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# SPECIES DELIMITATION IN THE GENUS *BYTHINELLA* (MOLLUSCA: CAENOGASTROPODA: RISSOOIDEA): A FIRST ATTEMPT COMBINING MOLECULAR AND MORPHOMETRICAL DATA

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## ABSTRACT

Within the springsnail genus *Bythinella*, few discontinuous morphological characters allow to unambiguously delineate species-level taxa. Opinions on the alpha-taxonomy of the group are divergent, with some authors recognizing every morphologically distinct local form as a species, while others interpreting such geographical forms as intraspecific variation. Because the value of morphological characters was rarely contrasted with molecular data, such opinions remained untested. In this work, variation between populations was studied through genetics isozymes, phylogeny (DNA), and morphometrics. Eleven populations representing five putative species were sampled from the French Pyrenees, a region where a high number of nominal species are classically recognized. Based on genetic and phylogenetic analyses, the material clusters into three groups, of which one consists of several nominal species. Environmental factors, sexual dimorphism, and genetic factors contribute to the significant morphological variation observed within the genetic groups. Thus, the number of species of *Bythinella* recognized in the Pyrenees appears probably overestimated, and the characters traditionally used for species delimitation should be re-evaluated.

Key words: hydrobioid springsnails, ITS-1 nuclear gene, isozyme, phylogeny, morphometry, multivariate analyses.

## INTRODUCTION

Among gastropods, hydrobioid springsnails are one of those taxa in which the lack of obvious discriminating morphological characters makes species delimitation difficult, resulting historically in the establishment of numerous nominal species and currently in a variety of specialist opinion on the taxonomic validity of these taxa (Kabat & Hershler, 1993; Herschler & Ponder, 1998; Wilke et al., 2001; Szarowska & Wilke, 2004). A consequence is that different specialists have different evaluations of the magnitude of biodiversity. The difficulty of resolving such differing opinions is also hampered by the lack of a solid theoretical and methodological framework, *de facto* rendering many taxonomic opinions untestable hypotheses. However, and although delimitating species boundaries is a central aim of alpha-taxonomy, there is no consensus concerning the meaning of the term "species" (as instances of the extensive literature on this question, see, e.g. Howard & Berlocher, 1998; Wilson, 1999; Winston, 1999; Wheeler & Meier, 2000; Hey, 2001a, b; Mallet, 2001; Noor, 2002).

The objective of this paper is to test the validity of the traditional delimitation of some species belonging to the genus *Bythinella* Moquin-Tandon, 1856, based upon a species concept that permits testable hypotheses. For this purpose, we use the Hennigian inter-nodal species concept recently developed and formalized by Samadi & Barberousse (2006). This species definition is close to the Evolutionary Species Concept (Simpson, 1961, modified by Wiley, 1981; Wiley & Mayden,

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2000), the Internodal Species Concept (Kornet, 1993) and the General Lineage Species Concept (De Queiroz, 1998, 1999). For De Queiroz (1998), most "species concepts" are ill-named, because they should rather be regarded as recognition criteria that, associated with a formal definition of the "species" that is independent of the mechanism by which species are recognized, permit delimitation of species in practice. As suggested by these authors, the various techniques used in systematics can be set in a coherent framework starting from species descriptions based on morphology and/or behavioural and ecological attributes. These descriptions are the *primary hypotheses* for species delimitation that correspond to alpha-taxonomy. Second, these hypotheses can be tested against criteria derived from the various so-called "species concepts", such as the Phylogenetic Species Concept (PSC) and/or the Biological Species Concept (BSC), corresponding respectively to the approaches of phylogenetic systematics (*sensu* Hennig, 1966) and evolutionary systematics (*sensu* Mayr, 1942). Finally, the results of these tests permit the selection of character sets corresponding to more robust primary descriptions. Here we attempt to delimit some *Bythinella* species, in a restricted geographical area, in coherence with the theory of evolution and to justify why, as argued for example by Sites & Marshall (2003, 2004), we need an integrated approach in taxonomy.

*Bythinella* species live in emerging groundwater springs and in the uppermost courses of small streams, where they can form large populations (1,000–10,000s individuals). Some taxa live exclusively in groundwater (Giusti & Pezzoli, 1980; Falniowski, 1987; Bernasconi, 2000; Bertrand, 2004; Bichain et al., 2004). The small (usually around 2.5 mm, maximum 4 mm), nondescript shell is characterized by a blunt apex, with an overall ovoid to conical-elongate shape, most often smooth, but occasionally with spiral keel(s) or one or more axial varices on the last adult whorl. Species of *Bythinella* are gonochoristic. Males are characterized anatomically by a penis-flagellum complex; females have a bursa copulatrix and a seminal receptacle. France is an area of high specific richness for the genus. Of the species currently recognized in Europe, 62% occur in France, of which 90% are endemic to France (Fauna Europaea, 2004).

In *Bythinella*, as in the majority of hydrobioid (*sensu* Davis, 1979) genera, species are primarily delimited based on non-discrete characters. A consequence of this continuous variation of character states is a range of taxonomic opinions among the various taxonomic authorities. For example, in Italy, based on shell morphology and reproductive anatomy, Alzona (1971) recognized eight species of *Bythinella*, whereas Giusti & Pezzoli (1980) regarded all Italian populations as belonging to two species. Based on shell characters, Radoman (1976) recognized about 12 species in the Balkans and Asia Minor. Based on shell morphology, soft part anatomy, and geographical distributions, Falkner et al. (2002) recognized 42 valid species in France, with several applications of species names differing from those of Bernasconi (2000). Thirteen of these species were restricted to the Pyrenees and their foothills. However, in Poland analyses of enzyme polymorphism and morphometric studies performed on shell and anatomical characters (Falniowski et al., 1998, 1999; Mazan, 2000; Mazan & Szarowska, 2000a, b) questioned the ability of the shell and anatomical characters used in alpha-taxonomy to delineate species of *Bythinella*. Consequently, because of these various opinions, which recognize these taxonomic entities alternatively as "good species" or not, we have no clear idea of the true diversity of this group.

Because of their narrow ranges and specialized habitat, hydrobioid springsnails in general, and *Bythinella* species in particular, are vulnerable to even small-scale habitat transformation, such as trampling by cattle or artificial diversion of springs. Several hydrobiid species are considered extinct, and many others are categorized as threatened (IUCN 2005, http://www.redlist.org). Of the species occurring in France, nine are nationally protected (statutory order October 7, 1992). Legal protection was bestowed upon them based on the state of taxonomic knowledge in the 1980s, and it is now fitting to evaluate whether the names involved in the legal texts apply to real units of biodiversity. Of the putative species analyzed in the present study, two are nationally protected.

Species delimitation in a number of nominal *Bythinella* species from the Pyrenees was explored according to criteria derived from the PSC and the BSC, and our purpose was to test if sets of morphological characters (traditional or newly developed) correspond to these criteria. For this purpose, we used populations representing nominal species sampled from their type localities or from the nearest pos-

sible area if the precise type locality could not be found or no longer existed. We performed multivariate analyses on shell parameters to evaluate whether the pattern of shell variation within and among populations permits non-ambiguously attributing each of the sampled population to a putative species, and we tested species delimitation resulting from traditional morphological approaches against BSC and PSC criteria. The BSC criterion amounts to delimitation of species as reproductively isolated groups that became genetically isolated from other such groups. Isolation was evaluated indirectly through analysis of gene flow among populations using electrophoretic variability of isozymes. In the most common view of the PSC, species are monophyletic groups. Monophyly of groups was identified using the pattern of variability of the ITS-1 nuclear gene. These three approaches were compared in order to evaluate the potential use of the morphological characters in the delimitation of *Bythinella* species.

## MATERIAL AND METHODS

### Taxa and Populations Studied

The taxa selected for the present study essentially originate from the Département Ariège, in the central/northeastern Pyrenees. The area has extensive karsts, with numerous springs where *Bythinella* species live in dense populations. Based on shell and reproductive anatomy, Bernasconi (2000) had proposed an alpha-taxonomy of *Bythinella* from this area, and we followed his treatment in how we applied specific names to each of the populations sampled.

Eleven populations were sampled that, based on Bernasconi (2000) and Falkner et al. (2002), correspond to five species (Table 1, Fig. 1). Ten of these populations, representing four putative species, are from the Pyrenean foothills, but *B. reyniesii* (Dupuy, 1851) is a higher altitude species. *Bythinella simoniana* (Moquin-Tandon, 1856), a species relatively well characterized by one or more axial varices on the body whorl, was sampled from three populations (Eng, Cat and Sou). Four populations (Tdl1, Tdl2, Suz and Roq) are attributable to *B. utriculus* (Paladilhe, 1874).

At Audinac, we sampled *Bythinella* from a thermal spring (ca. 18–20°C; Aud1), from a cold spring (ca. 13°C; Aud2) just 30 meters away, and from the confluence (Aud3) of the two springs. The thermal spring is the type locality of *B. rubiginosa* (Boubée, 1833), a nominal species considered to be restricted to this locality; the application of the species name is thus unambiguous. The population sampled from Aud2 had been identified by Bernasconi (2000) as *B. eurystoma* (Paladilhe, 1870). However, specimens do not match the original description of this nominal species well (type locality is St Jean-de-Fos, Département Hérault); we consequently do not follow Bernasconi in applying this name to that population, which we will from hereon designate as *B.* cf. *eurystoma*. The third population (Aud3) was not included in Bernasconi's dataset, and we could not easily place it in one of the nominal species.

As a geographical out-group within *Bythinella*, we used *B. viridis* (Poiret, 1801), the type species of the genus, which we sampled from the type locality (Che) in the east of the Paris basin. It has an ovoid shell that clearly sets it apart from all the Pyrenean *Bythinella* species included in our study.

Specimens were collected by washing small pebbles, aquatic vegetation and dead leaves over two sieves (2 mm and 450 µm mesh). For anatomical and biometrical studies, specimens were fixed in 70% ethanol; for molecular studies, specimens were frozen alive at -80°C. Given the very small size of the specimens, each of the different analyses (morphometric, isozyme and DNA sequence analyses) could not be carried out on the same individuals.

### Isozyme Electrophoresis

Protein extraction was carried out on whole animals (including the shell), and followed the protocol of Boisselier-Dubayle & Gofas (1999). Electrophoresis was done using vertical acrylamide gels on discontinuous systems. The running buffer was tris-glycine (4.95 mM, pH 8.3). Of the eight enzyme systems assayed, three gave scorable banding patterns and are used in this study (phosphoglucomutase: *Pgm*, EC 5.4.2.2; glucose-6-phosphate isomerase: *Gpi*, EC 5.3.1.9; aspartate aminotransferase: *Aat*, EC 2.6.1.1). Because of the small size of the specimens, it was technically not feasible to study all three enzyme systems on the same individual. We therefore extracted between 30 and 40 individuals per population, giving approximately 20 for each loci. In total, 439 individuals were used for the analyses (Table 1).

TABLE 1. *Bythinella* populations characteristics and number of individuals used for each analysis. All localities were cited by Bernasconi (2000). Tdl1 and Tdl2 are populations in two adjacent springs separated by 10 meters. Both fall within the generalized type locality of *Bythinella utriculus*. $N_{ea}$ = number of individuals used for enzymatic activities, $N_{sh}$ = number of shells measured.

| | Population information | | | | | | ITS-1 sequences | | |
|---|---|---|---|---|---|---|---|---|---|
| Nominal species | Code | Type Locality | Localities | Coordinates | Altitude (in meters) | $N_{ea}$ | DNA isolate | GenBank number | $N_{sh}$ and MNHN number |
| *B. viridis* | Che | yes | Chery-Chartreuve (02)<br>Moulin de Veau | 3°37'51"E<br>49°15'45"N | 130 | 25 | Che1<br>Che3 | DQ318901<br>DQ318902 | 27<br>Moll5959 |
| *B. rubiginosa* | Aud1 | yes | Audinac-les-Bains (09)<br>thermal spring | 1°10'56"E<br>43°00'22"N | 450 | 35 | Aud11<br>Aud12 | DQ318905<br>DQ318906 | 30<br>Moll5960 |
| *B. reyniesii* | Por | no | Boussenac (09)<br>Col de Port | 1°27'7"E<br>42°53'58"N | 1250 | 38 | Por1<br>Por2 | DQ318899<br>DQ318900 | 30<br>Moll5961 |
| *B. simoniana* | Cat | no | Alas (09)<br>Sainte-Catherine | 1°01'23"E<br>42°57'19"N | 510 | 36 | Cat3 | DQ318903 | 30<br>Moll5962 |
| | Eng | no | Engomer (09)<br>Arguilla | 1°04'17"E<br>42°56'53"N | 460 | 38 | No sequenced | | 30<br>Moll5963 |
| | Sou | no | Clermont (09)<br>La Souleille | 1°16'48"E<br>43°01'29"N | 300 | 40 | Sou2 | DQ318904 | 30<br>Moll5964 |
| *B.* cf. *eurystoma* | Aud2 | no | Audinac-les-Bains (09)<br>no thermal spring | 1°10'56"E<br>43°00'22"N | 450 | 38 | Aud23<br>Aud26 | DQ318907<br>DQ318908 | 30<br>Moll5965 |
| *Bythinella* sp. | Aud3 | no | Audinac-les-Bains (09)<br>confluence of AUD1 and AUD2 | 1°10'56"E<br>43°00'22"N | 450 | 34 | Aud32<br>Aud31 | DQ318909<br>DQ318910 | 30<br>Moll5966 |
| *B. utriculus* | Suz | no | La Bastide-de-Sérou (09)<br>Col de Suzan | 1°25'10"E<br>43°01'16"N | 410 | 34 | Suz3<br>Suz5 | DQ318914<br>DQ318915 | 30<br>Moll5967 |
| | Tdl1 | yes | La Bastide-de-Sérou (09)<br>Tour de Loly | 1°27'16"E<br>43°00'56"N | 460 | 37 | Tdl15<br>Tdl12 | DQ318912<br>DQ318913 | 30<br>Moll5968 |
| | Tdl2 | yes | La Bastide-de-Sérou (09)<br>Tour de Loly | 1°27'16"E<br>43°00'56"N | 460 | 42 | No sequenced | | 29<br>Moll5969 |
| | Roq | no | Roquefort les Cascades (09)<br>Cascades des Turasses | 1°45'44"E<br>42°57'22"N | 460 | 33 | Roq2 | DQ318911 | 59<br>Moll5970 |

Two polymorphic loci were interpreted for *Pgm*, only one for *Aat* and *Gpi*. Multiple loci encoding the same enzyme were numbered in order of decreasing mobility. The alleles were numbered with the same system.

For the four putative loci, data were scored in a matrix of individual genotypes. For each population, allele frequencies at each locus, percentage of polymorphic loci at 0.95 (*P*), mean number of alleles per locus (*A*), mean observed and expected heterozygosities (*Ho* and *He*), pairwise theta (Weir & Cockerham, 1984) among populations ($\theta$) were estimated using Genetix version 4.02 (Belkhir et al., 2001). Departures from Hardy Weinberg equilibrium (HWE) were tested in two steps: first a global test was performed using the exact HW test of Haldane (1954), Weir (1990), and Guo & Thompson (1992); second, heterozygote deficit or excess was tested with U tests. Genotypic linkage disequilibria were measured within each population and for each pair of locus, and exact tests of genotypic differentiation were performed per locus and for each population pair. All these tests were performed with Genepop version 3.4 (Raymond & Rousset, 1995). A sequential Bonferroni correction (Holm, 1979; Rice, 1989) was used when several statistical tests were performed simultaneously (for HWE, heterozygote deficit or excess, linkage disequilibrium).

A dendrogram of the 12 populations was constructed to analyse the distribution of genetic variation. The chord distance (Cavalli-Sforza & Edwards, 1967) was computed between all pairs of populations and the popu-

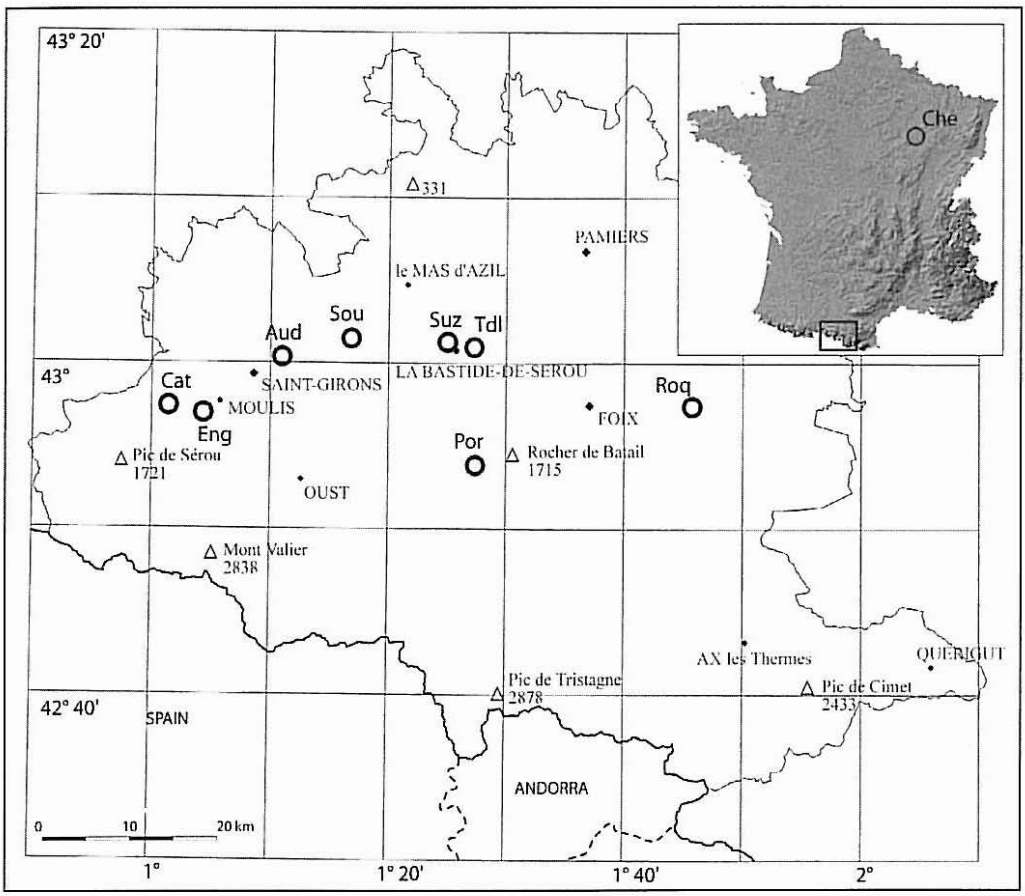


FIG. 1. Geographical location of *Bythinella* populations sampled. For details of the contiguous stations from Audinac (Aud) and La-Bastide-de-Sérou (TdL), see Table 1.

lations were clustered using the neighbour-joining (NJ) method. The chord distance assumes that allele frequency changes result from genetic drift alone; it does not assume that population sizes have remained constant and equal in size (Felsenstein, 1993). This analysis was conducted using Populations version 1.2.28 (Langella, 2002).

DNA Techniques and Phylogenetic Analyses

The ribosomal internal transcribed spacers (ITS-1 and ITS-2) are intergenic DNA sequences that, because of a relatively fast rate of evolution, are useful in resolving phylogenetic relationships of closely related taxa (see, for example, Schilthuizen et al., 2004, for molluscs, or for other zoological groups see (Hillis et al., 1996; Coleman, 2003; Chen et al., 2004). We here use the variability of ITS-1 that lies between ribosomal nuclear genes 18S and 5.8S.

DNA was extracted from whole individuals including shells, using the QIAGEN DNeasy®96 kit. The entire ITS-1 region was amplified using two universal primers ITS-2 (5'-GCT GCC TTC TTC ATC GAT GC-3') and ITS-5i (5'-AGG TGA CCT GCG GAA GGA TCA TT-3') (Hillis & Dixon, 1991).

PCR reactions were performed in a final volume of 50 µl, using approximately 5 ng of template DNA, 2.5 mM $MgCl_2$, 0.6 µM of each primer, 0.26 mM of each nucleotide, 5% DMSO and 1.5 unit of *Taq* polymerase (Qbiogene). Amplification products were generated by an initial denaturation step of 4 min at 94°C followed by 40 cycles at 94°C for 30 s, 50°C for 30 s and 30 s at 72°C, and a final extension at 72°C for 5 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced on a Ceq2000™ automated sequencer (Beckman), in both directions to confirm accuracy of each sequence. We obtained 17 sequences corresponding to one or two individuals analysed for each population (Table 1), aligned with the ClustalW algorithm (Thompson et al., 1994) implemented in the BioEdit 5.0.0 sequence alignment editor software package (Hall, 1999).

The DNA fragment sequenced included several highly variable regions with numerous insertions/deletions. These variable regions were removed from the data set and manually aligned following the procedure of Barriel (1994): number of indels, number of changes and number of modified sites are minimized in this order of priorities, a transition being preferred to a transversion in the ambiguous areas. Each plausible alignment for each of the highly variable regions was tested with PAUP* 4.0b (Swofford, 2000). The criterion used to choose an alignment among the different alignments tested was to obtain the most parsimonious tree.

After removing the first 5' ambiguous variable region which was located between the RNA18S gene and the first ITS-1 conserved region, the final data matrix was composed of 17 sequences and 360 bp. A maximum parsimony analysis was performed using PAUP*4.0b. Gaps were included as missing data. The analysis was performed using a heuristic search with 100 random-addition replications, branch swapping by the Tree Bisection and Reconnection (TBR) algorithm. To test the robustness of the results, the Bremer index (Bremer, 1994) was calculated and 100 bootstrap replicates were carried out, using the same heuristic search settings.

Morphometrical Analysis

*Shell Measurements* – Between 30 and 59 adult shells were used per population. Shells were washed in a 5% Chlorox solution for 30 min, then rinsed in distilled water, transferred to absolute ethanol and dried. The shells were placed on an adhesive support in a standardized position (for more details, see the legend of Fig. 2) and then digitized with a graduated scale using a stereomicroscope associated with a digital camera. An orthonormal frame (Ox, Oy) and the aperture center were overlaid on the digital picture, and 12 landmarks were then positioned using the TpsDig 1.23 software package (Rohlf, 2001). The coordinates of these landmarks within the orthonormal frame (Ox, Oy) were used to take 15 measurements characterizing individual shells (Fig. 2).

*Sex Determination* – Sexing the animal required dissolving the shell for dissection. After digitalization, specimens were removed from the adhesive support, placed for four min in a solution of 5% hydrochloric acid and 70% ethanol for shell dissolution, and then rinsed in distilled water. Dissections were done under a dissecting stereomicroscope. Sex was determined by the presence of the flagellum-penis complex for males and by the presence of the albumen and capsule glands for females.

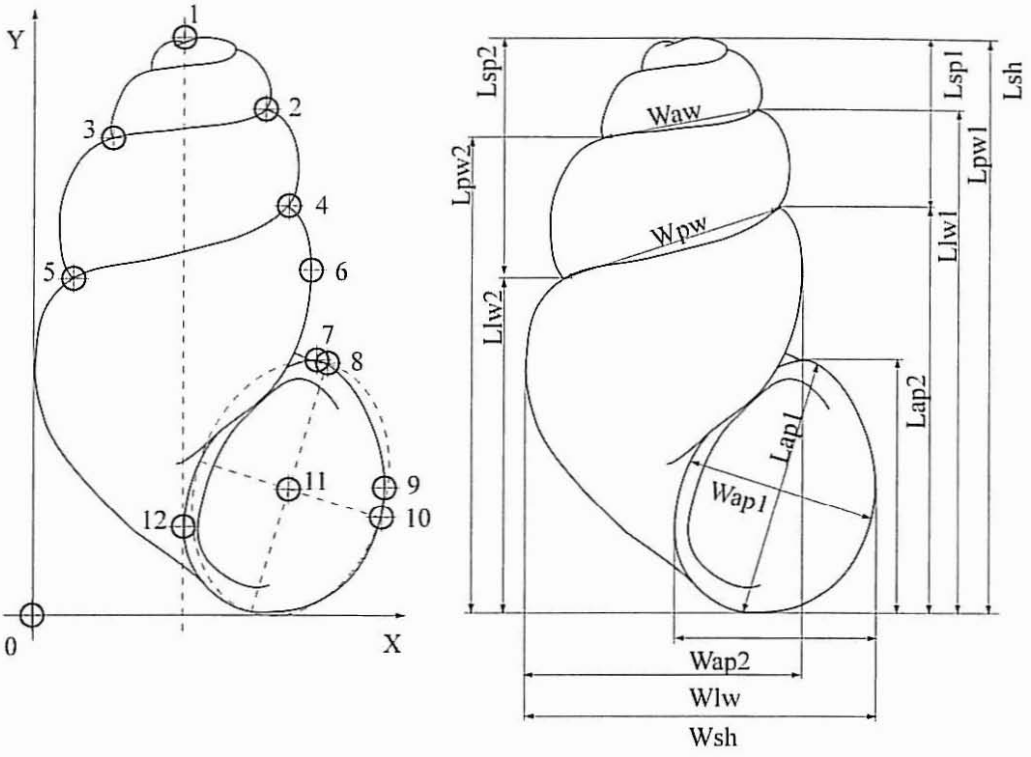


FIG. 2. Morphological parameters taken from shell of *Bythinella* in standard position: the collumelar axis and the aperture plane are parallel to the horizontal plane. Left – Landmarks locations. Orthonormal frame (Ox, Oy): the Oy axis is parallel to the collumelar axis and tangential to the left edge of the shell. The *Ox* axis is perpendicular to *Oy* axis and tangential to the lower edge of the aperture. The aperture center is the center of the *E* ellipse which is adjusted with the aperture edge. Right – Measurements taken. Shell height ($L_{sh}$), aperture length ($L_{ap1} = 2\sqrt{[x_8-x_{11})^2+(y_8-y_{11})^2]}$), aperture height ($L_{ap2}$), aperture diameter ($W_{ap1} = 2\sqrt{[x_{10}-x_{11})^2+(y_{10}-y_{11})^2]}$, aperture width ($W_{ap2} = x_9-x_{12}$), shell width ($W_{sh}$), width of last whorl ($W_{lw} = x_6$), width of penultimate whorl ($W_{pw} = \sqrt{[x_4-x_5)^2+(y_4-y_5)^2]}$), width of antepenultimate whorl ($W_{aw} = \sqrt{[x_2-x_3)^2+(y_2-y_3)^2]}$). Height measurements of the spire and of each whorl were taken on the left and on the right sides of the shell: left and right spire heights ($L_{sp1} = y_1-y_2$, $L_{sp2} = y_1-y_5$), left and right heights of penultimate whorl ($L_{pw1}$, $L_{pw2}$), left and right heights of last whorl ($L_{lw1}$, $L_{lw2}$).

*Analysis of Sexual Dimorphism* – Sexual dimorphism in *Bythinella* had been reported by Falniowski (1987) but never quantified. We assessed first how shell dimorphism might interfere with species delimitation within populations. For this, we analyzed shell parameters in 59 individuals from Roq. The input data in millimeters were transformed into Log-shape Ratio (LSR) data in order to limit an eventual size effect (Mosimann, 1970). We first performed a Principal Component Analysis (PCA) to explore how the range of shell parameters was distributed between males and females. Then a Discriminant Function Analysis (DFA) was used to test statistically the differences between males and females and then to identify which shell parameters reflect sexual dimorphism. Wilks' Lambda (WL) used in an ANOVA (F) test of mean differences was used to test if the discriminant model as a whole was significant. Then, if the global F test was significant, each variable was tested using Wilks' lambda to determine which variable differed significantly in mean between discriminated groups.

Second, we evaluated the effect of sexual dimorphism among populations, taxonomic groups and genetic groups. For this purpose, an analysis of variance (ANOVA) with two fixed factors (population and sex) was carried out on the parameters revealed by the DFA as significantly contributing to shell dimorphism in

the population analysed. This analysis permitted comparison of the relative effects of sex and population location on shell shape, and identification of the interaction between these two factors. Tukey's HSD *post hoc* test and its associated probability were used to examine the statistical significance of the differences between all pairs of means.

*Analysis of Shell Variation Between Populations and Taxa* – DFAs and associated Wilks' Lambda were performed on all 15 measurements after LSR transformation, and two discriminant factors were tested: (i) the *Taxon* discriminating factor: populations referred *a priori* to a nominal species based on the collection site were tested *a posteriori* to check whether they could be discriminated based on shell parameters (population Aud3 was excluded from the analysis because it was not assigned to a nominal taxon); (ii) the *Genetic* discriminating factor: clusters of populations resulting from the genetic and phylogenetic analyses were tested *a posteriori* to check whether specimens grouping together could also be discriminated based on their shell. We then compared the reclassification scores of the discriminating linear functions of the two grouping methods (i.e., taxon or genetic groups).

All analyses were performed with the software package STATISTICA 6.0 (Statsoft, 2001) and the level of significance used was 5%.

## RESULTS

### Isozymes Polymorphism

The three enzyme systems involving four putative loci revealed from three to five alleles per locus (Table 2), and the mean number of alleles per population ranged from 1.0 to 2.25 (Table 3). The *Aat-1* locus was the least polymorphic, with a total of three alleles overall and with each population being monomorphic. The one population (taxon) used as the out-group (*Bythinella viridis*) was monomorphic for all four loci, as was the Sou population. For the other populations, the mean observed heterozygosity (*Ho*) ranged from 0.015 to 0.241. Genotype

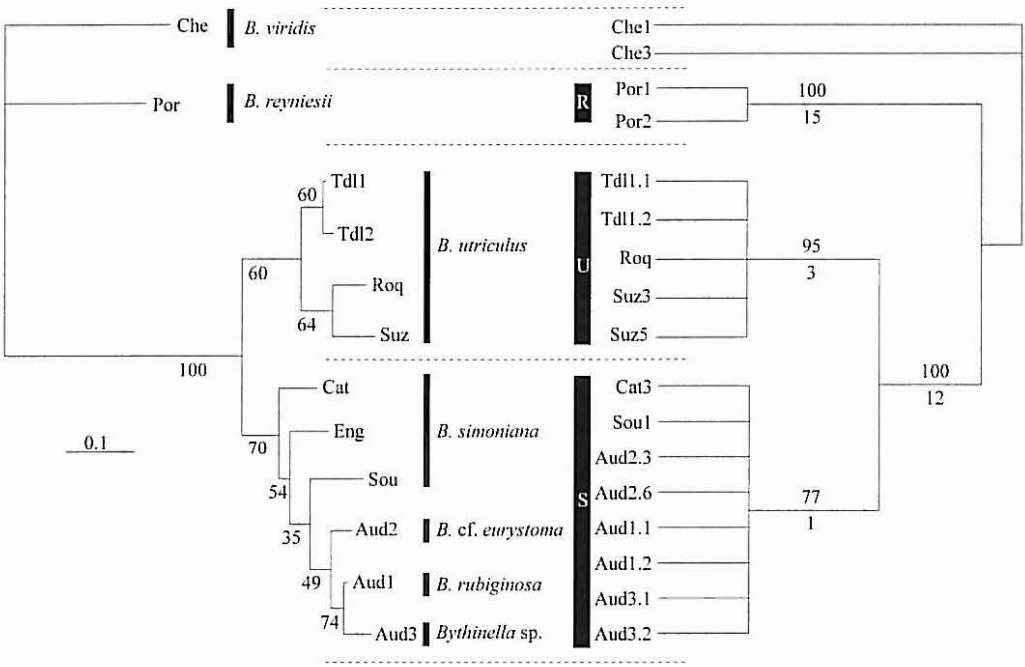


FIG. 3. NJ tree constructed from isozyme data on the D chord distance (Cavalli-Sforza & Edwards, 1967) between populations of *Bythinella* (left) with the Bootstrap values and parsimony tree (strict consensus) computed from ITS-1 sequences (right) with a heuristic search (Bootstrap values above, Bremer index below).

TABLE 2. Allele frequencies observed at 4 loci on 12 populations of *Bythinella*. N = number of individuals analysed for each locus.

| | | Aud1 | Aud2 | Aud3 | Cat | Eng | Sou | Tdl1 | Tdl2 | Roq | Suz | Por | Che |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Pgm-1* | (N) | (24) | (24) | (23) | (24) | (22) | (28) | (25) | (25) | (24) | (24) | (22) | (7) |
| | 1 | - | - | - | - | 0.023 | - | - | - | - | - | 1.000 | - |
| | 2 | 0.417 | 0.458 | 0.674 | 0.250 | 0.068 | - | 0.140 | 0.100 | - | 0.063 | - | - |
| | 3 | 0.563 | 0.458 | 0.326 | 0.688 | 0.886 | 1.000 | 0.340 | 0.300 | 0.854 | 0.208 | - | - |
| | 4 | 0.021 | 0.083 | - | 0.063 | 0.023 | - | 0.520 | 0.600 | 0.146 | 0.729 | - | - |
| | 5 | - | - | - | - | - | - | - | - | - | - | - | 1.000 |
| *Pgm-2* | (N) | (24) | (24) | (18) | (24) | (18) | (28) | (21) | (20) | (19) | (19) | (17) | (9) |
| | 1 | 1.000 | 1.000 | 1.000 | 0.646 | 0.889 | 1.000 | 0.262 | 0.225 | - | - | 0.029 | - |
| | 2 | - | - | - | 0.354 | 0.111 | - | 0.738 | 0.775 | 1.000 | 1.000 | - | - |
| | 3 | - | - | - | - | - | - | - | - | - | - | 0.971 | - |
| | 4 | - | - | - | - | - | - | - | - | - | - | - | 1.000 |
| *Gpi-1* | (N) | (24) | (25) | (22) | (27) | (22) | (30) | (27) | (30) | (26) | (26) | (24) | (19) |
| | 1 | - | - | - | - | - | - | - | - | - | - | 1.000 | 1.000 |
| | 2 | 0.958 | 1.000 | 0.955 | 0.981 | 0.932 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - | - |
| | 3 | 0.042 | - | 0.045 | 0.019 | 0.068 | - | - | - | - | - | - | - |
| *Aat-1* | (N) | (9) | (8) | (12) | (12) | (17) | (16) | (10) | (14) | (1) | (1) | (14) | (10) |
| | 1 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - | - |
| | 2 | - | - | - | - | - | - | - | - | - | - | 1.000 | 1.000 |

TABLE 3. Genetic variability computed on the four analysed loci on *Bythinella* populations. *Ntot* = total number of individuals used for isozyme activities; *n* = mean number of individuals analysed per locus; *P* = percentage of polymorphic loci; *A* = mean number of allele per locus; *Ho* = observed heterozygosity; *He* = expected heterozygosity; *HWE* = Hardy-Weinberg equilibrium tests (Fischer's method for test of significance, *significant at 0.05 level).

| Population | *Ntot* | *n* | *P* | *A* | *Ho* | *He* | *HWE* |
|---|---|---|---|---|---|---|---|
| Aud1 | 35 | 20.3 | 0.5 | 1.75 | 0.104 | 0.150 | 0.22 |
| Aud2 | 38 | 20.3 | 0.25 | 1.5 | 0.146 | 0.146 | 1 |
| Aud3 | 34 | 18.8 | 0.5 | 1.5 | 0.164 | 0.135 | 0.712 |
| Cat | 36 | 21.8 | 0.75 | 2.0 | 0.134 | 0.244 | 0.003* |
| Eng | 38 | 19.8 | 0.75 | 2.25 | 0.124 | 0.137 | 0.146 |
| Sou | 40 | 25.5 | - | 1.0 | - | - | - |
| Tdl1 | 37 | 20.8 | 0.5 | 1.75 | 0.241 | 0.251 | 0.179 |
| Tdl2 | 42 | 22.3 | 0.5 | 1.75 | 0.162 | 0.227 | 0.158 |
| Roq | 33 | 17.5 | 0.25 | 1.25 | 0.052 | 0.064 | 0.398 |
| Por | 38 | 19.3 | 0.25 | 1.25 | 0.015 | 0.015 | 1 |
| Suz | 34 | 17.5 | 0.25 | 1.5 | 0.073 | 0.107 | 0.089 |
| Che | 25 | 11.3 | - | 1.0 | - | - | - |

TABLE 4. F-statistics indices (Weir & Cockerham, 1984) calculated for the *Bythinella* populations analysed (excluding out-group and Por populations, see text for explanations).

| *Locus* | $F_{IS}$ | $F_{ST}$ | $F_{IT}$ |
|---|---|---|---|
| *Pgm-1* | 0.209 | 0.332 | 0.472 |
| *Pgm-2* | 0.179 | 0.718 | 0.769 |
| *Gpi-1* | -0.033 | 0.020 | -0.012 |
| *all* | 0.189 | 0.481 | 0.579 |

frequencies generally matched Hardy Weinberg expectations after a sequential Bonferroni correction, except for the Cat population, which had a heterozygosity deficit. No genotypic linkage disequilibrium between pairs of loci was found.

These four loci were together diagnostic for two of the six presumptive taxa: (i) individuals from the type-locality of *B. viridis* had diagnostic alleles for the two loci *Pgm-1* and *Pgm-2*; and (ii) individuals from Por, attributed to *B. reyniesii*, had a diagnostic allele for *Pgm-2*. Furthermore, individuals attributed to *B. viridis* and *B. reyniesii* together differed from all other *Bythinella* specimens by the same diagnostic alleles at the two loci *Gpi-1* and *Aat-1*. All other populations sampled from the Pyrenean foothills shared alleles with different frequencies at all the loci scored. Thus, gene flow between any of these three groups and any other population from our sample can be excluded, whereas gene flow among populations from the Pyrenean foothills cannot be excluded.

The *NJ* tree constructed on the chord distance illustrates the genetic relationships between populations (Fig. 3, left). Two populations, *B. viridis* and the Por population (attributed to *B. reyniesii*) were highly divergent from all others. For other populations, the topology of the tree revealed two main genetic groups. First, the four populations Tdl1, Tdl2, Suz, and Roq (attributed to *B. utriculus*) clustered together, with values of *D* ranging from 0.001 (Tdl1 *vs.* Tdl2) to 0.1 (Tdl1 *vs.* Roq). Second, the populations Aud1 (*B. rubiginosa*), Aud2 (*B.* cf. *eurystoma*), Aud3, Sou, Eng, and Cat (the last three attributed to *B. simoniana*) clustered together, with *D* ranging from 0.011 (Aud1 *vs.* Aud2) to 0.139 (Aud3 *vs.* Sou).

Because the pattern of shared alleles indicated that gene flow could not be excluded among the Pyrenean foothill populations, we analysed the genetic structure among them using F statistics (Table 4). The out-group and the Por population of *B. reyniesii* were not included in this analysis. The genetic variance among populations ($F_{ST}$ = 0.481) represented the principal component of the total genetic variance of the populations analysed ($F_{IT}$ = 0.579); the genetic variation within populations was relatively low ($F_{IT}$ = 0.189). Thus, these *Bythinella* populations are highly differentiated genetically. The $\theta$ values estimated between pairs of populations were significantly different from zero, except between populations collected in the same locality (Aud1, Aud2, Aud3; Tdl1, Tdl2) or nearby (Tdl2 with Suz). The genotypic differentiations calculated for each pair of populations and for all loci reveal significant *P* values, except between populations located in the same locality (Aud1 to 3 and Tdl1 and 2).

### Phylogenetic Analysis

Of the 360 bp analysed, 76 positions were variable and 48 were phylogenetically informative. Figure 3 (right) displays the strict consensus tree based on 2,700 equi-parsimonious trees resulting from the heuristic search. The tree length was 96 steps with CI = 0.96 and RI = 0.96.

The topology revealed three distinct clades. The first clade (clade R) included the individuals from Por referred to *B. reyniesii*. This clade was supported by strong Bootstrap values (= 100) and Bremer index (= 15). The second clade (clade U) included the individuals from populations referred to *B. utriculus* (Tdl1, Suz and Roq). The third clade (clade S) included the individuals referred to *B. rubiginosa* (Aud1), *B.* cf. *eurystoma* (Aud2), *B. simoniana* (Cat and Sou), and the Aud3 population. Clade U and S constituted a monophyletic group, which was also supported by strong Bootstrap values (= 100) and Bremer index (= 12).

The phylogenetic analysis corroborated the enzyme polymorphism studies. Indeed, the three monophyletic groups revealed corresponded to the *B. reyniesi* population and the two main groups revealed by the isozyme analyses. Therefore, in the following morphological analysis, we consider these groups as a hypothesis alternative to the classical taxonomy, which can be formulated as follows: four population groups of our dataset constitute four distinct species: Group V = Che (used as out-group); Group R = Por; Group S = Aud1, Aud2, Aud3, Cat, Sou and Eng; Group U = Tdl1, Tdl2; Suz and Roq.

Morphometrical Analysis

*Sexual Dimorphism* – Overall 358 specimens were successfully sexed, 55% males and 45% females. Within a single population, 59 specimens from Roq included 36 (61%) males and 23 (39%) females. PCA was carried out on the 15 shell parameters LSR transformed. The first (PC1) and second (PC2) principal components respectively account for 63.5% and 13.4% of the total variation (Fig. 4). Three groups of strongly correlated parameters were detected: (i) global shell size parameters ($L_{sh}$, $W_{sh}$, $W_{lw}$, $L_{pw1}$, $L_{pw2}$, $L_{lw1}$, $L_{lw2}$), (ii) spire parameters ($W_{aw}$, $W_{pw}$, $L_{sp1}$ and $L_{sp2}$) and (iii) aperture parameters ($L_{ap1}$, $L_{ap2}$, $W_{ap1}$, $W_{ap2}$). The first two sets of variables, which are linked to global shell size, were primarily associated with PC1. This first axis was thus interpreted as a size factor. The third set of parameters, related to shell aperture, were primarily associated with PC2. The projection of the individuals in the PC1 x PC2 factorial plane revealed that, whereas females and males had similar distribution patterns along PC1, they differed along PC2 (Fig. 4).

The DFA computed with *sex* as the discriminate factor was significant (WL = 0.4241, p = 0.0002). Scores of WL with the probability associated with each variable indicated that the two sexes differed significantly in one aperture parameter ($W_{ap2}$ with WL = 0.501 and p < 0.008). The global reclassification score of the discriminating linear functions was 86.44% (78.26% for females and 91.67% for males). These results suggest that females differ from males by having a larger aperture width ($W_{ap2}$), but not by shell size.

Between populations, the same DFA analyses were then performed on all populations referred, taxonomically and genetically, to the group *utriculus*: 136 specimens were sexed, 62% males and 38% females. This DFA was significant (WL = 0.725, p = 0.0004), but the two sexes differed significantly in the parameter $L_{ap1}$, another aperture parameter ($L_{ap1}$ with WL = 0.755, p = 0.028).

Finally, a DFA was performed with *sex* as the discriminate factor on the global data set of 358 sexed specimens. The analyses was globally significant (WL = 0.914, p = 0.011), indicating that the set of variables chosen allows discrimination between the sexes. Scores of WL with the probability associated with each variable suggested that the two sexes differed significantly only by one aperture parameter ($L_{ap1}$ with WL = 0.927 and p < 0.030).

In order to test for the respective effects of sexual dimorphism and taxonomic differences, two analyses of variance (ANOVA) were performed on the LSR global data set on the two aperture parameters $W_{ap2}$ and $L_{ap1}$ that significantly differed between males and females. First, we tested the effects of the two factors *taxonomic group* and *sex* and their interaction, and then the effect of the factors *genetic group* and *sex* and their interaction. These two ANOVAs revealed that both effects of *taxonomic group* and *genetic group* were significant for the two parameters whereas the effect of *sex* was not significant in any analysis.

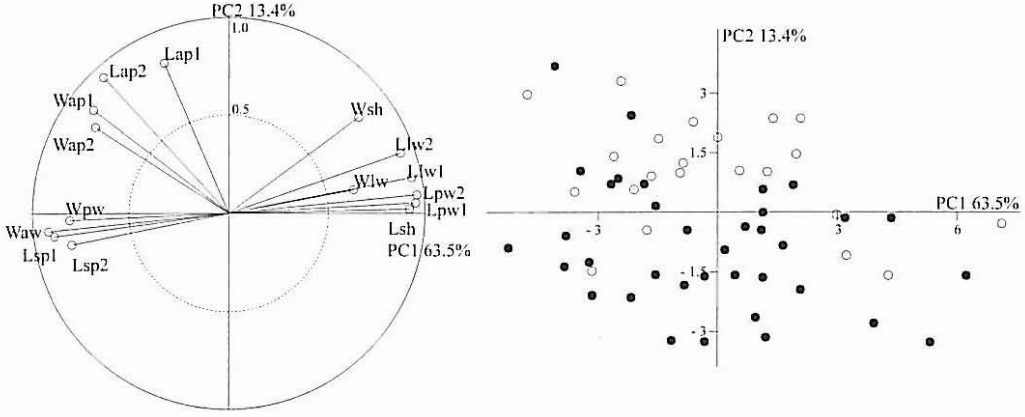


FIG. 4. Sexual dimorphism in Roq population of *Bythinella*: results of PCA. Left Correlation of the 15 parameters measured on the shell to the first two principal components PC1 and PC2. Right Projection of the individuals in the first factorial plan PC1 x PC2. Open circles are females and filled circles are males.

TABLE 5. Correlations matrix between the variables and the first two discriminate functions (F1 and F2) for the two alternative *Bythinella* hypotheses (Taxon group and Genetic group).

| | Taxon group hypothesis | | Genetic group hypothesis | |
|---|---|---|---|---|
| Shell parameters | F1 | F2 | F1 | F2 |
| Lsh | -0.027 | 0.189 | -0.058 | -0.243 |
| Wsh | -0.460 | 0.160 | -0.495 | -0.216 |
| Lap2 | 0.001 | -0.300 | 0.017 | 0.327 |
| Wap1 | 0.076 | -0.217 | 0.103 | 0.265 |
| Wlw | -0.504 | 0.084 | -0.407 | -0.008 |
| Lpw1 | -0.068 | 0.209 | -0.102 | -0.267 |
| Lpw2 | -0.090 | 0.228 | -0.125 | -0.289 |
| Llw1 | -0.108 | 0.200 | -0.147 | -0.267 |
| Llw2 | -0.188 | 0.184 | -0.237 | -0.264 |
| Waw | 0.263 | -0.184 | 0.307 | 0.256 |
| Wpw | 0.166 | -0.069 | 0.207 | 0.145 |
| Lsp1 | 0.368 | -0.179 | 0.418 | 0.256 |
| Lsp2 | 0.465 | -0.147 | 0.523 | 0.230 |
| Eingenvalue | 3.756 | 1.383 | 3.308 | 1.318 |
| Cum. Eigenv. | 53.8% | 73.7% | 63.3% | 88.5% |

For each of the two aperture parameters, the *taxonomic group* × *sex* interaction was not significant whereas the *genetic group* × *sex* interaction was significant but only for the $W_{ap2}$ parameter ($L_{ap1}$ F-test = 1.32 with p = 0.267 and $W_{ap2}$ F-test = 4.226 with p = 0.006). For this parameter, the *post hoc* test revealed that the *genetic group* × *sex* interaction was caused

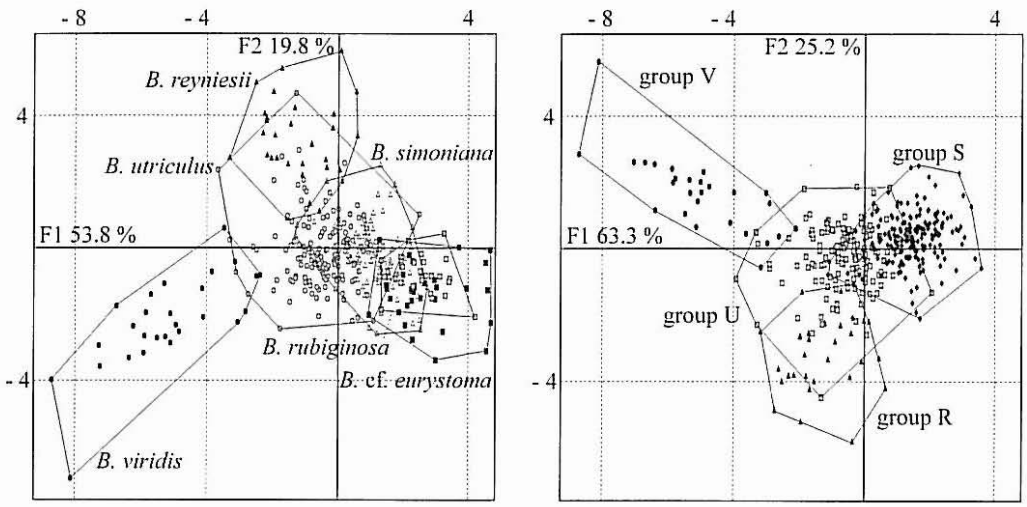


FIG. 5. DFA testing the two alternative hypotheses Taxon groups *vs* Genetic groups within *Bythinella* species. Left Taxon groups hypothesis. Filled circles: *B. viridis* (one population), open circles: *B. utriculus* (four populations), filled triangles: *B.* reyniesii (one population), open triangles: *B. simoniana* (three populations), filled squares: *B.* cf. *eurystoma* (one population), open squares: *B. rubiginosa* (one population). Right Genetic groups hypotheses. Filled circles: group V (*B. viridis*, one population), filled triangles: group R (*B. reyniesii*, one population), open squares: group U (*B. utriculus*, four populations), filled rhombus: group S (*B. simoniana*, *B. rubiginosa*, *B.* cf. *eurystoma* and Aud3 population, total six populations).

TABLE 6. Global reclassification scores of the discriminating linear functions for the two *Bythinella* hypotheses. Each line gives the number of specimens of a given taxon classified as any of the taxa, and the percentage of each that are correctly classified.

| | | Taxon group hypothesis | | | | | |
|---|---|---|---|---|---|---|---|
| *Bythinella* | % correct | *viridis* | *reyniesii* | *utriculus* | *simoniana* | cf. *eurystoma* | *rubiginosa* |
| *viridis* | 88.9% | 24 | 0 | 3 | 0 | 0 | 0 |
| *reyniesii* | 90.0% | 0 | 27 | 3 | 0 | 0 | 0 |
| *utriculus* | 87.2% | 0 | 5 | 129 | 13 | 1 | 0 |
| *simoniana* | 82.2% | 0 | 0 | 13 | 74 | 1 | 2 |
| cf. *eurystoma* | 83.3% | 0 | 0 | 1 | 0 | 25 | 4 |
| *rubiginosa* | 83.3% | 0 | 0 | 1 | 2 | 2 | 25 |
| Total | 85.6% | 24 | 32 | 150 | 89 | 29 | 31 |

| | | Genetic group hypothesis | | | |
|---|---|---|---|---|---|
| | % correct | Group V | Group R | Group U | Group S |
| Group V | 81.5% | 22 | 0 | 5 | 0 |
| Group R | 90.0% | 0 | 27 | 3 | 0 |
| Group U | 86.5% | 1 | 5 | 128 | 14 |
| Group S | 92.8% | 0 | 0 | 13 | 167 |
| Total | 89.4% | 23 | 32 | 149 | 181 |

by Por (group R, attributed to *B. reyniesii*), in which the sex ratio was 24% males and 76% females.

To summarize, the analysis shows that variation of aperture parameters is mainly explained by differences between "species" (defined on either the *taxonomic* or *genetic* delimitation hypotheses), and that sexual dimorphism within a population is manifest in aperture size. To minimize the effect of the sexual dimorphism, the two aperture parameters were removed in the subsequent analyses.

*Shell Variation Among Populations and Taxa* – To explore the pattern of shell variation, a PCA was carried out on the whole *LSR* dataset (385 individuals of both sexes). The first factorial plane accounted for 92.1% of the global variation with 80.3% by PC1 and 11.8% by PC2 (graph not presented here). Three groups of strongly correlated parameters were detected: (i) spire and aperture parameters negatively correlated with the PC1, (ii) height of the various whorls positively correlated with PC1, and (iii) shell width and width of the last whorl negatively correlated with PC2. The projection of the individuals in this factorial plane revealed a strong overlap between the various populations. On the PC2 axis, only individuals of *B. viridis* had a distinct position. Thus, except for this taxon, the parameters used in this analysis did not reveal any differences among groups of individuals when using either the taxonomic or the genetic hypotheses.

*Taxon Hypothesis* – The DFA on the LSR global data set (less the taxonomically doubtful Aud3 population and $L_{ap1}$ and $W_{ap2}$) performed with *Taxon* as discriminate factor was significant (WL = 0.023, $p < 0.05$). Scores of WL with probability associated with each variable showed that all parameters significantly discriminate the taxonomic groups. Projection of the individuals on the first factorial plane F1 x F2 (each axis respectively accounting for 53.8% and 19.8% of the total variance) allowed discrimination, mainly on the F1 axis, of a cluster of individuals attributed to *B. viridis*. The F1 axis was mainly correlated with spire parameters ($W_{pw}$, $L_{sp1}$ and $L_{sp2}$), whereas the F2 axis was mainly explained by a size effect (Table 5). Indeed, for this axis, all variables contributed the same weight and were strongly correlated (Fig. 5). In this F1 x F2 factorial plane, other taxonomic groups were poorly resolved with an extensive overlap among them. For example, individuals referred to *B. simoniana*, *B.* cf. *eurystoma* and *B. rubiginosa* were not separated.

The global reclassification score (Table 6) of the discriminating linear functions was 85.6%, with specific scores ranging from 83.3% (individuals attributed to *B.* cf. *eurystoma and B. rubiginosa*) to 90.0% (individuals attributed to *B. reyniesii*).

*Genetic Hypothesis* – DFAs performed on the LSR global data set (less $L_{ap1}$ and $W_{ap2}$) with *Genetic groups* as discriminate factor were also significant (WL = 0.062, $p < 0.05$). Scores of WL with probability associated for each variable showed that all parameters significantly discriminated the genetic groups. The F1 x F2 factorial plan accounted for 88.5% of the global variance, with 63.3% for F1 and 25.2% for F2 (Table 5). The spire parameters are positively correlated with F1, whereas $W_{sh}$ and $W_{lw}$ were negatively correlated with the same axis. The $W_{ap1}$ and $L_{ap2}$ parameters contributed essentially to F2. The projection of individuals in this factorial plane revealed three distinct groups on the F1 axis, corresponding to individuals assigned to groups V, U and S defined below (Fig. 5). On the other hand, F2 allowed separation of group R from all other groups. The global reclassification scores (Table 6) were 89.3% under the *Genetic* hypothesis, with reclassification scores ranging from 81.5% (group V) to 92.8% (group S).

From this result, we can identify four morphogroups based mainly on spire parameters: (i) group V with a small spire and a large last whorl that gives the shell an ovoid outline; (ii) group S with a large overall size and a high spire that gives the shell an elongate outline; (iii) group U, intermediate between V and S, with an outline that can be described as ovoid-elongate; (iv) group R with a spire height equal to the height of the last whorl and a large aperture, that gives the shell a pupoid outline.

## DISCUSSION AND CONCLUSION

Our analyses confirmed that all specimens attributed to *B. viridis* (Fig. 6A), which are used as out-group, were morphologically and genetically distinct from all Pyrenean specimens.

Within the Pyrenean sample, two species delimitations resulting from our analysis confirmed earlier traditional species delimitations (Bernasconi, 2000). Specimens attributed to *B. reyniesii* (Fig. 6B) were both morphologically and genetically distinct from all other specimens included in our study. The morphometric analysis describes the shell as having a spire equal to the height of the last whorl and a large aperture, resulting in a pupoid overall shape. However, since a single population was included in the study, this description lacks robustness and we do not know how the characters observed may be subject to phenotypic plasticity or to genetic variability.

The second validated species was *B. utriculus* (Fig. 6F), in which specimens from all populations clustered both as a monophyletic group (DNA sequences) and as a genetic group (isozymes). We hypothesize that the entire clade can be classified as a single species. As several populations are included within this species, our morphometric characterisation integrates at least part of the phenotypic plasticity component of shell shape variability.

All other populations formed a single genetic and phylogenetic group. Morphometric analysis describes the shell within this group as having a large overall size and a high spire that gives it an elongate shape. This group included the nominal *B. simoniana* (Fig. 6D), characterized in traditional taxonomy by axial varices on the last whorl – a character not confirmed in our analysis as of specific diagnostic value – and also included specimens from the two springs complex at Audinac, the cold spring (Fig. 6C) and the thermal spring type locality of *B. rubiginosa* (Fig. 6E), and their confluence. Thus, this cluster groups at least two nominal taxa that should be considered a single species, to be named *B. rubiginosa* based on the Principle of Priority (ICZN *Code* Art. 23.3). The high quantitative (size) or qualitative (varices) shell variability observed in this group may be due either to phenotypic plasticity or to intraspecific genetic variation.

However, although DFA analyses allow separation of these genetic groups, the PCA results highlighted overlaps among most populations, indicating that *a priori* determinations at the species level rank in *Bythinella* are still difficult even when using the shell characters here identified. Moreover, these shell characters cannot be validated for alpha-taxonomy without a robust test at the scale of the genus. We show here that it is possible to give an alternative species delimitation hypothesis to the traditional view in an evolutionary framework and that these newly defined entities exhibit distinct morphological features. We agree with Wilke et al. (2002) about the necessity to use molecular markers and morphometrical approaches in concert to study cryptic or closely related species.

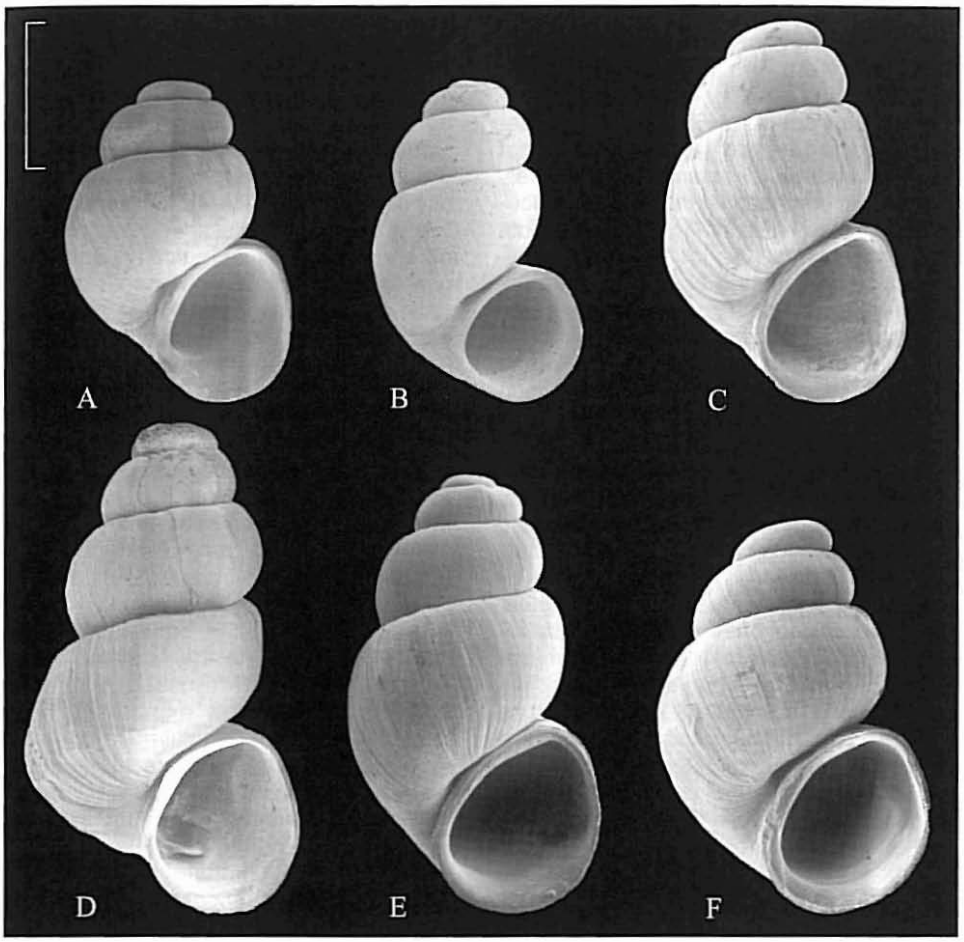


FIG. 6. Shells of the nominal species of *Bythinella* studied. A: *B. viridis* (Che); B: *B. reyniesii* (Por); C: *B.* cf. *eurystoma* (Aud2); D: *B. simoniana* (Eng); E: *B. rubiginosa* (Aud1); F: *B. utriculus* (Roq) (Scale 1 mm).

Genetic studies of Polish populations revealed weak genetic differentiation primarily correlated with geographic distance (Falniowski et al., 1998, 1999). The former suggested that "... the superspecies status of the genus (Giusti & Pezzoli, 1977) seems the most justified". The term superspecies was first used by Mayr (1931) and subsequently defined by Mayr & Ashlock (1991) as "a monophyletic group of closely related and entirely or largely allopatric species that are too distinct to be included in a single species or that demonstrate their reproduction isolation in a zone of contact." Our data don't refute this opinion within the Pyrenean area where a mosaic of populations probably belonging to different species more or less morphologically differentiated seems to coexist in allopatry.

Nevertheless, we show that some shell characters that were traditionally used to segregate species of *Bythinella* (see, for example, Bernasconi, 2000) do not necessarily have taxonomic significance, a result agreeing with that of others working on other hydrobioid groups (Falniowski & Wilke, 2001; Wilke & Falniowski, 2001; Szarowska & Wilke, 2004). The high intraspecific shell variability may be linked to biotic factors, such as food availability, parasite-induced gigantism (Jourdane, 1979; Gorbushin, 1997; Probst & Kube, 1999), or sexual dimorphism (Ponder et al., 1999; Kurata & Kikuchi, 2000; Velecka & Jüttner,

2000; Chiu et al., 2002). Our study corroborates Falniowski's (1987) observation that sexual dimorphism is rather low in *Bythinella*, females differing from males by their generally larger shell aperture. However, as sex ratio appears not fixed in populations of *Bythinella*, superficial morphological differences among populations may also be a consequence of different sex ratios, and contribute to fuzzy species delimitations. Shell variability may also be linked to abiotic parameters, such as temperature (Brown & Richardson, 1992). In the three Audinac populations of *B. rubiginosa*, specimens from the thermal spring are largest, specimens from the cold spring are smallest, and specimens from the confluence are intermediate.

To conclude, the present work offers an example of a three-step integrative taxonomy starting from (i) primary hypotheses (current species delimitations based on phenetic characters), to (ii) the test of these hypotheses by criteria that are conceptually sound. Population genetics approaches allow to identify reproductively isolated groups. Molecular phylogeny allows the recognition of monophyletic groups and thus the detection of groups that share ancestry. These two criteria, derived from the BSC and the PSC, permit new species delimitation, and finally (iii) a feedback to a new taxonomic hypothesis associated with new phenetic descriptors. Therefore, the final description corresponds to the most fitting hypothesis given the data at the time of the description.

A correct delimitation of species has important consequences both for a reliable estimation of biodiversity but also when drawing conservation priorities. Two of the nominal species in the present study (*B. viridis* and *B. reyniesii*) are categorized as Vulnerable in the IUCN Red List and are also protected under French law. Both emerged from the present study as probably distinct evolutionary units.

Our study was restricted to a small part of the geographical range of *Bythinella* including a subset of its nominal species and used a low number of isozyme loci and a short gene fragment. Thus, our results are of preliminary nature. In order to validate them, we need to expand this approach to a global revision of the genus, including supplementary mitochondrial and nuclear markers. However, the challenges to expanding this model are immense. The literature on European hydrobioids is replete with names of unknown significance (Davis, 2004) that blur evaluations of regional species richness, conservation priorities and evolutionary history. This is because many nominal species were established by 19th and 20th century authors that (i) worked on a local or regional basis, and/or (ii) worked outside a context of evolutionary systematics. Their approach to alpha-taxonomy has also occasionally injected in the literature names based on few, sometimes single, specimens only weakly differentiated conchologically. Because the present study shows that genetic or phylogenetic distinctiveness cannot be inferred from the degree of shell distinctiveness, the challenge to a comprehensive revision of *Bythinella* is that these nominal species have to be re-evaluated based on populations sampled alive from their type locality. At this moment, we do not know whether an integrative revision of *Bythinella* will result in just a few or several dozen species.

How do the present results impact the systematics of fossil *Bythinella*, and does the result of the present study preclude naming any new *Bythinella* species based on shell characters only? Despite the numerous nominal species of *Bythinella* described by 19th and 20th century authors, new exploration of little-known aquifers, in particular hypogean environments in southern France, still leads to the discovery of new morphotypes. Sometimes, only empty shells are known and living specimens escape collection despite intensive searches. We understand that it may be necessary, for communication or conservation purposes, to name such taxa; however, it is essential that the primary description is based on shell characters that have been tested for their robustness regarding species delimitation.

Stability in the alpha-taxonomy of hydrobioids will most likely be reached through an integrative taxonomy approach (Sites & Marshall, 2003, 2004). A possible approach would be the Hennigian inter-nodal species concept derived from the Theory of Evolution (Samadi & Barberousse, 2006) that here proved an efficient tool in the study of Pyrenean *Bythinella*.

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