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UNDISCLOSED TAXONOMIC DIVERSITY OF BATHYNELLACEA (MALACOSTRACA: SYNCARIDA) IN THE IBERIAN PENINSULA REVEALED BY MOLECULAR DATA

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ABSTRACT

The biodiversity of Bathynellacea, a globally important group of groundwater crustacean, remains poorly known and understood. The objectives of this work were to increase the molecular information of bathynellaceans in order to test: 1) its usefulness solving taxonomic problems; and 2) evaluate the extent of cryptic speciation in a morphologically constrained clades from populations that have already been studied using only morphological methodology, contributing in this way to estimate the real diversity of Spanish subterranean fauna. We employ the COI barcode region to provide a preliminary assessment of the genetic subdivision, mtDNA lineages, of the genus *Vejdovskybathynella*, Bathynellidae, which has a restricted distribution to a karst system of Burgos (Spain) and was initially identified as a single species by morphological evaluation: *Vejdovskybathynella edelweiss* Camacho, 2007. We also studied the mtDNA lineages within six morphospecies of Parabathynellidae, five species belonging to *Iberobathynella*, a genus of wider distribution in the Iberian Peninsula, and one species belonging to the cosmopolitan genus *Hexabathynella*. The analyses of molecular data demonstrate the presence of highly divergent genetic units. We identify three divergent mtDNA clades, that may represent cryptic species that had gone unnoticed and possibly correspond to undescribed new species. We present a first preliminary molecular phylogeny of Bathynellacea, using three genera of Parabathynellidae and one genus of Bathynellidae, and one member of Anaspididae Thomson, 1893 as an out-group. The results of this study provide the first molecular data complementing the existing morphological knowledge to try to resolve the relations among Spanish genera and species of Bathynellacea through phylogenetic studies. Based on the results, we conclude that the evolutionary scenario of this special group of subterranean crustaceans cannot be revealed using only morphological information due to the presence of cryptic species.

KEY WORDS: Bathynellacea, cryptic species, cytochrome oxidase I, Iberian Peninsula, stygofauna

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INTRODUCTION

Biodiversity of subterranean environments, especially groundwater, is poorly known. This is due to the fact that subterranean waters are difficult to access and that few researchers are devoted to their study. Nevertheless, the knowledge of groundwater fauna is still slowly advancing. Ground water, and its karstic habitat (consolidated rocks) in particular, is known to be an environment of exceptionally high level of endemism (Trontelj et al., 2009). In this environment, many animals have their distribution restricted to small areas and such is the case of Bathynellacea Chappuis, 1915, a group of groundwater Eumalacostraca. These animals live exclusively in fresh and brackish subterranean waters of all continents except Antarctica and have a very limited dispersion capacity because they lack any active dispersion mechanism, such as swimming larva (Camacho, 2006). Currently there are 250 species known worldwide, and most of them are only known from their type locality, or from a small region around it.

The Iberian Peninsula is one of the regions where the highest diversity of Bathynellacea has been found with a sampling effort that cannot be compared for example with France, where biospeleology has a much longer tradition. Currently, there are 39 species formally described and more that are pending. Within Parabathynellidae, the genus *Iberobathynella* Schminke, 1973 is endemic to the Iberian Peninsula and includes 22 species that can be identified through morphological characters, while the cosmopolitan genus *Hexabathynella* Schminke, 1972, is represented in Spain by four species (Camacho, 2006). On the other hand, Bathynellidae has not been thoroughly studied in the Iberian Peninsula, and currently only three species of the genus *Vejdovskybathynella* Serban and Leclerc, 1984 are well known through morphological descriptions.

In general, the taxonomy of all stygobitic organisms is very complex due to the convergent evolution of morphological characters that are associated with adaptations to the subterranean environment (Jones et al., 1992; Kane et al., 1994); this convergence confounds their true phyletic descent through the retention of primitive traits and loss of complex features (Guzik et al., 2008). In the case of Bathynellacea, the morphological simplification is further

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compounded by extreme progenetic development (Schminke, 1981). The isolation and strong selective pressures inherent to the adoption of an underground life can lead in opposite directions, resulting in both high genetic divergence and morphological convergence (Finston et al., 2007). The diagnosis of some specimens, particularly from the genera *Iberobathynella* and *Vejdoskybathynella*, using classical morphological taxonomic methods can be somewhat unsatisfying. Sometimes a specimen from a sample can be easily assigned to a certain species, while another one from that same sample may not fit so well; this has made us consider that we are failing to identify new species present in these localities, and thus, we might be in fact underestimating the real number of bathynellaceans species inhabiting subterranean waters in Spain. Taking into consideration that for stygobitic crustaceans the underestimation of species diversity based on morphological characters has been recently highlighted (Proudlove and Wood, 2003), we postulate that our populations of bathynellaceans in Northern Spain may include a number of cryptic species, a hypothesis that is tested here. A number of studies using DNA sequence data have already identified cryptic species in groundwater fauna (Jarman and Elliot, 2000; Finston and Johnson, 2004; Lefébure et al., 2006a, b; Guzik et al., 2008). Nowadays prediction of species diversity and boundaries with DNA sequence data are being increasingly investigated, particularly with the advent of DNA barcoding (Hebert et al., 2003). This method aims to identify and assign a single specimen to a certain animal species based on sequence diversity within one or more DNA barcode sequence(s). This method has the potential to: 1) help disentangling species complexes and separate sibling species (Porco et al., 2010), 2) highlight cryptic diversity and potential new species overlooked by morphological analysis (James et al., 2010), 3) unambiguously link juveniles to adults of the same taxon when they are morphologically different (Richard et al., 2010), and 4) allow species-level identifications in groups that require the use of characters that are only present in one sex (Stevens et al., 2011), such as in bathynellaceans. Currently, only a few bathynellacean DNA fragments have been published (Camacho et al., 2002; Guzik et al., 2008; Camacho et al., 2011), and thus, there are very few barcoding sequences available for comparison.

In this particular study we have two clear objectives: 1) increasing the limited molecular information in an important group in groundwater worldwide, the bathynellaceans, by extracting partial sequences of the mtDNA gene COI (507 bp) from 33 specimens collected in different sites of the Iberian Peninsula; and 2) testing its usefulness solving taxonomic problems, in particular identifying potential cryptic species in populations that have already been studied but that have gone unnoticed using only morphological methodology.

Materials and Methods

Taxonomic Sampling/Specimens Collection

The bathynellaceans are very difficult to collect because they are "rare creatures." They only live in ground water, sometimes in deep caves, and are not very abundant. Specimens for this study were collected at various sites using different sampling methods (see map of the Fig. 1): for gours and pools, in the epikartic zones of caves, we used hand or plankton haul nets (mesh size 0.100 mm), while gravel banks of the epigean and subterranean river were sampled using Karaman-Chappuis and Bou-Rouch methods (see Camacho, 1992). In total, 33 specimens of Spanish bathynellaceans were collected: 12 specimens belonging to Bathynellidae, and 21 belonging to Parabathynellidae (see Table 1; voucher number of the Tissues and DNA Collections from Museo Nacional de Ciencias Naturales, Madrid, Spain).

The 12 specimens of Bathynellidae were all morphologically identified as *Vejdovskybathynella edelweiss* Camacho, 2007 and were collected in different sites of the Ojo Guareña Karstic System. This system includes a main cave (Ojo Guareña) and several small cavities (Redonda Cave, Sima Huesos, Mina Cave, etc.) that are connected with the main cave totalling 110 km of underground galleries (Camacho et al., 2006a, 2011):

- eight specimens were found in three gours of the main cave, OG09 (22/09/2007 and 10/02/2007), OG01 (10/02/2007 and 21/02/2009), OG16 (10/02/2007)
- one specimen was found in a gour from Sima Huesos (12/05/2007)
- one specimen was found in shore of Erizos River of main cave (05/09/2009)
- two specimens were found in Redonda Cave (11/12/2009).

The specimens of Parabathynellidae were collected from eight populations and included 2 genera (*Iberobathynella* and *Hexabathynella*). Six species were identified morphologically:

- *I. imuniensis* Camacho, 1987: three specimens found in a pool in the Torca de los Morteros cave (TM) (Pt° de la Sía, Burgos), collected at −60 m (11/07/2007) and one specimen found in CO314 cave (Sierra de la Collada, Suarias, Asturias) (29/04/2006).
- *I. cantabriensis* Camacho and Serban, 1998: three specimens found in Torco Lobos cave (TL) (Sierra de la Collada, Herrerías, Cantabria) (5/06/1998); one specimen found in CO89 cave (Sierra de la Collada, Peñamellera Baja, Asturias) (03/10/2009) and three specimens found in CO314 cave (Sierra de la Collada, Suarias, Asturias) (29/04/2006).
- *I. magna* Camacho and Serban, 1998: four specimens found in Torcón Pelacristo cave (TP) (Sierra de la Collada, Merodio, Asturias) (01/06/1998)
- *I. celiana* Camacho, 2003, one specimen found in the gravel bank of the Viar stream (Castilblanco, Sevilla) (18/04/2007)
- *I. burgalensis* Camacho, 2005, three specimens found in Ojo Guareña cave (Cornejo, Burgos) in the gour OG53 (12/12/2009)
- *H. sevillaensis* Camacho, 2005, two specimens found in Santiago el Grande cave (SC) (Carmona, Sevilla) (17/10/2003).

To examine phylogenetic relationships among parabathynellids, we used partial DNA sequences of the mtDNA gene *COI* (507 bp).

DNA Extraction, Amplification, and Sequencing

Extraction was carried out with Chelex following Walsh et al. (1991). Fresh specimens, kept frozen at −4°C, were cut

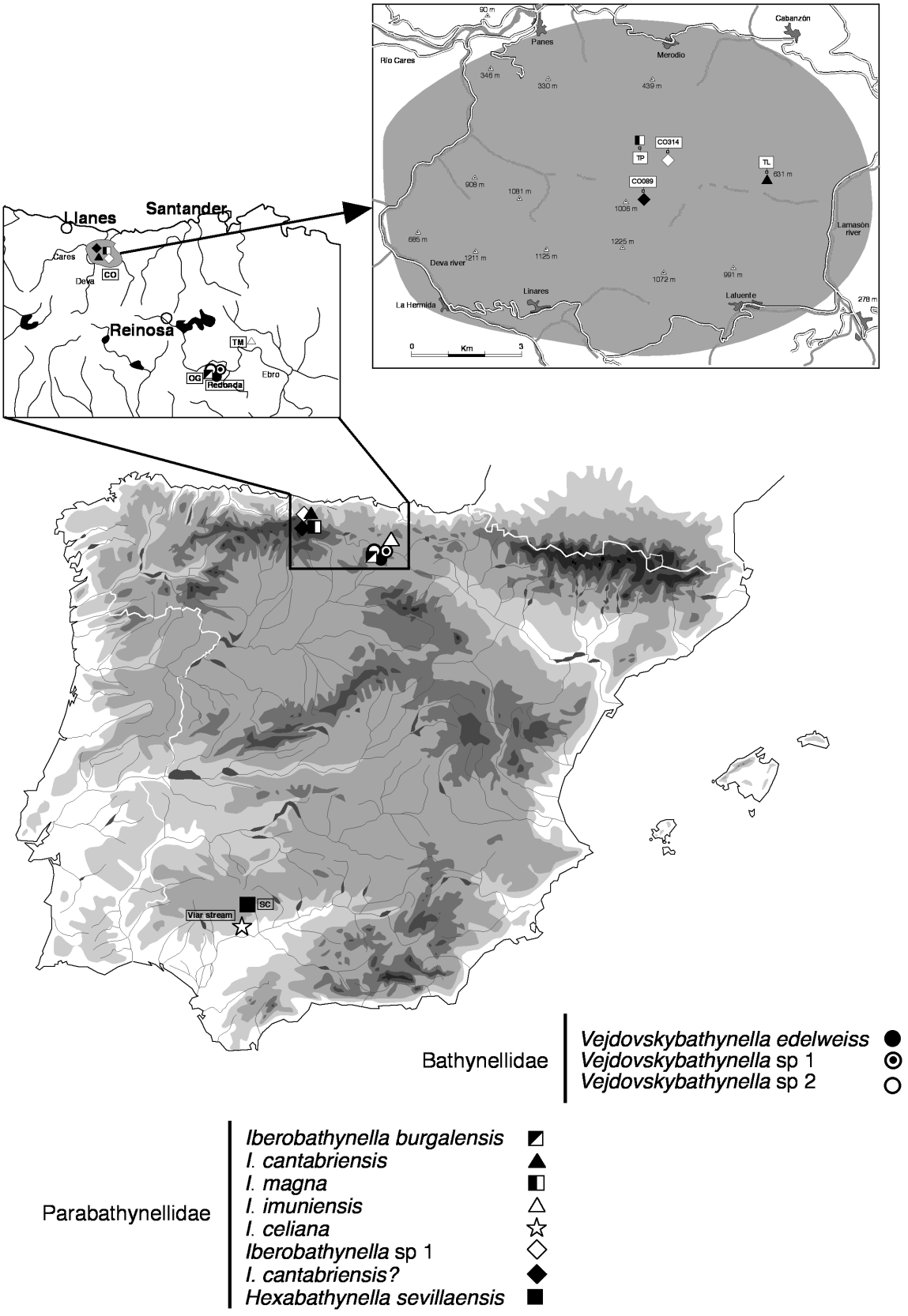

Fig. 1. Map of bathynellaceans Spanish species distribution of which COI sequences were obtained.

Table 1. Morphospecies identified and probably cryptic species found by mtDNA, localities of the studied populations, voucher numbers of specimens of the Tissue and DNA Collection of the Museo Nacional de Ciencias Naturales, CSIC (Spain), code used by each specimens in the Fig. 1, GenBank accession numbers and patristic distances within specimen of the same populations.

Order/Family/Species	Locality	Coordinates			Specimens voucher MNCN/ADN	Figures code	Accesion numbers	Patristic distance
		X	Y	Z				
BATHYNELLACEA CHAPPUIS, 1915								
Parabathynellidae Noodt, 1965								
Iberobathynella imuniensis Camacho, 1987	Torca Morteros Cave (TM), Imunía (Burgos)	451690	4777530	1280	29164	1TM	HQ659849	0-0.0024
I. imuniensis					29166	2TM	HQ659850	
I. imuniensis					29167	3TM	HQ659851	
I. cantabriensis Camacho and Serban, 1998	Torco Lobos Cave (TL), (Cantabria)	377681	4794064	492	29373	4TL	HQ659854	0-0.0023
I. cantabriensis					29371	5TL	HQ659852	
I. cantabriensis					29372	6TL	HQ659853	
I. magna Camacho and Serban, 1998	Torcón Pelacristo Cave (TP), (Asturias)	374456	4794725	720	29367	7TP	HQ659855	0
I. magna					29368	8TP	HQ659856	
I. magna					29151	9TP	HQ659857	
I. magna					29370	10TP	HQ659858	
I. burgalensis Camacho, 2005	Ojo Guareña Cave (OG53), Cornejo (Burgos)	446595	4764790	724	29520	11OG53	HQ659859	0
I. burgalensis					29521	12OG53	HQ659860	
I. burgalensis					29542	13OG53	HQ659861	0.0019
Iberobathynella celiana Camacho, 2003	Viar stream, Castiblanco (Sevilla)	246437	4178156	60	29452	14VR	HQ659862	
Iberobathynella sp. 1 (cryptic specie)	CO314 Cave, Suarias (Asturias)	375193	4795030	538	9001	15CO314	HQ659863	0-0.0020
I. sp. 1					9002	16CO314	HQ659864	
I. sp. 1					9003	17CO314	HQ659865	
Iberobathynella sp. **1?** (cryptic specie)	CO314 Cave, Suarias (Asturias)				29473	18CO314	HQ659866	**0.063**
Iberobathynella sp. 2 (cryptic specie)	Torca Sorios Cave (CO89) (Cantabria)	374336	4793707	869	29488	19CO89	HQ659867	
Hexabathynella sevillaensis Camacho, 2005	Santiago el Grande Cave (SC) (Sevilla)	245188	4213344	358	29544	20SC	HQ659868	0
H. sevillaensis					29545	21SC	HQ659869	
Atopobathynella wattsi Cho et al., 2007	Australia						EU350223	
Bathynellidae Grobben, 1904								
Vejdovskybathynella edelweiss Camacho, 2007	Ojo Guareña Cave (OG09), Cornejo (Burgos)	446595	4764790	724	29543	22OG09	HQ596568	0-0.0079
V. edelweiss					29413	23OG09	HQ596569	
V. edelweiss	Ojo Guareña Cave (OG01), Cornejo (Burgos)	446595	4764790	724	29415	24OG01	HQ596564	0-0.0039
V. edelweiss					29366	25OG01	HQ596563	0.015
V. edelweiss					29478	26OG01	HQ596565	
V. edelweiss					29479	27OG01	HQ596566	
V. edelweiss					29482	28OG01	HQ596567	
V. edelweiss	Ojo Guareña Cave (OG16), Cornejo (Burgos)	446595	4764790	724	29414	29OG16	HQ596570	
V. edelweiss	Sima Huesos Cave, Cornejo (Burgos)	448071	4764815	705	29440	30Huesos	HQ596571	
Vejdovskybathynella sp. 1 (cryptic specie)	Ojo Guareña Cave (Erizos river), (Burgos)	446595	4764790	724	29487	31Erizos	HQ596572	
Vejdovskybathynella sp. 2 (cryptic specie)	Redonda Cave, Cornejo (Burgos)	448858	4764811	668	29523	32Redonda	HQ596573	0
Vejdovskybathynella sp. 2 (cryptic specie)					29524	33Redonda	HQ596574	
ANASPIDACEA CALMAN, 1904								
Anaspididae Thomson, 1893								
Anaspides tasmaniae (Thomson, 1893)	Mount Wellington, Tasmania						AF048821	

into two pieces in distilled water, and were placed in the wall of a 1.5 ml microcentrifuge tube with a sterile needle. Each tube contained 100 μl Chelex 100 (Bio-Rad; 5% in distilled water) and 400 μl of distilled water. The specimens were incubated overnight at 56°C, followed by 10 minutes at 100°C and centrifuged at 16 000 g for 10 minutes.

A 510 base pair (bp) region of the COI gene was amplified with the primers C1-J-1718 (5′-GGAGGATTTGGAAATTG ATTAGTTCC-3′) and HCO2198 (5′-TAAACTTCAGGGTG ACCAAAAAATCA-3′) (Folmer et al., 1994; Simon et al., 1994) for all specimens. Three μl of the DNA solution was used as a template. Other components of the 25 μl PCR reaction were: 1× of the corresponding buffer (75 mM Tris HCl, pH 9.0; 50 mM KCl and 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$), 10 mM dNTPs mix, 0.1 μM of both primers, 0.02% BSA, and 0.125 units AmpliTaq Gold® DNA Polymerase (Applied Biosystems). Six microlitres of PCR products were electrophoresed through a 1.5% agarose gel and visualized with ethidium bromide under ultraviolet light. PCR products were purified by treatment with ExoSAP-IT (USB Amersham, Buckinghamshire, UK) in a 5:1 amplicon: enzyme ratio and incubated at 37°C for 45 min, followed by 80°C for 15 min to inactivate the enzyme. Purified PCR product was then used to sequence in both directions using the BigDye Terminator v3.1 sequencing kit (Applied Biosystems Inc., Foster City, USA) in a 10 μL volume, containing 15-20 ng purified product and 3 pmol primer. The sequences obtained were compared with sequences from GenBank, to verify that the sequence came from a bathynellaceans, using blast (Altschul et al., 1997). The alignment of all bathynellaceans COI gene sequences, generated in our lab and those from GenBank [*Atopobathynella watsi* Cho, Humphries, and Lee, 2006 and *Anaspides tasmaniae* (Thomson, 1893)] were edited and manually aligned using MEGA 4.0 (Tamura et al., 2007). Fine adjustments were made by eye, as the COI cannot present any gaps. All sequences were submitted to GenBank (see Table 1 for collection voucher number of each specimen and the GenBank Accession Number).

Phylogenetic and Mitochondrial DNA Sequence Analysis

To examine relationships between species, mtDNA COI sequences were analysed using a phylogenetic approach. As the monophyly of Parabathynellidae and Bathynellidae remains unconfirmed, a member of Anaspidacea was chosen as an out-group (*Anaspides tasmaniae*, GenBank accession number AF048821) and a sequence of *Atopobathynella wattsi* (GenBank accession number EU350223) from Australia was used to check the genetic distance between genera. Pairwise comparisons of observed proportional sequence divergence (p-distance) (Table 2) and corrected sequence divergence (Kimura-2-parameter model), were obtained using the computer program PAUP* 4.0b10 (Swofford, 2002). To test for possible saturation of nucleotide substitutions, we plotted p-distance (y) versus corrected estimates of proportional sequence divergence (x) for first, second, and third codon positions, as well as for transitions and transversions separately (not shown). We initially explored the dataset using distance analyses (neighbour joining, NJ) with the program PAUP* 4.0b10. Phylogenetic analyses were conducted using maximum likelihood (ML; Felsenstein, 1981, 1985) and Bayesian inference (BI) (Huelsenbeck and Ronquist, 2001). All characters were equally weighted. Modeltest 3.7 (Posada and Crandall, 1998) were used to identify the model of sequence evolution that best fit the data, based on Akaike information criteria (AIC), for use in the phylogenetic (ML) and distance analyses (NJ). The general time-reversible model of evolution (GTR) with gamma parameter and a proportion of invariable positions (GTR + G + I) was selected as the best fit and was used for ML (Yang, 1994; Gu et al., 1995; Swofford et al., 1996) and BI analyses. The proportion of invariable sites (I) was estimated to be 0.3772, and the gamma shape parameter (α) (Lanave et al., 1984) was estimated at 0.5369. ML analyses with empirical base frequencies were performed using Garli (Zwickl, 2006; Zwickl and Balhoff, 2006). We used nonparametric bootstrapping (500 pseudoreplicates) to assess the stability of internal branches in the resulting topologies (Felsenstein, 1985; Felsenstein and Kishino, 1993). BI analysis was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) assuming six discrete gamma categories. Bayesian analyses were initiated with random starting trees and run for 1 000 000 generations sampled every 100 generations. The convergence occurred during the first million generations, the likelihood values converged to relative stability after approximately 100 000 generations; subsequently we conservatively discarded all samples obtained during the first hundred thousand (10%) generations as burn-in. Robustness of the observed clades was assessed with Bayesian posterior probabilities.

Results

Nucleotide Analysis

The alignment of all bathynellaceans COI gene sequences (35 individuals in total) resulted in a consensus length of 507 bp, of which 54% were variable. The models of sequence evolution selected for the mtDNA were GTR + I + G. No stop codons or gaps were observed in any of the translated amino acid sequences suggesting that the genuine mtDNA COI gene was sequenced. On average, a bias towards A-T (70%) was observed in the bathynellacean sequences, which is consistent with previous results from parabathynellids and others crustaceans (Guzik et al., 2008). The COI sequences comprised 54% variable sites and 38% is the proportion of invarible sites. The base frequencies were as follows: A = 0.30, C = 0.14, G = 0.11 and T = 0.45. The observed proportion of transitions to transversions was high, Ti/Tv = 2.7.

Genetic Divergences

The uncorrected sequence divergence estimates between the specimens and the out-group taxa are summarized in Table 2. The genetic divergence is relevant at species level, with significant results found within the morphospecies studied the populations of *Vejdovskybathynella* and of the genus *Iberobathynella*.

In the case of Bathynellidae we identified only one morphospecies already described (Camacho, 2007b) as *V. edelweiss*. The genetic distance between specimens of *V. edelweiss* from the same population (OG01 or OG09) varies from 0.39 to 1.5%, and between different but geographically close populations (main cave and Huesos) the genetic

Table 2. COI genetic distances among and within species groups of the Spanish Bathynellacea.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Anaspides tasmaniae	0																																		
Atopobathynella watsi	28.1	0																																	
H. sevillaensis (20 CS)	28.4	22.5	0																																
H. sevillaensis (21 CS)	28.4	22.5	0	0																															
I. celiana (14 VR)	27.9	19.2	25.7	25.7	0																														
I. burgal. (11OG53)	26.8	18.6	20.8	20.8	16.9	0																													
I. burgal. (12OG53)	26.9	19.1	20.8	20.8	17.5	0	0																												
I. burgal. (13OG53)	26.9	18.8	20.8	20.8	17.1	0.19	0	0																											
I. sp. 1 (15CO314)	28.6	22.0	24.9	24.9	18.9	17.1	17.8	17.3	0																										
I. sp. 1 (16CO314)	28.6	22.4	24.9	24.9	19.2	17.4	17.8	17.6	0	0																									
I. sp. 1 (17CO314)	28.6	22.2	24.9	24.9	18.7	17.3	18.0	17.5	0.19	0.20	0																								
***I.* sp. 2 (19 CO89)**	27.3	19.0	23.2	23.2	16.9	16.2	16.7	16.4	16.7	17.0	16.9	0																							
I. magna (7 TP)	30.4	21.9	22.4	22.4	20.1	20.2	20.2	20.2	19.8	19.8	19.8	17.3	0																						
I. magna (8 TP)	28.6	20.9	20.3	20.3	18.9	18.6	18.7	18.7	18.4	18.4	18.4	16.5	0	0																					
I. magna (9 TP)	28.1	20.7	19.5	19.5	18.6	18.5	18.6	18.6	18.4	18.4	18.4	16.7	0	0	0																				
I. magna (10 TP)	28.6	20.9	20.3	20.3	18.9	18.6	18.7	18.7	18.4	18.4	18.4	16.5	0	0	0	0																			
I. cantabriensis (4 TL)	27.7	19.2	22.4	22.4	18.4	17.2	17.5	17.4	16.2	16.2	16.4	**6.7**	17.9	16.9	16.4	16.9	0																		
I. cantabriensis (5 TL)	24.9	19.9	20.4	20.4	18.8	17.7	17.7	17.7	16.5	16.5	16.5	**6.5**	17.9	16.7	16.2	16.7	0.20	0																	
I. cantabriensis (6 TL)	27.4	18.6	22.1	22.1	17.9	16.7	17.3	16.9	15.8	16.0	16.0	**6.5**	17.9	16.6	16.2	16.6	0.20	0	0																
I. imuniensis (1 TM)	24.3	22.9	25.4	25.4	23.6	19.8	19.8	19.8	19.0	19.1	19.0	20.4	20.7	20.6	20.5	20.6	20.4	20.4	20.4	0															
I. imuniensis (2 TM)	25.6	20.4	25.5	25.5	20.5	17.3	17.1	17.1	17.9	17.8	18.1	18.1	20.5	19.1	19.0	19.1	18.6	18.9	18.5	0.24	0														
I. imuniensis (3 TM)	25.6	20.6	25.3	25.3	20.3	17.1	16.9	16.9	17.7	17.6	17.9	17.9	20.7	19.3	19.2	19.3	18.4	18.7	18.3	0	0.19	0													
***I.* sp. 1 (18 CO314)**	28.3	21.2	23.6	23.6	19.7	17.9	18.2	17.7	**6.1**	**6.2**	**6.3**	17.5	18.9	17.7	17.7	17.7	17.6	17.4	17.1	18.3	17.3	17.1	0												
V. edelweiss (24OG01)	26.6	21.4	25.8	25.8	21.9	21.3	21.4	21.5	25.6	25.4	25.8	23.3	27.7	25.5	25.5	25.5	22.8	24.0	22.8	26.4	22.6	22.8	25.4	0											
V. edelweiss (25OG01)	26.8	21.6	26.0	26.0	21.5	21.1	21.4	21.3	26.0	26.0	26.2	23.7	28.0	25.9	26.0	25.9	23.0	24.2	22.8	27.4	23.2	23.4	25.8	1.1	0										
V. edelweiss (26OG01)	26.9	21.5	26.0	26.0	21.7	21.3	21.4	21.3	26.4	26.5	26.6	24.0	28.0	26.0	26.0	26.0	23.3	24.2	23.1	27.3	22.7	22.9	25.8	1.0	0	0									
V. edelweiss (27OG01)	26.9	21.2	26.0	26.0	21.1	20.7	21.4	20.9	25.6	26.0	25.8	23.3	28.0	26.0	26.0	26.0	23.0	24.2	22.5	27.4	22.9	23.1	25.4	1.5	0.39	0	0								
V. edelweiss (28OG01)	26.9	21.2	26.0	26.0	21.1	20.7	21.4	20.9	25.6	26.0	25.8	23.3	28.0	26.0	26.0	26.0	23.0	24.2	22.5	27.4	22.9	23.1	25.4	1.5	0.39	0	0	0							
V. edelweiss (23OG09)	26.7	21.6	25.9	25.9	22.1	21.5	21.5	21.7	25.9	25.5	26.1	23.5	27.7	25.6	25.6	25.6	22.9	24.1	23.1	26.4	22.5	22.7	25.7	0.1	1.38	1.01	1.78	0.17	0						
V. edelweiss (22OG09)	26.6	20.8	25.8	25.8	21.3	20.7	21.4	20.9	25.1	25.4	25.2	22.7	27.8	25.5	25.5	25.5	22.8	24.0	22.2	26.4	22.0	22.2	24.8	0.5	1.3	1.01	0.98	0.98	0.79	0					
V. edelweiss (29OG16)	26.6	21.2	25.8	25.8	21.7	21.1	21.4	21.3	25.4	25.4	25.6	23.1	27.8	25.5	25.5	25.5	22.8	24.0	22.6	26.4	22.4	22.6	25.2	0.1	0.98	1.01	1.38	1.38	0.39	0.39	0				
V. edelweiss (30Hues)	26.6	20.8	25.8	25.8	21.1	20.9	21.6	21.1	25.1	25.4	25.2	22.5	27.5	25.3	25.3	25.3	22.6	23.8	22.0	26.6	22.2	22.4	24.8	1.1	1.97	1.62	1.58	1.58	1.38	0.59	0.98	0			
Vejdosvk sp. 1 (31Eri.)	28.2	17.6	25.9	25.9	22.1	20.1	20.8	20.3	23.9	24.2	24.1	21.1	27.5	25.2	25.5	25.2	21.4	22.6	20.9	29.9	25.4	25.6	23.8	15.3	14.9	15.0	14.6	14.6	15.6	14.7	15.1	14.7	0		
Vejd sp. 2 298 (32Red.)	26.4	21.6	25.7	25.7	22.5	20.9	21.6	21.1	25.0	25.4	25.0	22.9	26.2	24.6	24.6	24.6	23.4	24.6	22.8	25.0	22.8	22.6	24.8	14.0	14.7	14.8	14.4	14.4	14.2	13.4	13.8	13.6	16.1	0	
Vejd sp. 2 299 (33Red.)	24.0	22.0	23.9	23.9	23.9	22.3	22.3	22.3	26.2	26.2	26.2	24.4	26.2	24.6	24.5	24.6	24.8	24.6	24.5	25.1	23.7	23.5	25.2	14.0	15.2	15.2	15.2	15.2	14.1	14.0	14.1	14.3	16.9	0	0

distance was 0.1 to 2% (Table 2). The specimens of the Erizos river showed a genetic distance of 15% to 17% when compared to other populations. Finally the specimens of Redonda Cave showed a genetic distance of 13.4% to 15.2% when are compared with the populations of the main cave and Huesos, and of 16.1% to 16.9% when compared with the specimen from Erizo River.

In the case of *Iberobathynella*, the genetic divergence between morphospecies varied between 15.8 and 23.6% while the genetic divergence found between the specimens of the same populations, morphologically identified as the same species, was never higher than 0.24%. Nevertheless, the four specimens from the CO314 cave population that were initially identified as *I. imuniensis*, showed a genetic distance between 17.6 and 19.1% with the specimens of *I. imuniensis* of the type locality (Torca Morteros), and up 19.8% with the other studied morphospecies. One of these specimens, "18CO314," presented a genetic distance between 6.1 and 6.3% with the other three specimens. The specimen from the CO89 cave, 19CO89, which was initially identified as *I. cantabriensis*, has a genetic distance between 6.5 and 6.7% with the specimens of Torco Lobos cave, which are confirmed *I. cantabriensis*.

Phylogenetic Analyses

The results of the phylogenetic analyses (ML, Bayesian) are condensed in Fig. 2. The COI mtDNA sequence data analysis produced a tree in which all samples of Bathynellacea are not clearly separated into families. Relationship amongst genera and families are not supported in ML and Bayesian analyses (>98% Bayesian posterior probability and 87% bootstrap values and >85% Bayesian posterior probability and 52% bootstrap values respectively). However, the phylogenetic reconstruction revealed that the major clades clearly grouped the different species into their respective genera.

The first robust group (100% bootstrap) is formed by the four populations of the bathynellid *V. edelweiss* (three of the Ojo Guareña main cave (OG01, OG09 and OG16) and one of the Sima Huesos). The population from Erizo River appears separated from the OG specimens, and the same is true for the Redonda Cave population that also appears in a different clade. Both of them appear as different from the well identified linage of *V. edelweiss*.

In the case of Parabathynellidae we find several subgroups: the Australian species *Atopobathynella watsi*, appears in one line, while on the other we find the six Spanish genetically distinct lineages identified from eight populations. One of the lineages belongs to the cosmopolitan genus *Hexabathynella*, while the other five belong to the Iberian Peninsula endemic *Iberobathynella*. One of the clades is that of the species morphologically identified as *I. celiana*, a species that lives in the South of the Iberian Peninsula, around Sevilla (see Fig. 1), an area very far from the northern populations studied. In a second clade, we find the populations of Burgos, from Ojo Guareña (OG53) and Torca Morteros (TM), that appear well separated into two subgroups (82% and 90% bootstrap, respectively). These two subgroups have been identified morphologically as *I. burgalensis* and *I. imuniensis*, respectively. The other three groups are from the populations of Asturias and Cantabria (Sierra de la Collada), which are 80 km from Burgos. The population of Torca Pelacristo (TP) that has been identified as *I. magna* appears separated from a group composed by the population of Torco Lobos (TL), identified as *I. cantabriensis*, and the population from the cave CO89, which has also been morphologically identified as *I. cantabriensis* but shows a certain genetic distance with the TL population. Separated from these last three populations we find the population of the cave CO314. In this population three of the specimens (15, 16, and 17) had been morphologically identified as *I. cantabriensis*, and a fourth specimen (18) had been assigned morphologically to the species *I. imuniensis*. Nevertheless based on the phylogeny built only with the mitochondrial gene COI, this population shows enough genetic distance to be separated into a completely different clade (see Table 2).

Discussion and Conclusion

The expected relationship amongst genera and families are not supported by bootstrap values (Fig. 2). This is not unexpected because the COI gene might not provide adequate information on the deep levels of the phylogeny, as acknowledged for other taxa (Hebert et al., 2003). Nevertheless these preliminary phylogenetic results do offer well-supported terminal clades at the species level.

Within Bathynellidae we have studied several populations of a single genus, *Vejdovskybathynella*. Before carrying out the preliminary analysis with mtDNA COI we morphologically identified the specimens from the karstic system Ojo Guareña, and all of them were assigned to the species *V. edelweiss*. All animals from the six populations studied had a remarkably similar morphology with only slight variations that were attributed to inter-population variability. In the molecular analysis, we found that the genetic distance between specimens of *V. edelweiss* from the same population, and even from different but geographically close populations, was very small (see Table 2). However, the specimens of the Erizos River and Redonda Cave showed a very large genetic distance (13.6%-17%) when compared to other populations. The genetic divergence between the three identified lineages is about 16%, a threshold proposed for interspecific relationships among crustaceans based on mtDNA COI (Lefébure et al., 2006a and Lefebure et al., 2006b). The phylogenetic results of our study show that what we initially identified as *V. edelweiss* probably includes three distinct genetic lineages (Fig. 2), and accepting the 16% threshold as valid, they could indeed be considered distinct sibling species, indicating the presence of two new cryptic species: *Vejdovskybathynella* sp. 1 from Erizos River, and *Vejdovskybathynella* sp. 2 from Redonda Cave. Although all of these taxa bear strong morphological similarities and live in a relatively small area, they appear clearly separated in the cladogram.

In the case of Parabathynellidae, the specimens collected all belong to the Iberian peninsula endemic genus *Iberobathynella*, which currently includes 22 species (Camacho, 2007a) all of which are very similar morphologically and mostly found only in a single cave or in very small geographic areas. Here we studied five morphospecies, four of them inhabiting in the North of Spain, and one more, *I. celiana*, found un the South of the country. Of the four

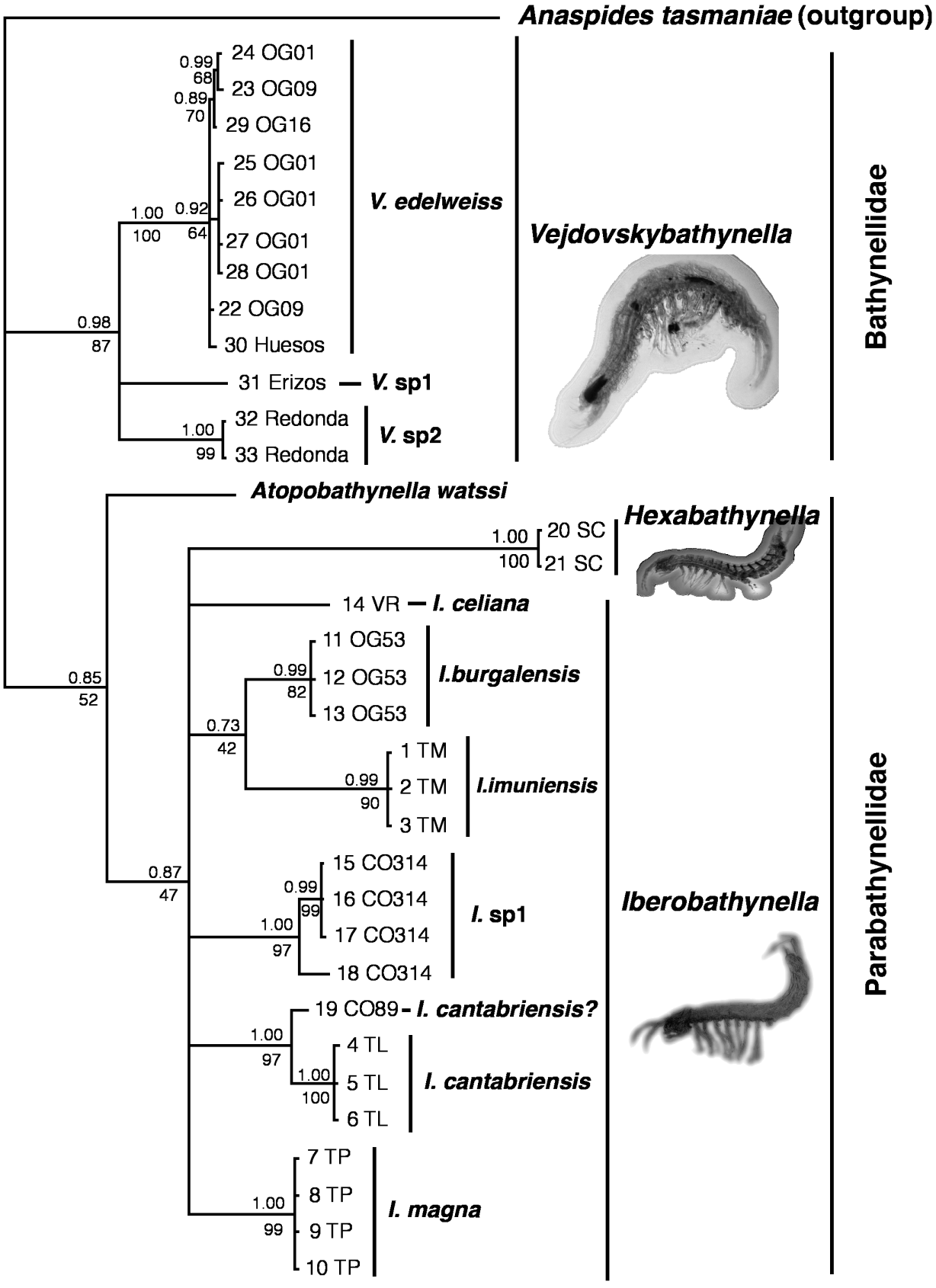

Fig. 2. Phylogenetic reconstruction of the mitochondrial lineages found in Spanish Bathynellacea. Posterior probability Bayesian consensus tree with re-estimated branch lengths using the GTR + I + G model of substitution. Bayesian posterior probabilities, credibility values, are shown above on corresponding nodes, and below the ML evolution bootstrap values. Sample codes are listed in Table 1.

species of the North, *I. burgalensis* has been found in Burgos, in small gours in the Ojo de Guareña cave system. In the Sierra de la Collada (Asturias-Cantabria), we find two more species restricted to a few caves in a small karstic area: *I. cantabriensis* (Torco Lobos, CO314 and CO89) and *I. magna* (Torcón de Pelacristo). Finally, *I. imuniensis* is one of the few species of the family that is supposed to have a wider distribution throughout the North of Spain (including type locality TM, Torca de los Morteros and CO314, among other places). In this study, we have found that the genetic distance between TM specimens and four specimens of the CO314 cave is higher (17.6 and 19.1%) than the threshold (16%) of genetic divergence for mtDNA COI used in crustaceans. These results suggest that this last population probably belongs to a diferent species, designated as *I.* sp. 1, which had gone undetected in classical taxonomic studies. In addition, we also found that one specimen from this last population (18CO314, see Fig. 2) shows a genetic divergence ranging from 6.1 to 6.3% for COI. The same is true for the specimen 19 from the CO89 cave (19CO89) that was identified morphologically as *I. cantabriensis*, which shows a genetic divergence ranging from 6.5 to 6.7% when compared to other specimens of the same species of Torco de los Lobos population (see Table 2). These genetic distances are much higher than what we normally found between specimens of a same species (rarely exceeding 1.5%), but are still low among the crustaceans to be identified as a different genetic linage. Nevertheless, these value suggest that the study should be scaled up by adding more specimens and more genes (not only mitochondrial) to properly establish the boundaries between interpopulational variability and the species limits. Particularly it would be desirable to add nuclear loci that diverge fast enough to distinguish closely related cryptic species, and those could be used to create a reference genomic library that could be employed to compare and identify bathynellaceans species from different regions of the world.

In other invertebrate groups, these divergence values would be sufficient to erect new species. For example, in butterflies, Hajibabaei et al. (2006) have suggested that a 4.5 to 6.0% divergence in COI mtDNA sequences is enough to discriminate between congeneric species. At the moment, we still lack clear taxon definitions, and the demarcation of species using genetic divergences based in a single mitochondrial sequence is still imprecise. Nevertheless, based on our results, the use of sequence data to tackle issues associated with cryptic species and convergent evolution may be effective and even quite necessary. More cryptic species are being detected in groundwater habitats each day (Jarman and Elliot, 2000; Proudlove and Wood, 2003; Lefebure et al., 2006a; Finston et al., 2007; Lefebure et al., 2007; Trontelj et al., 2009) and based on the interesting results obtained in this study, we have no doubts that Bathynellacea, a group of organisms exclusively found in groundwater habitats, will contribute towards increasing the number cases where crustacean biodiversity is being underestimated.

Although the number of specimens used in the study is not large and the molecular information we have produced is modest due to the difficulty of obtaining abundant material in good condition and extracting DNA in the groundwater samples, we still consider the results relevant for our colleagues dedicated to the study of groundwater organisms, filling a major gap in our knowledge of groundwater bathynellaceans. In previous several papers, we have studied the relationships between the different species of *Iberobathynella* based on morphological characters (Camacho and Serban, 2000; Camacho et al., 2000; Guil and Camacho, 2001; Camacho et al., 2006b). Inevitably, the conclusions have always been tentative pending the discovery of new species that would allow for more robust assessment of kin relationships. Now, with the confirmation of cryptic species by applying molecular analyses to populations that had already been studied, we probe the use of a new body of molecular data that will complement the morphological data in stygobiont systematics (Camacho et al., 2002, 2011; Guzik et al., 2008).

The taxonomy of groundwater animals needs to be pluralistic and integrate newest techniques for species delimitation if it is to become a modern evolutionary discipline. The time is ripe to create the necessary links between the activities of molecular biologists (ongoing DNA barcoding initiatives) and the efforts of "traditional" taxonomists to accelerate species descriptions, especially cryptic species. The use of both classical taxonomic tools and molecular techniques, will generate knowledge about a group of invertebrates that is proving to be much more diverse than previously considered. The study of groundwater habitat, traditionally considered as poor in biodiversity but including relict fauna and authentic living fossils, will gain new interest.

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REFERENCES

Altschul, S. F., T. L. Madden, A. A. Schaeffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped blast and psi-blast: a new generation of protein database search programs. Nucleic Acids Research 25: 3389-3402.

Camacho, A. I. 1987. A new subterranean Sincarid (Crustacea) fron Spain: *Iberobathynella imuniensis* n. sp. (Bathynellacea, Parabathynellidae). Archiv fur Hidrobiologia 111(1): 137-149.

———. 1992. Sampling the subterranean biota. Cave (aquatic environment), pp. 135-168. In, A. I. Camacho (ed.), The Natural History of Biospeology. Monografías del Museo Nacional de Ciencias Naturales, 7. CSIC, Madrid, Spain.

———. 2003a. Four new species of groundwater crustaceans (Syncarida, Bathynellacea, Parabathynellidae) endemic to the Iberian Peninsula. Journal of Natural History 37: 2885-2907.

———. 2003b. An overview of the distribution of the Parabathynellidae (Crustacea Syncarida Bathynellacea) on the Iberian Peninsula and Balearic Islands. Graellsia 59(1): 63-78.

———. 2005a. One more piece in the genus puzzle: a new species of *Iberobathynella* Schminke, 1973 (Syncarida, Bathynellacea, Parabathynellidae) from the Iberian Peninsula. Graellsia 61(1): 123-133.

———. 2005b. Expanding the taxonomic conundrum: three new species of groundwater crustacean (Syncarida, Bathynellacea, Parabathynellidae) endemic to the Iberian Peninsula. Journal of Natural History 39(21): 1819-1838.

———. 2006. An annotated checklist of Syncarida (Crustacea, Malacostraca) in the world. Zootaxa 1374: 1-54.

———. 2007a. A new species of *Iberobathynella* Schminke, 1973 (Syncarida, Bathynellacea, Parabathynellidae) in the South of the Iberian Peninsula. Graellsia 63(2): 339-347.

———. 2007b. The first record of the genus Vejdovskybathynella Serban and Leclerc, 1984 (Syncarida, Bathynellacea, Bathynellidae) in the Iberian Peninsula: three new species. Journal of Natural History 41(45-48): 2817-2841.

———, and E. Serban. 1998. Tribu des Iberobathynellini nov., diagnoses des taxa nouveaux (Parabathynellidae, Bathynellacea, Podophallocarida). Travaux de l'Institut de Spéologie "Émile Racovitza" 34: 15-75.

———, and ———. 2000. Revisión del grupo *Iberobathynella* (*Iberobathynella*) Camacho & Serban, 1998 (Crustacea, Syncarida, Parabathynellidae) endémico de la Península Ibérica. Graellsia 56: 35-48.

———, B. A. Dorda, and I. Rey. 2011. Identifying cryptic speciation across groudwater populations: first COI sequences of Bathynellidae (Crustacea, Syncarida). Graellsia 67(1): 7-12.

———, E. Serban, and N. Guil. 2000. Phylogenetical review and biogeographic remarks on the interstitial and subterranean freshwater iberobathynells (Crustacea, Syncarida, Parabathynellidae). Journal of Natural History 34: 563-585.

———, I. Rey, B. A. Dorda, A. Machordom, and A. G. Valdecasas. 2002. A note on the systematic position of the Bathynellacea (Crustacea, Malacostraca) using molecular evidence. Contributions to Zoology 71: 123-129.

———, T. Torres, C. J. Puch, J. E. Ortiz, and A. G. Valdecasas. 2006b. Small-scale biogeographical patterns in some groundwater Crustacea, the syncarid, Parabathynellidae. Biodiversity and Conservation 15: 3527-3541.

———, A. G. Valdecasas, J. Rodríguez, S. Cuezva, J. Lario, and S. Sánchez-Moral. 2006a. Habitat constraints in epikarstic waters of an Iberian Peninsula cave system. Annales de Limnologie-International Journal of Limnology 42: 127-140.

Chappuis, P. A. 1915. *Bathynella natans* und ihre Stellung System. Zoologisches Jahrbuch, Systematic (Systematik, Geographie u. Biologie der Tiere) 40(1/2): 147-176.

Cho, J.-L., W. F. Humphreys, and S.-D. Lee. 2006. Phylogenetic relationships within the genus *Atopobathynella* Schminke (Bathynellacea: Parabathynellidae). Invertebrate Systematics 20: 9-41.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. Journal of Molecular Evolution 17: 368-376.

———. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.

———, and H. Kishino. 1993. Is there something wrong with the bootstrap on phylogenies? A reply to Hillis and Bull. Systematic Biology 42: 193-200.

Finston, T. L., and M. S. Johnson. 2004. Geographic patterns of genetic diversity in subterranean amphipods of the Pilbara, Western Australia. Marine and Freshwater Research 55: 619-628.

———, ———, W. F. Humphreys, S. M. Eberhard, and S. A. Halse. 2007. Cryptic speciation in two widespread subterranean amphipod genera reflects historical drainage patterns in an ancient landscape. Molecular Ecology 16: 355-365.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit 1 from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294-299.

Grobben, K. 1905. Lehrbuch der Zoologie, begründet von C. Claus, 1 neubearbeitet von Dr Karl Grobben. Auflage (7. umgearbeitete Aufl.): 1-955. N. G. Elwert'sche Verlagsbuchhandlung, Marburg, Hessen.

Gu, X., Y. X. Fu, and W. H. Li. 1995. Maximum likelihood estimation of the heterogeneity of substitution rate among nucleotide sites. Molecular Biology and Evolution 12: 546-557.

Guil, N., and A. I. Camacho. 2001. Historical Biogeography of Iberobathynella (Crustacea, Syncarida, Bathynellacea), an aquatic subterranean genus of Parabathynellids, endemic to the Iberian Peninsula. Global Ecology and Biogeography 10: 487-501.

Guzik, M. T., K. M. Abrams, S. J. B. Cooper, W. F. Humphreys, J.-L. Cho, and A. Austin. 2008. Phylogeography of the ancient Parabathynellidae (Crustacea: Bathynellacea) from the Yilgarn region of Western Australia. Invertebrate Systematics 22: 205-216.

Hajibabaei, M., D. H. Janzen, J. M. Burns, W. Hallwachs, and P. D. N. Herbert. 2006. DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Science 103: 968-971.

Hebert, P. D., A. Cywinska, S. L. Ball, and J. R. deWaard. 2003. Biological identifications through DNA barcodes. Proceedings of Biological Science 270: 313-321.

Huelsenbeck, J. P., and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754-755.

James, S. W., D. Porco, T. Decaëns, B. Richard, R. Rougerie, and C. Erséus. 2010. DNA barcoding reveals cryptic diversity in *Lumbricus terrestris* L., 1758 (Clitellata): resurrection of *L. herculeus* (Savigny, 1826). PLoS ONE 5: e15629.

Jarman, S. N., and N. G. Elliot. 2000. DNA evidence for morphological and cryptic Cenozoic speciations in the Anaspididae, "living fossils" from the Triassic. Journal of Evolutionary Biology 13: 624-633.

Jones, R., D. C. Culver, and T. C. Kane. 1992. Are parallel morphologies of cave organisms the result of similar selection pressures? Evolution 46: 353-365.

Kane, T. C., D. C. Culver, and J. Mathieu. 1994. Biotic fluxes and gene flow, pp. 245-259. In, J. Gibert, D. L. Danielopol, and J. Stanford (eds.), Groundwater Ecology. Academic Press, New York.

Lanave, C., G. Preparata, C. Sacone, and G. Serio. 1984. A new method for calculating evolutionary substitution rates. Journal of Molecular Evolution 20: 86-93.

Lefébure, T., C. J. Douady, and J. Gibert. 2006b. Relationship between morphological taxonomy and molecular divergence within Crustacea: proposal of a molecular threshold to help species delimitation. Molecular Phylogenetics and Evolution 40: 435-447.

———, ———, F. Malard, and J. Gibert. 2007. Testing vicariance and cryptic diversity in a widely distributed groundwater amphipod (*Niphargus rhenorhodanensis*). Molecular Phylogenetics and Evolution 42: 676-686.

———, ———, M. Gouy, P. Trontelj, J. Briolay, and J. Gibert. 2006a. Phylogeography of a subterranean amphipod reveals cryptic diversity and dynamic evolution in extreme environments. Molecular Ecology 15: 1797-1806.

Noodt, W. 1965. Natürliches System und Biogeographie der Syncarida. Gewässer und Abwässer 37/38 (1964): 77-186.

Porco, D., A. Bedos, and L. Deharveng. 2010. Description and DNA barcoding assessment of the new species Deutonura gibbosa (Collembola: Neanuridae: Neanurinae), a common springtail of Alps and Jura. Zootaxa 2639: 59-68.

Posada, D., and K. A. Crandall. 1998. Model test: testing the model of DNA substitution. Bioinformatics 14: 817-818.

Proudlove, G., and P. J. Wood. 2003. The blind leading the blind: cryptic subterranean species and DNA taxonomy. Trends in Ecology and Evolution 18: 272-273.

Richard, B., T. Decaens, R. Rougerie, S. W. James, D. Porco, and P. D. N. Hebert. 2010. Re-integrating earthworm juveniles into soil biodiversity studies: species identification through DNA barcoding. Molecular Ecology Resources 10: 606-614.

Ronquist, F., and J. P. Huelsenbeck. 2003. MrBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

Schminke, H. K. 1972. *Hexabathynella halophila* gen. n., sp. n. und die Frage nach der marinen Abkunft der Bathynellacea (Crustacea, Malacostraca). Marine Biology 15: 282-287.

———. 1973. Evolution, System und Verbreitungsgeschichte der Familie Parabathynellidae (Bathynellacea, Malacostraca). Akademie der Wissenschaften und der Literatur Mainz, Mathematisch-Naturwissenschaftliche Klasse, Mikrofauna des Meeresbodens 24: 1-192.

———. 1981. Adaptations of Bathynellacea (Crustacea, Syncarida) to life in the interstitial ("Zoea Theory"). Internationale Revue gesamten Hydrobiologie 66: 578-637.

Serban, E., and P. Lecler. 1984. Cinq taxa nouveaux des Bathynellidés de France (Bathynellacea, Podophallocarida, Malacostraca). Travaux de l'Institut de Spéologie "Émile Racovitza" 23: 7-18.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87: 651-701.

Stevens, M. I., D. Porco, C. A. D'Haese, and L. Deharveng. 2011. Comment on "Taxonomy and the DNA Barcoding Enterprise" by Ebach. Zootaxa 2838: 85-88.

Swofford, D. L. 2002. PAUP*: Phylogeny Analysis Using Parsimony (*and Other Methods), Version 4.0b10. Sinauer Associates Inc., Sunderland, Massachusetts.

———, G. L. Olsen, P. J. Waddell, and D. M. Hillis. 1996. Phylogenetic inference, pp. 407-514. In, D. M. Hillis, C. Moritz, and B. K. Mable

(eds.), Molecular Systematics. 2nd Edition. Sinauer Associates, Sunderland, Massachussetts.

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599.

Thomson, G. M. 1893. Notes on Tasmania Crustacea, with description of new species. Papers and Proceedings of the Royal Society of Tasmania 1892: 45-76.

Trontelj, P., J. C. Douad, C. Fisher, J. Gibert, S. Goricki, T. Lefébure, B. Sket, and V. Zaksek. 2009. A molecular test for cryptic diversity in ground water: how large are the ranges of macro-stygobionts? Freshwater Biology 54: 727-744.

Walsh, P., D. Metzger, and R. Higuchi. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10: 506-513.

Yang, Z. 1994. Estimating the pattern of nucleotide substitution. Journal of Molecular Evolution 39: 105-111.

Zwickl, D. J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Unpublished Ph.D. Dissertation, University of Texas at Austin.

———, and J. Balhoff. 2006. GARLIv0.954.

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