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A gleam in the dark: Phylogenetic species delimitation in the confusing spring-snail genus *Bythinella* Moquin-Tandon, 1856 (Gastropoda: Rissooidea: Amnicolidae)

Jean-Michel Bichain [a,*], Philippe Gaubert [b], Sarah Samadi [c], Marie-Catherine Boisselier-Dubayle [c]

[a] *"Taxonomie et Collections", Département Systématique et Evolution (USM 602), Muséum National d'Histoire Naturelle, Case postale 51, 55 rue Buffon, F-75005 Paris CEDEX 05, France*
[b] *UR IRD 131, UMS MNHN 403, Département Milieux et Peuplements Aquatiques, 43, rue Cuvier, 75005 Paris CEDEX 05, France*
[c] *"Systématique, Adaptation et Evolution", UMR 7138 P6-IRD-MNHN-CNRS (UR IRD 148), Service de systématique moléculaire (CNRS, IFR101), Département Systématique et Evolution (USM 603), Muséum National d'Histoire Naturelle, Case postale 26, 57 Rue Cuvier, F-75231 Paris CEDEX 05, France*

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Abstract

We re-assess the value of morphological specific descriptors within the spring-snail genus *Bythinella* by sequencing mitochondrial COI and nuclear ITS1 gene fragments. Taxonomic coverage represents 16 nominal species sampled among 35 populations from France. Application of monophyly and cohesive haplotype networks as criteria to delineate species allow us to identify 10 mitochondrial species-level lineages, all but one of which are recovered by ITS1. COI species thresholds that are estimated from newly delimited species (ca. 1.5%) agree with values found among other hydrobioids. Our results strongly suggest that classical morphological descriptors may not constitute valid specific criteria within *Bythinella*. Our analyses support a complex scenario of invasions of subterranean habitats, as illustrated by the syntopy of several mitochondrial lineages or the conflicting evolutionary histories between COI and ITS1 in caves. In addition, morphological convergence related to subterranean ecological constraints that affect shell shape and size among the hypogean springsnails studied is suspected.

Keywords: Hydrobioid snails; COI; ITS1; DNA taxanomy; Species threshold; Hypogean species; Morphological convergence

1. Introduction

The amnicolid spring-snail genus *Bythinella* Moquin-Tandon, 1856 is one of the most diverse groups of European hydrobioids, with 80 valid terminal taxa (Bank, 2004). Members of this genus are minute (2–4 mm in length), gonochoristic snails with distributions ranging from north-eastern Spain to south-eastern Turkey; France is considered the centre of species-richness of the group with 42 putative species. *Bythinella* species live mainly in small springs and marginally in hypogean habitats (Boeters, 1979; Falniowski, 1987; Bichain et al., 2004), which are particularly sensitive to the impact of human activities (Szarowska, 2000; Hurt, 2004; Szarowska and Falniowski, 2004, 2006). Thirteen taxa are currently listed on the IUCN Red List of Threatened Species (World Conservation Union; www.redlist.org), including 6 species fully protected under French law.

Although the monophyly of the genus is well supported by molecular and anatomical characters (Wilke et al., 2001; Remigio and Hebert, 2003; Szarowska and Wilke, 2004),

* Corresponding author. Fax: +33 1 40 79 57 71.
E-mail address: jean-michel.bichain@educagri.fr (J.-M. Bichain).

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species delimitations within *Bythinella* are hotly debated. The alpha-taxonomy of this genus is based mainly on shell characters and/or anatomical features that are extremely variable (Falniowski, 1987; Mazan, 2000; Bichain et al., 2007). This problem is of particular concern in hydrobioids, in which morphological observations have led to taxonomic disagreements and have not permitted inferences of evolutionary relationships from the specific to the suprageneric level (Falniowski and Szarowska, 1995; Herschler and Ponder, 1998; Wilke et al., 2000, 2001, 2002; Wilke and Falniowski, 2001; Hershler et al., 2003). Recent studies using molecular approaches (Falniowski et al., 1998, 1999; Mazan and Szarowska, 2000a,b; Bichain et al., 2007) have drastically challenged the value of specific morphological descriptors used in *Bythinella*. As a consequence, Falniowski et al. (1998) gave the genus the status of superspecies (Mayr and Ashlock, 1991), a concept close to the non-adaptive radiation of Gittenberger (1991) and the morphostasic evolution of Davis (1992). *Bythinella* can therefore be considered as a monophyletic group of allopatric species that do not differ significantly in either morphological or ecological adaptive features.

The main objective of this paper is to test morphological species delimitation within the genus *Bythinella* following the molecular-based procedure presented by Bichain et al. (2007). For this purpose, we used a large subset of species from south-western France, the area that supports the highest number of endemic *Bythinella* species. We also included samples from several caves in order to discuss morphological evolution in subterranean habitats (Marmonier et al., 1993; Lefébure et al., 2006b; Buhay et al., 2007). *Bythinella* (morpho) species delimitations were re-assessed using an evolutionary framework based on two independent markers, the Cytochrome *c* Oxidase subunit 1 (COI) mitochondrial gene and the first Internal Transcript Spacer (ITS1) from the nuclear-encoded ribosomal gene region. Finally, we will (i) discuss the use of COI in the context of the barcoding approach (Hebert et al., 2003; Rubinoff and Holland, 2005) and (ii) re-define shell variability and geographical ranges of *Bythinella* species.

2. Material and methods

2.1. Taxonomic and geographic coverage

The core study area was located in south-western France, where 25 *Bythinella* species occur (Bank, 2004). We sampled specimens primarily from four geographical regions: the mountains located north of Montpellier, the Grands Causses of Lozère, the Dordogne-Quercy area and the Pyrenean area (Fig. 1). All are karstic (limestone) regions with numerous groundwater springs and small streams where species of *Bythinella* live in dense populations. This taxonomic sample set was enlarged by collecting additional specimens from springs in the department of Tarn-et-Garonne and in northeastern France (Fig. 1) and by adding eight COI sequences of five west-European *Bythinella* species from GenBank.

We mainly sampled in groundwater springs and the upper courses of small streams, but also in karstic networks. In this latter habitat, one locality was sampled in the Folatière cave and two localities, seven kilometers apart, were sampled in the Padirac subterranean network. Snails were collected by washing small pebbles, aquatic vegetation or dead leaves over two sieves (2 mm and 450 μm mesh). All specimens were fixed in 80% ethanol. In total, 32 epigean and three hypogean localities were sampled (Fig. 1 and Table 1).

Species names were attributed to specimens following currently accepted diagnostic shell characters available in the taxonomic literature (Boeters, 1998; Bernasconi, 2000; Falkner et al., 2002). In order to avoid doubtful species name application, we preferentially collected specimens from type localities. Our sample set thus included 16 nominal species, of which nine were from type localities. Nevertheless, seven individuals could not be assigned to named species, and were therefore identified *a posteriori* according to their phylogenetic positions. Overall, our study involved 21 nominal taxa, of which 15 were considered as strictly endemic to France.

2.2. DNA extraction, amplification and sequencing

Total DNA was extracted from whole individuals (shell included) using QIAGEN DNeasy kits (Qiagen Inc., Hilden, Germany). Partial COI mtDNA and the entire ITS1 nuclear DNA were amplified using, respectively, the universal primers H2198 and L1490 (Folmer et al., 1994) and two *Bythinella*-specific primers ITS1D (5′-GTG GGA CGG AGT GTT GTT-3′; first conserved region of ITS1) and ITS1R (5′-CCA CCG CCT AAA GTT GTT T-3′; initial 5′ domain of 5.8S rDNA). The latter primer pair was defined using the Primer3 software (Rozen and Skaletsky, 2000) from the sequences we obtained initially with the universal primers ITS2 and ITS5i (Hillis and Dixon, 1991).

PCRs were performed in a final volume of 25 μl, using ca. 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 U of *Taq* polymerase (MP Biomedicals Qbiogen, Illkirch, France). Amplification products were generated by an initial denaturation step of 4 min at 94 °C, followed (i) by 35 cycles at 94 °C for 30 s, 52 °C for 40 s and 72 °C for 40 s, and a final extension of 10 min at 72 °C for COI, or (ii) by 25 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final extension of 5 min at 72 °C for ITS1. PCR products were purified using QIAquick PCR purification kits (Qiagen Inc., Hilden, Germany).

Sequencing was performed with Beckman dye chemistry and a CEQ2000™ automated sequencer according to the manufacturer's instructions (Beckman Coulter Inc., Fullerton, California) in both directions to confirm accuracy of each sequence. For COI sequencing, we used the pair of internal specific primers COID (5′-CGG [AG]TT AGT

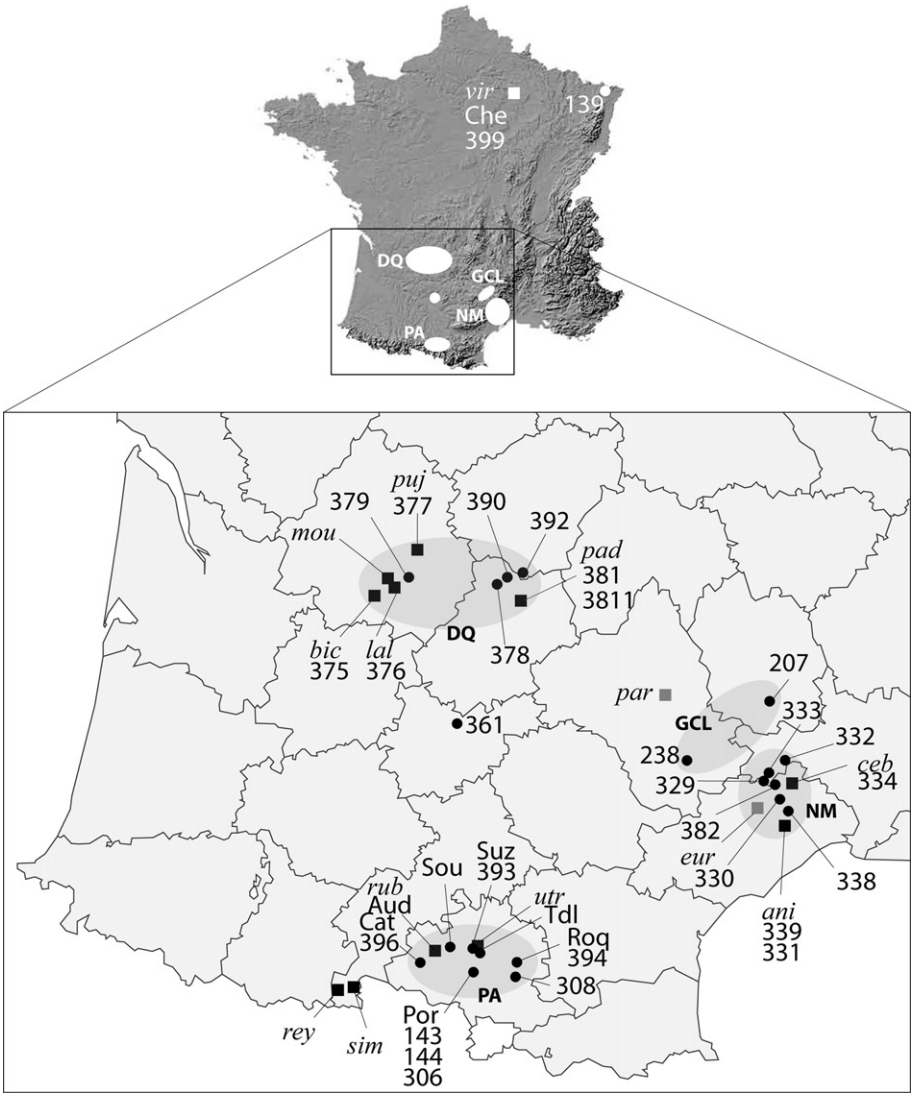

Fig. 1. Location of *Bythinella* sampling sites in France. Station codes and information on localities are given in Table 1. Filled circles correspond to sample sites and filled squares to type localities: ani, *B. anianensis* (Paladilhe, 1870); bic, *B. bicarinata* (Des Moulins, 1827); ceb, *B. cebennensis* (Dupuy, 1849); eur, *B. eurystoma* (Paladilhe, 1870); lal, *B. lalindei* (Bernasconi, 2000); mou, *B. moulinsii* (Dupuy, 1849); pad, padiraci Locard, 1903; par, *B. parvula* Locard, 1893; puj, *B. poujolensis* (Bernasconi, 2000); **rey**, *B. reyniesii* (Dupuy, 1851); rub *B. rubiginosa* (Boubée, 1833); sim, *B. simoniana* (Moquin-Tandon, 1856); utr, *B. utriculus* (Paladilhe, 1874); vir, *B. viridis* (Poiret, 1801). Grey squares indicate type localities imprecisely located in the original descriptions of nominal species involved in this study. DQ, Dordogne-Quercy area; GCL, Grands Causses of Lozère; NM, North of Montpellier; PA, Pyrenean Area.

[AGT]GG TAC AGC-3′) and COIR (5′-TGT ATT [AG]AA GTT TCG ATC TG-3′) that we generated from *Bythinella* sequences available on GenBank.

3. Data analyses

3.1. Alignment

COI sequences were aligned by eye using BioEdit© 5.0.0 Sequence Alignment Editor software package (Hall, 1999). No stop codons or shifts in the reading frame were detected when translated into amino acids. An initial alignment of the ITS1 sequences was performed on the total dataset using the ClustalW Multiple alignment algorithm (Thompson et al., 1994). Variable zones that could not be aligned unambiguously were then removed to avoid erroneous hypotheses of primary homology.

3.2. Phylogenetic inferences

We selected out-groups from within the family Amnicolidae, which belongs to a clade distinct from all other hydrobiid snails (Wilke et al., 2001). Three amnicolid COI sequences from GenBank were used to root our analyses. The only amnicolid ITS1 sequences available on GenBank (*Taylorconcha serpenticola*: DQ076028–DQ076088) were too divergent to be aligned with *Bythinella*. Consequently, the ITS1 phylogenetic analyses were rooted using *Bythinella viridis* sequences, which constitutes a well supported and distinct lineage within *Bythinella* (Bichain et al., 2007).

Table 1
Taxonomic sample set used in this study and GenBank Accession numbers

Nominal species	Population information							DNA isolate	COI gene	ITS1 gene
	Code	MNHN number	Type locality	Localities	Locality name	Biotope	region		GenBank number	GenBank number
B. eurystoma	329	Moll9511	No	Saint-Maurices-Navacelles		Spring	France/Hérault (34)	329-1	EF016231	
								329-2	EF016230	EF016129
								329-3		EF016128
								329-5	EF016229	
	333	Moll9512	No	Montdardier		Spring	France/Gard (30)	333-1	EF016220	
								333-3	EF016219	EF016132
								333-4	EF016218	
								333-5	EF016217	
	332	Moll9513	No	St-Julien-de-la-Nef		Spring	France/Gard (30)	332-1		EF016130
								332-3	EF016223	EF016131
								332-4	EF016220	
								332-5	EF016221	
B. anianenis	339	Moll9514	Yes	Aniane	Font-Cauquillade	Spring	France/Hérault (34)	339-1	EF016211	
								339-3		EF016141
								339-4	EF016210	
B. cebennensis	334	Moll9515	No	Brissac		Small stream in an urban park	France/Hérault (34)	334-2	EF016216	EF016138
								334-4	EF016215	
								334-5	EF016214	
	330	Moll9516	No	St-Jean-de-Buèges	La Buèges	Spring	France/Hérault (34)	330-1	EF016228	EF016135
								330-2	EF016227	
								330-3		EF016136
								330-5	EF016226	EF016137
Bythinella sp.	331	Moll9517	No	Aniane	Saint-Laurent	Spring	France/Hérault (34)	331-1	EF016225	
								331-2	EF016224	EF016139
								331-3		EF016140
Bythinella sp.	338	Moll9518	No	St-Guilhem-le-Désert	Cabrier	Spring	France/Hérault (34)	338-2	EF016213	
								338-3	EF016212	
Bythinella sp.	382	Moll9519	No	Rogues	Folatière	Subterranean water	France/Gard (30)	382-1	EF016183	
								382-2	EF016182	EF016134
								382-3	EF016181	
								382-4	EF016180	EF016133
								382-11	EF016179	
B. rubiginosa	*Aud1*	Moll5960	Yes	Audinac-les-Bains	Les Thermes	Thermal spring	France/Ariège (09)	Aud1-1		DQ318905
								Aud1-2		DQ318906
Bythinella sp.	*Aud2*	Moll5965	No	Audinac-les-Bains	Les Thermes	Spring	France/Ariège (09)	Aud2-3		DQ318907
								Aud2-6		DQ318908

Bythinella sp.	*Aud3*	Moll5966	No	Audinac-les-Bains	Les Thermes	Small stream confluence of 173 and 174	France/Ariège (09)	Aud3-1		DQ318910
								Aud3-2		DQ318909
B. utriculus	*Suz*	Moll5967	Yes	La-Bastide-de-Sérou	Suzan	Spring	France/Ariège (09)	Suz-3		DQ318914
								Suz-5		DQ318915
	393	Moll5967	Yes	La-Bastide-de-Sérou	Suzan	Spring	France/Ariège (09)	393-1	EF016170	
								393-2	EF016171	
	Roq	Moll5970	No	Roquefort-les-Cascades	near Cascade de la Turasse	Spring	France/Ariège (09)	Roq-2		DQ318911
	394	Moll5970						394-3	EF016175	
								394-5	EF016174	
	Tdl1	Moll5968	No	La-Bastide-de-Sérou	Tour de Loli	Spring	France/Ariège (09)	Tdl1-2		DQ318913
								Tdl1-5		DQ318912
B. simoniana	*Cat*	Moll5962	No	Alas	Ste-Catherine	Small stream	France/Ariège (09)	Cat-3		DQ318903
	396	Moll5962	No	Alas	Ste-Catherine	Small stream	France/Ariège (09)	396-1		EF016142
								396-3		EF016143
								396-4	EF016169	
	Sou	Moll5964	No	Clermont	La Souleille	Small stream	France/Ariège (09)	Sou2		DQ318904
Bythinella sp.	308	Moll9520	No	Montségur	Barrineuf	Spring	France/Ariège (09)	308-1	EF016232	EF016159
								308-2		EF016160
								308-3		EF016161
B. reyniesii	*Por*	Moll5961	No	Boussenac	Col de Port	Spring 1	France/Ariège (09)	Por-1		DQ318899
								Por-2		DQ318900
	143	Moll5961	No	Boussenac	Col de Port	Spring 1	France/Ariège (09)	143-1	EF016246	
								143-3	EF016245	
								143-4	EF016244	EF016154
								143-5	EF016243	
	144	Moll9521	No	Boussenac	Col de Port	Spring 2	France/Ariège (09)	144-1	EF016242	
								144-2	EF016241	EF016153
								144-3	EF016240	
	306	Moll9522	No	Boussenac	Col de Port	Spring 3	France/Ariège (09)	306-1	EF016234	EF016157
								306-2	EF016233	EF016156
								306-3		EF016158
B. viridis	*Che*	Moll5959	Yes	Chery-Chartreuve	Moulin de Veau	Spring	France/Aisne (02)	Che-1		DQ318901
								Che-3		DQ318902
	399	Moll5959	Yes	Chery-Chartreuve	Moulin de Veau	Spring	France/Aisne (02)	399-1	EF016165	
								399-2	EF016166	
								399-3	EF016167	
								399-4	EF016168	
B. dunkeri	139	Moll9523	No	Philisbourg	Falkensteinbach	Small stream	France/Moselle (57)	139-1	EF016248	
								139-5	EF016247	
B. bicarinata	375	Moll9524	Yes	Couze	Fontaine de la Vierge	Spring	France/Dordogne (24)	375-1	EF016209	
								375-2	EF016206	EF016144
								375-3	EF016205	

(*continued on next page*)

Table 1 (*continued*)

Nominal species	Population information							DNA isolate	COI gene	ITS1 gene
	Code	MNHN number	Type locality	Localities	Locality name	Biotope	region		GenBank number	GenBank number
B. lalindei	376	Moll9525	Yes	Lalinde	Source des Cannelles	Spring	France/Dordogne (24)	376-1	EF016204	
								376-2	EF016203	EF016145
								376-3	EF016202	
B. poujolensis	377	Moll9526	Yes	Sergeac	Poujol	Spring	France/Dordogne (24)	377-1	EF016201	
								377-2	EF016200	EF016146
								377-3	EF016199	
B. moulinsii	378	Moll9527	No	Martel	Gluges	Spring	France/Lot (46)	378-1	EF016198	
								378-2	EF016197	
								378-3	EF016196	
B. moulinsii	379	Moll9528	No	Mauzac	Fontblanque	Spring	France/Dordogne (24)	379-1	EF016194	
								379-2	EF016193	EF016147
								379-3	EF016195	
B. padiraci	381	Moll9529	Yes	Padirac	Grande Arcade	Subterranean water	France/Lot (46)	381-1	EF016184	EF016148
								381-2	EF016185	
								381-3	EF016186	EF016149
								381-4	EF016187	EF016150
	3811	Moll9530	Yes	Padirac	De Joly tributary	Subterranean water	France/Lot (46)	3811-1	EF016188	EF016151
								3811-2	EF016189	
								3811-3	EF016190	
								3811-4	EF016191	EF016152
								3811-5	EF016192	
B. parvula	238	Moll9531	No	Lapanouse-de-Cernon	Le Cernon	Small stream	France/Aveyron (12)	238-1	EF016236	
								238-3	EF016235	EF016155
B. parvula	207	Moll9532	No	St-Laurent-de-Trêves	Rau de Pèses	Spring	France/Lozère (48)	207-1	EF016239	EF016162
								207-2	EF016238	EF016163
								207-3	EF016237	EF016164
Bythinella sp.	361	Moll9533	No	Dufort-Lacapelette	St-Hubert	Spring	France/Tarn-et-Garonne (82)	361-1	EF016207	
								361-2	EF016208	
B. moulinsii	390	Moll9534	No	Martel (Courtils)	Les Courtils	Spring	France/Lot (46)	390-1	EF016178	
								390-2	EF016176	
								390-3	EF016177	
B. moulinsii	392	Moll9535	No	St-Denis-les-Martels	La Coste	Spring	France/Lot (46)	392-3	EF016173	
								392-5	EF016172	

B. austriaca	austriaca 1			Not documented	Autria (Hausdorf et al. 2003)		AF445333
	austriaca 2			Not documented	Austria (Wilke et al., 2000)		AF213349
B. compressa	compressa 1	Hessen	Altengrnonau	Not documented	Germany (Wilke et al., 2001)		AF367653
	compressa 2			Not documented	Not documented (Perez et al. 2005)		AY622474
B. pannonica	pannonica 1	Hrhov		Not documented	Slovakia (Szarowska and Wilke, 2004)	clone2639	AY222651
	pannonica 2	Hrhov		Not documented	Slovakia (Szarowska and Wilke, 2004)	clone2638	AY222650
B. schmidtii	schmidtii	Naselje Ivana Krivica		Not documented	Slovenia (Szarowska and Wilke, 2004)		AY222649
B. robiciana	robiciana			Not documented	Slovenia (Szarowska and Wilke, 2004)		AY273998
Erhaia jianouensis	erh			Not documented	China (Wilke et al., 2001)		AF367652
Marstoniopsis insubrica	mar			Not documented	Germany (Wilke and Falniowski, 2001)		AF322409
Amnicola limosa	amn			Not documented	USA (Wilke et al., 2000)		AF213348

Phylogenetic analyses were conducted using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference (BI) methods as implemented in PAUP*4.0b10 (Swofford, 2001), PhyML (Guindon and Gascuel, 2003) and MrBayes v3.1.1 (Ronquist and Huelsenbeck, 2003), respectively.

Unweighted MP analyses were run using a heuristic search option with 100 random-addition replicates, branch swapping by the Tree Bisection and Reconnection (TBR) algorithm and MAXTREES setting = 100,000. For ITS1, gaps were coded as fifth states, and the NOMAXTREES setting was activated. Robustness of nodes was assessed using 100 bootstrap pseudoreplicates (Felsenstein, 1985).

MrModeltest 2.2 (Nylander, 2004) selected the GTR+I+Γ model (Yang, 1994) and the HKY+G model (Hasegawa et al., 1985) as the best fit model of nucleotide substitution for COI and ITS1, respectively, using the AIC criterion. Parameters estimated for the GTR+I+Γ model were base frequencies: $A = 0.3597$, $C = 0.2011$, $G = 0.1026$, $T = 0.3367$; proportion of invariable sites = 0.5792; gamma distribution shape parameter = 1.1831. Parameters estimated for the HKY+G model were base frequencies: $A = 0.2187$, $C = 0.2290$, $G = 0.2949$, $T = 0.2574$; Ts/Tv = 3.0512; gamma distribution shape parameter = 0.1812.

ML phylogenetic analyses were run using 4 substitution rates. Gaps were coded as fifth states and clade support was assessed by non-parametric bootstrapping (100 replicates).

For BI, unambiguous indels in the ITS1 dataset were coded as binary characters, and analyzed using the standard discrete model implemented in MrBayes, which is based on a modified Jukes & Cantor model (Lewis, 2001). Markov chain Monte Carlo (MCMC) was run for 2 million generations. Flat priors were used for all parameters. Four chains (one heated, three cold) were run simultaneously and sampled every 100 generations after an initial burn-in period of 20,000 cycles. Final consensus trees were based only on the pooled samples from the stationary phase of the run. Node "robustness" was estimated through the Bayesian posterior probabilities.

3.3. COI haplotype network

COI haplotype networks were reconstructed in order to clarify genealogical relationships within some terminal clades in which ancestral polymorphism was suspected on the basis of low branching resolution (see Fig. 2). Haplotype networks were inferred using statistical parsimony (Templeton et al., 1992) implemented in TCS ver1.21 (Clement et al., 2000). The connection limit excluding homoplasic changes was set to 95%.

3.4. Species delimitations

We followed the Hennigian inter-nodal species concept formalized by Samadi and Barberousse (2006) to proceed to species delimitation. In this framework, species are

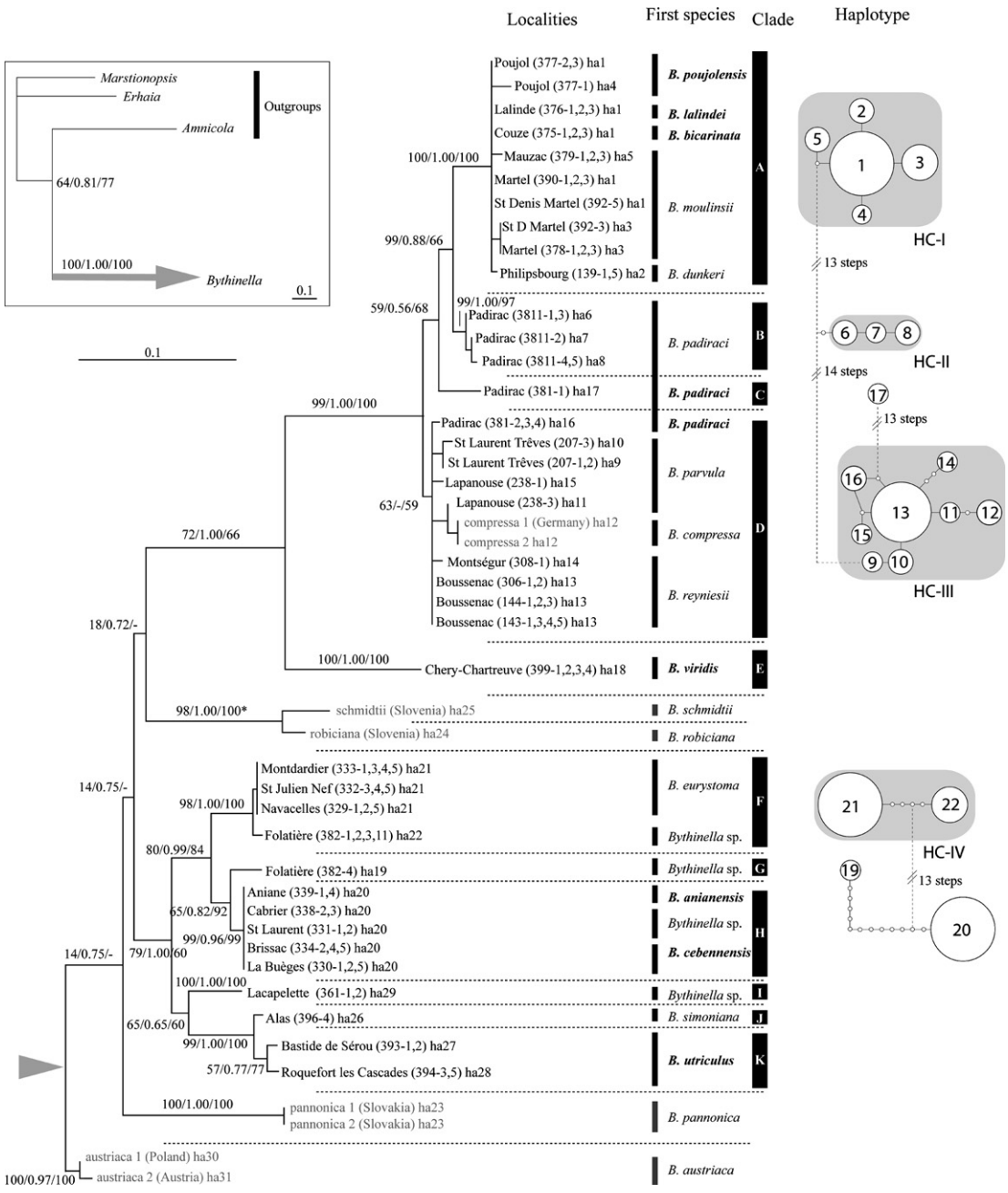

Fig. 2. Maximum likelihood phylogenetic tree of *Bythinella* specimens based on 95 COI sequences. (Left) Values of branch robustness for each phylogenetic analysis (ML, MP and BI) are indicated for each node (using - when node is absent and * for alternative branching). The out-group branching topology is shown in the box. *A priori* species identifications are indicated to the right of sampling codes; bold corresponds to type localities. ha = correspondence between haplotype codes and sampling localities. (Right) Haplotype network inferred from the TCS algorithm. Grey frames indicate haplotypes connected applying the 95% threshold. Inferred intermediate haplotypes, which were not sampled, are indicated by small empty circles or by dotted lines. Circles are proportional to the number of haplotypes. HC, haplotype cluster.

considered sets of organisms that have genealogic relationships (reticulation) and form isolated, irreversible evolutionary lineages. Consequently, we delineated species level taxa from (i) the cohesiveness of haplotype networks (Avise and Ball, 1990; Baum and Shaw, 1995) and (ii) the monophyly criterion derived from the Phylogenetic Species Concept (Cracraft, 1983), in which species constitute the smallest diagnosable monophyletic groups. To limit bias related to individual gene histories (Nichols, 2001; Sota and Vogler, 2001; Shaw, 2002; Funk and Omland, 2003; Avise, 2004), our molecular analyses included both mitochondrial and nuclear gene fragments.

3.5. COI genetic distances and species threshold value

The COI genetic distances were calculated within and among the re-assessed species delimitations based on the previously defined evolutionary framework. We used

genetic distances to evaluate potential overlap and threshold values within and among the delineated species. Genetic distances within *Bythinella* were calculated from 95 COI sequences using p-distance (Kumar et al., 2004) and the Kimura-2-parameters (K2p) model (Kimura, 1980), the latter having frequently been used to quantify intra- and inter-specific divergences. Given that both models yielded similar estimates, we used K2p to compare our results with published data. All the distance-based analyses were performed with MEGA 3.1 (Kumar et al., 2004).

4. Results

4.1. Phylogenetic analyses

We identified 34 haplotypes among the 95 COI sequences of our dataset. From a total of 536 aligned nucleotides, 172 were variable, of which 138 were parsimony informative. MP analysis of the COI matrix yielded 114 equi-parsimonious trees (494 steps, CI = 0.549, RI = 0.922). The ITS1 matrix included 54 sequences, ranging from 215 to 225 nucleotides (the first alignment procedure resulted in a matrix of 434 aligned characters identifying 6 highly variable zones that were subsequently removed). The final alignment consisted of 232 aligned characters, with 60 variable sites of which 57 were parsimony informative. MP analysis of the ITS1 matrix yielded 30 equi-parsimonious trees (96 steps, CI = 0.802, RI = 0.969). In general, mitochondrial and nuclear data recovered the same main monophyletic groups, whatever the methods of tree reconstruction used. However, taxonomic coverage was not identical between the two datasets (population 361 and *B. rubiginosa* not sequenced for ITS1 and COI, respectively). ITS1 was 3-fold less variable than COI and resolved terminal branches less well (Figs. 2 and 3).

The genus *Bythinella* was strongly supported as a monophyletic group (rooted COI tree: ML and MP bootstrap values = 100%; Bayesian posterior probability = 1.00;

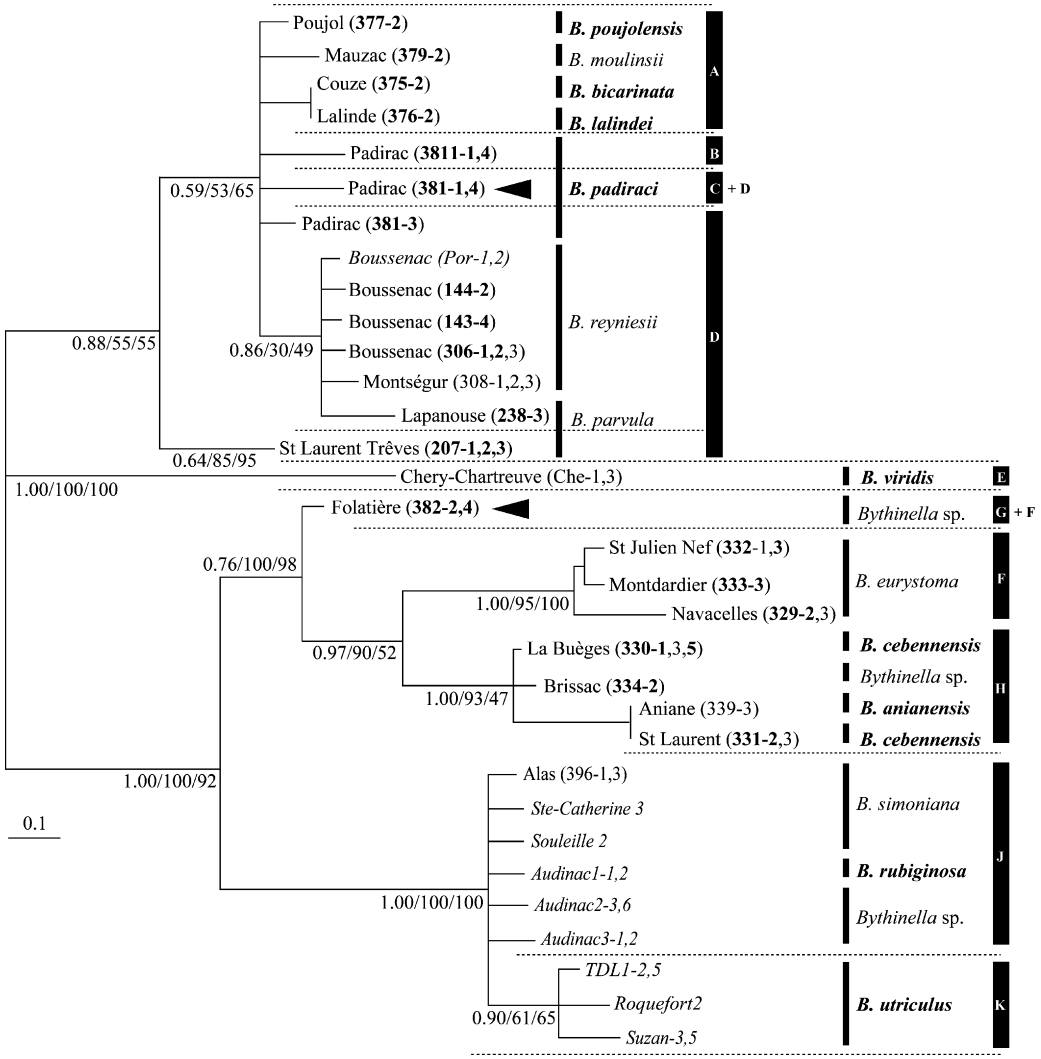

Fig. 3. Bayesian inference tree of *Bythinella* specimens based on 54 ITS1 sequences. Values of branch robustness for all phylogenetic analyses (BI, ML and MP) are indicated at each node (*null branch length). For BI, Ts/Tv = 4.474; gamma distribution shape parameter = 0.091. Specimens in bold were also analyzed using the COI gene. Clades inferred from the COI phylogeny are indicated on the ITS1 tree.

Fig. 2). Clade E corresponded to all the specimens sampled from the type locality of *B. viridis*. A second monophyletic group (Clades A–D) was composed of specimens from the Pyrenean mountains, Quercy-Dordogne area, the Grands Causses of Lozère, northeastern France and the GenBank sequences attributed to the German species *B. compressa*. Within this clade, clusters did not correspond to coherent geographical groupings. A third group (Clades F–K) included specimens from southwestern France only, i.e. the Pyrenean area (J, K), the northern Montpellier area (F–H) and the Tarn-et-Garonne department (I). The sequences from GenBank obtained from specimens attributed to Western European species formed three independent clades. A first clade included two distinct lineages corresponding to specimens attributed, respectively, to *Bythinella schmidtii* and *Bythinella robiciana*. A second clade grouped the sequences attributed to *Bythinella pannonica*, thus validating the position of this species within the genus *Bythinella* (Szarowska and Wilke, 2004). A third clade included all the sequences attributed to *B. austriaca*, which was the sister group to all the other spring-snails included in our study.

4.2. Haplotype network

We applied statistical parsimony analysis to clades A–D and F–H, in which some phylogenetic relationships were poorly resolved in the COI tree or conflicted with ITS1 topology (two individuals 382-2 and -4 from Folatière cave), but conflict concerned weakly supported nodes in both trees. We recovered three independent haplotype networks in clades A–D, separated by 13 or more mutational steps (Fig. 2). The network configuration in clades A and D suggested the presence of ancestral polymorphism, which was probably the cause of the general lack of resolution of terminal clades. The haplotype network also suggested an alternative evolutionary history for clade C (haplotype 17). Rather than being a distinct lineage, sister to clades A–B (a hypothesis weakly supported in the COI tree), haplotype 17 was more closely related to the clade D haplotype group (haplotypes 9–16). Moreover, this latter hypothesis was supported by the ITS1 phylogenetic analysis: COI haplotypes 17 and 16 (381-1 and 381-4, respectively) had the same ITS1 genotype (Fig. 3). The haplotype networks within clades F-H did not indicate close genealogical relationships between haplotypes 19 and 22 from Folatière cave, thus confirming the phylogenetic distinctiveness between these two lineages from a mitochondrial perspective (Fig. 2). However, the ITS1 analysis clustered the two specimens from the Folatière cave (382-2 and 382-4) in a monophyletic lineage (Fig. 3).

4.3. Species delimitations

Overall, after combining the criteria of (i) cohesiveness of haplotypes and (ii) smallest diagnosable monophyletic entities, we were able to identify and name a total of seven lineages from France that we hypothesize to be valid species (clades A, D, E, F, H, J and K; Fig. 2). Clades E, F and K supported the *a priori* morphological species assignments for *B. viridis*, *Bythinella eurystoma* and *Bythinella utriculus*, respectively. Conversely, the *a priori* species assignments were violated for nine nominal taxa that clustered in multi-species clades (A, D, H [COI and ITS1 trees] and J [ITS1 tree]). Following the Principle of Priority (ICZN, 1999: Article 23), we treat *Bythinella anianensis* as a junior subjective synonym of *Bythinella cebennensis* (clade H). We propose *Bythinella bicarinata* (with *Bythinella lalindei* and *Bythinella pujolensis* as subjective junior synonyms), *Bythinella reyniesii* and *Bythinella rubiginosa* as provisional names for clades A, D and J, respectively, pending study of additional topotypes (Table 2). The COI tree also suggested that clade C may constitute a distinct branch (*Bythinella padiraci*), but this hypothesis is

Table 2
Correspondence between monophyletic groups based on COI, the species names applied to these clades and proposed synonymies

Clade	Valid name	Junior synomym	Status of specimens studied	NP	IUCN
A	*bicarinata* Des Moulins, 1827 [*Paludina*]		Topotype	Yes	CR A1ce
		moulinsii Dupuy, 1849 [*Bithinia*]		No	No
		dunkeri Frauenfeld, 1857 [*Paludinella*]		No	VU B1+2c
		lalindei Bernasconi, 2000 [*Bythinella*]	Topotype	No	No
		poujolensis Bernasconi, 2000 [*Bythinella*]	Topotype	No	No
D	*reyniesii* Dupuy, 1851 [Hydrobia]			Yes	LR/lc
		compressa Frauenfeld, 1857 [*Paludinella*]		No	No
		parvula Locard, 1893 [*Bythinella*]		No	No
E	*viridis* Poiret, 1801 [*Bulimus*]		Topotype	Yes	Vu D2
F	*eurystoma* Paladilhe, 1870 [*Paludinella*]			No	No
H	*cebennensis* Dupuy, 1851 [*Bithinia*]		Topotype	No	No
		anianensis Paladilhe, 1870 [*Paludinella*]	Topotype	No	No
J	*rubiginosa* Boubee, 1833 [*Paludina*]		Topotype	No	No
		simoniana Moquin-Tandon, 1856 [*Bythinia*]		No	No
K	*utriculus* Paladilhe, 1874 [*Paludinella*]		Topotype	No	No

Abbreviations used in the Table 2: NP, nationally protected. IUCN categories of threat (Vu, vulnerable; CR, critically endangered; LR/lc, lower risk/least concern).

only weakly supported by the node robustness values and is not supported with ITS1 analyses. Therefore, at present we do not consider this clade as a putative distinct species. The type material of *B. padiraci* (Muséum National d'Histoire Naturelle, Paris; examined by JMB) is probably polytypic and subsequent molecular studies will be necessary to clarify its taxonomic status.

Our approach identified two new species of *Bythinella* (clades B and I) that will be described elsewhere. The COI tree showed an additional distinct lineage that lacks an available name (clade G) but conflict with the ITS1 tree topology (Fig. 3) prevents us from drawing a conclusion regarding its taxonomic status.

4.4. COI genetic distances

The COI pairwise distances between individuals, outgroup included, ranged from 0.000 to 0.216 (mean = 0.084, standard deviation = 0.049) using *p*-distance, and from 0.000 to 0.267 (mean = 0.092, standard deviation = 0.058) using K2p (Fig. 4). K2p and *p*-distances gave similar results for the smallest distance values (divergence mean value = 3.10^{-4}) but were more divergent for the largest distance values (divergence mean value = 0.017 for K2p values > 0.075) (data not shown).

A pairwise sequence gap occurred for values of K2p ranging between 0.145 and 0.171 (from 0.134 to 0.156 for *p*-distance). This gap corresponded to an absence of overlap between distances within *Bythinella* and distances between amnicolid genera (Fig. 4). The intra-sample genetic divergence ranged from 0 to about 0.043 (mean = 0.002).

K2p and p-genetic distances within the clusters identified as species (clades A to K) through our phylogenetic analyses ranged from 0 to 0.012 (mean = $1.625.10^{-3}$). Between species previously delimited, K2p distances ranged from 0.015 to 0.145 (0.015–0.134 for *p*-distances). No overlap occurred between intra- and inter-specific genetic distances. Therefore, we estimated that the species threshold within *Bythinella* using the mitochondrial COI gene was about 1.5%.

5. Discussion

5.1. Molecular species delimitations in Bythinella

COI results—which were partly congruent with ITS1 data- allowed us to propose re-assessed species boundaries in the genus *Bythinella* using the two complementary criteria derived from the species definition of Samadi and Barberousse (2006). We were able to recognize 10 putative evolutionary lineages from France, though only three of these conform to *a priori* definitions (Fig. 5). We also propose the delineation of two new species that will be described and named elsewhere (*Bythinella* sp. 1 and sp. 3; Fig. 5). One mitochondrial lineage conflicted with our results from ITS1 (*Bythinella* sp. 2; Fig. 5).

Analysis of the distribution of COI genetic distances between evolutionary lineages indicated that the mean genetic distances for COI within newly delineated species and the consecutive species threshold value were 0.016% and 1.5%, respectively. The latter value falls within the threshold range (0.5–3.4%) of other hydrobioid species (Hershler et al., 1999, 2003; Liu et al., 2003; Hurt, 2004). Moreover, this result supports the observation that the inter-specific threshold corresponds to ca. 10 times the mean intra-specific variation (Hebert et al., 2004). In this context, the COI threshold might be considered as an efficient tool for the rapid assessment of alpha-biodiversity in *Bythinella* as part of a barcoding approach. This result must be considered cautiously, since this threshold value was determined from the COI gene only and, consequently, needs to be confronted with additional data (e.g. nuclear genes and/or additional COI sequences).

Indeed, the analysis of ITS1 showed that conflicts concerning taxonomic delineations could occur between mitochondrial- and nuclear-based phylogenies (in our case among specimens from the Folatière cave, clades F-G), probably because of different gene evolutionary histories. Consequently, we suggest that the use of the barcoding approach alone, which relies on a single mtDNA gene

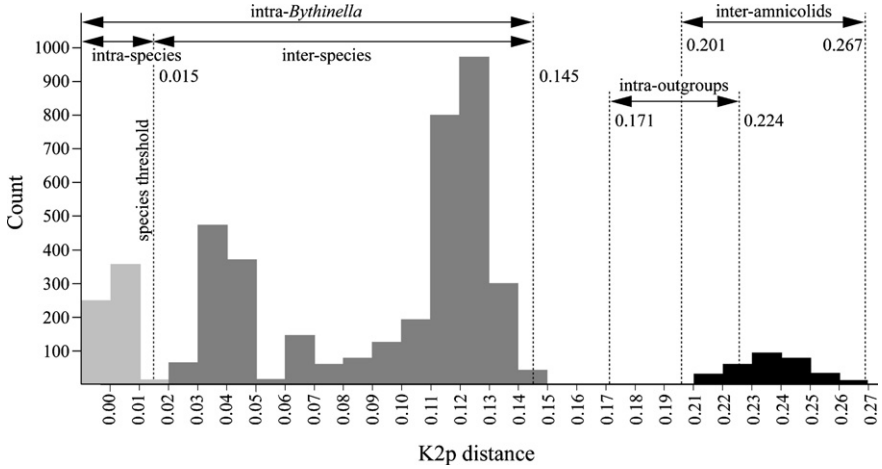

Fig. 4. Distribution (histogram) of the Kimura-2-parameters (K2p) genetic distances in the COI global dataset.

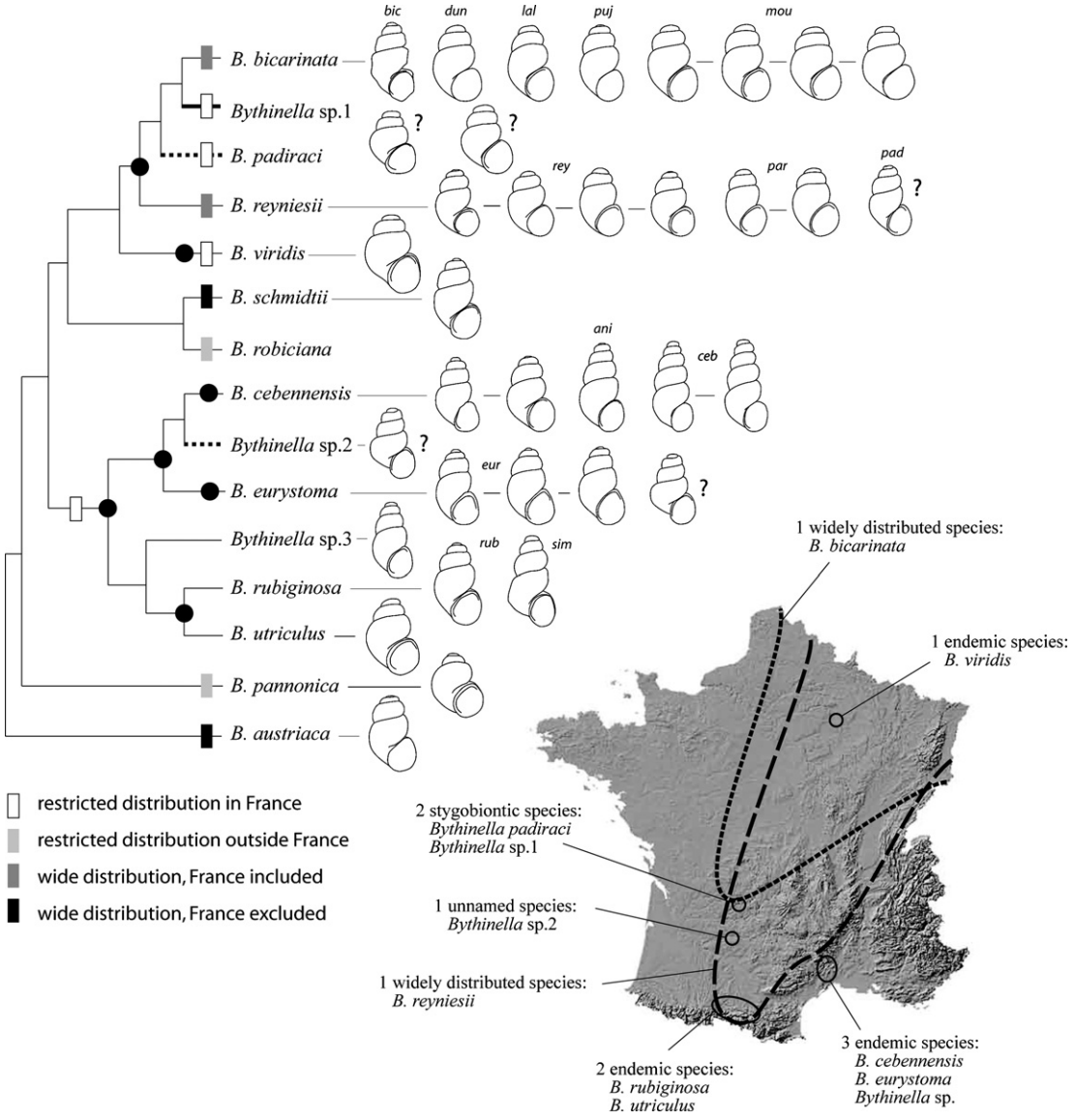

Fig. 5. Synthetic molecular tree and new species delimitations within *Bythinella*. The tree summarizes the phylogenetic relationships inferred from COI analyses. Black circles indicate lineages recovered in the ITS1 tree. Dashed lines represent COI lineages not recovered by ITS1 analyses. Shell outlines of one specimen per sampling site are illustrated. ? means that we could not assign a shell phenotype unambiguously. Bold lines indicate stygobite species. The putative distributions of the newly delineated taxa are shown on the map. See Fig. 1 for abbreviations above shell outlines.

history (COI), might be misleading in the assessment of species boundaries. We therefore consider that the use of barcoding and species thresholds are likely to be appropriate only after a preliminary exploration of taxonomic delineation based on phylogenetic/haplotype network procedures involving a number of markers (Lefébure et al., 2006a; Rubinoff, 2006; Rubinoff and Holland, 2005).

5.2. Re-assessment of shell variability and geographic ranges in epigean Bythinella

Our molecular analyses confirmed previously established species boundaries for *B. viridis*, *B. eurystoma* and *B. utriculus*, endemic to France. These species appear well characterized by an ovoid shell, with a low spire for *B. viridis* (Bichain et al., 2007), a conical shell with an angular aperture for *B. eurystoma* (Paladilhe, 1870), and an ovoid-elongated shell for *B. utriculus* (Bichain et al., 2007). Given the geographic coverage of our sampling, our study suggests that these three species have geographic distributions limited, respectively, to northern France, the mountains around Montpellier (North), and the Ariège area (Fig. 5).

Eight nominal taxa corresponded to just four species recognized in our analysis: *B. bicarinata*, *B. rubiginosa*, *B. cebennensis* and *B. reyniesii* (Table 2). Morphometric analyses (Bernasconi, 2000) that included specimens from localities we sampled, suggest great variability in shell size or in traditional discrete characters within these four re-assessed taxa. For example, within the lineage *B. bicarinata*, shells were either carinate or non-carinate, with length ranging from 2.19 to 2.61 mm ($n = 80$), a range much greater than

the level of intra-specific variability defined by Bernasconi (2000). However, we could associate a distinct, global shell shape to each re-assessed species delimitation (shell outlines showed in Fig. 5), namely a pupoidal shell in *B. bicarinata*, an elongated shell with a rounded aperture in *B. cebennensis*, a pupoid-cylindrical shell shape in *B. reyniesii*, and a conical elongated shell in *B. rubiginosa*. Whereas two of the newly delimited species exhibited restricted ranges in mountains in southern France (*B. cebennensis*, *B. rubiginosa*), the other two had wide distributions from the Pyrenees to Germany (*B. reyniesii*) and from south-western to north-eastern France (*B. bicarinata*) (Fig. 5). The new species from Tarn-et-Garonne (*Bythinella* sp. 2 = clade I) showed a peculiar morphology, including a conical shell shape and an elongated aperture.

Our results challenge the traditional view of widespread endemism and that species can be defined on the basis of narrow variation in morphological characters in *Bythinella*. Some authors (Radoman, 1976; Falniowski, 1987) have suggested that the combination of heritability of shell characters and allopatric speciation through habitat fragmentation was a scenario that could explain the morphological diversification of *Bythinella*. However, our analyses showed that the genus includes both geographically restricted species with low shell variability (e.g., *B. viridis* and *B. eurystoma*) and widely distributed species with high shell variability (e.g., *B. bicarinata* and *B. reyniesii*) implying inter-specific morphological overlap. Thus, classical specific descriptors such as presence/absence of a carina and shell size may not be valid criteria for species delimitation in *Bythinella*. According to Bichain et al. (2007), this significant level of intra- and inter-specific morphological variation could be linked to environmental variables (e.g. water temperature), parasitism or sexual dimorphism. Consequently, there is a crucial need to identify new diagnostic morphological traits in order to characterize species boundaries. The establishment of a molecular framework thus appears to be an appropriate prerequisite to test the taxonomic validity of morphological characters and to anchor the taxonomy of the group in its evolutionary history.

5.3. Colonization of hypogean habitats and convergence in shell shape in Bythinella

Mitochondrial DNA suggested the existence of several distinct phylogenetic lineages within the two caves under study (Padirac and Folatière). In both caves, we found individuals belonging to epigean species (*B. reyniesii* or *B. eurystoma*), confirming that colonisations of hypogean habitats by epigean individuals are not rare events in the genus (Boeters, 1979; Giusti and Pezzoli, 1982; Bole and Velkovrh, 1986; Boeters, 1992; Bernasconi, 2000; Velecka, 2000; Hlaváč, 2002). Although drifting with surface waters towards subterranean habitats seems the most probable way of invasion, we cannot exclude active colonisations of the karstic water from springs, which are interfaces between ground and surface waters.

We also identified distinct, exclusively hypogean, phylogenetic lineages (C and G; Fig. 2) that live in syntopy with individuals of *B. reyniesii* and *B. eurystoma*, respectively. This result supports independent invasions of subterranean habitats through time, resulting in the occurrence of multiple phylogenetic lineages with overlapping distributions (Soulier-Perkins, 2005; Zaknek et al., 2007). The simplest scenario of speciation within caves could follow three basic steps (Barr and Holsinger, 1985; Holsinger, 2000): (i) invasion by epigean individuals, (ii) isolation resulting from the complex spatial structure and dynamics of the karstic network, and (iii) genetic drift possibly leading to reproductive isolation. Results obtained for the individuals of clade B, which were found in a deep and isolated part of the Padirac karstic network, may illustrate the completion of such a scenario. However, the fluctuating nature of the hydrogeologic network dynamics might imply, in most cases, a more complex model with iterative introgressions between temporarily isolated hypogean populations. In addition, it is likely that genetic exchanges occur between epigean and hypogean populations through drifting of waters, probably introducing significant complexity into the evolutionary histories inferred within subterranean habitats, as illustrated by the conflicting signals between COI and ITS1 that we obtained for clade G.

Our results suggested that subterranean ecological constraints (e.g. absence of light and poor food supplies) led to similar shell shape among hypogean spring-snails of different species. Indeed, we could not distinguish between hypogean specimens belonging to epigean or to strictly subterranean lineages using morphometric analysis of shell characters (size and shape) (Bichain, unpublished data). For the first time, our study suggests morphological convergence in hypogean environment for hydrobioids. Such a phenomenon has already been reported for a wide spectrum of taxa inhabiting caves (Siluriformes: Wilcox et al., 2004; Coleoptera: Ortuño and Arillo, 2005; Decapoda: Zaknek et al., 2007; Amphipoda: Lefébure et al., 2006b).

We observed that hypogean *Bythinella* exhibited typical stygobite features (also called troglomorphy) such as the lack of tegument pigmentation, eye loss and small shell size. Further investigations are needed to ascertain whether other physiological features found in obligate or occasional cave animals, such as bigger eggs and reduced progeny numbers, extended embryonic development phases and greater adult longevity (Gibert and Deharveng, 2002), also characterize hypogean *Bythinella*.

6. Conclusion

Our results allowed a reassessment of the taxonomic validity of 16 French nominal *Bythinella* species using a molecular evolutionary framework and an explicit species delimitation procedure. The resulting delimitations challenged the traditional taxonomy of the genus, yielding 10 monophyletic groups of which four did not validate the *a priori* species identifications. Such results underline the

need for a taxonomic revision broadened to the European scale (80 terminal taxa) in order to further stabilize the taxonomy of *Bythinella*. In terms of conservation, our study confirmed species status for *B. viridis* (IUCN status, VU D2; nationally protected), but dramatically questioned the geographic and taxonomic boundaries of *B. bicarinata* (IUCN status, CR A1ce), *B. reyniesii* (IUCN status, LR/lc) and *B. dunkeri* (IUCN status, VU B1+2c), currently considered as threatened endemics.

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