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New data on the distribution of six morphologically cryptic species of *Niphargus stygius* species complex (Amphipoda: Niphargidae)

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Abstract. Subterranean amphipods, morphologically resembling *Niphargus stygius* species complex, were molecularly analysed. We isolated genomic DNA and amplified the subunit I of mitochondrial cytochrome oxidase gene (COI) for 94 specimens from 37 localities. We report on six new localities for *N. chagankae*, one for *N. cvajcki*, eight for *N. gottscheeanensis*, two for *N. kenki*, one for *N. malagorae* and two for *N. zagrebensis*. New data extend previously known distribution ranges of *N. gottscheeanensis* and fill the missing gap between the remote *N. kenki* occurrences. We report on new co-occurrence data for two species pairs, i) *N. chagankae* and *N. likanus*, and ii) *N. gottscheeanensis* and *N. podpecanus*.

Key words: cryptic species, *Niphargus stygius*, molecular taxonomy, distribution, COI, co-occurrence

Izvleček. Novi podatki o razširjenosti šestih morfološko kriptičnih vrst kompleksa *Niphargus stygius* (Amphipoda: Niphargidae) – Raziskovali smo razširjenost kriptičnih vrst v kompleksu *N. stygius*. Izolirali smo DNA in pomnoževali podenoto I mitohondrijskega gena za citokrom oksidazo (COI) 94 osebkov iz 37 lokalitet. Navajamo šest novih nahajališč vrste *N. chagankae*, eno za *N. cvajcki*, osem za *N. gottscheeanensis*, dve za *N. kenki*, eno za *N. malagorae* in dve za *N. zagrebensis*. Izboljšali smo znanje o razširjenosti *N. gottscheeanensis* in opisali novo lokaliteto *N. kenki* sredi obširnega območja med obema do sedaj znanima skupinama nahajališč. Opisali smo prvo sopojavljanje *N. chagankae* in *N. likanus* in novo lokacijo, kjer se sopojavljata vrsti *N. gottscheeanensis* in *N. podpecanus*.

Ključne besede: kriptične vrste, *Niphargus stygius*, molekularna taksonomija, razširjenost, COI, sopojavljanje

Introduction

The use of molecular methods has shown that morphology alone poorly reflects species composition of some taxa (Witt et al. 2006, Adams et al. 2014, Katouzian et al. 2016). This was thoroughly utilized to distinguish morphologically cryptic species which resemble each other to the point of being impossible to identify without usage of molecular approach (Bickford et al.

2007). Cryptic species often represent unique evolutionary lineages (Trontelj et al. 2009, Delić et al. 2017a) with much narrower distribution than nominal species (Trontelj et al. 2009, Eme et al. 2018). Therefore, they play an important role in understanding patterns of local or regional biodiversity and often appeal for additional conservational efforts (Delić et al. 2017a).

Cryptic species are common in the subterranean realm (Trontelj et al. 2009, Niemiller et al. 2012, Parimuchová et al. 2020). Fragmentation of subterranean environments, coupled with low dispersal abilities of subterranean fauna, increases the probability of allopatric speciation (Trontelj et al. 2009, Garcia-Machado et al. 2011). In addition, common properties of subterranean habitats, i.e. darkness and lack of food sources, enable acquisition of convergent morphologies through the processes of directional selection (Trontelj et al. 2009, Juan et al. 2010). Both mentioned processes, allopatric speciation and convergent evolution, lead to a cryptic speciation within subterranean habitats.

Genus *Niphargus*, the largest genus of freshwater Amphipoda in the world (Väinölä et al. 2008), comprises more than 430 described species (Fišer 2019). All representatives, including the three known fossil species, show morphological adaptations to subterranean habitats – troglomorphies, i.e. reduction of eye, body pigments, elongation of antennae and appendages (Fišer 2019). Many morphologically cryptic complexes of *Niphargus* species have been recognized (e. g. Lefébure et al. 2006, 2007, Fišer & Zagmajster 2009, Trontelj et al. 2009, Delić et al. 2017a, Eme et al. 2018), including *Niphargus stygius* sensu lato, consisting of 16 cryptic species (Delić et al. 2017a).

The *N. stygius* s.l. species are distributed in Slovenia, North-Western Croatia, and Northern Italy (Delić et al. 2017a). Individual species distribution is often poorly understood, six of them are known only from their type locality (Delić et al. 2017a). Although boundaries of species' ranges are not thoroughly explored, it has been established that ranges of several species from this complex overlap and that specimens can even co-occur. To improve our knowledge on factors limiting geographical distributions, as well as ecology and evolutionary history of these species, sequencing of all available individuals from every locality is of high importance. Therefore, we analysed additional samples of *N. stygius* s.l. and herein report on the obtained results.

Material and methods

We analysed the specimens from *Niphargus stygius* s.l. (as defined in Delić et al. 2017a) from the Zoological Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia, collected from 2002 to 2014. The samples were preserved in 96% or 70% ethanol. Samples were morphologically classified before the study. We performed DNA isolation and amplification of subunit I of mitochondrial cytochrome oxidase gene (COI) of 94 specimens from 37 localities.

We isolated DNA from one of the pereopods, using GenEluteTM Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). We amplified COI fragment, using LCO 1490 and HCO 2198 primers (Folmer et al. 1994) and PCR protocol as described in Švara et al. (2015). PCR products were purified using Exonuclease I and Fast AP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific Inc., USA). Purified PCR products were sequenced by Macrogen Europe (Amsterdam, Netherlands) with same amplification primers. We assembled and edited the resulting chromatograms in Geneious 6.0.5. The acquired sequences were then aligned and compared with the ones available in publicly available database GenBank using »blastn« (Boratyn et al. 2013) to assign the analyzed specimens to formally described species. A general threshold for determining the species was 98% similarity or, in case that sequence contained ambiguities, as close as possible to it. To reconstruct the species' distributions, we combined our species distribution data with those of Delić et al. (2017a).

Results and discussion

We obtained COI barcodes for 62 specimens (out of 94 in total) from 30 of the 37 original localities. On three localities we found only specimens that did not belong to *N. stygius* s.l. (*N. dobati* and *N. spoeckeri*). We acquired new presence data for six *Niphargus stygius* s.l. species from 20 localities: six new localities for *N. chagankae*, one for *N. cvajcki*, eight for *N. gottscheeanensis*, two for *N. kenki*, one for *N. malagorae* and two for *N. zagrebensis*. Sequences were submitted to GenBank under accession numbers MW370352–MW370416.

A new record from Cerkniško polje significantly extended the known distribution range of *N. gottscheeanensis* westward for more than 30 km. Additionally, we identified a new population of *N. kenki*. According to Delić et al. (2017a), this species has a disjunct range with known localities from Central Slovenia (Ljubljana, Škofja Loka) and Eastern Slovenia (Kozjansko). The newly discovered locality, Maroltova jama near Letuš, fills the gap between the two regions.

A new locality with co-occurring *N. podpecanus* and *N. gottscheeanensis* and a locality with the first-time reported co-occurrence of *N. chagankae* and *N. likanus* were recorded. *N. gottscheeanensis* co-occurs according to current records with *N. chagankae* (one locality), with *N. podpecanus* (four localities) and with *N. novomestanus* (one locality). Besides with *N. gottscheeanensis* (one locality mentioned above), *N. chagankae* co-occurs according to current records also with *N. likanus* (one locality). Syntopy between cryptic species, based on current knowledge, is rare (Delić et al. 2017a). However, the assembled data suggest that co-occurrences and syntopies between species of *N. stygius* species complex could be generally overlooked. The data from seven remaining localities correlate with Delić et al. (2017a). All results are summarized in Tab. 1 and Fig. 1.

Table 1. Analized samples of *Niphargus stygius* s.l. in SE Slovenia. Locality, coordinates (WGS84), number and species of individuals from samples are given, along with their legator and date of sampling. We were unable to classify any specimen from seven localities. Species marked with * are not *N. stygius* s.l. species. On localities marked with $^{+}$ we are reporting on the presence of *N. stygius* s.l. species for the first time. On some localities from Delić et al. (2017a), we are reporting on the presence of species that have previously not been reported from there. They are marked with **.

Tabela 1. Analizirani vzorci za *Niphargus stygius* s.l. v JV Sloveniji. Za vsak vzorec podajamo lokaliteto, koordinate (WGS84), število in vrste obravnavanih osebkov, datume vzorčenj ter legatorje. Iz vzorcev sedmih lokalitet nam ni uspelo določiti nobenega osebka. Vrst, označenih z *, ne uvrščamo v *N. stygius* s.l. Na lokalitetah, označenih s $^{+}$, smo prvič potrdili pojavljanje vrst *N. stygius* s.l. Na nekaterih že znanih lokalitetah *N. stygius* s.l. (Delić et al. 2017a) smo dodatno potrdili vrste, ki tam še niso bile zabeležene. Označene so z **.

Locality (Cave ID)	Lat. (N) Long. (E)	Species	Number of classified/ all specimens	Date	Legator
Izvir pri črpališču, Slovenska vas; Kočevje; SLO	45.66765 14.83022	*N. gottscheeanensis***	3/8	22.2.2007	B. Sket, G. Bračko
		N. podpecanus	3/8		
Jama Bedara (ID NA), Tihočaj; Žumberak; HRV	45.74657 15.54649	/	0/2	4.11.2005	S. Minihofer
				18.12.2005	M. Pavlek
Andrejčkov štibelc (1263), Zagozdec; Stari trg; SLO^{+}	45.52798 15.05823	*N. chagankae*	2/2	26.1.2002	P. Presetnik
Brezno presenečenj (4500), Krašica planina; Dobrovlje; SLO	46.26638 14.92125	/	0/2	12.5.2012	R. Kvas
Izvir Črnega potoka, Črni potok; Kočevje; SLO^{+}	45.59128 14.89939	*N. gottscheeanensis*	6/6	7.1.2007	B. Sket, M. Zagmajster
Izvir Ribjek, Osilnica; SLO^{+}	45.53333 14.73162	*N. gottscheeanensis*	1/1	28.4.2007	B. Sket, J. Jugovic
Tounjčica (ID NA), Tounj; HRV	45.24882 15.32263	/	0/2	27.1.2008	M. Pavlek
Potok Zege, Dolga vas, pri pokopališču; Kočevje; SLO^{+}	45.62472 14.87689	*N. gottscheeanensis*	1/1	9.2.2002	R. Verovnik
Jama Džud (3341), Lahinja; Belčji vrh; Črnomelj; SLO^{+}	45.50240 15.20062	*N. zagrebensis*	1/1	6.1.2007	V. Zakšek, B. Šarac
Kekčevo brezno (4672), Dolenja vas; SLO^{+}	45.68130 14.77650	*N. malagorae*	2/2	21.12.2013	T. Delić, M. Zagmajster, N. Sivec
Izvir v kamnolomu Vražji kamen, Miklarji; Črnomelj; SLO^{+}	45.55997 15.08765	*N. chagankae*	2/2	24.6.2013	T. Delić, J. Tramte, D. Šinigoj
Jama Trbušnjak (HR02378), Grahovljani; Pakrac; HRV	45.49513 17.25975	/	0/3	10.6.2007	M. Faller
Jama pod Zimzelom (ID NA), Potok Musulinski; Ogulin; HRV^{+}	45.28196 15.11917	*N. chagankae*	1/2	25.6.2008	B. Jalžić
				14.12.2008	K. Gašpić

Locality (Cave ID)	Lat. (N) Long. (E)	Species	Number of classified/ all specimens	Date	Legator
Jama v kamenolomu Tounj (HR00692), Tounj; HRV+	45.24800 15.31985	*N. chagankae*	2/2	7.10.2007	M. Pavlek
Jama Drobovnik (ID NA), Kunčanj; Radatoviči; Žumberak; HRV	45.72509 15.32496	/	0/2	20.4.2007	M. Pavlek
Izvir 200m S od Zajčjega Polja; Črni potok; Kočevje; SLO+	45.59635 14.89227	*N. gottscheeanensis*	2/2	7.1.2007	B. Sket, M. Zagmajster
Mala jama nad Trebnjem (394), Trebnje; SLO+	45.89980 15.00770	*N. cvajcki*	2/2	28.7.2009	M. Zagmajster
Jama pod gradom Luknja (575), Prečna; Novo mesto; SLO	45.81841 15.09978	*N. novomestanus*	3/3	3.9.2006	V. Zakšek, B. Šarac
				29.11.2006	B. Sket
Viršnica (571), Velika Račna; Grosuplje; SLO+	45.90392 14.70549	*N. gottscheeanensis*	1/1	17.7.2010	M. Zagmajster, L. Mrzelj, P. Dovč
Studenec Pahle, Črni potok; Kočevje; SLO+	45.58795 14.89512	*N. gottscheeanensis*	3/3	7.1.2007	B. Sket, M. Zagmajster
Zelške jame (576), Zelše; Unec; SLO	45.79066 14.30349	*N. spoeckeri**	4/4	8.8.2003	P. Trontelj, M. Zagmajster
				6.7.2011	T. Delić, J. Matičič
Pod Malim naravnim mostom, Rakov Škocjan; Rakek; SLO	45.79097 14.30554	/	0/2	23.3.2003	B. Sket
Izvir pri Kočevskih poljanah, Kočevske poljane; Dolenjske Toplice; SLO+	45.72466 15.05483	*N. zagrebensis*	2/2	27.2.2007	B. Sket, G. Bračko
Dekmanca-Lastnič, studenec ob cesti levo, Bistrica ob Sotli; SLO+	46.07968 15.63265	*N. kenki*	2/4	6.8.2002	B. Sket
				8.8.2002	unknown
Sustav Matešička-Popovača (HR00957), Matešiči; Slunj; HRV	45.10808 15.61389	/	0/2	11.4.2010	R. Baković
Svinjska jama (534), odtočni sifon, Cerknica; SLO	45.77179 14.32378	*N. dobati**	1/2	2.12.2006	M. Zagmajster, J. Jugovic
		*N. spoeckeri**	1/2		
Jama v Bobnaricah (11036), Otok; Cerknica; SLO+	45.75211 14.35560	*N. gottscheeanensis*	1/1	19.8.2011	J. Matičič, M. Matičič
Izvir pri Otovcu, Otovec; Črnomelj; SLO+	45.59228 15.16412	*N. zagrebensis*	1/3	30.6.2011	G. Bračko

Locality (Cave ID)	Lat. (N) Long. (E)	Species	Number of classified/ all specimens	Date	Legator
Maroltova jama (4895), Letuš; SLO+	46.30750 15.02148	*N. kenki*	2/2	8.2.2012	R. Kvas
Jama Lesina (1811), Lipa; Vinica; SLO+	45.47002 15.20277	*N. chagankae*	2/2	3.9.2014	C. Fišer
Planinska jama (748), Planina; Postojna; SLO	45.81989 14.24566	*N. spoeckeri**	3/8	16.9.2002	NA
				14.9.2004	R. Verovnik
				24.11.2009	J. Jugovic, A. Moškrič
				2.6.2011	V. Zakšek
Đulin ponor-Medvedica (HR00728), Ogulin; HRV	45.26704 15.22399	*N. chagankae***	1/3	25.2.2012	R. Baković, S. Minihofer
				25.2.2012	R. Baković, T. Mihoci, S. Minihofer
Podpeška jama (17), Podpeč; Videm; SLO	45.83926 14.68632	*N. gottscheeanensis*	1/1	6.11.2010	M. Zagmajster
Jama Gadina-Žopenca (235), Loka; Črnomelj; SLO	45.56461 15.18202	*N. zagrebensis*	1/1	6.1.2007	V. Zakšek B. Šarac
Velika jama nad Trebnjem (104), Trebnje; SLO	45.89989 15.00723	*N. cvajcki*	4/4	12.7.2011	S. Polak
Lukova jama pri Zdihovem (91), Suhor; Kočevje; SLO	45.52580 14.89410	*N. brachytelson*	2/2	20.11.2003	B. Sket
Jama v Kamnolomu (2950), Vinica; Črnomelj; SLO	45.45443 15.24431	*N. chagankae*	1/2	5.4.2007	S. Polak

Further targeted and wider sampling (filtering epikarst water, e.g., drips from crevices in the ceiling; traps, etc.) is needed for obtaining a better insight into the species' habitat preferences and distribution, a solid baseline for establishing conservational efforts.

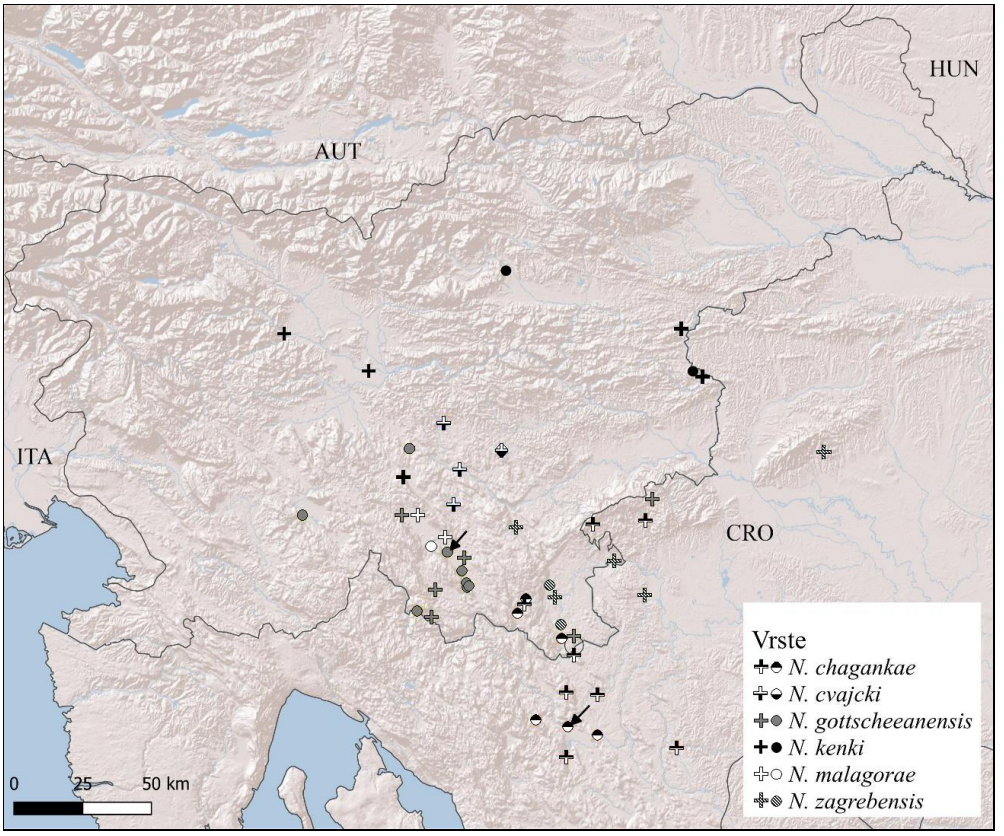

Figure 1. Distribution of *Niphargus stygius* s.l. species, based on the analyzed samples. Distributions of *N. stygius* s.l. species without any new localities found are not included (*N. brachytelson*, *N. goricae*, *N. iskae*, *N. kapelanus*, *N. karamani*, *N. kordunensis*, *N. likanus*, *N. novomestanus*, *N. podpecanus*, and *N. stygius*). Circles indicate new localities from our samples, crosses are older localities from Delić et. al. (2017b). Arrows point to the two new localities of syntopy of two species from *N. stygius* s.l. complex; co-occurrence of *N. podpecanus* and *N. gottscheeanensis* (grey circle) and co-occurrence of *N. chagankae* and N. likanus (circle with black upper half and white lower half).

Slika 1. Razširjenost vrst postranic *Niphargus stygius* s.l. ki smo jih določili v naših vzorcih. Razširjenosti vrst *N. stygius* s.l., za katere v raziskavi nismo opisali nobenega novega nahajališča, na sliki nismo prikazali (*N. brachytelson*, *N. goricae*, *N. iskae*, *N. kapelanus*, *N. karamani*, *N. kordunensis*, *N. likanus*, *N. novomestanus*, *N. podpecanus* in *N. stygius*). Krožci označujejo nove lokalitete iz naših vzorcev, križci pa lokalitete iz Delić et al. (2017b). S puščicama sta označeni novo znani lokaciji sopojavljanja dveh vrst tega morfološko kriptičnega kompleksa; *N. podpecanus* in *N. gottscheeanensis* (siv krožec) ter *N. chagankae* in *N. likanus* (krožec, katerega zgornja polovica je črna, spodnja pa bela).

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References

Adams M., Raadik T. A., Burridge C.P., Georges A. (2014): Global biodiversity assessment and hyper-cryptic species complexes: More than one species of elephant in the room? Syst. Biol. 63: 518-533.

Bickford D., Lohman D.J., Sohdi N.S., Ng P.K.L., Meier R., Winker K., Ingram K.K., Das I. (2007): Cryptic species as a window on diversity and conservation. Trends Ecol. Evol. 22: 148-155.

Boratyn G.M., Camacho C., Cooper P.S., Coulouris G., Fong A., Ma N., Madden T.L., Matten W.T., McGinnis S.D., Merezhuk Y., Raytselis Y., Sayers E. W., Tao T., Ye J., Zaretskaya I. (2013): BLAST: a more efficient report with usability improvements. Nucleic Acids Res. 41: W29-W33.

Delić T., Trontelj P., Rendoš M., Fišer C. (2017a): The importance of naming cryptic species and the conservation of endemic subterranean amphipods. Sci. Rep. 7: 339.

Delić T., Švara V., Coleman C.O., Trontelj P., Fišer C. (2017b): The giant cryptic amphipod species of the subterranean genus *Niphargus* (Crustacea, Amphipoda). Zool. Scr. 46(6): 740-752.

Eme D, Zagmajster M., Delić T., Fišer C., Flot J.-F., Konecny-Dupré L., Pálsson S., Stoch F., Zakšek V., Douady C.J., Malard F. (2018): Do cryptic species matter in macroecology? Sequencing European groundwater crustaceans yields smaller ranges but does not challenge biodiversity determinants. Ecography 41(2): 424-436.

Fišer C. (2019): Niphargus A model system for evolution and ecology. In: White W., Culver D., Pipan T. (Eds.), Encyclopedia of Caves. 3rd ed. Academic Press, Cambridge, pp. 746-755.

Fišer C., Zagmajster M. (2009): cryptic species from cryptic space: the case of *Niphargus fongi* sp. n. (Amphipoda, Niphargidae). Crustaceana 82(5): 593-614.

Folmer O., Black M., Hoeh W., Lutz R., Vrijenhoek R. (1994): DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3: 294-299.

García-Machado E., Hernández D.A., García-Debras P., Chevalier-Monteagudo C., Metcalfe L., Bernatchez L., Casane D. (2011). Molecular phylogeny and phylogeography of the Cuban cave-fishes of the genus *Lucifuga*: Evidence for cryptic allopatric diversity. Mol. Phylogenet. Evol. 61: 470-483.

Juan C., Guzik M.T., Jaume D., Cooper S.J.B. (2010): Evolution in caves: Darwin's 'wrecks of ancient life' in the molecular era. Mol. Ecol. 19: 3865-3880.

Katouzian A.-R., Sari A., Macher J.N., Weiss M., Saboori A., Leese F., Weigand A.M. (2016): Drastic underestimation of amphipod biodiversity in the endangered Irano-Anatolian and Caucasus biodiversity hotspots. Sci. Rep. 6: 22507.

Lefébure T., Douady C. J., Gouy M., Trontelj P., Briolay J., Gibert J. (2006): Phylogeography of a subterranean amphipod reveals cryptic diversity and dynamic evolution in extreme environments. Mol. Ecol. 15: 1797-1806.

Lefébure T., Douady C.J., Malard F., Gibert J. (2007): Testing dispersal and cryptic diversity in a widely distributed groundwater amphipod (*Niphargus rhenorhodanensis*). Mol. Phylogenet. Evol. 42: 676-686.

Niemiller M.L., Near T.J., Fitzpatrick B.M. (2012): Delimiting species using multilocus data: diagnosing cryptic diversity in the southern cavefish *Typhlichthys subterraneus* (Teleostei: Amblyopsidae). Evolution 66: 846-866.

Parimuchová A., Žurovcová M., Papáč V., Kováč Ľ. (2020): Subterranean *Deuteraphorura* Absolon, 1901, (Hexapoda, Collembola) of the Western Carpathians – Troglomorphy at the northern distributional limit in Europe. PLoS ONE 15(1): e0226966.

Švara V., Delić T., Rađa T., Fišer C. (2015): Molecular phylogeny of *Niphargus boskovici* (Crustacea: Amphipoda) reveals a new species from epikarst. Zootaxa 3994(3): 354-376.

Trontelj P., Douady C.J., Fišer C., Gibert J., Gorički Š., Lefebure T., Sket B., Zakšek V. (2009): A molecular test for cryptic diversity in ground water: how large are the ranges of macrostygobionts? Freshwater Biol. 54: 727-744.

Väinölä R., Witt J.D.S., Grabowski M., Bradbury J.H., Jazdzewski K., Sket B. (2008): Global diversity of amphipods (Amphipoda; Crustacea) in freshwater. Hydrobiologia 595: 241-255.

Witt J.D.S., Threloff D.L., Hebert P.D.N. (2006): DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. Mol. Ecol. 15: 3073-3082.