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The northernmost blind beauty rediscovered: The identity and cryptic diversity of the troglobiotic millipede *Typhloiulus polypodus* (Loksa, 1960) (Diplopoda, Julidae) from caves of the Western Carpathians

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Abstract

European blind forms of millipedes in the family Julidae constitute a taxonomically diverse ecological group of mostly cavernicolous species inhabiting primarily the Balkan Peninsula. Their northernmost endemic populations are confined to a few caves in two neighbouring karst areas in the Western Carpathians of Central Europe. The first study of these Carpathian millipedes focused on the description of *Allotyphloiulus polypodus* Loksa, 1960 from the Bükk Mountains, which was later transferred to the genus *Typhloiulus* Latzel, 1884. For more than half a century, the species was known only from its original description, as the type specimens were considered lost. We collected specimens of this blind and endemic millipede for a molecular study during long-term biospeleological research in caves of the Slovak and Aggtelek Karst and, more recently, also in the Bükk Mountains, two neighbouring karst areas with distinct geological histories. DNA barcoding based on three molecular markers (COI, 16S, and 28S rDNA) provided the first information on the population genetics and phylogeny of this millipede. We found that the populations of these two adjacent karst regions were significantly genetically different, even though morphological differences have not yet been observed. Thus, *Typhloiulus polypodus* could represent a complex of two cryptic lineages. Phylogenetic relationships along intra- and interpopulation genetic

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variability were assessed. The results suggest that the studied Carpathian blind Julidae are less closely related to *Typhloiulus* Latzel, 1884 and the tribe Typhloiulini Verhoeff, 1930 than to the phylogenetic line represented by the genus *Xestoiulus* Verhoeff, 1893 (Leptoiulini Lohmander, 1936) or some members of the tribe Julini Leach, 1814, which has comprised only epigeic, non-eyeless representatives. This conclusion is preliminarily supported by the structure of secondary sexual characters. Finally, we discuss negative impacts threatening the local populations in show caves.

Keywords
Obligate cave species, endemism, cryptic lineages, molecular phylogeny, habitus convergence, subterranean habitat threat

Introduction

Life in subterranean habitats differs from that on the surface in many ways. The absence of sunlight and primary producers (phototrophs) reduces food availability deeper underground, which limits the distributional opportunities of invertebrates and results in their low abundance and diversity, as well as infrequent reproduction and few offspring. However, some subterranean animals exhibit paradoxical adaptations – oversized bodies and long life cycles – which predispose them to long-term survival, even at latitudes where their surface relatives are already extinct due to climate change (Culver and Pipan 2019).

Based on the morphological and partly integrative classification of the taxonomically rich family Julidae Leach, 1814 (Enghoff et al. 2011; Decker et al. 2026), several lineages (subfamilies and tribes) and genera with exclusively blind representatives (usually troglobionts) were identified; however, some of them have unclear phylogenetic affiliations (Table 1). One or more blind species are also known in other genera, such as *Elbaiulus* Verhoeff, 1930, *Mesoiulus* Berlese, 1886 (Pachyiulinae), and *Cylindroiulus* Verhoeff, 1894 (Cylindroiulinae Verhoeff, 1930), along with epigean species with eyes (ommatidia).

Most European blind julid millipedes inhabiting the subterranean realm were formerly classified by taxonomists in the tribe Typhloiulini Verhoeff, 1930. The most diverse and geographically widespread genus is *Typhloiulus* Latzel, 1884, which currently includes 27 species and 3 subspecies (Decker et al. 2026). Despite the striking similarity in external morphology (Verhoeff 1899, 1930; Strasser 1962), the use of a comprehensive integrative taxonomic approach has recently provided evidence that this group (tribe Typhloiulini) of taxa is more deeply divergent. Thus, the tribe Typhloiulini and the genus *Typhloiulus* itself are, with high probability, not monophyletic, and the concept of the entire tribe is ambiguous (Makarov et al. 2017; Antić et al. 2018). Vagaliński et al. (2022) compared classical and molecular markers and state that the molecular-based phylogeny of Typhloiulini and blind Leptoiulini and Julini clearly indicates that *Typhloiulus* s. str. and the tribe Typhloiulini are poly- or paraphyletic in their current composition.

The morphological taxonomy, distribution, and habitat preferences of Typhloiulini are well known due to the intensive, long-term efforts of dozens of authors and detailed

Table 1. List of blind representatives of the family Julidae.

Species	Subfamily	Tribe
Buchneria Verhoeff, 1941	Julinae Leach, 1814	Typhloiulini Verhoeff, 1930
Inversotyphlus Strasser, 1962		
Leptotyphloiulus Verhoeff, 1899		
Serboiulus Strasser, 1962		
Stygiiulus Verhoeff. 1929		
Trogloiulus Manfredi, 1931		
Typhloiulus Latzel, 1884		
Titanophyllum Akkari, Stoev & Enghoff, 2011		Brachyiulini Verhoeff, 1909
Balkanophoenix Verhoeff, 1937		
Mammamia Akkari, Stoev & Enghoff, 2011		
Metaiulus Blower & Rolfe, 1956		Metaiulini Blower, 1958
Lamellotyphlus Tabacaru, 1976,	Oncoiulinae Verhoeff, 1909	Leucogeorgiini Verhoeff, 1930
Leucogeorgia Verhoeff, 1930		
Apfelbeckiella Verhoeff, 1901	Pachyiulinae Verhoeff, 1899	Pachyiulini Verhoeff, 1899
Chersoiulus Strasser, 1938		
Dolichoiulus Verhoeff, 1900		
Rhodopiella Strasser, 1966		
**Elbaiulus* Verhoeff, 1930		
**Mesoiulus* Berlese, 1886		
**Cylindroiulus* Verhoeff, 1894	Cylindroiulinae Verhoeff, 1930	Cylindroiulini Verhoeff, 1930

Genera marked with an asterisk are those where only some species are blind while the other species have developed ommatidia. Based on the original descriptions and redescriptions of genera and species. Their current systematic classification agrees with Millibase (Decker et al. 2026). Nevertheless, this classification is apparently not definitive, for example, Vagalinski et al. (2022) pointed out using an integrated taxonomic study that the genus *Stygiiulus* does not belong to the tribe Typhloiulini.

descriptions, such as the precise monograph by Strasser (1962). Nevertheless, descriptions of new species continue to appear (Vagalinski et al. 2015; Antić et al. 2017, 2018; Antić and Akkari 2023). The first molecular data on Typhloiulini were published by Makarov et al. (2017) and Vagaliński et al. (2022), among others, and supported the distinction and description of the new genus of blind julids, *Rhodopotyphlus* Vagalinski, 2021 (Vagalinski 2021). Yet the molecular taxonomy, phylogeny, phylogeography, and genetic variability remain largely unknown and unclear, and some taxa are still enigmatic due to the lack of material for DNA sequencing and adult specimens for detailed morphological study. Cryptic diversity and population structure/genetics in Diplopoda have been discussed in only a few papers (Bond and Sierwald 2002; Mwabvu et al. 2013, 2015; Reip and Wesener 2018; Shorter et al. 2018), especially when compared with other groups, such as springtails or mites. As the genetic variability of *Typhloiulus* species has not yet been studied, this study provides new insight into the molecular genetics of diplopods and new findings on genetic diversity within the genus *Typhloiulus*, specifically for *Typhloiulus polypodus* (Loksa, 1960).

The geographic distribution of blind Julidae covers mainly dry, warm territories around the Mediterranean and Black Seas and the islands of Macaronesia; two species are also found on the fringes of the Alps and one species in the Western Carpathians (Kime and Enghoff 2017). Most samples come from caves, but there are also some edaphic and epigeic records. Only a few species are partly or completely pigmented. Two main morphotypes are distinguished among blind Julidae: (a) semiaquatic, hygro-

petricolous dwellers of flooded microhabitats in caves, with short, poorly segmented bodies and modified mouthparts, and (b) dwellers of terrestrial subterranean environments and shaded surface habitats (the uppermost soil layer, leaf litter), with long, richly segmented bodies (Enghoff 1985).

Until the 1990s, records of blind Julidae were documented only from the Western Carpathians, the northwestern part of the Carpathian arc. Based on a male and a female collected with pitfall traps in Anna /Forrás Cave (Anna-barlang), Bükk Mountains, Hungary, in 1958–1959, Imre Loksa (1960) described the troglobiotic *Allotyphloiulus polypodus* Loksa, 1960. It should be noted that later, Loksa (1961) used two different names, *A. polypodus* and *A. bukkensis*, in the same paper for the same material (types) as in the original description in 1960. The latter is evidently a *lapsus calami*. As *Allotyphloiulus* Verhoeff, 1905 is a subgenus of *Cylindroiulus* Verhoeff, 1894 – a group of julids with completely different gonopods – Korsós (1994) more accurately assigned Loksa's species to the genus *Typhloiulus*, although in a later work Korsós and Lazányi (2020) admit that its generic allocation is still undecided. For the next four decades, no new findings were reported, and the types are considered lost (Korsós and Lazányi 2024). Subsequently, sporadic samples were documented from the Gombasecká and Domica caves (Mock et al. 2002), and later from the Baradla Cave (Papáč et al. 2014), all located in the Slovak and Aggtelek Karst (Slovakia and Hungary, respectively), which borders the geomorphological unit Bükk Mountains (Hungary). These samples were assumed to belong to a taxon identical to *T. polypodus*, but adult males and material from the type locality were missing, so the species determination remained incomplete (Košel 2012; Kováč et al. 2014).

Aims and hypothesis

This study aimed to investigate the genetic variability of *Typhloiulus polypodus* populations from two Western Carpathian regions in Hungary and Slovakia, and to reveal the phylogenetic relationships of this species within the family Julidae. Considering that the regions differ in origin and history and are separated by a geographical barrier (the Slaná River Basin), they have not communicated with each other for a long period. We hypothesized that the populations inhabiting these mountain ranges might clearly differ genetically from one another. We also discuss the background of the extremely rare occurrence of this species in caves and the reasons for the likely extinction of the population at the type locality.

Material and methods

Molecular research on *Typhloiulus polypodus* was carried out in six caves belonging to four hydrogeological (cave) systems located within two geomorphological regions. Following previous extensive exploration of a number of caves in the Bükk Mountains, Slovak Karst, Aggtelek Karst, and surrounding karst regions, this complex of blind

julid millipedes was confirmed in six caves. Four caves are located in the Slovak Karst and Aggtelek Karst, two in Slovakia (Gombasecká Cave – GOMC, Domica Cave – DOMC), two in Hungary (Baradla Cave – BARC, Vass Imre Cave – VIB), and two caves are located in the Bükk Mountains in Hungary (Szent István Cave – SZIC, Vesszős-gerinci Cave – VGC) (Fig. 1, Table 2).

Field research was conducted as part of long-term cooperation of the authors (AR, AM, ĽK) with the administrations of Bükk National Park, Aggtelek National Park, Slovak Karst National Park, and the Slovak Caves Administration. Initially, caves were explored, after which the most promising plots were selected for the installation of several types of pitfall traps, differing in fixative solution (water solution of ethylene glycol, ethanol (75%), or formaldehyde solution (4%)). Following the instructions of the Bükk Mountains National Park management, pitfall traps were not used in caves of this karst area. Trapping was carried out in the caves of the Aggtelek Karst and the Slovak Karst. At all localities, hand collecting and trapping were supplemented by baiting: sterilized wood material (usually fresh hardwood crushed in a garden branch chipper) was exposed for a long time and checked during subsequent visits to the cave. Part of the bait volume was heat-extracted in the laboratory. Following the pilot inventory of cave fauna, several caves were repeatedly surveyed, namely the Domica Cave and the Gombasecká Cave (Slovak Karst), where continuous monitoring has been carried out

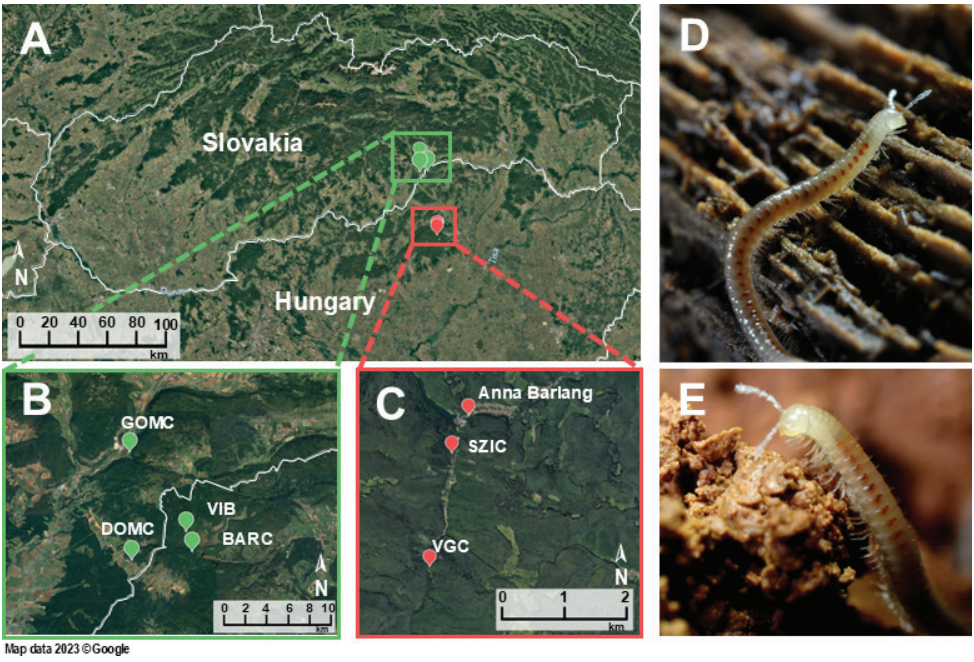

Figure 1. Locations of *Typhloiulus polypodus* populations in Slovakia and Hungary (**A**); Slovak and Aggtelek Karst (**B**); Bükk Mountains (**C**). *Typhloiulus polypodus*, Baradla Cave, Slovak and Aggtelek Karst. Photo: Ľ. Kováč and A. Mock (**D**). *Typhloiulus polypodus*, Vesszős-gerinci Cave, Bükk Mountains. Photo: Ľ. Kováč (**E**). For abbreviations of the sampling sites, see the Materials and Methods section.

Table 2. Sampling design of *Typhloiulus polypodus* populations: sites, collection dates, preferred habitats, and GenBank accession numbers of sequences.

Specimen code	Locality	State	Unit	Date	Habitat	Sex	Collector	GenBank accession number		
								28S	16S	COI
GOMC7	Gombasecká Cave	SK	Slovak Karst	13 Jun 2024	Rotten wood on clay bottom (stationary plot)	M	A. Mock; A. Rednošová	PX924953	PX939278	PX922004
GOMC8	Gombasecká Cave	SK	Slovak Karst	13 Jun 2024	Rotten wood on clay bottom (stationary plot)	F	A. Mock; A. Rednošová	PX924954	PX939279	PX922005
GOMC9	Gombasecká Cave	SK	Slovak Karst	13 Jun 2024	Rotten wood on clay bottom (stationary plot)	F	A. Mock; A. Rednošová	PX924955	PX939280	PX922006
GOMC10	Gombasecká Cave	SK	Slovak Karst	13 Jun 2024	Rotten wood on clay bottom (stationary plot)	F	A. Mock; A. Rednošová	PX924956	PX939281	PX922007
GOMC11	Gombasecká Cave	SK	Slovak Karst	13 Jun 2024	Rotten wood on clay bottom (stationary plot)	M	A. Mock; A. Rednošová	PX924957	PX939282	PX922008
GOMC12	Gombasecká Cave	SK	Slovak Karst	11 Aug 2020	Rotten wood on clay bottom (stationary plot)	F	M. Melega		PX939283	PX922009
BARC15	Baradla Cave	HU	Aggtelek Karst	1 Dec 2023	Rests of rotten wood on scree bottom	M	Ľ. Kováč; A. Mock	PX924958	PX939284	PX922010
DOMC17	Domica Cave	SK	Slovak Karst	25 May 2014	Rotten wood on clay bottom (stationary plot)	F	A. Mock	PX924959	PX939285	PX922011
VIB20	Vass Imre Barlang	HU	Aggtelek Karst	25 Oct 2014	Clay bottom at guano heap	M	V. Papáč	PX924960	PX939286	PX922012
SZIC13	Szent István Cave	HU	Bükk Mts.	19 Oct 2023	Rotten wood on clay bottom (remains of construction timber and stationary plot)	F	A. Mock	PX924961	PX939287	PX922013
SZIC14	Szent István cave	HU	Bükk Mts.	20 Oct 2023	Rotten wood on clay bottom (remains of construction timber and stationary plot)	M	A. Mock	PX924962	PX939288	PX922014
SZIC16	Szent István Cave	HU	Bükk Mts.	20 Oct 2023	Damp wall with clay coating	F	P. Ľuptáčik	PX924963	PX939289	PX922015
VGC18	Vesszős-gerinci Cave	HU	Bükk Mts.	20 Oct 2023	Wet clay bottom	F	A. Rendošová	PX924964	PX939290	PX922016
VGC19	Vesszős-gerinci Cave	HU	Bükk Mts.	20 Oct 2023	Wet clay bottom	F	A. Mock	PX924965	PX939291	PX922017

For abbreviations of sites, see the Materials and methods section.

for more than two decades. Despite numerous trapped invertebrates, none of them represented blind julid millipedes. Such specimens were collected only individually (frequently at the bait heaps) or through bait extraction in a Berlese-Tullgren funnels; they were preserved in pure 96% ethyl alcohol for subsequent molecular analysis.

Laboratory work

Individuals preserved in denatured 75% or pure (96%) ethyl alcohol were first studied under stereomicroscopes (Nikon CP-S, Olympus SZ 61). Specimens were initially sorted into adult males, females, and juveniles based on secondary sexual characters. General habitus was observed, and the shape of male gonopods was evaluated after dissection of the body (according to Loksa 1960; Strasser 1962; Vagaliński et al. 2015, 2022; Antić et al. 2018).

DNA extraction, amplification, and sequencing

Six legs from each of 14 specimens were used to extract total DNA with the QIAGEN DNeasy Blood and Tissue Kit (Valencia, CA, USA), following a modified manufacturer's protocol with 50 µl of elution buffer.

To reveal phylogenetic relationships and genetic diversity among populations of *Typhloiulus polypodus* from the Slovak and Aggtelek Karst and the Bükk Mts., we used two mitochondrial (16S rDNA and COI) and one nuclear (28S rDNA) molecular marker (Enghoff et al. 2013; Makarov et al. 2017). The following primers were used to amplify the 16S rDNA fragment: LR-J-12961 (Cognato and Vogler 2001) and LR-N-13398 (Simon et al. 1994); for 28S rDNA, the 28SH and 28SL primers (Muraji and Tachikawa 2000) were used. Degenerate LCO1490_JJ and HCO2198_JJ primers were used for the COI gene (Astrin and Stüben 2008). Primer sequences are available in Suppl. material 1: table S1.

PCR amplifications were performed with the Unis Taq system (TopBio s.r.o., Prague, Czech Republic) using standard volumes and were carried out in a TAdvanced Twin 48 G thermocycler (Biometra). PCR reactions were run at the annealing temperatures listed in Suppl. material 1: table S1 for 37 cycles. Following confirmation by agarose electrophoresis, the PCR products were enzymatically cleaned using ExoI-FastAP (Thermo Fisher Scientific) and subsequently sequenced by Sanger sequencing at Eurofins Genomics (Ebersberg, Germany).

Chromatograms were inspected and manually edited using Geneious Prime v.2024.1.1 (https://www.geneious.com/). Additionally, all sequences were verified as diplopod DNA using a BLASTn search in GenBank (the algorithm with default settings produced alignments only to other Diplopoda with an E value < 4×10^{-115}). All COI sequences were assumed to be genuine mitochondrial sequences and not nuclear copies (NUMTs) or pseudogenes, as their ORFs were intact and none contained stop codons or indels.

The sequences are available in GenBank for public use. Table 2 lists the voucher code and GenBank accession number for each specimen.

Molecular analysis

The ClustalW method (Thompson et al. 1994) in the MEGA X software (Kumar et al. 2018) was used to align all three markers, both individually and in combination (28S + 16S). Extended datasets were used for all molecular analyses. To elucidate phylogenetic relationships among *T. polypodus* populations and determine its position within Diplopoda, sequences (COI, 16S, and 28S rDNA) from various taxa of the order Julida were included. The selection consisted of a targeted cross-section of Julida clades into which the studied species could potentially be classified based on previous studies (Enghoff et al. 2011, 2013; Makarov et al. 2017). For the COI marker, to assess genetic variability among *T. polypodus* populations, the dataset included sequences from seven species of the genus *Typhloiulus*, as sequences for the species under study

were not available in the database. GenBank accession numbers and BOLD codes for the additional sequences are listed in Suppl. material 1: table S2.

Using the Maximum Likelihood (ML) method, we generated a phylogram to determine the phylogenetic relationships between *T. polypodus* populations from the Slovak Karst, the Aggtelek Karst and the Bűkk Mts., as well as to classify this species within the system of Diplopoda. For the concatenated dataset, the PhyML web server (Guindon et al. 2010, http://www.atgc-montpellier.fr/phyml/) was used to reconstruct the tree. The best-fit model, GTR+R (28S+16S rDNA), was selected using the Model Selection module (Lefort et al. 2017), and branch support was assessed by aLRT SH-like (1,000 replicates). For the COI dataset, the IQ-TREE 3 program (Wong et al. 2026) was used to construct the phylogenetic tree; node support was assessed using the bootstrap method (ultrafast, 5,000 replicates), and the TIM2+F+I+G4 model, selected using ModelFinder (Kalyaanamoorthy et al. 2017), was applied. Relevant sequences of *Proteroiulus fuscus* (Am Stein, 1857) (Julida, Blaniulidae) (GenBank accession number HQ966169.1) and *Thyropygus bearti* Enghoff & Panha, 2009 (Spirostreptida, Harpagophoridae) (GenBank accession numbers: JF321008 for 28S rDNA and JF320953 for 16S rDNA) were used as outgroups.

The final lengths of our alignments were 492 bp (28S), 397 bp (16S), and 615 bp (COI), and 1034 bp for the concatenated dataset (28S + 16S rDNA) with gaps.

Results

According to the concatenated ML phylogenetic tree (Fig. 2), the populations of *T. polypodus* from the Slovak and Aggtelek Karst were separated from those from the Bűkk Mts. with significant support. This separation was also observed in the COI-based tree (Fig. 3). Regarding the position of *Typhloiulus polypodus* within the system of Diplopoda, populations of this species were closest in both phylogenetic trees to species from the tribes Julini and Leptoiulini, particularly to the genus *Xestoiulus*. The concatenated phylogram showed a close relationship to *Xestoiulus imbecillus* (Latzel, 1884), while the COI phylogram displayed a relationship to *Xestoiulus laeticollis* (Porat, 1889).

The distances between populations of *T. polypodus* ranged from 0.0 to 12.083% and 13.445% (for p-dist and K2p, respectively; Suppl. material 1: table S3), while the genetic distances between *T. polypodus* and outgroup taxa were generally higher, ranging from 1.409% (p-dist) and 1.453% (K2p) to 24.953% and 30.894% (for p-dist and K2p, respectively) (Suppl. material 1: table S3). No genetic variability was observed within *T. polypodus* populations (Suppl. material 1: table S3). In terms of geographical regions, the genetic distance between individuals of *T. polypodus* from the Slovak and Aggtelek Karst and the Bűkk Mts. was 12.068% (p-dist) and 13.426% (K2p). In populations from the Bűkk Mts., we did not observe any genetic variability, while in populations from the Slovak and Aggtelek Karst the genetic variability reached 0.032% (p-dist and K2p).

For the other two markers, we observed the same pattern, i.e., lower genetic distances between *T. polypodus* populations than between outgroup taxa from the Typhloi-

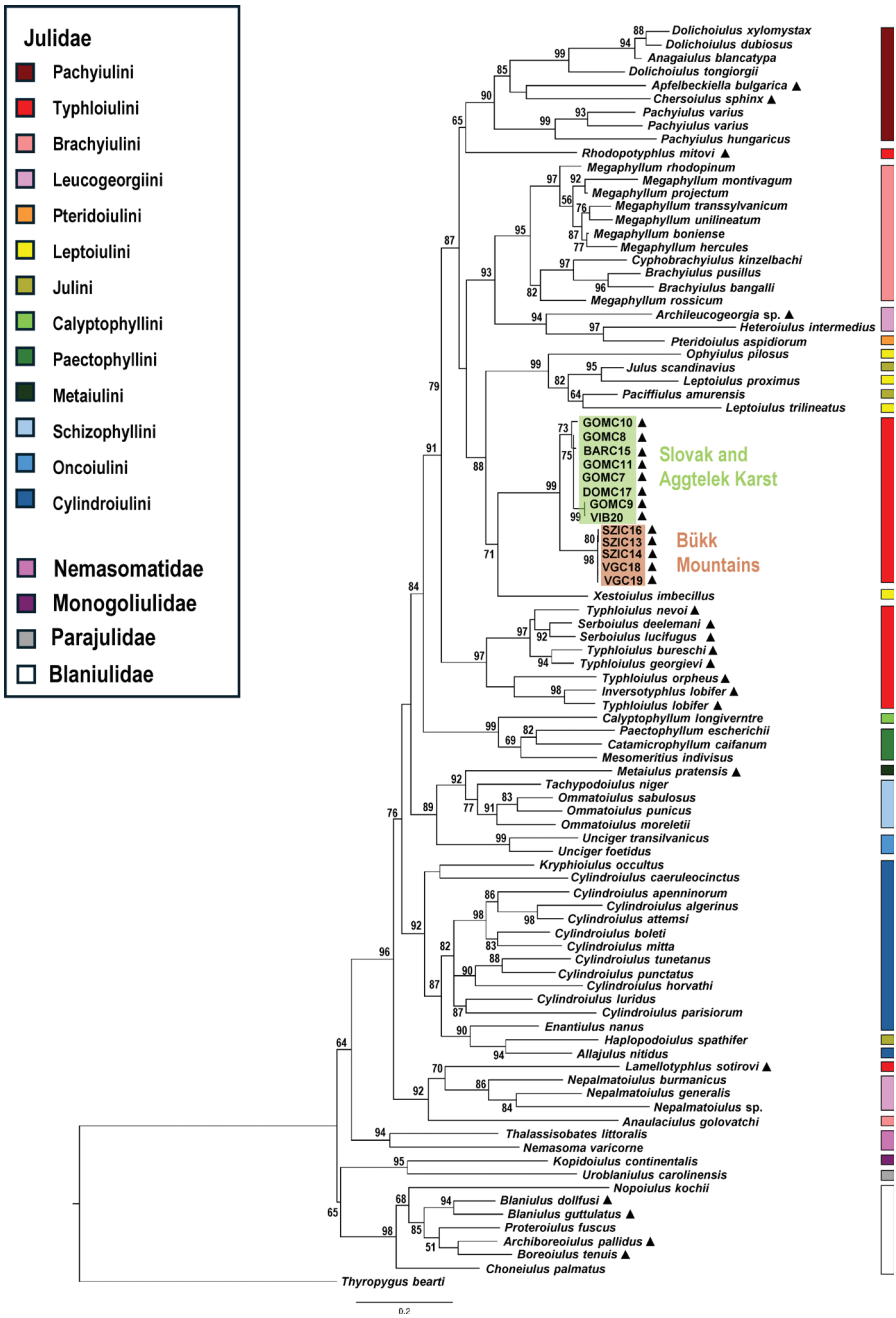

Figure 2. Maximum Likelihood (ML) phylogenetic tree reconstructed from the concatenated and extended dataset containing populations of *Typhloiulus polypodus*, selected representatives of the order Julida (28S + 16S rDNA genes), using the GTR+R model, and *Thyropygus bearti* as the outgroup (JF321008 for 28S rDNA, JF320953 for 16S rDNA). Values next to branches represent aLRT SH-like support (1,000 replicates; values > 50% shown). The classification of individual species into tribes/families is indicated by color; tribes correspond to the current system of Diplopoda (Decker et al. 2026). GenBank accession numbers of the included julids are listed in Suppl. material 1: table S2.

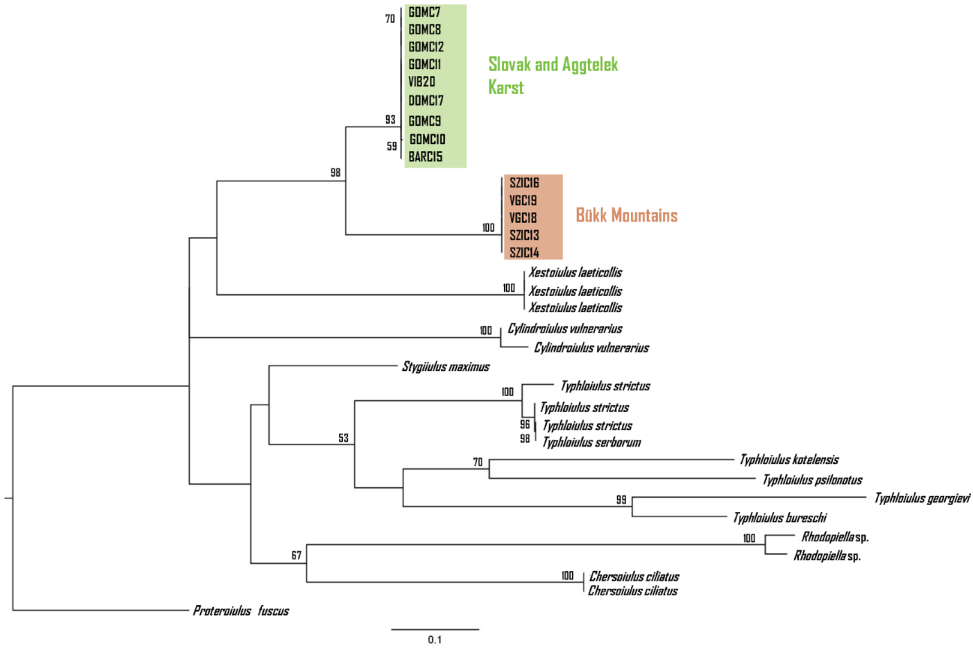

Figure 3. Maximum Likelihood (ML) phylogenetic tree reconstructed from extended *Typhloiulus polypodus* populations for the COI marker (TIM2+F+I+G4 model, 5000× bootstrap; values > 50% shown), with *Proteroiulus fuscus* (HQ966169.1) as the outgroup. The GenBank accession numbers of the included *Typhloiulus* species are listed in Suppl. material 1: table S2.

ulini tribe (Suppl. material 1: tables S4, S5), with values for the 28S rDNA marker generally lower than for the COI marker. For the 16S rDNA marker (Suppl. material 1: table S4), genetic variability within *T. polypodus* populations ranged from 0.0% to 0.112% (p-dist and K2p), and between populations from 0.000% (p-dist and K2p) to 9.192% (p-dist) or 9.597% (K2p). The genetic distances between outgroups for this marker ranged from 9.138% to 33.046% (p-dist) and from 9.938% to 43.306% (K2p). The genetic distances for the 28S marker are shown in Suppl. material 1: table S5; genetic variability within populations ranged from 0.0 to 0.138% (p-dist and K2p), and between populations from 0.0 to 0.832% (p-dist) or 0.836% (K2p), with lower values observed between populations from the same region and higher values between populations from different regions. For outgroup taxa, genetic distances ranged from 0.000% to 3.246% or 3.326% (p-dist and K2p, respectively).

Discussion

Phylogenetic analysis

The phylogenetic separation of the studied populations in both regions was observed, along with the interesting position of their MOTUs within the phylogenetic tree. Ac-

cording to the current classification and system of Diplopoda (Decker et al. 2026), we expected that *T. polypodus* would form a cluster with other species of this genus or within the tribe Typhloiulini. Conversely, based on the robust concatenated phylogenetic tree (Fig. 2), *T. polypodus* MOTUs formed a subcluster more closely related to the tribes Julini and Leptoiulini (based on phylogenetic analysis, these two tribes probably represent a single lineage), specifically to the genus *Xestoiulus* (represented by the species *Xestoiulus imbecillus* (Latzel, 1884)). The phylogenetic position of *T. polypodus* and the genus *Xestoiulus* (represented by the species *Xestoiulus laeticollis* (Porat, 1889)) was also supported by the analysis using only the COI marker. Although the explanation of the phylogenetic tree topology is not clear, poly- or paraphyly of the entire tribe Typhloiulini and the genus *Typhloiulus* within the family Julidae is suggested. This assumption is also supported by recent studies (Vagaliński et al. 2015, 2022; Makarov et al. 2017; Vagalinski 2021; Antić and Akkari 2023), in which, congruently with our results, the genus *Typhloiulus* was considered paraphyletic in relation to the genus *Serboiulus* (Makarov et al. 2017). However, increasing molecular data are helping to clarify these ambiguities, which result from the convergent evolution of subterranean lineages and the unreliability of morphological diagnostic characters in phylogenetic reconstructions (Enghoff et al. 2011; Vagaliński et al. 2022). Furthermore, based on phylogenetic analysis, it can be concluded that *Typhloiulus polypodus* (probably a complex of cryptic lineages, MOTUs) is not closely related to the other congeneric species of this genus and thus could represent a separate genus. Thus, *T. polypodus* is a candidate for assignment to the genus *Xestoiulus*, currently as the sole blind representative of this genus. A comprehensive redescription and taxonomic delineation of both lineages is necessary; however, this is outside the scope of this study.

Genetic variability of populations

Although DNA barcoding is widely and frequently used for species delimitation and for revealing genetic variability across nearly all groups of invertebrates (e.g., Hebert et al. 2003; Porco et al. 2012; von Saltzwedel et al. 2014; Sun et al. 2018; Striuchkova et al. 2022; Le Cadre et al. 2024), studies using this method in diplopods are scarce. This study, based on DNA barcoding in combination with two additional markers, mitochondrial 16S rDNA and nuclear 28S rDNA, provides important molecular and genetic information about troglobitic species within the class Diplopoda. For *Typhloiulus polypodus*, occurring in caves of the Western Carpathians, such information is currently lacking.

High genetic distances between *Typhloiulus polypodus* populations were observed for both mitochondrial markers, with significantly higher variability among morphologically distinct outgroup species. Genetic variability within populations, as well as within the Slovak Karst, Aggtelek Karst, and Bükk Mts. regions, was negligible or almost absent. The low variability among populations within the studied geographical regions suggests gene flow among these populations. The Domica and Baradla caves form the Domica–Baradla cave system, which may explain the lack of variability among these populations. Nevertheless, the difference between intrapopulation and

interpopulation distances was more than tenfold (COI), particularly among geographic regions. In other invertebrate groups, e.g., Collembola, individuals or populations showing such differences in genetic distances are considered distinct species, or are thought to harbor cryptic diversity (e.g., Hogg and Hebert 2004; Porco et al. 2014). Similar genetic distances to those reported here have been observed in a few Diplopoda studies (Bond and Sierwald 2002; Frederiksen et al. 2012; Mwabvu et al. 2015; Chen et al. 2024). For example, Frederiksen et al. (2012) focused on the genus *Pachyiulus* (Julida: Julidae), specifically the closely related species *Pachyiulus flavipes* C. L. Koch, 1847 and *Pachyiulus oenologus* Berlese, 1885. The authors observed divergence of approximately 13.2% for the COI marker and 16.9% for the 16S rDNA marker. However, similar values were also observed for closely related but morphologically distinct species (12.5–15% for COI and 8.7–15.5% for 16S). Furthermore, Bond and Sierwald (2002) provide a threshold for species differentiation within Diplopoda, ranging from 11 to 15% (p-distance), determined from COI sequences of 242 *Anadenobolus excisus* (Karsch, 1881) (Spirobolida: Rhinocricidae) individuals. Considering this threshold, the genetic distance values from previous studies, and the absence of differences in gonopod shape among individuals from the studied regions, it is assumed that the *T. polypodus* populations from the Slovak and Aggtelek Karst, and Bükk Mts. may represent two cryptic lineages. Thus, *Typhloiulus polypodus* from the Western Carpathians can be considered a complex of cryptic lineages (MOTUs, molecular taxonomical units).

No gene flow is supposed between the studied regions, which may have been caused by the geographical barrier formed by the Slaná River Basin. In addition, the Slovak Karst and Aggtelek Karst, and the Bükk Mountains differ in geology. Based on recent studies of plate tectonics in the Pannonian Sea and Carpathian regions, the Aggtelek Karst joined with the Slovak Karst developed independently from the Bükk Mountains and moved closer to one another only secondarily, from various directions, during the Early Miocene (Gaál 2008). Later, the terrestrial environments of both regions were separated by the former Pannonian Sea and rivers carrying massive sediment deposits. As a result, the limestone parts of these regions are isolated by sedimentary and non-sedimentary bedrock of diverse origins (Haas 2013; Gaál et al. 2022). The Bükk Mountains could serve as a kind of bridge or an important biogeographic refuge for Mediterranean fauna on the southern edge of the Western Carpathians, as geologically they are closer to the Dinaric Mountains and the geological units of the Pannonian region than to the Carpathians (Košel 2012). These regional differences are also reflected in the phylogenetic tree, which shows a clear separation of populations from the Slovak and Aggtelek Karst and the Bükk Mountains, supported by the absence of shared haplotypes (data not shown). In this case, the lack of gene flow can be considered additional evidence of the isolation of populations from these two regions, supporting the cryptic lineage complex hypothesis.

Notes on the phenotype of W. Carpathian cave julid millipedes

The diagnostic features of populations from the distributional range of the examined material show only slight intraspecific variability and basically correspond to the origi-

nal description of *Allotyphloiulus polypodus*, although the original illustrations are only schematic (Loksa 1960). Comparison of the morphological diagnosis of *T. polypodus* with the phylogenetic analysis of molecular data indicates the need for a detailed redescription of both molecular and geographic lineages of the taxon. We found relatively pronounced intraspecific and intrapopulation variability in the size and segmentation of both males and females, with the predominance of body size not being a clear case of sexual dimorphism. Considering intraspecific variability in the segmentation of adults of cave julid millipedes in general, it seems to increase in larger species with a higher number of body rings (Fig. 4). The etymology of the species name "polypodus" is apt: this species belongs within the family Julidae, and probably also within the entire order Julida, to those with the highest number of body rings, although it is not among the largest species (Enghoff et al. 2015). Intraspecific and interspecific variability of myriapod segmentation has been documented in many species and morphotypes, but a satisfactory evo-devo scenario is still missing (Minelli 2020).

Biogeography, ecology, conservation

Many years of field research have shown that cavernicolous julid millipedes in the Western Carpathians occur exclusively in caves within two karst regions of Slovakia and Hungary, separated by the 30 km wide plain of the Slaná/Sajó River, the Bűkk Mountains, and the Aggtelek Karst and Slovak Karst. Seven caves with occurrences of *T. polypodus* belong to four hydrogeological (cave) systems. The localities are grouped in the northeastern part of the Bűkk Mountains and the southwestern part of the Slovak Karst and Aggtelek Karst, in areas that were once geographically close to each other. The underground sites with *T. polypodus* share common characteristics: they are extensive underground systems formed in Mesozoic limestone, but their fragments are mostly horizontal, of fluviokarst origin, with a permanent or temporary underground stream. These caves are moist, occasionally flooded, and relatively warm (8.5–12.0 °C). Their main entrances are situated at elevations between 290 and 535 m a.s.l. (Gaál et al. 2022; Gruber et al. 2022; Vetési-Foith et al. 2022). The Anna Cave (type locality) differs in origin and bedrock; it formed in travertine as a complex of separate chambers (artificially connected into one system), making it a relatively young, Pleistocene complex of originally isolated cavities and an underground stream channel. However, this locality is not isolated and is hydrogeologically connected to the surrounding karst caves (the Szent-István and Vesszős-gerinci caves) (Vetési-Foith et al. 2022).

The distribution of *Typhloiulus polypodus* is the northernmost among cave julids in Europe; its endemic range is restricted to the Bűkk Mountains and the Slovak and Aggtelek Karst (Kime and Enghoff 2017). Košel (2012) assumed that the Bűkk Mountains and their surroundings (the Bűkkikum Unit) represented an important south–north land bridge in the Paratethys Sea between the Dinaric Mountains and the Western Carpathians during the Tertiary, which is also in agreement with the modern concept of an extinct continental Tisza-Dacia mega-tectonic unit (Haas 2013; Kováč et al. 2017; Vojtko et al. 2022). This assumption is supported by a large part of the

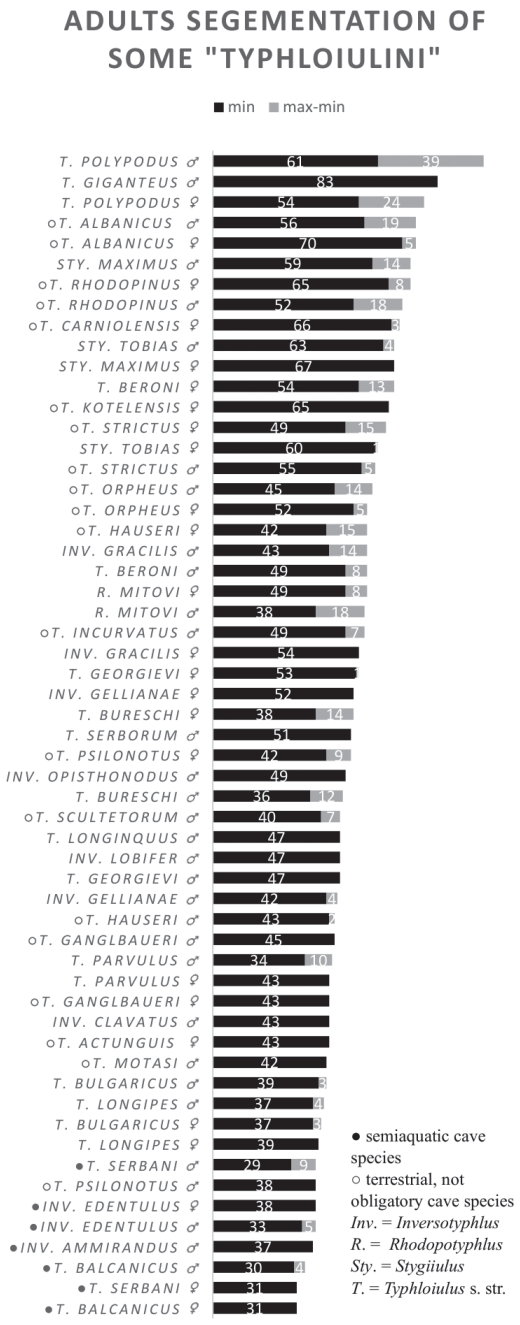

Figure 4. Metamerization of the body in some adult blind Julidae (Typhloiulini), sorted by total body segments in descending order, taking sex into account; a review (data mainly from original descriptions: Loksa 1960; Strasser 1962; Vagaliński et al. 2015, 2018; Antić et al. 2018; Vagalinski 2021; Antić and Akkari 2023; and original data). Legend: columns show total body segment counts: minimum (black), difference between minimum and maximum (gray), maximum (black + gray); • semiaquatic cave species, º terrestrial, non-obligate cave species. Unmarked species are terrestrial obligate cave dwellers. Abbreviations of generic names: Inv. = *Inversotyphlus*, R. = *Rhodopotyphlus*, Sty. = *Stygiiulus*, T. = *Typhloiulus* s. str.

recent subterranean fauna of the Western Carpathians, which has a southern (Mediterranean) origin (Košel 2012; Kováč et al. 2014).

Our observations suggest that this species is associated with more extensive fluviokarst systems that have a humid and relatively warm cave microclimate. "*Typhloiulus*" specimens were collected in various parts of caves, usually in the dark zone with a stable microclimate but relatively close to the surface (within a few tens to a maximum of several hundred meters). Such sites were often located in static zones of caves with sinking watercourses that transport organic substrates (wood, leaves, soil, animals). This environment is very sensitive to water management and chemical degradation of the surface reaches of streams. Most of our collection sites are located in show caves, accessible via concrete-lined paths that are regularly cleaned. Artificial lighting requires extensive electrical installations, which cause light and heat pollution and promote the growth of green plants (lamp flora) that are then removed using aggressive sprays, mostly chlorine-based cleaning products. Moreover, show caves have thousands of visitors per year, and protection of the subterranean ecosystem is secondary to visitor safety and comfort. Major interventions in the Anna (Forrás) Cave, a type locality of *T. polypodus*, probably led to the extinction of the local (sub) population of *T. polypodus* and other cavernicoles, when comparing recent data with those of Loksa (1961). These interventions included artificial drainage of systems, modification of the floor, disinfection of the underground space during the COVID-19 pandemic, chemical removal of lamp flora, and the high visitor traffic itself, which introduced non-native microorganisms and altered the microclimate (personal information from the cave managements). This example should serve as a lesson and lead to avoiding similar blunders in the management of show caves. Moreover, one of the local populations of this species disappeared in part of the Domica Cave due to ill-considered concreting of the floor in areas designated for speleotherapy. Local populations of *Typhloiulus polypodus* occurring in caves open to the public are thus under real threat. These caves are of exceptional natural value, especially in today's era of growing "cultural heritage tourism" (Chiarini et al. 2022; Piano et al. 2022, 2024). The presence of rare, endemic organisms in caves is part of the irreplaceable value of these peculiar habitats (Donato et al. 2014). We should consider biodiversity uniqueness not only in terms of morphologically well-defined species, but also distinct genetic lineages, which are also referred to as conservation units in modern nature protection (Coates et al. 2018; Allendorf et al. 2022). Our study showed that at least two such conservation units of blind julid millipedes occur in caves of the Western Carpathians.

Conclusion

The newly collected specimens of the obligate cave millipede provisionally assigned to *Typhloiulus polypodus* in Western Carpathian caves represent a complex of two cryptic lineages (MOTUs). We found that populations in two neighbouring but isolated mountain ranges in the southeastern part of the Western Carpathians are genetically distinct and form two separate phylogenetic lineages, although we have

not yet identified diagnostic morphological features that would confirm these two presumed species. In contrast, individuals from several caves within a single mountain range showed very low genetic variability, suggesting ongoing gene flow or a historical genetic bottleneck. Therefore, very detailed microscopic studies are needed to morphologically distinguish these two lineages and clarify their status. Phylogenetic analysis of the group of blind species from the tribe Typhloiulini has confirmed that the concept of the genus *Typhloiulus* is partly artificial and that the *T. polypodus* complex does not belong here genetically. Consequently, it is necessary to reconsider the taxonomy and phylogeny of the blind, subterranean species of the order Julidae in Central Europe. Field experience also shows that these cave-dwelling animals are sensitive to long-term human interventions in underground ecosystems, which most likely led to the permanent degradation of Anna Cave in the Bűkk Mountains, the type locality of the unique millipede *T. polypodus*, as well as the intensively visited parts of other show caves in both studied karst regions. A detailed investigation of the external morphology and alimentary tract physiology of the material, with taxonomic and evolutionary implications, is underway as the next phase of the study of these rare subterranean millipedes.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript, including the disclosure of any use of AI.
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Author contributions

MF: contributed to conceptualization, data acquisition, formal analyses, data interpretation, investigation, visualization, and writing – original draft. AR: contributed to data acquisition, formal analyses, and data interpretation. MŽ: contributed to formal analyses and data interpretation. AM: contributed to data acquisition, conceptualization, data interpretation, and writing – original draft. Ľ.K: acquired funding and contributed to data interpretation. All authors contributed to writing – review and editing of the final manuscript, commented on previous versions of the manuscript, and read and approved the final manuscript.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

Supplementary material 1

Supplementary tables

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Data type: docx

Explanation note: **table S1.** Primers and PCR reaction parameters for obtaining *Typhloiulus polypodus* sequences. **table S2.** GenBank accession numbers of the outgroup taxa used to calculate the genetic distances (28S rRNA) and to construct phylogenetic trees (28S rDNA and 16S rDNA). **table S3.** Intra- and interpopulation genetic distances (%) of *Typhloiulus polypodus* and outgroup taxa based on the COI marker. **table S4.** Intra- and interpopulations genetic distances (%) of *Typhloiulus polypodus* and the outgroup taxa for the 16S marker. **table S5.** Inter- and intrapopulations genetic distances (%) of *Typhloiulus polypodus* and the outgroup taxa for the 28S marker.

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