

## DESCRIPTION OF A NEW GENUS AND SPECIES AS THE FIRST GASTROPOD SPECIES FROM CAVES IN IRAN

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### ABSTRACT

We report on a new stygobiont truncatelloid gastropod from the sulfidic ponds of Tashan Cave in the Zagros Mountains of Southwest Iran. The hydrogen sulfide habitat resembles those hitherto known for gastropods from sulfide-rich caves in Romania, Italy and Greece. The newly described genus *Troglairanica* n. gen. with a newly described *T. tashanica* n. sp represent the first true stygobiont gastropods found in Iran. The phylogeny inferred from the mitochondrial (cytochrome oxidase subunit I) and the nuclear (histone 3) genomes, as well as anatomical evidence, place *T. tashanica* in the family Moitessieriidae Bourguignat, 1863. The new genus may represent an evolutionary relict from the post Miocene before the split of the families Moitessieriidae and Cochliopidae Tryon, 1866.

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### INTRODUCTION

The freshwater Mollusca of Iran have been a focus of research during past two decades (Mansoorian, 2001; Glöer and Pešić, 2009, 2012; Moghadam and Chegini, 2009; Mowlavi et al., 2009; Shahabuddin et al., 2012; Ektefa et al., 2013; Nouroozi, 2014), and the taxonomic position of the local Ponto-Caspian Hydrobiidae has been clarified only recently (Dellicado et al., 2016). Iran is situated at the interface of three zoo-geographic realms, Palaeartic, Oriental (Indo-Malayan), and Afrotropical (Ethiopian). The karstifiable carbonate formations cover around 11% of Iran's surface area with more than half (55.2%) being located at the Zagros Mountains in western Iran (Raeisi and Laumanns, 2012). Thus, it is not surprising that more than 90% of known Iranian troglobiont taxa are found in the Zagros karst formations (Malek-Hosseini and Zamani, 2017).

Tashan Cave, located near the Sarjooshar Village, harbors a unique ecosystem. The recent discovery of a blind fish, *Garra tashanensis* Mousavi-Sabet, Vatandoust, Fatemi and Eagderi, 2016, and of the isopod *Stenasellus tashanicus* Khalaji-Pirbalouty, Fatemi, Malek-Hosseini, Kuntner, 2018, has been followed by other discoveries including the new gastropod genus and species reported here. The Tashan Cave represents a specific hypogean habitat with several cave ponds containing groundwater rich in hydrogen sulfide. Earlier studies performed in sulfide-rich caves by Hose et al. (2000); Engel (2012); and Jones et al. (2014) have shown that chemoautotrophic sulfur-oxidizing microorganisms thrive in such environments using the redox interface between the sulfide in the water and the oxygen in the atmosphere to produce food *in situ*, independent of the external light energy. Sulfuric acid is a product of the bacterial sulfur oxidation and accelerates the dissolution of the limestone bedrock in the process called *sulfuric acid speleogenesis* (SAS), which results in the formation of *sulfide caves* (Engel et al., 2004; Porter et al., 2009). It is estimated that hydrogen sulfide-rich phreatic waters and microbially-generated sulfuric acid were involved in the speleogenesis of around 10% of worldwide known caves (Palmer, 2007). However, only a few of these caves remain active ducts containing hydrogen sulfide-rich groundwater. The sulfide caves host interesting ecosystems with unusually rich and diverse invertebrate communities dominated by crustaceans (Peterson et al., 2013; Por, 2014), insects (Tobler et al., 2013) and occasionally fishes (Reisch et al., 2010; Roach et al., 2011; Mousavi-Sabet et al., 2016). While sulfide-based ecosystems are usually well-recognized inside deep sea trenches (Deming and Baross, 1993), the groundwater sulfide ecosystems remain poorly studied (Engel, 2007). The sulfide cave ecosystems received attention with the studies of Sarbu et al. (1996). The first studied cave containing a sulfide ecosystem was Movable Cave in Romania (Sarbu and Popa, 1992; Sarbu et al., 1996; Sarbu, 2000; Engel, 2012), followed by the Frasassi caves and Grotto Azzurra in Italy (Macalady et al., 2007; Peterson et al., 2013), Tito Bustillo and Maltravieso caves in Spain (Schabereiter-Gurtner, 2002; Arzo et al., 1997), Fiume Coperto Cave in Italy (Latella et al., 1999), Melissotrypa Cave in Greece (Falniowski and Sarbu, 2015), El Hamma in Tunisia (Por, 1963), Ayalon Cave and the Tabgha Spring in Israel (Por, 1963; 2007; 2011), Kugitangtuo caves in Turkmenistan (Maltsev and Korshunov, 1998), Cueva de Villa Luz in Mexico (Hose et al., 2000; Engel, 2007), Bungonia and Nullarbor caves with Bunder Sinkhole in Australia (Holmes et al., 2001; Jaime et al., 2001), Lower Kane

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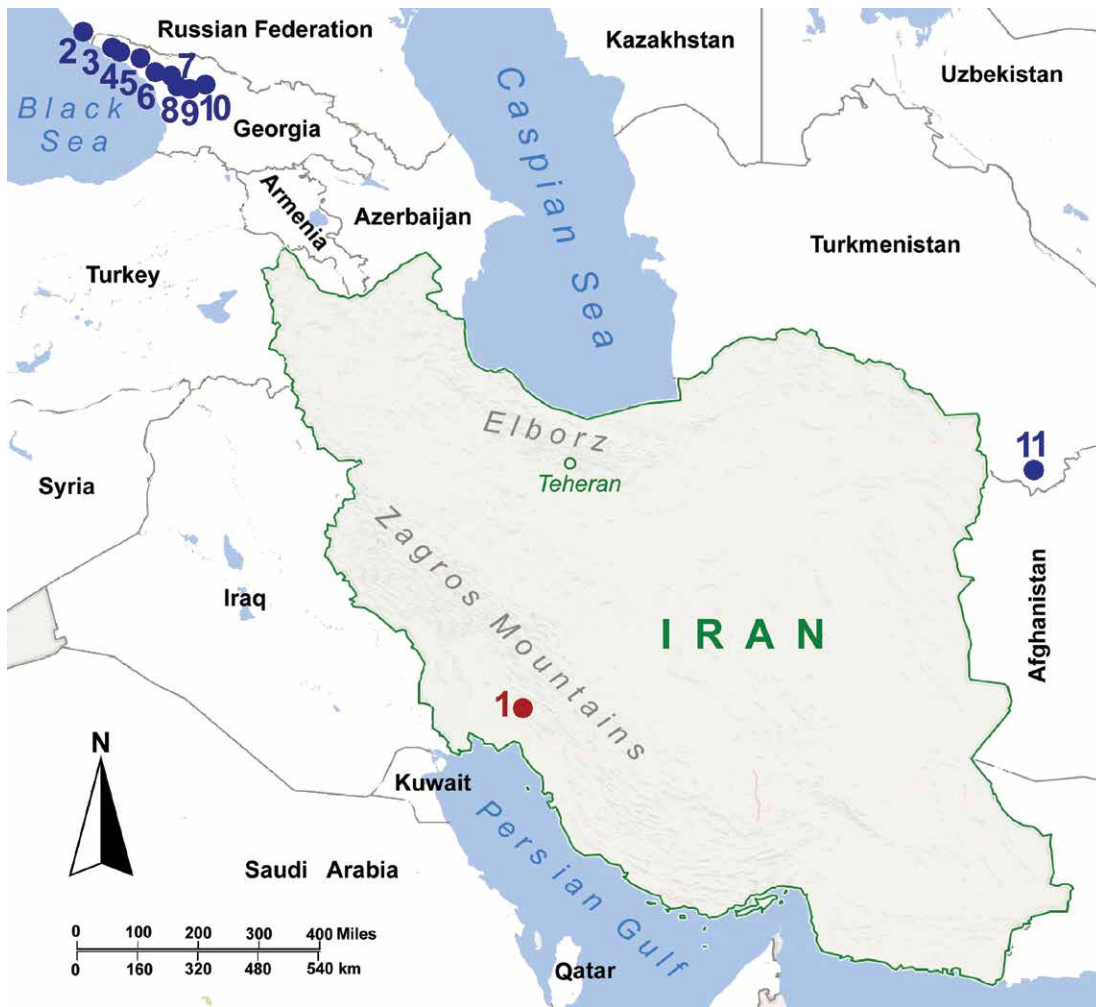
Cave in Wyoming, USA (Porter et al., 2002), Cesspool Cave in Virginia, USA (Engel et al., 2001), and the Mammoth and Parker Caves in North America (Hutchins et al., 2016).

Hydrogen sulfide is toxic for most organisms (Kelley et al., 2006), and only five of the around twenty so far known sulfide cave ecosystem sites host stygobiont gastropod species, as likely outcomes of extreme sulfide adaptation: *Heleobia dobrogica* (Grossu and Negrea, 1989) from Movile Cave (Falniowski et al., 2008), *Islamia sulfurea* Bodon and Cianfanelli, 2012 from the Frasassi caves (Bodon et al., 2009; Bodon and Cianfanelli, 2012), *Physella spelunca* Turner and Clench, 1974 from Lower Kane Cave in Wyoming (Porter, 2002; Wethington and Guralnick, 2004) as well as *Iglica hellenica* and *Daphiola magdalenae* (Falniowski and Sarbu, 2015) from Melissotrypa Cave in Greece. The discovery of a sixth sulfidic stygobiont gastropod species in the Tashan Cave of Iran, reported here, is thus of general ecological importance.

## MATERIAL AND METHODS

Snails were collected from pools in Tashan Cave, located under a limestone plateau close to Sarjooshar Village in the south-eastern part of the Zagros Mountains, Tashan district, Behbahan County, Khuzestan Province, southwest Iran (Fig. 1 and 2). Specimens were collected by using dropper pipettes and forceps and preserved in 96 % ethanol. A few specimens were transferred into a small rimstone basin at the cave pool shelf for photographing and subsequently returned to the pool.

The sampled snails were fixed in 80% ethanol. The dissections were done under a NIKON SMZ18 microscope with dark field, and the structures were photographed with a CANON EOS 50D digital camera. Frontal, ventral, and lateral images of the holotype were made using a Nikon SMZ25 microscope with a Nikon D200 camera and an AF-S Micro NIKKOR 60 mm lens at the Vienna Natural History Museum (NHMW), Austria. Morphological terms follow Hershler and Ponder (1998).



DNA was extracted from foot tissue, hydrated in Tris-EDTA (TE) buffer, using a Sherlock extraction kit (A&A Biotechnology), and dissolved in 20 mL of TE buffer. The extracted DNA was stored at  $-80^{\circ}\text{C}$  at the Department of Malacology of Institute of Zoology and Biomedical Research of the Jagiellonian University in Kraków. Our study targeted nucleotide data from the mitochondrial (cytochrome oxidase subunit I - COI) and the nuclear (histone 3 - H3) genomes, to produce phylogenetic inference from individual and combined gene datasets. Details of PCR conditions, primers used, and sequencing methods follow Szarowska et al. (2016). Sequences were aligned by MUSCLE (Edgar, 2004) implemented in MEGA 6

Figure 1. Map of Iran with the type locality of *Troglairanica tashanica* n. sp.: Khuzestan Province, Behbahan County, Tashan district, Sarjooshar Village, Tashan Cave (1 - Red ring) and geographically closest records of stygobiont gastropod species from SW Caucasus (2-10 - Blue dots) and from Turkmenistan, Kushka, Shar-Arab spring (11 - Blue dot).

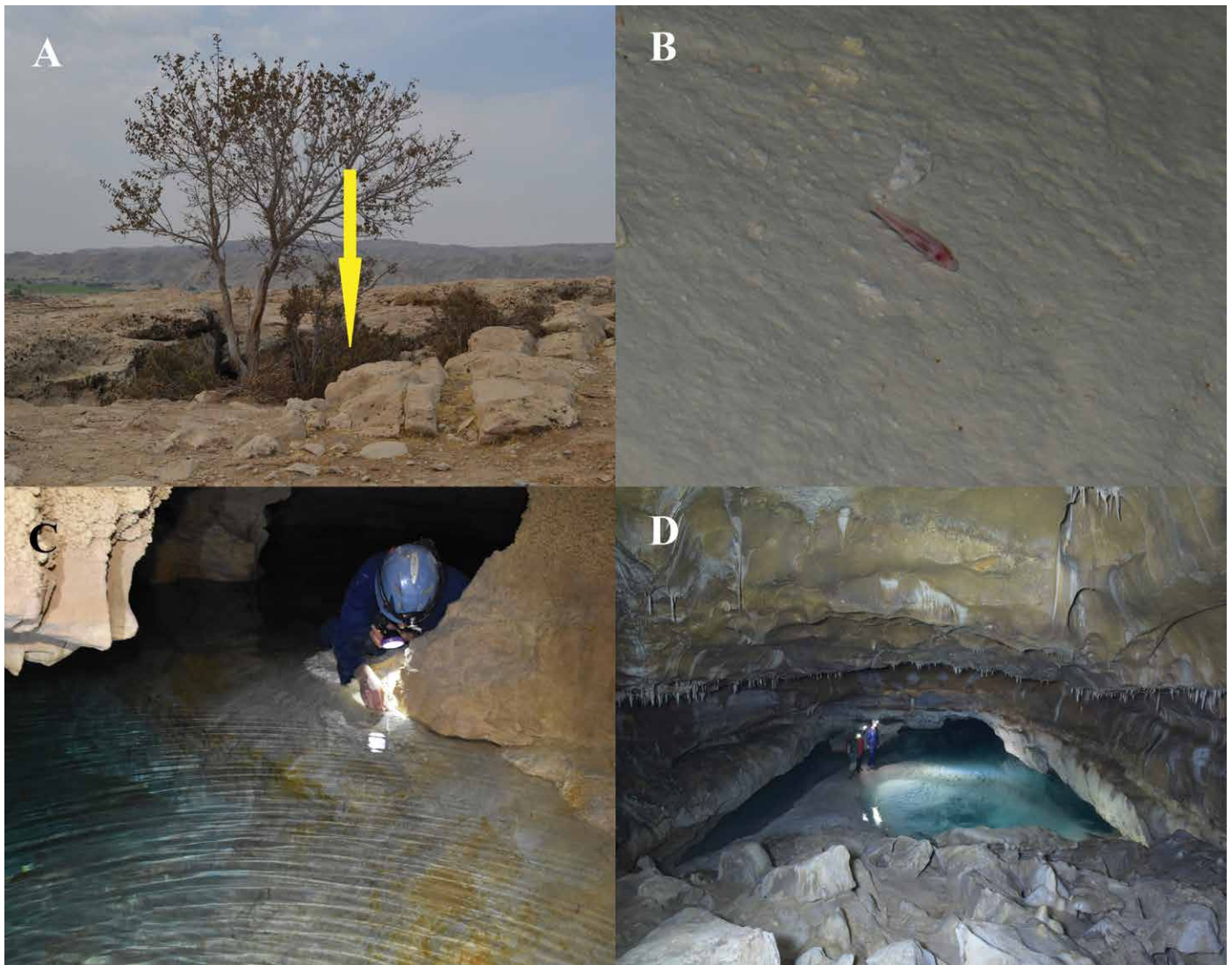


Figure 2. Photos of the type locality of *Troglairanica tashanica* n. sp.: A: Tashan Cave entrance on the semi-desert limestone plateau; B hydrogen sulfide-rich pond inside the Tashan cave showing *Garra tashanensis*; C and D: sampling at the type locality (photo: Mohammad Javad Malek-Hosseini and Yaser Fatemi).

(Tamura et al., 2013) and in Bioedit 7.1.3.0 (Hall, 1999). The saturation test (Xia, 2000, Xia et al., 2003) was performed using DAMBE (Xia, 2013). Additional sequences from GenBank were used in the phylogenetic analysis (Table S1). The phylogenies were inferred using Bayesian inference (BI) and maximum likelihood (ML).

The Bayesian analyses were run using MrBayes v. 3.2.3 (Ronquist et al., 2012) with the default priors. Two simultaneous analyses were performed, each of which lasted 10,000,000 generations, with one cold chain and three heated chains, starting from random trees and sampling the trees every 1,000 generations. The first 25 % of trees were discarded as burn-in. The analyses were summarized as a 50 % majority-rule tree. The ML approach was applied with RAxML v. 8.0.24 (Stamatakis, 2014). We applied the GTR model, whose parameters were estimated by the RAxML (Stamatakis, 2014). One thousand searches were initiated with starting trees obtained through the randomized step-wise addition maximum parsimony method. The tree with the highest likelihood score was considered as the best representation of the phylogeny. Bootstrap support was calculated with 1,000 replicates and summarized on the best ML tree. RAxML analyses were done in CIPRES Science Gateway (Miller et al., 2010).

#### Abbreviations

NMBE	Naturhistorisches Museum, Bern, Switzerland
NHMUK	Natural History Museum, London, UK
NHMW	Natural History Museum, Vienna, Austria
ZMUH	Zoological Museum, University of Hormozgan, Iran

SMF	Senckenberg Museum, Frankfurt, Germany
H	Shell height
W	Shell width
BH	Height of the body whorl
BW	Width of the body whorl
AH	Aperture height
AW	Aperture width
LT	Type locality

## RESULTS

The molecular and anatomical investigation of the live gastropods from the type locality in Tashan Cave revealed it to be a new species belonging to a new genus in Moitessieriidae Bourguignat, 1863.

### Superfamily Truncatelloidea Gray, 1840

### Family Moitessieriidae Bourguignat, 1863

### Genus *Trogloiranica* n. gen.

#### Diagnosis

The diagnostic features of the genus are the same as those of the type species, *Trogloiranica tashanica* n. sp. The oval shell shape with low spire is unique within the Moitessieriidae. Nevertheless, similar shell shapes seem to have convergently evolved in other truncatelloid families such as Hydrobiidae Stimpson, 1865, Cochliopidae Tryon, 1866, Pomatiopsidae Stimpson, 1865, and Tateidae Sacco, 1896.

#### Etymology

The name derives from a prefix troglo- referring to the cave habitat of the new taxon and suffix -iranica referring to the country of origin.

*Trogloiranica tashanica* sp. n.

#### Type locality

Iran, Khuzestan Province, Behbahan County, Tashan district, Sarjooshar Village, sulfidic freshwater ponds inside Tashan Cave, 30°51'54"N; 50°10'29"E (altitude 559 m a.s.l.).

#### Type material

Holotype: type locality: Yaser Fatemi and Mohammad Javad Malek-Hosseini leg. August 23, 2017 (holotype NMBE 558282).

Paratypes: type locality (same data) one paratype NMBE 558283; one paratype in each lot of ZMUH 123, 124 and 125; SMF 358125 one specimen, four specimens in coll. Grego; type locality Yaser Fatemi and Mohammad Javad Malek-Hosseini leg. August 27, 2017, two specimens in coll. Malek-Hosseini; type locality (same data); Yaser Fatemi and Mohammad Javad Malek-Hosseini leg. 17. March 2018 coll. 10 specimens in coll. Malek-Hosseini, two in coll. Jagiellonian University, Krakow.

#### Measurements

Holotype: H 2.39 mm; W 1.69 mm; BH 1.73 mm; NW 1.14; AH 1.69, AW 0.86 (holotype). Figure 3, A1-A6.

#### Diagnosis

The low spire with oval shell shape of the new species is rather unusual within the family Moitessieriidae, but a similar shape with more elevated spire is present in *Bythiospeum lamperti* (Geyer, 1907). Similar shell morphology can be found in representatives of other truncatelloid families such as: *Pseudamnicola hauffeni* Delicado and Ramos, 2012, Hydrobiidae from Spain; *Aroapyrgus passionensis* Goodrich & Van der Schalie, 1937, Cochliopidae from Guatemala; *Tricula spelaea* Grego, 2018, Pomatiopsidae from Laos; *Pseudotricula auriforma* Ponder, Clark, Eberhard and Studer, 2005, Tateidae from Tasmania, and in the hydrobiid *Turkmenamnicola lindholmi* (Zhadin, 1952) from Turkmenistan. The cochliopid *Heleobia dobrogica* (Grossu & Negrea, 1989) found in similar sulfidic habitats in Movile Cave, Romania, has much more elongate shell shape. However, the molecular and anatomical data distinguish the new species from all other morphologically convergent species.

#### Description

Oval shell with conical spire with four rounded slightly convex whorls with a semi-deep suture and a blunt apex. Shell surface smooth with faint transverse growth lines covered by horny yellowish periostracum and partly by precipitated reddish-brown mineral crystals especially at the suture and marginal part of the body whorl. Aperture elongate-oval,



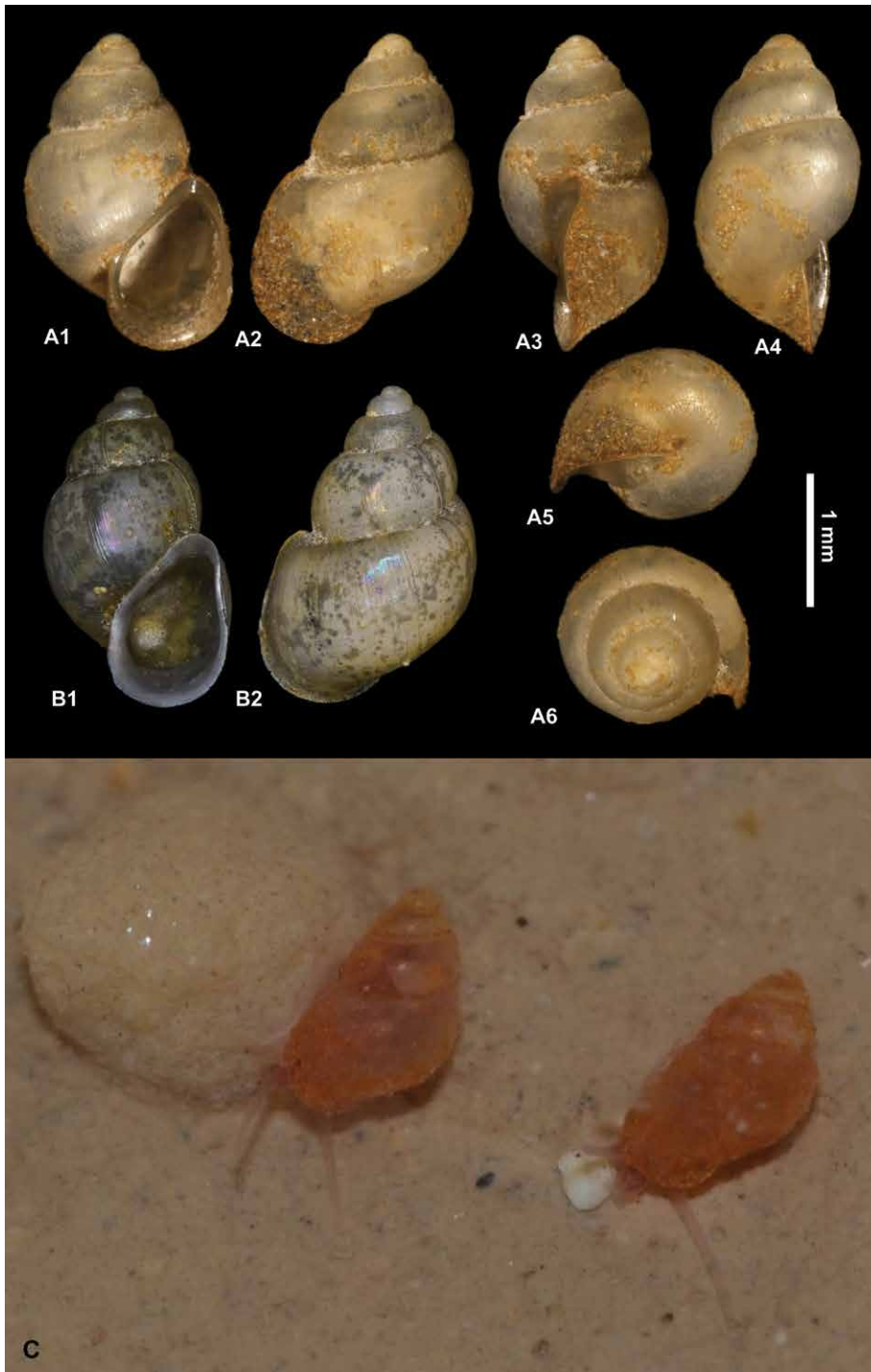


Figure 3. *Trogloiranica tashanica* n. sp., Iran, Khuzestan Province, Behbahan County, Tashan District, Sarjooshar Village, Tashan Cave. A1-A6: Holotype NMBE 558282; B1-B2: Paratype ZMUH 123; C: Live specimens of *Trogloiranica tashanica* n. sp., in their natural habitat within sulfide ponds of Tashan Cave (Specimens were transferred into a small stet basin at the pool shelf for photography) (photo: Jozef Grego, Yaser Fatemi and Mohammad Javad Malek-Hosseini).

clear H3 (Fig. 7) clearly demonstrated that *T. tashanica* belongs to the Moitessieriidae (Bootstrap support 86%). Likewise, the concatenated analysis supported this (Fig. 8).

**Etymology**

The species is named after the type locality, the Tashan Cave.

pear-shaped and slightly depressed form labral side, adapically separated from the body whorl by a weak furrow. Peristome margin expanded especially at its lower side and blunt along its outline. The labral lip characteristically sinuous in lateral view, columellar lip straight. Umbilicus closed and obscure.

**Anatomy**

Animal without eyes and pigment. Female reproductive organs (Fig. 4) typical of the Moitessieriidae: with rather big bursa copulatrix and one small receptaculum seminis (distal one (i.e., in the position of  $rs_1$  after Radoman 1983)). Penis (Fig. 5) simple, without any outgrowths. Loops of rectum (Fig. 5) with the faecal pellets arranged characteristically for the Moitessieriidae (Boeters & Gittenberger 1990), like a bandolier, not a chain.

**Molecular phylogenetic relationships**

We present two original COI (cytochrome oxidase subunit I) (552 bp, GenBank Accession numbers MK906039-MK906040) and two histone 3 sequences (283 bp, GenBank Accession numbers MK906041-MK906042). In both of these protein-coding loci the tests of Xia et al. (2003) revealed no saturation. Topology of the trees obtained with BI and ML analyses were identical. The obtained topologies using BI and ML were congruent. The phylogeny inferred with COI (Fig. 6) suggested a close relationship with the Moitessieriidae, but did not confirm the monophyly of the latter. Between the clade consisting of the *Bythiospeum* and the other grouping *Iglica* and *Paladilhopsis* there were representatives of the family Cochliopidae, and *T. tashanica* between the Cochliopidae and *Iglica*/*Paladilhopsis* clade. However, the low bootstrap supports are typical of deep nodes inferred with COI. On the other hand, the phylogeny inferred with nu-

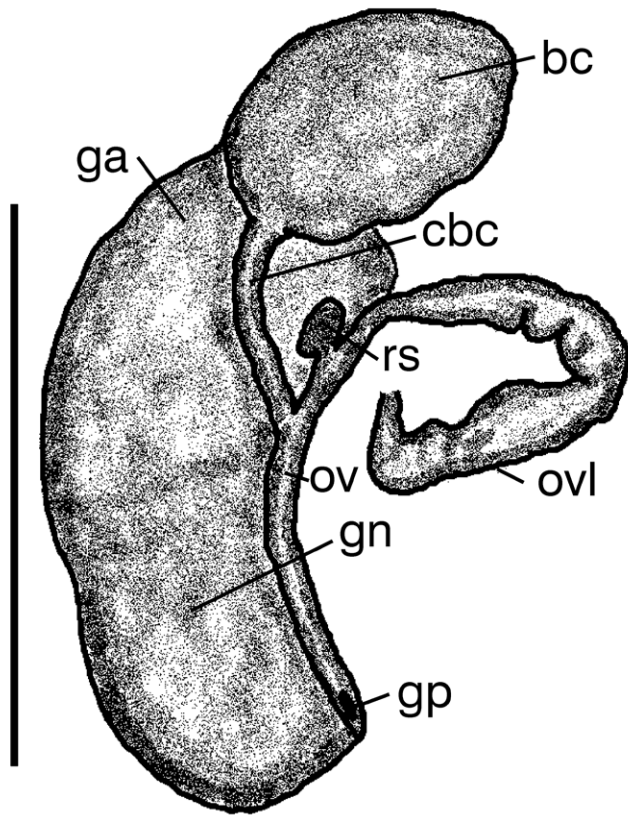


Figure 4. Renal and pallial part of the female reproductive organs of *Trogloiranica tashanica* n. sp. ((bc—bursa copulatrix, cbc—duct of bursa copulatrix, ga—albumen gland, gn—capsule gland, gp—gonopore, ov—oviduct, ovl—loop of oviduct, rs—seminal receptacle); bar equals 0.5 mm.

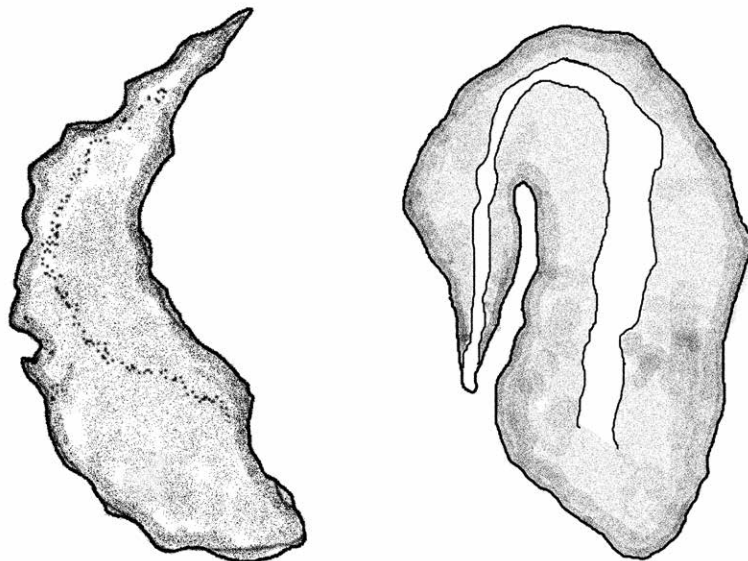


Figure 5. *Trogloiranica tashanica* n. sp., A. penis: at the left dorsally, in situ, in natural position, at the right ventrally, somewhat flattened, under the cover sleep. B. loops of the rectum, with the faecal pellets arranged characteristically for the Moitessieriidae, like a bandolier, not a chain.

*gloiranica tashanica* n. sp. is the first obligate cave-dwelling snail from Iran. The closest stygobiont gastropod records to this Iranian species can be found in the south-eastern part of Greater Caucasus in Georgia, in the vicinity of Sochi

## Distribution

Known only from the type locality.

## Ecology

Tashan Cave developed in Miocene limestones of Upper Red—Fars formation in the southeast part of the Zagros Mountain chain. The cave is situated under a semi-arid steppe plateau near the Sarjooshar village. The mostly horizontal cave with numerous passages and domes is several hundred meters long (detailed cave mapping is currently in progress). The morphology of the cave suggests a strong influence of sulfide-rich hydrothermal waters during the process of speleogenesis. Numerous pools and lakes inside the dark zone of the cave are rich in hydrogen sulfide, which is the main energy substrate for chemoautotrophic bacteria that utilize CO<sub>2</sub> and methane as a carbon source (Palmer, 2007). Preliminary stable isotope data (Sarbu, personal communication) suggest that the microbial biofilms that thrive in the cave pools represent the main source of food for the rich cave fauna including the *Trogloiranica tashanica* n. sp., the blind fish *Garra tashanensis* Mousavi-Sabet, Vatandoust, Fatemi and Eagderi, 2016, and the isopod *Stenasellus tashanicus* Khalaji-Pirbalouty, Fatemi, Malek-Hosseini, Kuntner, 2018 that were recently described from this cave. Specimens of *T. tashanica* n. sp. were found on the limestone walls, on the bottom rocks and on the mud covered by microbial biofilm in the cave pools. The water temperature was 25.4 °C and the pH was 7.8. The atmospheric conditions in the cave are almost constant throughout the year at 25.2 °C – 25.4 °C, 530 ppm – 755 ppm CO<sub>2</sub> saturation and 99.9 % relative humidity.

## DISCUSSION

Despite their ecological sensitivity and importance, subterranean ecosystems are very poorly studied worldwide and, except the areas of southern Europe, Balkans, southwestern Caucasus, eastern United States with north-east Mexico, Japan, Southeast Asia, New Zealand, and Tasmania, stygobiont gastropod records are extremely scarce (Bole and Velkovrh, 1986; Kabat and Hershler, 1993; Culver, 2012). This distribution represents only our present knowledge, while, most likely, the stygobiont Gastropoda inhabit all suitable habitats worldwide. The subterranean fauna of Iran is poorly known. Some cave-adapted groups of animals such as fishes (4 species), Crustacea (amphipods and isopods, more than 20 species), diplopods, and insects have been reported from Iran (Malek-Hosseini and Zamani, 2017). We suggest the putative presence of several groups of animals in hypogean environments of Iran, but a lot of field work is needed. *Tro-*

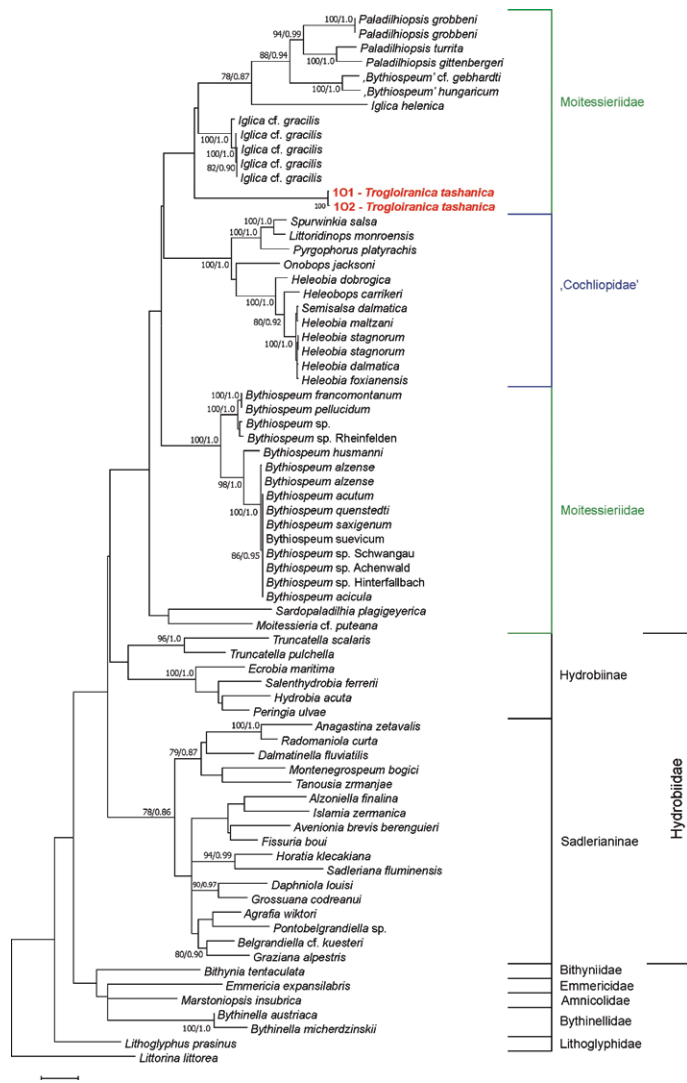


Figure 6. ML phylogeny based on COI. Bootstrap supports if > 70 % and Bayesian probabilities given. New sequences of *Troglairanica tashanica* are indicated in bold red; for the other sequences, see Table S1.

in Russia (Starobogatov, 1962; Vinarsky et al., 2014), and southern Turkmenistan (Zhadin, 1952), where we could expect the closest relations. The records from the Balkans (Glöer and Grego, 2015, Grego et al., 2017) in west and southeast Asia (Grego, 2018) towards the East, are likely not at all closely related to the Ponto-Caspian stygobionts, as it is proven for the related freshwater crenobionts of the family Hydrobiidae (Delicado et al., 2016). Of the five other known sulfide stygobiont gastropods, *Heleobia dobrogica* from Movile Cave represents the closest known lineage to *T. tashanica* n. sp. not only geographically, but also ecologically and by its three million-year old separation from its surface relatives (Falniowski et al., 2008). It appears that the other four known sulfidic stygobionts from the Frasassi caves in Italy, Lower Kane Cave in Wyoming, and Melissotrypa Cave in Greece could represent species with much younger adaptation history (Wethington and Guralnick, 2004). The communication of *Islamia sulfurea* with their surface relatives in the Frasassi caves (Bodon et al., 2009) is indicated by the distribution of the same species in other habitats and by the presence of the recent invader *Potamopyrgus antipodarum* Gray, 1843 at the same locality. This invasive species was found for the first time in Europe in 1889, and in Italy in 1961. Considering the special and rich biodiversity encountered in sulfidic caves and the estimation that 10% of the world's caves were formed by sulfidic speleogenesis (in some regions such as Greece these estimates reach up to 20 % of the accessible caves (Vaxevanopoulos, 2009), we hypothesize that the sulfide-induced high subterranean diversity persisted in most of these ex-sulfidic caves after the input of hydrogen sulfide faded out. According to our experience, the ex-sulfidic cave Tahm Nam Dôn in Khammouane, Laos, hosted significantly higher stygobiontic gastropod diversity than any other neighboring cave without traces of the sulfide corrosion in its vicinity (Grego, 2018). If so, the importance

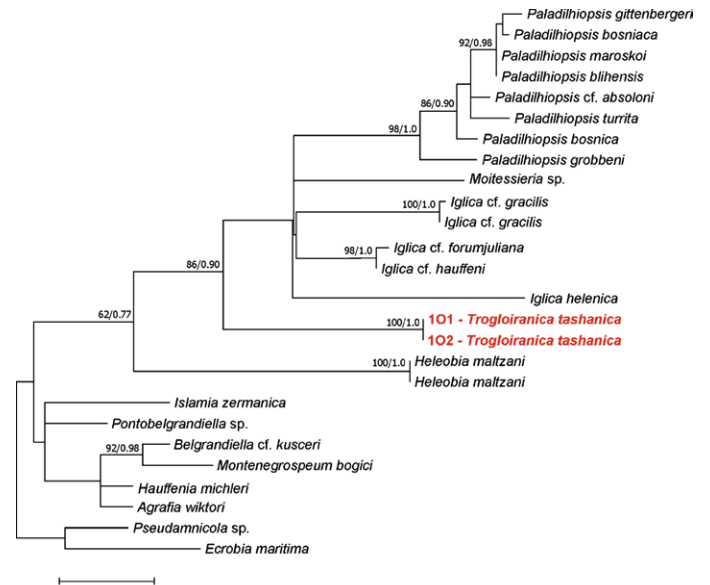


Figure 7. ML phylogeny based on H3. Bootstrap supports if > 60 % and Bayesian probabilities are given. New sequences of *Troglairanica tashanica* given in bold red for the other sequences see Table S1.

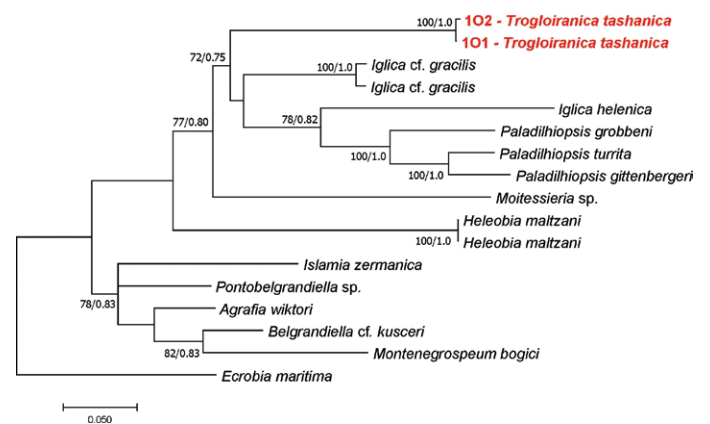


Figure 8. ML phylogeny based on concatenated data from COI and H3. Bootstrap supports if > 60 % and Bayesian probabilities are given. New sequences of *Troglairanica tashanica* given in bold red.

of the sulfidic processes in the formation of the recent stygobiont diversity would be significantly strengthened, and the high subterranean diversity of many isolated subterranean environment could be better explained.

## CONCLUSIONS

Tashan Cave likely represents another chemoautotrophically based cave ecosystem. The new species record is the sixth worldwide known stygobiont gastropod inhabiting subterranean sulfide-rich waters and the first records of stygobiont gastropod from Iran, indicating the large potential of the country for the presence of additional subterranean life forms. Although the whole ecosystem has not been well understood yet, at least we know that the water contains sulfide. The long natural history leads the subterranean ecosystems towards narrow, sometimes extreme sulfide specialization by ecological adaptation to the stable underground environment. Such a narrow specialization and long-term stable conditions reduced the adaptability of all the single ecosystem components and made it extremely sensitive against all, even very small changes, in the environmental conditions. The resulting very fragile web could be to some extent regarded as a multisymbiotic system. The disappearance of one component can lead to the collapse of the entire ecosystem. That is the main reason why the protection and conservation of such unique habitats as the Tashan Cave ecosystem have worldwide importance. Most of the caves worldwide hosting such rich sulfide ecosystems are heavily protected with restricted access, reduced to a small number of researchers per year. We believe the new locality also deserves such strict protection and that with the help of local environmentalists and cavers it can be soon achieved. Unfortunately, due to the negligence of authorities, cavers, locals, and even the research groups, a lot of habitat destruction has already occurred in this recently-discovered cave.

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