Molecular investigation and description of *Iberozospeum* n. gen., including the description of one new species (Eupulmonata, Ellobioidea, Carychiidae)

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Abstract

The subterranean realm of the Cantabrian-Pyrenean region of northern Spain harbours a rich diversity of *Zospeum*. Due to their tiny size and the difficulty of finding them alive, scarce animal material has been available for scientific investigation. Recent investigations of *Zospeum* shells have provided valuable, but limited insights towards our understanding of the evolutionary processes occurring within this taxon in northern Spain. In an integrative study, we investigate 57 populations of *Zospeum* from northern Spanish caves using two mitochondrial (COI and 16S) and two nuclear markers (H3 and 5.8 S rRNA + ITS2). Revealed is a separate radiation of the northern Spanish species for which the new genus, *Iberozospeum*, is proposed. The independent radiation of Dinaric *Zospeum* from that of northern Spain justifies the designation of *Iberozospeum* n. gen. Morphological evidence is provided via histological analysis of *Iberozospeum vasconicum* and SEM analyses of radulae of eastern Alpine, Dinaric and Iberian species. Important differences in morphological structure and character states are presented, including the first view of the sexually mature female and the presence of the giant albumen gland in an individual of the subterranean, troglobitic Carychiidae. Significant differences are revealed in superficial crystallographic structure of the columellar lamellae, the morphology of the columellar muscle and in the radula. Radular ribbon length, ribbon broadness, straightness of the ribbon base and cusp configuration are distinctive in the Iberian species. One new species is described corroborated by genetic and morphological characters.

Keywords Cave-dwelling species · Cryptic diversity · Histology · Intraspecific variability · Microgastropods · Subterranean land snail

Introduction

Tiny subterranean snails of the genus *Zospeum* (Bourguignat, 1856), are known to inhabit the broad network of caves underlying the Pyrenean-Cantabrian region of northern Spain (Fig. 1). Although recent collection efforts have provided significant finds, many new discoveries have remained parked in the lab due to doubts and complications regarding the taxonomic status of the oldest-described species from Spain, *Iberozospeum schaufussi* (von Frauenfeld, 1862; Jochum et al., 2019). Only until recently were these taxonomic issues clarified such that the description of new species could proceed unhindered in a region known to harbour rich “*Zospeum*” diversity (Jochum et al., 2019). We emphasise that northern Spanish material molecularly assessed by Weigand et al. (2013) as cf. *Z. suarezi* (Gittenberger, 1980) and deposited in the BOLD data base under this name was revised (Jochum et al., 2019) and is now in sync with this investigation, *I. schaufussi* (von Frauenfeld, 1862). Caves harbouring two to three different morphotypes are considered not unusual (Alonso et al., 2018). Despite this suggested richness, only eight species have been formally described, including *I. schaufussi* (von Frauenfeld, 1862), *I. bellesi* (Gittenberger, 1973), *I. bicaeniense* (Gómez & Prieto, 1983), *I. vasconicum* (Prieto et al., 2015 in Jochum et al., 2015a), *I. zaldívarae* (Prieto et al., 2015...
2015 in Jochum et al., 2015a), I. percostulatum (Alonso et al., 2018) and recently, I. gittenbergeri (Jochum et al., 2019) and I. praetermissum (Jochum et al., 2019).

Weigand et al. (2013) presented the first phylogenetic study incorporating Iberian “Zospeum” species, whereby nine populations of “Zospeum” are clustered into six evolutionary lineages (EL), with those from the Cantabrian Mountains being monophyletic. This study and additional phylogenetic investigations have since demonstrated the high incidence of intraspecific variability and cryptic diversity in Iberian, Eastern Alpine (Kruckenhauser et al., 2019; Weigand et al., 2011) and Dinaride (Inäbnit et al., 2019) Zospeum species.

In order to further understand zospeid evolutionary history in caves of northern Spain, we molecularly assess recent finds encompassing 57 populations. Anatomical perspectives of zospeid radulae and organ systems remain rare and few in number. Seven studies are known so far (see Jochum, 2011; Jochum et al., 2015b; Inäbnit et al., 2019). By studying histological sections of toptotypic I. vasconicum, toptypic Z. isselianum (Pollonera, 1887) and Z. amoenum (von Frauenfeld, 1856) (see Jochum et al. 2015b) and Z. spelaeum (Rossmässler, 1839), we compare the presence of specific structures and character states in the visceral mass of one northern Spanish and three Dinaride taxa. We additionally describe significant differences in radular morphology and crystallographic structure on the columnellar lamellae using scanning electron microscopy (SEM). Together, these investigations support the erection of the new genus, *Iberozospeum*.

**Material and methods**

Material is housed in the following collections:

AJC: Adrienne Jochum Collection, Kelkheim, Germany.
CAA: Collection of Alvaro Alonso, Spain.
CSQS: Collection of Sergio Quiñonero-Salgado, Spain.
MHNG: Muséum histoire naturelle Gèneve, Geneva, Switzerland.
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Analyses include the outgroup species *Zospeum spelaeum* (Kuščer, 1932), *Zospeum frauenfeldii* (Freyer, 1855), *Zospeum manitaense* (Inäbnit et al., 2019), *Zospeum robustum* (Inäbnit et al., 2019), *Zospeum pretneri* (Bole, 1960) from the Dinaric Alps (Inäbnit et al., 2019; Weigand et al., 2013). Additionally, sequences of *I. vasconicum* (No. 151 in Weigand et al., 2013), *I. schaufussi* (as *I. suarezi*, No. 140 in Weigand et al., 2013) and *I. zaldvareae* (No. 162 and 163 in Weigand et al., 2013) from their type localities were included. All images and DNA extracts of the specimens investigated in this study from their type localities were included. All images and DNA extraction, 200 µl Buffer AL was added. The mixture was vortexed and incubated at 56 °C for 10 min. Ethanol of 200 µl (100%) was added and vortexed again. The mixture was pipetted in a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8000 rpm (Centrifuge 5424, Eppendorf) for 1 min. The spin column was placed in a new 2 ml collection tube and 500 µl Buffer AW1 was added. It was centrifuged for 1 min at 8000 rpm. The spin column was placed in a new 2 ml collection tube, and 500 µl Buffer AW2 was added. It was centrifuged for 3 min at 14,000 rpm. Afterwards, the spin column was transferred to a new 1.5 ml Eppendorf tube. The DNA was then eluted by adding 200 µl Buffer AE. It was incubated for 1 min at room temperature (ca. 23 °C) and centrifuged for 1 min at 8000 rpm.

In this study, two mitochondrial (mtDNA) markers (COI and 16S) and two nuclear (nDNA) markers (H3 and 5.8 S rRNA + ITS2) were investigated. PCR mixtures consisted of 12.5 µl GoTaq G2 HotStart Green Master Mix (Promega M7423), 4.5 µl ddH₂O, 2 µl forward and reverse primer each and 4 µl DNA template. In Table 2 the respective primer pairs for the PCR are listed. The following PCR cycles were used as follows: for COI the admixture was heated 2 min at 94 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 40 °C and 1 min at 72 °C and, finally, 5 min at 72 °C; for 16S the admixture was heated 5 min at 95 °C, followed by 45 cycles of 30 s at 95 °C, 30 s at 48 °C and 45 s at 72 °C and, finally, 5 min at 72 °C; for H3 the admixture was heated 3 min at 95 °C, followed 40 cycles of 1 min at 95 °C, 1 min at 42 °C and 1 min at 72 °C and, finally, 10 min at 72 °C; and for 5.8 S rRNA + ITS2 the admixture was heated 1 min at 96 °C, followed by 45 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C and, finally, 10 min at 72 °C (SensoQuest Tabyclet and Techn e TC-512, witec AG, Littau, Switzerland). The purification and sequencing of the PCR products were performed by LGC (LGC Genomics Berlin, Germany).

Specimens collected in 2011 or later could be successfully sequenced. Since the DNA content in older specimens was too low for any analyses, sequences of those specimens could not be included in our study. Long-term storage in ethanol proved to be problematic for the successful sequencing of any small snails (<1.5 mm).

**Phylogenetic analyses**

The software package Geneious v9.1.8 (Biomatters Ltd) was used for sequence processing and editing. Maximum likelihood (ML) topology was estimated with the Geneious RAxML plug-in (Stamatakis, 2006) using rapid bootstrapping setting to compute the best scoring ML tree and 1500 bootstrapping replicates. The protein-coding gene fragments of COI was defined in two data blocks. The first and third codon positions were defined as one block and the second...
Table 1  Sampled northern Spanish caves with their geographic location and the GenBank accession numbers for the analysed mtDNA (COI, 16S) and nDNA (H3, 5.8 S rRNA + ITS2) sequences

| NMBE-No | ZUPV-No | Cave and Province          | Latitude | Longitude | Altitude [m] | GenBank accession number COI | GenBank accession number 16S | GenBank accession number H3 | GenBank accession number ITS2 |
|---------|---------|----------------------------|----------|-----------|--------------|-------------------------------|-------------------------------|-----------------------------|------------------------------|
| 540551_1 | 1847    | Akaitz Txiki (Gipuzkoa)    | 42.9818  | -2.0925   | 824          | MW626838                     | MW621911                     | MW622169                    | MW621272                     |
| 540552_1 | 2039    | Azkillar (Bizkaia)         | 43.0882  | -2.5912   | 1030         | MW626839                     | MW621915                     | MW622173                    | MW621275                     |
| 540552_3 | 2039    | Azkillar (Bizkaia)         | 43.0882  | -2.5912   | 1030         | MW626841                     | MW621917                     | MW622175                    | MW621277                     |
| 540553_1 | 1066    | Elorrea (Bizkaia)          | 43.0538  | -2.7956   | 1059         | -                            | MW621919                     | MW622177                    | -                            |
| 540554_4 | 1309    | Arrikrutz (Gipuzkoa)       | 42.9988  | -2.4265   | 495          | -                            | MW621920                     | MW622181                    | MW621282                     |
| 540555_2 | 937     | Basoxto (Navarra)          | 42.8798  | -2.246    | 705          | -                            | -                            | MW62183                     | -                            |
| 540556_1 | 874     | Haxondo (Bizkaia)          | 43.1835  | -2.862    | 300          | -                            | -                            | MW62184                     | -                            |
| 557136   | 4885    | Herreria (Asturias)        | 43.39784 | -4.75967  | 50           | -                            | MW621921                     | MW622188                    | MW621284                     |
| 557138   | 4702    | Baja (Cantabria)           | 43.40163 | -3.41371  | 51           | MW626842                     | MW621922                     | MW622189                    | MW621285                     |
| 557140   | 4862    | Collubina (Asturias)       | 43.39841 | -4.72264  | 45           | MW626843                     | MW621923                     | MW622190                    | MW621286                     |
| 557142   | 3671    | Cumbre (Asturias)          | 43.26893 | -4.89248  | 1761         | -                            | MW621924                     | MW622191                    | -                            |
| 557144   | 4136    | Udias (Cantabria)          | 43.34163 | -4.23989  | 109          | MZ620729                     | MW621925                     | MW622192                    | MW621287                     |
| 557146   | 3820    | Montosas (Cantabria)       | 43.22731 | -3.70042  | 725          | MW626844                     | MW621926                     | MW622193                    | MW621288                     |
| 557150   | 3405    | Txomenkoba (Gipuzkoa)      | 42.98131 | -2.47352  | 810          | MW626845                     | MW621927                     | MW622194                    | MW621289                     |
| 557152   | 3758    | San Valerio (Gipuzkoa)     | 43.08203 | -2.50105  | 444          | MW626846                     | MW621928                     | MW622195                    | MW621290                     |
| 557154   | 3849    | Penpelin (Gipuzkoa)        | 43.062   | -2.47655  | 690          | MW626847                     | MW621929                     | MW622196                    | MW621291                     |
| 557156   | 3856    | Artegi (Bizkaia)           | 43.09628 | -2.53847  | 540          | MW626848                     | MW621930                     | MW622197                    | MW621292                     |
| 557158   | 4601    | Saiturri-2 (Gipuzkoa)      | 42.96902 | -2.49111  | 1063         | MW626849                     | MW621931                     | MW622198                    | MW621293                     |
| 557160   | 4757    | Urkoba (Gipuzkoa)          | 42.97802 | -2.49103  | 1020         | -                            | -                            | MW621999                    | MW621294                     |
| 557162   | 4821    | Perusario-1 (Gipuzkoa)     | 42.95026 | -2.34416  | 1156         | -                            | MW621932                     | -                            | MW621295                     |
| 557166   | 4990    | Aizkizarri (Gipuzkoa)      | 42.99568 | -2.41728  | 600          | MW626850                     | MW621933                     | MW622200                    | MW621296                     |
| 557168   | 5005    | Arilaban (Gipuzkoa)        | 43.08629 | -2.53928  | 382          | MW626851                     | MW621934                     | MW622201                    | MW621297                     |
| 557174   | 1008    | Eskatxabel-2 (Bizkaia)     | 43.27509 | -3.0888   | 565          | -                            | -                            | MW622202                    | MW621298                     |
| 557178   | 1607    | Paules (Burgos)            | 42.94287 | -2.99363  | 830          | MW626852                     | MW621935                     | MW622203                    | MW621299                     |
| 557180   | 1740    | Lejazar (Araba)            | 42.93484 | -2.96285  | 836          | -                            | MW621936                     | MW622204                    | MW621300                     |
| 557182   | 874     | Haxondo (Bizkaia)          | 43.18162 | -2.86335  | 300          | -                            | MW621937                     | -                            | -                            |
**Table 1** (continued)

| NMBE-No | ZUPV-No | Cave and Province | Latitude  | Longitude  | Altitude [m] | GenBank accession number COI | GenBank accession number 16S | GenBank accession number H3 | GenBank accession number ITS2 |
|---------|---------|------------------|----------|-----------|--------------|----------------------------|----------------------------|----------------------------|----------------------------|
| 557187  | 1847    | Akaitz Txiki (Gipuzkoa) | 42.97995 | −2.09384  | 824          | MW626853                   | MW621938                   | MW622205                   | MW621301                   |
| 557188  | 1847    | Akaitz Txiki (Gipuzkoa) | 42.97995 | −2.09384  | 824          | MW626854                   | MW621939                   | MW622206                   | MW621302                   |
| 557189  | 2039    | Azkilar (Bizkaia)     | 43.08632 | −2.59251  | 1030         | MW626855                   | MW621940                   | MW622207                   | MW621303                   |
| 557198  | no number | Fuente de Estragueña (Cantabria) | 43.2997  | −4.6066   | -            | -                          | -                          | -                          | MW622208                   | MW621304                   |
| 557200  | 854     | San Juan-9 (Bizkaia)  | 43.28078 | −3.1001   | 637          | MW626856                   | MW621941                   | MW622209                   | -                          |
| 557207  | 3861    | Azkonar Zulueta (Gipuzkoa) | 42.98749 | −2.40171  | 790          | -                          | MW621942                   | -                          | MW621305                   |
| 557209  | 3904    | Iritegi (Gipuzkoa)    | 42.9798  | −2.40768  | 527          | MW626857                   | MW621943                   | MW622210                   | MW621306                   |
| 557211  | 4864    | Grazal (Bizkaia)      | 43.22227 | −3.05541  | 148          | MW626858                   | MW621944                   | MW622211                   | MW621307                   |
| 557213  | 5006    | Garcia (Burgos)       | 43.022   | −3.7057   | 935          | MW626859                   | MW621945                   | MW622212                   | MW621308                   |
| 557215  | 5091    | Arriqueras (Cantabria) | 43.07316 | −4.07478  | 660          | -                          | -                          | -                          | MW622213                   | MW621309                   |
| 557217  | 4930    | Mendikutxe (Gipuzkoa) | 43.14687 | −2.12069  | 710          | MW626860                   | MW621946                   | MW622214                   | MW621310                   |
| 557219  | 5087    | Munarrir Arrola (Bizkaia) | 43.33892 | −2.68541  | 63           | MW626861                   | MW621947                   | MW622215                   | MW621311                   |
| 557221  | 2874    | Irutxin (Navarra)     | 42.966   | −2.02518  | 1095         | -                          | -                          | -                          | MW622216                   | MW621312                   |
| 557223  | 3728    | Otxas (Bizkaia)       | 43.18599 | −2.74773  | 488          | MW626862                   | MW621948                   | MW622217                   | MW621313                   |
| 557225  | 4102    | Toyo (Cantabria)      | 43.28468 | −4.48524  | 190          | MW626863                   | MW621949                   | MW622218                   | MW621314                   |
| 557226  | 4102    | Toyo (Cantabria)      | 43.28468 | −4.48524  | 190          | MW626864                   | MW621950                   | MW622219                   | MW621315                   |
| 557227  | 4714    | Cuviñas Negras (Cantabria) | 43.25668 | −3.60984  | 250          | -                          | -                          | -                          | MW622220                   | -                          |
| 557229  | 4723    | San Juan de Socueva (Cantabria) | 43.26569 | −3.60993  | 430          | -                          | -                          | -                          | MW622221                   | MW621316                   |
| 557231  | 3078    | Valdebenci (Bizkaia)  | 43.24918 | −3.1663   | 188          | -                          | -                          | -                          | MW622222                   | MW621317                   |
| 557232  | 4017    | Princesa (Bizkaia)    | 43.2738  | −3.08898  | 620          | MW626865                   | MW621951                   | MW622223                   | MW621318                   |
| 557234  | 3371    | Lextota-2 (Navarra)   | 43.26819 | −1.55843  | 215          | MW626866                   | MW621952                   | MW622224                   | MW621319                   |
| 557236  | 3323    | Lezea (Navarra)       | 43.26853 | −1.57164  | 210          | MW626867                   | MW621953                   | MW622225                   | MW621320                   |
| 557238  | 2875    | Irutxin (Navarra)     | 42.96476 | −2.03077  | 1095         | MW626868                   | MW621954                   | MW622226                   | MW621321                   |
| 557240  | 3727    | Otxas (Bizkaia)       | 43.18165 | −2.74923  | 488          | -                          | MW621955                   | MW622227                   | MW621322                   |
| 557242  | 4057    | Cubija (Cantabria)    | 43.31812 | −3.61307  | 269          | MW626869                   | MW621956                   | MW622228                   | MW621323                   |
codon position as a second block. The non-coding regions from 16S and 5.8 S rRNA + ITS2 were defined as a single data block. The nucleotide model Gamma GTR I was used.

Partitionfinder-2.1.1 (Lanfear et al., 2016) was applied for searching optimal evolutionary models for the partitions using the corrected Akaike Information Criterion (cAIC).

Table 1 (continued)

| NMBE-No | ZUPV-No | Cave and Province | Latitude | Longitude | Altitude [m] | GenBank accession number COI | GenBank accession number 16S | GenBank accession number H3 | GenBank accession number ITS2 |
|--------|---------|------------------|----------|-----------|-------------|---------------------------|---------------------------|---------------------------|---------------------------|
| 557244 | 559     | Paules (Burgos)  | 42.94287 | −2.99363  | 840         | -                         | -                         | MW622229                 | MW621324                 |
| 557246 | 5210    | Valdemora (Asturias) | 43.47417 | −6.0556   | 310         | MW626870                 | MW621957                 | MW622230                 | MW621325                 |
| 557247 | 5302    | Valdemora (Asturias) | 43.47417 | −6.0556   | 310         | -                         | -                         | MW622231                 | MW621326                 |
| 557249 | 4915    | Llagar (Asturias) | 43.25613 | −6.08141  | 990         | MW626871                 | MW621958                 | MW622232                 | -                         |
| 557251 | 5211    | Caleru (Asturias) | 43.40077 | −5.1919   | 80          | MW626872                 | MW621959                 | MW622233                 | MW621327                 |
| 557253 | 5209    | Herreria (Asturias) | 43.39987 | −4.76584  | 45          | MW626873                 | MW621960                 | MW622234                 | MW621328                 |
| 557255 | 5208    | Busecu (Leon)   | 43.14277 | −5.03276  | 770         | MW626874                 | MW621961                 | MW622235                 | -                         |
| 559620 | 5257    | Puente Inguanzo (Asturias) | 43.31626 | −4.86252  | 225         | MW626875                 | MW621962                 | MW622236                 | MW621329                 |
| 559622 | 5258    | Zorra (Asturias) | 43.40259 | −4.88078  | -           | MW626876                 | MW621963                 | MW622237                 | MW621330                 |
| 559624 | 5203    | Iglesias (Cantabria) | 43.36562 | −3.69331  | 65          | MW626877                 | MW621964                 | MW622238                 | MW621331                 |
| 559626 | 3807    | Cesarea (Cantabria) | 43.32034 | −3.72279  | 258         | MW626878                 | MW621965                 | MW622239                 | MW621332                 |
| 559628 | 5175    | Soldados (Cantabria) | 43.29725 | −3.9894   | 370         | MW626879                 | MW621966                 | MW622240                 | MW621333                 |
| 559630 | 5171    | Buho (Cantabria)  | 43.29427 | −4.0028   | 420         | MW626880                 | MW621967                 | MW622241                 | MW621334                 |
| 559632 | 5213    | Arrigueras (Cantabria) | 43.07284 | −4.0806  | 660         | MW626881                 | MW621968                 | MW622242                 | MW621335                 |
| 559634 | 5200    | Murcielagos (Cantabria) | 43.36159 | −3.68115  | 70          | MW626882                 | MW621969                 | MW622243                 | MW621336                 |
| 559636 | 5196    | Prementera (Cantabria) | 43.37676 | −3.75574  | 125         | -                        | MW621970                 | MW622244                 | MW621337                 |

Table 2 Primer designs used for PCR reactions

| Gene  | Primer | Sequence | Sequence length (bp) | Reference            |
|-------|--------|----------|----------------------|----------------------|
| COI   | LCO1490 | 5'-GGTCAACAATAATCATAAAGATATTTGG-3' | 680 | Folmer et al. (1994) |
|       | HCO2198 | 5'-TAAACTTCAGGGTGACAAAAAATCA-3' |           |                      |
| 16S   | 16S ar  | 5'-CGC CTG TTT ATC AAA AAC AT-3' | 440 | Simon et al. (1994)  |
|       | 16S br  | 5'-CCG GTC TGA ACT ATG-3' |           |                      |
| H3    | H3AD   | 5'-ATGGCTCGTACCAAGCAGACCGC-3' | 380 | Colgan et al. (1998) |
|       | H3BD   | 5'-ATATCCCTTGGGCAATRATRGTGAC-3' |           |                      |
| ITS2  | ITS2ModA | 5'-GCTTGCGGAGATTATATGTGAA-3' | 900 | Bouaziz-Yahiatene et al. (2017) |
|       | ITS2ModB | 5'-GGTACCTTGGTCATCGGA-3' |           |                      |
Bayesian Inference (BI) was performed using Mr. Bayes v3.2.6×64 (Altekar et al., 2004; Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) through the HPC cluster from the University of Bern (http://www.id.unibe.ch/hpc). For the concatenated data set, Partitionfinder-2.1.1 was used for finding the optimal evolutionary models for each subset with the model = all function. The Monte Carlo Markov Chain (MCMC) parameter was set as follows: starting with four chains and four separate runs for 20 million generations with a tree sampling frequency of 1000 and a burn in of 25%.

Species delimitation

The species delimitation method, Automatic Barcoding Gap Discovery (ABGD) was applied via the web browser (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html). The ABGD method (Puillandre et al., 2012) sorts the sequences into hypothetical species based on the barcode gap. ABGD was conducted using the COI alignment of the investigated Iberozospeum specimens. The input variables were chosen according to Weigand et al. (2013) and Inábnit et al. (2019). The parameters were set as follows: Pmin = 0.001; Pmax = 0.1; Steps = 10; X = 1; Nb bins = 20; and distance: Jukes-Cantor.

Morphological analyses using scanning electron microscopy (SEM)

Radulae of the eastern Alpine and Dinaride Zospeum used in the comparative analyses are presented in Inábnit et al. (2019). For reasons of space, only images of the radular ribbons of Z. exiguum and Z. pretneri are presented here (Fig. 14). Topotypic I. vasconicum and I. zaldivarae were collected by AJ for radular investigation. Individuals from the westernmost sampled caves (Asturias Province), derived from the collection of Jos Notenboom, housed at the Naturalis Biodiversity Center (Leiden, NL) using the JEOL JSM-6480LV scanning electron microscope. Aluminium stubs were coated with gold–palladium using the Polaron Equipment LTD-E5100 SEM auto-coating sputter system. Shells of three potential new species, awaiting further investigation beyond our purposes here, derive from the J. Notenboom Collection: RMNH.MOL.234104 Cueva del Comediante; RMNH. MOL.234141 Cueva a Sul; and RMNH.MOL.234120, Cueva Refugio, Basinagré, Truegos. The former two shells were preserved in 75% ethanol by the collector (Notenboom & Meijers, 1985), and, thus, finer morphological structure is consequently eroded in these shells.

Histological sectioning and light microscopy follow Jochum et al. (2015b). Three individuals used in the comparative morphology include Z. isselianum (AJC 2287), Turjeva jama, Slovenia (46.2435, 13.5046); Z. spelaeum (AJC 848), Betalov Spodmol cave, Slovenia (45.7922, 14.1877); and I. vasconicum (AJC 1848), Cueva de la Ermita de San-Saldai (42.9994, 2.4381).

Results

The maximum likelihood and Bayesian inference tree (Fig. 2) shows the concatenated data set (COI, 16S, H3 and 5.8 S rRNA + ITS2) of 67 specimens of species from the west European radiation from 55 caves and seven Dinaride outgroup taxa. The dataset was supplemented with genetic data from Weigand et al. (2013) of topotypic specimens of I. vasconicum (No. 151 in Weigand et al., 2013), I. zaldivarae (No. 162 and 163 in Weigand et al., 2013) and I. schaufussi (designated as I. suarezi, No. 140 in Weigand et al., 2013).

The tree was rooted by two species of Carychium from the collection of AJ (in the NMBe). The topology reveals nine larger clades, which follow a geographic pattern (cf. Fig. 1).

The basic node separates Dinaride Zospeum taxa from taxa inhabiting northern Spanish caves. This node has a full ML and BI support and justifies the designation of the western Zospeum radiation to a separate new genus, Ibero-zospeum n. gen. The p-distances which show the numbers of base differences per site between sequences were calculated (Kumar et al., 2018). The mean p-distance from Iberozospeum n. gen. and Zospeum is 0.0767 (Table S1 in the supplementary material). The mean p-distance from Zospeum and Carychium is 0.1285. The mean p-distance of Ibero-zospeum n. gen. and Carychium is 0.1177.
In order to study the effect of incomplete genetic data, a reduced set comprising 42 individuals was computed (Fig. 3). Here, only specimens were used with a complete record of all four markers. The bootstrap support values and the Bayesian posterior probabilities are over all higher in the tree with complete marker sets (Fig. 3).

The *bisciainse-zaldivarae*-clade (grey clade in Figs. 2 and 3) harbours congeners from two different caves (Otxas 527400 and Leoxa 527230).
Molecular investigation and description of *Iberozospeum* n. gen., including the description of *Iberozospeum* and *Irutxin* which cluster with specimens of *zaldivarae* from the type locality (No. 162 and 163 in Weigand et al., 2013). The caves, Otxas and Irutxin, are about 100 km apart from each other and are non-contiguous. Congeners from these two caves are also found in the low-resolution vasconicum-clade 1 (pink clade in Figs. 2 and 3) and vasconicum-clade 2 (light blue clade in Figs. 2 and 3). From the external perspective, the investigated specimen (NMBE 557240) in the grey clade shows a typical *biscaiense* morph (Figs. 4a). It is found in the cave Otxas, which is the type locality of *I. biscaiense*. The specimen from the cave Irutxin (NMBE 557238) displays typical character states of *zaldivarae*. The specimens from the bellesi-clade (dark blue clade in Figs. 2 and 3) were collected in the caves Lezea and Lexotoa at the border of Spain and France. The caves are 1 km apart from each other and probably contiguous. This node is supported in our investigation (posterior probability of 0.97 and bootstrap value of 85 in Fig. 2, respectively, 0.94 and 80 in Fig. 3). In both phylogenetic trees (Fig. 2 and 3), the two investigated specimens have a bootstrap value of 100. The p-distance is 0.0039. From a morphological perspective, they have a similarly high-spired, conical shell form, but the aperture is clearly different. The aperture of the specimen from Lexotoa (NMBE 557234) (Fig. 5b) is oblique and has a moderately angular parietal shield. On the other hand, the parietal shield of the specimen from Lezea (NMBE 557236) (Fig. 5a) is compact and consists of thick callus, which is seemingly fused onto the body whorl. The peristome of the Lezea specimen is thick and roundish in form.

The Basque clade (Figs. 6 and 7) splits into two: a narrow, more centrally distributed vasconicum-clade 1 (pink clade in Figs. 2 and 3; Fig. 6) and a broader, western-reaching distribution comprising the vasconicum-clade 2 (light blue clade in Figs. 2 and 3; Fig. 7). The node of the split of these two clades has a low support (bootstrap value of 29 in Fig. 2, respectively 73 in Fig. 3). The split of these two clades is...
not supported by the Bayesian posterior probability. The
topotypic specimen of *I. vasconicum* (No. 151 in Weigand
et al., 2013) clusters within the pink clade. Morphologically,
shells from caves clustering in the pink *vasconicum*-clade 1
(Fig. 6) showed a commensurate similarity in shell shape
and apertural configuration with noticeable differences in
spire height in shells from Basotxo cave (Ton de Winter,
unpubl. data 2015). The two westernmost specimens NMBe
557138 and NMBe 557242 (cf. Fig. 1 and Fig. 7b, j) in the
light blue *vasconicum*-clade 2 differ morphologically from
the remaining *vasconicum*-like specimens in the clade.

The *vasconicum*-clade 3 (brown clade in Figs. 2 and 3)
has high posterior probability and bootstrap support only in
the terminal nodes. The deep nodes in the brown clade are
not supported in the ML analysis (bootstrap support of 53,
respectively, 57 in Fig. 3). Morphologically, they resemble
typical *I. vasconicum* due to shell shape and the apertural
configuration. The specimen in Fig. 8a is about 1.5 times
bigger than the smallest specimen from the brown clade,
but the shell shape is very similar to the other specimens in
this clade. Figure 8g is remarkable due to its broad conical
form, the deeply pronounced suture and the obvious, broadly
deepened umbilicus. We consider it a new species.

Support values in the *schaufussi*-clade (red clade in
Figs. 2 and 3) are high. In Fig. 9a, a specimen from the
cave Búho, Puente Viesgo, is illustrated, which is the
type locality of *I. suarezi* (Gittenberger, 1980). How-
ever, comparing our specimen with the holotype of *I.

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**Fig. 4** Grey clade; (a) *Iberozospeum bicaense* NMBe 557240, Durang, Igorre, Cueva Otxas, 23.3.2016, sh: 1.37 mm; (b) *Iberozospeum zaldi-varae* NMBe 557238, Aralar, Arbizu, Cueva Irutxin, 20.6.2015, sh: 1.59 mm. — All phot. ×40

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**Fig. 5** *Iberozospeum bellesi*. Dark blue clade; (a) NMBe 557236, Zugarram, Sare, Lezea, 13.12.2015, sh: 1.67 mm. (b) NMBe 557234, Zugarramurdi, Lexotoa-2, 20.12.2015, sh: 1.38 mm. — All phot. ×40
Molecular investigation and description of *Iberozospeum* n. gen., including the description...

Fig. 6 *Iberozospeum vasconicum*. Pink clade; (a) NMBE 557207, Aizkorri, Onati, Azkonar Zulueta, 28.5.2016, sh: 1.31 mm; (b) NMBE 557160, Aizkorri, Eskortatza, Urkoba, 8.3.2017, sh: 1.18 mm; (c) NMBE 557217, Ernio, Tolosa, Mendikute, 23.9.2017, sh: 1.5 mm; (d) NMBE 557219, Bust-Lea, Forua, Munarri Arrolla, 9.12.2017, sh: 1.56 mm; (e) NMBE 557209, Aizkorri, Onati, Cueva Iritegi, 28.5.2016, sh: 1.43 mm; (f) NMBE 557150, Aizkorri, Onati, Txomenkoba Goikoa, 16.12.2015, sh: 1.27 mm; (g) NMBE 557156, Udalaitz, Elorrio, Cueva Artegi, 17.5.2016, sh: 1.48 mm; (h) NMBE 557168, Aizkorri, Onati, Arlaban, 11.2017, sh: 1.27 mm; (i) NMBE 557154, Udalaitz, Aretxabaleta, Cueva Penpelin, 26.4.2016, sh: 1.34 mm; (j) NMBE 557166, Aizkorri, Onati, Aizkirri, 11.2017, sh: 1.59 mm; (k) NMBE 557223, Durang, Igorre, Otxas, 23.3.2016, sh: 1.24 mm; (l) NMBE 557152, Udalaitz, Arrasate, San Valerio (Galarra), 23.3.2016, sh: 1.25 mm. No picture of NMBE 540554.4, 540555.2, 540556.1 (shell destroyed). — All phot. × 40
suarezi (RMNH.MOL.55383) (Jochum et al., 2019: 72, Fig. 4a–c), it is evident that this is the same species. The specimens from the two Cantabrian clades have a more elliptical aperture compared with the vasconicum-clade specimens (pink, light blue and brown clades in Figs. 2, 3, 6, 7, and 8).

The Asturian clade splits into two sub-clades (purple and green clades in Figs. 2 and 3). Support values within these clades are moderate to high. Weigand et al. (2013: Fig. 5) sequenced two specimens from the Cueva del Bosque/Cueva Inganzo, which cluster within the purple clade (Figs. 2 and 3).

Moreover, Weigand et al. (2013) identified these two specimens as I. suarezi and considered them “evolutionary lineage Z14” (No. 140 in Weigand et al., 2013 in our trees). Jochum et al. (2019: 83–84, Fig. 15) explained that this cave is situated 1 km opposite of the cave, Cueva del Puente de Inguanzo, which is the cave Gittenberger (1980) considered to be the species I. suarezi. These two specimens originate from the same population in El Toyo in Cantabria; the two shells are morphologically quite different, illustrating shell variability within the lineage. The shell of the specimen from the Picos (Fig. 10k) is broadly conical, with a remarkably narrow-stepped coiling pattern of the teleoconch whorls and a large elliptical aperture. The group comprising Fig. 10l–n, forms another lineage in the green clade, which is characterised by broad conical shells resembling the specimen from the Picos, but with high and well-rounded teleoconch whorls.

The yellow clade (Figs. 2, 3, and 11) contains ribbed specimens with varying degrees of superficial striation. Although the specimen in Fig. 11a has less-pronounced ribs, they are clearly visible in apical view. This shell bears a deep suture and its aperture is elliptical and reverted. The specimen in Fig. 11b is a juvenile but the ribs are clearly visible. Although the shell morphology is different, it clusters together with specimen 11c, with a bootstrap support of 95% (Fig. 2). The p-distance of these individuals is 0 since only the nuclear marker H3 could be sequenced for both specimens and this sequence is identical. On the other hand, the strongly ribbed specimen in Fig. 10c constitutes the new species, Iberozospeum costalatum n. sp.

The ABGD assigned the 45 Iberozospeum COI sequences into 22 groups. The Barcode gap distance is 0.012. In Table 3 are the groupings of the ABGD analysis. Since we do not have the COI sequences of all investigated animals, not all specimens could be assigned to a group. The two animals from the same population from Toyo Cave (NMBE 557225 and 557226) were divided into different groups. Using ABGD, all animals from the pink vasconicum-clade were classified into group 1. Group 2 consists exclusively of animals from the light blue vasconicum-clade. Some animals from the light blue vasconicum-clade were classified into other groups (Table 3). The two animals from the scharfussi-clade (NMBE 559628 and 559630) were placed in group 6. The specimens from the praetermissum-clade were placed in group 7. The two specimens from the bellesi-clade (NMBE 557234 and 557236) were assigned in group 15. The remaining groups each contain only one animal.
Morphology

Although only one member of Iberozospeum is histologically sectioned here, sections of the upper visceral complex of toptype I. vasconicum (AJC 1848) show that the anatomy follows the general carychiid design (Morton, 1955; Harry, 1997–1998) and that described for Zospeum in Maier (1982), Dörge (2010), and Jochum et al. (2015b) as well as in Barker (2001) for aspects of the Ellobiidae (Fig. 12). The cross-sections reveal no phylogenetic differences but rather, seasonal differences in the specific individuals. The I. vasconicum individual, collected in June 2011, is aphalette. Euphally was detected by Jochum et al. (2015b) in Z. amoenum (von Frauenfeld, 1856) (species revised from “Zospeum sp.” in Inäbnit et al., 2019), collected in August 2008 from Konečka zjalka, Slovenia. The histological sections of the upper visceral complex of individuals of toptype Z. isselianum (Pollonera, 1887) (AJC 2287) and Z. spelaeum (Rossmässler, 1839) (AJC 848) from the Dinarides (Fig. 12a–b) in this work also show that these snails were aphalette during time of collection in June and October, respectively. The same upper visceral region of the snail (cuts 14–22) in our histological sections (Fig. 12) shows different perspectives due to the generally larger size of the two Dinaride individuals in this study. Visible in clockwise rotation from the outside in, Z. isselianum shows the mantle gland (mag), the cerebral ganglion (cg), the mantle cavity (mc), the pleural ganglion (pg), the foot (f), the pharynx (ph), the oesophagus (oe), the statocyst containing otoliths and the columellar muscle (cm) (Fig. 12a). For Z. spelaeum, the same clockwise perspective shows the mantle gland (mag), the foot (f), some haemolymph (hl), the pharynx (ph), the oesophagus (oe), a hump shaped upper, non-vascularized columellar muscle (cm) and a moderately long genital opening (go) (Fig. 12b). For I. vasconicum, a remarkably huge, glandular albumen gland (ag) is clearly

Fig. 8 Iberozospeum vasconicum. Brown clade; (a) NMBE 559634, Cantabria, Entrambasaguas, Murcielagos, 28.3.2018, sh: 1.56 mm; (b) NMBE 559624, Cantabria, Entrambasaguas, Iglesia, 28.3.2018, sh: 1.14 mm; (c) NMBE 557215, Cantabria, Santurde de Reinoso, Las Arriguera, 20.1.2018, sh: 1.39 mm; (d) NMBE 559632, ditto, 29.3.2018, sh: 1.36 mm; (e) NMBE 557213, Guareña, Merindad de Sotoscueva, Garcia, 26.11.2017, sh: 1.23 mm; (f) NMBE 557146, Miera, Soba, Las Montosas, 20.2.2016, sh: 1.23 mm; (g) NMBE 557229, Asón, Arredondo, San Juan de Socueva, 12.4.2017, sh: 1.41 mm; (h) NMBE 559636, Cantabria, Entrambasaguas, Prementera, 28.3.2018, sh: 1.3 mm. — All phot. ×40
visible (Fig. 12c). This is a compound, sac-like enlargement of the oviduct and is responsible for producing albumen for the formation of the eggs (Luchtel et al., 1997). The albumen gland is a female accessory sex gland, which enlarges with sexual maturation of the animal. The size of this gland is considered to determine the maximum number of eggs that can be produced at any one time (Barker, 2001). Due to the presence and large size of the albumen gland (indicative of the female phase) here, modification in existing structures and superposition of others on the fundamental carychiid pattern is clearly the case. Also, strikingly apparent is the large, well-developed, mucous gland (stained reddish violet) (mg). Additionally, visible in the clockwise perspective of the upper visceral complex of *I. vasconicum* is the kidney (k) with a prominent renopericardial passage and the heart (h) located at the base of the kidney, haemolymph (hl) at the foot section (f), the contractile pneumostome (p), the mantle gland (mag), the pharynx (ph) and oesophagus (oe), the vascularized, 2-humped columellar muscle (cm) and the long genital opening (go) (Fig. 12c). We remark that deeper analysis of anatomical aspects and individual structural analyses of *Iberozospeum* is beyond the scope of this paper and will be presented in a future work.

Significant however, for *Iberozospeum* n. gen., is that the columellar muscle shows prominent humps, which contain vasculature extending to the tips of these humps (Fig. 12c). In our investigation of the columellar lamellae of *Iberozospeum*, the dense, scaley crystallographic structure on localised zones of the lamellae correlates perfectly with the corresponding points of contact of these two hump-like elevations of the columellar muscle (Fig. 13). In sync with this observation, Barker (2001) emphasized there is a trend amongst the Ellobiidae that the columellar muscle becomes detached from the body wall from its origin on the columella and has become largely free in the haemocoel. The columellar muscle, thus, runs forward to attach on the cephalic organs and anterior body wall (Barker, 2001). In this case, rather than just simply retracting, the cephalopedal mass becomes inverted when the animal retracts into the shell (Barker, 2001). In *Iberozospeum*, the columellar muscle is indeed detached except at these two points of contact. In the Alpine and Dinaride species of *Zospeum*, crystallographic structure is not overlapping and not restricted to specific points. Moreover, it is comprised of low shoals of crystallographic structure (seen also in fossil Carychiidae in Jochum et al., 2015c, Fig. 5), randomly interspersed over the columellar lamellae deep in the shells of *Z. spelaeum* from two separate caves in Slovenia (MCBI CSR SASA 37049a, Velika Pasica and AJC 847, Betalov Spodmol Grotte) (Fig. 13a–b). Remarkable are the dense, localised, overlapping wedges of crystallographic structure on the complementary points of the columellar lamellae in the empty shells of the Iberian taxa: *Iberozospeum* sp. RMNH.MOL.234120 (Cueva Refugio, Trucios) (Fig. 13c–d), on the upper part of the lamella of *Iberozospeum* sp. RMNH.MOL.234104 (Cueva del Comediante) and on the lamella of *Iberozospeum* sp. RMNH.MOL.234141 (Cueva a Sul, Oviedo) (Fig. 13e–f) as well as on that of *I. vasconicum* (AJC 1849, Cueva Arríkrutz) (Fig. 13g–h). The lower lamella of *I. sp. RMNH. MOL.234120* (Cueva Refugio, Trucios) clearly shows the specific locality of the contact point of the columellar muscle (Fig. 13c–d). Shells of RMNH.MOL.234104 (Cueva del Comediante) and RMNH.MOL.234141 (Cueva a Sul, Oviedo) (Fig. 13e–f), which were initially preserved in 75% ethanol, still show the characteristic, rough crystallographic structure despite deterioration by the ethyl-alcohol treatment subjected to them by the collector (Notenboom & Meijers, 1985).

Our morphological investigations also included the radulae of four Dinaride and eastern Alpine taxa described and imaged in Inäbnit et al. (2019, Supplementary Figs. S17–21) including *Z. exiguum* (von Frauenfeld, 1854) (NMBE 553409) (Inäbnit et al., 2019, Fig. S17a–d); *Z. obesum* (von Frauenfeld, 1854) (NMBE 553384) (Inäbnit et al., 2019, Fig. S17a–d); *Z. pretneri* (Bole, 1960) (NMBE 553290) (Inäbnit et al., 2019, Fig. S19a–d); and *Z. spelaeum* (Rossmässler, 1839) (NMBE 553311) (Inäbnit et al., 2019, Fig. S20e–f). These were compared with radulae extracted from individuals collected from the westernmost-sampled caves by Oviedo (Asturias) and radulae from topotypic material of *Z. vasconicum* (Pietro et al., 2015 in Jochum et al., 2015a) (AJC 1847) and *Z. zaldivarae* (Pietro et al., 2015 in Jochum et al., 2015a) (AJC 1876).

In this comparative study, the narrow radular ribbons of Dinaride *Zospeum* are tapered to an obtuse or to a straight base as in *Z. exiguum* (Fig. 14a) or a straight base as in *Z. pretneri* (Fig. 14b). The Iberian radulae have a tapered
anterior end (velum), followed by a well-defined adhesive zone leading to a remarkably straight, carpet-like swath of longitudinal rows of smaller teeth and more of them per transverse row (Figs. 14c–h, 15c). The rachidian and lateral teeth of I. vasconicum demonstrate remarkable similarity to those of Carychium ibazoricum Bank & Gittenberger, 1985, imaged in Martins (2007, Fig. 138) (Fig. 15e). The rachidian and lateral teeth of the radula of totypic I. vasconicum (AJC 1848) show long endo- and ectocones flanking the mesocone by one half–three fourths the length of the mesocone (Fig. 15e). The radula of totypic Z. pretneri (NMBE 553290), I. vasconicum’s externally most similar phenotypic relative from the Dinarides, shows endo- and ectocones that are one third–one half the length of the mesocone (Fig. 15a). Medial grooves are visible on the mesocones of Z. pretneri and Z. isselianum (AJC 874, Turjeva jama, Slovenia) here (Fig. 15a–b) as well as in a number of other eastern Alpine and Dinaride species (see Inäbnit et al., 2019, Supplementary Figs. S17–21). The basal plates are generally more compact and shorter in the radulae of Iberian taxa (Figs. 15c–e, h) versus the longer and thinner versions of those of the eastern Alpine and Dinaride Zospeum species (Fig. 15a–b) (see also Inäbnit et al., 2019, Supplementary Figs. S17–21). Basal plates maintain the spacing of teeth and support them in the feeding process (Luchtel et al., 1997). The radular ribbon of totypic I. zaldivarae (AJC 1876) shows a rachidian tooth with a long pointed mesocone and very short endo- and ectocones that are about one fourth the length of the mesocone (Fig. 15g). The lateral teeth bear very short mesocones flanked by long, fang-like endo- and
ectocones on compact basal plates. The size and shape of teeth vary from row to row (Fig. 15h). As it is known for many ellobiids, the transition from the lateral teeth to the pectinate marginal teeth is abrupt (Fig. 15e) or gradual via a few intermediate, transitional teeth (Fig. 15c, h) (Martins, 1996, 2007). Within the central longitudinal rows of the radulae, the rachidian teeth of the individuals from Asturias (Fig. 15c–d) and Burgos (Fig. 15f) show a long mesocone.

### Table 3 Results of the species delimitation method ABGD

| NMBE-No | ZUPV-No | Cave and Province          | ABGD group | clade         |
|---------|---------|-----------------------------|------------|---------------|
| 557150  | 3405    | Txomenkoba (Gipuzkoa)       | 1          | vasconicum (pink) |
| 557152  | 3758    | San Valerio (Gipuzkoa)      | 1          | vasconicum (pink) |
| 557154  | 3849    | Penpelin (Gipuzkoa)         | 1          | vasconicum (pink) |
| 557156  | 3856    | Artegi (Bizkaia)            | 1          | vasconicum (pink) |
| 557166  | 4990    | Aizkiri (Gipuzkoa)          | 1          | vasconicum (pink) |
| 557168  | 5005    | Arlaban (Gipuzkoa)          | 1          | vasconicum (pink) |
| 557209  | 3904    | Iritegi (Gipuzkoa)          | 1          | vasconicum (pink) |
| 557217  | 4930    | Mendikute (Gipuzkoa)        | 1          | vasconicum (pink) |
| 557219  | 5087    | Munarri Arrola (Bizkaia)    | 1          | vasconicum (pink) |
| 557223  | 3728    | Otxas (Bizkaia)             | 1          | vasconicum (pink) |
| 540551_1| 1847    | Akaitz Txiki (Gipuzkoa)     | 2          | vasconicum (light blue) |
| 540552_1| 2039    | Azzilar (Bizkaia)           | 2          | vasconicum (light blue) |
| 557178  | 1607    | Paules (Burgos)             | 2          | vasconicum (light blue) |
| 557187  | 1847    | Akaitz Txiki (Gipuzkoa)     | 2          | vasconicum (light blue) |
| 557188  | 1847    | Akaitz Txiki (Gipuzkoa)     | 2          | vasconicum (light blue) |
| 559632  | 5213    | Arrugueras (Cantabria)      | 3          | vasconicum (brown) |
| 557189  | 2039    | Azzilar (Bizkaia)           | 4          | vasconicum (light blue) |
| 557138  | 4702    | Baja (Cantabria)            | 5          | vasconicum (light blue) |
| 559628  | 5175    | Soldados (Cantabria)        | 6          | schauffusi (red) |
| 559630  | 5171    | Buho (Cantabria)            | 6          | schauffusi (red) |
| 557140  | 4862    | Collubina (Asturias)        | 7          | praetermissum (purple) |
| 557246  | 5210    | Valdemora (Asturias)        | 7          | praetermissum (purple) |
| 557253  | 5209    | Herreria (Asturias)         | 7          | praetermissum (purple) |
| 557255  | 5208    | Baeuco (Leon)               | 7          | praetermissum (purple) |
| 559620  | 5257    | Puente Ingoguazu (Asturias) | 7          | praetermissum (purple) |
| 557249  | 4915    | Llagar (Asturias)           | 8          | spp. (green) |
| 557251  | 5211    | Caleru (Asturias)           | 8          | spp. (green) |
| 559626  | 3807    | Cesareo (Cantabria)         | 9          | costulatum (yellow) |
| 557242  | 4057    | Cubija (Cantabria)          | 10         | vasconicum (light blue) |
| 557213  | 5006    | Garcia (Burgos)             | 11         | vasconicum (brown) |
| 557200  | 854     | San Juan-9 (Bizkaia)        | 12         | vasconicum (light blue) |
| 557211  | 4864    | Grazal (Bizkaia)            | 12         | vasconicum (light blue) |
| 559624  | 5203    | Iglesias (Cantabria)        | 13         | vasconicum (brown) |
| 559634  | 5200    | Murcielegos (Cantabria)     | 13         | vasconicum (brown) |
| 557238  | 2875    | Irukin (Navarra)            | 14         | biscaiense (grey) |
| 557234  | 3371    | Lexotoa-2 (Navarra)         | 15         | bellesi (dark blue) |
| 557236  | 3323    | Lezea (Navarra)             | 15         | bellesi (dark blue) |
| 557146  | 3820    | Montosas (Cantabria)        | 16         | vasconicum (brown) |
| 557232  | 4017    | Princesa (Bizkaia)          | 17         | vasconicum (light blue) |
| 557158  | 4601    | Saiturri-2 (Gipuzkoa)       | 18         | vasconicum (light blue) |
| 557225  | 4102    | Toyo (Cantabria)            | 19         | spp. (green) |
| 557226  | 4102    | Toyo (Cantabria)            | 20         | spp. (green) |
| 557144  | 4136    | Udias (Cantabria)           | 21         | praetermissum (purple) |
| 559622  | 5258    | Zurra (Asturias)            | 22         | spp. (green) |
flanked by endo- and ectocones of varying lengths from short to long. A hint of a median groove is present on some of the lopsided transitional teeth of *I. zaldivarae* (AJC 1876) (Fig. 15h). In the transverse rows, there is an increased incidence of bi-cuspid, fang-like lateral teeth (i.e. RMNH.MOL. 234,116, Cueva a Sul, Oviedo) flanking both sides of the radicular tooth (Figs. 15c–d). As is considered for pulmonates (Luchtel et al., 1997), the teeth show constant form in the longitudinal rows but vary considerably in the transverse direction on the radular ribbon.

Individuals of *I. vasconicum* are generally smaller (Jochum et al., 2015a) than the Alpine and Dinaride species of *Zospeum*, except those comprising the *Z. pretneri* clade, which are about the same size and sometimes slightly larger (Inäbnit et al., 2019).

**Discussion**

In the molecular assessment, the topology generally did not change in the concatenated tree with all investigated specimens (Fig. 2) nor in the concatenated tree with complete marker sets (Fig. 3). In the latter, the support values are slightly higher and corroborate our interpretation of the phylogenetic and morphological findings. In order to understand the morphological traits in *Iberozospeum* n. gen., a figure is added here showing the type specimens of all hitherto described species (Fig. 16).

The *biscaiense-zaldivarae*-clade (grey clade in Figs. 2 and 3) contains sequences of two specimens (No. 162 and 163) from Weigand et al. (2013), who identified these...
specimens as lineage Z18. In 2015, Jochum et al. described the lineage Z18 as \textit{I. zaldivarae}. The sequences of the two specimens of \textit{I. zaldivarae} (Weigand et al., 2013) cluster with one specimen from the cave Iruxtu. The cave, Otxas, is the type locality of \textit{I. bicasiense} (Gomez & Prieto, 1983). We consider both species and supported entities; morphologically, the species differ by the mode of shell coiling (tighter in \textit{I. bicasiense} when compared with \textit{I. zaldivarae}) and presence of a small palatal denticle in \textit{I. bicasiense}, which is lacking in \textit{I. zaldivarae}.

The bellesi-clade (dark blue clade in Fig. 2) is not supported, although the complete marker set for both investigated specimens could be sequenced (dark blue clade in Fig. 3). A possible explanation for this finding is that \textit{I. bellesi} belongs to a young radiation. The species is morphologically separated from the other known species in the Pyrenees, but our selected genetic markers cannot reveal the genetic differentiation of the species. The known distribution of \textit{I. bellesi} spans the western Pyrenean region, including the French Basque Country and the Navarrese Pyrenees with the Sare-Zugarramuri massif constituting an isolated enclave (Prieto & Zuazu, 2018). While \textit{I. bellesi} is a unique Pyrenean species, all other known \textit{Iberozospeum} taxa derive from the Basque-Cantabrian Mountains. Genetically, \textit{I. bellesi} is clearly separated from the \textit{bicasiense-zaldivarae}-clade but close to the other known species in Spain. Maybe other genetic markers are needed to reveal the genetic differentiation of \textit{I. bellesi} from the other known species in Spain.

Within the Basque clades (pink and light blue clades in Figs. 2 and 3), the bootstrap support values and Bayesian posterior probabilities are low. This could be due to some missing markers in the concatenated tree (Fig. 2), because in the maximum likelihood tree, with complete marker sets (Fig. 3), the support values in the pink and light blue clades are much higher. The pink clade forms a polytomy with low support values. A possible explanation for this finding could be that these individuals form a young radiation. The selected genetic markers used in this study could not reveal the genetic differentiation of the specimens. The individuals in the light blue clade are genetically more differentiated than in the pink clade, but, bootstrap support values are also low. We included two specimens, NMBe 557178 and NMBe 557244 (Fig. 7k and m), which syntopically inhabit Las Paúles cave with \textit{I. zaldivarae}. Both specimens cluster within the vasconicum-clade 2 (light blue clade in Fig. 2 and 3). On the morphological front, they resemble individuals in Fig. 7b and j.

The brown clade is the sister clade to the two vasconicum-clades (pink and light blue in Figs. 2 and 3) with high support (posterior probability of 1 and bootstrap value of 86 in Fig. 3). The support values in the deep nodes are low, maybe due to missing mitochondrial markers for some specimens. Due to genetic and morphological similarities to the vasconicum-clades 1 and 2 (Figs. 2, 3, 6, and 7), the specimens in the brown clade are considered to be \textit{I. vasconicum}. We remain conservative with this clade because the subclades are not well supported. However, the type population of \textit{I. vasconicum} (Weigand et al., 2013) is included in the pink subclade. For this reason, we consider all three subclades as \textit{I. vasconicum}. Additional molecular methods like Next Generation Sequencing (NGS) are needed to resolve this group properly.

The schaufussi-clade (red clade in Figs. 2 and 3) contains a specimen from Cueva del Buho, which is the type locality for \textit{I. suarezi} (Gittenberger, 1980). The investigated specimens from the red clade have an elongate-conical shell, moderately tightly coiled whors and a roundish-lunate aperture typical for \textit{I. schaufussi} (Jochum et al., 2019).

The purple clade (Figs. 2 and 3) contains the specimen No. 140 from Weigand et al. (2013), which was described by Jochum et al. (2019) as \textit{I. praetermissum}. Morphologically, the individuals in the purple clade (Fig. 10a–f) show a uniform shell shape, except the specimen in Fig. 10f, which is a juvenile. The individual in Fig. 10d contains a clearly visible parietal tooth, which is typical for \textit{I. praetermissum} and differentiates this species from \textit{I. gittenbergeri} (Jochum et al., 2019). The individual in Fig. 10f cannot be clearly assigned to a species since the aperture is not fully grown. We remark, however, that this individual was found at the type locality of \textit{I. praetermissum} (Jochum et al., 2019). The genetic analyses revealed that it clearly belongs to the praetermissum-clade even if its morphology is not typical for \textit{I. praetermissum}, leading us to suggest that a certain spectrum of morphological variability within \textit{I. praetermissum} is the case.

The lineages within the green clade (Figs. 2 and 3) have moderate to high support values but differ morphologically. Each lineage is represented by a different morph.
The yellow clade (Figs. 2 and 3) is genetically clearly separated from the remaining clades, albeit that the complete marker set could be sequenced in only one specimen (Table 1 and Fig. 3). Morphologically, the three investigated individuals are completely different compared with the other known taxa in Spain. The specimen in Fig. 11b is a juvenile but resembles the specimen in Fig. 11c. Both specimens contain strong ribs. A new, ribbed species is described from this clade.

The caves of northern Spain show a high incidence of multiple species sympatry in contrast to those reported for the eastern Alpine and Dinaride regions (Inäbnit et al., 2019). In context, we remark here that Inäbnit et al.'s (2019) molecular investigation showed little congruency with Bole's (1974) earlier morphological interpretation of the eastern Alpine and Dinaride taxa, revealing that the incidence of multiple sympatric species is the exception, and not the rule in Dinaride caves. In Table 4, all investigated sympatric Iberozospeum species are listed.

The radulae of Iberian taxa show significant differences in radular ribbon form and dental morphology from those of eastern Alpine and Dinaride Zospeum. These differences reflect both odontophore and ribbon constitution in how it flattens out on the dorsal and lateral sections of the muscular odontophore. The ellobiid radula changes with age, with the very young specimens usually showing strongly denticulate crowns (Martins, 2007). Inäbnit et al. (2019) described four ribbon morphologies in eastern Alpine and Dinaride taxa, including those with an attenuated triangular base (Z. isselianum, Z. frauenfeldii) and those tapered to an obtuse to straight base as in Z. exiguum (Fig. 14a) or a straight base as in Z. pretneri (Fig. 14b) here. The form and composition of radular teeth vary according to their position on the radular membrane (referred in prepared SEM form here as ribbon) as well as diet, mineral composition of the environment, temperature and other factors (Luchtel et al., 1997). Within the ellobiids, Martins (1996, 2007) considered radular morphology a useful, distinguishing character at the generic level. For Iberozospeum here, the radular ribbons are clearly distinguishable from those of the Dinaride taxa. They are long and broad in form and very straight below the zone of the adhesive layer. They also bear more and smaller teeth per transverse row (Fig. 14c–h). Like those in their eastern Alpine and Dinaride relatives, they have a tapered anterior end (velum) above the adhesive layer. However, while the narrower ribbons of the latter taxa are tapered and sometimes straight at the base (i.e. Z. pretneri), all those of Iberozospeum are so far completely straight at the base, showing a flatter surface area aligned with very straight rows of teeth. We emphasize that this situation is
enhanced by the tendency towards increased length (i.e. double the ribbon length below the adhesive zone in those of the Pyrenean-Cantabrian individuals), the further westward (i.e. Asturias) the individuals were found, albeit the exception found in Burgos (RMNH.MOL.234108) (Fig. 14h). The radula of tootypic I. vasconicum (Fig. 14c) from the more eastern province of Gipuzkoa, for example, shows a moderately long ribbon in comparison to the exceptionally long ribbons extracted from individuals from Asturias and the one from Burgos. Moreover, since the radula is tensioned and moved over the muscular odontophore by muscles that derive from the buccal mass, radular ribbon form is a reflection of these muscles operating the odontophore and radula in concert. In addition, since the odontophore is moved by muscles extending from the body wall and the colleanor muscle (in shelled pulmonates) (Luchtel et al., 1997), it is apparent here that Iberozospeum has a different muscle constitution (i.e. muscles and modified muscle cells operating the odontophore, the odontophore itself and the colleanor muscle) than Zospeum. This situation is revealed here by the presence of crystallographic structure on specific points of muscle adhesion on the colleanor lamellae in shells of Iberozospeum (Fig. 13). Our observation is in sync with the trend in the Ellobiidae (Barker, 2001) that the colleanor muscle becomes detached from the body wall from its origin on the colleanor and has become largely free in the haemocoel. Moreover, the dense, localized surface structure most probably provides traction for the colleanor muscle. On a side note, but worth mentioning in context, is that during the manual preparation for SEM, ribbons from Iberozospeum mounted especially easily, like laying a carpet, onto the prepared SEM stub. Those of the eastern Alpine and Dinaride taxa, as well as from some Carychium species (AJ unpubl. data), tended to be generally more difficult to mount due to their flopping over and furling at the sides during the mounting procedure. This difference may be due to mineral composition or quality of the flexible chitin comprising the ribbon or to potential material thinness due to different or enhanced muscular action on the radular membrane in situ. In contrast to the long and broadly straight version of the Iberian taxa, the radular ribbons of the studied Alpine and Dinaride species are long and narrow and tapered on the anterior end and sometimes, on both ends (i.e. Z. isselianum and Z. frauenfeldii) (Inäbnit et al., 2019). We consider the consistency in shape, length, broadness and the straight-edged base of the radular ribbon systematically significant in Iberozospeum.

Ecologically, the westernmost Iberozospeum in our radula study were collected from caves characterised by certain aquatic biotopes (Notenboom & Meijers, 1985). These biotopes included infrequent caves (Cueva de Rales, Prov. Oviedo (now Asturias)) and temporary effluent caves (Cueva de La Foz, Cueva a Sul, Prov. Oviedo (now Asturias)), and caves with pools, puddles or gours (Cueva la Torcona, Prov. Burgos) or a combination of the latter two (Cueva la Torcona). These ecological situations play a role in diet and substrate composition as well as in the density of mud comprising Iberozospeum habitats (Jochum et al., 2012). As for the observed differences in Iberozospeum’s radular morphology, Jochum et al. (2015b) considered that the cusps of the radular teeth in Z. isselianum (Fig. 15b) interacted with substrate composition and structure (i.e. grain), causing adaptive moderations of morphological detail to evolve and correlate with substrate grain. In the case of Iberozospeum, it is probable that the longer, seemingly bi-cuspid lateral teeth of the westernmost sampled populations may well reflect environmentally induced adaptive factors in caves of the westernmost part of Iberozospeum’s range. We remark that these westernmost populations were not included in the molecular analyses comprising our study, but rather, collected by Jos Notenboom in 1983–1984 during a study of groundwater fauna in caves of northern Spain (Notenboom & Meijers, 1985).

The radiation of Iberozospeum aligns into the bigger context of Iberian geological and evolutionary considerations for which the southern peninsulas of Europe served as major refugia during the Pleistocene glaciation (Hewitt, 2004). Moreover, the Iberian Peninsula has been associated with climate stability over geologic time and is considered to be a historically climate-stable region encompassing high species diversity and endemism (Abellán & Svenning, 2014). Specifically, our study underscores the considerations of Abellán and Svenning (2014), in that the repeated range contractions and expansions experienced during the Pleistocene climatic oscillations may have resulted in the generation of multiple isolated lineages of fauna (in this case, Iberozospeum)
different refugial areas. The Cantabrian and Pyrenees Mountains, harbouring labyrinthine cave systems, well serve as current refugial areas for Iberozospeum species that may have been more widespread during colder periods during the Pleistocene. Moreover, with the Pyrenees-Cantabrian Mountains running east–west together with the main rivers flowing along a north–south axis, the following scenario can be proposed considering climatic, geological and physiographical characteristics of this geologically younger part of Spain (Notenboom & Meijers, 1985): Iberozospeum could have survived and spread via altitudinal shifts as cave systems gave way and sank into each other due to geologic processes and groundwater mechanisms, allowing the distribution of species and sympatry of species to occur over time.

**Taxonomic implications**

We found a well-supported, two-clade system in the genus Zospeum (Figs. 2 and 3) and propose here a new genus encompassing the northern Spanish radiation.

Genus **Iberozospeum** Jochum, Kneubühler, Prieto and Neubert, n. gen.

**Type species** Zospeum zaldivarae (Prieto et al., 2015 in Jochum et al., 2015a).

**Differential diagnosis** Iberozospeum, n. gen., differs from Zospeum by the generally smaller shell (on the average ca. 1.2 mm). The radula ribbon differs by its greater length,
regular broadness below the adhesive zone and its perfectly straight base. Radular teeth are smaller and more numerous per transverse row. The basal plates are more compact and shorter than the long, narrow versions in Zospeum species. At the microstructural level, it differs by localized, dense, rough, overlapping wedge-like scales of crystallographic structure on the surface of the columellar lamellae. These sites are located only at the points of contact with the columellar muscle. It also appears to differ by the correlating, vascularized humps of columellar muscle to the corresponding zones of microstructural texture on the lamellae.

**Etymology**
The name *Iberozospeum* derives from combining the region of origin of the type species, the Iberian Peninsula, and the generic name of *Zospeum* (Bourguignat, 1856).

Included taxa: all hitherto known species from the Iberian Peninsula:

- *Iberozospeum schaufussi* (von Frauenfeld, 1862).
- *Iberozospeum bellesi* (Gittenberger, 1973).
- *Iberozospeum bicaensiense* (Gómez & Prieto, 1983).
- *Iberozospeum vasconicum* (Prieto et al., 2015 in Jochum et al., 2015a).
- *Iberozospeum zaldivarae* (Prieto et al., 2015 in Jochum et al., 2015a).
- *Iberozospeum percostulatum* (Alonso et al., 2018).
- *Iberozospeum praetermissum* (Jochum et al., 2019).
- *Iberozospeum gittenbergeri* (Jochum et al., 2019).

**Iberozospeum costulatum Prieto and Jochum, n. sp.**

**Type locality** Mina del Pedreo (Bizkaia: Arcentales; 43.26800 -3.21402, 440 m).

**Holotype** A shell of 1.43 mm [MNCN 15.05/200128, ex. ZUPV 1952]; 22.02.2014, C. Prieto, A. Calvo, P. Jiménez leg. and other material.

| Table 4 | Investigated sympatric *Iberozospeum* species living in Spanish caves |
|---------|---------------------------------------------------------------|
| Cave    | Species 1                                           | Species 2                                           |
| Herrería | NMBE 557253 *praetermissum* (purple clade) | NMBE 557136 *percostulatum* (green clade) |
| Irutxin  | NMBE 557238 *bicaensiense* (grey clade)          | NMBE 557221 *vasconicum* (light blue clade)       |
| Las Paules | No. 162 and 163 Weigand 2013 *zaldivarae* (grey clade) | NMBE 557178 and NMBE 557244 *vasconicum* (light blue clade) |
| Otxas    | NMBE 557240 *bicaensiense* (grey clade)          | NMBE 557223 *vasconicum* (pink clade)              |
| Valdemora | NMBE 557246 *praetermissum* (purple clade)      | NMBE 557247 *gittenbergeri* (green clade)         |

**Paratypes** Mina del Pedreo [type locality]; 22.02.2014, C. Prieto, A. Calvo, P. Jiménez leg. [ZUPV 1952: 2 shells + 2 juvenile shells] 31.08.2014, C. Prieto, A. Calvo leg. [ZUPV 2578: 15 shells + 3 juvenile shells, ZUPV 2583: 1 shell] [MNCN 15.05/200129: 5 shells]. Cueva de Valdebeci (Bizkaia: Sopuerta: Beci; 43.24516 -3.17316, 188 m); 20.10.2015, A. Calvo leg. [ZUPV 3078: 6 shells] [NMBE 557231: 1 specimen, sequenced; Fig. 11c]. Cueva de Cuvias Negras (Cantabria: Soba: Asón; 43.25132 -3.60888, 250 m); 12.04.2017, C. Prieto, S. Quíñonero, A. Alonso, J. Ruiz-Cobo leg. [ZUPV 4714: 38 shells + 2 specimens] [NMBE 557227: 1 specimen, sequenced; Fig. 11b; NMBE 568196: 5 shells] [SMF 349,424: 5 shells] [MHNG-MOLL-013791: 5 shells] [MNCN 15.05/200130: 10 shells] [CAW-w/o n°: 19 shells] [CSQS-w/o n°: 25 shells].

**Other material** Cueva del Cesáreo (Cantabria: Liérganes: Extremera; 43.32034 -3.72279, 258 m); 21.03.2016, S. Quiñonero, J. Ruiz-Cobo, A. Alonso leg. [ZUPV 3807: 3 shells] [CAW-w/o n°: 1 shell] [NMBE 559626: 2 specimens, sequenced; Fig. 11a]. Cueva de Asunción (Cantabria: Ramales de la Victoria: Guardamino; 43.25837 -3.44820, 180 m); 21.03.2016, S. Quiñonero, J. Ruiz-Cobo leg. [ZUPV 3808: 4 shells]. Cueva del Comellante (Cantabria: Ruesga; 43.31111 -3.60806, 170 m); 30.03.2015, S. Quiñonero, J. Ruiz-Cobo leg. [ZUPV 3804: 1 shell] [CSQS-w/o n°:3 shells]. Cueva de Covallarco (Cantabria: San Roque de Riomiera: Merilla; 43.25654 -3.73412, 402 m); 18.06.2016, CP, J. Fernández leg. [ZUPV 3974: 1 shell]. Cueva de Cullalvera (Cantabria: Ramales de la Victoria; 43.25577 -3.45808, 95 m); 19.09.2014, C. Prieto, A. Calvo leg. [ZUPV 2604: 1 shell]. Torca de El Porrón (Cantabria: Ruesga: Porraco-lina; 43.25111 -3.66356, 920 m); 09.09.2016, M. Gutiérrez leg. [ZUPV 4180: 1 shell]. Cueva de La Puntida (Cantabria: Miera: Ajanedo; 43.25883 -3.71042, 500 m); 12.10.2015, C. Prieto, A. Calvo leg. [ZUPV 3032: 90 shells] [CSQS-w/o n°: 10 shells]. Fuente de La Revilla (Cantabria: Voto: San Miguel de Aras; 43.31972 -3.52036, 55 m); 30.03.2015, S. Quíñonero leg. [ZUPV 3806: 3 shells]. Cueva de Las Casajosas (Cantabria: San Roque de Riomiera; 43.25457...
Molecular investigation and description of *Iberozospeum* n. gen., including the description of *Iberozospeum costulatum* n. sp.

**Diagnosis** A medium-sized (average, 1.35 mm, \( n = 52 \)) and wide (average, 0.76 mm, \( n = 52 \)) *Iberozospeum* species with convex whorls bearing well-defined ribs, oblique, reniform aperture with broad straight-edged parietal callus and a strong columellar lamella clearly seen inside the aperture.

**Description** (Fig. 17). Shell of medium size (1.24–1.55 mm, \( n = 52 \)), conical. Spire formed by five (4.6–5.9, \( n = 52 \)) rather convex whorls separated by a deep suture. Protoconch (nucleus, 0.175 mm wide) and apical whorls smooth (Fig. 17f) with teleoconch bearing regularly spaced (5–6/0.5 mm; 27 in the body whorl, \( n = 52 \)), well-defined ribs, extending from one whorl to the next; ribs of the hind peristome region are more densely aligned and thinner. Body whorl large (holotype, 60% of the shell height), with upper convexity, ascending behind the aperture. Umbilicus closed, with ribs reaching the umbilical cavity. Aperture reniform, obliquely transverse (holotype, 47% of the shell height), attached to the body whorl by a thickly callused, broad, straight-edged parietal callus. Peristome wide and reflexed; lateral and palatal side regularly arched; columellar side almost straight and vertical. Inner lamella singular, large, oblique, (Fig. 17g) located above the parieto-columellar junction; lamella visible in oblique apertural view (Fig. 17c). Shell surface with micro sculpture of irregular spiral striae.

**Geographical distribution** *Iberozospeum costulatum* n. sp. inhabits caves throughout western Bizkaia and the eastern part of Cantabria (Fig. 18). In Bizkaia, *I. costulatum* is known from two caves located 4.2 km apart: Mina del Pedreo, where it was first discovered, and Cueva de Valdebeci (holotype, sequenced population). Both caves belong to the same limestone formation (Alén-Lujar) cut by the Barbadun river. The remaining localities are in Cantabria. They are separated from the Bizkaian caves by a distance of 20 km, with Cueva Cullalvera and Cueva de Asunción being the nearest. All Cantabrian localities are located in the limestone massifs belonging to the Miera and Asón river basins with Cueva del Cesáreo (sequenced, Fig. 11a) constituting the most north-western cave separated from this central cave. We remark that *I. costulatum* appears to be sympatric with *I. biscaiense* in the Montes de Triano massif west of Bilbao (Prieto & Calvo 2013; unpublished records) and that the east-westward geographic span recorded for *I. costulatum* likely represents the true geographic distribution of this species.

**Etymology** The specific epithet derives from the well-described ribbing of the last whorls of the shell.

**Variability** Ribbing is the most conspicuous feature on the shell of *I. costulatum*. Although the shells from the same cave show similar rib strength, spacing and length, a notable spectrum of variability is present in different cave populations. Shells deriving from populations from the Miera upper basin, such as La Puntida, PO-153 or Cuvias Negras (Fig. 17f–k), show poorly developed ribs and a weak columellar lamella, while individuals from Fuente de la Revilla bear strong and irregular ribs. On the other hand, shells from Asunción have well separated ribs (5/0.5 mm). Although obscured by wide intra-population variation, shell size also varies between populations. Shells from Cuvias Negras are the smallest and those from Valdebeci are the largest. The inner columellar lamella also varies somewhat in form in shells from Cueva del Cesáreo, whereby in addition to the columellar lamella, a small basal lamella can be seen from the opening in oblique view (Fig. 17g–i).

**Remarks** The conspicuous ribbing differentiates *I. costulatum* from all other iberozospeid taxa except *I. percostulatum*. The shell of *I. percostulatum* is higher (1.34–1.80 mm, average 1.55 mm) and narrower (1.32–1.65 mm, average 1.51 mm, \( n = 42 \)) and lacks inner lamellae (Alonso et al., 2018). Additionally, their geographic ranges are 80 km apart.
Conclusion

In this integrative study, we investigated 57 populations of cavernicolous zospeid snails using genetic and morphological data. Our study revealed a separate radiation for species inhabiting northern Spanish caves for which the new genus, *Iberozospeum*, has been defined. *Iberozospeum* populations show on-going speciation in situ within isolated cave systems of northern Spain. Our investigations corroborated the proposed ancestral area reconstruction of Weigand et al. (2013) indicating that the “Cantabrian Mountains + Alps” or “Cantabrian Mountains + Dinaric Alps” was the ancestral area of the *Iberozospeum* radiation. Coherent mitochondrial and nuclear sequence patterns as well as common morphological traits were observed in most of the clades. Three new species were molecularly revealed and one, *Iberozospeum costulatum*, is described using additional morphological data.

Our comparative histological investigations revealed the huge size of the albumen gland for the first time in a sexually mature member in the female phase within the subterranean Carychiidae. We found significant morphological and structural differences in both the columnellar muscle and the radulae of Dinaride *Zospeum* and *Iberozospeum* species. SEM investigations of the radular ribbon reveal that dentition form, size and alignment, consistency in ribbon shape, length, broadness and the straight-edged base are systematically significant in *Iberozospeum* species and that it morphologically differentiates these species from those of the Eastern Alpine and Dinaride *Zospeum* taxa.

Our investigations are ongoing. Future collection efforts and phylogeographic investigations will do well to reveal deeper patterns of phylogenetic relatedness and evolutionary processes in *Iberozospeum* populations.

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Molecular investigation and description of *Iberozospeum* n. gen., including the description…

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**Data availability** All data is published in the manuscript. Sequences are deposited in Genbank.

**Code availability** Software and programs are cited in the manuscript.

**Declarations**

**Ethics approval** Not applicable.

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**Conflict of interest** Not applicable.

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