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New records and unusual morphology of the cave hydrozoan *Velkovrhia enigmatica* Matjašič & Sket, 1971 (Cnidaria: Hydrozoa: Bougainvilliidae)

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Abstract. *Velkovrhia enigmatica* is the only freshwater hydrozoan living exclusively in groundwater. It is endemic to the Dinarides in the Balkan Peninsula, where it has been known from four caves only. Here we report on a new *V. enigmatica* population from cave Logarček near Laze in southwestern Slovenia. In addition, after almost 30 years since its last recorded presence, we confirm the current presence of numerous *V. enigmatica* individuals in the type locality, Planinska jama. Individuals from the two caves were morphologically different: polyps from the type locality had 4–9 tentacles, while the ones from Logarček had 14–36 tentacles. The mitochondrial DNA sequences in populations from both caves did not differ. Additional research is needed to provide further insights into species taxonomy, biology and distribution.

Key words: *Velkovrhia enigmatica*, Hydrozoa, Planinska jama, Logarček, morphology

Izvleček. Nove najdbe in nenavadna zgradba velkovrhije *Velkovrhia enigmatica* Matjašič & Sket, 1971 (Cnidaria: Hydrozoa: Bougainvilliidae) – *Velkovrhia enigmatica* je edini sladkovodni trdoživnjak, ki živi izključno v podzemeljskih vodah. Doslej so tega endemita Dinarskega krasa na Balkanskem polotoku našli le v štirih jamah. V prispevku poročamo o najdbi nove populacije v jami Logarček pri Lazah v jugovzhodni Sloveniji. Poleg tega lahko, po skoraj 30 letih, ponovno potrdimo pojavljanje velkovrhije na tipski lokaliteti v Planinski jami. Osebki iz obeh jam se medsebojno morfološko razlikujejo: polipi iz tipske lokalitete imajo 4–9 lovk v primerjavi s polipi iz Logarčka s po 14–36 lovkami. V zaporedjih mitohondrijske DNA obeh populacij ni razlik. Šele z dodatnimi raziskavami bi lahko izboljšali vpogled v taksonomijo, biologijo in razširjenost te vrste.

Key words: *Velkovrhia enigmatica*, Hydrozoa, Planinska jama, Logarček, morfologija

Introduction

Velkovrhia enigmatica Matjašič & Sket, 1971 belongs to the predominantly marine family Bougainvilliidae. It is the only freshwater hydrozoan living exclusively in groundwater, even though some surface-dwelling species of the family Hydridae can occasionally be found in groundwater as well (Zagmajster et al. 2011). The species was first discovered in the Rak channel in Planinska jama (near Planina, southwestern Slovenia; Matjašič & Sket 1971). Three caves were reported later: Krška jama (near Krka, southeastern Slovenia), Tounjčica (near Ogulin, central Croatia) and Vjetrenica (near Zavala, southern Bosnia and Herzegovina), all in the Dinarides in the Balkans (Velikonja 1986).

Velkovrhia enigmatica is a small colonial hydrozoan, with polyps from 1.3 to 1.7 mm in height, growing about 2–5 mm apart from the common stolon up to 15 mm long (Matjašič & Sket 1971). The stolon and polyps are covered with thin multilayered perisarc terminating below the hydranths, which have typically 5–10 weakly contractible tentacles (Matjašič & Sket 1971). With the exception of the type locality, only single records of the species are available for each of the other three caves (Velikonja 1986). In Tounjčica, live colonies were found on stones in small pools of water. In Krška jama, two pieces of stolons with single polyps were found in the basket of an artificial substrate sampler in the cave, while a report from Vjetrenica refers only to one polyp found in the tank where water and material collected in the cave was put (Velikonja 1986, Sket pers. comm.). In Planinska jama, individuals were found exclusively in the Rak channel. Most animals were found in a conglomerate, with their stolons overgrowing permanently submerged surfaces of the stone agglomerates. A few stolons were reported also from stones, exposed at low water levels (Matjašič & Sket 1971).

During the first 15 years after its discovery, studies on species ontogeny (Sket & Matjašič 1977, Velikonja 1986), morphology (Kuštor 1977), and seasonal dynamics (Velikonja 1986) were conducted. As the access to the animals in Planinska jama has often been limited due to high water levels, *V. enigmatica* was brought into the laboratory and successfully bred (Velikonja 1986). After this period, studies on the species biology stopped and there was no further confirmation of the species inhabiting the cave.

Here we report on a new locality of *V. enigmatica* with an unusual morphology as well as confirm the presence of the species at its type locality.

Material and Methods

Description of the caves

Logarček (Cad. No. 28; Slovenian Cave Register 2012) is a multilevel cave with over 4.4 km passage length and 120 m vertical extent, with permanent underground waters in the deepest corridors. Its entrance is located close to Laze village near Planina (Lat.: 45.864947, Long.: 14.268217, 499 m a.s.l.; Slovenian Cave Register 2012). The entrance is a 20 m vertical shaft, followed by about 200 m long corridor that splits into large southern and

northern corridors (Južni kanal and Severni kanal, respectively), each with several side channels (Ilič 2012). Both corridors contain water lakes and siphons at their deepest parts (Ilič 2012). Waters in the cave derive partly from Planinsko polje, a large karst polje close to Planina village, via various sinks of the river Unica, especially during floods in the polje (Gams 1963, 2003, Gabrovšek & Turk 2010).

Planinska jama (Cad. No. 748; Slovenian Cave Register 2012) is a horizontal cave with over 6.6 km passage length and 65 m vertical extent. Its entrance is situated at the southwestern part of Planinsko polje (Lat.: 45.819900, Long.: 14.245667, 453 m a.s.l.; Slovenian Cave Register 2012). The cave consists of two large channels with the underground Rak and Pivka rivers of different hydrological origins. The Pivka sinks underground in Postojnska jama near Postojna, about 5 km south-west from its underground siphon at the deepest point of the Pivka channel in Planinska jama. The Rak sinks underground in Tkalca jama, after its 2 km surface flow on the small karst polje Rakov Škocjan. Its waters originate from the intermittent lake Cerkniško jezero, 5 km north-west from Rakov Škocjan. The river reappears in the Rak channel in Planinska jama, together with the subterranean waters originating from the Javorniki mountains in the south. The subterranean flows of the Rak and Pivka rivers in Planinska jama meet and join 2.5 and 3 km from their respective siphons, to form the Unica river. The latter continues its subterranean flow for about 500 m and emerges from Planinska jama to Planinsko polje. The Rak and Pivka rivers differ in their yearly temperature fluctuations, as well as in the amount of organic material they contain: they are both lower in the Rak than in the Pivka river (Sket 1970).

Field work

We investigated both corridors of Logarček up to their siphons on 23. 2. 2012, while on 9. 3. 2012 we checked only the northern corridor up to the lakes in Skalni rov, about 600 m from the cave entrance. In Planinska jama, the entire Pivka and Rak channels were investigated on 2. 6. 2011 and 14. 2. 2012 up to the lake Dvojno jezero some 400 m upstream from the confluence of both rivers.

During the first visits to both caves, general inventory of the cave fauna was performed by visual inspection and macroscopic collecting methods. A detailed examination of various water bodies, like drip pools, sinking streams, shores of rivers and lakes was carried out. Some individuals of *V. enigmatica* were carefully collected from the rocks and stored in 96% ethanol at the collecting site. A few living individuals from Planinska jama were transferred to the speleobiological laboratory of the Department of Biology (Biotechnical Faculty, University of Ljubljana), where some of them were later preserved in 96% ethanol.

We photographed the animals *in situ* and measured some water characteristics during our second visit. In Logarček, only water temperature was measured in the lake in Skalni rov, using a portable hand thermometer. Conductivity, pH, oxygen saturation and particle concentration of the water were measured three days later in the laboratory, using portable multimeter Eutech PCD650. In Planinska jama, all parameters were measured in the cave using the same equipment.

Molecular work

Genomic DNA was extracted from two individuals from Planinska jama (BA001, BA002) and Logarček (BA003, BA004), using GenElute Mammalian Genomic DNA miniprep Kit (Sigma-Aldrich). About 600 bp of the large mitochondrial ribosomal RNA gene (16S) was amplified, using 16S SHA and 16S SHB primers (Cunningham & Buss 1993). PCR was performed using the following settings: 30 s at 94°C, 45 s at 50°C, 120 s at 72°C, for 35 cycles followed by final extension at 72°C for 10 min. After purification, the gene fragment was sequenced in both directions, using the same primers by Macrogen Europe. Sequence chromatograms were edited in Geneious ver. 5.5.6 (Biomatters Ltd.). The GenBank Accession number for *Velkovrhia enigmatica* is KF312212.

Results

We found numerous individuals of *V. enigmatica* in Logarček and Planinska jama, confirming a permanent presence of the species in both caves. Logarček is a new locality for the species, which is now known from five caves in the Dinarides (Fig. 1).

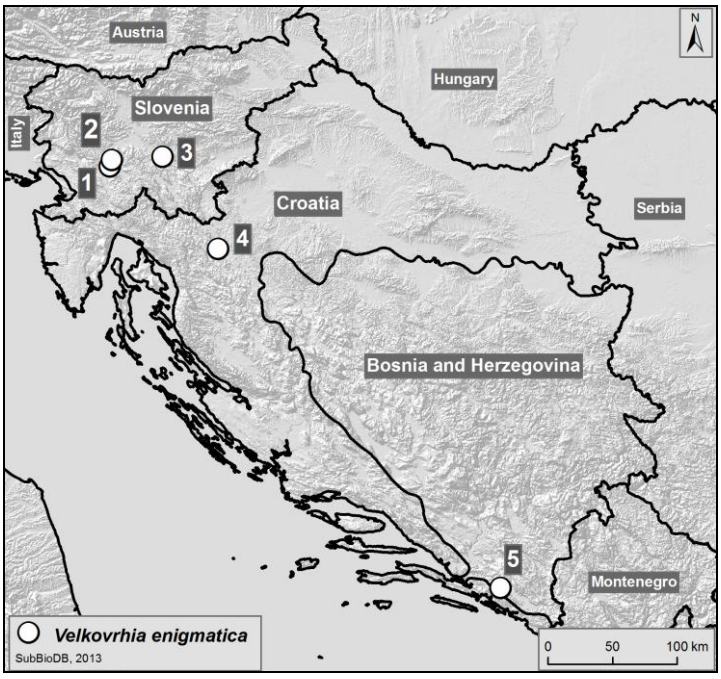

Figure 1. Caves where *V. enigmatica* was found: 1 – Planinska jama, 2 – Logarček, 3 – Krška jama, 4 – Tounjčica, 5 – Vjetrenica.
Slika 1. Jame, kjer je bila najdena velkovrhija: 1 – Planinska jama, 2 – Logarček, 3 – Krška jama, 4 – Tounjčica, 5 – Vjetrenica.

In Logarček, we found many polyps in the lake in Skalni rov, about 600 m from the cave entrance. In February 2012, they were attached to the submerged rocks, 10 cm or deeper below the lake surface. In March 2012, the water level was 10 cm higher. The lake was about 20 m long and 6 m wide, with the estimated maximum depth of about 1 m in its centre. Rocks with *V. enigmatica* in the southern and northern parts of the lake were partially covered by a thin layer of mud. During a thorough check of all accessible rocks in the lake, altogether 90 polyps were counted: 60 polyps growing from at least five stolons at the southern part of the lake, and 30 polyps growing from at least three stolons from its northern part (at the time we did not record the exact number of stolons).

In Planinska jama, *V. enigmatica* was found at two sites in the Rak channel. Only one polyp was found in a small water puddle on the rock that was separated from the main Rak flow due to the low water level. The rock was situated between Velika dvorana and Rudolfovo pristanišče, about 250 m from the confluence of the Rak and Pivka rivers. However, at least 50 stolons with polyps were found in the small cave water tributary, merging with the Rak river on its left bank in the close proximity to the lake Dvojno jezero, about 400 m upstream from the confluence. Stolons with polyps (Fig. 2) were overgrowing conglomeratic stones of different sizes, which lay in low water and in puddles, as well as the glass plates found in the tributary. Characteristics of water in the Rak river near Dvojno jezero were very similar to those of the lake in Skalni rov in Logarček (Tab. 1).

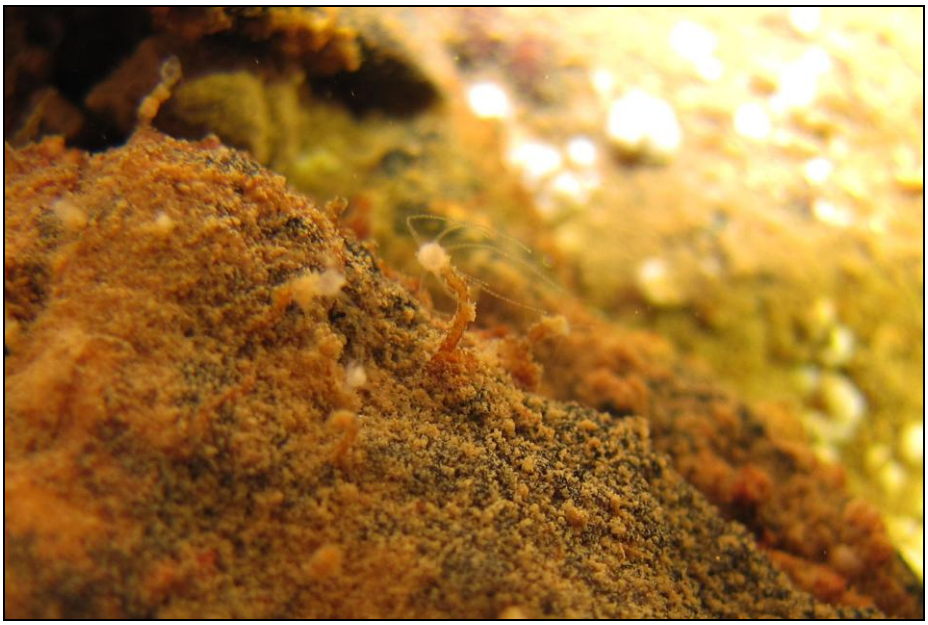

Figure 2. *Velkovrhia enigmatica* photographed in the small tributary of the Rak river in the Rak channel of Planinska jama (photo: Rodrigo L. Ferreira).
Slika 2. Velkovrhija, slikana v majhnem pritoku reke Rak v Rakovem rokavu Planinske jame (foto: Rodrigo L. Ferreira).

Table 1. Chemical characteristics of water of the Rak river near Dvojno jezero in Planinska jama and of the lake in Skalni rov in Logarček
Tabela 1. Kemične lastnosti vode v Raku pri Dvojnem jezeru v Planinski jami in v jezeru v Skalnem rovu v Logarčku

	Logarček	**Planinska jama**
Date	9. 3. 2012	14. 2. 2012
T (°C)	8.2	8.3
pH	7.67	7.72
Conductivity (µS)	314.8	361.8
Part. concentration (ppm)	282.9	/
O_2 (%)	/	91.3

Polyps collected in both caves differed substantially in the number of tentacles on their hydranths. While the ones from Planinska jama had up to 9 tentacles (4–9, N=19; Fig. 2), the count of tentacles in two polyps from Logarček was 36 in one and at least 14 in the other (damaged one). The maximum number of tentacles that could be counted from the photographs of 12 different polyps from the latter cave was about 22 (Fig. 3).

Figure 3. *Velkovrhia enigmatica* with large number of tentacles, photographed in the lake in Skalni rov in Logarček (photo: M. Zagmajster).
Slika 3. Velkovrhija z velikim številom lovk, slikana v jezeru v Skalnem rovu v Logarčku (foto: M. Zagmajster).

According to the molecular results, all individuals from Planinska jama and Logarček had exactly the same 16S sequence without indication of any genetic variability. Therefore, specimens from Logarček were identified as the same species.

Discussion

The discovery of *V. enigmatica* in Logarček represents the fifth locality of the species, whose distribution is still poorly known (Velikonja 1986). Besides waters coming to the cave from Planinsko polje, waters in the northern corridor may originate partly also from Cerkniško jezero, as is the case in the nearby cave Vetrovna luknja (Gabrovšek & Turk 2010). The new locality indicates the possibility that the species might be present also in other caves in the region between Cerkniško jezero and Planinsko polje, especially the ones that are drained by the subterranean waters of the same source, Cerkniško jezero (Gabrovšek & Turk 2010).

However, the difficulty in detecting the species poses a challenge in improving the knowledge on its distribution. *Velkovrhia enigmatica* is a small, hardly visible hydrozoan that can easily be overlooked in the caves during usual visual inspection. In addition to a thorough and careful search, low water level is also a prerequisite for a potentially successful sampling. In Planinska jama, we found *V. enigmatica* at the site that was known also at the time of its description, as we found also glass plates probably set in the stream by the late M. Velikonja during his studies of the species' biology (Sket, pers. comm.). As several individuals were observed on the glass plates, and there are no known reports on significant changes in the Rak river water quality, it is not likely that the absence of records in recent decades is due to temporary disappearance of this population from the site.

Individuals found in the type locality had the same number of tentacles as the ones in the original description by Matjašič & Sket (1971). Individuals from Logarček, however, had at least two but also more than three times higher number of tentacles, while no genetic differences between both populations was detected. This is the first observation of such great variation in tentacle number in *V. enigmatica*; so far, only annual variation in hydranth density has been reported (Velikonja 1986).

The number of tentacles in hydrozoans is known to depend on different factors. The Rak river in Planinska jama is low in organic matter (Sket 1970, Matjašič & Sket 1971), and it was shown that low food resources can lead to loss of tentacles in *Hydra* species (Berninger 1910, Kepner & Jester 1927). But, as waters in the Northern corridor of Logarček are very likely fed by the same source as in the Rak channel of Planinska jama, differences in the organic matter could be regarded as not substantial. Loss of tentacles in hydrozoans can result also from high temperatures (Annandale 1907), which can be excluded in the case of the subterranean Rak river with almost no seasonality (Sket 1970, Matjašič & Sket 1971). At both sites, chemical properties of water at the time of our second sampling were similar, so differences in the morphology cannot be easily explained by permanent differences of water. The increase in number of tentacles in Logarček seems to be site specific and could be also temporary, as there are no records of higher tentacle number in the other three populations of *V. enigmatica* in the Dinarides (Velikonja 1986). During both visits, we recorded a water drip with the increased salinity (13‰) in the side channel of the Northern corridor in Logarček, probably due to the salt spread on the highway above the cave in winter (Zagmajster et al., in prep). Even though that site is about 250 m straight line distance from the lake with *V. enigmatica*, a possibility of temporary salt inputs cannot be completely ruled out. It has been shown that unfavourable hydrogen ion concentration results in a changed number of tentacles in some

Hydra species (Threlkeld & Hall 1928). Also, in the case of the brackish water hydroid *Cordylophora caspia* (Pallas, 1771), both the morphology and ecology change drastically in varying salinities – in higher salinity the tentacle number is higher (Kinne 1957, Smith et al. 2002). At present, all of these possibilities are just speculations, which should be verified by further monitoring of species at both localities and by conducting experimental studies. Such findings of species indicate the lack of knowledge and warrant continuation of studies on taxonomy, biology and distribution of this world unique subterranean species.

Povzetek

Jamski trdoživ ali velkovrhija, *Velkovrhia enigmatica* Matjašič & Sket, 1971, pripada pretežno morski družini Bougainvilliidae. Je edini sladkovodni trdoživnjak, ki živi izključno v podzemeljskih vodah (Zagmajster et al. 2011), čeprav lahko tam najdemo tudi nekatere vrste iz družine Hydridae. Velkovrhija je bila najprej odkrita v Rakovem rokavu Planinske jame (pri Planini, jugovzhodna Slovenija), kasneje pa še v Krški jami (pri vasi Krka, jugovzhodna Slovenija), Tounjčici (pri Ogulinu, Hrvaška) in Vjetrenici (pri Zavali, Bosna in Hercegovina) (Velikonja 1986). Je majhen kolonijski trdoživnjak, s polipi, ki izraščajo iz skupnega stolona. Vse razen čašastega hidranta obdaja tanek perisark, iz hidranta pa izrašča 5–10 slabo krčljivih lovk (Matjašič & Sket 1971). Z izjemo tipske lokalitete predstavljajo podatke iz vseh ostalih jam le enkratne najdbe. Raziskovanje ontogenije (Sket & Matjašič 1977, Velikonja 1986), morfologije (Kuštor 1977) in sezonske dinamike (Velikonja 1986) je potekalo v prvih 15 letih po odkritju, predvsem na osebkih, gojenih v jamskem laboratoriju Oddelka za biologijo, Biotehniške fakultete, Univerze v Ljubljani (Velikonja 1986). Pozneje podatkov o velkovrhiji v naravi ni več moč najti.

Na podlagi terenskih raziskav v letih 2011 in 2012 podajamo nove podatke o razširjenosti velkovrhije v dveh jamah v okolici Planinskega polja. Logarček (kat. št. 28, Jamski kataster Slovenije 2012) je večnivojska jama, dolga 4,4 km, ki se 200 m za vhodnim breznom razdeli v Severni in Južni kanal (Ilič 2012). Planinska jama (kat. št. 748, Jamski kataster Slovenije 2012) je 6,6 km dolga jama, ki jo sestavljata dva velika podzemna rokava s ponikalnicama Rakom in Pivko, ki se v jami združita v reko Unico. V Logarčku smo 23. 2. 2012 raziskali Severni in Južni kanal, medtem ko smo 9. 3. 2012 pregledali le Severni kanal do jezerc v Skalnem rovu. Planinsko jamo smo raziskali 2. 6. 2011 in 14. 2. 2012, ko smo poleg Pivškega rokava raziskali tudi Rakov rokav do Dvojnega jezera. Živali smo nabirali ročno in jih spravljali v 96 % etanol. Pozneje smo izolirali mitohondrijsko DNA iz po dveh osebkov iz vsake jame.

Logarček je peta jama, od koder je znana velkovrhija. Veliko polipov smo našli v jezeru v Skalnem rovu, in sicer pritrjenih na potopljene skale, ponekod prekrite s tanko plastjo blata. Našli smo 90 polipov, ki so izraščali iz najmanj osmih stolonov (v času popisa stolonov nismo natančno šteli). V Planinski jami smo velkovrhijo našli na dveh mestih v Rakovem rokavu. En sam polip smo opazili v lužici na skali med Veliko dvorano in Rudolfovim pristaniščem. Vsaj 50 stolonov s številnimi polipi pa smo našli na konglomeratu in na steklenih ploščicah v manjšem levem pritoku reke Rak, v neposredni bližini Dvojnega jezera. Kemične lastnosti vode v jezeru v Skalnem rovu in v reki Rak pri Dvojnem jezeru se med seboj niso bistveno razlikovale. Število lovk na fiksiranih polipih iz obeh jam je bilo bistveno različno: tisti iz Planinske jame so imeli do 9 lovk (4–9; N=19), medtem ko je imel en polip iz Logarčka 36 lovk, poškodovani polip pa vsaj 14. Na fotografijah iz Logarčka je bilo mogoče na polipih prešteti do 22 lovk. Ker se populaciji velkovrhij iz obeh jam med seboj genetsko ne razlikujeta, je mogoče, da so razlike v številu lovk posledica različnih biotskih ali abiotskih faktorjev. Le dodatne raziskave bi omogočile boljši vpogled v taksonomijo, biologijo in razširjenost vrste, kot tudi razumevanje ozadja opaženih morfoloških razlik.

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