella species live mainly in springs and in the uppermost courses of small streams (Falniowski 1987). However, five taxa from France are stygobite, i.e. living strictly in underground waters: *B. pupoides phreaticola* Bernasconi, 1989 and *B. geisserti* Boeters & Falkner, 2003 were described from hyporheic zones in the French Jura region; *B. padiraci* Locard, 1903 was described from the Padirac karstic network in the Lot (see Bichain *et al.* 2004); *B. bouloti* Girardi, Bichain & Wienin, 2002 and *B. galerae* Girardi, Bichain & Wienin, 2002 are both restricted to a few caves in the Grands Causses of Lozère.

Because access to, and sampling in, hypogean habitats are difficult, *Bythinella* species have been described mainly from very few living animals or from empty shells only, sometimes collected outside the subterranean networks. Consequently, species delimitations and/or ecological status (stygobite vs. stygoxene, i.e. strict vs. occasional subterranean species) of hypogean *Bythinella* populations are still unclear and subject to various opinions. For example, Bertrand (2004) recognized five stygobite

Bythinella taxa whereas Falkner (*in* Ferreira *et al.* 2003) recognized seven. Additionally, Bernasconi (2000) recognized *Bythinella eutrepha* (Paladilhe, 1867) as an occasional hypogean species, widespread in southeastern France whereas Falkner (*in* Ferreira *et al.* 2003) considers it as a strictly stygobite species endemic to the Lez catchment.

One of us (VP, August 2003) collected *c.* 45 living *Bythinella* specimens in the underground waters of the Folatière cave (Fig. 1A, B) in southern France. Morphologically, they did not correspond to any described species. Subsequently, empty shells of this morph were also sampled in 21 other subterranean localities of the Larzac and Blandas plateaus, a large karstic zone located north of Montpellier (France). No living specimens were found in any of these localities.

In order to explore the taxonomic status of this morph, five specimens collected in the Folatière cave were first included in a molecular phylogenetic study that focused on species delimitation problems within the genus *Bythinella* (Bichain *et al.* 2007a). The results highlighted two divergent mitochondrial lineages among syntopic specimens sampled in the cave. Four specimens clustered with specimens unambiguously attributed to the epigean species *B. eurystoma* (Paladilhe, 1870) and one specimen constituted a distinct and unnamed lineage. No shell characters allowed morphological discrimination among these syntopic hypogean specimens that would support this mitochondrial lineage divergence.

In contrast, the nuclear ITS1 sequences obtained from two hypogean specimens were deeply divergent from those of epigean specimens attributed to *B. eurystoma*. ITS1 thus indicated that *B. eurystoma* would be a species restricted to epigean habitats whereas all hypogean specimens from Folatière cave would belong to a distinct stygobite species.

The few individuals analyzed so far from the Folatière cave, coupled with the fact that substitution rate is higher in the mitochondrial genome than in the nuclear one, may suggest that the genetic break observed by Bichain *et al.* (2007a) could be due to a sampling effect: two divergent haplotypes may have been randomly sampled in the population.

Here, we increased the dataset of Bichain *et al.* (2007a) by including sequences from 16 additional specimens sampled in the Folatière cave. Our objectives were 1) to test if the two divergent mitochondrial lineages still persist when further individuals are analyzed, and 2) to examine divergences/similarities in shell morphology between the epigean and hypogean populations and within the hypogean population.

Abbreviations

a.s.l.	above sea level;
BI	Bayesian Inference;
COI	cytochrome oxydase subunit I;
DFA	Discriminant Function Analysis;
ITS1	Internal Transcriber Spacer 1;
mtDNA	mitochondrial DNA;
nDNA	nuclear DNA;
PCA	Principal Component Analysis;
PC	Principal Component;
L	lens;
L_A	aperture height;
L_{sh}	shell height;
L_{lw}	last whorl height;
R	retina;
W_a	aperture width;
W_{LW}	last whorl width;
W_{sh}	shell width;
WN	whorls number;
WL	Wilks' lambda;
MNHN	Muséum national d'Histoire naturelle, Paris.

MATERIAL AND METHODS

Study area and sampling methods

The study area is located in the Lez and Hérault catchments, southern France (Fig. 1). In order to collect hypogean *Bythinella*, sampling was performed in caves, springs and in the hyporheic zone. For each of these biotopes, we used several collecting methods: 1) in caves, living animals were collected on the spot by sight and sediment was sampled; in the Folatière cave, divers collected sediment down to a depth of 22 m; 2) in springs, sediment was collected and drifting fauna was sampled using drifting nets during flood events; and 3) the hyporheic zone was investigated with the Bou-Rouch pumping method (Bou & Rouch 1967). Cave and spring sediments were first soaked in tap water and left in the dark for a few days in order to leave enough time for live animals to reach the surface of the sediment.

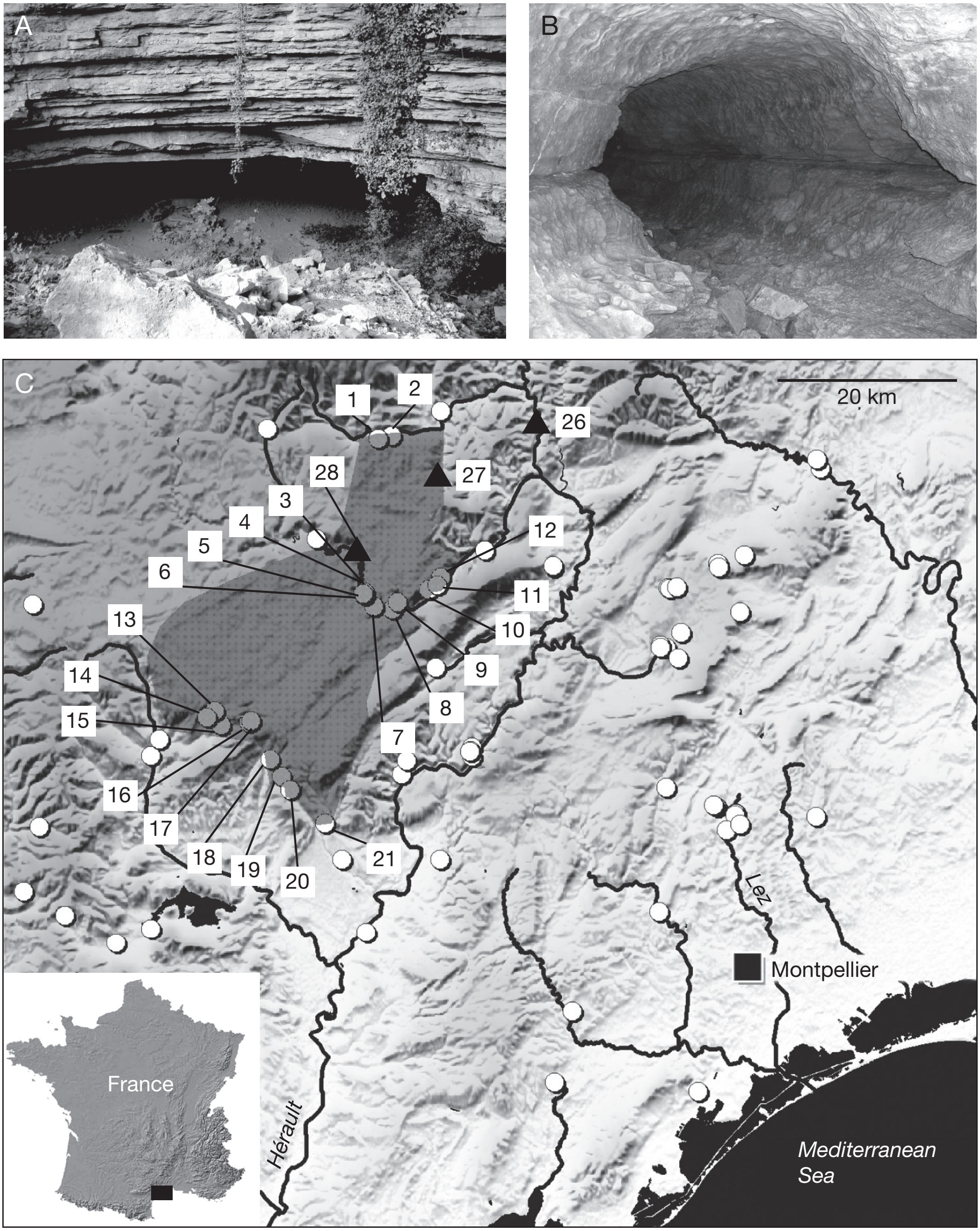

Fig. 1. — Study area and sampling sites: **A**, view of the Folatière cave entrance; **B**, view inside the Folatière cave (type locality of *Bythinella navacellensis* n. sp.) showing the typical erosion resulting from the active network; **C**, location of sampling sites (station code and details on localities are given in Table 1); **Thick lines**, main rivers; ○, sampling sites (sites with occurrence of *B. navacellensis* n. sp. are numbered, data from rivers alluvium are not shown); ▲, sampling sites of *B. eurystoma* (Paladilhe, 1870). The geographical range of *B. navacellensis* n. sp., inferred from hydro-geological data (see text), is darkened.

TABLE 1. — List of *Bythinella navacellensis* n. sp. and *B. eurystoma* (Paladilhe, 1870) sampling sites. Site numbers from 1 to 21 and 26 to 28 are plotted on Figure 1.

Sites no.	Species	Sample sites description	Latitude	Longitude	Hydrogeological zone
1	*B. navacellensis* n. sp.	Springs of Lasfons (1)	696 400	1 885 700	Causse de Blandas (north)
2	*B. navacellensis* n. sp.	Springs of Lasfons (2)	697 800	1 885 900	Causse de Blandas (north)
3	*B. navacellensis* n. sp.	Folatière cave (locality 382)	695 750	1 874 280	Causse de Blandas (south)
4	*B. navacellensis* n. sp.	Spring of Folatière cave	695 652	1 874 182	Causse de Blandas (south)
5	*B. navacellensis* n. sp.	Gourneyras cave	695 600	1 874 110	Causse du Larzac méridional (north)
6	*B. navacellensis* n. sp.	Gourneyrou cave	695 720	1 874 040	Causse du Larzac méridional (north)
7	*B. navacellensis* n. sp.	Small spring on the side Vis river	696 440	1 873 240	Causse du Larzac méridional (north)
8	*B. navacellensis* n. sp.	Magnanerie cave	697 830	1 873 010	Causse de Blandas (south)
9	*B. navacellensis* n. sp.	La Tuilède cave	698 180	1 873 510	Causse de Blandas (south)
10	*B. navacellensis* n. sp.	Spring of Grenouillet	697 550	1 872 670	Causse du Larzac méridional (north)
11	*B. navacellensis* n. sp.	Springs of Mas-Neuf	701 200	1 874 800	Causse de Blandas (south)
12	*B. navacellensis* n. sp.	Calavon cave	701 400	1 875 400	Causse de Blandas (south)
13	*B. navacellensis* n. sp.	Spring in the Cirque du bout du monde	684 700	1 865 500	Causse du Larzac (south)
14	*B. navacellensis* n. sp.	Spring of Saint-Laurent	684 000	1 865 000	Causse du Larzac (south)
15	*B. navacellensis* n. sp.	Banquier cave	685 100	1 864 400	Causse du Larzac (south)
16	*B. navacellensis* n. sp.	Spring of Parlatge (1)	687 100	1 864 600	Causse du Larzac (south)
17	*B. navacellensis* n. sp.	Spring of Parlatge (2)	687 300	1 864 800	Causse du Larzac (south)
18	*B. navacellensis* n. sp.	Springs of Ruisseau de la Tour	689 600	1 860 600	Causse du Larzac (south)
19	*B. navacellensis* n. sp.	Springs of Valrousse	688 800	1 861 900	Causse du Larzac (south)
20	*B. navacellensis* n. sp.	Springs of Saint-Saturnin de Lucian	690 200	1 859 600	Causse du Larzac (south)
21	*B. navacellensis* n. sp.	Spring of Arboras	692 800	1 857 000	Causse du Larzac (south-east)
22	*B. navacellensis* n. sp.	Alluvium of the Vis river	695 000	1 876 900	Unknown
23	*B. navacellensis* n. sp.	Alluvium of the Vis river	699 490	1 873 230	Unknown
24	*B. navacellensis* n. sp.	Alluvium of the Vis river	694 700	1 877 800	Unknown
25	*B. navacellensis* n. sp.	Alluvium of the Lergue river	679 880	1 862 120	Unknown
26	*B. eurystoma*	Spring near St-Julien-de-la-Nef (locality 332)	–	–	Unknown
27	*B. eurystoma*	Spring near Montdardier (locality 333)	–	–	Causse de Blandas (north)
28	*B. eurystoma*	Spring near Saint-Maurice-Navacelles (locality 329)	–	–	Causse de Blandas (south)

After collecting the live specimens, the sediment was dried and poured into water again, thus allowing empty shells to surface. Sampling involved more than 60 localities of which only one revealed living hypogean animals (Folatière cave, Fig. 1A, B) and 21 (from caves and springs only) revealed empty shells attributable to the *B. navacellensis* n. sp. morphotype (Fig. 1C; Table 1). All live specimens were fixed in 95% ethanol.

Sixteen living animals sampled from the Folatière cave were analyzed both genetically and morphometrically. For the latter analyses, we included empty shells from the Folatière cave and specimens of *B. eurystoma* from three epigean localities in the Larzac region (Fig. 1C; Table 1).

MOLECULAR ANALYSES

Total DNA was extracted from whole individuals (shell included) using the QIAGEN DNeasy kit (Qiagen Inc., Hilden, Germany). Partial COI mtDNA was amplified using the universal primers H2198 and L1490 (Folmer *et al.* 1994). PCR reactions were performed in a final volume of 25 µl, using *c.* 2.5 ng of template DNA, 1.25 mM $MgCl_2$, 0.3 µM of each primer, 0.13 mM of each nucleotide, 5% DMSO and 1.5 unit of *Taq* polymerase (MP Biomedical Qbiogene Inc., Illkirch, France). Amplification products were generated by an initial denaturation step of 4 min at 94°C, followed by 35 cycles at 94°C for 30 s (denaturation), 52°C for 40 s (annealing) and 72°C for 40 s (extension), and a final extension of 10 min at 72°C. PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc.). Sequencing was performed by Genoscreen Inc. (Lille, France) on an ABI prism 3730XL (Applied Biosystems, Foster City, California) in both directions to confirm authenticity of each sequence.

We added 16 COI sequences (GenBank numbers EF372562-EF372577) to the global dataset of Bichain *et al.* (2007a) (92 sequences, GenBank numbers EF016165-EF016248). Three amnicolid COI sequences from GenBank (AF367652, AF322409, AF213348) were used to root our analyses. COI sequences were aligned unambiguously by eye using BioEdit 5.0.0 (Hall 1999). No stop codon or shifts in the reading frame were detected when translated into amino acids.

Phylogenetic analyses were conducted using Bayesian inference (BI) as implemented in MrBayes v3.1.1 (Ronquist & Huelsenbeck 2003). Using the AIC criterion, the GTR + I + Γ model (Yang 1994) was selected by MrModeltest 2.2 (Nylander 2004); we subsequently used this model for all the analyses. Flat priors were used for all parameters. Four Markov chain Monte Carlo simulations (one heated, three cold) were run for 2 million generations and parameters were sampled every 100 generations. The first 2×10^5 generations (2000 trees) were discarded ("burn-in" period) and the posterior probabilities were estimated from the remaining sampled generations. We checked that the potential scale reduction factor approached 1.0 for all parameters. We used Tracer v1.4 (Rambaut & Drummond 2007) to ascertain that our sampling of the posterior distribution has reached enough effective sample size.

MORPHOLOGICAL ANALYSES

Shells were placed on an adhesive support in a standard position (Bichain *et al.* 2007b) and then digitalized with a graduated scale using a stereomicroscope connected to a digital camera. Seven parameters were recorded on each picture using ImageTool 3.00 (Wilcox *et al.* 2002): height and width of the shell, height and width of the last whorl, height and width of the aperture, the whorls number visible on each picture. Four ratios (W_{sh}/L_{sh}, L_{lw}/L_{sh}, L_a/L_{sh}, W_a/L_a) commonly used in the alpha-taxonomy of hydrobioid spring-snails (Bernasconi 2000; Haase *et al.* 2005) were calculated.

We performed multivariate analyses on these 11 shell parameters measured on: 1) 72 epigean shells attributed to *B. eurystoma*; 2) the 16 new specimens from the Folatière cave involved in our molecular study, which were digitized before the DNA extractions and; 3) 19 empty shells from this cave that could not be specifically attributed.

We first performed a Principal Component Analysis (PCA) to explore the distribution of the shell parameters between epigean and hypogean specimens. Then Discriminant Function Analyses (DFA) were used to test statistically: 1) the differences between hypogean shells attributed genetically to divergent haplotypes, and 2) the differences between hypogean and epigean individuals. Wilks' lambda used in an ANOVA (F) test of mean differences was used to test if the discriminant model as a whole was significant. In such cases, each variable was then tested using WL to determine which variable differed significantly in mean value between discriminated groups.

All analyses were performed with Statistica® 6.0 and the level of significance used was set to 5%.

RESULTS

PHYLOGENETIC ANALYSES

The Bayesian tree obtained from 111 COI sequences agreed with the phylogenetic results of Bichain *et al.* (2007a) and confirmed the strong genetic divergences between the two hypogean mitochondrial lineages from the Folatière cave. Eight new COI sequences clustered (Fig. 2A) in the clade A (*B. navacellensis* n. sp. and *B. eurystoma*) and eight others in the clade B (*B. navacellensis* n. sp. and *B. cebennensis* (Dupuy, 1849)).

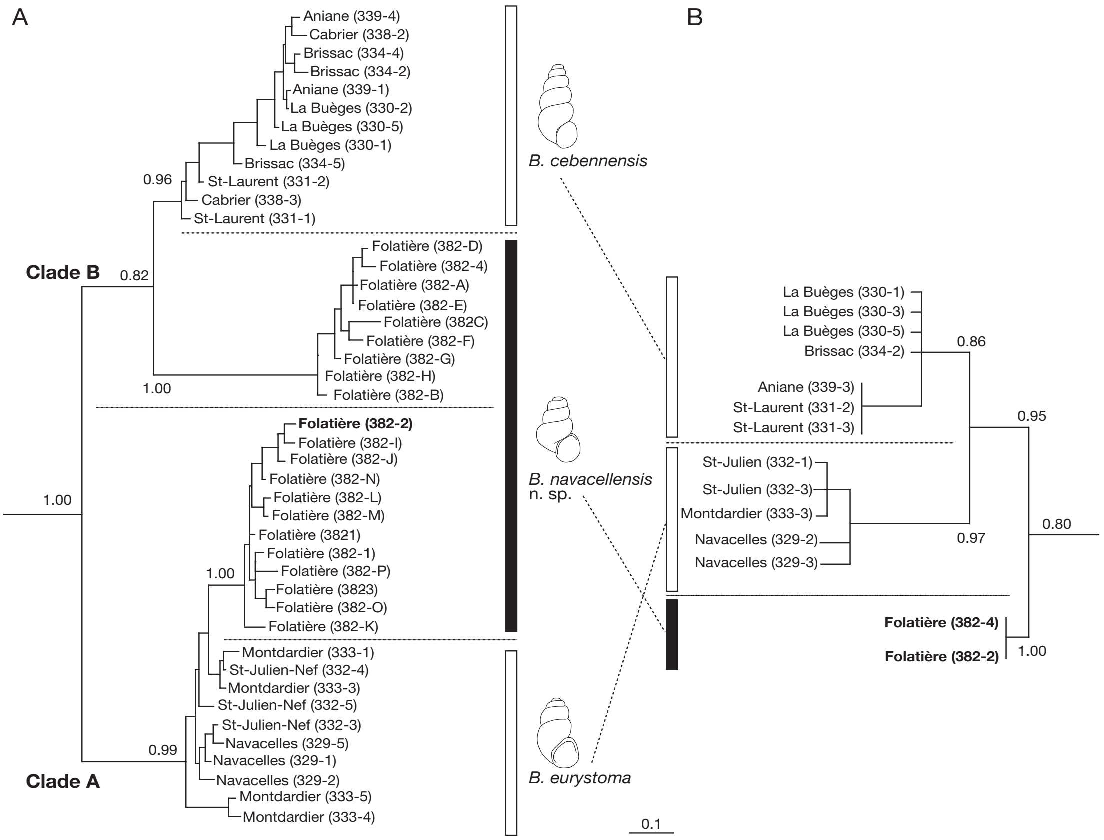

FIG. 2. — Bayesian phylogenetic tree of *Bythinella* specimens based on 111 COI sequences: **A**, detail of the COI global Bayesian tree implying the clade A (*B. navacellensis* n. sp. and *B. eurystoma* (Paladilhe, 1870)) and clade B (*B. cebennensis* (Dupuy, 1849) and *B. navacellensis* n. sp.) (shell outlines represented). Name and code of each locality are indicated in Table 1 for *B. eurystoma* and *B. navacellensis* n. sp. **Thick bars**, exclusively hypogean species. Values of branch robustness are indicated for each node; model parameters for BI analyses: I = 0.228; Γ_4 = 0.124; [AC] = 0.035; [AG] = 0.459; [AT] = 0.050; [CG] = 0.048; [CT] = 0.291; [GT] = 0.026; A = 0.327; C = 0.221; G = 0.115; T = 0.327; **B**, detail of the ITS1 global Bayesian tree modified from Bichain *et al.* (2007a). The individuals of *B. navacellensis* n. sp. for which we obtained both COI and ITS1 sequences are indicated in bold. Values of branch robustness are indicated for each node.

Within the clade A, there were two haplotypes diverging by four substitutions. Each haplotype corresponded respectively either to the epigean (*B. eurystoma* lineage, 22 sequences) or hypogean (*B. navacellensis* n. sp. lineage, 12 sequences) individuals. Within the *B. navacellensis* n. sp. lineage belonging to the clade B (nine sequences), three haplotypes diverged by two substitutions at most. The intra-clade p-distance ranged from 0.1% (within clade A) to 0.4% (within *B. navacellensis* n. sp. lineage from clade B) and the inter-clade p-distance [clade A vs. *B. navacellensis* n. sp. lineage from clade B] reached 4.7%.

MULTIVARIATE ANALYSES

Our global dataset included 11 parameters measured on 107 shells. From epigean localities, 72 shells were attributed to *B. eurystoma,* a species genetically and morphologically distinct from *B. cebennensis*. From the Folatière cave 16 were attributed to *B. navacellensis* n. sp. and 19 empty shells were not specifically attributed. Basic statistics (mean and standard error) of the 11 shell parameters are given in the Table 2.

To explore the pattern of shell variation, a PCA was carried out on the entire dataset (107 epigean

TABLE 2. — Basic statistics of 11 shell parameters for *Bythinella eurystoma* (Paladilhe, 1870) and *B. navacellensis* n. sp. Measurements in mm.

Bythinella eurystoma											
N = 72	**L_{sh}**	**L_{lw}**	**L_a**	**W_{lw}**	**W_{sh}**	**W_a**	**W_{sh}/L_{sh}**	**L_a/L_{sh}**	**L_{lw}/L_{sh}**	**W_a/L_a**	**WN**
mean	2.666	1.906	1.210	1.292	1.574	0.999	0.592	0.455	0.717	0.827	3.361
Minimum	2.269	1.678	1.064	1.106	1.379	0.904	0.537	0.409	0.636	0.726	3.000
Maximum	3.108	2.141	1.366	1.476	1.812	1.119	0.665	0.489	0.780	0.918	4.000
Standard error	0.198	0.102	0.075	0.073	0.091	0.050	0.034	0.018	0.029	0.039	0.319
Specimens from the Folatière cave [16 living animals and 19 empty shells attributed to *B. navacellensis* n. sp.]											
N = 35	**L_{sh}**	**L_{lw}**	**L_a**	**W_{lw}**	**W_{sh}**	**W_a**	**W_{sh}/L_{sh}**	**L_a/L_{sh}**	**L_{lw}/L_{sh}**	**W_a/L_a**	**WN**
mean	2.460	1.703	1.038	1.350	1.591	0.867	0.650	0.423	0.695	0.835	3.689
Minimum	2.038	1.489	0.864	1.193	1.335	0.675	0.575	0.389	0.646	0.762	3.500
Maximum	2.960	1.961	1.209	1.591	1.914	1.057	0.758	0.475	0.740	0.909	4.000
Standard error	0.272	0.141	0.092	0.099	0.126	0.085	0.041	0.022	0.026	0.035	0.246
Specimens from the Folatière cave [16 living animals attributed to *B. navacellensis* n. sp.]											
N = 16	**L_{sh}**	**L_{lw}**	**L_a**	**W_{lw}**	**W_{sh}**	**W_a**	**W_{sh}/L_{sh}**	**L_a/L_{sh}**	**L_{lw}/L_{sh}**	**W_a/L_a**	**WN**
mean	2.613	1.781	1.085	1.417	1.648	0.908	0.633	0.416	0.684	0.837	3.844
Minimum	2.078	1.520	0.864	1.275	1.384	0.719	0.575	0.389	0.646	0.766	3.500
Maximum	2.960	1.961	1.209	1.591	1.914	1.057	0.691	0.451	0.732	0.909	4.000
Standard error	0.271	0.141	0.100	0.093	0.138	0.096	0.031	0.016	0.023	0.041	0.239

and hypogean shells). The first (PC1) and second (PC2) principal components respectively account for 44.9% and 27.4% of the total variation (Fig. 3A). Two groups of strongly correlated parameters were detected: 1) global size parameters (L_{sh}, W_{sh}, W_{lw}, L_{lw}, L_a, W_a); and 2) ratios (L_{lw}/L_{sh}, L_a/L_{sh}) representing the relative size of the aperture and of the last whorl. The first set of variables was primarily associated with PC1. This first axis was thus interpreted as a size factor. The second set of parameters was primarily associated with PC2. Additionally, W_{sh}/L_{sh} and W_a/L_a were weakly negatively correlated with PC1 and the whorl number (WN) was strongly correlated with PC2. The projection of the individuals in the PC1 × PC2 factorial plane revealed that *B. eurystoma* and all hypogean individuals from Folatière cave differed along PC1 and PC2 (Fig. 3B). PCA failed to reveal any obvious difference between individuals of *B. navacellensis* n. sp. belonging to the two mitochondrial lineages revealed by the phylogenetic analyses. The PCA suggested that epigean *B. eurystoma* differed from all the hypogean specimens sampled in the Folatière cave in having larger shells, larger last whorl, aperture different in shape and size, spire formed by a smaller number of whorls.

The DFA carried out on the 16 living individuals from the Folatière cave (eight attributed to clade A and eight attributed to clade B) and computed as "species" as the discriminant factor was not significant (WL = 0.144 and $p = 0.239$). The DFA carried out on the overall dataset and computed with "hypogean/epigean origin" (35 hypogean and 72 epigean) as the discriminant factor was significant (WL = 0.063 and $p < 0.05$). Scores of WL with the probability associated with each variable indicated that both shell samples differed significantly in six parameters (L_{sh}, L_a, W_{sh}, L_a/L_{sh}, L_{lw}/L_{sh}, WM). The overall reclassification score of the discriminating linear functions was 100%.

The DFA carried out on the overall dataset, but excluding the 19 subterranean specimens not taxonomically attributed and computed with "species attribution" (72 *B. eurystoma* and 16 *B. navacellensis* n. sp.) as the discriminant factor was highly significant (WL = 0.569, $p < 0.05$). The overall reclassification score of the discriminating linear functions was 100%.

The DFA results corroborate the PCA analysis. DFA indicated that morphological differences between hypogean and epigean shells were statistically significant, whereas shell differences between

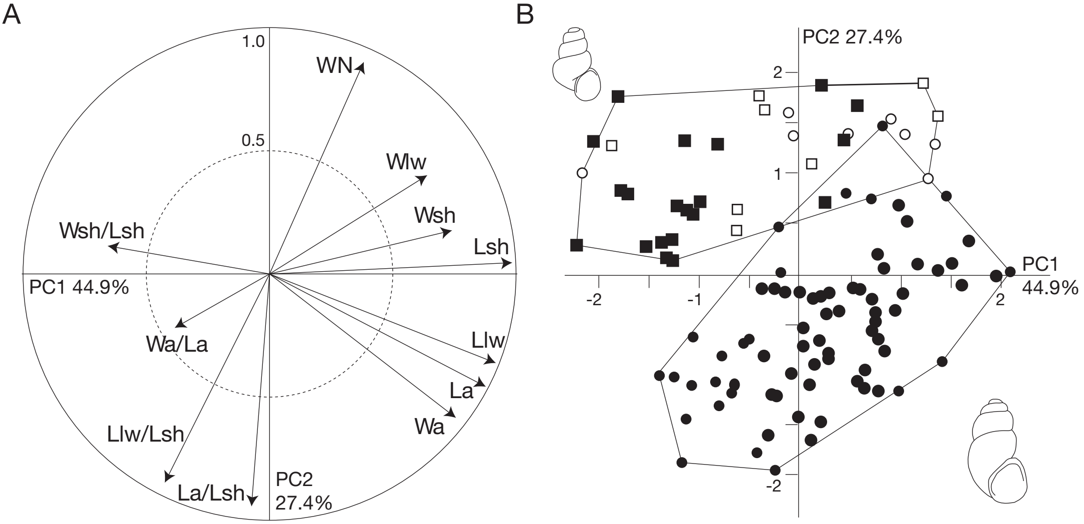

FIG. 3. — Multivariate analyses on shell parameters of *Bythinella eurystoma* (Paladilhe, 1870) and *B. navacellensis* n. sp.: **A**, correlation of the 11 parameters measured on the shell to the first two principal components, PC1 and PC2; **B**, projection of the individuals in the first factorial plane PC1 × PC2 (○, individuals of *B. navacellensis* n. sp. [from clade A]; □, individuals of *B. navacellensis* n. sp. [from clade B]; ●, individuals of *B. eurystoma*; ■, undetermined individuals sampled in the Folatière cave. All the hypogean individuals were sampled in the Folatière cave, type locality of *B. navacellensis* n. sp.).

hypogean mitochondrial lineages of *B. navacellensis* n. sp. were not.

Hypogean individuals of the Folatière cave (Fig. 4) seem to differ from epigean ones in having smaller shells and a more stocky shell shape, expressed by the lower values of L_{lw}/L_{sh} and L_a/L_{sh} and the greater value of W_{lw}.

SPECIES DELIMITATION

Our approach to species delimitation was based on a combination of morphological characters, ecological traits and genetic data. The population of the Folatière cave exhibits a markedly peculiar shell and a clear ecological specificity. Additionally, previously published phylogenetic reconstruction based on the ITS1 nuclear gene (Fig. 2B) suggests that this population constitutes a monophyletic lineage (Bichain *et al.* 2007a). The paraphyly suggested by the COI gene is interpreted as a consequence of introgression by hybridization (see Discussion). Based on nDNA tree, shell characters, anatomical features and ecological traits, we therefore consider this population as belonging to a distinct and new species named *B. navacellensis* n. sp.

SYSTEMATICS

Family AMNICOLIDAE Tryon, 1863
Genus *Bythinella* Moquin-Tandon, 1856

Bythinella navacellensis n. sp.

Bythinella sp. – Bichain *et al.* 2007a: figs 2, 3, clade F + G (and *Bythinella* sp. 2 on the figure 5).

Bythinella sp. – Prié 2008: fig. 1H.

HOLOTYPE. — France, Folatière cave,VII.2003, V. Prié collector, 1 specimen (MNHN-9315; Fig. 5A).

PARATYPES. — Same data as holotype, 19 specimens (MNHN-9316).

TYPE LOCALITY. — France, Grotte de la Folatière (Folatière cave), town of Rogues, Gard département, 3.5264E, 43.8636N.

ETYMOLOGY. — The specific epithet refers to the grandiose Cirque de Navacelles located near the Folatière cave.

VERNACULAR NAMES. — Bythinelle de Navacelles (French), Navacelles' cave snail (English).

OTHER MATERIAL EXAMINED. — See Table 1.

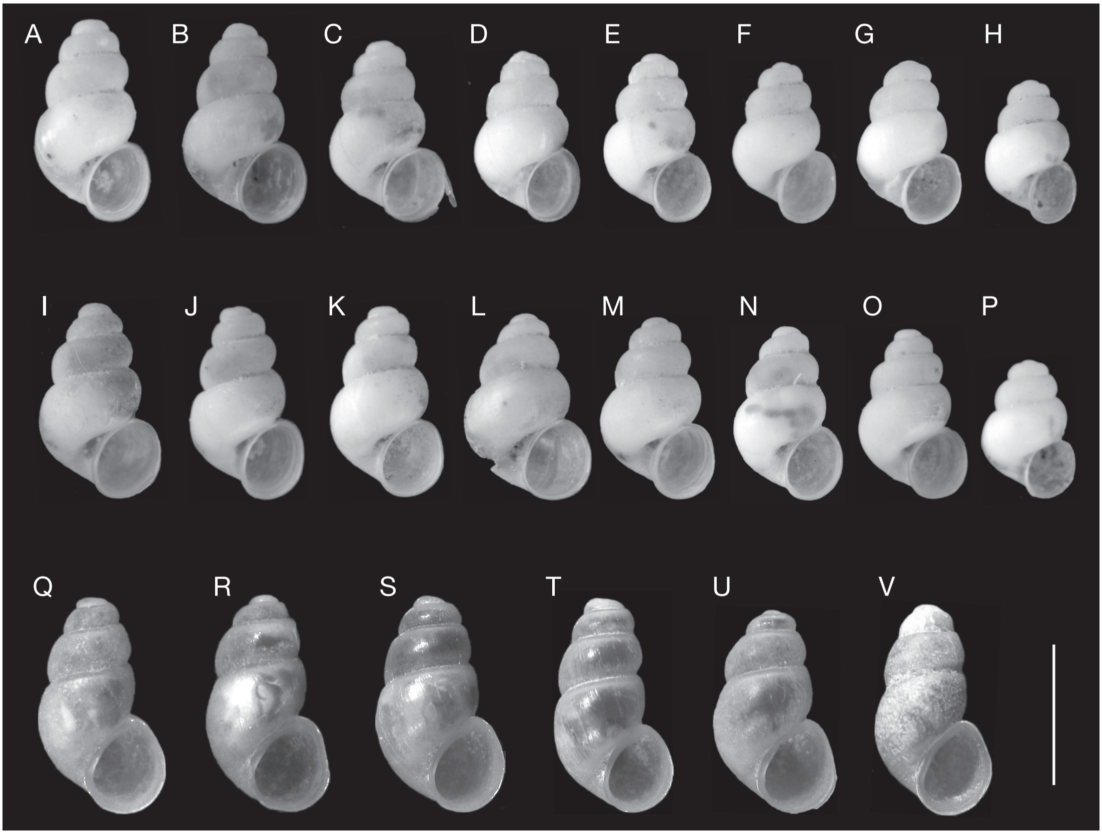

FIG. 4. — Intra- and inter-specific shell variability of *Bythinella eurystoma* (Paladilhe, 1870) and *B. navacellensis* n. sp.: **A**-**H**, individuals sampled in the Folatière cave and attributed subsequently to *B. navacellensis* n. sp. (from clade B) after COI sequencing; **I**-**P**, individuals sampled in the Folatière cave and attributed subsequently to *B. navacellensis* n. sp. (from clade A) after COI sequencing; **Q**-**V**, individuals of an epigean population (Saint-Julien-de-la-Nef, Hérault, France) of *B. eurystoma*. Scale bar: 1 mm.

DISTRIBUTION. — The species is present in the subterranean aquifers of the Blandas and southern Larzac plateaus, France, Gard and Hérault departments. Sample sites details are given in Table 1 and inferred distribution is shown in Figure 1.

DESCRIPTION

Shell

Teleoconch conical and variably elongate, spire formed by *c.* 4 well convex whorls, gradually increasing, separated by deep sutures (Figs 4; 5). The shell width is *c.* ⅔ of the shell length and the last whorl is more than ⅔ of the shell length. Shell is transparent, with marked growth lines in the form of collabral grooves but no visible spiral micro-sculpture. Aperture holostome, oval; external margin of peristome slightly fluted. Umbilicus is widely open. Aperture could be rejected on the side, giving the shell a distinct globular shape. Operculum is thin, pliable, transparent and oval. Protoconch of 1¼ whorls, almost smooth, but exhibits thin spiral micro-sculptures in the form of shallow grooves. Basic statistics of 11 shell parameters from 35 shells are given in Table 2.

Anatomy

The entire body is unpigmented. Eyes are invisible externally; however, a lens may be present but pigmented retinal cells were not observed (Fig. 5C). Snout is about as long as wide, approximately parallel-sided or slightly tapered, with a weak but noticeable distal lobation. Tentacles are longer than the snout, more

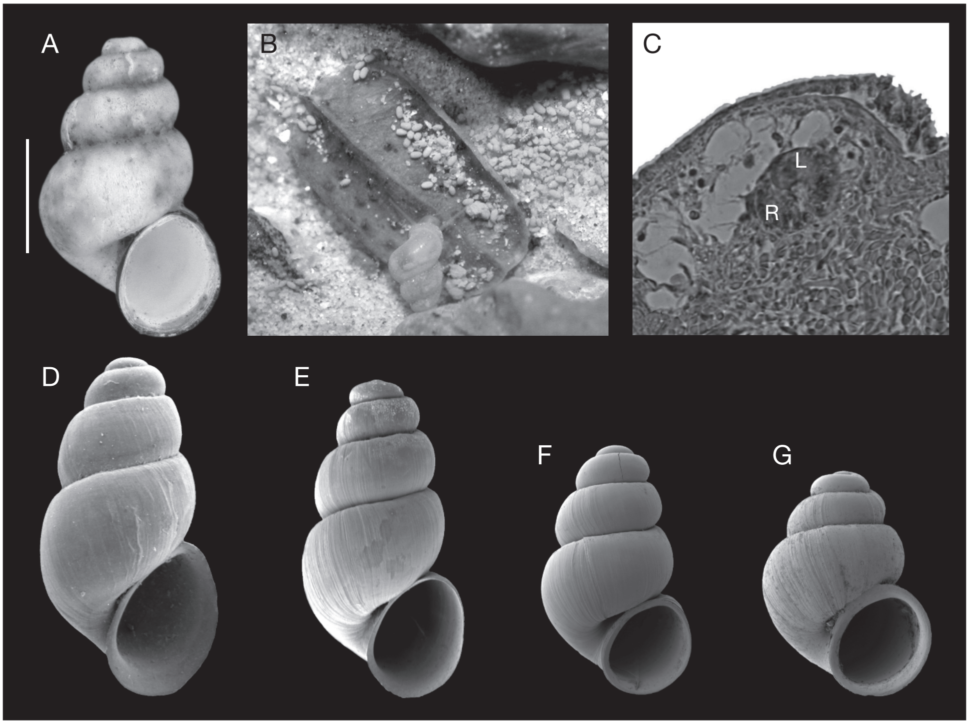

FIG. 5. — Hypogean and epigean species of the *Bythinella* Moquin-Tandon, 1856 genus: **A**, holotype of *B. navacellensis* n. sp. (MNHN 9315) from the Folatière cave; **B**, live specimen of *B. navacellensis* n. sp. *in situ* from the type locality, with scattered fecal pellets visible on the ground and a drawn *Buxus sempervirens* L. leaf; **C**, histological section of the eye of a specimen from the Folatière cave; **D**, shell of *Bythinella eurystoma* (Paladilhe, 1870); **E**, syntype of *Bythinella padiraci* Locard, 1903 (MNHN); **F**, holotype of *Bythinella bouloti* Girardi, Bichain & Wienin, 2002 (MNHN); **G**, holotype of *Bythinella galerae* Girardi, Bichain & Wienin, 2002 (MNHN). Abbreviations: **L**, lens; **R**, retina without pigmented layer. Scale bar: 1 mm.

than 10 times as long as they are wide with the distal end slightly expanded. Under an optical microscope no ciliation is visible on the tentacles (Fig. 5B).

On dissection, the anatomy of the male genitalia shows the typical organization found in *Bythinella*: penis bi-armed, the left arm (the penis proper) cylindrical and the right arm (usually referred to as the flagellum) short, blunt and thinner. The intestine makes a loop visible through the translucent shell. Content of stomach and intestine consists of clay and sand particles.

Ecology

The only known living population comes from the Folatière cave. Living animals were found in shallow water, in streams and puddle in the active hydro-geological karstic network. In the Folatière cave, about 67% of the individuals were found by sight trapped in puddle and 33% were collected on the submerged gallery walls, from the surface to about the depth of one meter. Only empty shells were collected by divers in deep water, i.e. more than one meter below the water surface. Since this species, which exhibits troglomorphic features, has only been sampled alive in hypogean habitats, we consider *B. navacellensis* n. sp. as a strictly stygobite species.

DISCUSSION

INTROGRESSION BY HYBRIDIZATION

According to Haase *et al.* (2007), genetic variability is usually low within the rissooidean populations and specimens belonging to a single population cluster in the same clade after phylogenetic reconstruction. Our phylogenetic results, based on the COI mitochondrial gene, highlighted that the hypogean population of the Folatière cave was distributed across two well-supported clades separated by *c.* 4.7% of genetic divergence. A value higher than the level of inter-specific divergence (from 0.5% to 3.4%) commonly admitted for hydrobioids (Hershler *et al.* 1999, 2003; Liu *et al.* 2003; Hurt 2004). In contrast, phylogeny based on the ITS1 nuclear gene clustered the Folatière cave sequenced specimens in the same clade.

The topology conflict between nDNA and mtDNA trees could be explained by a scenario of introgression by hybridization, i.e. a foreign genetic material transfer by interspecific backcross (Arnold 1997). Mitochondrial genome is considered as particularly susceptible to introgression events (Ballard & Whitlock 2004). This hypothesis implies that the misleading phylogenetic tree is given by the mitochondrial gene(s), and that the "true" species tree is given by the nuclear genes. In our analyses, the hypothesis of introgressive hybridization involves only one evolutionary event corresponding to the mtDNA transfer from "*B. eurystoma*" to "*B. navacellensis* n. sp." Following this scenario, the nuclear phylogenetic tree – the "right" evolutionary signal – suggested that the Folatière cave population constitutes a single and divergent evolutionary lineage. Shell characters, anatomic features (eye regression and unpigmented body) and ecological traits (cave habitat) corroborated this hypothesis as well. However, we admit that our scenario of introgression is based on an unbalanced molecular dataset. In order to test this hypothesis, new ITS1 sequences should be produced to complement the current dataset.

Distinction from other hypogean *Bythinella* species

Bythinella navacellensis n. sp. is readily distinguishable on both morphological and molecular characters from the stygobite species *B. padiraci* (Fig. 5E) from the Padirac karstic network and morphologically from *B. bouloti* and *B. galerae* (Fig. 5F, G), all hypogean taxa belonging to the Atlantic drainage.

Concerning the putative stygobite species *B. eutrepha*, Bernasconi (2000) applied this name to epigean populations from southeastern France, but Falkner *et al.* (2002) restricted the name *B. eutrepha* to the hypogean population from the Lez basin. The latter considered that the material stored in the Bourguignat collection (Muséum d'Histoire naturelle de Genève) is composite with a specimen attributable to *B. eurystoma* (from St-Guilhem-le-Désert, Hérault) (Fig. 5D) and a specimen corresponding to the "true" *B. eutrepha* (from Lez alluviums). Consequently, Falkner *et al.* (2002) designated this specimen as the lectotype of *B. eutrepha*. However, this type specimen was collected in the Lez rivers' alluvium, making it impossible to assign it to any precise habitat. This species has never been sampled alive and its taxonomic status is still unclear. One of us (VP) has checked the *Bythinella* specimens in the Paladilhe collection (University of Montpellier) and collected recent shells from the alluviums of the Lez. Shells labeled "*B. eutrepha*" in the Paladilhe collection seem composite with specimens from the Hérault basin attributable to *B. eurystoma* and nine specimens from the Lez basin exhibiting distinct shell features attributable to the distinct morph *B. eutrepha* as it can still be sampled at present in the Lez alluviums. Overall, this material is morphologically very different from the new species described here.

Distribution and IUCN category

Among the 60 subterranean localities sampled, 21 sites revealed empty shells attributable to the *B. navacellensis* n. sp. morphotype. The morph "*B. navacellensis*" is found in the karstic networks of the Hérault basin, only in the karstic aquifers of the Causse de Blandas (Gard) and the Causse du Larzac méridional (Hérault). It has not been found at altitudes below 300 m a.s.l. It seems to be absent from the Buèges river's catchment and the upstream waters of the Hérault catchment. No specimen was found in the hyporheic zone, even near the caves in which *B. navacellensis* n. sp. occurs (e.g., in front of the Folatière cave). Its distribution seems therefore to be limited by 1) the Atlantic drainage on the north-west side of its range; 2) the acid substrate of the Cévennes mountains in the north; 3) the Hérault river and the karstic network of the Buèges river in the East; and 4) lower altitude regions in the south, which are hydrogeologically isolated by a layer of marl. The infered distribution range (Fig. 1C) is a small delimited and cohesive area of *c.* 300 km^2 located around the southern Larzac.

Hydrological systems can be viewed as continental islands made of a single water body, isolated from adjacent ones. According to Prié (2008), within a single water body snails appear to be evenly distributed in space and time in the whole hydrological system. Therefore, the distribution of subterranean snails (1) includes the whole water body they are found in and (2) can be delimited by adjacent isolated systems. The distribution map shown in Figure 1C is based on these assumptions.

Population size or trend are unknown but presumably stable in this relatively intact environment. However, short-range *B. navacellensis* n. sp. can be considered to occur in three locations only *sensu* IUCN (2001). According to the IUCN guidelines, a location is defined as “a geographically or ecologically distinct area in which a single event (e.g., pollution) will soon affect all individuals of the taxon present”. Given the regional hydrological context, we reckon that three distinct locations harbour the whole species populations: 1) the Blandas plateau water body, which cohesiveness has been shown by fluorescein dye (Caumont 1992); and the 2) northward and 3) southward drainages of the Larzac plateau, which may constitute two separated water bodies. *Bythinella navacellensis* n. sp. can therefore be categorized as “Vulnerable, VU D2” (few locations) according to the IUCN criteria (IUCN 2001).

Human activities are currently very limited in this area, with *c.* 1 inhabitant per km^2, the main activity being extensive cattle farming. The aquifer is thus relatively free of pollution from human activities. Nevertheless, human pressure on southern France aquifers for drinking water is becoming a prominent issue and we cannot predict the consequences of such increasing pressure on either the aquifers or the stygobite fauna. This is a potential threat for 10 species of stygobite snails found in the area (Prié unpublished data), all of which being endemic to France and four of them endemic to the study area.

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