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Wolbachia prevalence and diversity in selected riverine predatory beetles (Bembidiini and Paederini)

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Abstract

Despite the many studies on *Wolbachia* in Coleoptera, data about the prevalence of this bacterium among ground and rove beetles are missing. This study describes *Wolbachia* distribution and diversity in predatory beetles co-habiting the same environment - submontane river channels. Three species of Paederini (Staphylinidae) and four Bembidiini (Carabidae) were collected from six river catchments of the Carpathians. *Wolbachia* was absent in three species: *Paederidus rubrothoracicus* (Goeze), *Bembidion decorum* (Zenker in Panzer), *Bembidion modestum* (F.) - but detected in four others. *Paederus limnophilus* Erichson and *Paederidus ruficollis* F. were infected at only some sites by different strains of supergroup A, *Bembidion punctulatum* Drapiez was infected at all sites by a single strain from supergroup B, and *Bembidion varicolor* F. was single or double infection by three strains from A and B supergroups. The patterns of infection prevalence and strain diversity were very complex and only in some cases support common *Wolbachia* infection and strain similarity among different species of predatory beetles sharing the same environment. Moreover, the similar genetic patterns observed in all examined beetles cannot be associated with the infection status of particular species (distinct mitochondrial and nuclear haplotypes fixed in distant populations; small diversity within populations).

Key words: α-proteobacteria, endosymbionts, Coleoptera, rove beetles, ground beetles, riverine, predators.

Introduction

Recently, population genetic and ecological studies on insects have often considered the infection status and strain diversity of intracellular bacteria (Saridaki and Bourtzis, 2010). Incorporating data about the microbiota of investigated species is especially important when endosymbiotic bacteria that affect host development, diversity and demography are detected (Stouthamer *et al.*, 1999; Jiggins, 2003; Hurst and Jiggins, 2005; Werren *et al.*, 2008). Notable examples of such endosymbionts are the maternally inherited bacteria from the genus *Wolbachia* (Saridaki and Bourtzis, 2010), which infect Arthropoda and Nematoda. *Wolbachia* is widespread endosymbionts of insects (Hilgenboecker *et al.*, 2008) and responsible for numerous disorders in host reproduction (Stouthamer *et al.*, 1999). *Wolbachia* is often considered in genetic studies on insects for a variety of reasons, one of which is that it can be used as an additional marker for understanding host diversity and relations (Kajtoch *et al.*, 2012). Moreover, evidence has emerged that *Wolbachia* can be horizontally transmitted across species that share environments (Vavre *et al.*, 1999; Caspi-Fluger *et al.*, 2012; Mazur *et al.*, 2016), food resources - e.g., plants or fungi; (Sintupachee *et al.*, 2006; Stahlhut, 2010; Kolasa *et al.*, 2017), prey (Johanowicz and Hoy, 1996; Le Clec'h *et al.*, 2013), or in parasitoid relations (Dedeine *et al.*, 2005; Raychoudhury *et al.*, 2009; Brucker and Bordenstein, 2012). On the other hand, there are studies that contradict the hypothesis that common habitat or prey could act as the vector - e.g. (Cordaux *et al.*, 2001; Yun *et al.*, 2011). The issue of whether *Wolbachia* can spread among insects associated with particular habitats and/or food resources could be highly important for understanding the ecological relations between infected (and uninfected) species - eg. a predator vs prey. This phenomenon is highly underestimated according to recent review of Bailly-Bechet *et al.* (2017), which shows high dynamic of *Wolbachia* in Arthropods populations (species acquire and lose bacteria more frequently than it was expected). Moreover, *Wolbachia* cause extreme consequences ranging from sex-ratio distortion and mating incompatibilities to protection against viruses (Martinez *et al.*, 2014).

Groups of beetles (Coleoptera) from the families Carabidae and Staphylinidae are some of the most diverse predators in the world of invertebrates (Fisher, 1988; Pakaluk and Ślipiński, 1995). Ground and rove beetles are also often the most abundant predators in environments (Hurka, 1996; Szujecki, 2008). Moreover, the ecological properties of predatory beetles (their strict affinity to environments of natural state and the role of top predators) make them good indicators of environment quality (Abellán *et al.*, 2005; Skalski *et al.*, 2012). Genetic studies on Carabidae and Staphylinidae mainly cover issues related with species delimitation, barcoding, phylogenetics or phylogeography (Naomi, 1985; Maddison *et al.*, 1999). Surprisingly, despite much research on *Wolbachia* infections among various beetles - e.g. (Lachowska *et al.*, 2010; Sontowski *et al.*, 2015), there is only one study that reports preliminary data for a single species of ground beetle (Frank *et al.,* 2009) and two studies for rove beetles (Yun *et al.*, 2011; Bili *et al.*, 2016).

Here, we focus on predatory beetles that inhabit riverine habitats: ground beetles of the tribe Bembidiini and rove beetles of the tribe Paederini. Members of both groups express high affinity for specific riverine or riparian habitats (natural gravel or stony alluvia) and sensitivity to alterations of flow regimes (Kleinwächter and

Rickfelder, 2007; Schatz, 2007; Skalski *et al*., 2012). *Bembidion* carabids are well studied with respect to their phylogenetic relations and barcoding (Raupach *et al*., 2011; Maddison, 2012; Raupach *et al*., 2016). Similarly, only few phylogenetic, species delimitation and phylogeographic studies were published on rove beetles (Pfeiler *et al*., 2013; Frisch, 2015; Marcelino *et al*., 2016). As was highlighted above, there are no data about *Wolbachia* infections in Bembidiini and only Yun *et al*. (2011) have reported this bacterium in Chinese *Paederus fuscipes* Curtis.

Horizontal transmission of bacteria (e.g. *Wolbachia*) among riverine beetles is possible as all these species inhabit the same habitat (gravel alluvia) (Paetzold and Tockner, 2005; Lambeets *et al*., 2009) and are predators of riverine invertebrates, mainly insects (Paetzold and Tockner, 2005), which could act as vectors for bacteria transmission. Therefore, interesting would be to verify whether all these species are infected by *Wolbachia* and whether infected species harbour similar strains.

Estimations of riverine beetles populations diversity and connectivity should be important for understanding *Wolbachia* prevalence and strains diversity. Considering the ecological and morphological features of riverine beetles and environmental constraints, two sets of factors could impact the genetic diversity and connectivity of beetle populations. First, the affinity of beetle species to a particular type of environment (in this study - gravel and stony alluvia in mountainous areas) could suggest that their mobility is restricted, e.g. they move along watercourses and generally avoid or are not able to cross barriers like mountain ridges. This would imply that their populations are structured geographically and consequently different *Wolbachia* strains should infect species/populations living in the isolated river system. On the other hand, annual inundations and occasional floods could prone riverine beetles to frequent and quick movements that would enable re-colonization of areas covered by waters during periods of the year (Bonn *et al*., 2000). This would lead to homogenization of genetic diversity of beetles and facilitate spread of *Wolbachia* strains, at least across some areas (e.g. within catchments, drainages).

To solve the primary goal of the study, we investigated the *Wolbachia* prevalence, diversity and population genetics of several potential host species strongly associated within the same environment (riverine gravel alluvia from mountainous areas). We tested the hypothesis that co-habiting beetles (all of which are predators) also share the endosymbiotic bacterium *Wolbachia*.

Materials and methods

Study area

For the purpose of this study, we sampled beetles from six selected river catchments within the Carpathian Mountains belonging to different drainages: the i) Raba and ii) Dunajec rivers in southern Poland (Western Carpathians; both belong to the Vistula river basin and Baltic Sea drainage basin), iii) Oder river in Czechia (the Baltic Sea drainage basin), iv) Topľa river in Slovakia (within the Danube river basin and Black Sea drainage basin), v) Wiar (Vihor) river in Ukraine (within the Vistula river basin and Baltic Sea drainage basin) and vi) Moldova river in Romania (within the Danube river basin and Black Sea drainage basin) (figure 1).

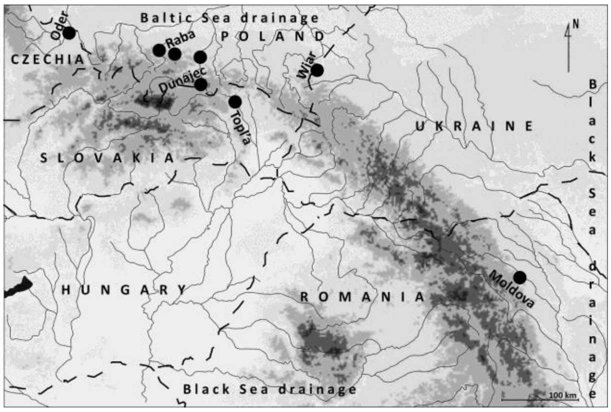

Figure 1. Simplified map of the Western and Eastern Carpathians with the network of major rivers and localization of sampling sites in selected river catchments (black dots).

Species characterization and selection

Seven species of riverine beetles characteristic for mountainous areas and associated with river channels were selected for this study. Three of them belong to the rove beetles (Staphylinidae): *Paederus limnophilus* Erichson 1840; *Paederidus rubrothoracicus* (Goeze 1777); *Paederidus ruficollis* (F. 1781), and four to ground beetles (Carabidae): *Bembidion (Ocydromus) decorum* (Zenker in Panzer 1799); *Bembidion* (*Bembidionetolitzkya*) *varicolor* F. 1803; *Bembidion* (*Ocydromus*) *modestum* F. 1801; *Bembidion* (*Princidium*) *punctulatum* Drapiez 1820. All these species exclusively or mostly inhabit mountain and sometimes highland river and stream channels. Some can also be found in other moist rocky and stony habitats. All these beetles are distributed widely in suitable habitats across Eurasia, and all are common in the Carpathian range; however, locally, their ranges overlapped only in some of selected river catchments.

Sampling

Specimens were collected with an exhauster directly from stony or gravelly river banks (usually with the help of a flush of water), preserved in 99% ethanol and then stored at −22 °C. Specimens of the seven studied species were collected during several field expeditions between 2014 and 2016 in Poland, Czechia, Slovakia, Ukraine and Romania (figure 1). Several specimens of each available species were sampled from each location. Each site was a 0.5 km transect of river along which beetles were randomly collected in suitable habitats (stony banks and gravel alluvia). There were some differences in sampling because not all species could be found and collected in the same time from each rivers (occurrence of species did not overlap everywhere) (table 1). Then,

Table 1. Numbers of examined beetles per sampling site (N) and number of *Wolbachia* infected specimens (W). In brackets - *Wolbachia* supergroups identified (A and/or B).

Species	Raba1		Raba2		Dunajec1		Dunajec2		Wiar		Oder		Topľa		Moldova	
	N	W	N	W	N	W	N	W	N	W	N	W	N	W	N	W
Paederus limnophilus	5	2 (A)	5	0	5	1 (A)	5	1 (A)	5	0	-	-	-	-	5	0
Paederidus ruficollis	5	5 (A)	5	5 (A)	5	5 (A)	5	5 (A)	5	0	-	-	-	-	5	0
Paederidus rubrothoracicus	5	0	5	0	5	0	5	0	5	0	-	-	-	-	5	0
Bembidion varicolor	5	5 (B)	5	5 (B)	5	5 (B)	5	5 (B)		-	-	-	5	5 (A/B)	5	5 (A/B)
Bembidion decorum	5	0	5	0	5	0	5	0		-	-	-	5	0	5	0
Bembidion modestum	5	0	5	0	5	0	5	0		-	5	0	-	-	5	0
Bembidion punctulatum	5	5 (B)	5	5 (B)	5	5 (B)	5	5 (B)		-	5	5 (B)	-	-	5	5 (B)

taking into account the species distribution and success of the sampling. Five specimens were randomly selected from each site that the species was collected from. For each target species we selected six sites for analyses, two in the Raba catchment, two in Dunajec catchment, one in Moldova catchment and another site in one of the three other catchments: Oder, Wiar or Topľa (table 1). This resulted in 210 analyzed individuals (30 individuals per chosen beetle species).

Laboratory procedures

Whole beetle bodies were used for DNA extraction using a Nucleospin Tissue kit (Macherey-Nagel). Prior to DNA extraction, beetles were washed several times in 99% ethanol and distilled water to remove all external contamination. Five standard MLST housekeeping genes were used for *Wolbachia* screening: Cytochrome C Oxidase, Subunit I (*coxA*), Spartyl/glutamyl-tRNA(Gln) Amidotransferase, Subunit B (*gatB*), Conserved Hypothetical Protein (*hcpA*), Fructose-bisphosphate Adolase (*fbpA*) and the Cell division protein (*ftsZ*) according to methods described by Baldo *et al.* (2006). All DNA isolates obtained from the beetles were amplified. Additionally all PCRs were run with two positive controls (*Polydrusus inustus* Germar and *Eusomus ovulum* Germar weevils, known to be infected by *Wolbachia*; (Mazur *et al.*, 2016) and a negative control (distilled and autoclaved water). Moreover, amplification of fragments of hosts genes was performed. Mitochondrial Cytochrome Oxidase I (*COXI*) gene was amplified using primer pairs: B1490-Bcoi2R (Maddison, 2014), LCO1490-HCO2198 (Vrijenhoek, 1994) or newly designed pair: Paed-F2: GATCAGGAATAGTTGGAAC-ATCAT and Paed-R2: TGTTGGTATAAAATTGGGT-CT. Nuclear protein-coding gene - Arginine Kinase (*ArgK*) was amplified using primers AK168F and AK939R (Wild and Maddison, 2008). The reagent concentrations used for the amplifications and PCR cycling profiles of both markers were as in Kajtoch *et al.* (2012) (annealing temperatures: 50 °C). After purification, the PCR fragments were sequenced using a BigDye Terminator v.3.1. Cycle Sequencing Kit (Applied Biosystems) and run on an ABI 3100 Automated Capillary DNA Sequencer. All newly obtained sequences were deposited in GenBank (supplemental material table S1).

Data analysis

Wolbachia genotyping

The sequences of presumed *Wolbachia* genes were compared with the online NCBI databank using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) to check if primers specifically amplified the targeted α-proteobacteria.

Allelic profiles of MLST genes were generated for each infected individual. Next, an approach similar to that of Montagna *et al.* (2014) was utilized to compare the allelic profiles generated from the ground and rove beetles with some representative sequence types from other species that harbored bacteria belonging to the same supergroups: A (ST-1) from *Drosophila melanogaster*

Meigen, B (ST-15) from *Drosophila simulans* Sturtevant, D (ST-35) from nematode, F (ST-8) from *Cimex lectularius* L., and H (ST-90) from *Zootermes angusticollis* (Hagen). Next, the generated alignment of MLST genes was used for the construction of a phylogenetic network. Unrooted phylogenetic networks were prepared separately for each MLST gene in SplitsTree4 (Huson and Bryant, 2006) with neighbor-net algorithm distance estimates. Contrary to traditional phylogenetic trees, this allows for visualization of multiple connections between the examined sequences, which can represent, e.g., recombination events. MLST sequences were compared against *Wolbachia* sequences deposited in the MLST database (http://pubmlst.org/wolbachia/) and also to GenBank (https://www.ncbi.nlm.nih.gov/genbank/) using the Basic Local Alignment Search Tool (Altschul *et al*., 1990) to find other hosts harbouring similar *Wolbachia* strains (with maximum identity of sequences).

Beetle markers

The sequences were checked using BioEdit v.7.0.5.2 (Hall, 1999) and aligned with MAFFT v.7 7 (Katoh and Standley, 2013). Sequences were verified with respect to the presence of stop codons (which were not detected) and eventual heterozygous sites in nuclear genes (which were also not detected due to the general low level of polymorphism and presence of single alleles in populations). After trimming of ambiguous fragments, the *COXI* alignment comprised 645 bp, except for *P. rubrothoracicus*, for which shorter sequences were generated (of 591 bp) due to problems with using universal primers. The same carried out for *ArgK* resulted in 704 bp for all the ground and rove beetles.

COXI and *ArgK* haplotypes were identified and standard genetic indices - e.g. the number of variable sites (V), number of segregating sites (S), haplotype diversity (Hdiv), nucleotide diversity (π) and number of private haplotypes (Hpriv) - for populations were calculated in DnaSP v.5 (Librado and Rozas, 2009). Incongruence between the phylogenetic signals from *COXI* and *ArgK* was assessed by statistically evaluating the ILD index (Farris *et al*., 1994) by using the partition homogeneity test implemented in PAUP* 4.9b10 (Swofford, 2003). The ILD test rejected incongruence between both markers (p = 0.100); therefore phylogenetic analyses were executed on a concatenated dataset. Unique genotypes per species and site (identified for joined *COXI* and *ArgK* sequences) were used for phylogenetic tree reconstruction with Maximum Likelihood (ML) method implemented in PhyML 3.0 (Guindon *et al*., 2010) - using the web-page interface http://www.atgc-montpellier.fr/phyml/. The beta version of the program was used, which includes automatic selection of the best DNA substitution model - Smart Model Selection (http://www.atgc-montpellier.fr/sms/) with the use of the Akaike Information Criterion (AIC). Branch support was obtained using the approximate Likelihood-Ratio Test (aLRT SH-like) (Anisimova and Gascuel, 2006).

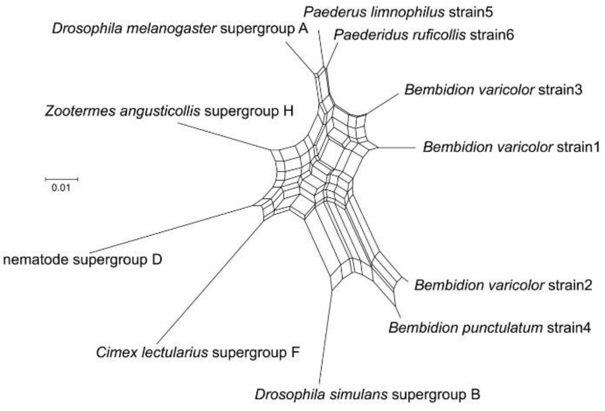

Figure 2. Neighbour-net networks constructed for identified *Wolbachia* strain genotypes identified on the basis of few housekeeping genes of Multilocus Sequence Typing system in examined riverine beetles shown with reference strains belonging to identified *Wolbachia* supergroups.

Results

Wolbachia prevalence and diversity

Three species were found to be totally uninfected: *P. rubrothoracicus*, *B. decorum* and *B. modestum.* A single *Wolbachia* strain from supergroup A was found in only 25% of *P. limnophilus* specimens from the Raba and Dunajec populations (figure 2, table 1), and populations from Wiar and Moldova were uninfected. A strain from supergroup A was detected in the entire Raba and Dunajec populations of *P. ruficollis*, while other populations were uninfected (figure 2, table 1). All examined individuals from all populations of *B. varicolor* were *Wolbachia* positive. This species harboured the most complex diversity of *Wolbachia* as it was infected by three different strains: one (from supergroup B) characteristic for Raba and Dunajec, and two other strains (from both supergroups) found in Moldova and Topľa (figure 2, table 1). All individuals from all populations of *B. punctulatum* were infected, but only by a single *Wolbachia* strain, belonging to B supergroup (figure 2, table 1).

Some of the *Wolbachia* genes found in the infected beetles were most similar to sequences known from hosts such as other Coleoptera (including aquatic Hydraenidae), Hymenoptera (mainly parasitoid wasps) and single representatives of Diptera and Araneae (supplemental material table S2). MLST profiles identified in the infected beetles were similar at just 1-3 loci to profiles from some Lepidoptera and Hymenoptera (supplemental material table S2).

Genetic diversity of beetles

Despite geographical and environmental isolation the populations do not much differ from each other, both in their mitochondrial and nuclear genomes (supplemental material table S3, figure 3). Almost all of the selected beetles harboured slightly distinct *COXI* and *ArgK* haplotypes (differed by only 1-2 substitutions) between their populations (supplemental material table S3, figure 3). The exceptions were *P. ruficollis*, *P. rubrotho-*

racicus, *B. varicolor* and *B. punctulatum*, collected from sites within the Raba and Dunajec river catchments: at all of these sites individuals of particular species shared the same *COXI* and *ArgK* genes. Within populations (sites), the genetic pattern was opposite, as almost all individuals from particular sites (especially from Raba and Dunajec rivers) were monomorphic with respect to *COXI* and *ArgK* genes. Rare exceptions were particularly found in Moldova, where *P. ruficollis*, *P. rubrothoracicus* and *B. modestum* possessed 2 *COXI* haplotypes. *P. ruficollis* also had 2 *COXI* haplotypes in Wiar and *B. decorum* had 2 *ArgK* haplotypes in Raba (supplemental material table S3, figure 3).

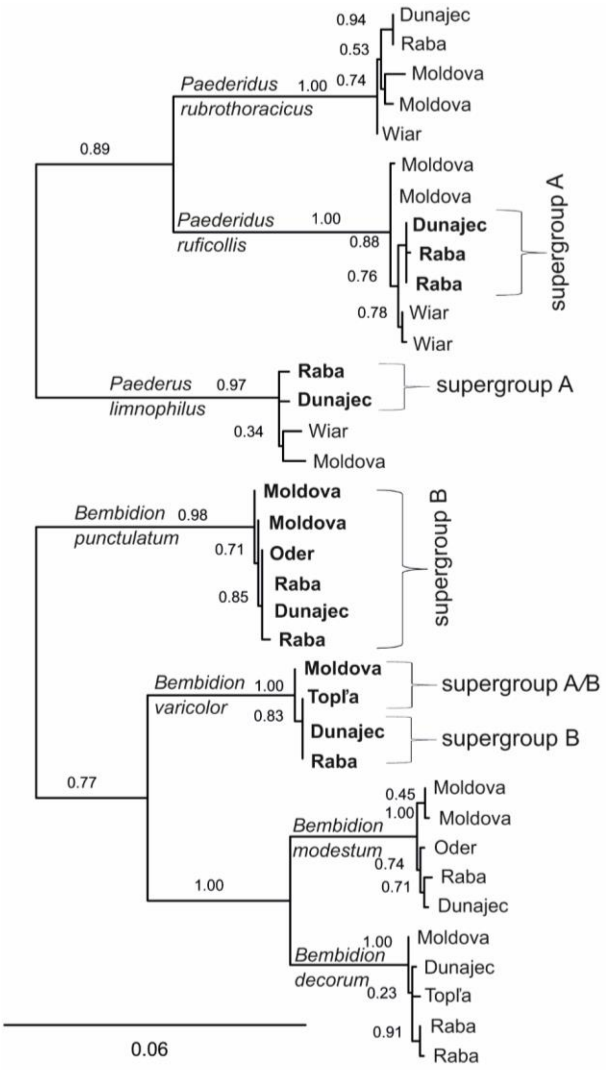

Figure 3. Maximum likelihood phylogenetic trees of the three examined Paederini and four Bembidiini species inferred on the basis of concatenated sequences from two markers: Cytochrome Oxidase subunit I gene of mitochondrial DNA and Arginine Kinase gene. Genotypes from *Wolbachia* infected beetles in bold with strains affinity to supergroups presented to the right. Numbers above the branches indicate statistical measures of nodal support.

Discussion

Despite numerous studies on *Wolbachia* prevalence and relations with numerous species of beetles, such data for predatory species have been restricted mainly to aquatic beetles (Dytiscidae, Gyrinidae, Haliplidae, Hydraenidae, Hydrophilidae) (Sontowski *et al*., 2015). Among terrestrial-predatory beetles *Wolbachia* infection was found in some ladybirds (Coccinellidae) (Elnagdy *et al*., 2013; Goryacheva *et al*., 2015). Only single ground beetle species, *Pheropsophus aequinoctialis* (L.) (Frank *et al*., (2009) and three rove beetles, *Aleochara bilineata* Gyllenhal, and *Aleochara bipustulata* (L.) (Bili *et al*., 2016) and *P. fuscipes* (Yun *et al*., 2011) were reported as to be infected. Thus, this study extend substantially knowledge about *Wolbachia* prevalence and diversity in representatives of a Carabidae and Staphylinidae.

Wolbachia infection in the riverine beetle populations was highly complex but rather unpatterned. There were species infected across the whole of their examined range (*B. varicolor* and *B. punctulatum*), in only some populations (*P. limnophilus*, *P. ruficollis*), or that were bacteria-free (*P. rubrothoracicus*, *B. decorum* and *B. modestum*). Also, the frequency of infected specimens in populations varied, as some were found to be totally infected (*B. varicolor* and *B. punctulatum*) and in others only a small number of individuals were infected (*P. limnophilus*). In most of the species a single strain of *Wolbachia* was found, with the exception of *B. varicolor*, which harboured three strains, including two strains in double infected individuals from the Moldova and Wiar rivers. An even more interesting phenomenon was that the infected species harboured different *Wolbachia* strains from each other, despite their co-occurrence in the same habitat and foraging on potentially common prey. This observation consequently led us to reject the hypothesis that cohabiting riverine beetles share their endosymbiotic *Wolbachia*. This is in contrast to some studies on other beetles that share a habitat, but all these previous works were only executed on either herbivorous - e.g. steppic weevils and leaf beetles (Mazur *et al*., 2016; Kolasa *et al*., 2017) - or cambioxylophagous species, bark beetles (Kawasaki *et al*., 2016). The reason why predatory beetles do not share *Wolbachia* strains needs further study. It could be explained in respect to niche displacement between these species and the foraging of particular ground and rove beetles on different preys, which would mean that there are no common prey that could act as vectors for *Wolbachia* transmission. Unfortunately, examination of prey of studied beetles would be difficult, if feasible, task. Foraging habits and strategy of hunt of examined beetles are poorly known (Paetzold and Tockner, 2005) and most probably these species take various preys. Another possible explanation is that the predator-prey route cannot transfer *Wolbachia* because the symbionts may not be able to survive in the predators' digestive tracts (Cordaux *et al*., 2001), however this route has been supported, at least for infected isopods (Le Clec'h *et al*., 2013). The observed lack of shared bacteria strains among predatory beetles is in agreement with a preliminary study on spiders and insects inhabiting crops in China (Yun *et al*., 2011).

The examined riverine predatory beetles have different strains of *Wolbachia*, but some genes are similar to those, which have been found in other beetles and other insects. The most pronounced is the similarity of some bacteria genes to those found in Hydraenidae, which inhabit freshwaters and thus live in close proximity to the Paederini and Bembidiini. Moreover, Hydraenidae are known to be frequently infected by *Wolbachia* (Sontowski *et al*., 2015). Among other species that have similar *Wolbachia* genes to those found in the riverine beetles in this study, parasitoid wasps are interesting and are suspected to be *Wolbachia* vectors (Dedeine *et al*., 2005).

It is interesting that all examined ground and rove beetles share similar population genetic structures despite their different *Wolbachia* infection status. The genetic diversity of the uninfected species does not differ from the infected ones at either the intra- or inter-population levels. All distant populations (from different river catchments) possessed distinct mitochondrial and nuclear haplotypes, but within populations, or more accurately-sampling sites, from particular river catchments, these species expressed very low or even a lack of genetic diversity. Moreover, the partially infected species (*P. limnophilus*, *P. ruficollis*) show that there are no clear differences in population genetic structure between infected and uninfected populations. Also, distant populations of *B. varicolor*, which differ with respect the *Wolbachia* strains infecting them, generally have similar genetic patterns. We can consequently hypothesize that *Wolbachia* has no effects on the genetics of the examined predatory beetles and that other factors are responsible for their similar population genetic patterns - e.g. common history of origin and colonization of the examined areas in the Carpathians, as has been shown for other freshwater species (Pauls *et al*., 2006; Dénes *et al*., 2016), and/or parallel responses of these species to the frequent inundations that shape habitats in mountain rivers (O'Callaghan *et al*., 2013; Skalski *et al*., 2016).

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