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# New *Zospeum* species (Gastropoda, Ellobioidea, Carychiidae) from 980 m depth in the Lukina Jama – Trojama cave system (Velebit Mts., Croatia)

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## Abstract

A new species of the eutroglobiont gastropod taxon *Zospeum* Bourguignat, 1856 is described. *Zospeum tholussum* sp. n. is characterized based on a population from the Lukina Jama–Trojama cave system (Velebit Mts., Croatia). A single living specimen occurred at 980 m depth. The species is morphologically related to *Zospeum amoenum* (Frauenfeld, 1856), but can be readily distinguished from the latter by the presence of a weak columellar fold and its dome-like structured 2nd whorl. DNA barcoding is capable to clearly delineate *Z. tholussum* from other *Zospeum* spp. as well.

## Keywords

DNA barcoding, cryptic species, biospeleology, eutroglobiont gastropod, cave-dwelling species, microgastropoda

## Introduction

Taxonomic research distinguishes eleven morphospecies (no subspecies considered) of the microgastropod taxon *Zospeum* Bourguignat, 1856 (Ellobioidea, Carychiidae) for the region of the Dinaric Alps (Bole 1974, Slapnik and Ozimec 2004, de Jong 2012). Their taxonomic history has been shaped by confusing and sometimes contradicting (sub-)species assignments, primarily as a result of the general problem to distinguish intraspecific and interspecific shell variability (Bole 1974), which for decades was the main source of taxonomic information. Recently, Weigand et al. (2011) implemented DNA barcodes to molecularly identify species (i.e. DNA barcoding) based on a fragment of the mitochondrial-encoded (mtDNA) Cytochrome C Oxidase Subunit I (COI) locus (Hajibabaei et al. 2007). In a subsequent study and by addressing the evolutionary history of Carychiidae, Weigand et al. (2013) revealed several morphologically unrecognized (cryptic) *Zospeum* species and demonstrated the suitability of molecular data for taxonomic purposes in this group.

All known *Zospeum* have lost visual orientation and are considered true eutroglobionts (see Sket 2008). Information on their biology is scarce, but species seem to possess a limited active movement (Slapnik 2001) and to prefer muddy and permanently wet subterranean microhabitats (Jochum et al. 2012), as generally found along the drainage system of a cave. Weigand et al. (2013) proposed that dispersal may primarily occur via passive transportation (e.g. by water or larger mammals).

Here, a new *Zospeum* population from the Lukina Jama–Trojama cave system situated in the Velebit mountain range of Croatia is characterized (Fig. 1). On the basis of molecular and conchological data, a new species from 980 m depth–*Zospeum tholussum* Weigand, sp. n.–is described.

## Methods

### Conchological measurements

Specimen shells were measured by taking individual images with 456 pixels corresponding to 1 mm. The total shell height (SH), shell width (SW), aperture height (AH), aperture width (AW) and the number of whorls were measured. Moreover, ratios for SH/SW and AH/AW were calculated.

### Non-invasive DNA isolation, PCR and sequencing

Specimen shells of live-collected *Zospeum tholussum* sp. n. (single specimen) and *Zospeum amoenum* (Frauenfeld, 1856) (two specimens) were kept intact by using a protocol for a non-invasive DNA isolation method originally described in Schizas et al. (1997) and partially modified after Böttger-Schnack and Machida (2011). Each specimen

was cleaned and then dried in a 0.2 mL PCR-tube by raising the temperature once up to 94 °C. A volume of 9 µL dd$H_2O$ and 1 µL 10x PCR-buffer were added. The mixture was heated for 2 min at 94 °C; 1.3 µL Proteinase K were added and the solution homogenized by gentle mixing. An incubation step was performed for 15 min at 55 °C followed by 10 min at 70 °C. The incubation was repeated once. 10 µL of Gene Releaser (Bioventures Inc.) were added and the following thermocycler protocol used: 30 s at 65 °C, 30 s at 8 °C, 1.5 min at 65 °C, 3 min at 97 °C, 1 min at 8 °C, 3 min at 65 °C, 1 min at 97 °C, 1 min at 65 °C, 5 min at 80 °C and storage at 4 °C. The reaction mixture including the intact shell was centrifuged for 1 min using a table centrifuge. The clean phase containing the DNA was collected and transferred to another 0.2 mL PCR-tube. The shell was cleaned from the remains of the Gene Releaser chemicals by alternate rinsing in water and ethanol. 10–15 µL of AE Buffer (DNeasy Kit, Qiagen) were added to the isolated DNA.

PCR conditions were the same as described in Weigand et al. (2011), sequencing was performed at Source BioScience (LifeSciences, Berlin, Germany) following the companies instructions.

### DNA barcoding

DNA barcodes, chromatograms, images, geographic data and further information of the genetically analysed *Zospeum tholussum* (BOLD-ID BARCA210-13) and *Z. amoenum* (BOLD-IDs BARCA211-13 and BARCA212-13) specimens are deposited in the Barcode of Life Database (BOLD) (Ratnasingham and Hebert 2007) in the project "Barcoding Carychiid Microsnails" [BARCA]. The obtained COI-sequences were compared with other *Zospeum* species in BOLD.

## Results

**Family Carychiidae Jeffreys, 1830**
**Genus *Zospeum* Bourguignat, 1856**

***Zospeum tholussum* Weigand, sp. n.**
http://zoobank.org/A3B380AD-B918-451B-866D-7FA59642A733
http://species-id.net/wiki/Zospeum_tholussum
Figs 1–4

**Material examined.** A single living specimen was collected on 31.07.2010 (leg. Jana Bedek), which is designated as the holotype specimen (BOLD-ID BARCA210-13) (Fig. 1, life image; Fig. 2, upper row). Eight shells comprise paratype specimens, of which one was partially broken and used to investigate the form of the columellar fold. Shells were collected on several days during the caving expedition from 29.07.

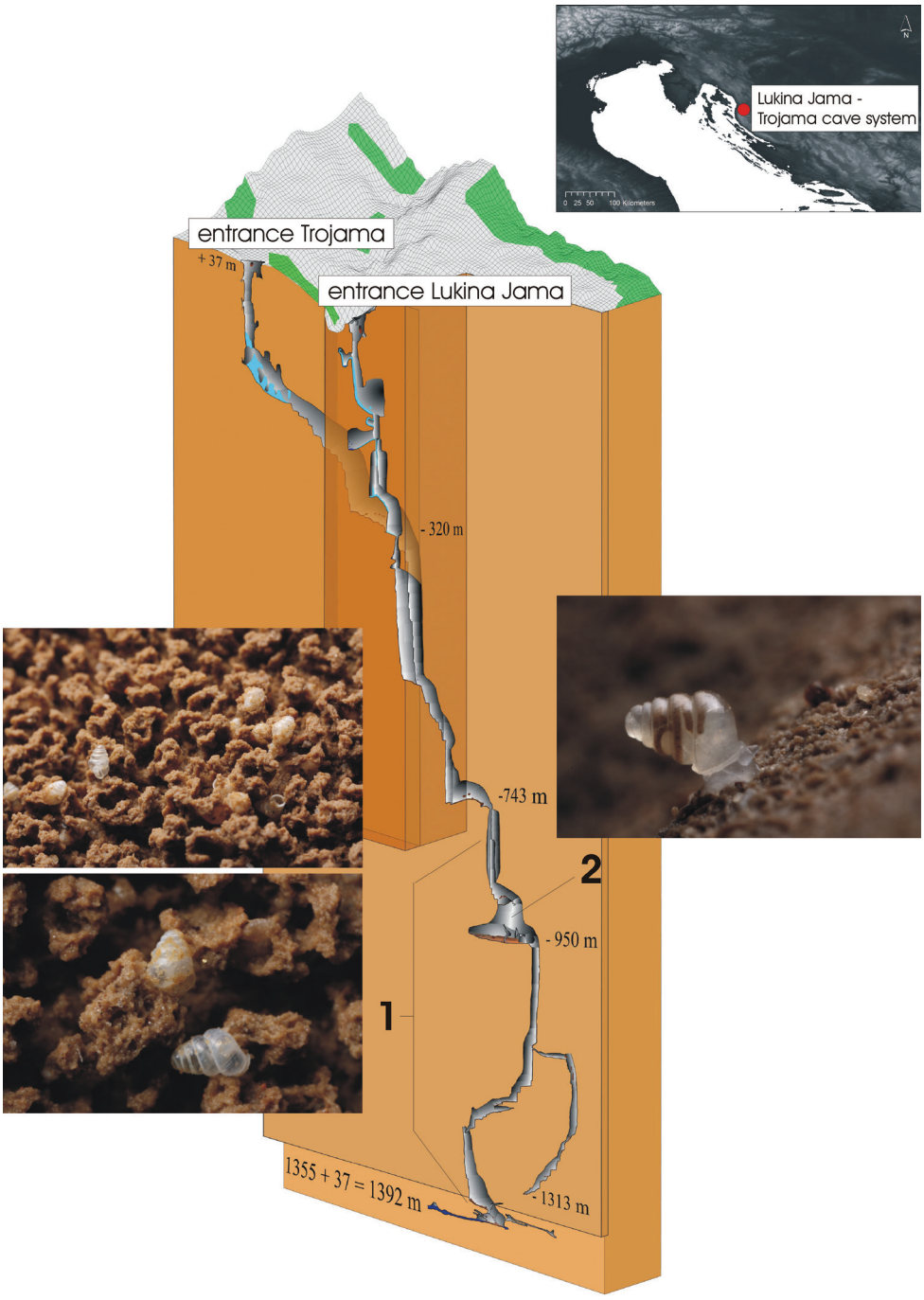


**Figure 1.** The Lukina Jama–Trojama cave system. Overview of the geographical position and 3D cave cross-section. In the latter, the region of collected shells (1) and the collection site of the living specimen of *Zospeum tholussum* (2) are indicated. The 3D cross-section was provided by D. Bakšić et al. (2010), Croatian Speleological Server, http://www.speleologija.hr/lukinajama. Photos were taken by J. Bedek.

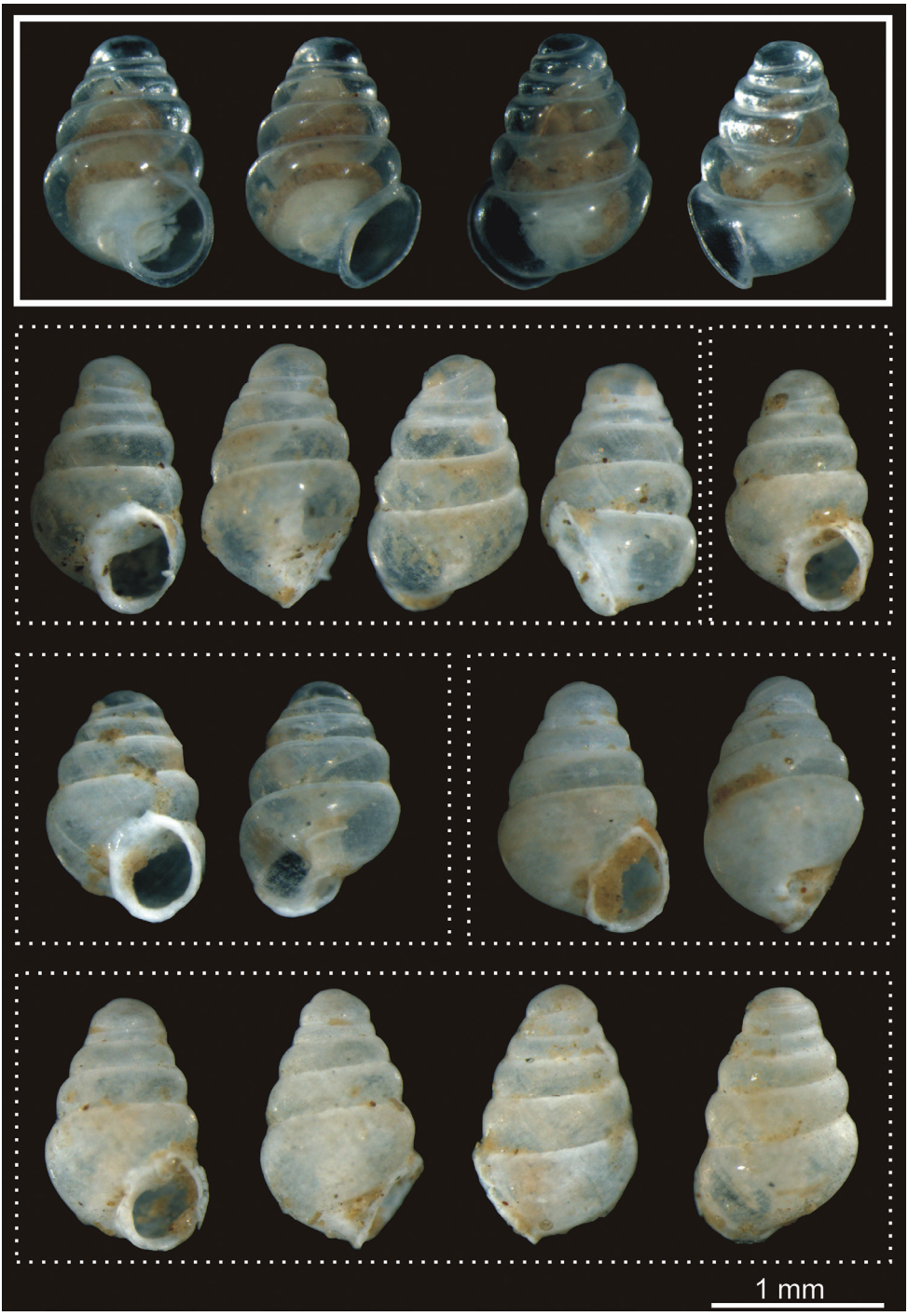


**Figure 2.** Holotype and paratypes of *Zospeum tholussum*. The holotype (former living specimen) is marked with a solid line; five paratype specimens (shells) are surrounded by dotted lines.

**Table 1.** Conchological measurements for *Zospeum tholussum*. Seven paratypes (Ind. 1–7) and the holotype specimen (Ind. 8) were measured. All measurements are given in millimeter. Additionally, ratios of shell height / shell width (sh/sw) and aperture height / aperture width (ah/aw) were calculated. The range of variability is given for all parameters.

| Ind. | sh/sw | ah/aw | shell height | shell width | aperture height | aperture width |
|---|---|---|---|---|---|---|
| 1 | 1.60 | 1.16 | 1.49 | 0.93 | 0.44 | 0.38 |
| 2 | 1.42 | 1.30 | 1.54 | 1.08 | 0.52 | 0.40 |
| 3 | 1.46 | 1.16 | 1.41 | 0.97 | 0.46 | 0.39 |
| 4 | 1.34 | 1.05 | 1.44 | 1.07 | 0.47 | 0.44 |
| 5 | 1.50 | 1.31 | 1.63 | 1.09 | 0.54 | 0.41 |
| 6 | 1.53 | 1.23 | 1.61 | 1.05 | 0.51 | 0.41 |
| 7 | 1.62 | 1.15 | 1.81 | 1.12 | 0.53 | 0.46 |
| 8 (holotype) | 1.48 | 1.12 | 1.47 | 0.99 | 0.44 | 0.39 |
| range | 1.34–1.62 | 1.05–1.30 | 1.41–1.81 | 0.93–1.12 | 0.44–0.54 | 0.38–0.46 |

- 03.08.2010 (Fig. 2). All investigated specimens are deposited in the Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt am Main, Germany (museum voucher SMF 341633).

**Diagnosis.** The general shape of *Zospeum tholussum* sp. n. resembles larger specimens of *Zospeum amoenum* (Frauenfeld, 1856) from which it can be best delineated by the dome-like structured 2$^{nd}$ whorl (Fig. 2), the weak but present columellar fold (Fig. 3) and by means of their DNA barcodes (11.7 %–12.1 % genetic p-distance between both species).

**Description.** The eight conchologically investigated specimens (the holotype and seven paratypes) demonstrate moderate shell variability (Table 1). Shells are generally smooth, very thin and fragile. Fresh shells or those of living specimens are translucent, older ones adopt a milky white color. The number of whorls are 5–6, SH ranges from 1.41–1.81 mm, SW from 0.93–1.12 mm, AH from 0.44–0.54 mm and AW from 0.38–0.46 mm. The ratio of SH/SW is between 1.34–1.62 and AH/AW between 1.05–1.30. The 2$^{nd}$ whorl is remarkably enlarged possessing a height of 2/3–5/6 the height of the 3$^{rd}$ + 4$^{th}$ whorl. The columellar is only weakly developed (Fig. 3) and no teeth (i.e. parietalis, palatalis or columellaris) are present. The DNA barcode of the holotype, when compared with other DNA barcodes of *Zospeum* species deposited in BOLD, shows its lowest interspecific genetic distance to *Zospeum pretneri* Bole, 1960 (5.6 %; BOLD-ID: BARCA120-10). This result is well above the barcoding gap of 3.2 % for Carychiidae, which is suitable to separate between-species (> 3.2 %, interspecific) and within-species genetic diversity (< 3.2 %, intraspecific) in *Zospeum* (Weigand et al. 2011, 2013).

**Habitat.** The single living specimen was found in an unnamed large chamber at 980 m depth (85 m long, 70 m wide) with lots of stones, rocks and sand (Fig. 1, right site). A temporal small stream of running water was present close to the collecting site. Air temperature was between 3.3–3.5 °C (depending on the measurement device),

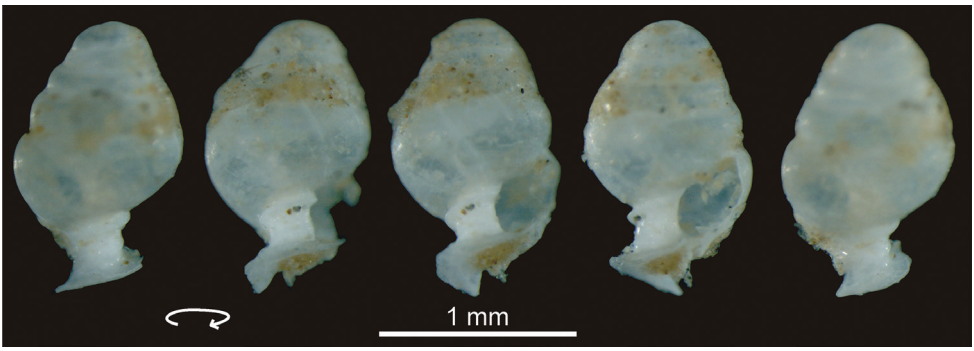


**Figure 3.** Columellar fold views of *Zospeum tholussum*. The columellar fold of the broken paratype specimen shell is shown in clockwise rotation.

water temperature 5.1 °C and air humidity 100 %. Shells were observed beginning from 800 m depth till the bottom of the cave. Shells were generally found on layers of mud (Fig. 1, left site). The first 200 m of the entrance passage of Lukina Jama are permanently covered by varying levels of snow and ice.

**Etymology.** The Latin word *tholus* means dome or cupola and refers to the remarkable dome-like shape of the 2nd whorl.

**Remarks.** In addition to the newly described *Zospeum tholussum*, a second *Zospeum* species is present in the Lukina Jama–Trojama cave system (Fig. 4C). This species can be differentiated from *Z. tholussum* by the presence of a tooth, its general shell shape, more prominent columellar fold and absence of the characteristic dome-like structured 2nd whorl. Because only shells were found, no DNA barcodes are available for this species.

So far, *Zospeum tholussum* is only known from the Lukina Jama–Trojama cave system. However, this cave system is situated in the distribution range of the morphologically related species *Zospeum amoenum* (Frauenfeld, 1856), which inhabits caves of the West Balkan of North Slovenia, West Croatia, Bosnia and Herzegovina and South to Montenegro (Bole 1974). Absolon (1916) named but did not describe another morphologically related species - *Zospeum troglobalcanicum*. Since Weigand et al. (2013) revealed that *Zospeum* spp. with an inferred large distribution range are particularly prone to host additional, morphologically unrecognized species, some of the distribution records for *Z. amoenum* may very well refer to *Z. tholussum*. Moreover, the intraspecific genetic and conchological variability of the new species should be investigated in greater detail, thus to judge about the taxonomic information content of the diagnostic characters applied within this description.

The preliminary information on the habitat of *Zospeum tholussum* is in congruence with previous findings for *Zospeum*. Interestingly, the grazing-labyrinth-like structure, in which most of the shells were embedded (Fig. 1, left site), has been already observed during caving expeditions in Northern Spain (Jochum et al. 2012). Finally, the presence of a temporal stream at the collecting site of the living specimen further supports a passive dispersal scenario as suggested by Weigand et al. (2013).

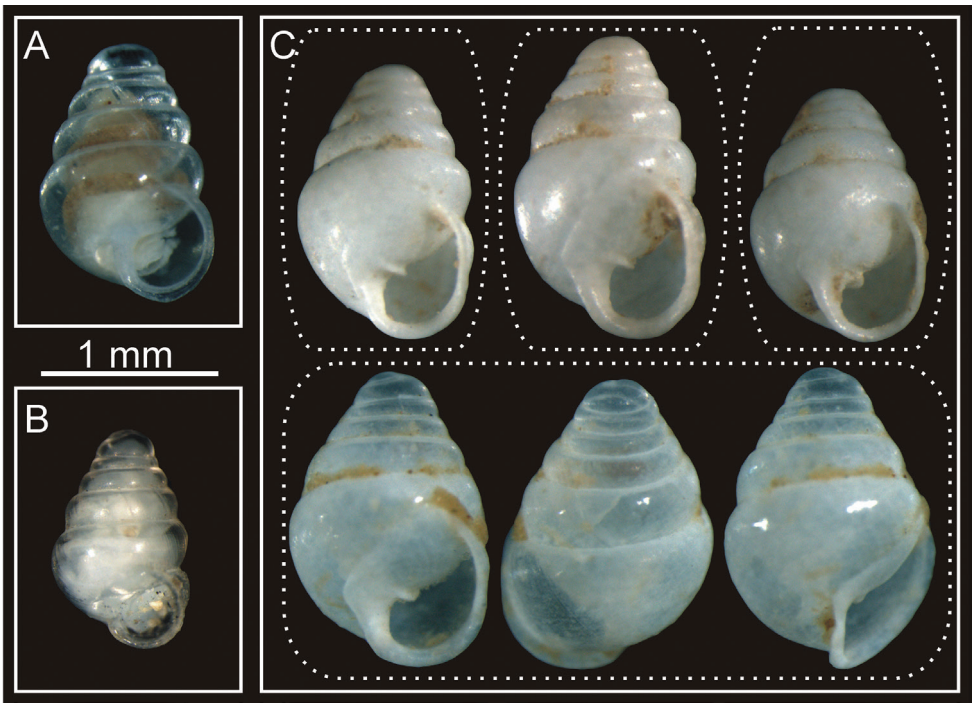


**Figure 4.** *Zospeum* species. **A** *Zospeum tholussum* holotype **B** *Zospeum pretneri* from *locus typicus*, which demonstrates the lowest genetic distance to *Z. tholussum* **C** Second *Zospeum* sp. from the Lukina Jama–Trojama cave system.

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