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First insight into molecular diversity and DNA barcode library of epikarst-dwelling invertebrates in the Western Carpathians

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ABSTRACT

DNA barcoding represents a handy tool for species identification. In addition, it serves as a complementary approach that improves the characterisation of evolutionary lineages and facilitates the detection of potentially undescribed and cryptic species. Based on the case study in the Western Carpathians, which belong to the Carpathian biodiversity hotspot, we have compiled the first DNA barcode reference library for molecular identification of invertebrates associated with epikarst, a unique, yet understudied, shallow subterranean aquatic habitat that extends at the interface between the soil and carbonate rocks. We analysed invertebrates collected in 2019-2020 from epikarst water that continuously seeps into four caves of the Demänovský Cave System in northern Slovakia. The standard barcode marker of the mitochondrial COI gene was amplified in more than 920 individuals of aquatic, semi-aquatic, and terrestrial invertebrates. The final data set consisted of 784 barcode sequences representing 36 morphospecies, the majority (98.3%) belonging to Arthropoda. Automated cluster delineation using the Barcode of Life Data System (BOLD) revealed 60 Barcode Index Numbers (BINs), of which 43 BINs were new to BOLD, representing mostly typical subterranean species. Almost 20% of the morphospecies displayed high intraspecific variation (>2.2%), suggesting the need for further investigation to assess potential taxonomic problems or cryptic diversity. Our results also indicated the existence of several yet undescribed invertebrate species and possible heteroplasmy or COI numts in the collembolan *Megalothorax* sp. (*incertus* species group). The resulting DNA barcode library represents a significant advance not only in the characterisation of epikarst biodiversity but also in the understanding of subterranean biodiversity in general, paving the way for future complex evolutionary and biogeographical studies.

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1. Introduction

Over the past two decades, DNA barcoding has become a handy tool for taxonomic identification of organisms, particularly in the case of Metazoa. It involves sequencing approximately 658 base pairs (bp) long fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and comparing it with a reference library (Hebert et al., 2003). Generally, the DNA barcode region is relatively conserved at the intraspecific level, allowing for reliable species designation in many animal taxa (Rubinoff, 2006; Antil et al., 2022). It also helps to detect genetic divergence gaps that delineate interspecific boundaries and, in many cases, to differentiate between divergent phylogenetic lineages within conventionally recognised morphospecies (Barrett and Hebert, 2005; Čandek and Kuntner, 2015). In recent decades, DNA barcoding has revolutionised the fields of ecology, biodiversity, and conservation by providing an affordable and accurate approach for species identification and conservation efforts that greatly contribute to illumination of biodiversity and understanding its dynamics (Gostel and Kress, 2022). In this way, it helps to build an important bridge that allows an enhancement of the environmental potential of the biosphere, which can be achieved only by considering the multidimensional goal of sustainability: Water, Biodiversity, Services from ecosystems, Resilience, and Cultural heritage (WBSRC) (Zalewski et al. 2016). Furthermore, DNA barcoding effectively identifies species regardless of the life stage, sex, or even the completeness of an individual, allowing for the taxonomic assignment of body remnants, feathers, fur, faeces, etc. (Antil et al., 2022). Finally, it often reveals morphologically similar but genetically distinct cryptic species, aiding in the discovery and description of new species (Ekrem et al., 2010; Park et al., 2011; Venter and Bezuidenhout, 2016). Molecular genetic tools can serve as a complementary technique to significantly enhance species identification efficiency and overcome global taxonomic impediment (Schindel and Miller, 2005). Nevertheless, the effectiveness of DNA barcoding relies on well-curated and publicly available reference libraries that contain DNA sequences of species accurately identified based on morphological traits. Currently, the most comprehensive and reliable public DNA barcode library is the Barcode of Life Datasystems (BOLD) (https://www.boldsystems.org/) (Ratnasingham and Hebert, 2007; Weigand et al., 2019). Many taxonomic groups are still unavailable or underrepresented in these libraries, what can limit species discrimination and detection capabilities, thereby leading to a possible misinterpretation of biodiversity assessment results (Duarte et al., 2020; Rey et al., 2020). This is particularly true for subterranean ecosystems, where unlike in the epigean environments, molecular genetic tools, including DNA barcoding, are still underutilised in biodiversity assessments due to limited accessibility and cryptic nature of the local biota (Saccò et al., 2022).

On a global scale, subterranean ecosystems are among the most widespread yet least understood. They are characterised by specific environmental conditions, such as the absence of light, oligotrophy, and a stable climate, which make them ideal natural laboratories for investigating the ecology and evolution of their inhabitants (Culver and Pipan, 2013; Griebler et al., 2014; Mammola, 2019; Mammola et al., 2020). An integral component of subterranean ecosystems is the epikarst, which is defined as the uppermost zone of the karst landscape at the interface between soil and carbonate rocks. It consists of a complex network of extensive cavities and channels that retain water from precipitation. This water gradually drains downward through an underlying infiltration fissure zone and eventually reaches the water table over an impermeable phreatic zone (Bakalowicz, 1995, 2012; Williams, 2008). In addition to serving as a water reservoir, the epikarst also represents a unique subterranean aquatic habitat that harbours various groups of minute invertebrates. These organisms are commonly carried by vertical currents down the infiltration zone and subsequently enter the caves through seeping water (Culver and Pipan, 2014; Pipan and Culver 2013). However, knowledge of the organisms occurring in epikarst is still lacking in most parts of the world. So far, invertebrates collected from epikarst water that seeps into cave corridors have been studied in a limited number of mainly European countries, including Italy (Bruno et al., 2017, 2018), Romania (Meleg et al., 2011; Moldovan et al., 2007, 2011), Spain (Camacho et al., 2006) and most extensively in Slovenia (e.g. Brancelj, 2006; Papi et Pipan, 2011; Pipan, 2005; Pipan and Culver, 2007; Pipan et al., 2006, 2018). Studies employing a conventional morphological approach to identify invertebrate species have revealed that epikarst waters are rich in aquatic fauna, often characterised by hypogean species with narrow distribution ranges restricted to small karst areas.

The primary objective of this study is to establish a first comprehensive and taxonomically curated DNA barcode reference library for the molecular identification of epikarst invertebrates. This is based on the case study sites in the Demänovský Cave System, which is the longest known cave system in the Carpathian Arch, recognised as a prominent biodiversity and endemism hotspot in Europe (Mráz and Ronikier, 2016). Our results provide the first integrative insight into the taxonomic and molecular diversity of sparsely known metazoan organisms that inhabit this distinctive environment. Additionally, our study will serve as an initial reference and a blueprint for future biodiversity assessments of epikarst invertebrates, as well as of scarce and little-known endemic species, and will enable their effective monitoring through non-invasive eDNA and metabarcoding techniques. Ultimately, our findings have the potential to spark future complex evolutionary and biogeographical studies in epikarst ecosystems.

2. Material and Methods

2.1. Study site and invertebrate sampling

In this study we focused on the epikarst of the Demänovská Valley, located beneath the main ridge of the Low Tatras, approximately 10 km south of the town of Liptovský Mikuláš (Western Carpathians, Slovakia). The allogenic karst of the Demänovská Valley, which extends at an elevation of approximately 740-1200 m, has been

formed in the Triassic carbonate rocks by a transiting allochthonous Demänovka Brook, the activity of which created various karst formations, including dozens of different types of caves (Bella et al., 2021).

Due to the physical inaccessibility of the epikarst, invertebrates present in this habitat were indirectly sampled from May 2019 to July 2020 using 27 devices designed following the methodology of Pipan (2005). These devices enabled us to filter the epikarst water seeping from the ceilings of four caves: the Demänovská jaskyňa mieru Cave (5 filtering devices), the Demänovská jaskyňa slobody Cave (12 filtering devices), both of which constitute a significant portion of the 48 km long Demänovský Cave System (Bella et al., 2021), the Beniková Cave (5 filtering devices), and the Okno Cave (5 filtering devices), both of which share common speleogenesis with the Demänovský Cave System (Figure 1).

Each filtering device consisted of a plastic funnel that directed the seeping epikarst water into a cubic-shaped plastic vessel with a fine sieve (mesh 60 µm) covering square openings in two adjacent walls. A perforated plastic container was used to stabilise both components (Figure 2). The cubic-shaped vessel was removed from the filtering devices once a month. A small volume of water at the bottom of the vessel, containing live invertebrates, was poured through a modified plankton net (mesh 60 µm). The invertebrates caught in the net were directly washed down the tube, fixed in 96% ethanol, and stored in a freezer at -20°C until further processing in the laboratory. The sampling of epikarst invertebrates was carried out under an official permit (no. 266112017-6.3) issued by the Ministry of Environment of the Slovak Republic for biological investigations in the aforementioned caves.

2.2. DNA isolation, PCR amplification, and sequencing

A total of 941 individuals of invertebrates were subjected to DNA extraction out of the total number of 1,630 individuals captured in four caves using 27 filtering devices. Total genomic DNA was extracted from the studied invertebrates by the standard phenol-chloroform method (Hillis and Moritz, 1996) (in the case of Nematoda and Clitellata) or by one of the two commercial silica column-based DNA extraction kits: (1) Genomic Mini kit for genomic DNA purification (A&A Biotechnology, Poland) (Arachnida, Collembola) and (2) GeneMATRIX Tissue DNA Purification Kit (EURx, Poland) (all other taxa), following the manufacturer's protocols. The clitellates were dissected in the anterior and posterior halves prior to DNA extraction. The posterior part was used to extract DNA, while the anterior part was used for further morphological identification. The DNA of a few individuals of Amphipoda and Plecoptera with body size $>$ 2 mm was extracted from the legs (two from each individual). In the case of invertebrates with body size $<$ 2 mm, total DNA was extracted from the whole individual, leaving its exoskeleton intact. The exoskeletons, except for the nematodes, were recovered immediately after lysis by pulling them out of the lysate using a sterile microscope loop. Subsequently, the exoskeletons were rinsed with distilled water, transferred to 70% ethanol, and stored at room temperature for further morphological identification. Nematoda was the only taxon that we were unable to identify morphologically. Morphological identification of nematodes, based on microscopic analysis of morphological and anatomical traits, does not allow for subsequent DNA extraction. On the other hand, lysis generally dissolves the collagen exoskeleton of nematodes, making morphological identification impossible after DNA extraction. After excluding 17 individuals, in which neither morphological identification (due to loss of their exoskeletons after DNA extraction) nor COI amplification was successful, 924 individuals of invertebrates were finally subjected to DNA barcoding (Table 2).

The standard barcode fragment of the cytochrome *c* oxidase subunit I (COI) gene (Hebert et al., 2003) was amplified using three different sets of primers: (1) LCO1490-JJ and HCO2198-JJ (Astrin and Stüben, 2008), (2) coxf and coxr2 (Cheng et al., 2013) and (3) cop-COX1+20 (Chang, 2007) and HarCO1-2189R (Rossel and Martínez Arbizu, 2018). Polymerase chain reactions (PCR) were carried out in 10–20 µl PCR mix containing DreamTaq^{TM} Hot Start Green PCR Master Mix (Thermo Fisher Scientific), the relevant pair of 5 µM primers, and nuclease-free water. The PCR conditions depended on the invertebrate taxon studied and are specified in the more detail in Table 1.

PCR products (5µl) were cleaned with Exonuclease I (2 U, EURx) and alkaline phosphatase Fast Polar-BAP (1 U, EURx) treatment, according to the manufacturer's guidelines. Sanger sequencing of the purified PCR products with forward primer, and in the case of collembolan *Megalothorax* sp. (*incertus* species group) with both forward and reverse primers, was outsourced to Macrogen Europe BV (The Netherlands). The obtained sequences were edited, aligned, and trimmed using Geneious R11 (https://www.geneious.com/). The same software was used to check for the absence of frameshifts, double peaks, and stop codons. We used BLAST (Altschul et al., 1990) to verify the identity of each obtained sequence. To build the Neighbour-Joing tree, the COI sequences were collapsed into haplotypes with FaBox ver. 1.61 (https://birc.au.dk/~palle/php/fabox/) (Villesen, 2007). All relevant voucher information, taxonomic classification, photos (taken before DNA extraction), and DNA barcode sequences were deposited in Barcode of Life DataSystems (BOLD) (http://www.boldsystems.org/, Ratnasingham and Hebert, 2007) and are available under the data set DS-EDVINV (DOI: dx.doi.org/10.5883/DS-EDVINV).

2.3. Taxonomic identification

Invertebrates captured in devices that filter percolating epikarst water were first sorted to the class level using a stereomicroscope (Motic SMZ-168). Subsequently, under the microscope Olympus BX53, each individual was identified to the lowest possible taxonomic level using the available literature, i.e. Arslan et al., 2007; Martinez-Ansemil et al., 2012; Timm, 2009; van Haaren and Soors, 2013 (Clitellata); Janetzky et al., 1996; Pesce et al., 1987; Wells, 2007 (Copepoda); Weigmann, 2006; Zacharda, 1980 (Acarina); Bretfeld, 1999; Papáč and Kováč, 2013; Pomorski, 1998; Potapov, 2001;

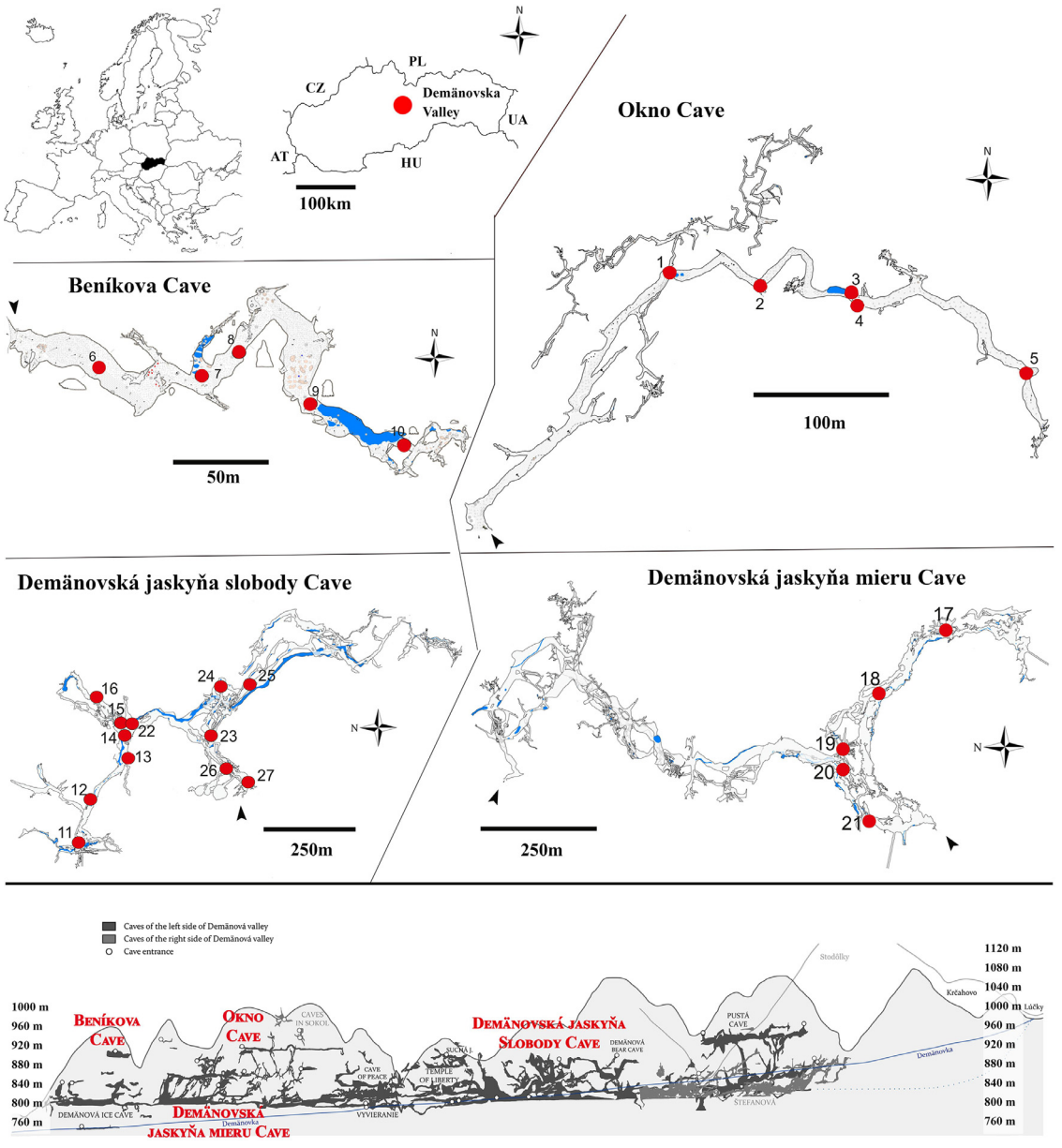

Figure 1. The location of the the Demänovská Valley within Europe and Slovakia (top left corner of the figure), cross-section through the karst of the Demänovská Valley with the indicated altitudes (bottom of the figure) and ground plans of the four studied caves with 27 sampling points (red circles). The blue areas in the ground plans of the caves represent the cave river or lakes. Cross-section and ground plans of the caves were compiled by Pavel Herich.

Table 1
COI primer sequences and PCR thermal settings used for molecular phylogenetic analysis.

Primer	Primer sequence	Direction	PCR thermal profile	Amplified taxa
LCO1490-JJ	5′-CHACWAAYCATAAAGATATYGG-3′	forward	94°C: 3 min; 5[a]x (94°C: 30 s, 45°C: 90 s,	Clitelata, Copepoda,
HCO2198-JJ	5′-AWACTTCVGGRTGVCCAAARAATCA-3′	reverse	72°C: 60 s); 35[a]x (94°C: 30 s, 51°C: 90 s, 72°C: 60 s); 72°C: 5 min	Malacostraca, Arachnida, Collembola, Insecta
coxf	5′-GGTCCTGTAATCATAAAGAYATYGG-3′	forward	94°C: 5 min; 40[a]x (94°C: 45 s, 54°C: 75 s,	Copepoda
coxr2	5′-TCTATCCCAACTGTAAATATRTGRTG-3′	reverse	72°C: 75 s); 72°C: 2 min	
cop-COX1+20	5′-GACTAATCATAAAGATATTGGTAC-3′	forward	94°C: 5 min; 40[a]x (94°C: 45 s, 51°C: 75 s,	
HarCO1-2189R	5′-GGGTGRCCRAARAATCARAA-3′	reverse	72°C: 75 s); 72°C: 2 min	

[a] Numbers of cycles.

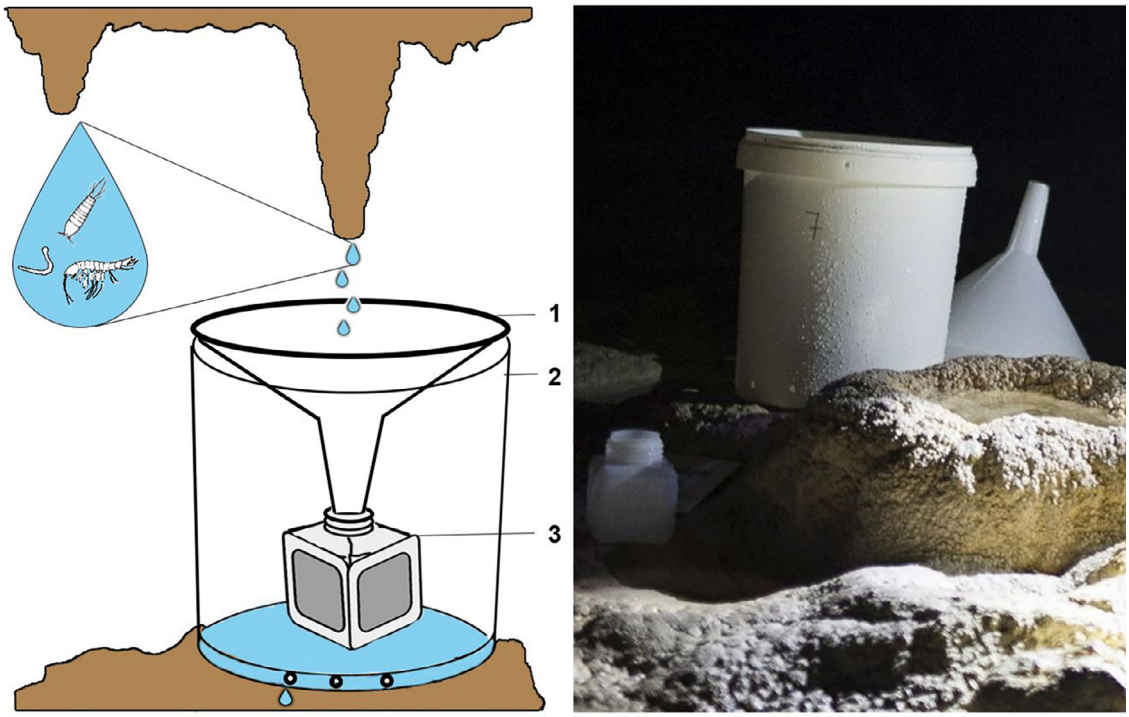

Figure 2. Device filtering seeping epikarst water. A. Diagram of device components: 1 - funnel, 2 - perforated container, 3 - cubic-shaped vessel with a fine mesh (modified after Pipan, 2005); B. One of the devices set in a cave corridor (Photo by Pavol Staník).

Schneider, 2022 (Collembola) and Klink and Moller Pillot, 2003 (Diptera).

Based on knowledge obtained from various biospeleological inventories conducted in Western Carpathian caves over the past decades (Košel, 2012; Kováč et al., 2014), the identified genera and species have been categorised into three ecological groups, which reflect their affinity for the subterranean environment (Howarth and Moldovan, 2018): (1) stygobiont/troglobiont - taxa that are exclusively restricted to subterranean habitats, (2) stygophiles/troglophiles - taxa that can inhabit both subterranean and epigeic environments, without being necessarily limited to either, but capable of establishing stable subterranean populations, and (3) stygoxenes/trogloxenes - strictly epigeic taxa that occasionally occur in or visit subterranean habitats but are unable to establish a stable subterranean populations, unlike the previous two categories. For the categories stygobiont/troglobiont and stygophiles/troglophiles, we use the collective designation "subterranean" throughout the text.

2.4. DNA barcode analysis

To analyse the data set, we implemented the basic analytical functions of the workbench provided by BOLD. Sequence divergence (maximum intraspecific variation and minimum genetic distance to the nearest-neighbour species) was estimated using "Barcode Gap Analysis" and "Distance Summary Tools" based on the Kimura-2-Parameter (K2P) distance. It is important to note that BOLD applies both functions exclusively to barcodes within the dataset obtained in this study. Therefore, barcodes divergence is analysed only within the dataset and not across the entire BOLD database. The Barcode Index Number (BIN) system (Ratnasingham and Hebert, 2013) was used as a delimitation criterion to assign molecular operational taxonomic units (MOTUs) across the entire set of DNA barcodes deposited in BOLD. This method uses a 2.2% sequence divergence as a threshold for the rough clustering of DNA barcodes into unique BINs considered tentative species equivalents. This threshold has been widely used in other DNA barcoding-based studies (Havemann et al., 2018; Pentinsaari et al., 2019; Raupach et al., 2014, 2016). Then, separately in each class, the diversity of MOTUs was estimated with two additional species delimitation methods. The Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al., 2021), classifying sequences into putative species clusters based on the distribution of intraspecific and interspecific genetic divergence (barcode gap), was run online (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html). The Kimura 2-parameter (Nishimaki and Sato, 2019) was selected as a nucleotide substitution model, and other settings were left as default. The Bayesian implementation of the Poison Tree Processor (bPTP) (Zhang et al., 2013) was run online (https://species.h-its.org/) for 500 000 generations, with a thinning of 100 and a 0.1 burn-in. For the bPTP method, we used the ML phylogenetic tree generated by the PhyML software package (Guindon et al., 2010) available at http://www.atgc-montpellier.fr/phyml/. Branch support was inferred using the non-parametric bootstrap algorithm based on 1 000 replicates (Minh et al., 2013). In the case of the

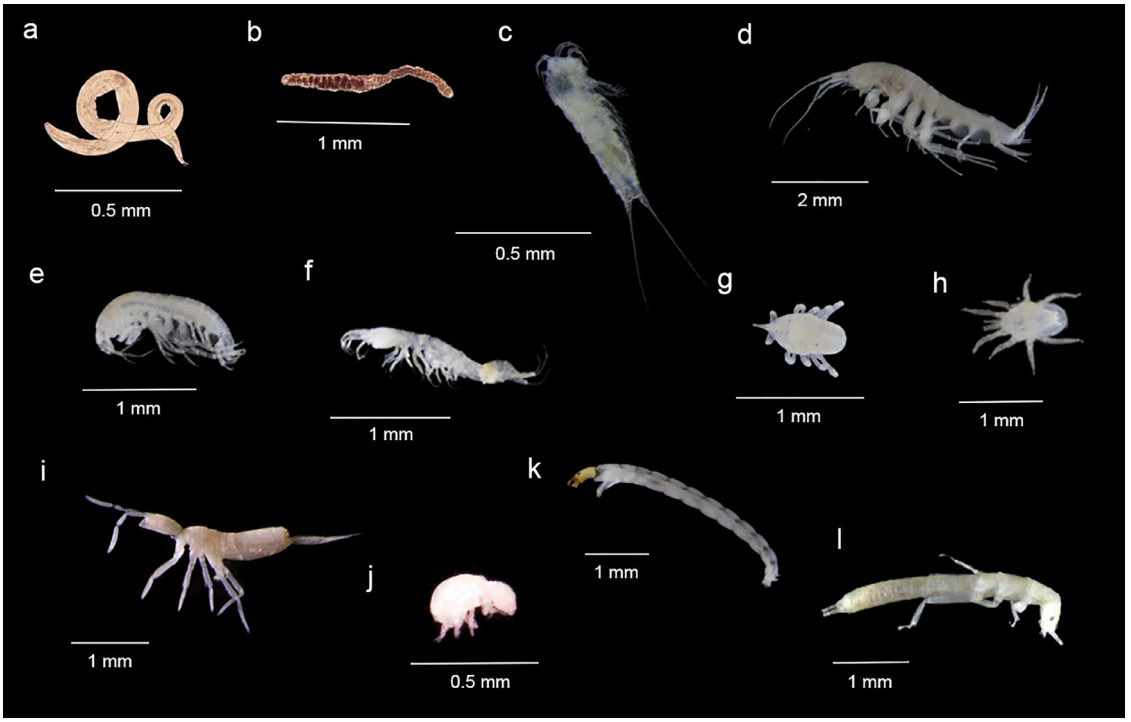

Figure 3. Examples of invertebrates caught in the devices filtering seeping epikarst water: a - Nematoda indet., b - stygobitic clitellate *Parvidrilus* sp., c - stygobitic copepod *Elaphoidella phreatica*, d - stygobitic amphipod *Niphargus* sp., e - stygobitic representative of the order Amphipoda, f - representative of the stygobitic order Bathynellacea, g - terrestrial mite of the suborder Prostigmata, h - terrestrial mite of the Gamasina cohort, i - troglobitic collembolan *Pseudosinella paclti*, j - troglobitic collembolan *Megalothorax hipmani*, k - aquatic larva of dipteran *Brillia bifida* and l - aquatic larva of plecopteran *Leuctra pseudosignifera*.

Collembola, *Megalothorax* sp. (*incertus* species-group) was excluded from these two additional delimitation methods, as all of its COI sequences had multiple ambiguous bases.

To illustrate the molecular and taxonomic diversity of the studied epikarst dwelling taxa and the phylogenetic relationships among haplotypes, we built a Neighbour-Joining tree (Saitou and Nei, 1987) based on the K2P model of nucleotide substitution (Kimura, 1980), with a bootstrap test based on 10,000 replicates, using MEGA 11 (Tamura et al., 2021). The resulting phylogenetic tree was visualised and edited using iTOL ver. 5 (Letunic and Bork, 2021).

3. Results

We successfully generated 784 DNA barcodes (sequencing success rate of 84.8 %) representing 36 morphospecies from 17 orders and 6 animal classes, most of which (98.3 %) belonged to Arthropoda (Figure 3). The highest sequencing success rate (92.6%) was obtained for copepods, while the lowest (41.4%) was obtained for arachnids (Table 2, Table 3). Copepoda accounted for the highest number of barcodes (68.4 % of all sequences), followed by Collembola (21.9 %), other crustaceans and insects (6.5 %). Nematoda was the only taxon for which we failed to obtain any DNA barcode. We morphologically identified 10 obligatory aquatic species, of which 4 species were stygobionts limited to groundwaters (i.e., clitellate *Parvidrilus* sp., harpacticoids *Elaphoidella phreatica* and *Elaphoidella* sp., amphipod *Niphargus* sp.) and 9 amphibiotic species (aquatic larval stages of Plecoptera and Diptera). Furthermore, we identified 17 terrestrial species, of which 5 species, all collembolans, were troglobionts associated with the cave environment (that is *Deuteraphorura kratochvili, Megalothorax hipmani, Pseudosinella paclti* and others) (Table 3).

The length of the DNA barcodes ranged between 515 and 622 bp. One sequence of *Megalothorax* sp. was identified as a putative pseudogene and thus discarded from subsequent analysis. The sequences were characterised by high A+T content, with mean nucleotide compositions G=18.29%, C=15.89%, A=27.35% and T=38.47%, and were grouped into a total of 177 haplotypes (Table 2). Ambiguous nucleotides were observed only in the case of *Megalothorax* sp. that displayed 3 to 5 double peaks per sequence. Of these, nearly 60% of individuals were characterised by non-synonymous mutations (max. 2 per each sequence) (Figure 4). The sequences deposited in BOLD were automatically split into 60 Barcode Index Numbers (BINs), of which 43 BINs were new to BOLD. A majority (24) of these new BINs comprised subterranean genera and species (e.g., stygobitic copepods of the genus *Elaphoidella*, troglobitic collembolans of the genus *Megalothorax*), while only 4 new BINs represented epigeic species (e.g., clitellate *Cognettia* sp., chironomid dipteran *Polypedilum laetum* or plecopteran *Nemoura* sp.). The remaining 15 new BINs corresponded to taxa identified only to the higher taxa level (e.g., Clitellata indet., Amphipoda indet., Prostigmata indet., and others), making it impossible to define their ex-

Table 2
Summary of invertebrate higher taxa caught in the filtering devices with indication of DNA barcoding efficiency and diversity of invertebrate lineages.

Taxa	Individuals subjected to DNA extraction	Individuals with exoskeletons lost after lysis, COI unsuccessfully amplified	Individuals subjected to DNA barcoding	COI Barcodes generated	Haplotypes	Morphospecies	BIN	ASAP	bPTP
Nematoda	6	6	0	NA	NA	0	NA	NA	NA
Clitellata	22	NA	22	13	13	3	9	10	9
Copepoda	586	7	579	536	34	5	8	7	9
Malacostraca	20	0	20	18	6	1	3	4	3
Arachnida	30	1	29	12	12	6	9	9	9
Collembola	217	3	214	172	88 (33*)	10	16 (15**)	11**	11**
Insecta	60	0	60	33	24	11	15	16	15
Total	941	17	924	784	177 (122*)	36	60 (59**)	57**	56**

* number of haplotypes after excluding all sequences of collembolan *Megalothorax* sp. (incertus group) characterised by multiple ambiguous nucleotides.
** number of MOTUs after excluding the collembolan *Megalothorax* sp. (incertus group) from species delimitation the sequences of which had all multiplate ambiguous nucleotides.

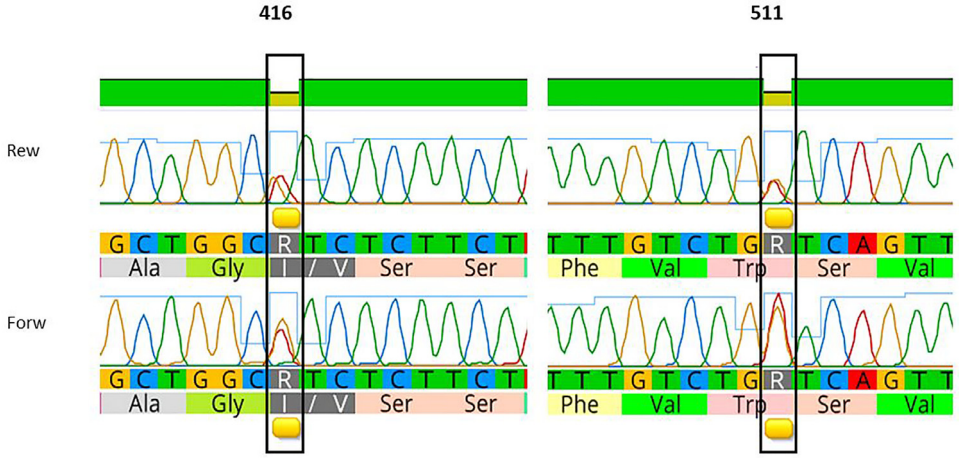

Figure 4. Non-synonymous (left) and synonymous (right) ambiguity in nucleotide sequences of *Megalothorax* sp. (*incertus* group).

act affinity to the subterranean environment. In contrast, most BINs already present in BOLD (13) comprised epigeic species (e.g., clitellate *Cognettia chlorophila*, oribatid mite *Chamobates spinosus* and several insects), while only 4 such BINs were represented by terrestrial subterranean species (e.g., troglobitic colembolan *Deuteraphorura kratochvili* and troglophilic dipteran *Trichocera maculipennis*).

Twenty-six morphospecies (72.2%) were assigned to a single BIN, each. The copepods *Bryocamptus* (*R.*) *spinulosus* and *B. typhlops*, collembolans *Pseudosinella paclti* and *Deuteraphorura kratochvili* and dipteran *Brillia bifida* were each assigned to 2 BINs. The copepod *Elaphoidella phreatica,* as well as the collembolans *Megalothorax carpaticus* and *M. tatrensis,* were assigned to 3 BINs. Twenty-two BINs were represented by a single sequence, each. No BIN sharing was found between different morphospecies (Table 3). The two additional molecular delimitation methods yielded slightly different numbers of species. Specifically, ASAP detected 57 MOTUs based on the best partitioning schemes with respect to the ASAP score, whereas the highest Bayesian supported solution of the tree-based bPTP found a total of 56 MOTUs.

The K2P distance ranged from 0 to a maximum of 10.10 in *Brillia bifida*, the chironomid dipteran for which two BINs were detected. On the other hand, the interspecific distances between the nearest BINs in our dataset varied from 10.93 (distances between copepods *Elaphoidella phreatica* and *Elaphoidella* sp.) to 38.53% (distances between watermite *Atractides rivalis* and chironomid dipteran *Polypedilum laetum*) (Table 3). The K2P-based neighbour joining phenogram revealed well-defined clades, with bootstrap support values > 70%, for 17 morphospecies (47.2%). The diversity and rough phylogenetic affiliations of the collected invertebrates are illustrated in Figure 5.

4. Discussion

Our paper provides the very first insight into the diversity of invertebrates associated with epikarst of the Western Carpathians and, for the first time ever, applies DNA barcoding in this type of aquatic subterranean habitat. Compiling a comprehensive DNA barcode reference library, one of the pivotal outputs of the present study, signifies an important advancement in the molecular characteri-

Table 3
Species delimitation and K2P distances of COI sequences within and between Western Carpathian epikarst invertebrates.

Class	Order	Species	BI	NB	BIN	MaxISD	Nearest neighbor	DNN	ASAP	bPTP
Clitellata			2	2	**AES6438**, **AES6439**	NA	NA	NA	2	2
	Enchytraeida	*Cognettia chlorophila* [A]	1	1	AAT8936	NA	*Cognettia* sp.	22.94	1	1
		Cognettia sp. [A]	1	1	**AES6442**	NA	*Parvidrilus* sp.	21.87	1	1
	Parvidrilida	*Parvidrilus* sp.[A**]	1	1	**AET8807**	NA	*Cognettia* sp.	21.87	1	1
	Tubificida	*Gianius* indet.	17	8	**AES5188**, **AES6441**, **AET8805**, **AEU3974**	NA	NA	NA	5	4
Copepoda	Cyclopoida	*Paracyclop* sp. [A]	1	0	NA	NA	NA	NA	NA	NA
	Harpacticoida	*Bryocamptus* (*R.*) *spinulosus* [A*]	10	5	**AEP1390**, **AEP1393**	2.85	*Elaphoidella phreatica*	17.12	1	2
		Bryocamptus typhlops [A*]	4	2	**AEP1398**, **AES9779**	2.66	*Bryocamptus* (*R.*) *spinulosus*	18.70	2	2
		Elaphoidella phreatica [A**]	410	375	**AEP1377**, **AEP1381**, **AEP1382**	4.14	*Elaphoidella* sp.	10.93	3	4
		Elaphoidella sp. [A**]	154	154	**AEP1380**	1.23	*Elaphoidella phreatica*	10.93	1	1
Malacostraca	Amphipoda		11	11	**AER9180**	NA	NA	NA	2	1
		Niphargus sp. [A**]	8	6	**AET4029**	0.48	*Rheocricotopus effusus*	25.79	1	1
	Bathynellacea		1	1	**AEU1545**	NA	NA	NA	1	1
Arachnida	Actinedida	Rhagidiidae indet.	1	1	**AES8987**	NA	NA	NA	1	1
		Poecilophysis (*P.*) *spelaea* [T*]	2	2	**AEU1274**	0.33	*Chamobates spinosus*	32.38	1	1
	Mesostigmata	Gamasina indet.	14	2	**AES4285**	NA	NA	NA	1	1
		Uropodina indet.	1	0	NA	NA	NA	NA	NA	NA
	Sarcoptiformes	*Chamobates spinosus* [T]	1	1	ACF9437	NA	*Phauloppia nemoralis*	24.74	1	1
		Metabelba (*P.*) *sphagni* [T]	1	1	**AES8986**	NA	*Phauloppia nemoralis*	29.32	1	1
		Phauloppia nemoralis [T]	1	1	ACG5108	NA	*Chamobates spinosus*	24.74	1	1
		Tricheremaeus travei [T]	3	0	NA	NA	NA	NA	NA	NA
	Trombidiformes	*Atractides rivalis* [A]	1	1	ADG8744	NA	*Polypedilum laetum*	38.53	1	1
		Prostigmata indet.	4	3	**AES8988**, **AEU1275**	NA	NA	NA	2	2
Collembola	Entomobryomorpha	*Oncopodura crassicornis* [T*]	1	1	**AEQ7633**	NA	*Protaphorura janosik*	21.43	1	1
		Plutomurus sp. [T*]	4	4	**AEQ7597**	0.00	*Megalothorax* sp. (incertus-group)	25.93	1	1
		Pseudosinella paclti [T**]	12	11	**AER3210**, **AER3213**	1.96	*Protaphorura janosik*	22.76	2	1
	Neelipleona	*Megalothorax* sp. (incertus-group) [T*]	111	83	**AEQ7580**	0.79	*Megalothorax hipmani*	22.50	NA	NA
		Megalothorax carpaticus [T*]	21	16	**AEQ7585**, **AEQ7596**, **AEQ7622**	4.84	*Megalothorax* sp. (incertus-group)	22.58	1	1
		Megalothorax hipmani [T**]	9	8	**AEQ7604**	0.20	*Megalothorax* sp. (incertus-group)	22.50	1	1
		Megalothorax tatrensis [T**]	12	9	**AER3208**, **AER3209**, **AEQ7614**	2.96	*Protaphorura janosik*	22.58	1	1
	Poduromorpha	*Deuteraphorura kratochvili* [T**]	36	34	AED8018, AED8021	6.28	*Protaphorura janosik*	21.66	2	3
		Protaphorura janosik [T**]	4	4	ADW2809	0.00	*Oncopodura crassicornis*	21.43	1	1
		Tullbergiidae indet.	1	0	NA	NA	NA	NA	NA	NA
	Symphypleona	*Pygmarrhopalites pygmaeus* [T*]	2	2	**AEQ7618**	0.39	*Megalothorax* sp. (incertus-group)	29.77	1	1
		Sminthuridae indet.	1	0	NA	NA	NA	NA	NA	NA
Insecta	Diptera	Ceratopogonidae indet.	2	2	**AET7713**, **AEU5426**	NA	NA	NA	2	2
		Chironomidae indet.	2	2	**AET7231**	NA	NA	NA	1	1
		Brillia bifida [SA]	2	2	AER0386, AES3000	10.10	*Parametriocnemus stylatus*	15.97	2	2
		Corynoneura lobata [SA]	8	8	AAD1162	1.76	*Polypedilum laetum*	17.71	2	1
		Eukiefferiella tirolensis [SA]	6	4	ACT5781	0.52	*Rheocricotopus effusus*	17.97	1	1
		Parametriocnemus stylatus [SA]	1	1	AAB4494	NA	*Brillia bifida*	15.97	1	1
		Polypedilum laetum [SA]	1	1	**AES2344**	NA	*Rheocricotopus effusus*	17.27	1	1
		Rheocricotopus effusus [SA]	1	1	ACH2822	NA	*Brillia bifida*	17.25	1	1
		Thaumalea testacea [T]	1	1	ACU7701	NA	*Brillia bifida*	18.83	1	1
		Trichocera maculipennis [T*]	31	7	ACA0770	0.00	*Rheocricotopus effusus*	18.84	1	1
		Tvetenia calvescens [SA]	2	1	AAF6377	NA	*Rheocricotopus effusus*	17.37	1	1
	Plecoptera	*Leuctra pseudosignifera* [SA]	1	1	ACB2495	NA	*Thaumalea testacea*	20.52	1	1
		Nemoura sp. [SA]	2	2	**AET7981**	0.16	*Tvetenia calvescens*	21.66	1	1

Captions: BI - total number of individuals subjected to DNA barcoding, NB - number of COI barcodes generated, BIN - Barcode Index Number, MaxISD - maximum intraspecific distance, DNN - distance to nearest neighbour in our dataset, A - obligatory aquatic species, [T] terrestrial species, [SA] larval stage exclusively inhabiting the aquatic environment, [*] stygophile/troglophile, [**] stygobiont/troglobiont, ASAP - number of MOTUs detected by distance-based ASAP delimitation method, bPTP - number of MOTUs detected by phylogeny-based bPTP delimitation method. BINs that are new in BOLD are written in bold.

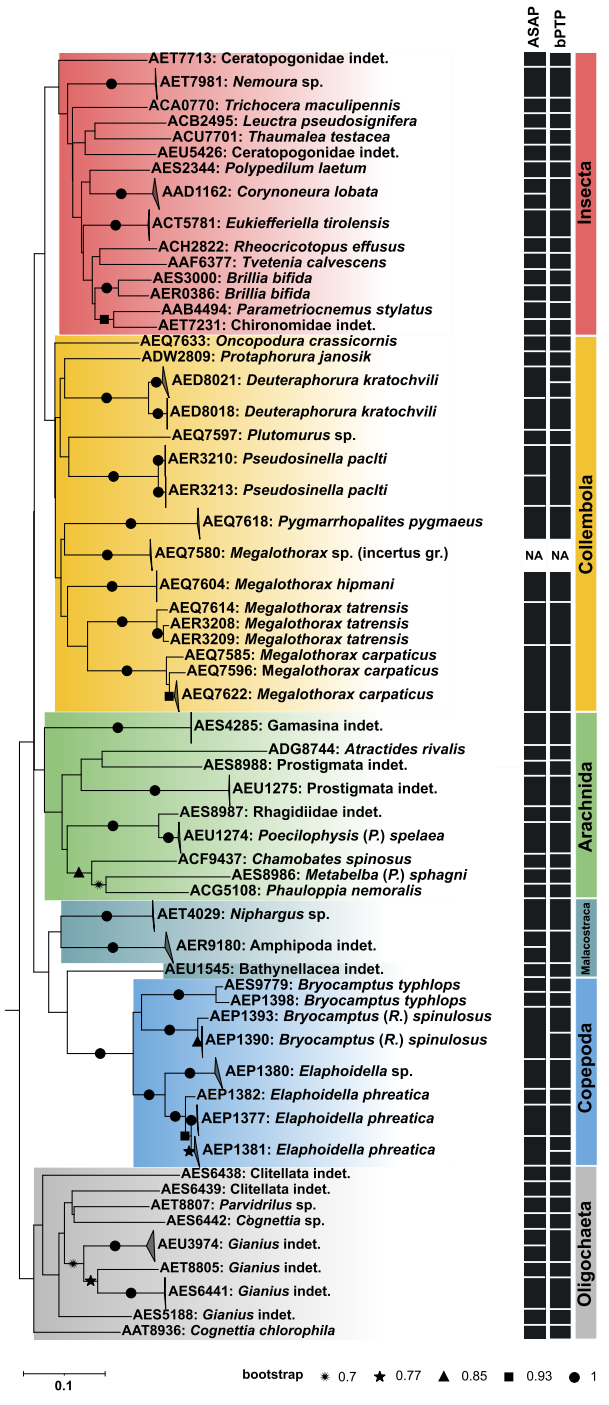

Figure 5. Phylogenetic tree of invertebrate species caught in 27 devices filtering epikarst water seeping into four caves of Demänovská Valley, inferred using Neighbor-Joining analysis of 177 haplotypes.

zation of invertebrates occurring in seeping epikarst water. Furthermore, it sheds light on the understudied realm of groundwater biodiversity and sets the stage for future extensive investigations into subterranean life diversity in the Western Carpathians and adjacent regions. Groundwater, recharged mainly from rainwater and snowmelt, contributes as much as ca. 30% of all readily available freshwater on Earth and is a critical resource for both the functioning of natural ecosystems and the development of human societies (Frappart and Merwade 2022). Epikarst remains a crucial part of the groundwater recharge system, but, due to its high dependency on precipitation, it is highly vulnerable to profound alterations in hydrological mesocycles caused by human activity (discussed thoroughly by Zalewski, 2015). Despite its importance and vulnerability, the ecological processes in this peculiar ecosystem remain practically unstudied. Such knowledge, starting with the biodiversity survey that integrates Linnaean taxonomy with a molecular approach to define reference conditions, is fundamental in accordance with the modern concept of ecohydrology. It aims to comprehend the underlying interactions between water and biota, thereby offering a novel tool for the conservation and governance of water resources (Zalewski, 2015, 2021).

Our study revealed a unique documentation of the genetic and species diversity of the Metazoa occupying the epikarst. More than 70% of the BINs (43 of 60) detected in the epikarst of the Demänovská Valley were new to BOLD. The vast majority (24 out of 28) of morphologically identified subterranean species have not yet had sequence coverage in BOLD, apart from two collembolans, *Deuteraphorura kratochvili* and *Protaphorura janosik*, and the dipteran *Trichocera maculipennis.* The troglobionts *D. kratochvili* and *P. janosik* represent widespread and abundant cave species in the Western Carpathian (Parimuchová and Kováč, 2016a, 2016b). Genetic variability, based on the mitochondrial COI marker, was only recently studied in several populations of the aforementioned troglobitic collembolans, including the population occurring in the Demänovský Cave System (Parimuchová et al., 2017, 2020). The result showed that populations of *D. kratochvili* inhabiting different caves were isolated from each other with no haplotype sharing, and their divergence was higher than between the populations of *P. janosik*, indicating longer isolation and evolution of *D. kratochvili*, which is apparently a troglobiont representing an old phyletic lineage. In fact, the genetic distance between BINs observed in our data set and other BINs (AED6524, AED8020) present in BOLD suggests that *D. kratochvili* may be a group of distinct cryptic or pseudocryptic species. In contrast, the troglophilous dipteran *Trichocera maculipennis* which is widely distributed in the Holarctis (Potocka and Krzemińska, 2018) is characterised by a low intraspecific divergence of the mitochondrial COI and 16S genes in Europe (Potocka et al., 2020). In BOLD it is represented by only two very closely related BINs ACA0770 and ABA7148, the former widespread from Svalbard to the Alps and the latter, including populations from the subterranean habitats of the Western Carpathians, barcoded within the current study. Such substantial variations in interpopulation divergence levels observed among populations inhabiting different caves can be readily explained by the contrasting dispersal capabilities of the wingless, obligate cave-dwelling, stenoecious collembolan versus the euryoecious dipteran, which can fly well and has unintentionally been introduced to Antarctica, successfully establishing populations even in its harsh environmental conditions (Potocka and Krzemińska, 2018).

Among all the invertebrates analysed in this study, Nematoda was the only taxon for which we were unable to obtain any DNA barcode. It is recognized that the conventional DNA barcode marker, the COI gene (Hebert et al., 2003), employed in this study, demonstrates restricted applicability due to pronounced sequence variability in the primer regions (Vogt et al., 2014). In the future studies, to comprehensively characterize the diversity of Nematoda within the epikarst, it will be necessary to utilize one of the alternative nuclear DNA markers that have gained prominence in the past decade for nematode identification (e.g., Creer et al., 2010; Félix et al., 2014; Pereira et al., 2010).

We observed several notable cases of intraspecific divergence. Specifically, the intraspecific distance > 2.2% and two corresponding BINs were found in four species, mostly subterranean taxa (Table 2) (MaxISD ranging from 2.66 % in copepod *Bryocamptus typhlops* to even 10.10 % in chironomid dipteran *Brillia bifida*). Additionally, three subterranean species were assigned up to three BINs (MaxISD was increased from 2.96% in collembolan *Megalothorax tatrensis* to 4.84% in collembolan *M. carpaticus*). Various historical, geographical, and ecological factors determining phylogeographic processes, such as retention of ancestral polymorphisms, incomplete lineage sorting, founder and bottleneck effects, secondary contacts resulting in hybridisation and introgression, may provide a background for intraspecific genetic structure in aquatic invertebrates reflected in distinct lineages of the COI barcoding fragment (e.g., Grabowski et al., 2017; Mamos et al., 2016; Sworobowicz et al., 2020; Ye et al., 2016) and even lead to (pseudo)cryptic speciation (e.g., Mamos et al., 2021; Wattier et al., 2020). Based on this very first data on invertebrate diversity in the Western Carpathians epikarst and given the limitations of one marker approach in DNA barcoding (e.g., Kress et al., 2015), it is not possible to elucidate the reasons for the observed nucleotide distances and divergent lineages. However, we assume that deep intraspecific divergence might indicate the potential for existence of morphologically indistinguishable cryptic species. These species may evolve in response to the fragmented nature of the karst system, which is characterized by recurrent and prolonged isolation of populations, resulting in intrinsic vicariance and dispersal patterns (e.g., Culver et al., 2009; Meleg et al., 2013). Alternatively, the cryptic diversification could be attributed to postglacial colonization originating from multiple refugia and asynchronous waves of dispersal, as observed, for instance, in groundwater isopods of the genus *Proasellus* (Eme et al., 2013), or even adaptive radiation, which facilitates rapid diversification within a lineage driven by heritable ecological versatility, as recently documented in groundwater amphipods of the genus *Niphargus* (Borko et al., 2021). It must be underlined that the Carpathian Arch, being the longest and one of the most prominent mountain chains in Europe, characterised by a complex geological setup and climate history, is also known as a key biodiversity and endemism hotspot for terrestrial and aquatic fauna (Mráz and Ronikier, 2016), the latter both in the epigean (e.g. Bozáňová et al., 2020, 2021; Copilas-Ciocianu et al., 2017, 2019; Copilas-Ciocianu and Petrusek, 2017; Dénes et al., 2016), and underground waters (Meleg et al., 2013). It can be expected that the dynamic geological history of the highly fragmented Carpathian karst has also promoted diversification in the epikarst-dwelling organisms. However, to verify our assumptions, further complex studies of integrative nature are needed. So far, our data gives evidence for the presence of other yet undescribed species in some crustacean genera (i.e., the harpacticoid copepod *Elaphoidella* sp., the amphipod malacostracan *Niphargus* sp.), associated with epikarst water. Based on the analysis of the COI sequences, there is also an indication of the occurrence of an unknown, possibly new-to-science family within the order Amphipoda. However, further molecular analyses using nuclear markers and detailed morphological examination are required to confirm its distinctiveness.

Analyses of DNA barcodes revealed ambiguity in the nucleotide sequences of a minute troglophilic collembolan *Megalothorax* sp. (*incertus* species group). DNA barcoding usually assumes that the PCR-amplified COI fragments from genomic DNA represent orthologous copies of mitochondrial DNA (Rubinoff et al., 2006). However, various molecular evolutionary processes can impede the accurate amplification of mtDNA orthologs. One such process is heteroplasmy, which involves the coexistence of two mtDNA haplotypes within a single individual (Avise, 2000). Heteroplasmy has been documented in several invertebrate species, including mollusks (Passamonti and Scali, 2001), crustaceans (Rodríguez-Pena et al., 2020), and particularly insects (Nunes et al., 2013; Meza-Lázaro et al., 2018). Several factors contribute to heteroplasmy in the mtDNA of a natural population, such as *de novo* mutations in germ line cells, recombination events, paternal leakage, or doubly uniparental inheritance (Breton and Stewart, 2015). While heteroplasmy is relatively rare and limited to specific organisms, another evolutionary process known as capture and integration of mtDNA fragments in nuclear genome, resulting in the presence of nuclear mitochondrial pseudogenes (COI numts), is a widespread phenomenon observed in various eukaryotic clades (Bensasson et al., 2001; Richly and Leister, 2004). Usually, such pseudogenes accumulate mutations making them visibly non-functional, however in some cases they can be very difficult to distinguish from the mtDNA genes (Hawlitschek et al. 2017). Determining which of these molecular evolutionary processes led to sequence ambiguity in the studied *Megalothorax* individuals requires additional and more complex analyses, which is beyond the scope of this study.

Previous studies on epikarst fauna, employing the methodology of filtering seeping epikarst water (Pipan, 2005), found several taxa of terrestrial invertebrates in addition to obligate aquatic and semiaquatic invertebrates. These were usually considered to be accidental, passively floated by water seeping from the soil surface to epikarst and, subsequently, deeper into the karst system (see, e.g., Meleg et al., 2011; Moldovan et al., 2007, 2011; Pipan et al., 2018). Likewise, a relatively high number of terrestrial microinvertebrate taxa (17 terrestrial species vs. 9 aquatic-semiaquatic invertebrate species) was captured by filtering devices we installed along the Demänová caves. Apart from the epigean terrestrial microinvertebrates, such as moss-dwelling mites, *Chamo-*

bates spinosus and *Tricheremaeus travei* (Seniczak et al., 2019, 2020), we caught several subterranean terrestrial invertebrates (troglobionts and troglophiles) generally associated with cavernous moist microhabitats. For example, we detected two species of troglobitic collembolans of the genus *Megalothorax* commonly feeding on biofilm formed on the surface of water pools (Papáč and Kováč, 2013) as well as larvae of the troglophilic dipteran *Trichocera maculipennis*, which require a constantly moist and chilly subterranean environment for their development (Potocka and Krzemińska, 2018). It is not evident whether, besides the cave environment, the terrestrial subterranean fauna occupies the fissures within the upper infiltration zone, from where it could have been drifted deeper into the karst system and thus reached the filtering devices. Alternatively, filtering devices may serve as potential attractants for terrestrial hygrophilous invertebrates living in the cave environment, causing their mixing with fauna originating in epikarst. To clearly distinguish the origin of terrestrial fauna, it will be necessary to modify the devices by adding a barrier, which would eliminate the possibility of active penetration of cave terrestrial fauna to the devices.

In conclusion, our study has generated the first DNA barcode reference library for the molecular identification of epikarst invertebrates in the Carpathian biodiversity and endemism hotspot (for review see Mráz and Ronikier, 2016). In the example of the case study sites in the Demänovský cave system, we have shown that genetic and species diversity of invertebrates associated with seeping epikarst waters is largely understudied. In several morphospecies, we revealed deep genetic divergence indicating the existence of morphologically indistinguishable cryptic species that most likely evolved within the fragmented karst system. Our study unveiled sequence ambiguity in all individuals of the Collembola species *Megalothorax* sp. (incertus-group), suggesting potential heteroplasmy or the presence of nuclear mitochondrial pseudogenes (COI numts). As such, this study represents a significant milestone in characterizing invertebrates inhabiting the epikarst and provides a novel DNA-based perspective for future comprehensive investigations into subterranean biodiversity. Furthermore, it marks the initial phase of a broader research endeavour focused on the biodiversity of the epikarst, with the aim of expanding the study to various karst regions. Initially, the focus will be on the Western Carpathians (e.g., the Malé Karpaty Mts. or Slovenský kras National Park). Subsequently, the investigation will expand to encompass other areas within the Carpathian Arch.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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