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Distinct phylogeographic patterns in populations of two oribatid mite species from the genus *Pantelozetes* (Acari, Oribatida, Thyrisomidae) in Central Europe

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Abstract
Oribatid mites are important decomposers of dead organic matter in soils across the world. Their origin dates back at least 380 Mya. Multiple severe climatic changes during Late Pliocene and Pleistocene shaped the migration patterns of these organisms and should be reflected in the genetic variability of their current populations. In this study, we examined the genetic diversity and phylogeographic structure as well as the evolutionary history of populations of two ecologically different oribatid mite species. *Pantelozetes cavaticus* is a troglophile oribatid mite known mainly from Central European caves, whereas *Pantelozetes paolii* is a common surface eurytopic species with Holarctic distribution. We used two molecular markers—mitochondrial cytochrome c oxidase subunit I (COI) and the nuclear D3 region of the 28S rDNA gene—to reveal phylogenetic relationships between contemporary populations. Whereas the D3 region showed minimal or no variability within populations, COI appeared to be a relevant marker for population studies. Phylogeographic analysis based on COI detected two lineages of *P. cavaticus* ('Czech' and 'Slovak'), which separated during the Late Pliocene (2.9 Mya) and revealed the existence of one new species. In contrast, three identified genetic lineages of *P. paolii* (radiation time 2.9 and 1.2 Mya, respectively) uncovered in this study were found to coexist in the distant sampling localities, suggesting a connection between populations even over long distances.

Keywords Oribatid mites · Genetic diversity · Phylogeography · COI · D3 region

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Introduction

Oribatid mites (Acari: Oribatida) are mostly a soil-dwelling and very diverse group of microarthropods with more than 11,000 species described so far (Subías 2020). Their representatives can be found in almost every terrestrial habitat from tropics to poles, being the most abundant group in the first few centimeters of soil, with a density up to hundreds of thousands of individuals per m^2 in temperate forests with a thick humus layer (Maraun and Scheu 2000). They play an important functional role in soils as decomposers of dead organic matter and stimulators of fungal and bacterial growth (Luxton 1972).

Oribatid mites are among the oldest terrestrial animals, with earliest fossil record from Devonian sediments dated at 380 Mya (Norton et al. 1988) and molecular analyses dating their origin to at least 580 Mya (Schaefer et al. 2010). In Europe, climatic oscillations and consequential biome changes over the past 3 million years caused considerable shifts in distributional patterns of surface as well as soil-living animals (Hewitt 2004). During the cold periods, the warm-adapted fauna and flora either went extinct or were forced to look for more favourable habitats, i.e., refugia. However, active dispersal skills of the soil mesofauna, such as Oribatida or Collembola, are quite limited (Lehmitz et al. 2012) and more recent climatic and habitat changes during Pliocene and Pleistocene defined the migration patterns of the individual species, which should be still reflected in the genetic composition of their current populations. Such effects on the genetic diversity have been studied only scarcely in soil-dwelling arthropods (Beheregaray 2008; Rosenberger 2010), although present-day distributional patterns of intraspecific genetic diversity and estimations of its degree have been shown to provide important insights into the phylogeography and evolutionary history of species (Beebee and Row 2008).

Yosii (1956) pointed out that caves with constant microclimatic conditions in the northern temperate zone can serve as refugia, especially for small soil-dwelling species. Subterranean ecosystems are generally considered as habitats where species from different external ecological conditions can successfully cohabit during hostile surface conditions (Kováč et al. 2016).

The troglophile *Pantelozetes cavaticus* (Kunst) is a sexually reproducing oribatid mite that has been reported mainly from Central European caves, in eastern Czech Republic (Starý 2008), in the Western Carpathians and the Slovak Karst in Slovakia (Ľuptáčik and Miko 2003), in eastern Austria (Bruckner 1995), in southern Poland (Żbikowska-Zdun et al. 2009) and in some caves in Germany, Belgium and Hungary (Ľuptáčik 2004). The species does not possess any obvious troglobiomorphic adaptations (e.g., depigmentation, elongated antennae and legs), but it shows a strong affiliation to the cave environment. Only two findings have been reported from surface environments (Starý 2008; Żbikowska-Zdun et al. 2009). It is considered coprophilous (guanophilous) with frequent occurrence on rotten wood (Ľuptáčik and Miko 2003). The distribution pattern along with absent adaptations to cave life of *P. cavaticus* indicates that it could be a glacial relict, most likely a descendant of an old Pleistocene fauna, or even older Tertiary fauna.

Żbikowska-Zdun et al. (2009) examined populations of *P. cavaticus* from two isolated caves in Poland and found no significant morphological differences between them. Here we take an approach of investigating possible differences between populations at the molecular level. Deep genetic divergences in soil-dwelling arthropods at the population level have been previously reported and well documented in oribatid mites (Heethoff et al. 2007; Rosenberger 2010; Saltzwedel et al. 2014; Schäffer et al. 2019).

To evaluate whether the association of *P. cavaticus* with subterranean habitats caused a geographical isolation and consequently a reproductive isolation, we compared its genetic variability with that of populations of a closely related surface species *Pantelozetes paolii* (Oudemans). In contrast to *P. cavaticus*, *P. paolii* is a widespread and abundant eurytopic pan-phytophagous species with sexual reproduction and Holarctic distribution. *Pantelozetes paolii* is a type species of the genus *Pantelozetes*, which now comprises 22 species and one subspecies based on morphological characters (Subías 2020); phylogenetic relationships among these species are unknown.

In the present study, we used the combination of two DNA markers—the mitochondrial cytochrome c oxidase subunit I (COI, the barcoding region) and the nuclear D3 region of the 28S rDNA gene—as both markers were previously successfully used in other studies aimed at phylogeny and phylogeography of oribatid mites (COI: Rosenberger 2010; Schäffer et al. 2010, 2019; Pfingstl et al. 2019; D3: Lehmitz and Decker 2017; Pachl et al. 2017). Furthermore, Kreipe et al. (2015) suggested that the combination of these two genes should provide a reliable insight into the phylogeny and species radiation within oribatid mites.

Our objectives were (1) to investigate the genetic variability within and between the populations of the troglobiotic *P. cavaticus* and to determine whether the studied caves were reproductively isolated; (2) to compare the genetic variability of two congeneric oribatid mite species with a different ecology—*P. cavaticus* and *P. paolii*; and (3) to trace the evolutionary history of potential lineages of both species. Assuming that the active dispersal abilities of oribatid mites are limited, we expected similar patterns of genetic variability, with genetic isolation more pronounced in the troglobiotic species even over shorter geographical distances.

Materials and methods

Specimen sampling, DNA extraction and sequencing

Organic litter, decaying wood or bat guano samples for the extraction of *P. cavaticus* were taken from sediments in 10 caves between 2015 and 2017 (three caves in Czech Republic, seven in Slovakia; see Fig. 1), where the presence of this species was demonstrated in previous research (Ľuptáčik 2004; Starý 2008). Nová Amatérská Cave and Sloupsko-Šošůvské Caves are in the Moravian Karst, which is the largest karst area in Czech Republic based in the Middle Devonian limestone. These two cave systems are connected via a subterranean stream. A third cave sampled in Czech Republic, the Javoříčské Caves, is situated in a small outcrop of Devonian limestone in the Špraněk Hill. Five of the caves sampled in Slovakia are part of the Slovak Karst (the largest karst area in Central Europe composed of several layers of Mesozoic limestone and dolomite) and are relatively close to each other—500 m to 40 km. Belianska Cave is the largest cave in the Slovak part of the Tatra Mountains established in Mesozoic limestone. The last cave sampled in Slovakia, Andrejová Cave II, located in the Čierna Hora Mountains (part of the Slovak Ore Mountains), is a small joint cave in a limestone rock cliff.

For the common soil living *P. paolii*, soil samples to the depth of 5 cm were collected at 12 already established study sites of the Institute of Soil Biology (Biology Centre CAS, České Budějovice) between 2016 and 2017 (Czech Republic, Slovakia, and Poland; Fig. 1).

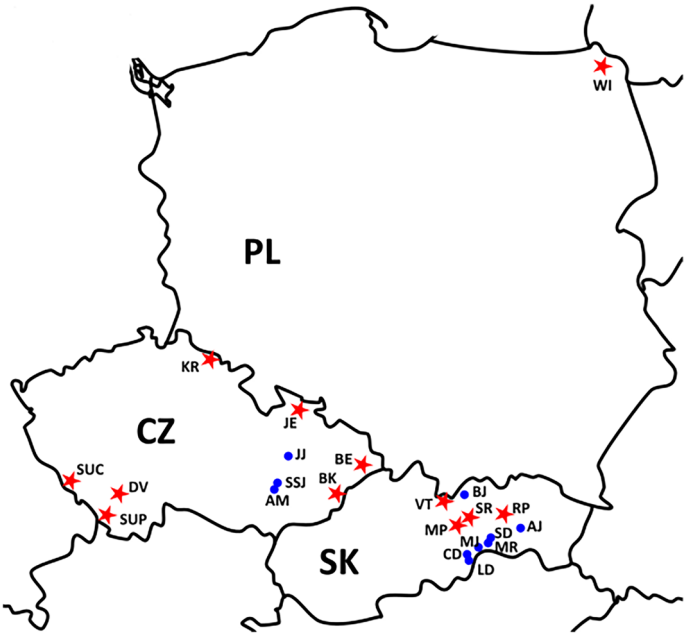

Fig. 1 Map of Czech Republic, Slovakia and Poland with the sampling localities; dots for *Pantelozetes cavaticus* and stars for *P. paolii* (for detailed information about each sampling locality see Table 1, Online Resource 1)

The name of the sampling locality and its abbreviation, number of collected specimens as well as the number of sequences obtained for each locality and GenBank reference numbers are provided for all records in Table 1. More detailed information about sampling localities (GPS, altitude, material collected, etc.) are given in Online Resource 1.

Soil arthropods were extracted in a high-gradient extractor by Marshall (1972) for 5 days with increasing temperatures. *Pantelozetes cavaticus* and *P. paolii* specimens were identified to species level under a light microscope after Weigmann (2006) and stored in 96% ethanol at −20 °C until preparation.

Genomic DNA was extracted from single whole individuals (20 specimens of *P. cavaticus* and 10 of *P. paolii* from every sampling locality if such a number was available—see Table 1 for details) using the Exgene Tissue SV mini kit following the manufacturer' protocol for insects with final elution in 50 µl instead of 200 µl (GeneAll® Biotechnology). A fragment of cytochrome c oxidase subunit I (COI) was amplified using the primers OriLCO (5′-TCAACAAATCATAAAGAYATYGG-3′; slightly modified primer COIarchI used in Heethoff et al. 2007), and standard HCO2198 (5′-TAAACT GGGTGACCAAAAAATCA-3′; Folmer et al. 1994). For the amplification of the D3 fragment of nuclear 28S rDNA gene, the forward primer D3A and the reverse primer D3B were used as described in Kreipe et al. (2015).

The polymerase chain reaction (PCR) contained 0.75 µl of each primer (0.5 pmol/µl), 1 µl of dNTPs (2.5 mM of each dNTP), 1.25 µl of 10× reaction buffer, 0.1 µl of TaKaRa Ex Taq® polymerase, 7.75 µl of PCR water and 2 µl (for COI) and 1 µl (for

Table 1 List of sampling locality names in Czech Republic (CZ), Slovakia (SK) and Poland (PL) with their abbreviations and specifications of obtained DNA sequences

Species	Locality name	Country	Abbreviation	No. of specimens collected/isolated	No. of seq COI/D3	Accession no COI	Accession no D3
Pantelozetes cavaticus	Javoříčské Caves	CZ—Špraněk Hill	JJ_CZ	176/20	20/10	MW034736-755	MW581985-994
	Nová Amatérská Cave	CZ—Moravian Karst	AM_CZ	42/20	20/10	MW034678-697	MW581965-974
	Sloupsko-Šošůvské Caves	CZ—Moravian Karst	SSJ_CZ	31/25	16/10	MW034817-832	MW581975-984
	Belianska Cave	SK—Belianske Tatry	BJ_SK	17/17	17/10	MW034698-714	MW582045-054
	Majkova Cave	SK—Slovak Karst	MJ_SK	27/21	21/10	MW034776-796	MW582005-014
	Marciho Cave	SK—Slovak Karst	MR_SK	50/20	20/10	MW034797-816	MW582015-024
	Snieżna diera Cave	SK—Slovak Karst	SD_SK	50/25	25/10	MW034833-857	MW581995-2004
	Čertova diera Cave	SK—Slovak Karst	CD_SK	122/21	21/10	MW034715-735	MW582025-034
	Liščia diera Cave	SK—Slovak Karst	LD_SK	48/20	20/10	MW034756-775	MW582035-044
Pantelozetes sp.	Andrejová Cave II	SK—Slovak Ore Mountains	AJ_SK	65/23	23/10	MW649874-896	MW581955-964
P. paolii	Dlouhá Ves	CZ	DV_CZ	25/15	3/5	MW193960-962	MW646033-037
	NP Šumava	CZ	SUP_CZ	25/15	6/5	MW193900;903;933-936	MW646087-091
	Šumava PLA	CZ	SUC_CZ	17/15	5/5	MW193901-902;930-932	MW646038-042
	Jeseníky PLA	CZ	JE_CZ	12/12	4/5	MW193926-929	MW646043-047
	NP Krkonoše	CZ	KR_CZ	15/15	9/5	MW193917-924	MW646048-052
	Beskydy PLA	CZ	BE_CZ	8/8	6/5	MW193911-916	MW646053-057
	Bílé Karpaty PLA	CZ	BK_CZ	9/9	7/5	MW193904-910	MW646058-062
	NP Muránska planina	SK	MP_SK	14/14	6/5	MW193954-959	MW646063-067
	Ružín water dam	SK	RP_SK	19/15	5/5	MW193949-953	MW646068-072
	NP Slovenský ráj	SK	SR_SK	21/15	4/5	MW193945-948	MW646073-077
	Tatranský NP	SK	VT_SK	9/9	3/5	MW193942-944	MW646078-082
	NP Wigry	PL	WI_PL	19/15	5/5	MW193937-941	MW646083-087

NP national park, *PLA* protected landscape area

D3), respectively, of template DNA. The PCR conditions consisted of initial 94 °C for 1 min; 40 cycles at 94 °C for 15 s, 47 °C for 40 s and 72 °C for 50 s; and final extension at 72 °C for 2 min. PCR products were visualized on 2% agarose gel and samples with positive products were enzymatically purified with a mixture of 0.5 µl Exonuclease I, 1 µl FastAP (ThermoFisher Scientific, USA) and 0.5 µl PCR water per sample (incubated at 37 °C for 30 min followed by 15 min at 80 °C) before direct sequencing. The purified PCR products were sequenced by GATC Biotech (Germany).

A surface-dwelling oribatid mite *Nothrus silvestris* (Nicolet) (Crotonioidea) was used as an outgroup taxon. Its COI and D3 sequences were taken over from Rosenberger (2010) and Lehmitz and Decker (2017), respectively (GenBank acc. nr. JF263835 and KY681356, respectively).

All sequences generated for this study were checked to be consistent with oribatid mite DNA via Blast searches (Altschul et al. 1997); no contaminations were discovered. All sequences generated for this study are publicly available from GenBank.

Data analysis

Sequences were manually edited: ambiguous positions were corrected by hand and unreadable short stretches were trimmed using the chromatograms (ca. 30 bp at the 5′ and 3′ ends) with BioEdit v.7 (Hall 1999). Both COI and D3 sequences together with the outgroup taxon *N. silvestris* were aligned with the MEGA X software (Kumar et al. 2018) by Muscle algorithm with default parameters. The final alignment of the COI fragment contained 203 sequences with the length of 630 bp for *P. cavaticus* and 63 sequences with the length of 626 bp for *P. paolii*, respectively. The final alignment of the D3 gene fragment contained 100 sequences (462 bp) for *P. cavaticus* and 60 sequences (456 bp) for *P. paolii*. Mitochondrial gene sequences were translated into amino acid sequences using the Invertebrate Mitochondrial Gene Code implemented in MEGA and as there were no stop codons and the alignments were gap-free, all of them were considered as true mitochondrial and not nuclear copies and were reverse-translated into nucleotide sequences for further analyses. All alignments are available from the authors upon request.

The number of synonymous and non-synonymous mutations in the COI alignment were calculated in MEGA, nucleotide (π) and haplotype diversity (Hd) were calculated in DnaSP v.5.10 (Librado and Rozas 2009). The number of protein haplotypes was determined using the online tool DNAcollapser in FaBox v.1.5 (Villesen 2007). The independent analysis of molecular variance (AMOVA) was performed only for *P. cavaticus* in ARLEQUIN v.3.5 (Excoffier et al. 2005), to investigate within- and between-populations structure based on uncorrected p-distances selecting Czech Republic and Slovakia as groups. Isolation by distance was tested by Mantel Test (10,000 permutations) implemented in ARLEQUIN.

To measure the effect of demographic changes on the DNA sequences of the populations, the neutrality tests Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997) were performed in ARLEQUIN. Haplotype networks were constructed in PopART v.1.7 (Leigh and Bryant 2015) using Median Joining Network, separately for *P. cavaticus* and *P. paolii*, to determine and visualize the relationships and history between haplotypes.

The best-fitting model for phylogenetic analyses of COI alignments of both species was selected using the Bayesian Information Criterion (BIC) in the ModelFinder (Kalyaanamoorthy et al. 2017) implemented in IQ-TREE v.1.6.12 (Nguyen et al. 2015). The model of sequence evolution was HKY + F + G4 for *P. cavaticus* and HKY + F + I for *P. paolii*, respectively. Phylogenetic trees of both species were calculated separately using Maximum

Likelihood (ML) and Bayesian Inference (BI) methods, implementing the models selected by ModelFinder. ML analyses were conducted in IQ-TREE with default parameter settings and 10,000 ultrafast bootstrap replicates (Hoang et al. 2018). BI was conducted in MrBayes v.3.2.7 (Ronquist et al. 2012) and BEAST v.2.6.0 (Bouckaert et al. 2019). The Bayesian Markov Chain Monte Carlo simulations in MrBayes were performed in two independent runs with four chains for each species separately, each for 10 million generations sampled every 1000th generation, a burn-in of 2500 was applied. To assess run convergence, the Tracer v.1.7.1 (Rambaut et al. 2018) was used.

Molecular divergence times of major lineages were estimated separately for *P. cavaticus* and *P. paolii*. Prior to this analysis, substitution saturation of the COI sequences was measured in DAMBE 6 software (Xia 2017) with the test by Xia et al. (2003)—the sequences experienced little substitution saturation. The relaxed clock log-normal analysis was performed in two runs for both species with BEAUti, BEAST and TreeAnnotator, all v.2.6.0 (Bouckaert et al. 2019). We set a fixed substitution rate of 0.0115, which corresponds to a standard arthropod mutation rate of COI, 2.3% sequence divergence per million years (Avise 1994; Brower 1994). The site model was HKY + F + G4 for *P. cavaticus* and HKY + F + I for *P. paolii*, respectively, and 'Coalescent Constant Population' was used as tree prior (as recommended for population-level studies by the authors of BEAST). The population size was set as log normal; all the other priors were estimated by the software. The convergence of the MCMC chain after 10,000,000 generations with every 1000th generation sampled and a burn-in of 1000 was confirmed using the Tracer.

Results

DNA extraction and subsequent PCR amplification of the COI gene was successful in 203 out of 212 *P. cavaticus* specimens and in 63 out of 157 *P. paolii* specimens analysed (Table 1); various sets of primers and PCR conditions were tested on the problematic individuals, albeit without success.

PCR amplification of the D3 region worked for all processed samples; however, it showed only a very low molecular variation between the two studied species (uncorrelated p-distances were 0.6%), and none within them. Only four positions of the 462 bp fragment varied in the 100 analysed individuals of *P. cavaticus* and no position of the 456 bp fragment varied in the 60 analysed individuals of *P. paolii*. Accordingly, the phylogenetic trees had no structure and sampling localities were mixed (ML phylogenetic tree of studied species together with an outgroup based on the D3 is shown in Online Resource 2a). Therefore, D3 sequences were not used for further analyses.

Phylogenetic analysis of COI sequences split the analysed species into three lineages and suggested the existence of a new species independent of *P. cavaticus* and *P. paolii*—hereafter it is referred to as *Pantelozetes* sp. The new species showed a closer relationship to *P. cavaticus*, values of uncorrected p-distances in the COI gene were 20.4% for *P. cavaticus* vs. *Pantelozetes* sp. and 22.7% between *P. paolii* and *Pantelozetes* sp. (Table 2a). D3 sequences failed to distinguish *Pantelozetes* sp. from *P. cavaticus*. *Pantelozetes* sp. was found only at the sampling locality Andrejová Cave II. Subsequent re-inspection under the light microscope revealed distinctive morphological features of the new species and its detailed description is currently in progress and will be published separately. Intraspecific p-distances were 0.2% and five haplotypes were detected in the 23 analysed *Pantelozetes* sp. COI sequences, which were not used for the reconstruction of phylogenetic trees and

Table 2 Intra- and interspecific distances (uncorrected p-distances, %) for the studied COI gene marker: (**a**) for all studied *Pantelozetes* species, (**b**) for *P. cavaticus* populations, and (**c**) for *P. paolii* populations, according to sampling locality and lineages

(a)

Species	*P. cavaticus*	*P. paolii*	*Pantelozetes* sp.
P. cavaticus	*2.4*		
P. paolii	21.7	*3.7*	
Pantelozetes sp.	20.4	22.7	*0.2*
Nothrus silvestris	22.6	23.4	25.9

(b)

Locality	AM_CZ	BJ_SK	CD_SK	JJ_CZ	LD_SK	MJ_SK	MR_SK	SSJ_CZ	SD_SK
AM_CZ	*0.5*								
BJ_SK	4.7	*0.6*							
CD_SK	3.9	2.1	*0.8*						
JJ_CZ	1.0	4.8	3.7	*0.04*					
LD_SK	4.1	2.5	1.0	4.1	*0.1*				
MJ_SK	3.9	2.1	0.7	3.7	1.7	*0.03*			
MR_SK	4.1	2.5	1.2	4.1	0.7	1.7	*0.8*		
SSJ_CZ	0.4	4.6	3.7	0.7	3.9	3.7	3.9	*0.1*	
SD_SK	4.2	2.6	0.9	4.1	0.4	1.4	0.7	4.0	*0.2*

(c)

Locality	BE_CZ	BK_CZ	DV_CZ	JE_CZ	KR_CZ	RP_SK	SR_SK	MP_SK	SUC_CZ	SUP_CZ	VT_SK	WI_PL
BE_CZ	*4.3*											
BK_CZ	3.9	*3.4*							Lineage	1	2	3
DV_CZ	3.7	5.7	*0*						1	*0.1*		
JE_CZ	3.9	3.6	4.1	*5*					2	2.6	*0*	
KR_CZ	3.8	4.8	1.9	4	*3.3*				3	7	6.7	*0.1*
RP_SK	3.8	4	3.3	3.8	3.6	*4.6*						
SR_SK	3.6	3.9	3.5	3.8	3.7	3.7	*4.7*					
MP_SK	3.6	3.9	3.5	3.8	3.7	3.7	3.5	*4.2*				
SUC_CZ	3.6	4.2	2.8	3.9	3.3	3.6	3.5	3.5	*4.2*			
SUP_CZ	3.6	4.5	2.3	3.9	3.1	3.6	3.5	3.5	3.3	*3.7*		
VT_SK	4.8	4.8	2.5	4	3.3	3.7	4.6	4.6	4.2	3.9	*0*	
WI_PL	3.6	4.2	2.8	3.9	3.3	3.6	3.5	3.5	3.4	3.3	4.2	*4.2*

Intraspecific and intrapopulation distances are given in italics

haplotype network of the studied species. ML phylogenetic tree of all studied species (*P. cavaticus*, *P. paolii*, *Pantelozetes* sp.) with an outgroup *N. silvestris* is shown in the Online Resource 2b.

Pantelozetes cavaticus

In each of the phylogenetic trees *P. cavaticus* was monophyletic and separated with high support from the outgroup taxon *N. silvestris* (as well as from *P. paolii* and *Pantelozetes* sp.; Online Resource 2b). Phylogenetic reconstructions based on BI and ML methods of the COI nucleotide alignment revealed very similar topologies with slightly different resolution. Only the BI tree (created in BEAST) is shown in Fig. 2a (see Online Resource 3 for the other trees). COI haplotypes generally clustered according to sampling locations and separated with high support into two main lineages—'Czech' (1) and 'Slovak' (2) (Fig. 2a). Applying a standard invertebrate mitochondrial substitution rate of 2.3% per million years, 'Czech' and 'Slovak' lineages diverged in the Late Pliocene, 2.9 ± 1.6 Mya (Fig. 2a).

The COI nucleotide haplotype network also showed a strong cave-related structure once more with an obvious separation of Czech and Slovak caves (Fig. 3a). Individuals from nearby located sampling localities (caves) shared identical or closely related haplotypes. Haplotypes of the individuals from Belianska Cave (SK) and Javoříčské Caves (CZ) were clearly separated and not shared with any other individual from another caves. In total, 37 nucleotide haplotypes were sampled within the 180 individuals sequenced. The estimated haplotype diversity was relatively high within the populations and lineages (average values 0.502 and 0.802, respectively), whereas nucleotide diversity was considerably lower in both cases (on average 0.004 in populations and 0.009 in lineages) (Table 3). As for the amino acid haplotypes, 16 were identified, two (most abundant) shared among the individuals from Czech and Slovak caves, while the remaining 14 were detected only in Slovak caves (ML phylogenetic tree based on amino acid sequencies is shown in the Online resource 4a).

Genetic distances between the populations from discrete caves were moderately higher and ranged between 0.4 and 4.8% for the COI gene (Table 2b). Within-population genetic distances were generally low (Table 2b). Accordingly, as indicated by AMOVA, genetic variance was highest between groups (Czech and Slovak caves: 70.5%), markedly lower among populations within groups (21.1%) and lowest within populations (8.5%) (Table 4). A significant correlation between genetic and geographical distances of populations, isolation by distance, was revealed using Mantel test ($R^2 = 0.877$, $p = 0.004$). The neutrality analyses did not show any significant differences from zero within the populations ($p > 0.05$ for both D and F_S), except for two caves—Majkova Cave and Sniežná diera Cave—indicating a population expansion after a bottleneck event (Table 3).

Pantelozetes paolii

The phylogenetic trees based on BI and ML methods of the COI nucleotide alignment showed similar topologies and only the BI tree (MrBayes) is shown in Fig. 2b (ML tree is shown in Online Resource 5). *Pantelozetes paolii* was monophyletic and separated with high support from the outgroup taxon *N. silvestris* (as well as from *P. cavaticus* and *Pantelozetes* sp., Online Resource 2b) and the COI haplotypes clustered into three main lineages independent of the sampling site (Fig. 2b; for detailed information about the number and geographic origin of the individuals from each lineage see Online resource

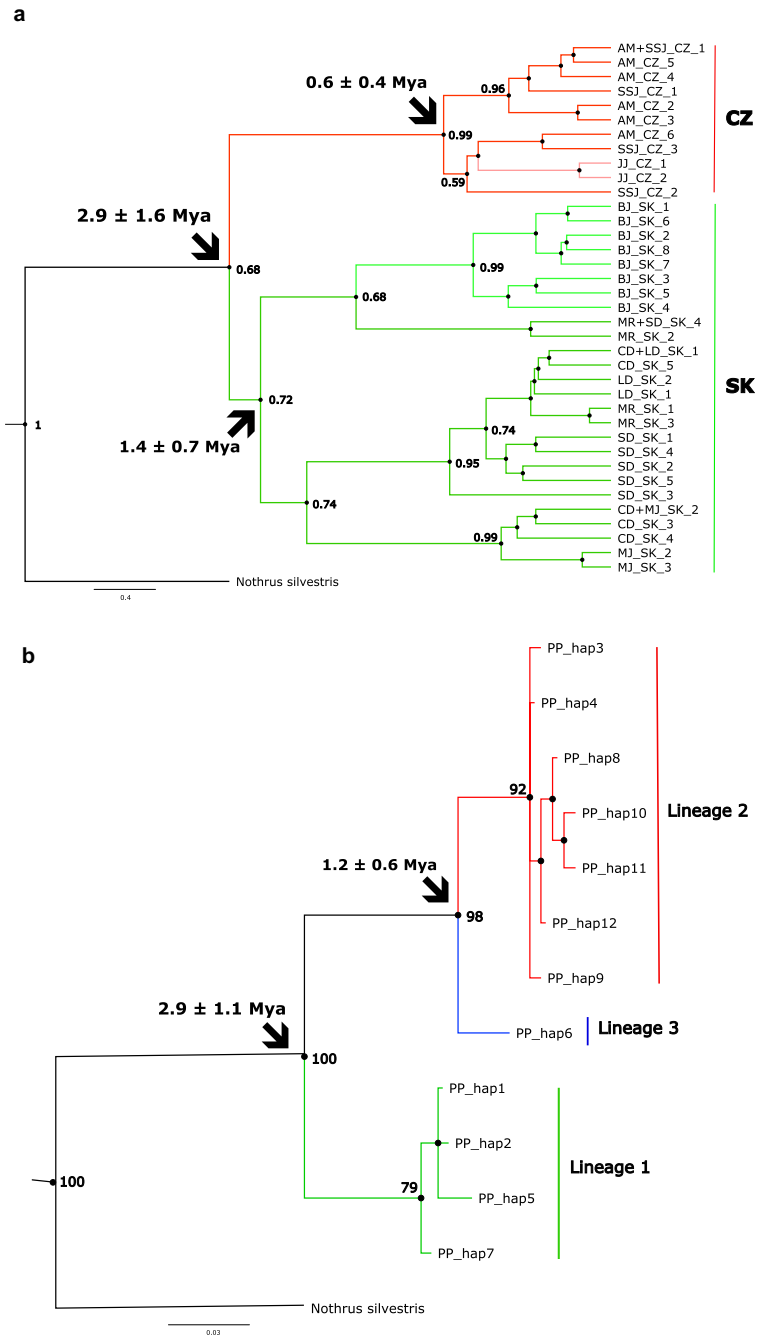

Fig. 2 Bayesian inference trees based on the COI gene showing the relatedness among individuals of (**a**) *Pantelozetes cavaticus* (calculated in BEAST) and (**b**) *P. paolii* (calculated in MrBayes). Different branch colors indicate genetic lineages identified within the species (see color version online). Numbers at the nodes represent posterior probabilities, bold numbers are median estimated divergence times ± 95% HPD (highest posterior density) calculated in BEAST

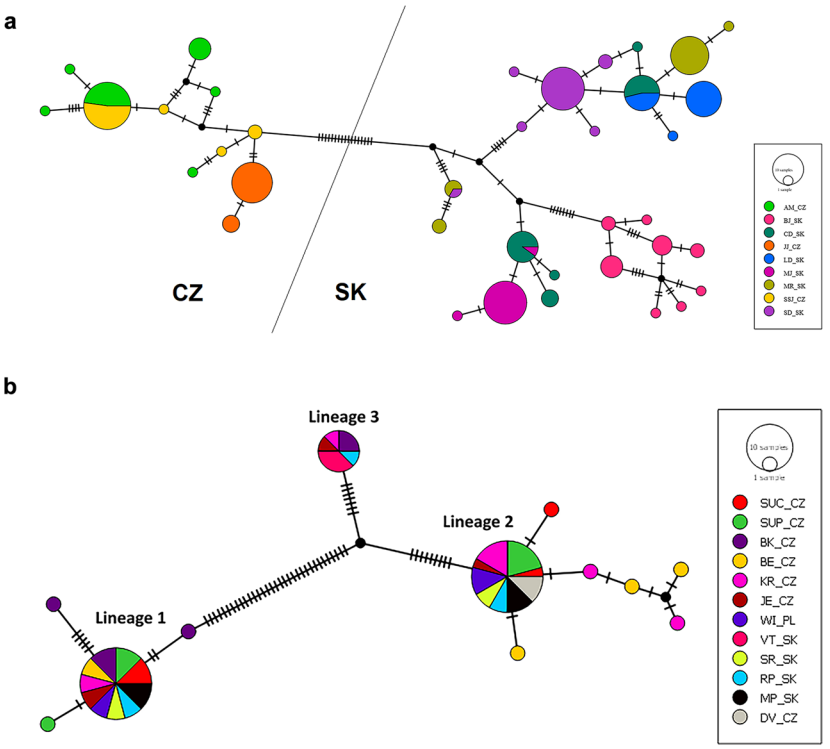

Fig. 3 Median Joining Haplotype Network of COI sequences of (**a**) *Pantelozetes cavaticus* and (**b**) *P. paolii* (see color version online). The size of the circles is proportional to the number of sequences per haplotype, bars on the lines represent number of mutation steps separating haplotypes

6). Applying a mitochondrial substitution rate of 2.3% per million years, the lineage 1 diverged from the rest 2.9 ± 1.1 Mya during the Late Pliocene, and lineages 2 and 3 split during the Pleistocene 1.2 ± 0.6 Mya (Fig. 2b).

The COI haplotype network showed a clear structure of three dominant haplotypes, i.e., three lineages shared among individuals from often very distant sampling localities (Fig. 3b). The network showed individuals from multiple sampling localities in all lineages, i.e., in the sites Ružín Water Dam (RP_SK), NP Krkonoše (KR_CZ) and Jeseníky PLA (JE_CZ) individuals with all three haplotypes coexisted, and in the rest of the sampling sites, a mix of two of the three main haplotypes was found. In total, 12 haplotypes were found in the 63 individuals sequenced. The most abundant haplotypes 1 and 2 were associated with some other haplotypes (represented only by one individual) by a mutational step. Contrary to *P. cavaticus*, the estimated haplotype diversity was relatively high within the populations (an average value 0.545) but much lower within the lineage (an average value 0.198), whereas nucleotide diversity was low in both cases (and markedly lower within the lineages) (Table 3). We identified six amino acid haplotypes, two abundant: first shared only between the individuals from lineage 1, second shared between individuals from lineages 2 and 3; the rest of the amino acid haplotypes were detected only in one individual on different sampling localities (ML phylogenetic tree is shown in Online Resource 3b).

Table 3 Basic molecular diversity parameters and neutrality tests for the studied *Pantelozetes* species, sampling localities (see Table 1 for code explanation) and lineages

	N	H	Hd	π	Tajima's D	Fu's Fs
P. cavaticus						
All individuals	180	37	0.933	0.024	0.738	0.806
AM_CZ	20	6	0.658	0.005	−0.736	1.058
JJ_CZ	20	2	0.268	0.001	−0.086	0.381
SSJ_CZ	16	4	0.442	0.002	0.227	−0.316
BJ_SK	17	8	0.878	0.006	−0.152	−0.663
CD_SK	21	5	0.714	0.008	2.083	4.030
LD_SK	20	3	0.511	0.001	−0.526	0.382
MJ_SK	21	3	0.186	0.001	−1.514*	−1.920*
MR_SK	20	4	0.437	0.008	0.738	5.656
SD_SK	25	6	0.427	0.002	−2.179*	−1.220
Lineage CZ	56	11	0.738	0.006	0.035	−0.086
Lineage SK	124	26	0.912	0.013	−0.097	−1.200
P. paolii						
All individuals	63	12	0.726	0.037	1.306	6.085
DV_CZ	3	1	0	0	0	
BE_CZ	6	4	0.800	0.044	2.078	4.966
BK_CZ	7	4	0.810	0.034	0.901	5.678
JE_CZ	4	3	0.833	0.049	1.315	4.649
KR_CZ	9	5	0.806	0.033	0.162	5.449
SUC_CZ	5	4	0.900	0.042	1.812	3.245
SUP_CZ	6	2	0.533	0.037	1.392	11.584
MP_SK	6	2	0.600	0.042	2.365	12.112
RP_SK	5	3	0.800	0.046	1.455	6.519
SR_SK	4	2	0.667	0.046	2.309	8.541
VT_SK	3	1	0	0	0	–
WI_PL	5	2	0.600	0.042	1.883	10.274
Lineage 1	25	4	0.230	0.001	−2.216*	−0.806*
Lineage 2	30	7	0.366	0.001	−1.582*	−3.484*
Lineage 3	8	1	0	0	0	–

N number of sequenced individuals, *H* number of observed haplotypes, *Hd* haplotype diversity, π nucleotide diversity

*Significant differences from zero, suggesting population expansion after a bottleneck event

Genetic distances detected between populations from discrete sampling localities were high and ranged between 1.9 and 5.7% for the COI gene. Intrapopulation genetic distances in the COI gene were high as well (3.3–5%), except for two populations where the value was zero (possibly the result of the small number of successfully sequenced individuals). After redefining the groups of individuals according to their assignment to the genetic lineage, the genetic distances among the lineages ranged between 2.6 and 7% and between 0 and 0.1% within the lineages, respectively (Table 2c).

Isolation by distance was rejected, Mantel test being not significant ($R^2 = 0.034$, $p = 0.14$). The neutrality analyses did not show any significant differences from zero within

Table 4 Result of AMOVA based on the uncorrected p-distances for *Pantelozetes cavaticus*

Source of variation	Between groups (CZ and SK)	Within groups	Within populations
Degrees of freedom	1	7	171
Sum of squares	764.87	393.28	189.53
Variance components	9.21 Va*	2.75 Vb*	1.11 Vc*
Variation %	70.46	21.05	8.49
Fixation indices	Fst 0.915*		

Fst F-statistics

*Indicate significant differences ($p < 0.05$)

the populations ($p > 0.05$ for both D and F_S) leading to the assumption that there is no evidence of size changes in the populations. However, after this analysis was carried out on every identified lineage (except lineage 3 with only one haplotype), values significantly different from zero were observed ($p > 0.05$ for both D and F_S) indicating that the lineages may have undergone a process of population expansion after a bottleneck event (Table 3).

Discussion

The results of our study provide the first insight into the genetic diversity and population structure of two oribatid mite species from the genus *Pantelozetes*. In agreement with our hypothesis, the cave-associated species, *P. cavaticus*, is represented by several phylogeographically subdivided populations that are reproductively well isolated between discrete karst areas, falling into two main genetic lineages ('Czech' and 'Slovak'). Conversely, the other investigated species, the common soil-living *P. paolii*, lacks any geography-related population structure, i.e., the three identified main genetic lineages coexist on individual sampling locations, suggesting good dispersal abilities of this species.

The intraspecific variation of the COI gene was quite high between the identified lineages of both species (ranging from 2.6 to 7%). The standard COI barcoding gene shows in general more variability between the populations of flightless or less mobile wide-spread taxa (Papadopoulou et al. 2009), which was already documented also for the populations of soil-living microarthropods (Heethoff et al. 2007; Schäffer et al. 2010; Rosenberger et al. 2013; Kreipe et al. 2015; Lehmitz and Decker 2017) and which corresponds with our results. The phylogenetic analysis of *P. cavaticus* COI sequences further revealed existence of new species *Pantelozetes* sp., which was clearly separate from *P. cavaticus* (20.4% interspecific distance). A morphological re-inspection of the samples from a small, isolated locality in Čierna Hora Mts. (Andrejová Cave II), where the genetic analysis indicated the presence of the new species, revealed individuals with minor yet distinctive morphological features.

The D3 region was considered as a possible species marker in several studies (Maraun et al. 2003; Laumann et al. 2007; Lehmitz and Decker 2017), although some have reported it to fail to separate closely related species (Lehmitz and Decker 2017; Schäffer et al. 2019). In our study we found only little variation in D3 between *P. cavaticus* and *P. paolii* (0.6%); furthermore, the locus also failed to distinguish the new species, with its sequences being

identical to those of *P. cavaticus*. It seems that the D3 fragment is too short and conserved to be reliably used as the sole species marker for oribatid mites.

Pantelozetes cavaticus

The phylogeographic analysis of *P. cavaticus* sampled in the mid-point of its distribution revealed deep genetic differences between the individual populations. Considering the limited active migration possibilities of the species and, more importantly, the discontinuous nature of the cave environment to which the species is bound, the geographic isolation was expected. Two main lineages of *P. cavaticus* were identified, 'Czech' and 'Slovak'. Both lineages had specific, non-synonymous substitutions in the COI gene and were never found to coexist in a single cave, indicating selection followed by a subsequent spread of the most competitive genotype. This is consistent with the results of several other studies that revealed high intraspecific genetic diversity in COI of widespread oribatid mite species (Rosenberger 2010; Schäffer et al. 2010, 2019; von Saltzwedel et al. 2014; Pfingstl et al. 2019).

Mutation rate of the mitochondrial COI gene is relatively high, which could lead to the formation of a significant inter-population diversity over a short evolutionary timescale (Hebert et al. 2003). Therefore, the presence of individuals from different caves that shared the same haplotypes (i.e., AM_CZ + SSJ_CZ; LD_SK + MJ_SK + CD_SK; SD_SK + MR_SK) points to a continual gene flow between these populations or to a recent colonization of one cave from the other. This could be easily explained by a short geographical distance between these caves and their potential connection via underground streams or crevices (e.g., Nová Amatérská Cave and Sloupsko-Šošůvské Caves are connected by a subterranean stream, and all the caves from Slovak Karst are close to each other).

In contrast, no shared haplotypes and presumably no gene flow between the populations from distant and not inter-connected karst areas indicate effective reproductive isolation of these populations from each other. A very similar trend of COI genetic variability increasing with geographical distance was detected by Parimuchová et al. (2017) in the populations of the troglophile collembolan *Protaphorura janosik* in Slovak caves.

Molecular divergence estimates based on *P. cavaticus* COI sequences indicated that the separation of 'Czech' and 'Slovak' lineages substantially predated Quaternary glaciations (Pleistocene) and happened during the Late Pliocene (2.9 Mya). This radiation event coincides with the climatic and biotic changes occurring in Europe during this epoch, i.e., global cooling, less precipitation, and the spread of grasslands (Retallack 2001). This might have forced the common ancestor of extant *P. cavaticus* lineages to escape from the changing surface conditions to cave ecosystems with more stable conditions. Despite this relatively ancient radiation, the lineages have remained morphologically consistent and no clear morphological differences were evident between individuals during the identification prior to the DNA extraction. The stable conditions of the subterranean environment may have contributed to the morphological consistency of the emerging genetic lineages. Homogenous habitat conditions were suggested to enforce stabilizing selection, which can maintain a constant phenotype across the range of the group, resulting in conserved morphologies on a diverse genetic background (Colborn et al. 2001; Pfingstl et al. 2019).

Nevertheless, long-term geographical and genetic isolation of the lineages could be expected to provoke, through the growing genetic distances, an evolution of new species differing even in their morphology. Indeed, our discovery of a new species in Andrejová Cave II, seems to support this theory. Based on the molecular divergence estimates,

Pantelozetes sp. separated from the ancestor of *P. cavaticus* 10.1 (± 1.8) Mya, during the Late Miocene.

Pantelozetes paolii

Pantelozetes paolii is a eurytopic and abundant species that can be found in a variety of surface habitats (with Holarctic distribution), indicating that this species can cope with a wide range of environmental conditions. Unfortunately, the number of specimens obtained during sample collection was limited and PCR amplification of the COI gene did not work very well in this species, therefore the number of successfully sequenced individuals from some sampling localities was low (i.e., only three individuals from DV_CZ and VT_SK).

We assumed that given its small size and limited active locomotion powers, the populations from distant areas would create a distinct phylogeographic pattern as this was already described in many other studies investigating the genetic structure of populations of small widespread soil arthropods (Schäffer et al. 2010; Rosenberger et al. 2013; Saltzwedel et al. 2016, 2017). Waters et al. (2013) suggested that a founder effect may play a major role in the colonization of new habitats, which results in a low genetic variance within the populations but a high one between them.

Contrary to this, in 63 sequenced individuals we found three main genetic lineages with deep genetic differences (genetic distances between the lineages ranged from 2.6 to 7%) cohabiting at distinct sampling localities. High haplotype diversity indicated ancient separation and independent evolution of these lineages. It was common that individuals from at least two lineages (and in a few cases even all three—in Ružín Water Dam, Jeseníky PLA and NP Krkonoše) coexisted at one locality (one haplotype was shared among the individuals from distinct localities). This points to an effective long-distance dispersal ability of this species.

Poor active dispersal, even on distances of only couple of centimeters, is characteristic for oribatid mites (Lehmitz et al. 2012). However, given that many species have huge geographical distribution ranges, efficient dispersal pathways must exist. This topic has not yet been completely resolved. Passive dispersal by wind, though documented, is species-specific and not very common over a longer distance in oribatid mites given their small size and susceptibility to dehydration (Lehmitz et al. 2011; Schuppenhauer et al. 2019). Transport by running water and on larger animals, especially on birds, is more common and well documented (Lebedeva and Krivolutsky 2003; Krivolutsky and Lebedeva 2004; Schuppenhauer et al. 2019). *Pantelozetes paolii* has been sporadically found in the feathers of waterfowl (Krivolutsky and Lebedeva 2004), which is probably the most likely dispersal manner of this species.

High haplotype diversity (Hd, on average 0.545 and 0.198 for populations and lineages, respectively) and relatively low nucleotide diversity (π, on average 0.035 and 0.001 for populations and lineages, respectively) of *P. paolii* obtained in this study is consistent with what has been described for some other species of oribatid mites. For example, Schäffer et al. (2010), in their analysis of the COI region of two *Scutovertex* species (sampled also in Central Europe), observed values of Hd between 0.818 and 0.989 and values of π between 0.018 and 0.051.

These differences in Hd and π can indicate recent population growth (Korstian et al. 2015), which is consistent with the results of the neutrality tests performed within the detected lineages of *P. paolii* in our study. It seems that the species experienced an event that caused a drastic reduction in its abundance (bottleneck effect) followed by a rapid

population growth. First of these events might have happened during the Late Pliocene (2.9 Mya per molecular divergence time estimates) when the climatic and habitat conditions were rapidly changing—similarly to the situation in *P. cavaticus* as described above. The second event of radiation (1.2 Mya) was probably caused by strong climatic oscillations during the Pleistocene Epoch (Hewitt 2004). Moreover, bottleneck events followed by demographic expansions have been consistently shown to leave a genetic mark in the current populations in the pattern of some haplotypes being broadly shared and others, less frequent, differing by only a few mutations (Bas 1995).

Initial morphological examination of specimen did not reveal substantial differences, however an in-depth morphometric study of individuals from different genetic lineages may reveal relevant morphological traits. Additional coupled genetic and morphological data from representative sampling sites across the *P. paolli* distribution range are necessary to clarify the relationships among its populations.

Conclusions

This study provides the first insight into the genetic diversity, population structure and evolutionary history of two ecologically different species from the genus *Pantelozetes.* We show that populations of the cave-dwelling *P. cavaticus* are effectively confined to the respective karst areas, whereas the populations of the surface-dwelling *P. paolii* from comparably distant localities are intermixed, demonstrated as three main lineages that coexist on multiple sampling sites, suggesting effective long-distance dispersal of the latter species. The estimated divergence of the genetic lineages of *P. cavaticus* and *P. paolii* of 2.9 Mya coincides with the onset of major climatic changes during Late Pliocene, when the European climate began to cool rapidly. In addition, we confirmed that COI is a good marker for studies of population structure, but found the D3 fragment too conserved to distinguish populations or even closely-related species. Finally, we report a new candidate species, *Pantelozetes* sp., from one of the sampled caves in Slovakia (Andrejová Cave II); its description will be published separately.

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Data availability All the sequences obtained for this study are publicly available from the GenBank (accession numbers are listed in Table 1). The datasets (alignments) generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflicts of interest The authors have no conflict of interest to declare that are relevant to the content of this article.

Ethics approval No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

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