ARTHROPOD SYSTEMATICS
& PHYLOGENY

75 (3): 351–362
20.12.2017

SENCKENBERG

A glacial relict in the Carpathian caves – population variability or a species complex?

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Accepted 17.v.2017.
Published online at www.senckenberg.de/arthropod-systematics on 11.xii.2017.

Editors in charge: Julia Goldberg & Klaus-Dieter Klass

Abstract

The collembolan *Protaphorura janosik* Weiner, 1990, is a widespread and abundant troglobiotic species with a distribution range limited to the Western and Eastern Carpathian Mountains, in Central Europe. Owing to limited dispersal ability, its populations are probably isolated to some extent in the subterranean environments of different geomorphological units. In five of nine populations examined for morphological variability, genetic analyses were also carried out. Analysis of 10 measurable or countable morphological traits by non-metric multidimensional scaling (NMS) showed a slight separation of neighbouring localities; however, no clear geographical pattern was evident among distant populations. In contrast, genetic analysis based on partial sequences of the mitochondrial COI gene showed a different pattern. Although only eight haplotypes out of 88 sequences were detected, their geographical distribution points towards a high population differentiation. One haplotype was shared by two populations from adjacent caves (the Nová Kresanica and Mylna caves), while all the others were unique to different populations. A Mantel test showed a significant correlation of the geographical and genetic distances. Genetic distances (K2P) between the populations ranged from 0.1% to 3.1%, suggesting the existence of geographical isolates. The bacterial genus *Wolbachia* was detected only in one population from a pseudokarst (sandstone) cave, while it was absent in the remaining populations occupying karst caves.

Key words

Collembola, geographic isolation, haplotypes, morphological variability, troglobiont, *Wolbachia*.

1. Introduction

Changes of biota composition during the Pleistocene climatic periods have been the focus of attention of biologists for many decades. The Carpathian Mountains, a mountain range system forming an arc across Central and Eastern Europe, are among the most valuable areas in Europe in terms of biodiversity (Mráz et al. 2016). Many studies documented the high diversity of terrestrial cave fauna in this territory, with the presence of numerous obligate subterranean species, especially in Romanian caves (Decu & Racovitza 1994; Moldovan & Rajka 2007). But whether such specialized forms of fauna also occur in the Western Carpathians, since this territory was in close contact with continental glaciers during periods of Pleistocene glacial maxima, was still in question (Culver et al. 2006; Zasadni & Kłapyta 2014). Kováč et al. (2014, 2016) provided examples of several troglobionts that occupy caves in this territory, especially among arthropods. It is hypothesized that these forms represent a relict fauna, likely descendants of old Pleistocene fauna, some of them even pre-Pleistocene, Tertiary fauna. These

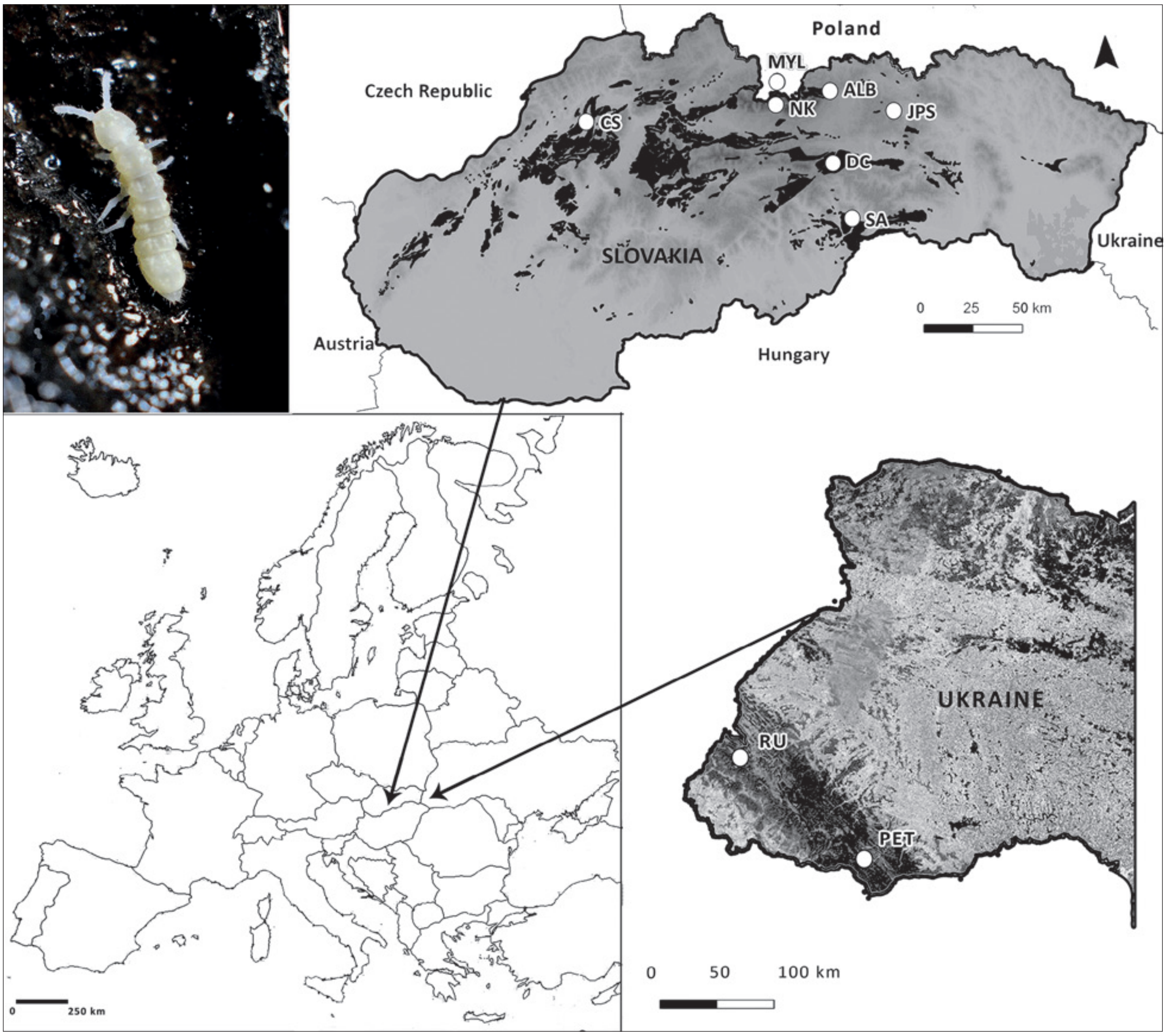


Fig. 1. Sampling localities of *Protaphorura janosik* cave populations in Slovakia and Ukraine. Upper left: specimen in its habitat.

assumptions are mainly based on morphological traits but must be supported by molecular data to be specified.

In this study, we focused on *Protaphorura janosik* Weiner, 1990, a collembolan troglobiont that is distributed in many caves across the Western Carpathians and in at least two caves of the adjacent Eastern Carpathians. Its distribution range and habitat preference (cold caves) indicate that it is a glacial relict species. The genus *Protaphorura* Absolon, 1901, distributed in the entire Holarctic, is the most diverse taxon of the subfamily Onychiurinae, with more than 150 species described to date (Bellinger et al. 2015). All representatives of the genus are euedaphic, characterized by a depigmented body, elongated and flattened in shape, and the absence of eyes (anophthalmia) and spring apparatus. A high morphological variability associated especially with body chaetotaxy and body pseudocellar pattern is characteristic for the congeners (Pitkin 1980; Pomorski 1990). The number of pseudocelli may vary within the species depending on instar (Hale 1964), and their position on the body may be asymmetrical (Bödvarsson 1970). Taxonomy of the genus is principally based on the combination of this pseudocellar pattern and chaetotaxy, and due to frequent variability its use as a diagnostic trait is often problematic, e.g. *P. tundricola, P. elenae* (Babenko & Kaprus' 2014; Kaprus' & Pomorski 2008). Such differences in adult instars may stand for morphological aberrations or indicate a separate species. Molecular markers, such as the barcode region of the COI mitochondrial gene, are useful in revealing hidden diversity hardly distinguishable by morphological examinations (Hogg & Hebert 2004; Stevens et al. 2011). The molecular approach potentially enables the detection of a cryptic species irrespective of its distribution range size (e.g. Porco et al. 2012, 2014; Raschmanová et al. 2017).

Based on recent observations, *P. janosik* Weiner, 1990 is a species endemic to the Western and Eastern Carpathians, inhabiting both karst and pseudokarst caves. It is a relatively large species, reaching 4.2 mm in length. It is peculiar due to its shallow furcal cuticular fold, long post-antennal organ (PAO) consisting of 36–46 simple vesicles, the presence of anal spines on

Table 1. List of studied populations of *P. janosik*. ● – pseudokarst cave; WC – Western Carpathians, EC – Eastern Carpathians; SK – Slovakia, PL – Poland, UA – Ukraine; AP – Andrea Parimuchová, ĽK – Ľubomír Kováč, RV – Robert Vargovitsh. Sources: Bella et al. (2007), Vargovitsh (2010), http://jaskiniepolski.pgi.gov.pl/.

Cave/Abyss	Abbrev.	Altitude (m a.s.l.)	Length (m)	Depth (m)	T (°C)	Region	Orographic unit	Type of bedrock	Collectors
Alabastrová	ALB	1390	543	44	3.5 – 4.5	WC	Belianske Tatry Mts (SK)	L	ĽK
Četníkova svadba	CS	1075	1276	70	6.0 – 6.2	WC	Strážovské vrchy Mts (SK)	L	AP, ĽK
Duča	DC	995	319	20	0.9 – 5.7	WC	Slovak Paradise (SK)	L	AP
Jaskyňa pod Spišskou ●	JPS	1022	746	25	5.7 – 6.6	WC	Levočské vrchy Mts (SK)	S	AP, ĽK
Jaskynia Mylna	MYL	1084	1615	46		WC	Západné Tatry Mts (PL)	L	AP, ĽK
Nová Kresanica	NK	2016	820	194	2.3 – 3.1	WC	Západné Tatry Mts (SK)	L	AP, ĽK
Šingliarova priepasť	SA	680	383	65	5.1 – 6.7	WC	Slovak Karst (SK)	L	AP, ĽK
Petros	PET	1900	185	28	3.5 – 4.5	EC	Chornohora Mts (UA)	S	RV
Runa	RU	1270	190	9	4.0 – 5.0	EC	Polonya Runa Mts (UA)	S	RV

distinct papillae, the presence of macrochaeta p3 on thoracic tergum I, the absence of a male ventral organ and a dorsal pseudocellar formula of 33/023/33343. According to Kováč et al. (2016) the species does not possess any obvious troglobiomorphic adaptations to cave life (e.g. distinctly elongated antennae, legs and claws), but it is characterized by a larger body compared to most congeners and has not been found outside of caves. The most *Protaphorura* species inhabit deeper soil layers, while only a few occupy the Western Carpathian caves (Parimuchová & Kováč 2016); the phylogenetic relationships between them are not clear due to lack of available barcode sequences.

In the present paper, a combined morphological and molecular approach is applied to reveal variability in and between populations of troglobiotic *P. janosik* occupying mostly cold caves within its distribution range. We also aimed to examine the bacterial endosymbiont *Wolbachia* as a potential force in the isolation and diversification of the particular collembolan populations. Since the species distribution range covers habitats presumed not to be connected, we expected to find molecular and morphological differences between the populations even within relatively small areas, which could represent a complex of closely related species.

2. Materials and methods

2.1. Studied area and collecting of material

The Carpathian Mountains arch, with a total area of approximately 210,000 km², extends substantially across Slovakia, Ukraine and Romania, while also reaching into Poland, Hungary, Austria, Czech Republic and Serbia. It consists of the Western, Eastern and Southern Carpathians and the Apuseni Mts (Lóczy et al. 2012). The total area of the Western Carpathians is approximately 70,000 km^2 (Plašienka et al. 1997). The central units of this mountain range are geologically related to the central zones of the Eastern Alps, whereas the internal units, mostly submerged below the Neogene fill of the Pannonian Basin, are closely related to the Southern Alps and Dinarides. The central Western Carpathians represent the most important units in terms of cave development due to the occurrence of chemically pure Mesozoic limestones. Non-karst (pseudokarst) caves are created in sandstones, conglomerates, travertines or andesite volcano-clastic rocks mostly in the southern and eastern geomorphological units (Kováč et al. 2014).

Individuals from nine populations were included in the morphological study of *P. janosik* (Table 1). We examined specimens from seven Western Carpathian caves (6 in Slovakia and 1 in Poland – the type locality) and specimens collected in two caves of the Eastern Carpathians (western Ukraine) (Fig. 1). Twenty individuals were examined from each of the seven populations of *P. janosik* from the Western Carpathians caves to determine morphological variability. From the Eastern Carpathians, 11 individuals were analyzed from the Runa Cave population and six individuals from the Petros Cave (Tables 1, 2). The diagnostic characteristics of the Eastern Carpathian specimens corresponded with *P. janosik* (Weiner 1990; Parimuchová & Kováč 2016) and were included in the population analysis of morphological traits. The specimens were caught by pitfall trapping or hand collecting during 2012 – 2014 and preserved in 75% ethanol.

2.2. Morphological analyses

In the laboratory, the specimens were separately mounted on permanent slides in Swann medium (Liquido de Swann) modified after Rusek (1975) and studied in a phase-contrast Leica DM 2500 microscope. We examined 20 measurable/countable, binary or qualitative morphological traits (Table 2). Measurements were made using an ocular micrometer.

Morphological similarity between the populations was evaluated by Non-metric multidimensional scaling (NMS) analysis using PC-ORD software (McCune & Mefford 2011) based on 10 measurable or countable traits, specifically: size (mm); number of vesicles in the

Table 2. Morphological characteristics of *P. janosik* populations from the Western and Eastern Carpathian caves (for abbreviations of caves see Table 1). ● – trait used in discrimination analysis, LT – long thin, ST – short thick, bm – basomedian, prox. – proximal, postlab. ch. – postlabial chaetae, add. – additional, l/w – length/width ratio, a,b,c – pseudocelli on Abd. V according to Pomorski (1990), percentages in brackets indicate frequency of the feature in the population.

	ALB	CS	DC	JPS	MYL	NK	PET	RU	SA
Number of studied individuals	20	20	20	20	20	20	6	11	20
● Size (mm)	2.94–3.98	2.5–3.78	3.34–4.2	3.43–4.3	3.3–4.6	2.86–3.67	3.2–3.8	2.7–3.4	3.3–3.92
● PAO vesicles	38–49 (51)	45–58	44–55 (58)	44–53 (59)	43–49	44–50 (53)	47–50	36–53	35–47 (49)
● Ant. I chaetae	15–23	12–21	15–19	14– 20	13–16	11–14	18–19	11	12–16
Pso per half body									
Th. III dorsally	2 (95%)	3 (95%)	2 (85%)–3	2–3 (50%)	2–3 (64%)	2 (78%)–3	3	2	2–3 (50%)
Head ventrally	1	1	1	1	1	1	NO	1	1
Head									
● p1–p1 granules	10–13	11–14	11–13	11–14	12–15	10–14	10–12	11–14	10–12
Labial chaetae									
bm field	4	4	4–5	4–5	4	4	4	4	4–5
prox. field	7–8 (9)	8	8–9	8–9 (11)	7–8	7–8	7	7–8	8–9
postlab. ch.	4–6 (7)	4	3–6	4–6	4–6	5–6	6–8	6–8	4
Tita I chaetae									
● verticil C	3–5	4–5	5 (4–6)	5–6 (4–7)	5	3	6	5	5
additional	YES (15%)	YES (5%)	YES (7%)	YES (50%)	YES (15%)	NO	YES (50%)	NO	YES (5%)
Tita II chaetae									
verticil C	5–6	5–6	5–7	6–7	6–7	5	6–7	4–5	5–7
● additional	YES (15%)	YES (60%)	YES (60%)	YES (50%)	YES (60%)	NO	YES (100%)	YES (9%)	YES (25%)
Tita III chaetae									
verticil C	4–6	4–6	4–7	5 (6)	5–7	5 (4)	5–6	4–5	5–6 (7)
● additional	YES (35%)	YES (55%)	YES (92%)	YES (84%)	YES (60%)	NO	YES (100%)	YES (18%)	YES (85%)
Claws									
● l/w claw I	1.9–2.5	2–2.4	1.9–2.4	2–2.5	1.7–2.4	2.1–2.5	2.5–2.7	2.4–2.8	2–2.4
● l/w claw II	1.9–2.5	1.9–2.4	1.9–2.4	2–2.5	1.7–2.7	2–2.4	2.4–2.6	2.2–2.8	2–2.5
● l/w claw III	1.9–2.5	1.8–2.3	1.9–2.5	1.8–2.6	1.8–2.8	2–2.6	2.5–2.7	2.4–2.7	2–2.4
Abdomen									
Abd. I,II– *s* chaetae	ST	LT	ST	LT	LT	LT	LT	LT	ST
Abd. V– position of *s*	b	b	b–c	b–c, c	b	b	c	c	b, b–c
Abd. V– chaetae nr. between M–M′	2–3	1–2	2 (1)	2–3	1–3	1–3	1	1	1 (2)
Abd. VI– kx chaeta	NO	YES (15%)	YES (30%)	YES (20%)	YES (70%)	YES (30%)	YES (30%)	NO	YES (60%)

PAO; number of chaetae on Ant. I; number of chaetae on Tita I; number of additional chaetae between the B-C verticil of Tita II and Tita III, respectively; width / length ratio of claw I, II and III, respectively; and number of granules between chaetae p1-p1 on the head. Missing values for these traits (9% of the total) were substituted by the mean/median of every population. An autopilot with slow and thorough mode and relative Euclidean distances were used. After randomization runs, a three-dimensional solution was accepted as optimal. The pattern of chaetae *s*, *s′* and *s″* on the abdominal terga and chaetotaxy of Th. I and Abd. VI was applied according to Pomorski (1990). We followed Fjellberg (1998/99) in chaetotaxy of the labium, while chaetotaxy of the tibiotarsus is presented after Deharveng (1983) and chaetotaxy of the head after Jordana et al. (1997). The claw length was measured as the distance from the distal part of the pretarsus to the top of claw, and the claw width as the width of the claw complex at the conjunction of the pretarsus and tibiotarsus.

Abbreviations for Collembola morphology: Ant. – antennal segment, Th. – thoracic tergum, Abd. – abdominal tergum, Tita – tibiotarsus, PAO – postantennal organ, pso – pseudocellus, psx – parapseudocellus.

2.3. Molecular data analyses

Additional 20 specimens of *P. janosik* were collected in five Western Carpathian caves and stored in pure ethanol at 4°C until analyzed. To prevent contamination, all DNA laboratory work was conducted under sterile conditions with the use of barrier tips. Total DNA was extracted with the Thermo Scientific GeneJET PCR Purification Kit. Entire specimens were digested in lysis buffer + proteinase K for 3 hrs; extraction was then carried out as advised by the manufacturer. Final elution of DNA was conducted twice with 50 µL of the elution buffer (for the 1^{st} and 2^{nd} elutions). A polymerase chain reaction (PCR) (Saiki et al. 1988) was carried out using a 12.5 µL reaction volume consisting of 1 µL of template DNA (not quantified), 10 × PCR Buffer (VWR), 12.5 mM of dNTP mix, 5 µM of each primer and 0.125 units of Taq polymerase (VWR) on a GenePro (Bioer Co., Ltd, China) thermal cycler.

A fragment (667 bp) of the **COI gene** was amplified using the universal primers LCO1490 (5'-ggt caacaaat cataaagatattg g-3') and HCO2198 (5'-taa act gggtgac caaaaaat ca-3'; Folmer et al. 1994). Thermal cycling conditions were as follows: 94°C for 1 min followed by 35 cycles of (94°C for 30 sec, 45°C for 40 sec and 72°C for 1 min), followed by 2 min in 72°C. After verification on agarose electrophoresis, reaction products were purified using Exo I / FastAP (Thermo Fisher Scientific). Sequencing of purified products was performed using LCO1490 at the Lambda a.s. company in Bratislava, Slovakia, or SEQme s.r.o. in Dobris, Czech Republic, using the Sanger method. In cases when primer failed to produce high quality chromatograms, reverse primer sequencing was employed.

Sequences were manually edited and trimmed of unreadable short stretches (ca 30 bp at the 5' and 3' ends) with Bioedit v.7 (Hall 1999). Since none of them contained stop codons or indels if ORF was set correctly, all were considered to be true mitochondrial and not nuclear copies. Sequences were aligned with the MEGA v.6 (Tamura et al. 2013) software by Muscle (Codons) algorithm using the Invertebrate Mitochondrial Gene Code and default parameters. All the sequences were verified as being consistent with collembolan DNA using the GenBank BLASTn search (the Mega Blast algorithm with the default setting brought alignments only to other *Protaphorura* congeners with an E value of 0.0). Standard DNA barcoding distance analysis was conducted using the Kimura 2-parameter method (Kimura 1980). An unrooted tree was constructed using a neighbour-joining algorithm (Saitou & Nei 1987), and the robustness of the tree nodes was assessed by bootstrap analysis with 1000 pseudoreplications. Correlation between the genetic and geographical distance of populations was evaluated by the Mantel test (999 permutations) using the GenAlEx 6.5 program, and genetic and geographical distances, respectively, were displayed by PCoA. Haplotype diversity (h) was calculated using DnaSP 5; then a haplotype network for *P. janosik* was constructed using the Network 5.0.0.0 (results not shown). All new sequences are publicly available in GenBank (see Electronic Supplement 1).

Populations from the Western Carpathians were examined for the presence of the bacterial endosymbiont *Wolbachia.* A PCR was carried out using a 12.5 µL reaction volume consisting of 1 µL of template DNA (not quantified), 10 × PCR Buffer (TopBio), 12.5 mM of dNTP mix, 5 µM of each primer and 0.325 units of Taq polymerase (TopBio) and 1.5 µL BSA on a GenePro (Bioer Co., Ltd, China) thermal cycler.

A prokaryotic fragment (878 bp) of the 16S gene) and a fragment (480 bp) of the fbpA gene were amplified using 16Sf (5'-ttgtagcctgctatggtataact-3') and 16Sr (5'-gaataggttatgattttcatgt-3') primers (O'Neill et al. 1992) and fbpAf (5'-gctgctccrcttggywtgat-3') and fbpAr (5'-ccrccagaraaaayyactattc-3') (Baldo et al. 2006). Thermal cycling conditions were as follows: 94°C for 2 min followed by 37 cycles of (94°C for 30 sec, 52°C for 40 sec for 16S; 54°C for 40 sec for fbpA and 72°C for 1 min 30 sec), followed by 10 min at 72°C. After verification on agarose electrophoresis, reaction products were processed as in the COI sequences. All 16S and fbpA sequences were verified as being consistent with *Wolbachia* endosymbiont using a GenBank BLASTn search (with E value 0.0 and 4x10-114 for 16S and fbpA, respectively).

3. Results

3.1. Intra-specific morphological variability

In studied populations of *Protaphorura janosik* morphological variability was observed mainly in body size, number of chaetae on the first antennal segment and number of vesicles in PAO. On the other side, granulation of the body was rather fine and uniform in all populations, with 10–15 granules between the chaetae p1 on the hind margin of the head. Unlike the labial palp morphology of the genus *Protaphorura* specified by Fjellberg (1998/99), the blunt-tipped sensillum was not found on papilla A in the vast majority of the studied *P. janosik* specimens.

In the studied populations body chaetotaxy was often asymmetrical, with macrochaetae weakly differentiated, except for the PET population with well recognizable abdominal macrochaetae. Chaeta p3 on thorax I was thickened as well as chaetae *s* on abdominal terga. Chaetae *s* on Abd. I–III were located closest to pso *a* with a variable shape: thin and long equally to chaeta p1 (populations NK, JPS, CS, MYL and both Eastern Carpathian populations) or thick and short (localities DC, SA, ALB). Chaeta *s* on Abd. V was located above pso *b* (NK, ALB, CS, MYL) or close to pso *c* (DC, JPS, SA). Along with chaeta *s*, one or two other chaetae (*s′*, *s″*) appeared between M–M′ in the Western Carpathian populations.

A full set of chaetae on verticils T+A (11 chaetae) and verticil B (7 chaetae + chaeta "M") on the tibiotarsi was found in all populations. In contrast, the arrangement of chaetae on the C-verticil on the tibiotarsi was unstable among populations, as well as presence of additional chaetae or chaetal verticil(s). The marked interpopulation difference was registered in chaetotaxy of the first leg. Except for NK, all the populations had 5 or more chaetae on verticil C and additional chaetae with floating positions between verticils B and C on all legs. The Eastern Carpathian population from PET had 4–5 distinct verticils of chaetae on Tita II–III, with additional chaetae between verticils B and C, and above verticil C. The length/width ratio of the claws displayed a troglobiomorphic

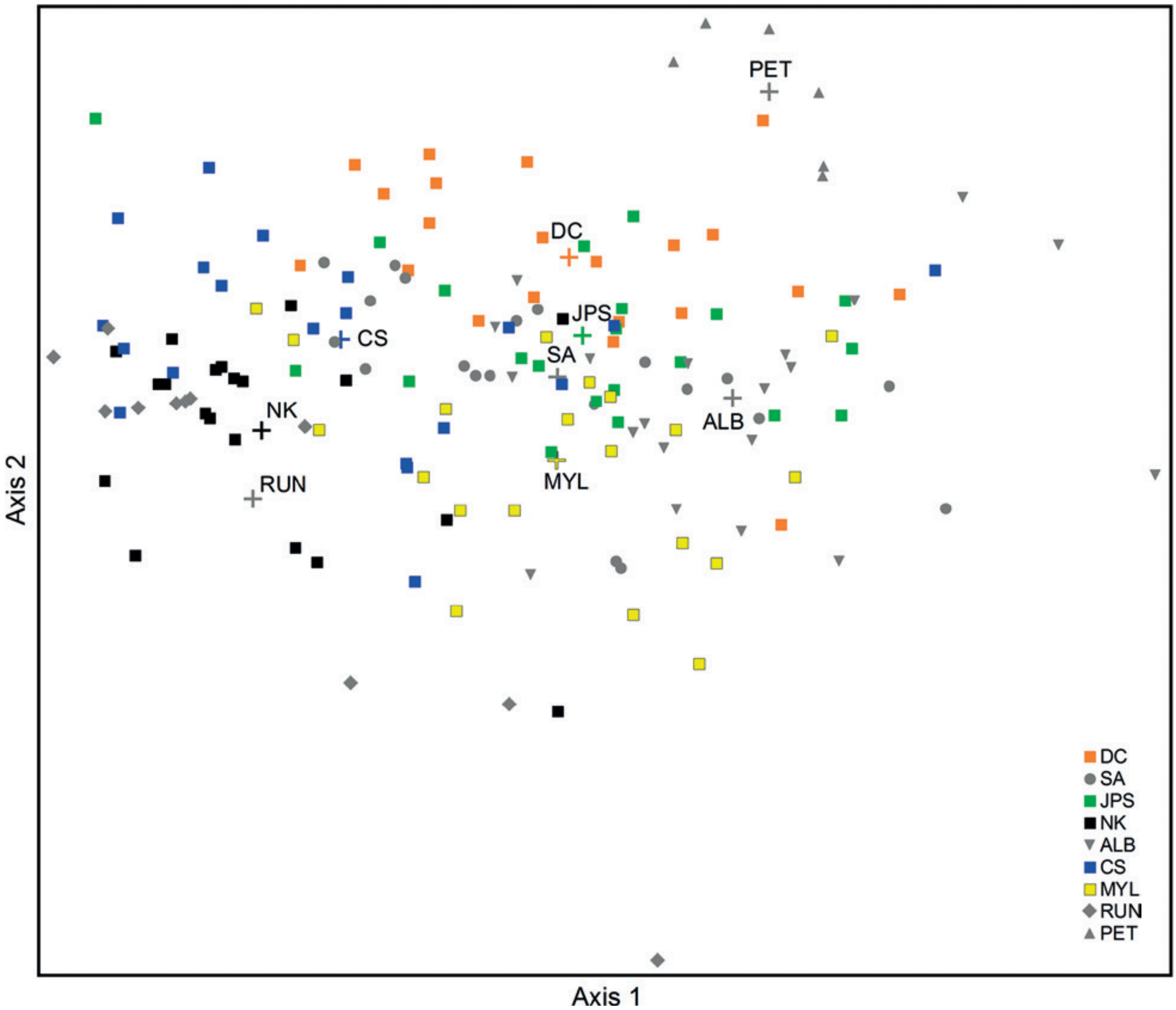


Fig. 2. Non-metric multidimensional scaling (MNS) analysis of quantitative morphological traits in *P. janosik* populations from the Western and Eastern Carpathian caves (for abbreviations of caves see Table 1, for traits see Table 2)

character in all the studied populations, with a difference between the Western Carpathian (2.0 – 2.5) and Eastern Carpathian populations (2.5 – 2.8). In Eastern Carpathian populations, especially in individuals from PET, the empodial filament reached the length of the claw, while in the Western Carpathian populations it reached ¾ of the length of the inner edge of the claw.

A strong variability was revealed in the dorsal pseudocellar pattern, especially in the number of pseudocelli (pso) on thoracic tergum III, with left-right asymmetry. Two pso were found on this segment in the NK, DC and ALB populations, while 3 pso were observed more often in those of the CS and MYL caves. In JPS and SA, specimens with 2 and 3 pso occured in equal proportion (50%). Individuals with 2 – 3 pso on Th. III (left-right asymmetry) were thus considered as aberrant and this character as variable in this species. On the ventral side of head 1 pso appeared in all populations except for PET, where psx was present instead of pso.

Morphological characteristics were analysed by NMS ordination. A three-dimensional solution was recommended by Autopilot and confirmed by the Monte Carlo permutation test, with a significance of $p = 0.004$ and a mean stress of 4.24 for real data and 250 runs for both real and randomised data. The best three-dimensional solution had a final stress of 4.22, $p < 0.00001$, after 109 iterations. An NMS ordination diagram (Fig. 2) plotted against the first two axes (variance explained 72.1% and 20.5%, respectively) did not show any distinct clustering of specimens from different caves, except for those from PET, which were clearly separated from the other populations.

3.2. Molecular analyses

Molecular analysis based on a mitochondrial *cytochrome oxidase I* (COI) fragment was carried out in five populations from the Western Carpathians. Populations from the Eastern Carpathians were not sequenced successfully. A 645 bp fragment (215 codons) of the COI gene was used in all analyses, and no insertions, deletions or stop

Table 3. Intra- and interpopulation diversity in *P. janosik* populations, bootstrap method with 1000 replications, K2P parameter, standard errors in italics. For abbreviations see Table 1.

Population	Intrapopulation diversity		Interpopulation diversity				
	CS		DC	JPS	MYL	NK	
CS	0,000	*0,000*		*0,006*	*0,006*	*0,007*	*0,007*
DC	0,000	*0,000*	0,028		*0,002*	*0,004*	*0,004*
JPS	0,000	*0,000*	0,026	0,002		*0,004*	*0,004*
MYL	0,001	*0,001*	0,031	0,014	0,013		*0,001*
NK	0,000	*0,000*	0,029	0,013	0,011	0,001	

Fig. 3. Principal coordinates analysis (PCoA) plot generated from genetic and geographical distances of *Protaphorura janosik* populations from the Western Carpathian caves. Amounts of the explained total variation are in parentheses.

codons were detected. Nucleotide composition of all sequences was biased for A and T (A = 26%, T = 38%, C = 19%, G = 16%). Across the 645 sites, 25 were variable and 23 parsimony-informative. Genetic diversity was calculated within and between populations. Almost zero intrapopulation diversity was observed, while it ranged from 0.1 – 3.1% between populations (Table 3). Genetic and geographical distances between sequenced populations were evaluated by Principal Coordinates Analysis (PCoA) (Fig. 3). The percentages of variation explained by the first two axes were 60.64% and 35.04% for genetic distance, and 60.2% and 26.32% for geographical distance, respectively. A significant correlation between genetic and geographical distances of populations was calculated using Mantel test ($p = 0.037$).

Out of 88 sequences, eight haplotypes were detected, seven of which were limited to a single population and one shared between the two populations (NK, MYL). Two haplotypes were detected in JPS and DC, respectively, with the less frequent one represented by a single sample each.

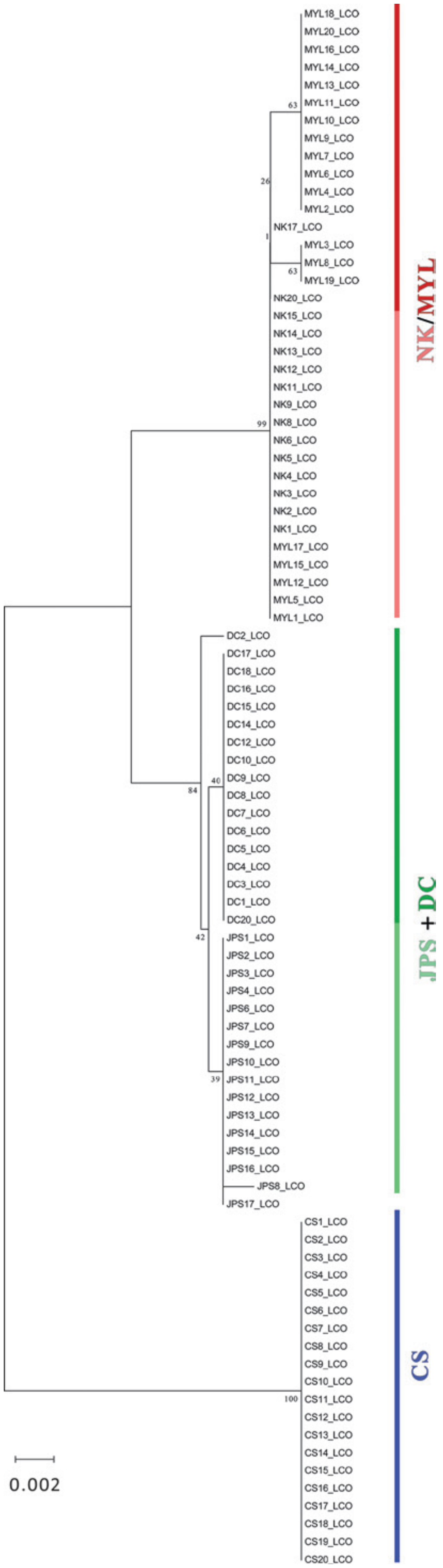
An unrooted neighbour-joining tree based on the COI mitochondrial gene divided the *P. janosik* populations into three distinct clusters: (1) a cluster corresponding to cave CS, (2) a cluster including JPS and DC, and (3) a cluster consisting of a mixture of specimens from adjacent caves MYL and NK (Fig. 4).

The presence of *Wolbachia* was detected only in a population from a sandstone cave (JPS), in which 50% of specimens were infected. Strains 16S were consistent with *Wolbachia* proved in *Paratullbergia callipygos*, *Mesaphorura italica* and *Folsomia candida*.

4. Discussion

In the present study, we combined classical morphological appraisal with DNA barcoding to reveal the diversity of *P. janosik*, populations, which occupy the caves of the Western Carpathian Mountains in Central Europe. To examine the morphological variability between populations we selected traits without high taxonomic value (Table 2). *P. janosik* is an exception from the symplesiomorphy of Poduromorpha (Weiner 1996; D'Haese 2003), with B7 chaeta always present on the metathoracic tibiotarsi. In contrast to the genus-specific labial palp, no blunt-tipped sensillum was found on papilla A in the vast majority of the studied specimens of this species. Fjellberg (1998/99) noticed a constriction and pointed tip of A sensillum in specimens during ecdysis. It seems that these tips do not break off and the sensilla remain acuminate in *P. janosik*.

We selected only adult specimens for morphological examination, since species-specific morphological characteristics used in taxonomy, especially chaetotaxy, are not fully developed in juveniles (Hopkin 1997). In term of adults, several difficulties can appear in sex-determination of a particular species. As sexual maturity and maximum body size in most Onychiurinae is attained after the seventh moult (Pomorski 1998), it is difficult to recognize this stage on microscopic slides. We estimated the age of specimens based on body size and developmental stage of the male or female genital plate. Nevertheless, we registered large females of *P. janosik* with a genital plate more weakly developed than in smaller females, suggesting that the developmental stage of the genital plate is not proportional to body size.



P. janosik is an obligate subterranean species endemic to the Western Carpathians (Kováč 2000; Kováč et al. 2014, 2016). Based on study of material from Ukraine, its distributional range is likely more extensive, involving the Eastern Carpathian caves. Unfortunately, we had only a limited number of specimens from the Eastern Carpathian caves available for molecular analyses and the sequencing of COI fragment was not successful. Therefore, only morphological examination was carried out in these populations. We found only minor differences between Ukrainian specimens and well defined *P. janosik*. Since the populations of the same species from different geographical regions may vary in some minor characteristics, e.g. in *P. taimyrica* from Taimyr and China, respectively (Babenko & Kaprus' 2014; Sun et al. 2015), we considered the Eastern Carpathian specimens to be consistent with *P. janosik*. Barcoding data are necessary for verifying the species affiliation of these specimens. Here, we have been unable to evaluate the genetic distance between Eastern and Western Carpathian populations. The equal distance of Ukrainian populations and Slovakian CS population from those of central Slovakia indicate similar genetic distance (~ 3%). Geographically isolated Ukrainian populations may potentially represent a cryptic species of *P. janosik*.

A morphological similarity between the nine populations, based on measurable or countable traits, was evaluated by NMS analysis. The studied populations were not clearly separated from one another and the high intrapopulation morphological variability was obvious especially among the Western Carpathian populations. The Eastern Carpathian populations (RU and PET) were strikingly separated from each other in the ordination diagram. The population from PET differed from RU and the Western Carpathian populations by having psx instead of pso on the ventral side of the head, well differentiated abdominal macrochaetae and an empodial filament on the third leg as long as the inner edge of the claw. The morphology of the claw complex is one of the major indications of troglobiomorphism in Collembola (Christiansen 1961 – as "troglomorphism"), as long, slender claws facilitate movement on wet and slippery cave surfaces. According to the present study, specimens from Ukraine had more elongated claws in comparison with *P. janosik* from the Western Carpathians, and this may suggest a longer evolution in a cave environment. Equally, two Western Carpathian populations (NK and MYL) from the same mountain range formed partly overlapping clusters. Conversely, a comparison of molecular data confirmed our hypothesis on genetic similarity in geographically related localities.

The effectiveness of reproductive isolating mechanisms is unclear in Onychiuridae. Thus, for the purpose of molecular study, we selected caves where *P. janosik* is

Fig. 4. Unrooted neighbour-joining tree based on COI fragments of *P. janosik* populations from the Western Carpathian caves, constructed in Mega 6 software using the Kimura 2-parameter model, bootstrap method with 1000 replications, scale: 0.002.

the only representative of the genus due to the probability of inter-specific hybridization among congeners living at the same site (Skarżyński 2004). We documented genetic differences between populations of *P. janosik* that were expected from its presumed low dispersal ability in a subterranean environment. Gene flow among the studied populations was extremely low, as shown by the lack of shared haplotypes between distant populations. Similar to our findings, only a few or even no common haplotype was found in isolated populations of the Antarctic soil-living collembolan *Isotoma klovstadi* based on the COII gene (Frati et al. 2001). Several studies have documented high genetic diversity within collembolan species (Timmermans et al. 2005; Torricelli et al. 2010; Zhang et al. 2014) and the existence of cryptic species. Previous studies (see below) have outlined low sequence divergences within a species (< 1%), but the authors have diverged in the threshold for species delimitation. For example, interspecific distances among 19 collembolan species distributed in 12 genera were 8% (Hogg & Hebert 2004), while only 2–3% among closely related species of various arthropod groups (Hebert et al. 2003a,b).

As the mitochondrial COI gene has a relatively high mutation rate resulting in diversity within and between populations over relatively short evolutionary timescales (Hebert et al. 2003a), the MYL+NK cluster with a shared haplotype points to continual gene flow between these populations and/or very recent colonization from a common source or colonization of one cave from the other. Due to short geographical distance and potential connection between these caves, the molecular similarity of both populations was expected. Surprisingly, we found some morphological differences between these populations. On the other hand, minimal gene flow between populations from presumably not inter-connected karst areas indicates their effective isolation. Similarly, Fanciulli et al. (2001) suggested a low level of gene flow and differentiation of populations over very small geographical distances. Moreover, the genetic and geographical distance between the studied *P. janosik* populations correlated positively, suggesting the importance of geographical isolation for population differentiation. Populations DC and JPS, forming a common cluster, are more distant geographically than genetically. However, no shared haplotype was found, and sequence divergences between DC and JPS were very low (0.2%). In contrast to the uniform CS population, both DC and JPS were represented by two unique haplotypes. Considering three clusters of *P. janosik*, sequence divergences between the distant ones (2.6%–3.1%: DC+JPS–CS, MYL+NK–CS) approximate the threshold of a species (Hebert et al. 2003a), suggesting that these populations possibly represent geographic isolates (Hebert et al. 2003b). The most often used calibration for COI as a molecular clock, namely 2.3% sequence divergence per million years (Brower 1994; Knowles 2000), suggests that these populations diverged between 1.3 million and 800,000 years ago (Early Pleistocene – Ehlers & Gibbard 2008). Based on distinct genetic variation, we assume that in the long-term scale, geographical and genetic isolation would lead to evolution of independent species from the particular populations.

Frati et al. (2004) confirmed the presence of *Wolbachia* endosymbiont in parthenogenetic populations of *F. candida* but obtained negative results in a population of *F.* cf. *candida* in which males were also recognized. Indeed, the genetic divergence between these populations was high, indicating different species. A wide range of invertebrates is infected by endosymbiotic proteobacteria of the genus *Wolbachia*. Among arthropods, six supergroups have been defined: A, B and E–H (Werren et al. 1995; Vandekerckhove et al. 1999; Lo et al. 2002; Rowley et al. 2004; Bordenstein & Rosengaus 2005). *Wolbachia* infection induces several reproductive alterations in their host species (Werren et al. 2008), which may lead to elimination of males from reproduction. We examined the presence of the bacterial endosymbiont *Wolbachia* in *P. janosik* populations from the Western Carpathians. However, as we observed, *P. janosik* is a sexually reproducing species with a balanced sex ratio. We detected collembolan-specific strains of *Wolbachia* (supergroup E) (Vandekerckhove et al. 1999) in one population from a sandstone cave, where 50% of specimens were infected. Unfortunately, the sex of these specimens was not examined. Contrary to our results, *Wolbachia* was not detected in another sexually reproducing congener, *P. fimata* (Czarnetzki & Tebbe 2004). As already mentioned, *P. janosik* populations from distant caves appeared to be isolated, and gene flow between them reduced. *Wolbachia* may enhance the speciation process by inducing incompatibility and cutting-off gene flow (Charlat et al. 2003). This leads to the question of why only one of the studied populations was *Wolbachia*-positive. Yet, we cannot be conclusive about the role of *Wolbachia* in these *P. janosik* populations.

Weakly developed troglobiomorphic characters and occurrence in both karst and pseudokarst caves support the idea that *P. janosik* is a relatively young troglobiont (Kováč et al. 2016). Thus, it is a glacial relict species predominantly associated with colder caves. This idea is supported by distribution of morphologically similar species with a larger body confined to cold climatic zones – *Megaphorura arctica* distributed in the Arctic islands of the Palaearctic region (Fjellberg 1998) and *P. macrodentata* in Canadian tundra (Hammer 1953). A congener living in a very similar habitat as *P. janosik* was recently discovered in a cave of the very distant Baikal area in Siberia, Russia, however representing a morphologically and genetically well separated species from *P. janosik* (Parimuchová & Kováč 2017). Based on the very similar morphology and affinity to habitats with colder microclimate, we suppose that the common hypothetical ancestor of these psychrophile forms was distributed over a large area during the Pleistocene glacial periods. Thus, contemporary distribution of these recent forms could be explained by the climatic relict hypothesis (e.g. Holsinger 2000). According to this model, caves served as refugia against climatic alteration for the ancestors of troglobionts. Warming in interglacial periods caused extinction of the remaining epigean ancestral populations, whereas hypogean ones were isolated and inclined to evolve into obligatory cave fauna. After the present study, the morphological and genetic differences observed between populations of *P. janosik* in caves of the Western Carpathians indicate the existence of geographic isolates, probably representing an allopatric species at the beginning of speciation. However, further molecular study of Eastern Carpathian populations is necessary to clarify the phylogeny of *P. janosik* and relationships between its populations.

5. Acknowledgements

The study was supported by grant 1/0199/14 from the Slovak Scientific Grant Agency VEGA, VVGS-2014-224 grant from the Pavol Jozef Šafárik University in Košice and by Operational Programme "Research & Development" funded from ERDF (Code ITMS:26220120022, rate 0.4). We are grateful to Robert S. Vargovitsh (Schmalhausen Institute of Zoology, Kiev, Ukraine) for kindly providing us with *Protaphorura* specimens from the Eastern Carpathians, and Olga Ivanovna Kadebskaja (Perm State University, Perm, Russia) for the specimens from Ochotnichya Cave in the Baikal area, Siberia, Russia. We thank our colleagues Michal Rendoš, Peter Ľuptáčik and Andrej Mock (P.J. Šafárik University in Košice) for their important help with collecting Collembola specimens in caves. Mr. David McLean is acknowledged for the linguistic corrections of the manuscript draft. We are especially grateful to one of the two anonymous reviewers for the very constructive comments.

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File 1: parimuchova&al-carpathiancollembolan-asp2017-electronicsupplement.doc – Accession numbers of sequences publicly available in GenBank.