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A systematic review of the Iberian springsnail subgenus *Alzoniella* (*Navarriella*) (Caenogastropoda: Hydrobiidae), with the description of a new potentially relict subfamily

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ABSTRACT

The threatened springsnail subgenus *Alzoniella* (*Navarriella*) in the Iberian Peninsula has been suggested to be an old and relict lineage of the family Hydrobiidae. The subgenus is represented by two morphological species, both endemic to the Pyrenees and their southern foothills. We conducted phylogenetic analyses based on mitochondrial and nuclear gene fragments of topotypes and other populations, four molecular species delimitation methods, and morphological examinations to clarify the uncertain systematic position of the subgenus within the family, assess its species diversity, and understand the population genetic structure of the two geographically restricted species. Our phylogenetic results revealed that *Alzoniella* (*Navarriella*) is distantly related to all other species of *Alzoniella*, even belonging to an independent subfamily-level clade, for which we introduce the new genus *Navarriella* and the new subfamily Navarriellinae subfam. nov. Molecular methods and geometric morphometric analysis of shell shape identified a single species in the new genus. The significant phylogenetic distance from other hydrobiid taxa, narrow distribution, and limited gene flow among its populations (estimated from mitochondrial cytochrome *c* oxidase subunit I sequences) highlight *Navarriella* as an isolated lineage within the family that requires urgent conservation attention. Furthermore, our results cast a new light on the northern Iberian Mountains as a dispersal barrier for ancient spring lineages.

Keywords: freshwater gastropods; molecular phylogenetics; geometric morphometric analyses; integrative taxonomy; conservation; Pyrenees.

INTRODUCTION

The Iberian Peninsula is recognized as an evolutionary centre and glacial refugia for freshwater microgastropods belonging to the family Hydrobiidae W. Stimpson, 1865 (Arconada and Ramos 2003). This region harbors a significant number of imperilled species that are not found elsewhere (Miller *et al.* 2018). With over 90 described species (MolluscaBase 2023) representing 23 genera, the family Hydrobiidae is likely the most species-rich group of freshwater gastropods known in the Iberian Peninsula to date. These species are primarily found in headwater springs within the Iberian River basins and often exhibit specialized microhabitat preferences, with limited dispersal capabilities. Consequently, their geographic range is restricted to a few isolated springs (Verdú and Galante 2009, Verdú *et al.* 2011). This limited dispersal ability leads to reduced gene flow and, ultimately, to the risk of extinction. However, identifying hydrobiid species presents challenges due to their small size (shell size 0.5–5 mm) and simple morphology, which hampers the establishment of their taxonomic and conservation status.

The application of DNA-based phylogenies has significantly altered our understanding of the systematics and diversity of 13 hydrobiid genera inhabiting the Iberian Peninsula, as well as their actual geographic distribution in the region (Delicado and Ramos 2012, Delicado *et al.* 2013, 2019, Miller *et al.* 2022). For instance, the European genus *Arganiella* Fo. Giusti & Pezzoli, 1980 has recently been redefined to include only its type species, which occurs in the Italian Peninsula. The Iberian representative was transferred to the endemic genus *Aretiana* Delicado & Ramos, 2021 (Delicado *et al.*

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2021). Another case is *Deganta azarum* (Boeters & Rolán, 1988), which was initially classified under the genus *Islamia* Radoman, 1973 due to their morphological similarities. Consequently, the systematics of the remaining ten genera with species on the Iberian Peninsula needs to be reassessed using molecular tools.

The spring snail *Alzoniella* Fo. Giusti & Bodon, 1984 (subfamily Islamiinae Radoman, 1973) is another example of a widespread hydrobiid genus found in the Iberian Peninsula, where only morphological information has been utilized for the taxonomic identification of its species. Initially, the genus was erected to accommodate three hydrobiid snails with unique anatomical features and occurring on the Italian Peninsula, with *Alzoniella finalina* Fo. Giusti & Bodon, 1984 designated as the type species (Giusti and Bodon 1984). Subsequently, the known geographical range of the genus was expanded to encompass the Iberian Peninsula, southern France, Slovenia, and Hungary (Lozek and Brtek 1964, Rolán 1991, Boeters 2000, Arconada, Bolán & Boeters, 2007, Cianfanelli and Bodon 2017, Birindelli *et al.* 2020, Varga 2021).

Currently, the genus *Alzoniella* is divided into two subgenera and consists of more than 40 species, as recognized based on morphological characters (Glöer 2022, MolluscaBase 2023). Boeters (2000) classified the genus *Alzoniella* into two subgenera: *Alzoniella* (*s.s.*) and *Navarriella* Boeters, 2000 (type species: *Paludinella elliptica* Paladilhe, 1874). The first subgenus contains 41 species (14 of which are endemic to the Iberian Peninsula), while the second subgenus consists of only two species (i.e. the type species and *Alzoniella* (*Navarriella*) *pellitica* (Arconada, Bolán & Boeters, 2007), both Iberian endemics. It is noteworthy that the northern region of the Iberian Peninsula hosts species of both subgenera. Within this region, *Alzoniella* (*Navarriella*) is distributed from the Navarra Province to the Basque Country in the Spanish Pyrenees (Rolán 1991, Boeters 2000, Arconada, Bolán & Boeters, 2007) while the Iberian *A.* (*Alzoniella*) species are mainly found from Galicia to the Basque Country (Arconada, Bolán & Boeters, 2007, Rolán *et al.* 2009, Rolán and Boeters 2015). There are only five localities where both subgenera cohabit (Arconada, Bolán & Boeters, 2007). Currently, approximately 20 populations of *A.* (*Navarriella*) have been discovered on the Iberian Peninsula (Rolán 1991, Arconada, Bolán & Boeters, 2007). *Alzoniella* (*Navarriella*) was originally distinguished from the nominal subgenus according to its Z-shaped intestinal loop and pedunculated seminal receptacles (Boeters 2000, 2001). However, the penis of *A.* (*Navarriella*) was originally illustrated as having several lobes on its base, which were not observed in species of *Alzoniella s.s.* (Giusti and Bodon 1984). Arconada, Bolán & Boeters (2007) confirmed the differences in the shape of the intestinal loop and the number of penial lobes. They indicated that differences between the subgenera exist in the position of the distal end of the oviduct in relation to the mantle edge, rather than the seminal receptacles. The differences in the shape and position of the seminal receptacles, bursa copulatrix, penis, and intestinal loop are considered to determine the genus level in Islamiinae. Additionally, the presence of none, one, or two seminal receptacles and bursa copulatrix are taken into account. Moreover, the number and position of the penial lobes are observed to determine the genus level (Bodon *et al.* 2001).

The observed anatomical differences between the two subgenera and unpublished genetic analyses by T. Wilke led Bodon and Cianfanelli (2004) to propose elevating the subgenus *Navarriella* to the genus level. However, due to the absence of a formal description of the newly proposed genus, subsequent studies have placed it as a subgenus of *Alzoniella* (Arconada, Bolán & Boeters, 2007, Rolán *et al.* 2009, Rolán and Boeters 2015). Recently, Delicado *et al.* (2023) identified a high genetic distance between a population from the Pyrenees, referred to as *Alzoniella* (*Navarriella*) *elliptica*, and *A. finalina*. This finding even suggested that the two species belong to different subfamilies, with *A.* (*N.*) *elliptica* being one of the oldest lineages within the Hydrobiidae. However, genetic studies of the topotypical population of this species are required to confirm its classification within the family, the taxonomic status of the two *Navarriella* species, and their geographic distribution. Furthermore, it is necessary to confirm the potential relict nature of the *A.* (*Navarriella*) species, as the Iberian Peninsula is recognized as a biodiversity hotspot with numerous endemic species and glacial refugia (Hewitt 1999, Arconada and Ramos 2003, Benke *et al.* 2009, Schmitt 2017, Miller *et al.* 2018).

Here, we generated mitochondrial and nuclear genetic data for *A.* (*N.*) *elliptica* and *A.* (*N.*) *pellitica* to (1) clarify their uncertain systematic position within the Hydrobiidae, (2) assess their species status, and (3) gain insight into population genetic structure and diversity of these geographically restricted species. Additionally, we investigated potential overlaps in shell morphology among *A.* (*Navarriella*) populations and examined whether patterns of shell variation align with the genetic data. Furthermore, we present new morphological data for this subgenus and discuss its taxonomic status based on our molecular and morphological findings.

MATERIAL AND METHODS

Material examined

A total of 17 populations of *A.* (*Navarriella*) were collected and genetically analyzed (Fig. 1), while 15 of these populations and the type material of *A.* (*N.*) *pellitica* were used for morphological studies. They were collected across the known geographic range of the subgenus between 2018 and 2020. The type localities of *A.* (*N.*) *elliptica* and *A.* (*N.*) *pellitica* were both sampled.

Snails were collected by hand from stones and dry leaves over a watercourse or by sieving sediment. All specimens were relaxed and fixed following the protocol of Araujo *et al.* (1995). Specimens were preserved in either 80% or absolute ethanol for genetic analyses, and in 80% ethanol for anatomical studies. All specimens were then stored at -20 °C. All collected material and DNA samples were deposited in the Malacology Collection and the Tissues and DNA Collection at the National Museum of Natural Sciences (MNCN-CSIC), Madrid, Spain, respectively (Supporting Information, Tables S1, S2).

DNA isolation, amplification, and sequencing

One to five specimens from each population were processed for DNA sequencing. Genomic DNA was extracted from the whole animal using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Fragments of two mitochondrial genes, cytochrome *c* oxidase subunit I (*COI*) and the large ribosomal subunit (16S), and two nuclear genes, histone 3 (*H3*) and the large ribosomal subunit (28S), were generated. The primer pairs used for PCR

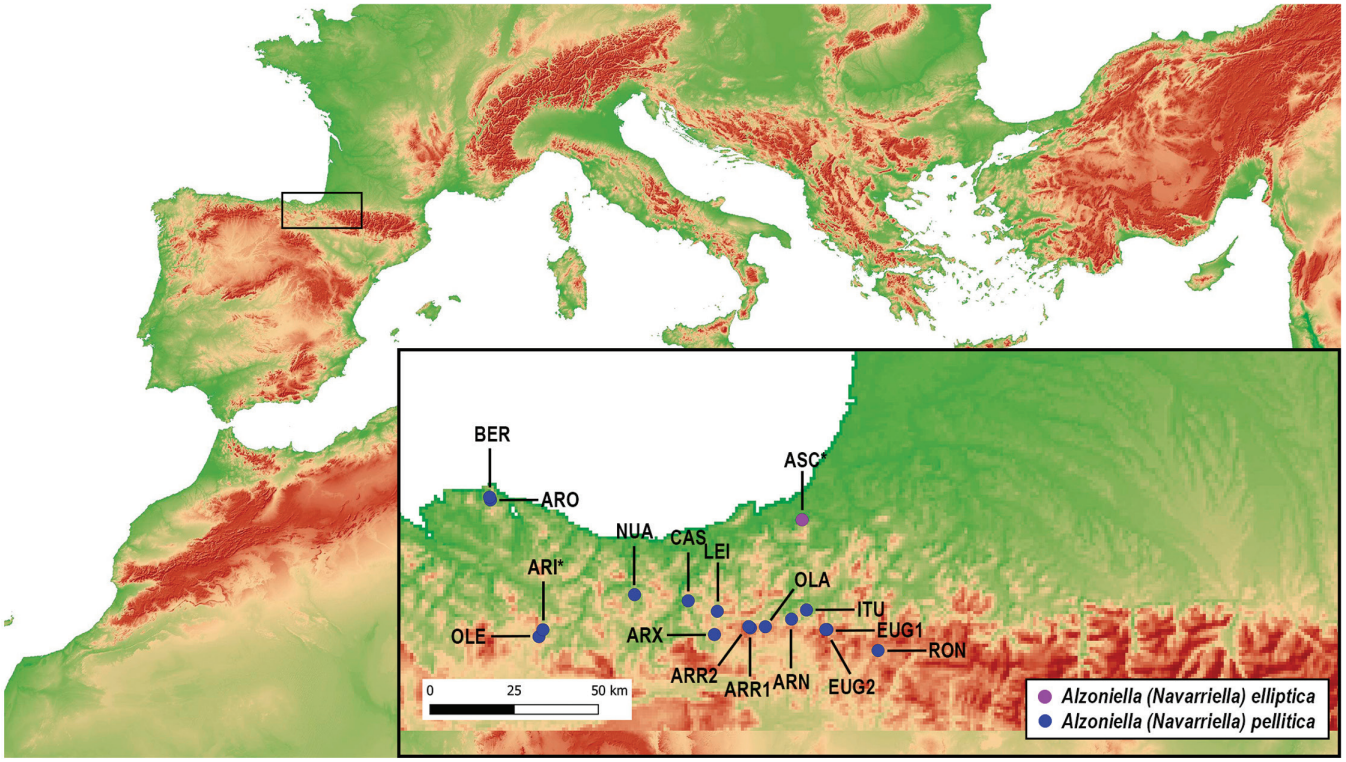

Figure 1. Distribution of the studied populations of *Alzoniella* (*Navarriella*) in the north of the Iberian Peninsula. Label abbreviations are explained in Supporting Information, Table S1. * indicates the sampled topotypes.

amplification were: COF14 (Folmer *et al.* 1994) and COR722b (Wilke and Davis 2000) for *COI*; 16Sar-L and 16Sbr-H (Palumbi *et al.* 1991), modified by Delicado *et al.* (2019), for 16S; H3F and H3R (Colgan *et al.* 2000) for *H3*; 18SF and 18SR (Holland *et al.* 1991) for 18S; and F63 and LSU3 (Park and Foighil 2000), modified by Benke *et al.* (2009), for 28S. These gene fragments have been successfully used to detect genetic differences among hydrobiid species (Delicado *et al.* 2015, 2019, Grego *et al.* 2020, Hofman *et al.* 2022, Miller *et al.* 2022). However, the 18S fragment is a conserved DNA region in the family Hydrobiidae (Wilke *et al.* 2001). For this reason, a single sequence was used to portray all individuals of the same species in the analyses.

Each PCR tube contained 1–5 μL of DNA, 2.5 μL of 10× Buffer, 0.6 μL of dNTPs mix, 0.6 μL of each primer (10 mM), 0.4 μL of *Taq* DNA polymerase (5U/μL – Takara), 0.25 μL of $MgCl_2$, and 19.65–15.65 μL of purified distilled water. The following PCR conditions were used: an initial step at 94 °C for 4 min; followed by 35 cycles at 94 °C for 45 s, this temperature varying according to the gene fragment used (50 °C for *COI*, 16S, and 18S, 54 °C for *H3*, and 51 °C for 28S) for 45 s (except 90 s for 28S) and 75 °C for 45 s; and a final extension at 72 °C for 10 min. PCR products were sequenced by the Macrogen Service Centre (Madrid, Spain).

Alignment and molecular data analyses

The amplified sequences (Supporting Information, Table S2) were edited using SEQUENCER v.5.4.6 (Gene Codes Corp., Ann Arbor. MI, USA). We subjected two datasets to phylogenetic analyses: (1) a concatenated dataset (*COI* and 18S) of the family, which included the sequences of the species representing the known subfamilies within the Hydrobiidae available in GenBank (Supporting Information, Table S3), and the sequences of *Bythinella austriaca* (Frauenfeld, 1857) from the related family Bythinellidae Locard, 1893 as an outgroup (GenBank accession number: AF213349 for *COI* and AF212917 for 18S); (2) a concatenated dataset (*COI*, 16S, 28S, and *H3*) of *Alzoniella* (*Navarriella*) individuals from the 17 studied populations, and the outgroups *Corrosella navasiana* (Fagot, 1907) (GenBank accession number: JX081861 for *COI*, JX081963 for 16S, and JX081752 for 28S) and *Mercuria similis* (Draparnaud, 1805) (GenBank accession numbers: OK360825 for *COI*, OK359340 for 16S, and OK359149 for 28S). The *H3* gene fragment of *Mercuria similis* was obtained for this study (GenBank accession number OR105647) using the same DNA voucher that was previously extracted by Miller *et al.* (2022).

The sequence code used in this paper was arranged according to the following criteria: the first four numbers correspond to the locality code, and the fifth number corresponds to the analyzed specimen. If the sampled locality could host more than one species, a letter was added next to identify a priori the species (i.e. A = *Alzoniella*, N = *Navarriella*, De = *Deganta*). The next three letters correspond to the name of the collected species, and the last three letters correspond to the name of the sampled locality.

The protein-coding mitochondrial *COI* and nuclear *H3* sequences were unambiguously aligned in MEGA v.7.0.14 (Kumar *et al.* 2016). The alignments of the ribosomal 16S, 18S, and 28S sequences were performed in MAFFT v.7.312 (Katoh and Standley 2013), with default settings [gap opening penalty (GOP) = 1.53]. Gblocks v.0.91b (Castresana 2000) was used to remove the hypervariable regions of the 16S, 18S, and 28S alignments. Sequence divergences (uncorrected *p*-distances) were calculated in MEGA v.7.0.14.

For phylogenetic analyses of the family, the combined dataset was 1024 base pairs (bp) in length and was composed of the mitochondrial gene *COI* (658 bp) and the nuclear gene 18S (366 bp). For phylogenetic analyses of *A.* (*Navarriella*) populations, the concatenated dataset (*COI*, 16S, *H3*, and 28S) consisted of a 2557 bp alignment from a total of 46 specimens (Supporting Information, Table S2). A total of 1165 bp was obtained for the mitochondrial genes *COI* (658 bp) and 16S (507 bp), and 1392 bp for the nuclear genes *H3* (342 bp) and 28S (1050 bp). The best-fit substitution models for the family datasets, identified by jModelTest v.2.1.6 (Darriba *et al.* 2012), were: TPM2uf (Kimura 1981)+I (invariable sites) +G (rate variation among sites) for *COI* and K80 (Kimura 1980)+G for 18S. For the dataset of *A.* (*Navarriella*) individuals, the best-fit substitution models were: TIM2 (Posada 2008)+I for *COI*, TIM2+I for 16S, TIM3 (Posada 2008)+I for 28S, and HKY (Hasegawa *et al.* 1985)+G for *H3*.

The phylogenetic relationships within single-gene and concatenated datasets were estimated using Bayesian inference (BI) and maximum likelihood (ML). BI analyses were performed using the best-fit substitution models identified by jModelTest v.2.1.6 for each gene partition. The analyses were conducted using MrBayes v.3.2.7a (Ronquist *et al.* 2012) on the Cyber Infrastructure for Phylogenetic Research project (CIPRES, www.phylo.org). Markov chain Monte Carlo (MCMC) analyses were run with four parallel chains for 5 million generations with a sampling frequency of a tree every 1000 generations. The convergence of the MCMC simulations was assessed by ensuring an average standard deviation of split frequencies lower than 0.01. Ten percent of the sampled trees were discarded as burn-in. The robustness of the inferred tree was quantified using Bayesian posterior probabilities (BPP > 0.95). ML analysis was conducted using RAxML-NG v.1.0.2 (Kozlov *et al.* 2019), applying the best-fit substitution model for each partition and 100 random starting trees. Branch supports were assessed by heuristic bootstrapping (BS) with a stopping threshold of 0.03 and later quantified using the transfer bootstrap expectation (TBE; Lemoine *et al.* 2018).

The morphology-based taxonomy of *A.* (*Navarriella*) was assessed using the dataset consisting of only *A.* (*Navarriella*) individuals and four molecular species delimitation methods: the distance-based automatic gap discovery (ABGD) method (Puillandre *et al.* 2012), the Bayesian single-rate Poisson Tree Processes (bPTP) method (Zhang *et al.* 2013), the multi-rate Poisson Tree Processes (mPTP) method (Kapli *et al.* 2017), and the single-threshold generalized mixed Yule-coalescent (ST-GMYC) method (Pons *et al.* 2006). These methods are based on genetic distance or Bayesian and maximum likelihood approaches, and have proven to be relevant tools for species delimitation in other hydrobiid groups (Delicado *et al.* 2019, Miller *et al.* 2022).

For the ABGD approach, we uploaded the *COI* alignment to the ABGD server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) and selected a Kimura 2-parameter (Kimura 1980) option to calculate the distance matrix. The remaining settings were: Pmin = 0.001, Pmax = 0.1, steps = 10, relative gap width (X) = 1.00, and Nb bins = 20.

For the PTP method, we used an unrooted phylogeny of the concatenated mitochondrial (*COI* and 16S) dataset estimated with RAxML after trimming identical haplotypes with the R v.4.3.0 (R Core Team 2023) package ape (Paradis and Schliep 2018). Both bPTP and mPTP analyses were implemented in mPTP v.0.2.4 (downloaded from GitHub at https://github.com/Pas-Kapli/mptp). Both approaches were run with 50 million MCMC generations, sampling every 100 000 iterations and the first million discarded as burn-in.

For the ST-GMYC approach, we used an ultrametric tree previously obtained using BEAST v.1.8.4 (Drummond *et al.* 2012) with the concatenated mitochondrial (*COI* and 16S) dataset. For the tree prior, we used the Yule speciation process (Yule 1925), 100 million MCMC generations, sampling every 2000 iterations and discarding the first 10% as burn-in. An effective sample size (ESS) > 200 for each parameter was confirmed in Tracer v.1.7.1 (Rambaut *et al.* 2018) to ensure stationarity in the posterior distribution. TreeAnnotator v.1.8.4 was used to identify the maximum clade credibility (MCC) tree. We removed all zero branch lengths using the R package ape. Finally, the ultrametric MCC tree obtained was uploaded onto the GMYC web server (https://species.h-its.org/gmyc/) as input.

We evaluated the performance of the species delimitation methods using the match ratio as in Ahrens *et al.* (2016). The match ratio formula is $Match\ ratio = 2 \times Nmatch/(Ndelimited + Nmorph)$, whereby *Nmatch* denotes the number of delimited species that exactly match a defined morphospecies, *Ndelimited* is the total number of species delimited by the method, and *Nmorph* refers to the total number of morphospecies. An overestimation of the species delimitation method is indicated by lower values of this formula.

To gain some insights into the population structure and genetic diversity of *Alzoniella* (*Navarriella*) species, we built a haplotype network using the *COI* alignment and the Templeton, Crandall and Sing (TCS) algorithm (Clement *et al.* 2000) implemented in PopART v.1.7 (Leigh and Bryant 2015). The geographic areas were delimited according to the mountainous systems named in the geographic maps of the Basque Country and Navarra provinces. The correlation between geographical distances and sequence distances among populations was evaluated with the Mantel test (Mantel 1967). For this purpose, the *COI* alignment in FASTA format and a table with sequence codes and latitude and longitude coordinates were used as inputs. The significance was tested based on 9999 permutations. The script was coded using the R packages geosphere (Hijmans 2022), vegan (Oksanen *et al.* 2022), seqinr (Charif and Lobry 2007), and ape.

Morphometry and anatomy descriptions

A total of 342 specimens from 16 populations of the two *A.* (*Navarriella*) species were analyzed to explore variation in shell shape using geometric morphometric (GM) analyses. The type material of *A.* (*N.*) *pellitica* deposited at MNCN-CSIC (MNCN 15.05/60162) was also included in the GM analyses. Images were taken with a Leica MZ16A stereomicroscope and a Leica DFC550 camera in frontal view and the spiral axis on the y-axis. Only mature specimens with a similar number of whorls and a well-developed inner lip were selected to set homologous points. For this reason, two of the populations included in the molecular studies were discarded.

Twenty shell variables (8 landmarks and twelve semilandmarks) were scored (Fig. 2A) in all specimens using

TPSdig v.2.31 (Rohlf 2018) to provide coordinate data for each point. Landmarks corresponded to fixed anatomical features present on the shells (i.e. apex, beginning and end of a whorl, or base of the shell), whereas semilandmarks were determined based on a mathematical criterion (i.e. medial point in a whorl, intersection of the line of maximum width of a whorl with the columellar axis, or the line of maximum length/width of the aperture). This configuration of landmarks and semilandmarks has demonstrated to be effective for characterizing hydrobiid shell shape (Miller *et al.* 2023). In order to compile the TPS file that stores the information of the digitization, the folder containing the pictures of all the selected localities was processed using TPSUtil v.1.76 (Rohlf 2007). During the imaging digitizing process, a small variation in position may occur. To avoid this variation, the data matrix was subjected to Procrustes superposition analysis to remove size differences and the effects of rotation and to minimize errors. Variation in shell shape was characterized by principal component analysis (PCA) and linear discriminant analysis (LDA). To visually inspect the variation in shell shape between species, the consensus of each species was calculated. A thin plate spline (TPS) plot was created for each consensus against the medium shape using PAST v.4.5. (Hammer *et al.* 2022). This analysis allows us to determine which areas of the morphospace have accounted for the greater variability by indicating in a colour scale the contraction and expansion factors.

Traditional measurements of shells (Fig. 2B, C) and anatomical structures were recorded on shell and dissection

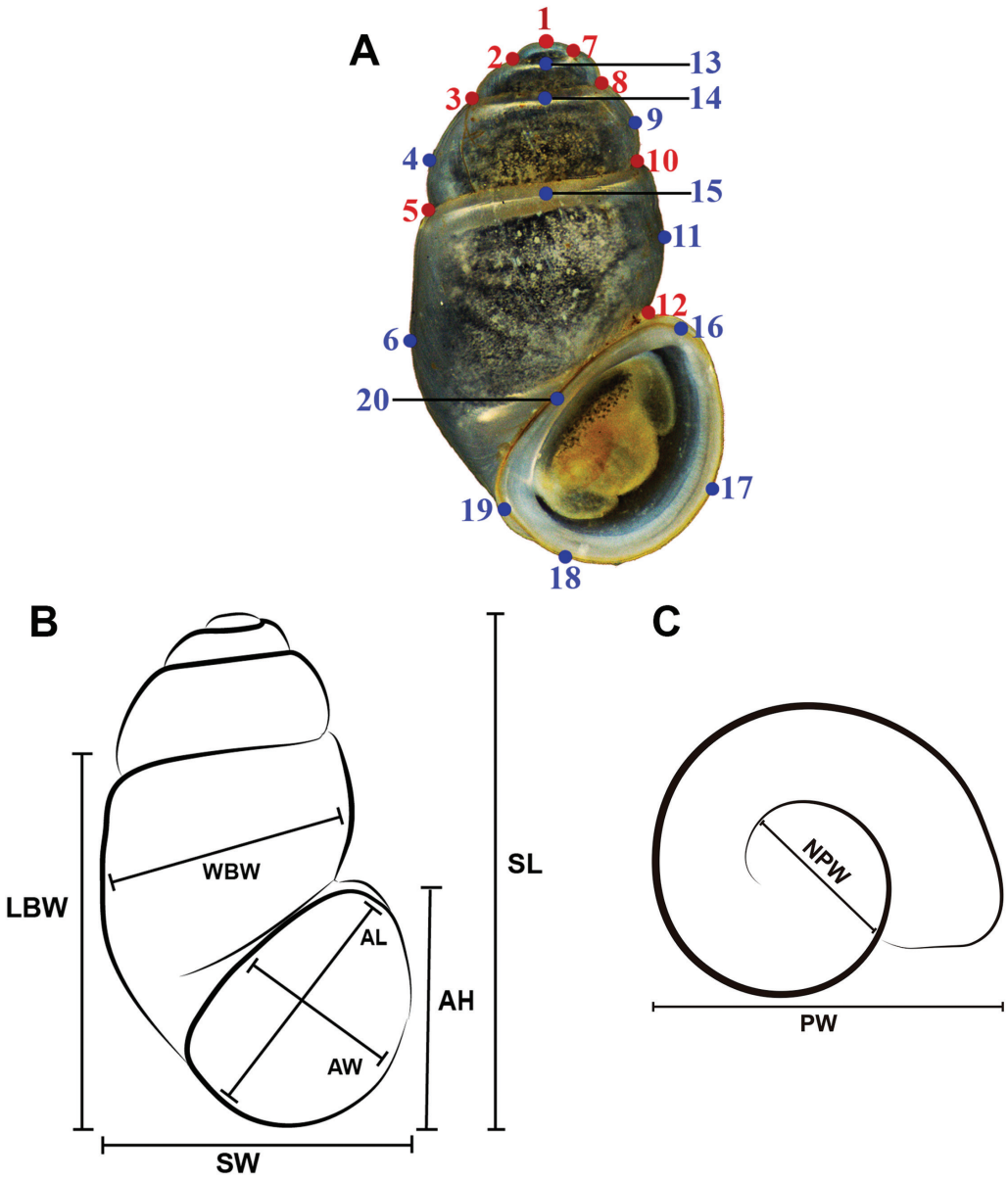

Figure 2. Shell morphometric variables. A, Image of a specimen of *Alzoniella* (*Navarriella*) *elliptica* showing the landmarks (red) and semilandmarks (blue) designated for the geometric morphometric analyses (Principal Component Analysis / Linear Discriminant Analysis). B, C, Drawings of the shells of *Alzoniella* (*Navarriella*), depicting the linear measurements recorded on the shell and protoconch. Variable abbreviations are described in the Material and Methods section.

images using the Leica Application Suite (LAS) v.4.6. The total number of whorls was included in these data following Ramos *et al.* (2000). In addition, descriptive statistics such as mean, standard deviation, and minimum and maximum values were used to summarize intra- and interspecific variation (Supporting Information, Tables S4–S6).

To carry out the dissection of specimens, the shells were treated in an aqueous solution of 5% ethylenediaminetetraacetic acid (EDTA) overnight to eliminate the calcareous part of the shell. Then, they were treated with several successive washes in distilled water for 1 h. To observe the protoconch, its microsculpture, and operculum details, the shells and opercula were subjected to a commercial solution of sodium hypochlorite until the periostracum and the soft part of the operculum were dissolved. For radula examinations, buccal bulbs were immersed in 30% bleach dilution until the soft part of the buccal bulb was removed. Lastly, shells and radulae were covered with a gold conductive layer to scan them in a FEI INSPECT Environmental Scanning Electron Microscope (ESEM; FEI Company, the Netherlands).

We followed the terminology proposed by Hershler and Ponder (1998) for the shell and anatomical features, except in cases where a character needed to be defined or specified in more detail. The Right Pleural Ganglion (RPG) ratio is an index provided by Davis *et al.* (1976) to observe the degree of concentration of ganglia and connectives in the perioesophageal nervous ring. This ratio is traditionally used to determine species or genera. The formula for the RPG ratio is $RPG\ ratio = L.Psc/(L.Rp + L.Psc + L.Sug)$, where *L.Psc* symbolizes the length of the pleuro-supraoesophageal connective, *L.Rp* refers to the length of the right pleural ganglion, and *L.Sug* indicates the length of the supraoesophageal ganglion. According to this ratio, the perioesophageal nervous ring can be: concentrated (RPG ≤ 0.29), moderately concentrated (0.30–0.49), elongated (0.50–0.67), or extremely elongated (≥ 0.68) (Davis and Pons da Silva 1984, Davis *et al.* 1986, 1992).

ABBREVIATIONS USED IN TEXT, TABLES, AND FIGURES

Shell measurements

AH, aperture height; AL, aperture length; AW, aperture width; LBW, length of body whorl; SL, shell length; SW, shell width; WBW, width of body whorl; NPW, nucleus width of protoconch; PW, protoconch width.

Anatomy

Ag, albumen gland; Bc, bursa copulatrix; Cg, capsule gland; Ct, ctenidium; Os, osphradium; Pl, penial lobe; P, penis; Pr, prostate gland; Sr1, seminal receptacle distal; Sr2, seminal receptacle proximal; Ss, style sac; St, stomach.

RESULTS

Phylogenetic relationships and intraspecific genetic diversity

The multilocus phylogenetic analyses of the family, conducted using ML and BI, yielded similar topologies and relationships among the subfamilies included in our study. Therefore, only the BI tree is presented in Figure 3A. Both analyses consistently placed the subgenus *Alzoniella* (*Navarriella*) outside of Islamiinae, indicating a distant relationship to *A. finalina* (Fig. 3A). The subgenus is basal to a clade (BPP = 0.98, BS = 0.91), which is further formed by all subfamilies studied except Islamiinae. The genera *Avenionia*, *Arganiella*, *Corbellaria*, and *Kerkia* could not be assigned to any of the recognized subfamilies. The ML and BI phylogenetic analyses based on *COI* showed similar topologies to those of the multilocus analyses, whereas phylogenetic relationships based on 18S were poorly supported. Sequence divergence (measured as uncorrected pairwise distance) between *A.* (*Navarriella*) and *A. finalina* was 19.54% for *COI* and 0.61% for 18S.

The BI and the ML approaches for *A.* (*Navarriella*) populations based on the concatenated dataset revealed that *A.* (*N.*) *elliptica* and *A.* (*N.*) *pellitica* are not differentiated genetically. Both species conform to a well-supported group (BPP > 0.95, BS > 75), including the 17 populations analyzed (Fig. 3B). All molecular species delimitation methods were congruent delimiting just a single putative species, which resulted in the same match ratio across methods (0.67).

Interspecific mean sequence divergences between *A.* (*N.*) *elliptica* and *A.* (*N.*) *pellitica* were 1.52% for *COI*, 0.34% for 16S, 0.45% for *H3*, and 0.44% for 28S. Both species were indistinguishable in the single-gene analyses (Supporting Information, Figs S1–S8).

The TCS haplotype network of *A.* (*Navarriella*) provided higher spatial resolution than the poorly phylogenetic relationships inferred among populations in the *COI* analyses (Supporting Information, Figs S1, S5). It detected 14 haplotypes from the 46 *COI* amplified sequences, which were grouped in seven geographic areas (Fig. 4). One haplotype is exclusive to *A.* (*N.*) *elliptica*, while the remaining haplotypes belong to *A.* (*N.*) *pellitica*. These two previously described species differed in five mutations. The haplotype network reinforced the idea that we are dealing with a single species. The Navarrese Pyrenees area is the most haplotype diverse, showing the presence of eight haplotypes. The most different haplotypes H01 and H02 are located south-east of the Navarrese Pyrenees (i.e. belonging to the Roncesvalles and Eugi populations), while the other six are placed central and northwest. One of the localities in Eugi harbors two very distant haplotypes (H02 and H13). The haplotype with the greatest spread is H08 and this one may be the oldest haplotype. A Mantel test disclosed no statistical correlation between genetic and geographic distances (r = -0.01, $P = 0.538$).

Geometric morphometrics of shell shape

In the PCA analysis of the specimens studied, the first two components explained 47.8% of the variation in shell shape. When the third component was included, it accounted for a total of 57.82% of the variation. Both the PCA and the LDA revealed high variability in shell shape within populations, resulting in an overlap between the two nominal species (Fig. 5A–C). The TPS plots illustrated that *A.* (*N.*) *elliptica* tends to have a more rounded aperture and last whorl compared to *A.* (*N.*) *pellitica*, whereas the latter exhibits greater roundness and width in the penultimate whorl and antepenultimate whorl (Fig. 5D).

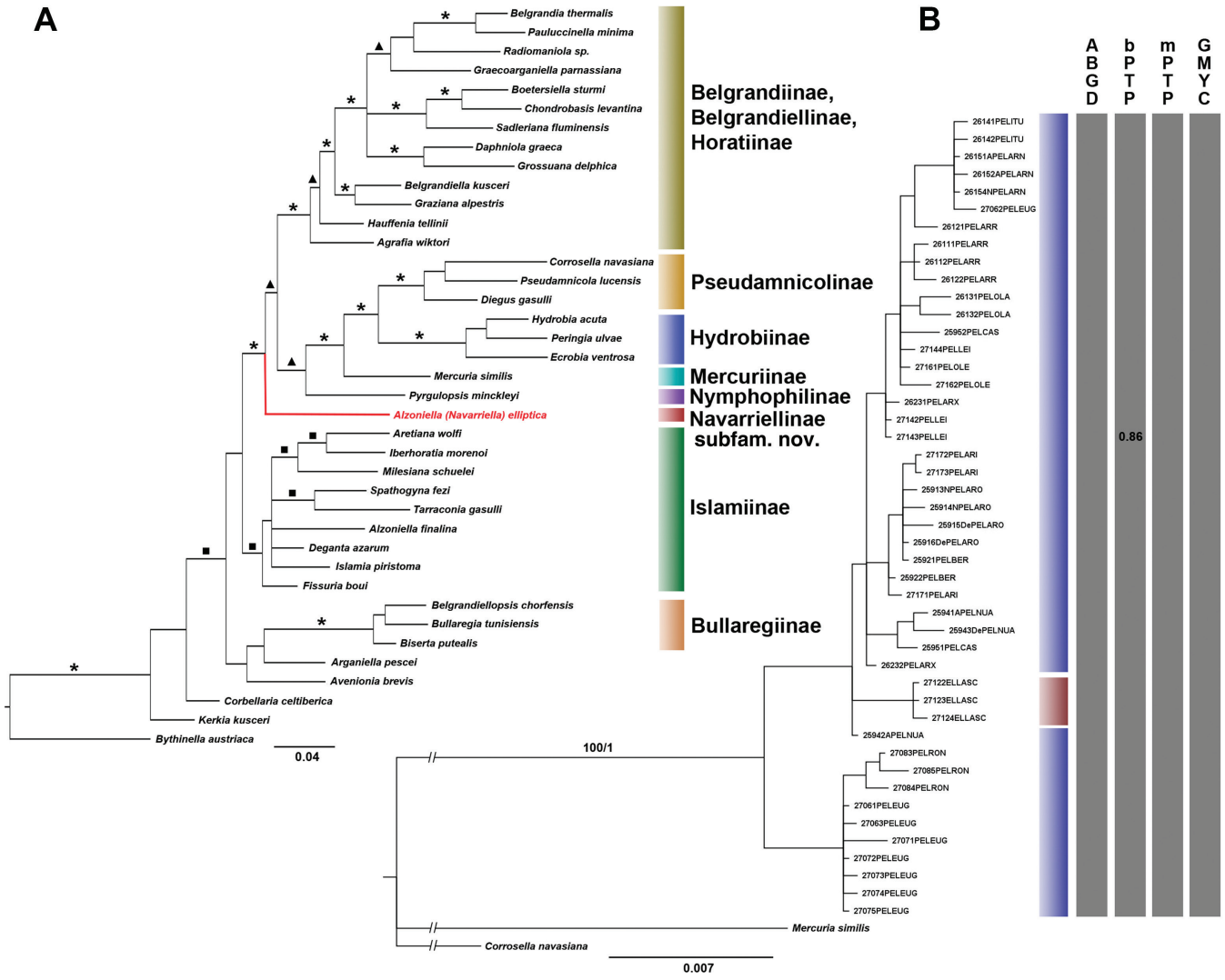

Figure 3. Phylogenetic relationships of hydrobiid taxa incorporating the species of *Alzoniella* (*Navarriella*). A, Bayesian tree of the family Hydrobiidae based on the concatenated dataset (*COI* and 18S), including some selected species of the hydrobiid subfamilies (highlighted with vertical bars). Branches with bootstrap support (BS) values from the maximum likelihood (ML) analysis and Bayesian posterior probabilities (BPP) greater than 75% and 0.95, respectively, are indicated with asterisks. Branches only with BS > 75% are denoted with a triangle. Branches only with BPP > 0.95 are shown with a square. B, Bayesian phylogenetic tree of specimens of *Alzoniella* (*Navarriella*) based on the concatenated dataset (*COI*, 16S, *H3*, and 28S). BS and BPP are provided above branches when greater than 75% and 0.95, respectively. The first vertical bar represents the taxonomic species of each population assigned in previous studies as *Alzoniella* (*Navarriella*) *elliptica* (in red) and *Alzoniella* (*Navarriella*) *pellitica* (in blue). The remaining vertical bars refer to the species delimitation methods: ABGD, distance-based automatic gap discovery; GMYC, single-threshold generalized mixed Yule-coalescent; bPTP, Bayesian approach of the Poisson Tree Processes; mPTP, multirate Poisson Tree Processes. The bPTP bar is displayed with its Bayesian support value. Label abbreviations are explained in Supporting Information, Table S2. Scale bars: expected change per site.

TAXONOMIC REVIEW

Class Gastropoda Cuvier, 1795

Subclass Caenogastropoda Cox, 1960

Order Littorinimorpha Golikov & Starobogatov, 1975

Family Hydrobiidae W. Stimpson, 1865

Navarriellinae subfam. nov. García-Guerrero, Miller and Ramos

ZooBank registration: urn:lsid:zoobank.org:act:FF20DC48-F862-4456-B5FC-960275C7ED78.

Type genus: Navarriella Boeters, 2000.

Diagnosis

Shell cylindrical with rounded apex, whorls moderately convex and umbilicus covered by the inner lip. Periostracum pale yellow to whitish. Operculum corneous, orangish, thin, pliable, oval, paucispiral, with a submarginal nucleus. Radula taenioglossate; two pairs of basal cusps on the central radular tooth. Bursa copulatrix large in size. Two seminal receptacles with a long duct. Penis strap-like to gradually tapering with several penial lobes.

Remarks

Navarriellinae is a monotypic subfamily and represents a highly divergent lineage within the Hydrobiidae, distantly related to the other 13 formally recognized subfamilies (Delicado *et al*

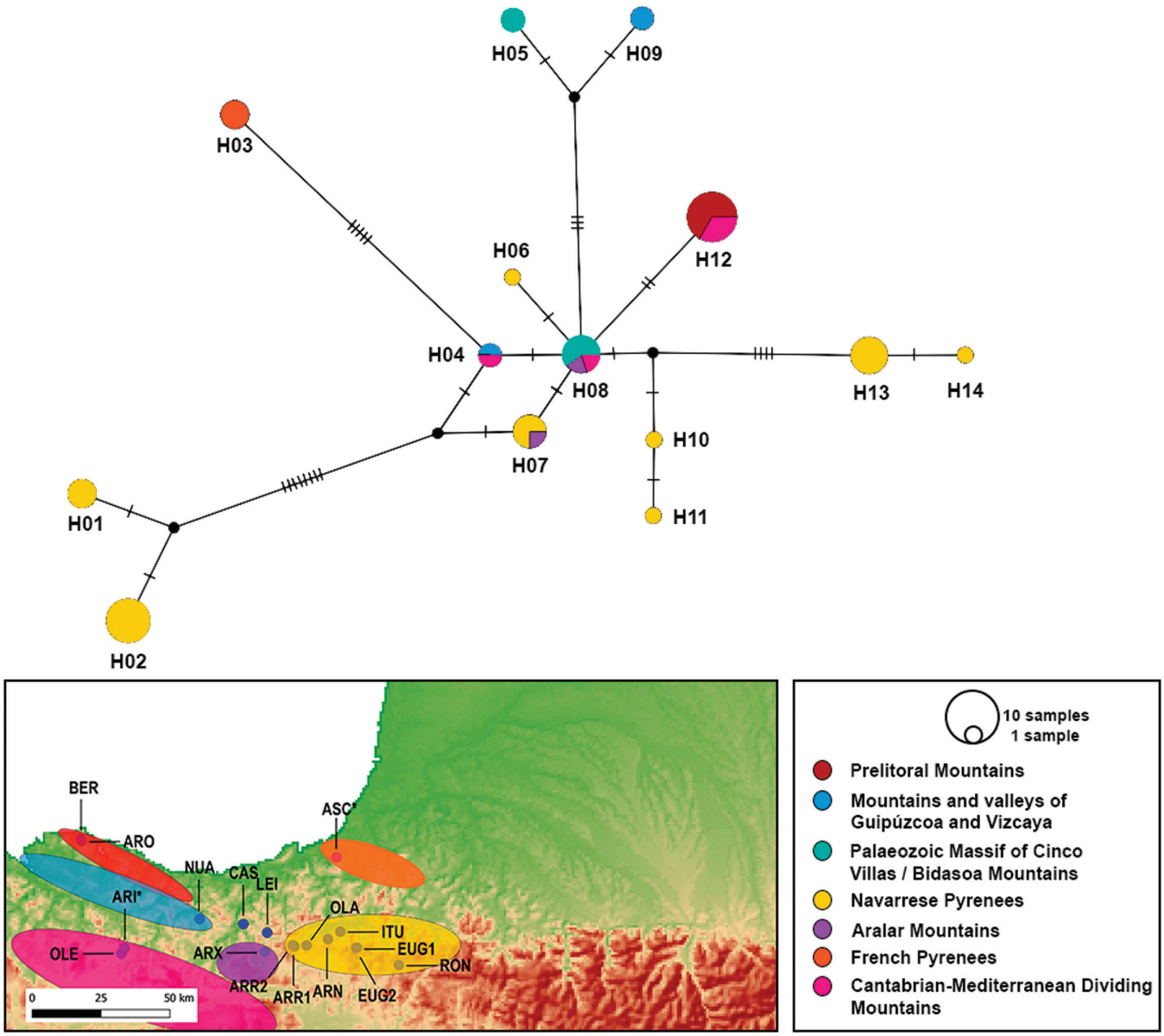

Figure 4. Statistical parsimony network based on *COI* haplotypes for the sampled populations of *Alzoniella* (*Navarriella*) *elliptica* and *Alzoniella* (*Navarriella*) *pellitica*. The circles are colour-coded by population and represent the number of haplotypes. Each vertical bar represents one mutation. The codes of the figure are explained in Supporting Information, Tables S1, S2. Asterisks indicate the sampled topotypes.

2023; fig. 2). While its species may have shell shapes similar to those of other hydrobiid subfamilies, they can be anatomically distinguished. All Islamiinae (including *Alzoniella*) differ from Navarriellinae by the presence of one or two sessile seminal receptacles and one or two penial lobes (Radoman 1973, 1983, Giusti and Bodon 1984, Arconada and Ramos 2006); all Belgrandiellinae differ from Navarriellinae by a single Sr1 and one penial lobe (Radoman 1983); all Belgrandiinae differ from Navarriellinae by two sessile seminal receptacles and one penial lobe (Boeters 1988, Haase 2000); and all Bullaregiinae differ from Navarriellinae by a Sr1 and one penial lobe (Khalloufi *et al.* 2017, Delicado *et al.* 2023). Navarriellinae also differs from the phylogenetically closely related subfamilies Hydrobiinae W. Stimpson, 1865, Mercuriinae Boeters & Falkner, 2017, Nymphophilinae D.W. Taylor, 1966, and Pseudamnicolinae Radoman, 1977 according to its cylindrical shell, narrower cusps on the central and lateral radular teeth, and the presence of more than one seminal receptacle on the renal oviduct and various penial lobes.

Navarriella Boeters, 2000

Synonyms

Alzoniella (*Navarriella*) Boeters, 2000: 160–161.

Type species: Paludinella elliptica Paladilhe, 1874. Designated by Boeters (2000).

Diagnosis

Shell cylindrical with a rounded apex; aperture obliquely ovate; periostracum pale yellow to whitish; protoconch low and dome shaped. Operculum corneous, orangish, thin, pliable, oval, paucispiral, with a submarginal nucleus. Two pairs of basal cusps on the central radular tooth. Ctenidium well

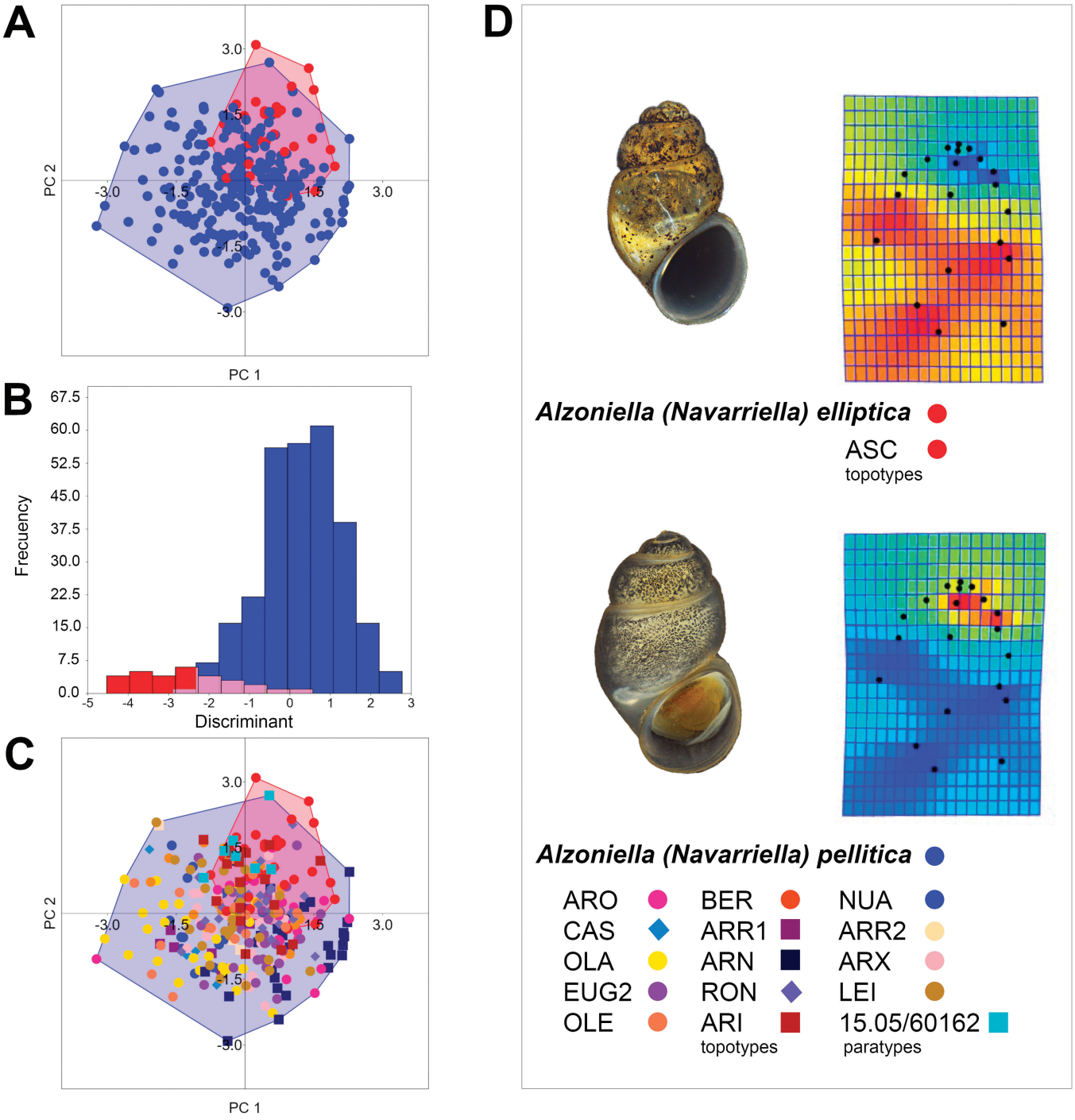

Figure 5. Geometric Morphometric analyses for the shells of *Alzoniella* (*Navarriella*) species based on 20 coordinates (eight landmarks and 12 semilandmarks). A, Principal Component Analysis (PCA) for species. B, Linear Discriminant Analysis (LDA) for species. C, PCA for populations. D, Thin-plate spline (TPS) plot displaying expansion (in red) and contraction (in blue) of the shell shape. Label abbreviations are explained in Supporting Information, Table S1.

developed. Bursa copulatrix large, pyriform, pedunculated, and lying against the posterior section of the albumen gland; two seminal receptacles with a long duct; Sr2 smaller than Sr1 and arising at the renal oviduct loop. Penis unpigmented, strap-like; distal end of the penis gradually tapering; more than two penial lobes. Nervous system scarcely pigmented, moderately concentrated with cerebral ganglia roughly equal in size.

Remarks

Navarriella is a monospecific genus, belonging to an independent lineage separate from *Alzoniella*, and it is unclassified within the subfamily Islamiinae (Fig. 3A). The *COI* average sequence divergence with the type species *A. finalina* is 18.54%. Morphologically, *Alzoniella* differs from *Navarriella* by the presence of two sessile seminal receptacles and two penial lobes. *Navarriella* has cylindrical shells with a height of 1.5–2.2 mm

(Boeters 2000, 2001, Arconada, Bolán & Boeters, 2007), whereas the shells of *Alzoniella* are conic to cylindrical-conic with a height of 0.7–2.5 mm (Giusti and Bodon 1984). *Guadiella* Boeters, 2003 differs from *Navarriella* by having a single seminal receptacle, a slender penis without penial lobes, and narrow, cylindrical to slightly conical shells with a height of 1.40–1.70 mm (Boeters 2003, Arconada, Bolán & Boeters, 2007).

Navarriella elliptica (Paladilhe, 1874) **comb. nov.**

(Figs 6–7; Supporting Information, Tables S4–S6)

Synonyms

Paludinella elliptica Paladilhe, 1874: 33, pl. 3, figs 11–12. Type locality: 'les environs d'Ascain (Basses-Pyrénées)'.

Microna elliptica (Paladilhe, 1874) – Boeters 1970: 132, pl. 9, fig 34. Syntype PA/7, Bou/1, SMF 141895.

Litthabitella elliptica (Paladilhe, 1874) – Boeters 1974: 90, figs 5–7. Topotype: BOE 358 'Mas Pascoulin in Serres bei Ascain, Dép. Basses-Pyrénees'.

Belgrandiella elliptica (Paladilhe, 1874) – Boeters 1988: 227, pl. 3, fig. 45; figs 198, 232–234. BOE 355.

Belgrandiella elliptica (Paladilhe, 1874) – Rolán 1991: 112, pl. 7, figs 1–5.

Alzoniella (*Navarriella*) *elliptica* (Paladilhe, 1874) – Boeters 2000: 161, figs 10–11, 17, 24, 31.

Alzoniella (*Navarriella*) *elliptica* (Paladilhe, 1874) – Arconada, Bolán & Boeters, 2007: 135, figs 110–114, 118, 119, 121, 122.

Alzoniella (*Navarriella*) *pellitica* Arconada, Bolán & Boeters, 2007: 136, figs 13, 16, 17, 64, 65, 68, 69, 92, 115–117, 120. Type locality: 'Santa Agueda area, Arriola, spring about 250m from the houses at brook'. Holotype MNCN 15.05/60162H, paratypes MNCN 15.05/60162P.

Type material: Syntypes PA/7, Bou/1, SMF 141895.

Type locality: 'Les environs d'Ascain', Basses-Pyrénées, France (Paladilhe 1874).

Material studied: Spring in Chemin d'Andienea, Ascain, Basses-Pyrénees, France (FW2712); spring in Arriola, Alava, Basque Country, Spain (FW2717); spring in Olaeta, Alava, Basque Country, Spain (FW2716); spring in Castillo, Gipuzkoa, Basque Country, Spain (FW2595); spring in Nuarbe Auzoa from Urrestilla to Beizama, Gipuzkoa, Basque Country, Spain (FW2594); spring near Mañu Auzoa, Bermeo, Vizcaya, Basque Country, Spain (FW2592); spring in Arronategi Auz, Vizcaya, Basque Country, Spain (FW2591); spring from Leitza to Tolosa, Navarre, Spain (FW2714); spring next to Araxes River, Navarre, Spain (FW2623); watercourse from Roncesvalles to Valcarlos, Navarre, Spain (FW2708); two springs near Eugi, Navarre, Spain (FW2707 and FW2706); spring in Arrantza, Navarre, Spain (FW2615); Iturriotz Spring, Almandoz, Navarre, Spain (FW2614); spring in Ola, Navarre, Spain (FW2613); two springs near Arrarat, Navarre, Spain (FW2612 and FW2611).

Description

Shell cylindrical, whorls 4–5, height 1.6–2.1 mm, width 1.1–1.4 mm (Fig. 6A–N; Supporting Information, Table S4); periostracum whitish; protoconch of 1.5 whorls, *c.* 350 μm wide and nucleus *c.* 200 μm wide (Fig. 6Q, R); protoconch microsculpture pitted (Fig. 6S–V); teleoconch whorls convex separated by a noticeable and no convex suture; body whorl occupies about two-thirds of the total shell length; aperture obliquely ovate and complete; inner lip thicker than outer lip; aperture margin straight; inner lip touching the shell wall; rounded apex; umbilicus covered by the inner lip.

Operculum corneous, orangish, thin, pliable, oval, paucispiral, with a submarginal nucleus, about two whorls; muscle attachment oval, located near the nucleus (Fig. 6O, P).

Radula taenioglossate with a central tooth formula 5–C–5/2–2, basal-tongue broadly 'V' shaped, cutting-edge concave (Fig. 7A, B). Lateral tooth formula (5)3–C–3(5), central cusp 'V' shaped. Inner marginal teeth having ≥ 24 cusps (Fig. 7C); outer marginal teeth having ≥ 25 cusps (Fig. 7D). Radular data were collected from the specimens of the following localities: spring in Chemin d'Andienea, Ascain, Basses-Pyrénees, France (FW2712); spring from Roncesvalles to Valcarlos, Navarre, Spain (FW2708).

Some animals partially pigmented (Fig. 6A–L). Ctenidium occupying two-thirds of the total length of the pallial cavity; 10–13 gill filaments; filaments well developed, taller than broad (Fig. 7E). Osphradium of intermediate width, two to three times as long as wide (Supporting Information, Table S5), positioned opposite approximate middle of ctenidium. Stomach almost as long as wide with two chambers almost equal in size, style sac slightly longer than wide (Fig. 7F; Supporting Information, Table S5). Nervous system scarcely pigmented, moderately concentrated (mean RPG ratio = 0.40; Supporting Information, Table S5); cerebral ganglia roughly equal in size; pleuro-supraoesophageal connective *c.* three times longer than the pleuro-suboesophageal one (Fig. 7G).

Female genitalia with a capsule gland longer than albumen gland (Fig. 7H–J; Supporting Information, Table S6); bursa copulatrix large, pyriform, about twice as long as wide; bursal duct shorter than bursa copulatrix; renal oviduct unpigmented with a single loop; two seminal receptacles; Sr1 pyriform with a long duct, placed just above the junction with the bursal duct; Sr2 pyriform with a short duct, situated just behind the single loop (Fig. 7H–J).

Male genitalia with a prostate gland bean-shaped about three times longer than wide (Fig. 7K; Supporting Information, Table S6). Penis unpigmented, strap-like, distal end of the penis gradually tapering and attached to the neck behind the right eye; several penial lobes, four proximal and one large distal (Fig. 7L–Q).

Habitat and distribution

Most of the studied specimens were found in water with conductivities ranging from 140 to 740 μS/cm, except in the type locality of Ascain (932 μS/cm). *Navarriella elliptica* cohabits with other molluscs such as *Pisidium* spp. and *Ancylus* spp. It is also found alongside *Potamopyrgus antipodarum* (J.E. Gray, 1843) in Ascain and species of *Bythinella* Moquin-Tandon, 1856 in Bermeo and Arrantaza (Basque Country).

The species is distributed in springs and watercourses in the Basque Country and Navarra provinces in the north of the Iberian Peninsula, as well as Ascain in the French Pyrenees (Fig. 1). It has also been reported in other areas of the French Pyrenees (Boeters 1974). Most of the specimens were found under rocks and leaves, except in Eugi where they were among the mosses.

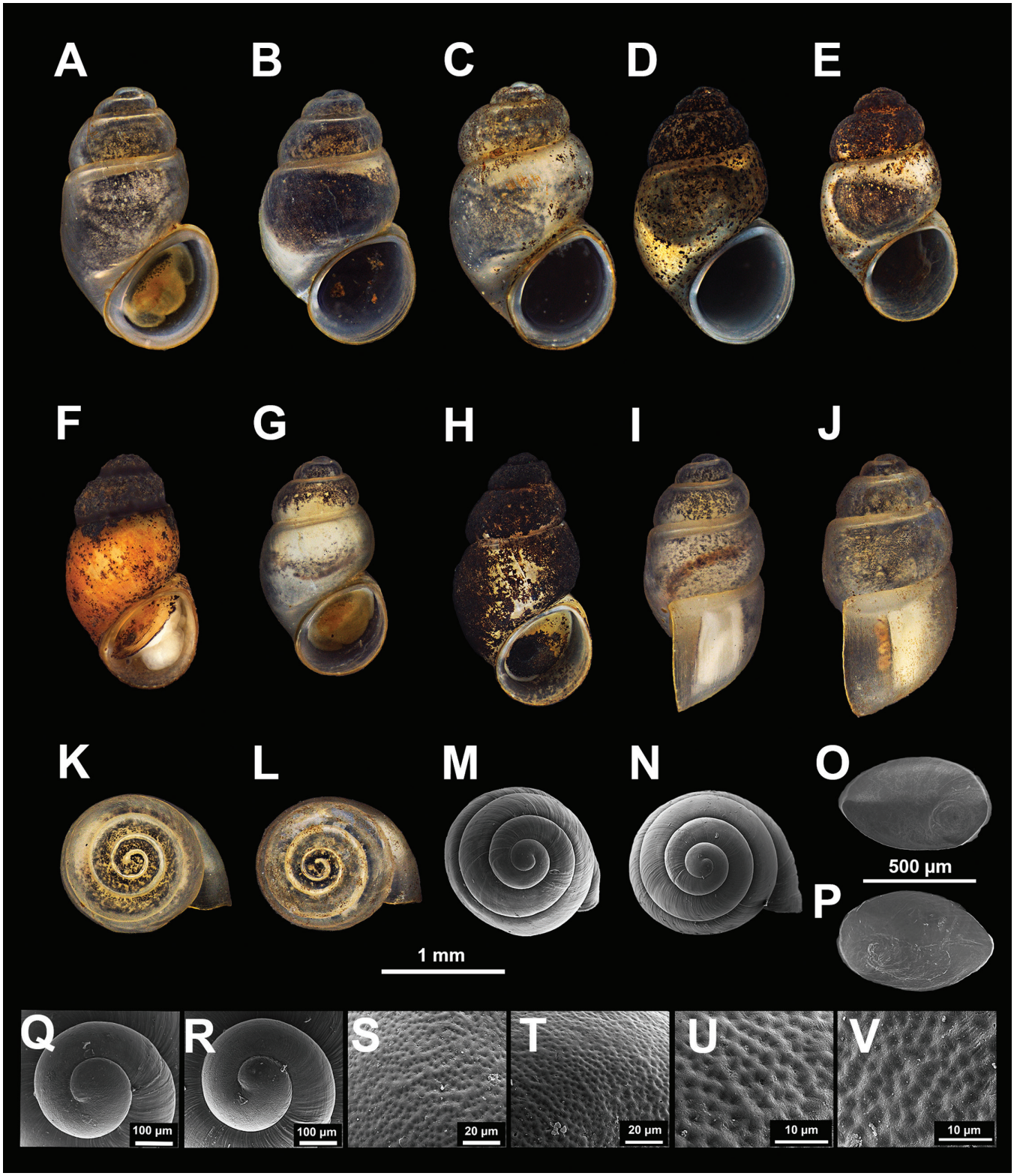

Figure 6. Intraspecific variation of the shell shape, protoconch and opercula of *Navarriella elliptica*. A–N, shells. O–P, operculum. Q–V, protoconch and details of protoconch. A, I, K, M, T, and U, FW2611—spring in Arrarats, Navarra, Spain. B, N, O, P, and R, FW2623—spring next to Araxes River, Navarra, Spain. C, FW2708—watercourse from Roncesvalles to Valcarlos, Navarra, Spain. D and V, FW2712—spring in Chemin d'Andienea, Ascain, France. E, FW2717—spring in Arriola, Navarra, Spain. F, FW2591—spring in Arronategi Auz, Vizcaya, Spain. G, FW2594—spring in Nuarbe Auzoa, Guipúzcoa, Spain. H, spring in Ola, Navarra, Spain. J and L, FW2615—spring in Arrantza, Navarra, Spain. Q and S, FW2592—spring near Mañu Auzoa, Bermeo, Vizcaya, Spain.

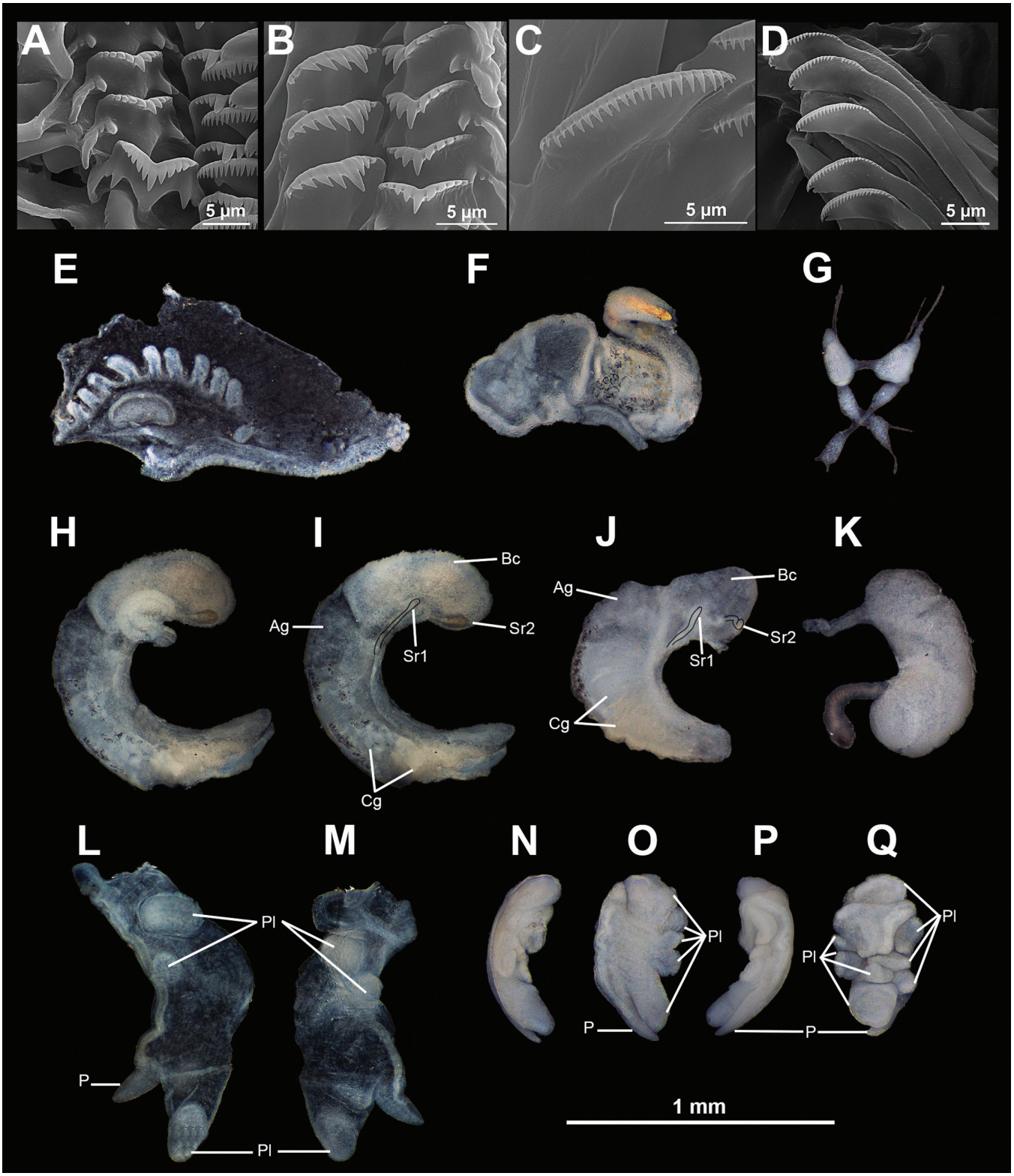

Figure 7. Radulae and anatomy of *Navarriella elliptica*. A, central radular teeth; B, lateral and central radular teeth; C, inner marginal teeth; D, outer marginal teeth; E, ctenidium and osphradium; F, stomach; G, perioesophageal nervous ring; H, J, female genitalia; K, prostate gland; L, M, penis relaxed; N–Q, penis contracted. A–D, FW2708—watercourse from Roncesvalles to Valcarlos, Navarra, Spain. E–I, K–M, FW2591—spring in Arronategi Auz, Vizcaya, Spain. J, FW2717—spring in Arriola, Navarra, Spain. N–Q, FW2623—spring next to Araxes River, Navarra, Spain. Ag, albumen gland; Bc, bursa copulatrix; Cg, capsule gland; Sr1, distal seminal receptacle; Sr2, proximal seminal receptacle; P, penis; Pl, penis lobes.

Remarks

Arconada, Bolán & Boeters (2007) reported that *A.* (*N.*) *pellitica* differs from *A.* (*N.*) *elliptica* primarily in the size and shape of Sr2. However, considering our molecular species delimitation methods and examining topotypical (or near topotypical) specimens of *A.* (*N.*) *elliptica* (FW2712) and *A.* (*N.*) *pellitica* (FW2717), we find additional evidence supporting the conspecific nature of these two taxa (Fig. 3B, 4, 5A–C; Supporting Information, Figs S1–S8, Tables S4–S6). The large Sr1 of *A.* (*N.*) *pellitica* might have been misinterpreted as the bursa copulatrix by Arconada, Bolán & Boeters (2007, fig. 13C, D). Our dissected specimens from the type locality of *A.* (*N.*) *pellitica* (Fig. 7J) exhibit similar bursa copulatrix, Sr1, and Sr2 characteristics to specimens from the remaining populations and those described in the original description of *A.* (*N.*) *elliptica* in Boeters (2000: fig. 31) and Boeters (2001: figs 5–7). The observed anatomical differences in the penis between the Arronategi Auz population and the other populations were probably caused by the relaxation of the organ at the time of ethanol fixation (Fig. 7L–Q; Supporting Information, Table S6). The relaxed penises correspond to the illustrations in Boeters (2001: figs 2, 3), whereas the contracted ones align with the drawings in Boeters (2000: fig. 24) and Arconada, Bolán & Boeters (2007: fig. A, B). The shells of *A.* (*N.*) *elliptica* and *A.* (*N.*) *pellitica* are nearly indistinguishable based on the PCA and DLA results (Fig. 5A–C). The TPS plot reveals two different morphotypes, one being wider and the other more cylindrical. However, this variation could be considered as intraspecific variation attributable to the shape of the penultimate, antepenultimate, and last whorl, as well as the aperture (Fig. 5D). In terms of the linear measurements, the studied specimens exhibit low variability in shell size, with a shell height ranging from 1.80–2.39 mm (Supporting Information, Table S4).

DISCUSSION

Our multilocus phylogeny and morphological descriptions support the proposal of Bodon and Cianfanelli (2004) that the subgenus *Alzoniella* (*Navarriella*) should be recognized as a separate genus. The newly erected genus is suggested here as a monospecific taxon belonging to a new subfamily called Navarriellinae Its low species diversity, phylogenetic position within the Hydrobiidae, and significant phylogenetic distance from other hydrobiid taxa highlight *Navarriella* as an isolated lineage distinct from all other Hydrobiidae, yet susceptible to extinction. Furthermore, these findings emphasize the role of the northern Iberian Mountains as a dispersal barrier for ancient spring lineages.

Systematic position and taxonomic status of *Navarriella*

Our results indicate that *Alzoniella* is not monophyletic; at least two different genera may be present in the genus, based on our molecular analyses, including sequences from its type species (Fig. 3A). Our finding that *Navarriella* is excluded from *Alzoniella* opposes with an earlier hypothesis suggesting a closer relationship of the subgenus with species of *Alzoniella* based on morphological characters (Boeters 2000, 2001, Arconada, Bolán & Boeters, 2007). Therefore, a redefinition of the diagnostic characters distinguishing the two taxa is necessary for a more reliable systematic interpretation. As observed in previous studies (Boeters 2000, 2001, Arconada, Bolán & Boeters, 2007), differences in the shape of seminal receptacles (pedunculated or sessile) and intestine loop (U-shaped bend or Z-shaped bend) can be observed between the subgenus *Navarriella* and the type species *A. finalina*. We also found differences between these taxa in terms of the number and position of the penial lobes, as indicated by Bodon and Cianfanelli (2004). The presence/absence, shape, and size of the bursa copulatrix, the presence/absence, number, shape, and position of the seminal receptacles, the shape and size of the penis, and the presence/absence, number, and position of the penial lobes are considered at genus level in studies of hydrobiid systematics (Giusti and Bodon 1984: fig. 1, Bodon *et al.* 2001, Falniowski 2018). All these pieces of evidence led us to consider the subgenus *Navarriella* as a full genus.

In agreement with previous analyses (Wilke *et al.* 2001, Delicado *et al.* 2023), our phylogenies depict *A. finalina*, and thus the genus *Alzoniella s.s.*, as closely related to *Islamia* and *Fissuria* Boeters, 1981 within the Islamiinae clade. Comparing with our *COI* sequences, we confirm that the *COI* sequence of *A. elliptica* published in Delicado *et al.* (2023) belongs to *Navarriella elliptica*. Consequently, both studies indicate that the lineage of *Navarriella* is strongly divergent within the family, even being independent of other subfamily-level clades. Based on this evidence, we redescribed the genus *Navarriella* as a member of a new subfamily, Navarriellinae. Its phylogenetic position within the Hydrobiidae, which was unresolved in previous studies (Delicado *et al.* 2023), was well supported by our analyses, placing the new subfamily as a basal lineage of a group containing morphologically and ecologically diverse subfamilies such as the Nymphophilinae, Horatiinae D.W. Taylor, 1966, or Hydrobiinae.

The evolutionary scenario we have inferred can be further refined by including more species and genera in the analysis for which there is currently no sequenced material, especially those associated with underground waters or springs in the Iberian Peninsula (such as *Guadiella* and *Plesiella* Boeters, 2003). The species of these genera may belong to this subfamily or as-yet-undiscovered subfamilies. Our results provide the most robust evolutionary hypothesis at present, although the sister group of *Navarriella* remains unknown.

Species diversity of *Navarriella*

Alzoniella (*Navarriella*) was hitherto considered a subgenus comprising two species with a limited geographic distribution in the north of the Iberian Peninsula and southern France (Boeters 2000, Arconada, Bolán & Boeters, 2007). The phylogenetic analyses, haplotype network, and species delimitation methods conducted in our study, which included multiple localities within the distribution range and the topotypes of the two traditional species, revealed a single entity that should be regarded as *Navarriella elliptica* (Figs 3B, 4). Our anatomical examination of *A.* (*N.*) *pellitica* suggests a potential misinterpretation of the diagnostic characters previously identified by Arconada, Bolán & Boeters (2007) (see the Taxonomic review) and confirms the classification of the DNA-based analyses. Nonetheless, we did observe high variation within the species in other morphological traits, such as shell shape (Figs 5, 6). Variation within species is common in other hydrobiid species, such as *Mercuria* Boeters, 1971, *Hydrobia* W. Hartmann, 1821,

Ecrobia W. Stimpson, 1865, or *Peringia* Paladilhe, 1874 (Wilke *et al.* 2000, Barszcz 2004, Miller *et al.* 2023). These differences are attributed to various environmental factors (such as substrate and water physiochemistry) and parasitism (Wilke *et al.* 2000, Barszcz 2004, Verhaegen *et al.* 2019).

Navarriella represents one of the oldest lineages of the family Hydrobiidae, making it particularly intriguing to further sample and study in detail population dynamics, gene flow, and biogeography within the genus. Three hypothetical scenarios could explain the current low species diversity observed today in the genus: (i) *Navarriella* may consist of several non-detected extinct species, and the populations of the extant species survived as relicts; (ii) there may be additional extant species of *Navarriella* that have yet to be discovered; (iii) *Navarriella* may comprise a single species with a broader historical distribution range that has been reduced during glacial periods, currently restricted to the Pyrenean region as a refugium.

Genetic variation and conservation

Our study indicates that *Navarriella elliptica* survived the Late Pleistocene glaciations in one of the European southern refugia, as did many other springsnail species (Falniowski and Wilke 2001, Benke *et al.* 2009). Our phylogenetic analyses of the family (Fig. 3) also suggest *in situ* long-term survival. However, the current known distribution of *N. elliptica*, although expanded by our study, is still very limited within the Iberian Peninsula. This narrow range and the high genetic diversity identified by our interpopulation analysis (Fig. 4) underline the role of the Iberian geographical barriers (such as the Pyrenees) restricting the dispersal of springsnails living in upland headwater habitats. This is not the case for hydrobiid species occurring in lowland continental waters [e.g. *Mercuria tachoensis* (Frauenfeld, 1865)], which have been evidenced to spread out of Iberia during interglacial periods (Miller *et al.* 2022). Our results, however, suggest some long-distance dispersal over intermediate zones (e.g. between the Prelitoral Mountains and Cantabrian-Mediterranean Dividing Mountains), which may explain the lack of population structure and low correlation between genetic divergence and geographic distance. Long-distance dispersal within groups with no population structure has been associated with the island model of Wright (1931) for hydrobiid mud snails (Wilke and Davis 2000), which opposes the isolation by distance model detected when there is a correlation between genetic and geographic distances. On the other hand, the relatively low number of individuals per population studied in this paper and potential unsampled snails in some regions such as the Mountains of Gipuzkoa and Vizcaya may also explain the little population structure observed in *N. elliptica*.

The network also identifies the Navarrese Pyrenees as the genetically most diverse area, with many unique haplotypes in localities where Roncesvalles and Eugi appear to be undergoing an initial allopatric fragmentation process. Our analyses did not directly identify ancestral haplotypes, but this pattern also suggests that the Navarrese Pyrenees might be an ancestral region for the crown node of the species since ancestral regions likely have a higher diversity than newly colonized areas (Nevill *et al.* 2010). A pattern of past fragmentation followed by range expansion in the glacial refugium of the Pyrenees could have occurred in species of the springsnail genus *Bythinella* (Benke *et al.* 2009).

Concerning the conservation status of this species, the IUCN Red List of Threatened Species (www.iucnredlist.org) has classified *A. elliptica* as vulnerable because it was recorded from only nine locations (Arconada and Prié 2010). However, our study demonstrates that the species is found in more locations. Despite this, we recommend revising its status to a more stringent threatened category due to its independent and ancestral phylogenetic characteristics, low population structure, and high genetic diversity, with special consideration given to the Pyrenees as a protected area.

CONCLUSION

Navarriella is an enigmatic group of springsnails found only in the northern mountains of the Iberian Peninsula (western Palaearctic). For over 20 years, it has been considered a subgenus of the widespread European *Alzoniella* based on several shared anatomical characteristics. However, multilocus phylogenies of this and an earlier study recovered this subgenus as an independent lineage within the Hydrobiidae, distantly related to the type species *A. finalina*. *Navarriella* consists of a unique species, making the genus monospecific. Its distribution encompasses several genetically isolated populations, such as Roncesvalles and Eugi, which are of particular interest to conservation efforts.

This study demonstrates that the Iberian Peninsula harbors ancient and potentially relict lineages of freshwater snails, thereby characterizing it as a refugium for this mollusc group in the face of global climatic changes. However, its distinct geographical features lead to disruptions in gene flow, which can generate speciation events and loss of intraspecific genetic diversity.

SUPPLEMENTARY DATA

Supplementary data is available at *Zoological Journal of the Linnean Society* Journal online.

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DATA AVAILABILITY

The DNA sequence data underlying this article are available in GenBank (Table S2). All samples, specimens, and dissections are deposited in the MNCN collection.

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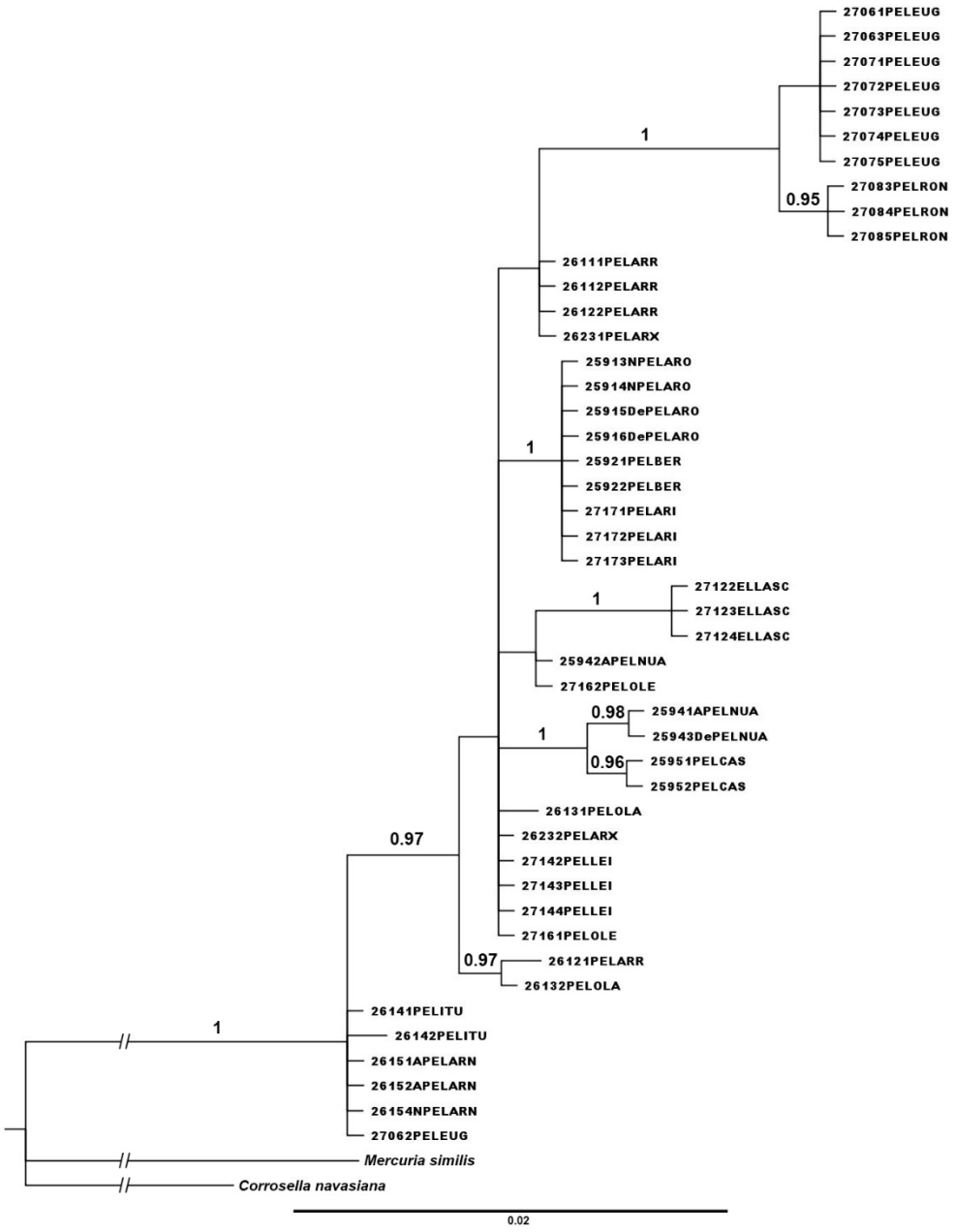

Figure S1. Bayesian phylogenetic tree of *Navarriella* populations based on the COI dataset. Bayesian posterior probabilities are provided above branches. Scale bar: expected change per site. Sequence codes are described in the Material and Methods section.

Figure S2. Bayesian phylogenetic tree of *Navarriella* populations based on the 16S dataset. Bayesian posterior probabilities are provided above branches. Scale bar: expected change per site. Sequence codes are described in the Material and Methods section.

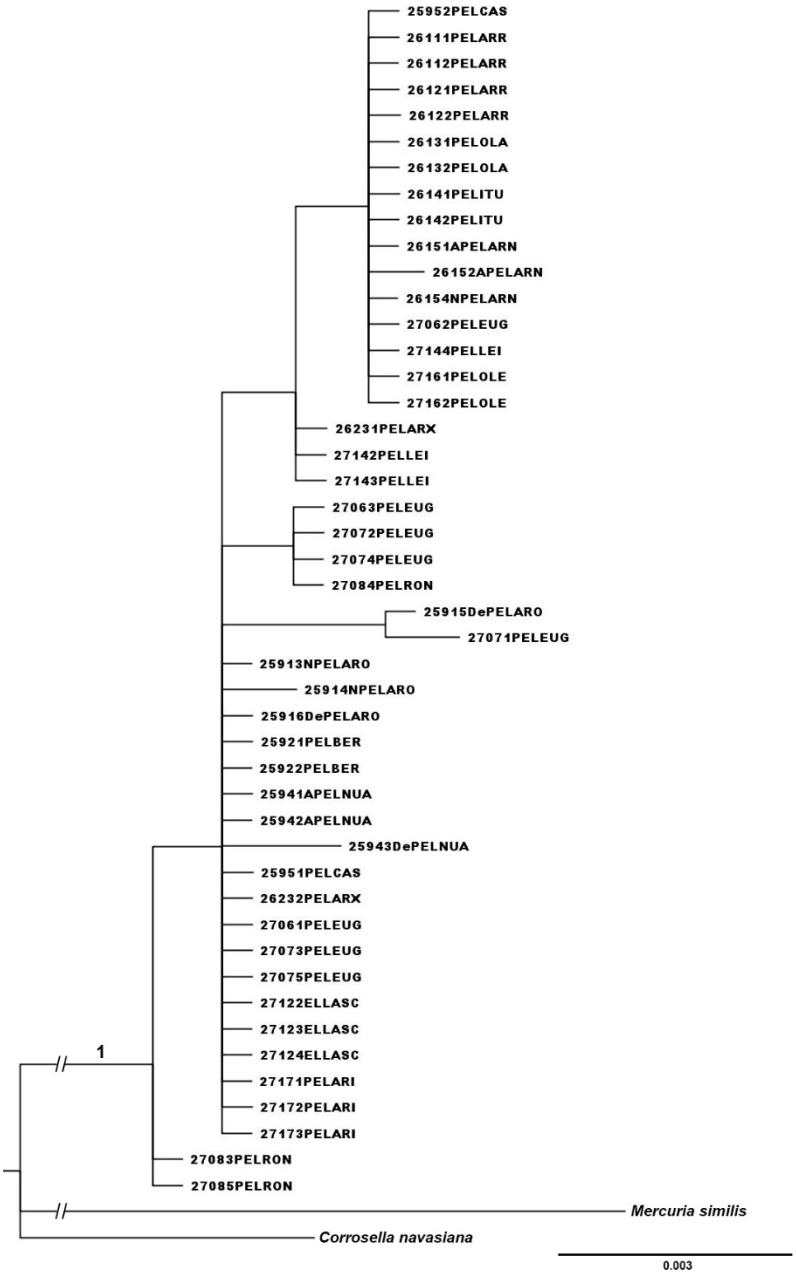

Figure S3. Bayesian phylogenetic tree of *Navarriella* populations based on the 28S dataset. Bayesian posterior probabilities are provided above branches. Scale bar: expected change per site. Sequence codes are described in the Material and Methods section.

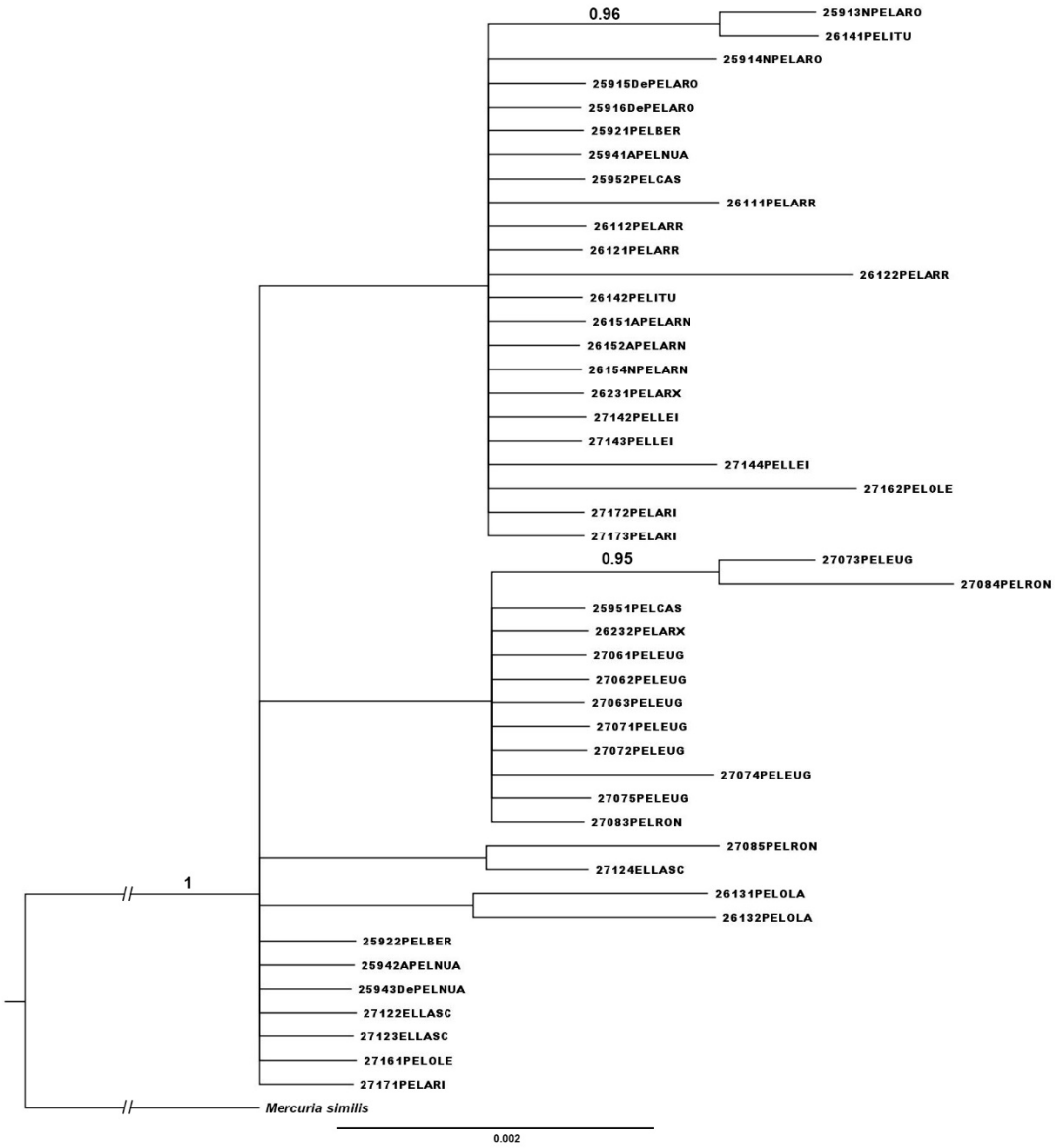

Figure S4. Bayesian phylogenetic tree of *Navarriella* populations based on the H3 dataset. Bayesian posterior probabilities are provided above branches. Scale bar: expected change per site. Sequence codes are described in the Material and Methods section.

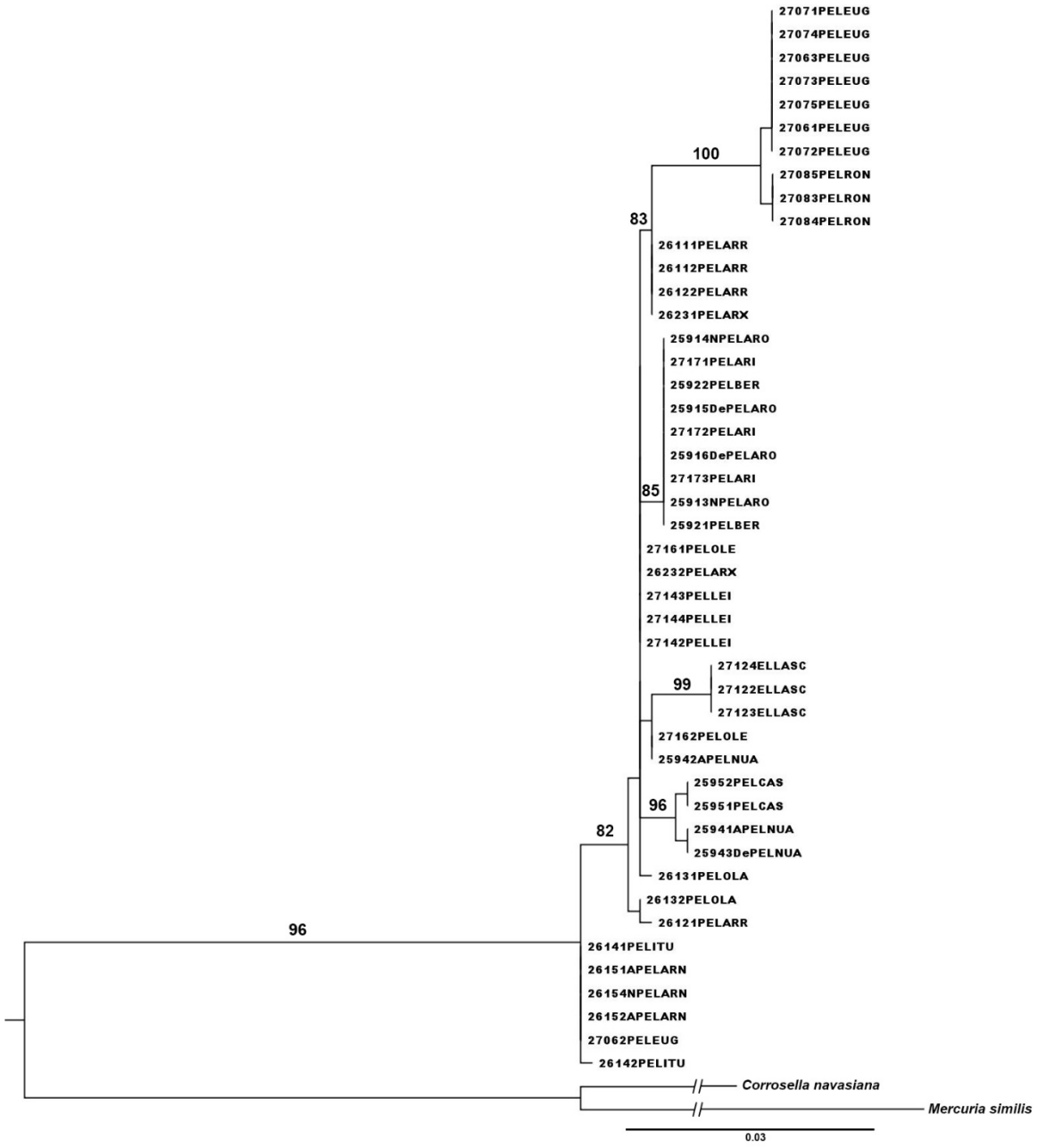

Figure S5. Maximum likelihood phylogenetic tree of *Navarriella* populations based on the COI dataset. Bootstrap support values are provided above branches. Scale bar: expected change per site. Sequence codes are described in the Material and Methods section.

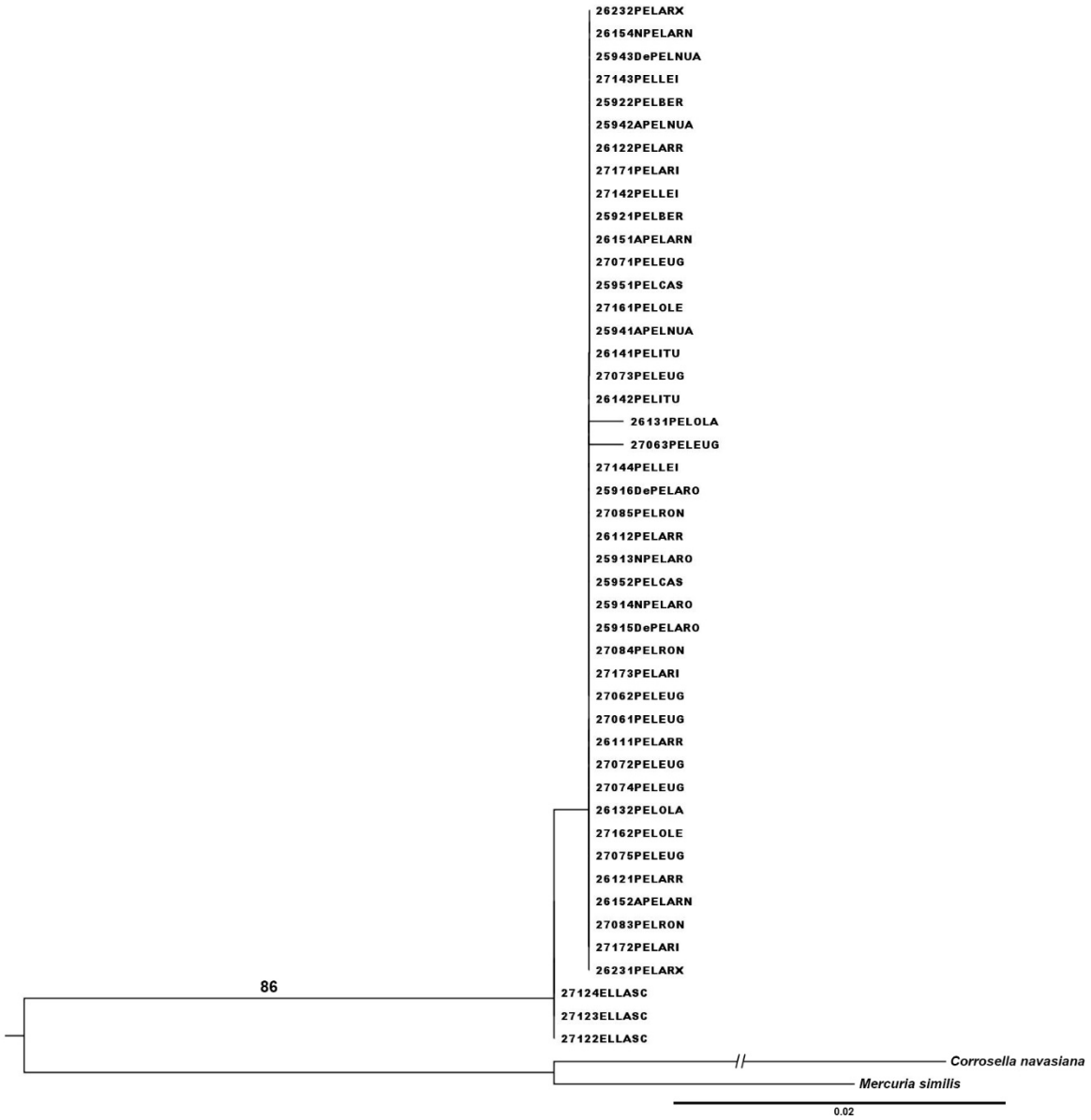

Figure S6. Maximum likelihood phylogenetic tree of *Navarriella* populations based on the 16S dataset. Bootstrap support values are provided above branches. Scale bar: expected change per site. Sequence codes are described in the Material and Methods section.

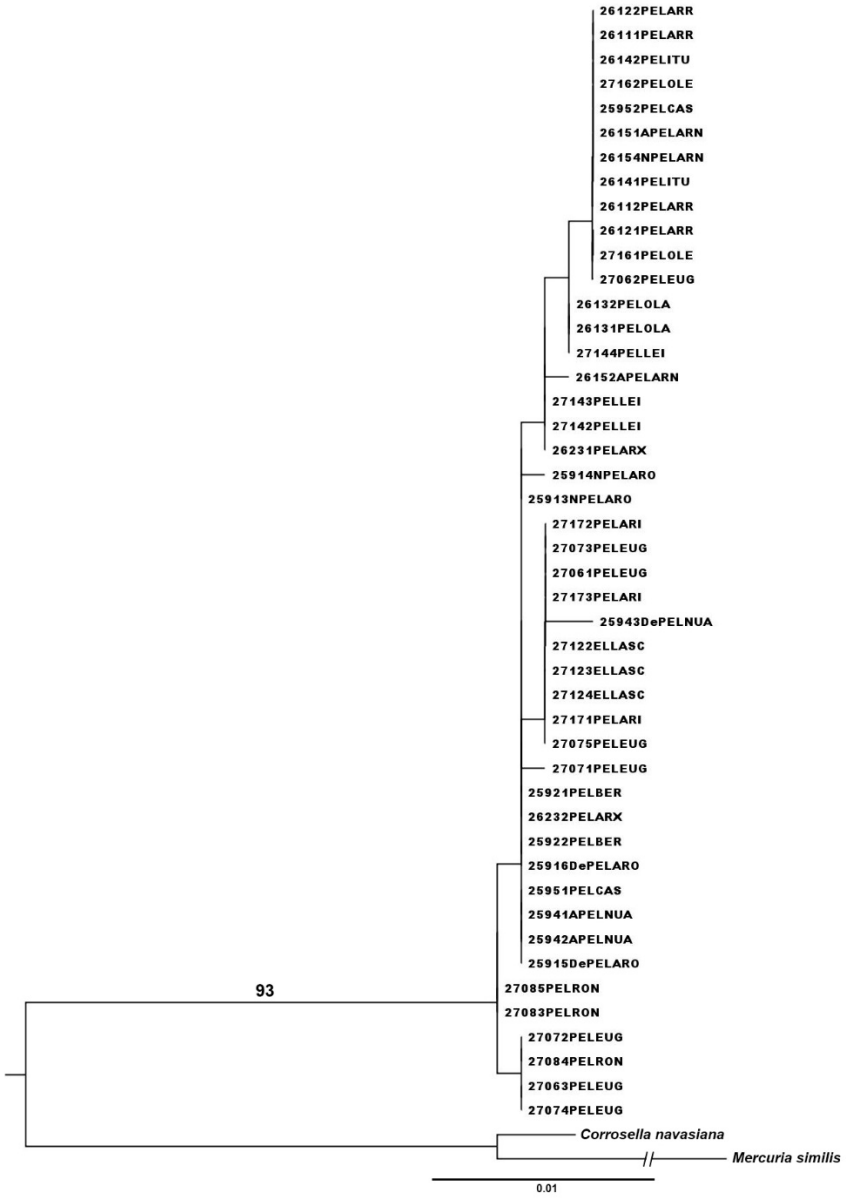

Figure S7. Maximum likelihood phylogenetic tree of *Navarriella* populations based on the 28S dataset. Bootstrap support values are provided above branches. Scale bar: expected change per site. Sequence codes are described in the Material and Methods section.

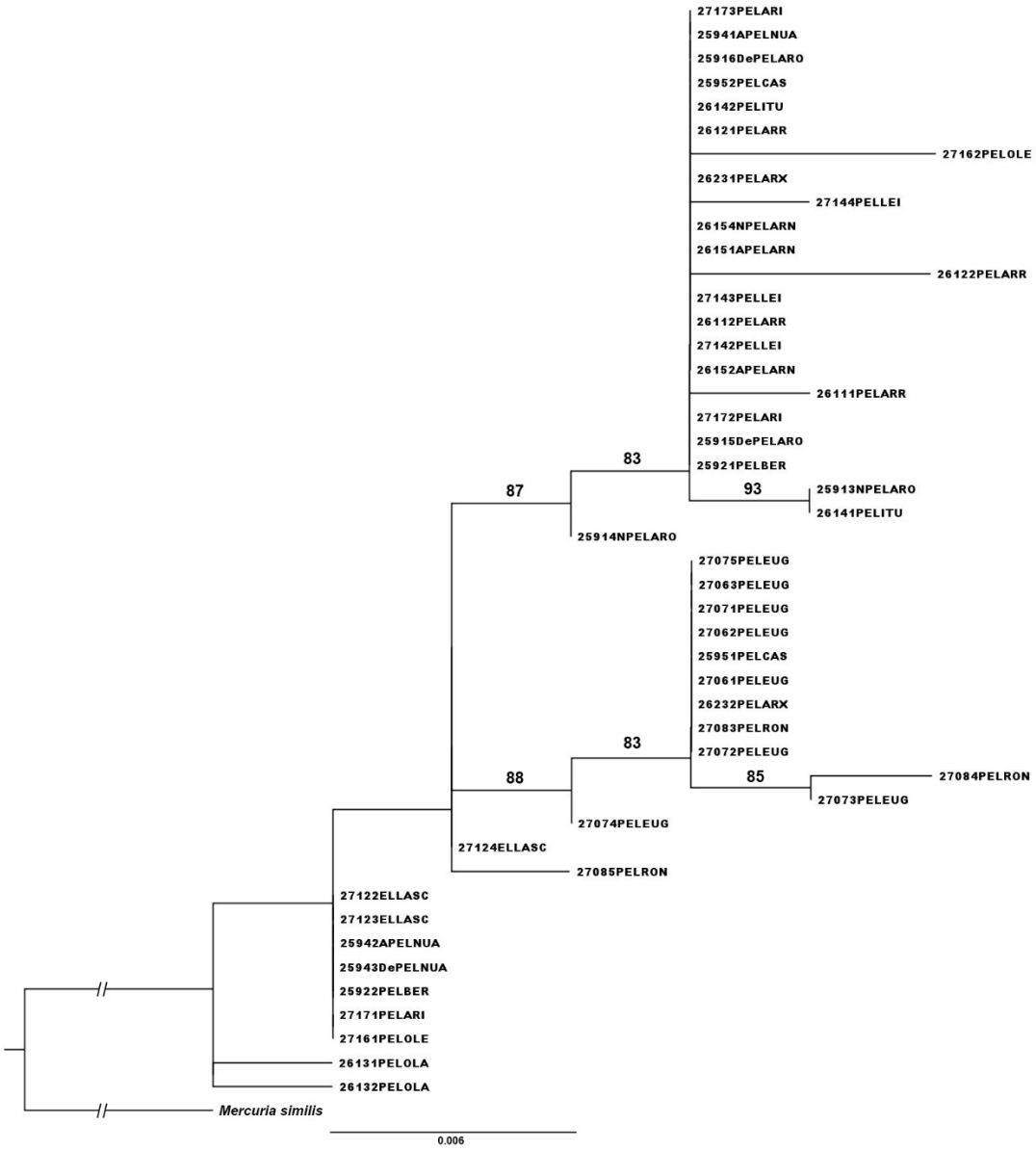

Figure S8. Maximum likelihood phylogenetic tree of *Navarriella* populations based on the H3 dataset. Bootstrap support values are provided above branches. Scale bar: expected change per site. Sequence codes are described in the Material and Methods section.

Table S1. Sampling codes, locality codes, Malacology Collection numbers from the National Museum of Natural Sciences (MNCN) in Madrid (Spain), species names, locality information and collectors for the specimens of *Alzoniella (Navarriella)* examined in this study. Collectors: M. C. P., Miguel Carrillo Pacheco; J. P. M., Jonathan Pereira Miller; F. G. G., Fernando García-Guerrero; P. Z., Pantxo Zuazu. –, insufficient specimens and not deposited, only used for DNA. N.D., no data.

Sampling code	Species	Locality	Province	Country	Locality code	X_Longitude	Y_Latitude	Conductivity (µS/cm^2)	Collectors	Date	Malacology Colection #
FW2591	*Alzoniella (Navarriella) pellitica*	Spring in Arronategi Auz	Vizcaya	Spain	ARO	-2.755304	43.393163	140	M. C. P.; J. P. M.; F. G. G.	28/07/2018	15.05/95458
FW2592	*Alzoniella (Navarriella) pellitica*	Spring near Mañu Auzoa, Bermeo	Vizcaya	Spain	BER	-2.758338	43.400573	255	M. C. P.; J. P. M.; F. G. G.	28/07/2018	15.05/95459
FW2594	*Alzoniella (Navarriella) pellitica*	Spring in Nuarbe Auzoa	Guipúzcoa	Spain	NUA	-2.229916	43.137534	220	M. C. P.; J. P. M.; F. G. G.	29/07/2018	15.05/95460
FW2595	*Alzoniella (Navarriella) pellitica*	Spring in Castillo	Guipúzcoa	Spain	CAS	-2.034156	43.119636	441	M. C. P.; J. P. M.; F. G. G.	29/07/2018	15.05/95461
FW2611	*Alzoniella (Navarriella) pellitica*	Spring in Arrarats	Navarra	Spain	ARR1	-1.808518	43.0452	208	M. C. P.; F. G. G.	31/08/2018	15.05/95462
FW2612	*Alzoniella (Navarriella) pellitica*	Spring in Arrarats	Navarra	Spain	ARR2	-1.814412	43.04875	208	M. C. P.; F. G. G.	31/08/2018	15.05/95463
FW2613	*Alzoniella (Navarriella) pellitica*	Spring in Ola	Navarra	Spain	OLA	-1.75396	43.047695	N.D.	M. C. P.; F. G. G.	31/08/2018	15.05/95464
FW2614	*Alzoniella (Navarriella) pellitica*	Iturriotz Spring, Almandoz	Navarra	Spain	ITU	-1.602919	43.090704	168	M. C. P.; F. G. G.	31/08/2018	–
FW2615	*Alzoniella (Navarriella) pellitica*	Spring in Arrantza	Navarra	Spain	ARN	-1.658216	43.066857	307	M. C. P.; F. G. G.	31/08/2018	15.05/95465
FW2623	*Alzoniella (Navarriella) pellitica*	Spring next to Araxes River	Navarra	Spain	ARX	-1.940032	43.028676	611	M. C. P.; P. Z.; F. G. G.	02/09/2018	15.05/95466
FW2706	*Alzoniella (Navarriella) pellitica*	Spring near Eugi	Navarra	Spain	EUG1	-1.529473	43.037525	230	J. P. M.; F. G .G.	02/09/2020	15.05/95467

Table S1 (cont.). Sampling codes, locality codes, Malacology Collection numbers from the MNCN in Madrid (Spain), species names, locality information and collectors for the specimens of *Alzoniella (Navarriella)* examined in this study. Collectors: M. C. P., Miguel Carrillo Pacheco; J. P. M., Jonathan Pereira Miller; F. G. G., Fernando GarcíaGuerrero; P. Z., Pantxo Zuazu. –, insufficient specimens and not deposited, only used for DNA. N.D., no data.

Sampling code	Species	Locality	Province	Country	Locality code	X_Longitude	Y_Latitude	Conductivity (µS/cm^2)	Collectors	Date	Malacology Colection #
FW2707	*Alzoniella (Navarriella) pellitica*	Spring near Eugi	Navarra	Spain	EUG2	-1.533449	43.037594	253	J. P. M.; F. G .G	02/09/2020	15.05/95468
FW2708	*Alzoniella (Navarriella) pellitica*	Watercoruse from Roncesvalles to Valcarlos	Navarra	Spain	RON	-1.344664	42.978967	217	J. P. M.; F. G .G.	03/09/2020	15.05/95469
FW2712	*Alzoniella (Navarriella) pellitica*	Spring in Chemin d'Andienea	Ascain	France	ASC	-1.613516	43.33166	932	J. P. M.; F. G .G.	04/09/2020	15.05/95470
FW2714	*Alzoniella (Navarriella) pellitica*	Spring from Leitza to Tolosa	Navarra	Spain	LEI	-1.92866	43.0907	740	J. P. M.; F. G .G.	07/09/2020	15.05/95472
FW2716	*Alzoniella (Navarriella) pellitica*	Spring in Olaeta	Álava	Spain	OLE	-2.579891	43.027215	293	J. P. M.; F. G .G.	08/09/2020	15.05/95473
FW2717	*Alzoniella (Navarriella) pellitica*	Spring in Arriola	Álava	Spain	ARI	-2.565152	43.045613	717	J. P. M.; F. G .G.	08/09/2020	15.05/95474

Table S2. Sampling codes, phylogenetic tree code, DNA & Tissue Collection numbers from the MNCN in Madrid (Spain), species names, locality and GenBank accession numbers for the specimens of *Alzoniella (Navarriella)* sequenced in this study.

Sampling code	Species	Locality	Province	Country	Tree code	Haplotype code	DNA & Tissue Collection #	COI GenBank #	16S GenBank #	28S GenBank #	H3 GenBank #	18S GenBank #
FW2591	*Alzoniella (Navarriella) pellitica*	Spring in Arronategi Auz	Vizcaya	Spain	25913NPELARO	H12	150383	OR106057	OR096609	OR096657	OR105693	
					25914NPELARO	H12	150384	OR106056	OR096608	OR096656	OR105692	
					25915DePELARO	H12	150385	OR106055	OR096607	OR096655	OR105691	
					25916DePELARO	H12	150386	OR106054	OR096606	OR096654	OR105690	
FW2592	*Alzoniella (Navarriella) pellitica*	Spring near Mañu Auzoa, Bermeo	Vizcaya	Spain	25921PELBER	H12	150387	OR106053	OR096605	OR096653	OR105689	
					25922PELBER	H12	150388	OR106052	OR096604	OR096652	OR105688	
FW2594	*Alzoniella (Navarriella) pellitica*	Spring in Auzoa	Guipúzcoa	Spain	25941APELNUA	H09	150389	OR106051	OR096603	OR096651	OR105687	
					25942APELNUA	H04	150390	OR106050	OR096602	OR096650	OR105686	
					25943DePELNUA	H09	150391	OR106049	OR096601	OR096649	OR105685	
FW2595	*Alzoniella (Navarriella) pellitica*	Spring in Castillo	Guipúzcoa	Spain	25951PELCAS	H05	150393	OR106048	OR096600	OR096648	OR105684	
					25952PELCAS	H05	150394	OR106047	OR096599	OR096647	OR105683	
FW2611	*Alzoniella (Navarriella) pellitica*	Spring in Arrarats	Navarra	Spain	26111PELARR	H07	150395	OR106046	OR096598	OR096646	OR105682	
					26112PELARR	H07	150396	OR106045	OR096597	OR096645	OR105681	

Table S2 (cont.). Sampling codes, tree code, DNA & Tissue Collection numbers from the MNCN in Madrid (Spain), species names, locality and GenBank accession numbers for the specimens of *Alzoniella (Navarriella)* sequenced in this study.

Sampling code	Species	Locality	Province	Country	Tree code	Haplotype code	DNA & Tissue Collection #	COI GenBank #	16S GenBank #	28S GenBank #	H3 GenBank #	18S GenBank #
FW2612	Alzoniella (Navarriella) pellitica	Spring in Arrarats	Navarra	Spain	26121PELARR	H11	150397	OR106044	OR096596	OR096644	OR105680	
FW2613	*Alzoniella (Navarriella) pellitica*	Spring in Ola	Navarra	Spain	26122PELARR	H07	150398	OR106043	OR096595	OR096643	OR105679	
					26131PELOLA	H06	150399	OR106042	OR096594	OR096642	OR105678	
FW2614	*Alzoniella (Navarriella) pellitica*	Iturriotz Spring, Almandoz	Navarra	Spain	26132PELOLA	H10	150400	OR106041	OR096593	OR096641	OR105677	
					26141PELITU	H13	150401	OR106040	OR096592	OR096640	OR105676	
FW2615	*Alzoniella (Navarriella) pellitica*	Spring in Arrantza	Navarra	Spain	26142PELITU	H14	150402	OR106039	OR096591	OR096639	OR105675	
					26151APELARN	H13	150403	OR106038	OR096590	OR096638	OR105674	
					26152APELARN	H13	150404	OR106037	OR096589	OR096637	OR105673	
					26154NPELARN	H13	150405	OR106036	OR096588	OR096636	OR105672	
FW2623	*Alzoniella (Navarriella) pellitica*	Spring next to Araxes River	Navarra	Spain	26231PELARX	H07	150408	OR106035	OR096587	OR096635	OR105671	
					26232PELARX	H08	150409	OR106034	OR096586	OR096634	OR105670	

Table S2 (cont.). Sampling codes, tree code, DNA & Tissue Collection numbers from the MNCN in Madrid (Spain), species names, locality and GenBank accession numbers for the specimens of *Alzoniella (Navarriella)* sequenced in this study.

Sampling code	Species	Locality	Province	Country	Tree code	Haplotype code	DNA & Tissue Collection #	COI GenBank #	16S GenBank #	28S GenBank #	H3 GenBank #	18S GenBank #
FW2706	*Alzoniella (Navarriella) pellitica*	Spring near Eugi	Navarra	Spain	27061PELEUG	H02	150411	OR106033	OR096585	OR096633	OR105669	
					27062PELEUG	H13	150412	OR106032	OR096584	OR096632	OR105668	
					27063PELEUG	H02	150413	OR106031	OR096583	OR096631	OR105667	
FW2707	*Alzoniella (Navarriella) pellitica*	Spring near Eugi	Navarra	Spain	27071PELEUG	H02	150414	OR106030	OR096582	OR096630	OR105666	
					27072PELEUG	H02	150415	OR106029	OR096581	OR096629	OR105665	
					27073PELEUG	H02	150416	OR106028	OR096580	OR096628	OR105664	
					27074PELEUG	H02	150417	OR106027	OR096579	OR096627	OR105663	
					27075PELEUG	H02	150418	OR106026	OR096578	OR096626	OR105662	
FW2708	*Alzoniella (Navarriella) pellitica*	Watercoruse from Roncesvalles to Valcarlos	Navarra	Spain	27083PELRON	H01	150421	OR106025	OR096577	OR096625	OR105661	
					27084PELRON	H01	150422	OR106024	OR096576	OR096624	OR105660	
					27085PELRON	H01	150423	OR106023	OR096575	OR096623	OR105659	

Table S2 (cont.). Sampling codes, tree code, DNA & Tissue Collection numbers from the MNCN in Madrid (Spain), species names, locality and GenBank accession numbers for the specimens of *Alzoniella (Navarriella)* sequenced in this study.

Sampling code	Species	Locality	Province	Country	Tree code	Haplotype code	DNA & Tissue Collection #	COI GenBank #	16S GenBank #	28S GenBank #	H3 GenBank #	18S GenBank #
FW2712	*Alzoniella (Navarriella) elliptica*	Spring in Chemin d'Andienea	Ascain	France	27122ELLASC	H03	150426	OR106022	OR096574	OR096622	OR105658	
					27123ELLASC	H03	150427	OR106021	OR096573	OR096621	OR105657	OR096702
					27124ELLASC	H03	150428	OR106020	OR096572	OR096620	OR105656	
FW2714	*Alzoniella (Navarriella) pellitica*	Spring from Leitza to Tolosa	Navarra	Spain	27142PELLEI	H08	150431	OR106019	OR096571	OR096619	OR105655	
					27143PELLEI	H08	150432	OR106018	OR096570	OR096618	OR105654	
					27144PELLEI	H08	150433	OR106017	OR096569	OR096617	OR105653	
FW2716	*Alzoniella (Navarriella) pellitica*	Spring in Olaeta	Álava	Spain	27161PELOLE	H08	150435	OR106016	OR096568	OR096616	OR105652	
					27162PELOLE	H04	150436	OR106015	OR096567	OR096615	OR105651	
FW2717	*Alzoniella (Navarriella) pellitica*	Spring in Arriola	Álava	Spain	27171PELARI	H12	150439	OR106014	OR096566	OR096614	OR105650	
					27172PELARI	H12	150440	OR106013	OR096567	OR096613	OR105649	
					27173PELARI	H12	150441	OR106012	OR096566	OR096612	OR105648	

Table S3. GenBank accession numbers for the species that best represent the known subfamilies within the family Hydrobiidae used in this study.

Subfamily	Taxon	Locality	COI Genbank #	18S Genbank #	Reference
Belgrandiinae	*Agrafia wiktori*	Agrafa Mountains, Sikiá, Evrytania, Greece	JF906765	JF906758	Szarowska & Falniowski (2011)
	Belgrandia thermalis	Thermal channel near S. Giuliano, Tuscany, Pisa, S. Giuliano Terme, Italy	AF367648	AF367684	Wilke, Davis, Falnoowski, Guisti, Bodon & Szarowska (2001)
	Daphniola graeca	Spring at the bottom of a natural pool rich in vegetation, about 30 km north of Larissa, Greece	EU047764	EF070624	Falniowski, Szarowska & Grzmil (2007)
	Grossuana delphica	Kastalia Spring at Delphi (type locality), Greece	EF061922	EF061917	Szarowska, Grzmil, Falniowski & Sirbu (2007)
	Hauffenia tellinii	Isonzo River near Sagrado Spring, Friuli Venetia Julia, Gorizia, Italy	AF367640	AF367672	Wilke *et al*. (2001)
Belgrandiellinae	*Arganiella pescei*	Santa Susanna Springs, Rivodutri, Latium, Italy	MW553909	MW561453	Delicado, Pešić & Ramos (2021)
	Belgrandiella kusceri	Rakovski potok [Crab stream], near Rakovski Skocjan, Rakek, Slovenia	JX970610	JX970574	Wilke, Haase, Hershler, Liu, Misof & Ponder (2013)
	Graziana alpestris	Spring at the Porra River, Liguria, Savona, Molino, Italy	AF367641	AF367673	Wilke *et al*. (2001)
	Kerkia kusceri	Cave Krska jama, Krka, Ivancna Gorica, Slovenia	KY087867	KY087833	Rysiewska, Prevorčnik,Osikowski, Hofman, Beran & Falniowski (2017)
	Pauluccinella minima	Lago di Piediluco, S. Egidio, Italy	JX970612	JX970578	Wilke *et al*. (2013)
Horatiinae	*Boetersiella sturmi*	La Mata Spring, Mata Bejid, Jaén, Spain	MH350199	MH348097	Delicado, Arconada, Aguado & Ramos (2019)
	Chondrobasis levantina	Mina Spring, Jarafuel, Valencia, Spain	MH350206	MH348098	Delicado *et al*. (2019)
	Graecoarganiella parnassiana	Spring N of Kalania, Parnassus Mountains, Greece	JN202352	JN202345	Falniowsk & Szarowska (2011)
	Radiomaniola sp.	Spring of Vrana River, between Vrana and Radosinovci, Croatia	AF367637	AF367669	Wilke *et al*. (2001)
	Sadleriana fluminensis	Main spring of Ljubljanica River, Mocilnik near Vrhnika, Slovenia	AY273996		Szarowska & Wilke (2004)
				EU573996	Ponder, Wilke, Zhang, Golding, Fukuda & Mason (2008)

Table S3 (cont.). GenBank accession numbers for the species that best represent the known subfamilies within the family Hydrobiidae used in this study

Subfamily	Taxon	Locality	COI Genbank #	18S Genbank #	Reference
Hydrobiinae	*Ecrobia ventrosa*	Snettisham lagoon RSPB bird reserve, The Wash, Norfolk, United Kingdom	AF118335		Wilke & Davis (2000)
				AF367681	Wilke *et al*. (2001)
	Hydrobia acuta	Etang du Prévost, Hérault, France	AF278808	AF367680	Wilke *et al*. (2001)
	Peringia ulvae	Logoon ‘‘Levin navolok’’, White Sea, Russia	AF118302	AF367679	Wilke *et al*. (2001)
Islamiinae	*Alzoniella finalina*	Spring at the Porra River Molino, Liguria, Savona, Italy	AF367650	AF367686	Wilke *et al*. (2001)
	Aretiana wolfi	Virgen de los Ángeles hermitage, Alajar, Huelva, Spain	MH350193	MH348095	Delicado *et al*. (2019)
	Avenionia brevis	Spring of the fountain of St.Victor La Coste, Gard, France	AF367638	AF367670	Wilke *et al*. (2001)
	Corbellaria celtiberica	Manubles River, Soria, Spain	MH350207	MH348099	Delicado *et al*. (2019)
	Deganta azarum	La Fontona Spring, Borondes, Asturias, Spain	MH350208	MH348100	Delicado *et al*. (2019)
	Fissuria boui	Spring near La Prouveresse, Alpes Maritimes, France	AF367654	AF367690	Wilke *et al*. (2001)
	Iberhoratia morenoi	Prado del Rey Spring, Cádiz, Spain	MH350211	MH348101	Delicado *et al*. (2019)
	Islamia piristoma	Spring on the right bank the Magra River near Romito, Liguria, La Spezia, Arcola, Italy	AF367639	AF367671	Wilke *et al*. (2001)
	Milesiana schuelei	Spring in Berchul, Félix, Almería, Spain	MH350244	MH348108	Delicado *et al*. (2019)
	Spathogyna fezi	Roble Spring, Yémeda, Cuenca, Spain	MH350251	MH348109	Delicado *et al*. (2019)
	Tarraconia gasulli	La Esperanza Spring, Navajas, Castellón, Spain	MH350254	MH348110	Delicado *et al*. (2019)
Mercuriinae	*Mercuria similis*	La Puebla, Mallorca, Spain.		AF212913	Wilke, Davis, Gong & Liu (2000)
			JX081888		Delicado, Machordom & Ramos (2013)
Nymphophilinae	*Pyrgulopsis minckleyi*	Cuatro Cienegas Basin, Laguna Churince, Coahuila, Mexico	AF354771		Liu, Hershler & Thomspon (2001)
				JX970577	Wilke *et al*. (2013)

Table S3 (cont.). GenBank accession numbers for the species that best represent the known subfamilies within the family Hydrobiidae used in this study

Subfamily	Taxon	Locality	COI Genbank #	18S Genbank #	Reference
Pseudamnicolinae	*Corrosella navasiana*	Fonnueva Spring, Bulbuente, Spain	JX081861	JX081801	Delicado *et al*. (2013)
	Diegus gasulli	Stream at Barranco de las Negras, Almería, Spain	KF060736		Delicado, Machordom & Ramos (2014)
	Pseudamnicola lucensis	Thermal spring, Bagni Caldi, Bagni di Lucca, Lucca, Tuscany, Italy	AF367651	AF367687	Wilke *et al*. (2001)
	Bullaregia tunisiensis	Spring in Djebba, Province Béja, Tunisia	KX821683		Khalloufi, Béjaoui & Delicado (2017)
				MN575709	Khalloufi, Béjaoui & Delicado (2020)
	Belgrandiellopsis chorfensis	Chorfa Spring, ca 15 km west of Joumine, Béja Province, Tunisia	MN580416	MN575710	Khalloufi *et al*. (2020)
	Biserta putealis	Soudene Well, Bizerte Province, Tunisia	MN580420	MN575713	Khalloufi *et al*. (2020)

Table S4. Shell dimensions (in mm) of the *Navarriella elliptica* n. comb. populations: **1,** FW2712 – Spring in Chemin d'Andienea, Ascain, France; **2,** FW2717 – Spring in Arriola, Navarre, Spain; **3,** FW2591 – Spring in Arronategi Auz, Vizcaya, Spain; **4,** FW2623 – Spring next to Araxes River, Navarre, Spain; **5,** FW2707 – Spring near Eugi, Navarre, Spain; **6,** FW2708 – Watercourse from Roncesvalles to Valcarlos, Navarre, Spain. Variable abbreviations are described in the Material and Methods section.

	1 Mean ± SD; CV (Max Min) n=30	**2** Mean ± SD; CV (Max Min) n=17	**3** Mean ± SD; CV (Max Min) n=16	**4** Mean ± SD; CV (Max Min) n=24	**5** Mean ± SD; CV (Max Min) n=30	**6** Mean ± SD; CV (Max Min) n=30
SL	1.98 ± 0.07; 0.04 (2.14 1.85)	1.90 ± 0.08; 0.04 (2.03 1.80)	2.00 ± 0.11; 0.05 (2.21 1.76)	2.01 ± 0.05; 0.03 (2.11 1.93)	1.72 ± 0.08; 0.04 (1.90 1.56)	2.00 ± 0.13; 0.07 (2.39 1.82)
AH	0.94 ± 0.04; 0.04 (1.00 0.84)	0.89 ± 0.04; 0.04 (0.98 0.84)	0.92 ± 0.05; 0.06 (1.03 0.80)	0.90 ± 0.04; 0.04 (0.96 0.80)	0.77 ± 0.04; 0.05 (0.85 0.71)	0.89 ± 0.07; 0.07 (1.07 0.80)
AL	0.94 ± 0.04; 0.04 (1.02 0.82)	0.89 ± 0.03; 0.04 (0.97 0.84)	0.93 ± 0.05;0.06 (1.01 0.79)	0.94 ± 0.03; 0.03 (1.00 0.88)	0.81 ± 0.04; 0.04 (0.89 0.75)	0.94 ± 0.06; 0.06 (1.12 0.86)
SW	1.40 ± 0.04; 0.03 (1.47 1.34)	1.32 ± 0.04; 0.03 (1.42 1.27)	1.35 ± 0.07; 0.05 (1.46 1.24)	1.34 ± 0.05; 0.03 (1.46 1.27)	1.16 ± 0.05; 0.05 (1.27 1.05)	1.36 ± 0.09; 0.06 (1.61 1.25)
WBW	1.10 ± 0.04; 0.04 (1.19 1.04)	1.09 ± 0.16; 0.15 (1.71 1.01)	1.08 ± 0.04; 0.04 (1.13 1.00)	1.08 ± 0.04; 0.04 (1.16 1.00)	0.93 ± 0.04; 0.04 (0.99 0.85)	1.11 ± 0.09; 0.08 (1.41 1.01)
AW	0.72 ± 0.03; 0.04 (0.77 0.66)	0.69 ± 0.03; 0.04 (0.75 0.66)	0.7 ± 0.04; 0.05 (0.76 0.64)	0.70 ± 0.03; 0.05 (0.77 0.63)	0.63 ± 0.03; 0.05 (0.71 0.58)	0.73 ± 0.05; 0.07 (0.92 0.66)
LBW	1.46 ± 0.06; 0.04 (1.59 1.34)	1.41 ± 0.06; 0.05 (1.58 1.33)	1.41 ± 0.07; 0.05 (1.51 1.29)	1.45 ± 0.07; 0.05 (1.58 1.19)	1.25 ± 0.06; 0.05 (1.37 1.15)	1.45 ± 0.11; 0.07 (1.79 1.18)

Table S5. Dimensions of the ctenidium, osphradium and digestive system (in mm) and RPG Ratio recorded in the *Navarriella elliptica* n. comb. populations: **1,** FW2712 – Spring in Chemin d'Andienea, Ascain, France; **2,** FW2717 – Spring in Arriola, Navarre, Spain; **3,** FW2591 – Spring in Arronategi Auz, Vizcaya, Spain; **4,** FW2623 – Spring next to Araxes River, Navarre, Spain; **5,** FW2707 – Spring near Eugi, Navarre, Spain; **6,** FW2708 –Watercourse from Roncesvalles to Valcarlos, Navarre, Spain. Variable abbreviations are described in the Material and Methods section.

	1 Mean ± SD; CV (Max Min) n = 5	**2** Mean ± SD; CV (Max Min) n = 5	**3** Mean ± SD; CV (Max Min) n = 4	**4** Mean ± SD; CV (Max Min) n = 7	**5** Mean ± SD; CV (Max Min) n = 7	**6** Mean ± SD; CV (Max Min) n = 7
CtL	0.64 ± 0.07; 0.10 (0.70 0.55)	0.50 ± 0.10; 0.19 (0.64 0.44)	0.73 ± 0.05; 0.07 (0.80 0.68)	0.73 ± 0.09; 0.13 (0.85 0.63)	0.62 ± 0.05; 0.07 (0.66 0.56)	0.65 ± 0.015; 0.25 (1.00 0.54)
OsL	0.19 ± 0.06; 0.29 (0.25 0.10)	0.21 ± 0.03; 0.14 (0.24 0.17)	0.24 ± 0.01; 0.06 (0.38 0.26)	0.23 ± 0.04; 0.18 (0.28 0.17)	0.22 ± 0.03; 0.14 (0.25 0.18)	0.27 ± 0.11; 0.41 (0.50 0.19)
OsW	0.09 ± 0.03; 0.26 (0.12 0.06)	0.09 ± 0.02; 0.23 (0.26 0.19)	0.12 ± 0.01; 0.07 (0.13 0.11)	0.10 ± 0.02; 0.19 (0.12 0.07)	0.08 ± 0.02; 0.29 (0.10 0.05)	0.08 ± 0.02; 0.30 (0.49 0.27)
StL	0.38 ± 0.01; 0.03 (0.39 0.37)	0.38 ± 0.04; 0.10 (0.44 0.34)	0.45 ± 0.03; 0.06 (0.48 0.43)	0.38 ± 0.02; 0.06 (0.41 0.35)	0.40 ± 0.03; 0.08 (0.43 0.36)	0.41 ± 0.03; 0.07 (0.46 0.37)
StW	0.34 ± 0.06; 0.17 (0.39 0.28)	0.36 ± 0.02; 0.06 (0.40 0.35)	0.35 ± 0.06; 0.17 (0.38 0.26)	0.40 ± 0.05; 0.13 (0.48 0.34)	0.33 ± 0.02; 0.07 (0.43 0.36)	0.34 ± 0.04; 0.13 (0.41 0.27)
SsL	0.32 ± 0.03; 0.08 (0.35 0.30)	0.28 ± 0.02; 0.05 (0.30 0.26)	0.36 ± 0.06; 0.18 (0.43 0.30)	0.34 ± 0.04; 0.11 (0.40 0.31)	0.36 ± 0.09; 0.25 (0.49 0.30)	0.33 ± 0.02; 0.06 (0.36 0.31)
SsW	0.25 ± 0.05; 0.18 (0.30 0.21)	0.22 ± 0.03; 0.13 (0.26 0.19)	0.28 ± 0.10; 0.35 (0.42 0.21)	0.24 ± 0.04; 0.16 (0.29 0.18)	0.23 ± 0.02; 0.11 (0.25 0.20)	0.25 ± 0.04; 0.14 (0.30 0.20)
RPG Ratio	0.42	0.44	0.39	0.40	0.33	0.39

Table S6. Male and female genitalia measurements (in mm) recorded in the *Navarriella elliptica* n. comb. populations: **1,** FW2712 – Spring in Chemin d'Andienea, Ascain, France; **2,** FW2717 – Spring in Arriola, Navarre, Spain; **3,** FW2591 – Spring in Arronategi Auz, Vizcaya, Spain; **4,** FW2623 – Spring next to Araxes River, Navarre, Spain; **5,** FW2707 – Spring near Eugi, Navarre, Spain; **6,** FW2708 – Watercoruse from Roncesvalles to Valcarlos, Navarre, Spain. Variable abbreviations are described in the Material and Methods section.

	1 Mean ± SD; CV (Max Min) n = 5	**2** Mean ± SD; CV (Max Min) n = 5	**3** Mean ± SD; CV (Max Min) n = 4	**4** Mean ± SD; CV (Max Min) n = 7	**5** Mean ± SD; CV (Max Min) n = 7	**6** Mean ± SD; CV (Max Min) n = 7
PrL	0.61 ± 0.07; 0.11 (0.69 0.56)	0.58 ± 0.05; 0.09 (0.63 0.53)	0.61 ± 0.16; 0.26 (0.72 0.50)	0.67 ± 0.09; 0.14 (0.75 0.58)	0.60 ± 0.04; 0.07 (0.65 0.57)	0.59 ± 0.12; 0.20 (0.70 0.47)
PrW	0.22 ± 0.05; 0.23 (0.27 0.17)	0.20 ± 0.20; 0.08 (0.22 0.19)	0.22 ± 0.08; 0.25 (0.27 0.19)	0.26 ± 0.04; 0.14 (0.30 0.22)	0.21 ± 0.02; 0.10 (0.23 0.19)	0.19 ± 0.02; 0.08 (0.20 0.17)
PL	0.43 ± 0.05; 0.11 (0.48 0.40)	0.40 ± 0.08; 0.21 (0.48 0.32)	0.98 ± 0.12; 0.12 (1.06 0.89)	0.55 ± 0.02; 0.04 (0.57 0.52)	0.41 ± 0.03; 0.06 (0.44 0.39)	0.49 ± 0.01; 0.03 (0.50 0.48)
PlL	0.52 ± 0.08; 0.16 (0.61 0.45)	0.46 ± 0.05; 0.11 (0.50 0.39)	1.05 ± 0.16; 0.15 (1.16 0.94)	0.61 ± 0.05; 0.09 (0.69 0.57)	0.50 ± 0.07; 0.13 (0.56 0.43)	0.58 ± 0.06; 0.10 (0.62 0.54)
PlW	0.17 ± 0.02; 0.10 (0.19 0.16)	0.15 ± 0.02; 0.15 (0.18 0.13)	0.27 ± 0.01; 0.02 (0.28 0.26)	0.19 ± 0.03; 0.15 (0.21 0.15)	0.16 ± 0.01; 0.06 (0.17 0.15)	0.19 ± 0.03; 0.15 (0.21 0.17)
AgL	0.31 ± 0.12; 0.39 (0.39 0.22)	0.27 ± 0.03; 0.10 (0.29 0.25)	0.31 ± 0.01; 0.05 (0.32 0.30)	0.24 ± 0.04; 0.18 (0.29 0.21)	0.28 ± 0.05; 0.18 (0.33 0.21)	0.26 ± 0.05; 0.19 (0.33 0.322)
CgL	0.61 ± 0.05; 0.08 (0.64 0.57)	0.52 ± 0.06; 0.12 (0.56 0.47)	0.74 ± 0.014; 0.19 (0.84 0.64)	0.62 ± 0.05; 0.08 (0.67 0.57)	0.61 ± 0.05; 0.05 (0.64 0.57)	0.52 ± 0.03; 0.05 (0.56 0.50)
Sr1L	0.30 ± 0.03; 0.09 (0.32 0.28)	0.19 ± 0.01; 0.04 (0.19 0.18)	0.22 ± 0.01; 0,06 (0.24 0.22)	0.19 ± 0.02; 0.08 (0.21 0.18)	0.26 ± 0.07; 0.27 (0.34 0.18)	0.22 ± 0.03; 0.12 (0.25 0.20)
Sr2L	0.20 ± 0.04; 0.21 (0.23 0.17)	0.12 ± 0.01; 0.12 (0.13 0.11)	0.17 ± 0.03; 0.06 (0.19 0.15)	0.17 ± 0.03; 0.16 (0.20 0.15)	0.24 ± 0.03; 0.14 (0.28 0.20)	0.17 ± 0.02; 0.09 (0.25 0.20)
BcL	0.50 ± 0.08; 0.17 (0.56 0.44)	0.39 ± 0.04; 0.11 (0.42 0.36)	0.54 ± 0.01; 0.03 (0.55 0.53)	0.37 ± 0.08; 0.21 (0.46 0.32)	0.43 ± 0.11; 0.26 (0.53 0.28)	0.38 ± 0.08; 0.20 (0.43 0.27)

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