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INVERTEBRATE MICROBIOLOGY

Relations of *Wolbachia* Infection with Phylogeography of *Philaenus spumarius* (Hemiptera: Aphrophoridae) Populations Within and Beyond the Carpathian Contact Zone

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Abstract *Wolbachia* is the most widespread intracellular α-proteobacteria maternally inherited endosymbiont of insects and nematodes. These bacteria are associated with a number of different reproductive phenotypes of their hosts. Relatively few studies have dealt with distribution of infections across populations and with the influence of these bacteria on host genetic diversification and speciation. The aims of this study are to determine the distribution and rate of infection and to characterize the *Wolbachia* strains associated with *Philaenus spumarius* spittlebug (Hemiptera) by using multilocus sequencing typing (MLST) analysis and host phylogeography. The results showed that infection rate was significantly different between members of both main mitochondrial phylogenetic lineages of *P. spumarius*. We detected much higher infection rates of *Wolbachia* in *P. spumarius* populations from the north-east clade than the south-west clade. Moreover, the frequency of these infections varied within and outside the contact zone known from the Carpathians. Given the reproductive alterations which are often associated with this endosymbiont, *Wolbachia* probably maintain genetic differentiation of its hosts in its contact zone in the Carpathians. This is one of the first studies demonstrating the presence of *Wolbachia* across a large part of the range of insect species, including the contact zone. The spread of *Wolbachia* in *P. spumarius* populations can potentially cause speciation by compromising the potential reproductive barrier between infected and uninfected populations. We discuss possible implications of *Wolbachia* infection inducing cytoplasmic incompatibility in the population dynamics of this spittlebug but confirm that more studies are also required.

Keywords Rickettsia · Endosymbiont · Hemiptera · The Carpathians · Speciation · MLST

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Introduction

Wolbachia is the most widespread intracellular α-proteobacteria. This maternally inherited endosymbiont is known to infect 15-76 % of insect species [37, 87]. It is found also in many non-insect invertebrates: spiders, mites, crustaceans, and nematodes [29, 37, 64, 65, 89], whereas it is apparently absent in, e.g., mollusks [70]. *Wolbachia* exists in 13 monophyletic clades: A to N, referred as supergroups [19]; however, supergroups A and B are known to be the most frequent in insects [64, 87]. *Wolbachia* lives inside the cytoplasm in reproductive tissues and is associated with a number of different reproductive phenotypes in its hosts, such as cytoplasmic incompatibility (CI) [9, 34, 36, 72], feminization, parthenogenesis inducing (PI), male killing, and modifying fecundity [5, 33, 77]. These modifications of the host breed impart a selective advantage, thus allowing *Wolbachia* to spread efficiently and rapidly into host populations [19, 29]. Furthermore, the ability to manipulate the reproductive properties may have an effect on the host's biology, ecology, and evolution [4].

Many studies argue that *Wolbachia* promote rapid speciation by causing reproductive incompatibility between mates, usually from different populations belonging to distinct mitochondrial lineages [10, 62, 77, 82, 94]. The molecular basis of cytoplasmic incompatibility is unknown, but it has been confirmed that the genetic determinants are maternally inherited [62] and correlate with the presence of rickettsia endosymbionts in the arthropods' gonad tissue (ovaries and testes) [88]. This phenomenon is expressed when an infected male mates with a female that is not infected, when male and female are both infected with two different *Wolbachia* strains, or when the male is infected with two strains and the female is infected with a single strain of *Wolbachia* [24]. Examples of CI were found in a diverse range of insects, including flour beetles, *Tribolium confusum* [61, 84], alfalfa weevils, *Hypera postica* [31, 46], parasitic wasps, *Nasonia* [67], planthoppers, *Laodelphax striatellus* [58, 59], flour moths, *Ephestia cautella* [13], mosquitoes, *Aedes scutellaris* [81], and fruit flies, *Drosophila simulans* [8, 49]. It is also known that *Wolbachia* may be transmitted horizontally between different hosts (species or members of distant phylogenetic lineages) [88, 90], which can also play a role in speciation.

Wolbachia infections are relatively common in insects although little information is available about the effects of *Wolbachia* infections in population scale. Populations of insects might not be infected at all: e.g., weevil *Centricnemus leucogrammus* [40] and *Cryptocephalus* leaf beetles [51]. On the other hand, some species seemed to be infected across an entire range by the same supergroup and even the same strain: e.g., weevil *Polydrusus inustus* [41], Chinese rice leafroller *Cnaphalocrocis medinalis* [15], Indian butterflies *Talicada nyseus* [2], and leaf beetle *Oreina cacaliae* [51]. An alternative example concerns species with populations co-infected by different supergroups and/or strains: e.g., grasshopper *Chorthippus parallelus* [92], bean beetles *Callosobruchus chinensis* [43], leaf beetle *Crioceris quatuordecimpunctata*, and *Crioceris quinquepunctata* [44, 56]. Also, there are known species that some populations infected and others did not: e.g., flower bug *Orius strigicollis* [86], little fire ant *Wasmannia auropunctata* [66], and leaf beetle *Diabrotica virgifera* [24]. In some species, only a portion of individuals are infected within populations: e.g., grasshopper *C. parallelus* [92]. The latter two examples could concern situations in which all infected populations harbor the same bacteria (single or multiple infection) or each of these populations is infected by another supergroup and/or strain of *Wolbachia*. A reason for these infection variants could possibly be found in the natural horizontal transmission of *Wolbachia* by consumption of infected or contaminated food, e.g., plant phloem [18] and/or from parasitoids, e.g., parasitoidal wasps [27, 32]. However, knowledge about infection patterns in populations is relatively poor due to difficulties in studying large numbers of populations and specimens across species ranges and possible diverse compositions of *Wolbachia* strains in infected populations. Moreover, it is hard to determine if some populations are really uninfected because in some of them, a very low number of individuals could harbor bacteria [89]. Similar problems concern identification of all strains present in some multiple-infected populations, as some strains could infect low numbers of individuals or some strains could dominate others even within a single individual. However, these issues have not been satisfactorily studied thus far.

In recent years, very few studies report cumulative data about *Wolbachia* infections in populations within contact zones. The paradigmatic work for our studies was performed by Zabal-Aguirre et al. [92], who described two subspecies of the meadow grasshopper *C. parallelus* that arose in allopatry and next form a secondary contact zone in the Pyrenees. Zabal-Aguirre et al. [92] reported that *Wolbachia* infection is widespread in *C. parallelus* populations, where most of the samples featured 76-100 % infected individuals. Moreover, all the analyzed populations of *C. parallelus* were infected with *Wolbachia*, although there were differences in infection type. Their studies indicated a significant infection influence on the origin, maintenance, and dynamics of this contact zone. Bella et al. [7] executed consistent studies with *C. parallelus*. These studies show that *Wolbachia* may be involved in speciation phenomenon in this grasshopper that is produced by a reproductive barrier. Additional research of three species of the *Allonemobius socius* complex of crickets from North America in zones of secondary contact including CI caused by the *Wolbachia* was described by Marshall [52, 53]. Giordano et al. [24] presented the role of *Wolbachia* bacteria in reproductive incompatibilities and hybrid zones of *Diabrotica* beetles and *Gryllus* crickets. The examples mentioned above suggest that the role of *Wolbachia* in inducing CI and the resulting speciation in insects could be underestimated. However, most of this research was executed on orthopteran, which is due to intensive studies on this group of insects rather than special association of *Wolbachia* with these insects. On the other hand, almost nothing is known about the influence of *Wolbachia* on reproductive barriers, contact zone origin, and speciation process in other groups of insects. Recently, the contact zone has been described for the spittlebug *Philaenus spumarius* (L.) (Hemiptera: Aphrophoridae) [48, 55], which has been one of the most intensively studied bugs in recent years [20, 21, 25, 48, 54, 55, 68, 71], making it a model species in evolutionary studies of insects.

Currently, genus *Philaenus* is believed to consist of nine or ten species [20, 54, 80]. Most of them are distributed in the Mediterranean area. Only *P. spumarius* is widespread and occurs naturally throughout the entire temperate and warm Holarctic region [20, 21]. It therefore seems to be an excellent subject of research for the spread of *Wolbachia* infection. Recent studies on the phylogeny and population genetics of

spittlebugs of the genus *Philaenus* show that *P. spumarius* is divided into two highly distinct mitochondrial clades: northeastern (north-central Europe and Asia) and southwestern (Western Europe and the Mediterranean region, also introduced/invaded populations to the North America), which meet along European mountain ranges [48, 54, 55]. Similarly, analysis of a nuclear marker (elongation factor 1 alpha gene (EF1-α)) suggests that there are three main clades: northeastern (Eurasian), southeastern (east Mediterranean-Caucasian), and southwestern (Italo-Iberian), which probably overlap along European mountain ranges [48, 55]. Moreover, detailed examination of genetics for populations in the contact zone of main phylogenetic lineages in the Carpathians demonstrated that some of these populations consist of individuals belonging to different clades and, even more, that some individuals showed signs of hybrid genotypes, including examples of heteroplasmy [48]. These findings strongly suggest that *P. spumarius* is a complex of evolutionary units with uncertain taxonomic status. Therefore, it is an excellent subject for further population genetic and speciation studies. Because our preliminary analyses confirmed that some of *P. spumarius* populations are infected by *Wolbachia,* we decided to perform detailed studies on these bacteria occurrence across the range of *P. spumarius*, including populations in contact zones.

The principal aims of this study were to determine infection diffusing of *Wolbachia* and identify supergroups of this microorganism in *P. spumarius* populations in the entire range and in the contact zone of the main phylogenetic lineages. As a result, we will try to verify the following hypotheses: (i) *Wolbachia* infects only one of *P. spumarius* mitochondrial phylogenetic lineages and consequently (ii) *Wolbachia* infection is associated with the beginning of allopatric speciation of the host and with limited hybridization between genetically distinct populations of the *P. spumarius*. As this work is the first step in understanding interactions between *Wolbachia* and *P. spumarius*, it does not include examination of cytoplasmic incompatibility or other mechanisms influencing reproduction of the host.

Materials and Methods

Sampling Area

Individuals from 49 populations of *P. spumarius* were collected from 2003 to 2011 (Fig. 1). Thirty-one populations (102 individuals) covering nearly the entire range of *P. spumarius* (locality symbols S) and eighteen populations (72 individuals) from six transects across the Carpathian arc (locality symbol CM) were sampled (Table 1). Additionally, six specimens of *Philaenus tesselatus* (from Portugal) and single representatives of *Philaenus italosignus* (from Sicily), *Philaenus signatus* (from Greece), *Philaenus arslani* (from Lebanon),

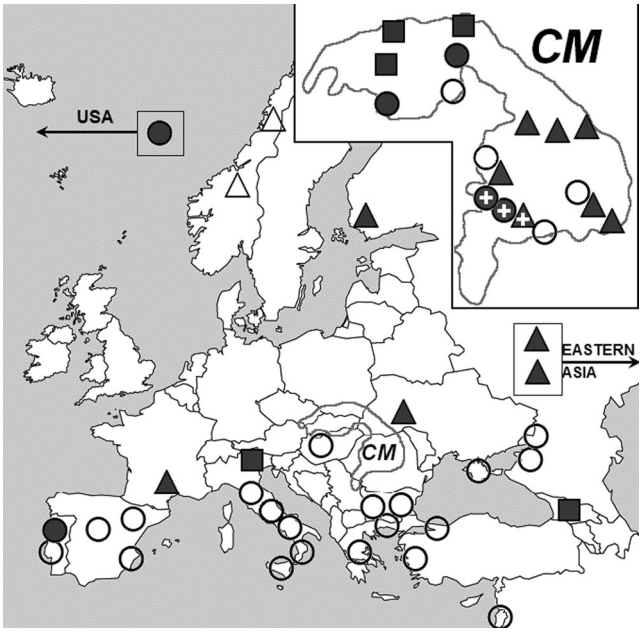

Fig. 1 Localization of *P. spumarius* sampling sites and distribution of *Wolbachia* infections (including Carpathian Mountains (*CM*)). *Circles* represent populations harboring haplotypes belonging to SW mitochondrial clade; *triangles* represent populations with NE mitochondrial haplotypes; and *squares* represent populations including haplotypes from both clades. *Empty marks* indicate lack of evidence for *Wolbachia* infection; *grey marks* represent populations in which infection was detected (strains belonging to B supergroup). Populations infected with local strain of A supergroup are marked additionally with a *plus mark*

Philaenus loukasi (from Greece), *Philaenus tarifa,* and *Philaenus maghresignus* (from Southern Spain) were tested for *Wolbachia* presence (we missed the newly described species *Philaenus elbursianus* and *Philaenus iranicus* due to the unavailability of these Iranian taxa [80]). Samples of these species, as well as the majority of individuals of *P. spumarius*, were previously used in phylogenetic and phylogeographic studies [54, 55], whereas Carpathian samples were used for examination of the contact zone [48]. The spittlebugs were caught in a sweep-net, instantaneously preserved in 99 % ethanol, and stored at −20 °C. All tested specimens were damaged during extraction of DNA procedure. The remaining voucher specimens are preserved at the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences.

Wolbachia Detection

DNA was extracted from the whole body of the individuals. Amplification, purification, and sequencing of multilocus sequence typing (MLST) genes were performed using the standard protocols with different sets of primers (available at: http://www.pubmlst.org/wolbachia/) [6]. A total of 174 specimens of *P. spumarius* and 12 specimens of other *Philaenus* species were screened for the presence of

Table 1 Symbols and localization of sampled populations of *P. spumarius* used in the study

Locality symbol	Locality
CM-5	Vélke pole
CM-7	Malatina
CM-17	Kamienica River Valley
CM-32	Jaśliska
CM-35	Olka
CM-38	Regec
CM-42	NE from Baia Mare
CM-45	Tihuta Pass
CM-47	Petru Voda Pass
CM-53	Predeal
CM-54	Sinaia
CM-55	Sinca Veche
CM-59	Voineasa
CM-61	Crasna
CM-65	Băiţa
CM-67	Buceş
CM-68	Marişel
CM-69	Şuncuiuş
Spain-S1	Sierra dela Penya Rossa
Spain-S2	Sierra de Guadarama
Spain-S3	Sierra del Madero
Portugal-S4	São Pedro de Manuel
France-S5	Saillagouse
Italy-S6	Gemona del Friuli
Italy-S7	Passo de Muraglione
Italy-S8	Lagonegro
Italy-S9	Santa Agata di Eboli
Italy-S10	Reserva Naturale Aurunci
Italy-S11	Nebrodi Mts, Sicily
Greece-S12	Delphi
Lebanon-S13	Lebanon Mts
Finland-S14	Turku
U.S.A.-S15	Illinois
Portugal-S17	Serra de São Mamede
Russia-S18	Kunashir Island
Russia-S19	Sakhalin
Ukraine-S20	Chatyr-Dag Plateau
Georgia-S21	Guria
Greece-S23	Alexandropoulos
Turkey-S24	Ayvaçik
Turkey-S25	Boz Dagi
Bulgaria-S26	south Pirin Mts
Bulgaria-S27	central Rhodope
Hungary-S28	Üllö
Russia-S29	Kamennaya Balka
Russia-S30	Semibalki
Ukraine-S31	Chartova Gora
Norway-S32	Geiranger
Norway-S33	Dombas

CM Carpathian Mts

Wolbachia strains. Detection was based on the *Wolbachia* ftsZ gene and results in the amplification of an approximately 528-base pair-long DNA fragment with the *Wolbachia* primers ftsZ_F and ftsZ_R [6]. Next, all the ftsZ-positive specimens were subjected to amplification for the other MLST genes (gatB, coxA, hcpA, and fbpA). To rule out the possibility of poor quality or degraded DNA templates used for amplification, the cytochrome B gene of mitochondrial DNA (CB-J10747 and CB-N11526 primers by Stewart i Beckenbach [74]) was additionally amplified. Polymerase chain reaction (PCR) products were checked on agarose gel. Sequences of the same gene (not generated de novo but obtained from previous studies, Maryańska-Nadachowska et al. [48, 54, 55] were used in further analyses.

PCR was performed in 30 μl reaction volumes with 3.0 μl of 10× PCR buffer, 3.0 μl of 25 mM $MgCl_2$, 0.6 μl of a dNTP mixture, each in a 10mM concentration, 0.6 μl of each 15 mM forward and reverse primers, 3.0 μl of 100 ng of genomic DNA, 0.2 μl of Taq DNA polymerase (Qiagen, Germany) and sterile and deionized water (up to 30.0 μl). PCR conditions for standard primers were as follows: 4 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 1 min at 54 °C, and 2 min at 72 °C, with 10 min at 72 °C after the last cycle. To obtain a full set of MLST sequences for individuals infected with local strain (found in specimens located in the inner side of the southern Carpathians, see "Results"), some genes need to be amplified by specific primers, with the annealing temperature adjusted respectively from protocols available at: http://www.pubmlst.org/wolbachia/. PCR products were subjected to electrophoresis on 1.5 % agarose gels and stained with Midori Green DNA Stain from ABO. For the MLST gene sequencing of infected *P. spumarius*, specimens were obtained with the same primers used during amplification (available at http://www.pubmlst.org/wolbachia/).

MLST Analyses of *Wolbachia* Strains

The sequences were compared with the online NCBI databank using the Basic Local Alignment Search Toot (BLAST) [1] option to check if primers specifically amplified the targeted α-proteobacteria. Next, sequences were edited using the BioEdit Sequence Alignment Editor 5.0.9. [26] and aligned using ClustalX 1.8 [79]. Haplotype (allele) reconstruction of MLST genes from genotype data was conducted using the algorithms provided in PHASE as implemented in DnaSP 5.0 [47].

The information about host and *Wolbachia* haplotypes has been submitted to the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BankIt/). The nucleotide sequences have been deposited in the GenBank database at accession numbers for hcpA, KM377676–KM377688; coxA, KM377689–KM377736; fbpA, KM377737–KM377764; ftsZ, KM377765–KM377773; and gatB, KM377774–KM377832.

The *Wolbachia* strains were assigned a sequence type (ST) and defined as the combination of five integers corresponding to the allele numbers at the five MLST loci (allelic profile) with the help of START2 software [38]. A strain is defined as a *Wolbachia* isolate from a single host population. Strains identical to the five alleles were assigned the same ST.

Sequences of MLST genes were compared against *Wolbachia* sequences deposited in GenBank (which used BLAST) to find other hosts harboring the most similar (maximum identity) *Wolbachia* strains sequences. A dataset of the five concatenated *Wolbachia* MLST gene (i.e., *gatB, coxA, hcpA, fbpA,* and *ftsZ*), belonging to 87 different *Wolbachia* STs were retrieved from the MLST Website and used in comparative analysis with the STs isolated from *P. spumarius.*

Statistical Analyses

The differences of frequencies of positive and negative spittlebug for *Wolbachia* from the whole species range, contact zone in the Carpathians, and all populations outside this zone were analyzed by using the Chi-square test. Relationships between *Wolbachia* strains were featured by building phylogenetic trees and networks for MLST genes. Trees and networks were made of original sequences for each MLST gene separately and for a combined dataset of all MLST sequences. Phylogenetic trees were constructed using MEGA5.0 software [78] with neighbor-joining algorithm. Bootstrap analysis was done with 1000 replications; bootstrap values were calculated using a 50 % majority rule. Construction of networks was done in SplitsTree4 [35]. This software use of median-joining algorithm distance estimates to compute unrooted phylogenetic networks from molecular sequence data. Contrary to traditional phylogenetic trees, it allows for visualization of multiple connections among examined sequences which could represent, e.g., recombination events. Moreover, mitochondrial DNA (mtDNA) networks from previous phylogeographic studies on *P. spumarius* [55, 48] were used for visualization connections with *Wolbachia* infections against a background of *P. spumarius* main phylogenetic lineages. The mtDNA network was build with the use of TCS 1.21 software [17], and symbols of haplotypes (circles) were colored according to *Wolbachia* infection prevalence (lack of infection, single or all individuals infected). The same relationship was visualized on the mitochondrial phylogeographic map of species range. The pairwise homoplasy test (PHI) [14] has been shown to identify the presence/absence of recombination within a wide range of sequence samples with a low false-positive rate [14]. PHI test was used to analyze genetic recombination within and among MLST genes. Results were obtained in part by using SplitsTree4 [35].

Results

Wolbachia Detection in *Philaenus* Species

The screening of *Wolbachia* infections among eight *Philaenus* species was initially executed using amplification of *ftsZ* gene. *Wolbachia* infections were found in *P. spumarius* and *P. italosignus* specimens. However, in the other *Philaenus* species - *P. signatus*, *P. arslani*, *P. loukasi*, *P. tarifa*, *P. maghresignus*, and *P. tesselatus* - we did not ascertain infections of these bacteria. However, it is important to highlight that only for *P. spumarius* did we screen many samples of specimens across species range; for the rest of species, we tested only single specimens for *Wolbachia* infections.

Infection Rate of *Wolbachia* in Different *P. spumarius* Populations

In 23 different *P. spumarius* populations, we obtained an approximately 528-base pair-long DNA fragment out of the ftsZ gene for 72 of 174 individuals. This indicates that 41.4 % of the individuals from all examined populations were positive for infection (Table 2).

Additionally obtained results clearly show differences in infections among populations and main mitochondrial clades. When considering all sampled populations, 70.3 % of individuals from the NE clade were found to be infected, whereas only 20.0 % of the individuals from the SW possessed these bacteria, respectively (Table 2). But these infection frequencies varied within and outside contact zone.

In the identified contact zone in the Carpathians, 93.9 % individuals belonging to the NE clades were infected but two times fewer (46.2 %) individuals from the SW clade were *Wolbachia* positive (Table 2). In total, 68.1 % of all Carpathian specimens were positive for *Wolbachia* infections.

In the rest of species range (excluding Carpathians), there were in total 51.2 % of infected specimens from the NE clade; only 3.3 % of individuals from the SW clade possessed these bacteria (Table 2).

The infection rate was significantly different between members of both main mitochondrial phylogenetic lineages when considering the whole range of species (χ^2=45.5, p<0.0001), contact zone in the Carpathians (χ^2=58.4, p<0.0001), and all populations outside this zone (χ^2=50.3, p<0.0001).

Table 2 Assignment of *P. spumarius* sampled populations to mitochondrial clades (SW and NE) and *Wolbachia* infection frequencies in these populations of the host

Locality symbol	mtDNA clade	Number of screened specimens	Number of infected specimens	Infection frequency (%)	
CM-5	SW	4	4	**In the contact zone (Carpathians) NE: 93.9 % SW: 46.2 % Total: 68.1 %**	**In all studied populations across species range NE: 70.3 % SW: 20.0 % Total: 41.4 %**
CM-7	SW/NE	3/1	3/1		
CM-17	SW/NE	1/3	1/2		
CM-32	SW/NE	3/1	3/1		
CM-35	SW	4	4		
CM-38	SW	4	0		
CM-42	NE	4	3		
CM-45	NE	4	4		
CM-47	NE	4	4		
CM-53	NE	4	4		
CM-54	NE	4	4		
CM-55	SW	4	0		
CM-59	NE	4	4 [1]		
CM-61	SW	4	0		
CM-65	SW	4	1 [1]		
CM-67	SW	4	2 [2]		
CM-68	NE	4	4		
CM-69	SW	4	0		
Spain-S1	SW	2	0	**In the rest of species range NE: 51.2 % SW: 3.3 % Total: 22.5 %**	
Spain-S2	SW	3	0		
Spain-S3	SW	3	0		
Portugal-S4	SW	5	1		
France-S5	NE	3	2		
Italy-S6	SW/NE	2/2	0/2		
Italy-S7	SW	3	0		
Italy-S8	SW	3	0		
Italy-S9	SW	3	0		
Italy-S10	SW	3	0		
Italy-S11	SW	3	0		
Greece-S12	SW	3	0		
Lebanon-S13	SW	3	0		
Finland-S14	NE	4	4		
U.S.A.-S15	SW	3	1		
Portugal-S17	SW	3	0		
Russia-S18	NE	4	4		
Russia-S19	NE	4	3		
Ukraine-S20	SW	3	0		
Georgia-S21	SW/NE	1/3	0/3		
Greece-S23	SW	3	0		
Turkey-S24	SW	3	0		
Turkey-S25	SW	3	0		
Bulgaria-S26	SW	3	0		
Bulgaria-S27	SW	3	0		
Hungary-S28	SW	3	0		
Russia-S29	SW	3	0		
Russia-S30	SW	3	0		
Ukraine-S31	NE	4	3		
Norway-S32	NE	4	0		
Norway-S33	NE	4	0		

Distribution of *Wolbachia* in *P. spumarius*

The sequence analyses of all MLST genes indicates that supergroup B occurred in most of the infected populations in the range of *P. spumarius*, although there were differences in infection type and variations of strains.

In the group of all infected specimens, nearly 75 % harbored more than one strain of supergroup B, in the Carpathians, and over 76 % were double infected. Most individuals were from the eastern Carpathians (from the outer and central arch of the mountains) (Fig. 1).

In the rest of the range, 36 % were single infected by a strain that was not defined in the MLST database. They occurred mostly in populations where single individuals were infected (Portugal-S4, France-S5, U.S.A.-S15, and Georgia-S21) (Fig. 1).

Throughout the Carpathian's contact zone, most specimens were infected by the same *Wolbachia* supergroup like other infected populations from Europe and Asia; moreover, there were only four populations without *Wolbachia* infections, all from the southwestern clade (Fig. 1). In three closely located populations (CM-59, CM-65, and CM-67) from southern Carpathians (Fig. 1), unique strains of these bacteria were identified as belonging to supergroup A. Of all of the obtained, only approximately 5 % of all infections were from a distinct supergroup, and all were from the inner side of the southern Carpathians (Fig. 1). This different supergroup has been detected in all infected individuals from CM-65 and CM-67 belonging to the southwestern mitochondrial clade and one specimen from the population CM-59 belonging to the northeastern clade. In the CM-59 population, only one of four specimens harbored this local strain; three other infected specimens harbored similar strains like other multi-infected individuals from SW. Moreover, three belonging to supergroup A/B populations were single infected with a distinct strain (not detected in MLST database). Only the CM-67 specimen was double infected with different strains of this supergroup. Moreover, analysis of MLST allelic profiles (Table 1) indicated only two identical sequence types from the 87 obtained. This testified to the very high variability of the *Wolbachia*-analyzed genes.

Additionally, there were identified hosts harboring similar strains of *Wolbachia* as *P. spumarius*, obtained from the GenBank (Table 2). The strains of this bacterium in *P. spumarius* were closely related to those in wasps (*Vespidae*), flies (*Drosophilidae*), hemipterans (*Aleyrodidae*), and also in beetles (*Chrysomelidae, Curculionidae*).

Phylogenetic Analysis of the *Wolbachia* MLST Sequences

The phylogenetic analysis results of the *Wolbachia* MLST sequences from *P. spumarius* and *P. italosignus* are shown in Fig. 2. The neighbor-joining analysis revealed two major

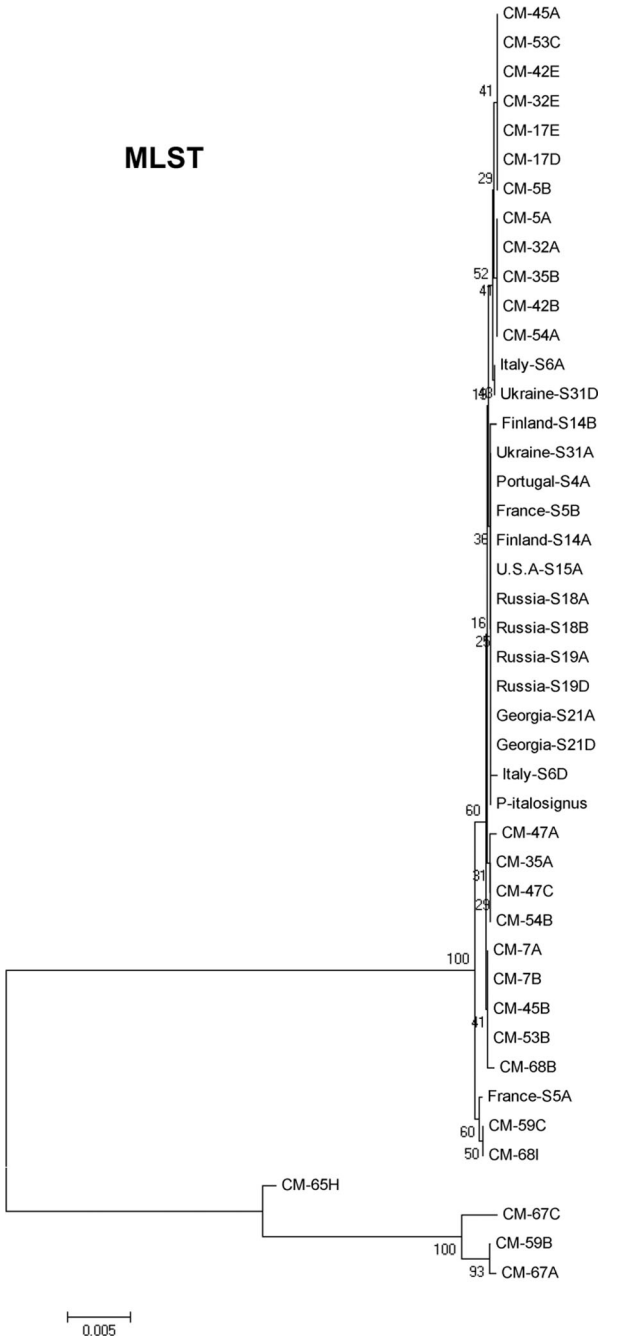

Fig. 2 Neighbor-joining phylogenic tree of *Wolbachia* strains in *P. spumarius* obtained with the use of MEGA5 for joined MLST genes

branches in the phylogenetic trees based on *Wolbachia* MLST sequences separately for each gene (Fig. S1a-e) and collectively for all MLST genes (Fig. 2). These two characteristic branches clustered *Wolbachia* sequences from the *P. spumarius* populations into two main supergroups (Fig. 2). At the first branches are nearly 95 % of populations which harbored strains belonging to the B supergroup. The second branch included *Wolbachia*-infected populations from the southern Carpathians, which suggested that approximately 5 % were infected from an alternative source by strains

belonging to supergroup A. Additionally, the possible relationships between the *Wolbachia* haplotypes were estimated using the median-joining networks shown in Figs. 3 and S2a-e, which show congruent patterns with neighbor-joining trees. Additionally this network brought more information than traditional phylogenetic tree as it showed also multiple connections among examined *Wolbachia* haplotypes (MLST strains) which could correspond to, e.g., recombination events.

Moreover, the networks in Fig. 4 demonstrated results of relations within the *P. spumarius* mtDNA clades and infections of *Wolbachia* bacteria. This figure clearly shows that infections dominate in northeastern clade, and only in population from Norway (belonging to this clade) we did not find this microorganism. In the southwestern clade, the mtDNA network the most infected populations were from the Carpathian Mountains where the contact zone of the main phylogenetic *P. spumarius* clades occurs. This is where specimens can mix with the northeastern clade. Another single exception of the infected populations belonging to the southwestern clade is from Portugal and the USA. About one fifth of the infected individuals was in the population from Portugal; in the USA, one-third of the examined individuals harbored the *Wolbachia*.

Recombination Analysis

The phi test executed by SplitsTree4 [35] indicated statistically significant evidence for recombination of three of five analyzed MLST genes: gatB, coxA, and fbpA but not for hcpA and ftsZ (Table 3). Moreover, recombination was detected among joined sequences of all MLST genes (Table 3).

Discussion

This study provides the first evidence of *Wolbachia* infections in *Philaenus* spittlebugs; moreover, it represents primary research that includes distribution of these bacteria among *P. spumarius* populations across almost the entire species range and in the contact zone of the main phylogenetic lineages of this insect in the Carpathians.

Since first discovered in *Culex pipiens* [28], *Wolbachia* have been described as a widespread and common bacteria infecting insects all over the world (e.g., Neotropics [83], Palaearctics [91], and Nearctics [89]). Most research on *Wolbachia* focused on screens of species, but single representatives of each species were usually investigated. Due to known effects of *Wolbachia* on reproduction of its hosts (e.g., cytoplasmic incompatibility (CI) [10, 11, 45, 62–64], parthenogenesis [3, 75, 76], male killing [23, 33] and feminizing of genetic males [30, 69]), it can be hypothesized that these bacteria have influenced on diversification and speciation of its hosts. Unfortunately, such studies are not easy to perform due to problems with identifying contact zones of species/phylogenetic lineages that harbor distinct strains or when only one species/phylogenetic lineage is infected. Consequently, there were only single studies dealing with infections in the contact zone. Example of such recent research might be work done by Zabal-Aguirre *et al.* [92, 93], which

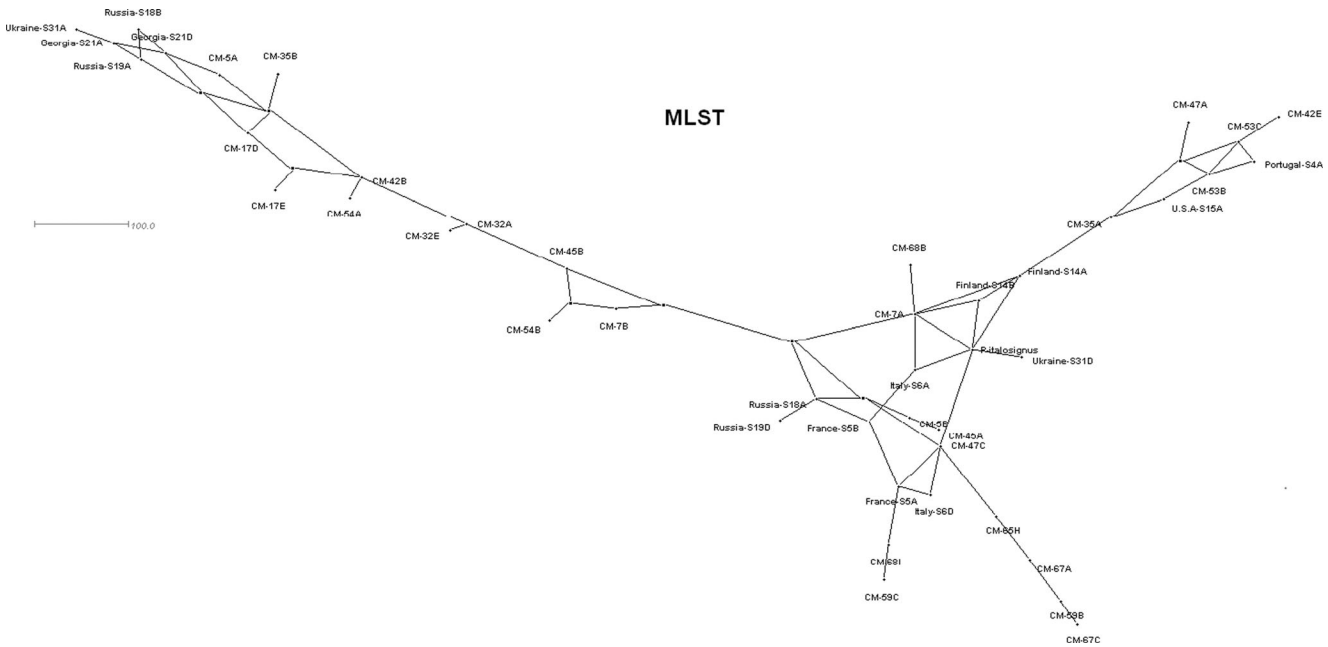

Fig. 3 Median-joining network of *Wolbachia* strains in *P. spumarius* obtained by SplitTree4 for joined MLST genes

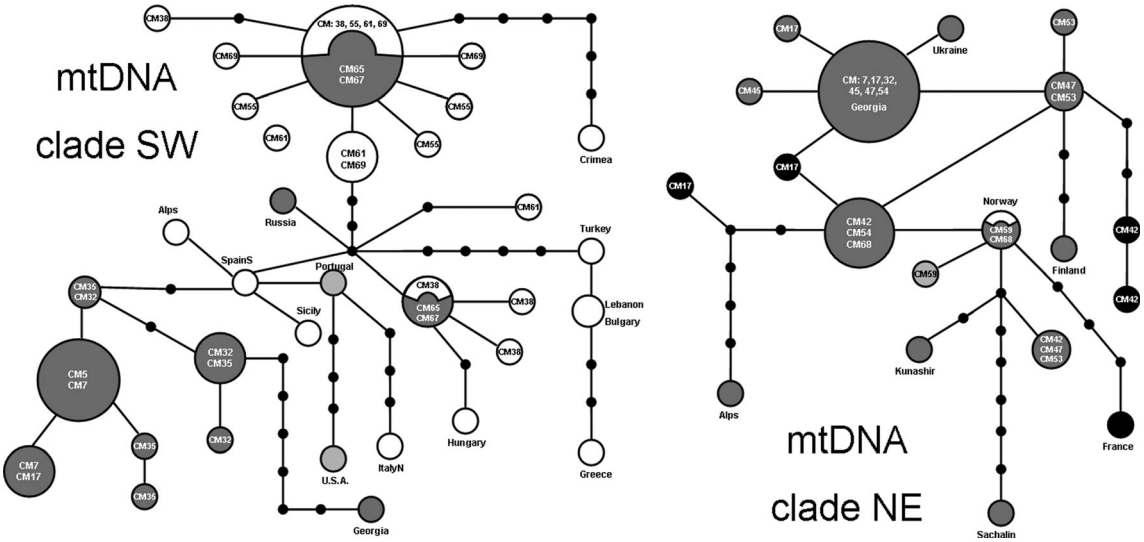

Fig. 4 Haplotype cytochrome B networks of *P. spumarius* and association of *Wolbachia* infections with haplotypes belonging to the NE and SW mitochondrial clades from the species range, including contact zone in the Carpathians (symbols of populations like in Table 1). *White circles*, not infected; *gray circles*, all population infected; *black circle*, some individuals infected of population

shows the genetic structure of *Wolbachia* infection in the Pyrenean contact zone of the widely investigated Meadow grasshopper *C. parallelus* subspecies: European *C. p. parallelus* and *C. p. erythropus*.

In this study, we discovered that approximately 41 % specimens of *P. spumarius* spittlebug across its entire range are *Wolbachia* positive, but the infection rate differs significantly between members of mitochondrial clades (c. 51 % infected in NE clades versus only c. 3 % in the SW clade) and in contact zone (c. 93 % from the NE clade and c. 46 % from the SW clade in this zone). These frequencies support the idea that *Wolbachia* is widespread in *P. spumarius* just like in numerous other arthropods [19, 29, 37, 64, 87], but that these bacteria are not distributed uniformly. Moreover, the higher frequency of infections in the contact zone compared with other parts of the species range, especially noticeable for the SW clade, indicates that *Wolbachia* could spread between members of both mitochondrial clades in areas where they meet. This implies that mitochondrial phylogenetic lineages of *P. spumarius* have not yet formed sufficient reproductive barriers, which has also recently been proven on the basis of population genetic studies [48]. It is probable that some environmental factors (basically, host plants that can mediate *Wolbachia* infection in phytophagous insect populations [12, 16, 85]), could be responsible for the observed pattern of these bacteria distribution and diversity in *P. spumarius* range. *P. spumarius* is a polyphagous species, but it is possible that its populations from NE and SW could feed on different plant species due to differences in vegetation between the temperate flora of lowland central and eastern Europe and north Asia compared with Mediterranean flora of southern Europe (where most *Philaenus* species feed exclusively on *Asphodelus*) [50]. The contact zone in the Carpathians simultaneously forms a potential primary contact zone for *Wolbachia* diffusion into the SW clade. Outside this zone, an almost strict division was observed: infection in most populations belonging to the NE clade and almost no infection in populations belonging to the SW clade.

The increased level of infection in *P. spumarius* NE clade and only isolated infections of these bacteria in the SW clade indicates that the ancestors of the lineage were presumably infected by *Wolbachia* after the split of its main mitochondrial lineages or during this split (which possibly has not finished

Table 3 Recombination analysis of MLST genes by PhiTest executing in SplitTree4 software

PhiTest						
Gene	coxA	gatB	hcpA	fbpa	ftsZ	MLST
Informative sites	66	50	60	56	4	238
Mean	0.032	0.041	0.001	0.092	0.333	0.083
Variance	2.204	5.516	1.006	9.790	0.037	1.703
Observed	0.021	0.025	0.0	0.030	0.333	0.022
P value	0.007093	0.01608	0.13	1.969E−10	0.5	0.0

yet). The distribution of *Wolbachia* could also be associated with the presence of some host plants in the *P. spumarius* range (see explanation above). The demographic and spatial expansion of this species may have occurred earlier than Holocene [55, 68]. Also, high genetic diversity detected in *P. spumarius* populations from northern Europe (Scandinavia) indicate that the north of Europe was colonized by populations that may have survived in several extra-Mediterranean glacial refugia in addition to the "classical" Mediterranean refuges [68]. That may explain non-infected populations from Norway; however, loss of infection in these populations cannot be ruled out. Also interesting are the non-infected populations from Crimea and southern Russia (belonging to distinct lineage within the SW clade), which apparently avoided infection despite close proximity to populations belonging to the infected NE clade. The possible source of the infection is directed to the eastern locations (possibly from Asia) and derived from there to the west, particularly to central Europe but not to northern and southeastern Europe. This infection wave has not invaded southern populations. However, single infections in southwestern *P. spumarius* populations from Portugal and probably descendent population in the USA (according to mitochondrial studies [55]) can be explained by accidental human translocations. Moreover, lack of infection in southern populations of *P. spumarius* is consistent with lack of infection in other *Philaenus* species that inhabit mostly Mediterranean regions. The single infection in *P. italosignus* might have come from feeding on plant phloem [18], which could have mediated *Wolbachia* transmission from *P. spumarius* or other infected insect.

It is probable that the observed pattern of *Wolbacha* infection in the contact zone may have caused cytoplasmic incompatibility between isolated populations from the northeast and the southwest. In this study, we have a few examples of populations where individuals from two main mitochondrial clades were in one population collectively, and infections were detected only in specimens from NE clades. That support of cytoplasmic incompatibility may have also occurred between infected and non-infected *P. spumarius* individuals. Therefore, this phenomenon may also play a role with allopatric speciation in *P. spumarius.* This phenomenon needs further studies, including mating of hosts belonging to distinct mitochondrial lineages and investigation of *Wolbacha* transmission to future generations. Moreover, this phenomenon should also be tested with respect to feeding preferences of *P. spumarius*, which are only broadly known as polyphagous species, but no detailed studies have been undertaken to verify differences in host plant composition across its range, e.g., with the use of host plant barcoding from insect guts (e.g., [42, 57]). *Wolbachia* could be transmitted by means of plant phloem, so this route of infection should also be considered in further studies on *P. spumarius.*

As was described in the "Results," different infection types show that *P. spumarius* were infected by more than one supergroup of *Wolbachia*. Moreover, different strains were detected in both B and A supergroups, which feature advanced states of infection of this microorganism and a high degree of recombination events. Supergroup B is a main group of *Wolbachia* in *P. spumarius* populations, but local infections of supergroup A in the southern Carpathians showed that infections is not homogeneous in all populations. That state can be explained by different sources of infections (e.g., from other insect species) that occurred only locally in southern Carpathians and have not spread to other populations of *P. spumarius.* This local distribution of supergroup A could additionally be explained by the cytoplasmic incompatibility of individuals harboring this supergroup with bugs infected (or not infected) by supergroup B. As we show in Table 2, *P. spumarius* would have received *Wolbachia* from other insect species. The main strain of these bacteria probably might come from wasps (*Vespidae*), flies (*Drosophilidae*), or hemipterans (*Aleyrodidae*), so infection could be transmitted either by predation (some injury, e.g., by wasp) or more probably by parasitoids, which may function as a vector for *Wolbachia* bacteria and transfer it to other arthropods [39, 60]. Instead of supergroup A in the local area in the Carpathians, beetles (Chrysomelidae, Curculionidae) were identified as the most presumable donor of *Wolbachia*.

In this study, we acknowledge our main hypothesis that *Wolbachia* infects in the majority the NE mitochondrial clade of *P. spumarius*. Our results pointed out single infections in SW clade, which could have been caused by man-made translocation of these bugs. Although, individuals from the Carpathians belonging to the SW clade harbored different supergroup of *Wolbachia*. There is very likely that between two main mitochondrial *P. spumarius* clades, we are dealing with cytoplasmic incompatibility. The presented results indicate that *Wolbachia* may play a significant role in the maintenance of the limited hybridization between genetically distinct populations of the *P. spumarius* in the contact zone via probable cytoplasmic incompatibility. This phenomenon in the Carpathians should be considered to probably exist in the other *P. spumarius* contact zones such as in the Alps, the Pyrenees and the Caucasus. We also verified the correlation of mitochondrial variability with *Wolbachia* infection through this contact zone. *Wolbachia* are associated with mtDNA, as both are maternally inherited within cytoplasm in reproductive tissue and can therefore result in a homogenization of mtDNA haplotypes (selective slippage [22, 72, 73]). Consequently, this would have implications for important evolutionary processes along with speciation. There was evidence presented in other research of *Nasonia* of reproductive isolation among species driven by *Wolbachia* [92]. Our results clearly show the distribution of *Wolbachia* in the *P. spumarius* range and reveal geographical patterns of

distribution of the bacterial strains that may also appear in other organisms. But our results do not represent complete evidence that *Wolbachia* acts as a barrier to reproduction in the contact zone of *P. spumarius* by inducting cytoplasmic incompatibility, although overall they give a view that is consistent with such a possibility.

Conclusions

This work presents the hypothesis that *Wolbachia* infection is associated with limited hybridization between genetically distinct populations of the *P. spumarius* and also with allopatric speciation in progress in the secondary contact zone in the Carpathians. Yet, further study is also required for (i) infections in other contact zones in the Alps, the Pyrenees, and the Caucasus, (ii) laboratory crossing of individuals from different populations (mtDNA and infection) to detect signs of cytoplasmic incompatibility and reinforcement of reproductive barriers, and (iii) artificial trials of infection of individuals from uninfected populations and removal of bacteria from infected populations to check influence of such experiments on host survival and reproduction. This data overall supports the hypothesis that *Wolbachia* infections are associated with only one *P. spumarius* clade. Overall, this suggests ongoing partial cytoplasmic incompatibility in the hybridization events among the *P. spumarius* clades in the Carpathians and an allopatric speciation in progress.

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