Received: 9 October 2022 | Accepted: 15 July 2023

DOI: 10.1111/1462-2920.16473

RESEARCH ARTICLE

ENVIRONMENTAL MICROBIOLOGY Applied Microbiology International

Till evolution do us part: The diversity of symbiotic associations across populations of *Philaenus* spittlebugs

Michał Kolasa[1,2] | **Łukasz Kajtoch**[2] | **Anna Michalik**[3] | **Anna Maryańska-Nadachowska**[2] | **Piotr Łukasik**[1]

[1]Institute of Environmental Sciences, Faculty of Biology, Jagiellonian University, Krakow, Poland

[2]Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Krakow, Poland

[3]Department of Developmental Biology and Morphology of Invertebrates, Institute of Zoology and Biomedical Research, Faculty of Biology, Jagiellonian University, Krakow, Poland

Correspondence
Michał Kolasa, Institute of Environmental Sciences, Faculty of Biology, Jagiellonian University, Krakow, Poland.
Email: michal.r.kolasa@uj.edu.pl and michal.r.kolasa@gmail.com

Funding information
Narodowa Agencja Wymiany Akademickiej, Grant/Award Number: PPN/PPO/2018/1/00015; Narodowe Centrum Nauki, Grant/Award Numbers: 2018/30/E/NZ8/00880, 2018/31/B/NZ8/01158

Abstract

Symbiotic bacteria have played crucial roles in the evolution of sap-feeding insects and can strongly affect host function. However, their diversity and distribution within species are not well understood; we do not know to what extent environmental factors or associations with other species may affect microbial community profiles. We addressed this question in *Philaenus* spittlebugs by surveying both insect and bacterial marker gene amplicons across multiple host populations. Host mitochondrial sequence data confirmed morphology-based identification of six species and revealed two divergent clades of *Philaenus spumarius*. All of them hosted the primary symbiont *Sulcia* that was almost always accompanied by *Sodalis*. Interestingly, populations and individuals often differed in the presence of *Sodalis* sequence variants, suggestive of intra-genome 16S rRNA variant polymorphism combined with rapid genome evolution and/or recent additional infections or replacements of the co-primary symbiont. The prevalence of facultative endosymbionts, including *Wolbachia*, *Rickettsia*, and *Spiroplasma*, varied among populations. Notably, *cytochrome I oxidase* (COI) amplicon data also showed that nearly a quarter of *P. spumarius* were infected by parasitoid flies (*Verralia aucta*). One of the *Wolbachia* operational taxonomic units (OTUs) was exclusively present in *Verralia*-parasitized specimens, suggestive of parasitoids as their source and highlighting the utility of host gene amplicon sequencing in microbiome studies.

INTRODUCTION

Symbiosis with microorganisms has been a critical force driving eukaryotic evolution. Through their effects, ranging from the biosynthesis of nutrients, through defence against pathogens, to the manipulation of reproduction (Feldhaar, 2011; Moran et al., 2008), symbiotic microorganisms have repeatedly enabled the emergence of significant insect clades feeding on specialized foods, as well as allowing for more dynamic responses to spatial heterogeneity, natural enemy pressure, and changing environmental conditions (Jaenike et al., 2010; Smith et al., 2021). Although our knowledge expanded significantly during the last two decades, we are still far from understanding the relationship dynamics between symbionts and their hosts.

When considering symbiotic interactions, it is crucial to consider their nature and stability. The recent classification of symbioses into closed, open, and mixed (Perreau & Moran, 2022) has created a helpful framework for such comparisons. Closed symbioses, usually characterized by their ancient origin, genome reduction of the symbiont, and its strict vertical transmission across host generations (Bennett & Moran, 2015),

include heritable nutritional symbionts of sap-feeding hemipterans that provide them with essential amino acids and other nutrients deficient in their unbalanced diet (Douglas, 2009, 2016; McCutcheon et al., 2009). Mixed symbioses are not fixed on evolutionary time scales: in addition to maternal transmission, these microbes are able to colonize new hosts and spread in populations rapidly thanks to their fitness effects, including protection against a wide range of natural enemies and abiotic stressors, but also the ability to manipulate host reproduction (Gerardo & Parker, 2014; Ju et al., 2019; Truitt et al., 2019). Finally, open symbioses, including diverse microbes hosted within digestive tracts and on the body surfaces of hosts, do not generally form long-term associations with hosts, but can still have important nutritional or defensive functions (Gerardo & Parker, 2014; Ju et al., 2019). Host clades and species differ in the relative contribution of these categories. However, even those primarily known for their closed symbioses can display substantial variability at evolutionary, but also shorter timescales.

For example, Auchenorrhyncha, a hemipteran suborder that dates back some 300 million years and includes planthoppers, leafhoppers, treehoppers, spittlebugs, and cicadas, associated ancestrally with a Bacteroidetes, *Candidatus* Sulcia muelleri (further referred to as *Sulcia*). Together with its co-symbionts, *Sulcia* produces essential amino acids and vitamins deficient in their hosts' diet of plant sap. However, both *Sulcia* and its co-symbionts have been repeatedly replaced, or sometimes complemented, by other bacteria or fungi in different host clades, resulting in a diversity of obligatory nutritional endosymbioses across extant Auchenorrhyncha. On top of that, these insects frequently associate with facultative endosymbionts ('mixed' symbioses) such as *Wolbachia* and *Rickettsia* and may be colonized by gut or surface bacteria. Surprisingly few studies have investigated spatio-temporal distribution of these symbiotic associations.

The primary focus of the present study is the characterization of the diversity and distribution of the microbiota of the meadow spittlebug *Philaenus spumarius* (Hemiptera: Aphrophoridae). This polyphagous xylem-feeding insect of Palearctic origin was recently introduced in North America, the Azores islands, Hawaii, and New Zealand (Rodrigues et al., 2014). As a vector of *Xylella fastidiosa*, a recently emerged and dangerous pathogen of crops including olives, grapevines, citrus trees, and coffee, *P. spumarius* has become an economically important species, especially in Europe (Godefroid et al., 2021; Saponari et al., 2014). Its microbiota, critical to spittlebugs' nutrition and likely affecting other functions, perhaps including interactions with vectored plant pathogens, have thus attracted researchers' attention.

Members of the Philaenini tribe of spittlebugs were found to be universally infected by *Sulcia* and *Sodalis* symbionts (Koga et al., 2013) which together produce 10 essential amino acids deficient in the xylem sap diet (Koga & Moran, 2014). Koga and Moran (2014) postulated the establishment of *Sodalis* as a nutritional symbiont in the ancestor of the whole *Philaenini* tribe. However, they comprehensively characterized only a single *P. spumarius* population, and their work was limited to a few samples. On the other hand, much attention has been paid recently to understanding the distribution and dynamics of the facultative symbionts such as *Wolbachia*, *Rickettsia*, and *Cardinium* across different geographic populations of the *Philaenus* group, especially in the Mediterranean region (Formisano et al., 2022; Kapantaidaki et al., 2021; Lis et al., 2015). Despite this, the picture of microbiota across the *Philaenus* diversity, including the stability of the association with nutritional symbionts, the distribution of facultative symbionts, or the presence of other bacterial categories, is far from complete. Novel methodological and conceptual developments can help consolidate our understanding of these interactions; however, numerous methodological caveats associated with tools such as 16S rRNA amplicon sequencing are not often systematically addressed (Knight et al., 2018).

For example, wild insect microbiome studies tend to neglect issues such as molecular confirmation of the host identity or the presence of parasites and parasitoids due to usually focusing on small number of species (e.g., Paddock et al., 2022; Tinker & Ottesen, 2021). A completely different problem is reagent- and cross-contamination, which can be a major problem, especially in samples with low bacterial load (Salter et al., 2014), and is hard to tackle without negative controls for different laboratory steps. However, we can largely avoid these issues by amplifying host marker genes alongside those of microbes, using negative controls and well-thought bioinformatic pipelines (Knight et al., 2018).

Here, we aim to characterize the diversity and distribution of symbiotic bacteria in six species belonging to the genus *Philaenus*, with a strong emphasis on different populations of *P. spumarius*. We use insect and symbiont marker gene amplicon sequencing to address questions about the stability of obligatory nutritional 'closed' symbioses and the diversity and distribution of facultative endosymbionts across species and populations. We discuss the symbiont diversity and distribution patterns, with emphasis on the roles of parasitoids and the process of symbiont replacement.

EXPERIMENTAL PROCEDURES

Sampling

Philaenus species were collected during field trips across Europe, the Middle- and the Far East between 2000 and 2011 (Figure 1, Table S1). Specimens were preserved in 96% ethanol immediately after collection

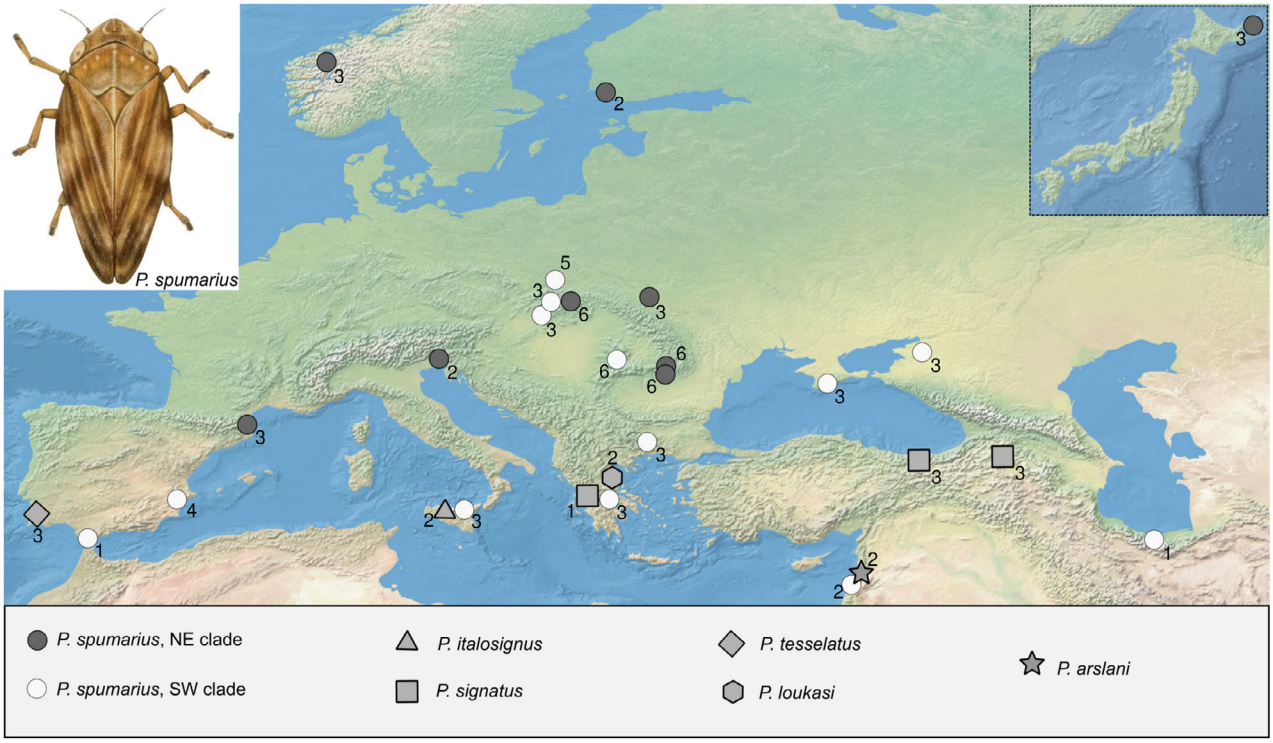

FIGURE 1 The sampling sites of specimens from the *Philaenus* group classified into species based on mitochondrial sequences. Circles—*P. spumarius*, triangle—*P. italosignus*, rectangle—*P. signatus*, diamond—*P. tesselatus*, hexagon—*P. loukasi*, five-pointed star—*P. arslani*. The box in the upper right corner shows the sampling site of *P. spumarius* on Kunashir Island, Japan. Colours indicate mitochondrial clades of *P. spumarius*: white—NE, dark grey—SW. Digits indicate the number of samples used for this study from each population/species.

and stored at −20°C until processing. Morphology-based identifications by expert taxonomists revealed eight species: *P. spumarius* (22 populations/72 specimens), *P. signatus* (3 populations/7 specimens), and a single population of other species: *P. tesselatus* (3 specimens), *P. arslani* (2 specimens), *P. italosignus* (2 specimens), *P. loukasi* (2 specimens), *P. maghresignus* (1 specimen), and *P. tarifa* (1 specimen). In total, 90 specimens from eight species were chosen for amplicon-based characterization. However, as explained later, molecular barcodes, which we decided to use as the primary means to determine species, did not support some of these identifications.

Host diversity and microbiome screening

Library preparation and sequencing

We characterized the selected insects using a custom two-marker-gene amplicon sequencing approach. We simultaneously targeted the insect *mitochondrial cytochrome I oxidase (COI)* gene, allowing for the confirmation of the host identity, and the *V4 hypervariable region of the bacterial 16S rRNA* gene, providing a picture of its microbiota.

DNA was extracted from whole insects using the Nucleospin Tissue kit (Macherey-Nagel), following the manufacturer's instructions. The extraction was conducted in two batches: some samples were processed back in 2009 and others in December 2019 (Table S1). Samples and negative controls (DNA extraction control, as well as molecular-grade water as a PCR control) for both batches were used for amplicon library preparation following a modified two-step PCR library preparation approach as outlined by Glenn (2011) (method 4). In the first step, two marker regions of interest were amplified using template-specific primers 515F/806R (Apprill et al., 2015; Parada et al., 2016) and COIBF3/COIBR2 (Elbrecht et al., 2019) with Illumina adapter tails. The PCR products were purified using SPRI magnetic beads and used as templates for the second indexing PCR reaction. Pooled libraries were sequenced on an Illumina MiSeq v3 lane (2 × 300 bp reads) at the Institute of Environmental Sciences of Jagiellonian University. The primer sequences and detailed protocols for amplicon library preparation are provided in Table S2.

Analyses of amplicon sequencing data

We processed 16S rRNA and COI amplicon data using a custom pipeline based on USEARCH/VSEARCH (available and described in detail at https://github.com/Symbiosis-JU/Philaenus-Microbiota-Project). Aware of the limitations of Illumina sequencing, including sequencing errors, chimera formation and cross-contamination

among samples (Kircher et al., 2011), we have invested much effort in the development and optimization of workflows that mitigate these challenges. Initially, all amplicon datasets were split into bins corresponding to the two target genes based on primer sequences. Using PEAR, we assembled quality-filtered forward and reverse reads for both bins into contigs (Zhang et al., 2014). Next, contigs were de-replicated (Rognes et al., 2016) and denoised (Edgar, 2016); this was done separately for every library to avoid losing information about rare genotypes that could happen during the denoising of the whole sequence set at once (Prodan et al., 2020). The sequences were then screened for chimaeras using USEARCH and then classified by taxonomy using the SINTAX algorithm and customized databases: SILVA for bacterial 16S rRNA (version 138 SSU) (Quast et al., 2013) and MIDORI (version GB 239) (Leray et al., 2018) custom-screened for misclassified *Wolbachia* sequences, for COI. Finally, the sequences were clustered at a 97% identity level using the UPARSE-operational taxonomic unit (OTU) algorithm implemented in USEARCH. The product of our custom pipeline were tables with two levels of classification: ASVs (Amplicon Sequencing Variant) (also known as zOTUs—zero-radius Operational Taxonomic Units) describing genotypic diversity and OTUs (Operational Taxonomic Unit)—clustering genotypes based on a similarity threshold. ASVs have been used in the past for the comparison of the microbiota of different bees based on ASVs or 99.5% OTUs (e.g., Amiri et al., 2023; Kwong et al., 2017). Also, a similar approach was implemented by using the information on genotype distribution within OTUs to support some of the conclusions about the biology of ant symbionts (Łukasik et al., 2017). The resulting tables were then used for a series of custom steps. Bacterial 16S rRNA gene data were screened for putative DNA extraction and PCR reagent contaminants using negative controls (blanks) for DNA extraction and PCR steps as a reference. We used a custom python script that based on taxonomic assignment recognizes and filters out OTUs assigned as mitochondria, chloroplast, Eukaryote or Archaea. COI data were screened for bacterial and parasite reads using custom references. Specimens with a low number of COI or 16S V4 reads after decontamination (not included in the counts above) were excluded from further analysis. The minimum spanning network for COI haplotypes of *Philaenus spumarius* was generated using PopArt (Leigh & Bryant, 2015). All statistical analyses were run using SAS 9.4 version (SAS Institute, 2013).

Microscopic analyses of symbiont morphology and localization

Light and transmission electron microscopy

Abdomens of *P. spumarius* females collected in Krakow (Poland) were fixed and stored in 2.5% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4) at 4°C for 3 months and, after this time, washed with the same buffer with the addition of sucrose (5.8%). Next, samples were post-fixed in a 1% solution of osmium tetroxide, dehydrated in ethanol and acetone series, and embedded in epoxy resin Epon 812 (SERVA, Heidelberg, Germany). The resin blocks were cut into serial, semithin sections for histological analyses, stained in 1% methylene blue in 1% borax, and observed under the Nikon Eclipse 80i light microscope. Ultrathin sections for ultrastructural analyses were contrasted with lead citrate and uranyl acetate and observed under the JEOL JEM 2100 electron transmission microscope.

Fluorescence microscopy

For fluorescence in situ hybridization (FISH), ethanol-preserved specimens of *P. spumarius* (from Spain) were rehydrated, postfixed in 4% paraformaldehyde for 2 h, and then dehydrated again through incubation in increasing concentration of ethanol and acetone. The samples were then embedded in Technovit 8100 resin (Kulzer, Wehrheim, Germany) and cut into semithin sections. Hybridization using *Sulcia*- and *Sodalis*-targeting probes labelled with Cy3 and Cy5 fluorochromes (sequences in Table S2) was performed overnight at room temperature. Slides were washed three times in PBS solution, dried, covered with ProLong Gold Antifade Reagent (Life Technologies), and examined using a confocal laser scanning microscope Zeiss Axio Observer LSM 710.

RESULTS

COI amplicon data permit validation of species identity and reveal parasitoid infections

After all analysis steps, the total number of COI reads taxonomically assigned to Auchenorrhyncha was 570,638, or 6341 per sample on average. We identified 34 amplicon sequence variants (ASVs) that clustered to six OTUs with a 97% identity cutoff. The vast majority of examined specimens were characterized by a dominant COI genotype confidently classified as representing the genus *Philaenus*. Out of 90 libraries, in 71, the dominant sequence variant from that OTU represented over 95% of total COI reads; in further eight, it represented 90%–95% of reads and in the remaining 11, >70%. Widespread secondary ASVs with the same species-level assignments were found in *P. spumarius* (2 ASVs) and *P. signatus* (one ASV). They were much less abundant and their relative abundances were similar in different specimens, suggestive of them representing nuclear pseudogenes, or numts (Dong et al., 2021) (Figure 2, Table S3).

Analyses of COI marker gene sequences for the experimental specimens confirmed their morphology-based identifications in most cases. We decided to retain the morphology-based delimitation of *P. tesselatus* and *P. spumarius*, previously shown to share the same COI haplotype (Maryańska-Nadachowska et al., 2012) (Figure 2A). However, specimens labelled as *P. maghresignus* and *P. tarifa* were indistinguishable from *P. spumarius* based on their COI gene fragment (despite previously being shown to be distinct); therefore, we classified them as *P. spumarius*. We later verified that their microbiota did not depart from those of *P. spumarius* from the same or nearby sites.

The final dataset contains 74 specimens assigned as *P. spumarius*, seven as *P. signatus*, three as *P. tesselatus*, and two specimens per species as *P. arslani*, *P. loukasi* and *P. italosignus*. The haplotype

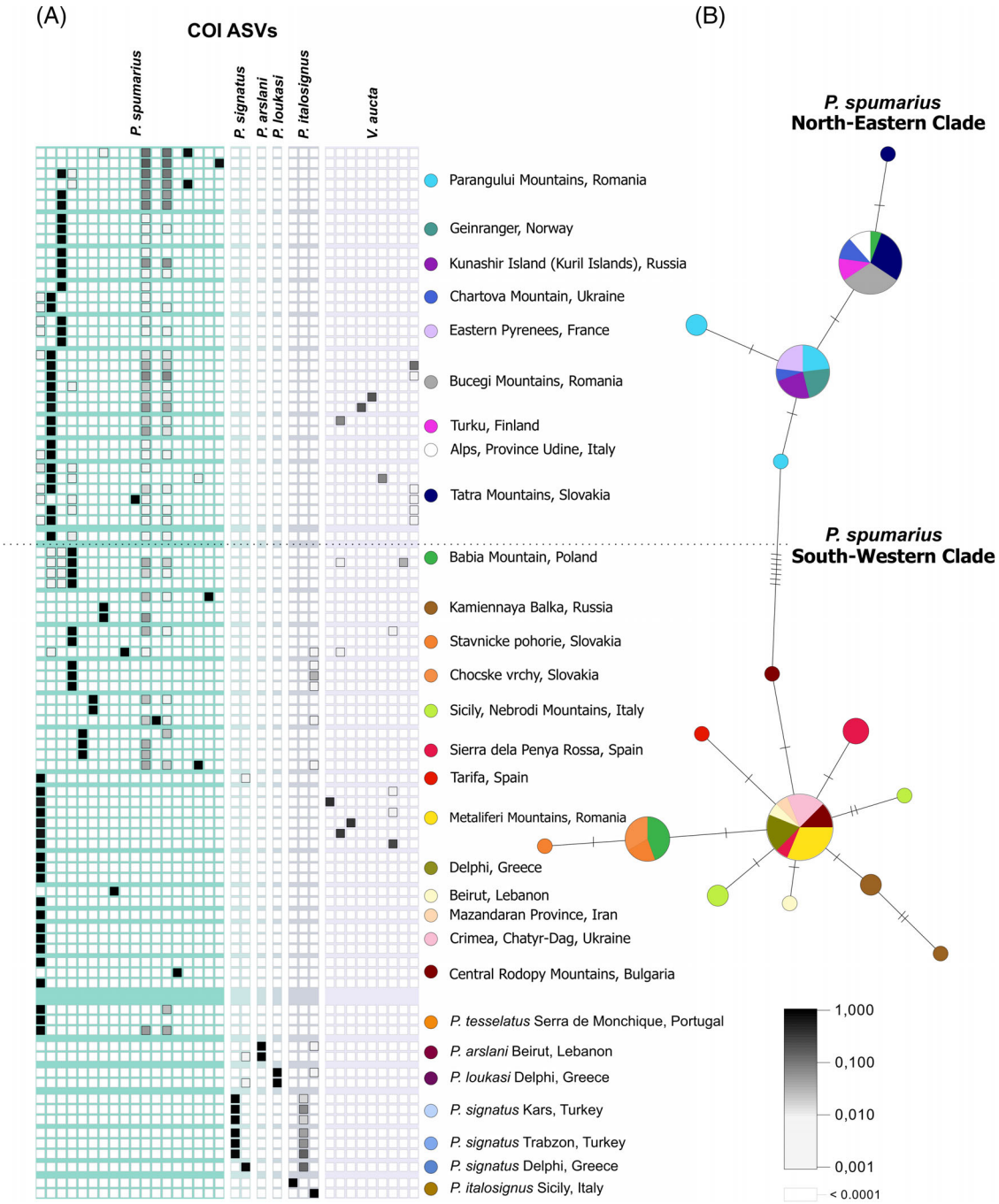

FIGURE 2 (A) Distribution of cytochrome oxidase subunit I (COI) genotypes (ASVs) among the studied species. (B) Minimum spanning network of COI haplotypes of *P. spumarius*. The dashed line indicates the separation between NE clade and SW clade of *P. spumarius*. Coloured circles indicate populations from which samples were collected and congruent with the haplotype network colours.

networks revealed two geographically disjunct clades of *P. spumarius* (Figure 2B) with hybridization zones in the Carpathian arc, in agreement with previous studies (Lis et al., 2014).

In a subset of specimens, including all individuals of *P. signatus*, we also discovered COI ASVs corresponding to other species of *Philaenus* than the dominant genotype—specifically, to *P. italosignus*. In all but one case, their cumulative relative abundance did not exceed 1%. In *P. signatus*, the sequence is different from those present in studied *P. italosignus*, suggestive of being a numt (Song et al., 2008), but in other cases, it matches the barcode of one *P. italosignus* specimen. We verified that there was no apparent cross-contamination in bacterial 16S rRNA amplicon data generated in the same PCR reactions and conclude that the observed cross-specific signal is likely a biological phenomenon (Figure 2A).

Interestingly, in 19 samples of *P. spumarius*, in addition to the spittlebug signal, we found one of nine ASVs belonging to a single 97% OTU identified as representing the parasitoid fly *Verralia aucta* (Diptera: Pipunculidae). In nine of those samples, parasitoid reads exceeded 1% of the total.

Clear patterns in the distribution of bacterial genera

The comparison of 16S rRNA gene V4 region data for spittlebugs against PCR and extraction blanks showed that, on average, 99.83% of reads in the library represented actual insect-associated microbes rather than contaminants (Table S5). The highest contamination level observed in the sample was 3.7%. ASVs classified as representing reagents or laboratory contaminants—that is, those that were not substantially more abundant in experimental samples than in blanks—were removed.

The total number of 16S rRNA reads after all analysis steps were 1,488,428, or 16,538 per sample on average (range 1098–47,801). Within these data, we identified 217 ASVs (zOTUs) clustered to 89 OTUs with a 97% identity cutoff and exceeding at least 1% relative abundance in the any of the libraries. The dominant microbial OTUs represented taxa previously reported from *P. spumarius* or other insects. All studied *Philaenus* individuals hosted *Sulcia*, and 88 out of 90 hosted *Sodalis*. Together, these obligate symbionts comprised 78.9% of all bacterial reads in a library on average. Other bacteria known as insect-associated were present in some specimens. We detected *Wolbachia* in 46.6%, *Rickettsia* in 15.5%, *Spiroplasma* in 6.6% and *Pectobacterium* in 15.5% of individuals. Together, these six clades comprised 96.5% of reads in a library on average. Other, less prevalent and abundant microbes, including the genus *Pseudomonas* and the families Oxalobacteraceae and Rhizobiaceae, represent taxa reported from a variety of environments, including insects.

Pectobacterium, an enterobacterial symbiont known to colonize various invertebrates as a nutritional heritable mutualist (Martinson et al., 2020), was found in a small fraction of *P. spumarius* individuals from several locations. In most cases, it accompanied *Sodalis*. Noteworthy, the single characterized specimen from Iran hosted *Pectobacterium* but not *Sodalis*, hinting at a possibility of a replacement of the ancestral obligatory nutritional mutualist. The low abundance of *Sodalis* but high abundance of *Pectobacterium* in one of *P. spumarius* individuals from Kamiennaya Balka, Russia and one of *P. italosignus* from Sicily, could potentially indicate a similar ongoing process (Figure 3A). Similarly, *Sodalis* was absent from a single Greek specimen of *P. signatus*, which in turn hosts abundant *Rickettsia*, an alphaproteobacterium that may contribute to the host nutrition in some cases (Driscoll et al., 2017). Interestingly, *Rickettsia* was abundant in almost all populations of *Philaenus* species other than *P. spumarius*, but in this dominant species, only a single individual from Spain was infected. In contrast, *Spiroplasma* was found only in *P. spumarius* and only in the South-Western mitochondrial clade, with high relative abundance (>10%) in the six infected specimens.

In our dataset, we found two OTUs of *Wolbachia*, one of which was much more prevalent. This dominant OTU showed a specific geographic pattern in *P. spumarius*, where in the North-Eastern clade it infected 25 out of 35 specimens (65.7%), but in the South-Western clade, only nine examined specimens out of 39 (23%) (as positive, we conservatively scored individuals where *Wolbachia* exceeded 1% relative abundance, thus, discarding possible false-positives resulting from cross-contamination). *Wolbachia* infection was significantly more common in the North-Eastern clade (GLM, $F_{1,20.01} = 9.25$, $p = 0.0064$). We also detected *Wolbachia* in one specimen of *P. signatus* and two of *P. italosignus* (Table S4). A particularly interesting case was the second *Wolbachia* OTU, much less widespread or abundant when present. In all specimens, hosting this OTU we identified COI reads representing the parasitoid fly *Verralia aucta*, strongly suggesting that this less abundant *Wolbachia* OTU is of parasitoid origin (Figure 3A). Specifically, 5 out of 10 parasitoid-positive specimens in the North-Eastern clade and 3 out of 9 in the South-Western clade contained reads of the second *Wolbachia* OTU.

The remaining bacteria in our dataset show less clear patterns. Two OTUs assigned to the family Morganellaceae were present at low abundances in two specimens of *P. spumarius* from Crimea. Surprisingly, we found reads assigned to *Buchnera* in five specimens of *P. spumarius* from three localities and one *P. tesselatus* from Portugal, verifying the sequence

identity to the obligatory nutritional endosymbiont of *Aphis fabae* through BLAST searches against the NCBI nt database. However, with its scattered distribution and the maximum abundance in any library not exceeding 2.5%, we conclude that this is environmental contamination. Other OTUs were generally both relatively rare and low-abundance when present (maximum abundance in any of the libraries <5%) and often

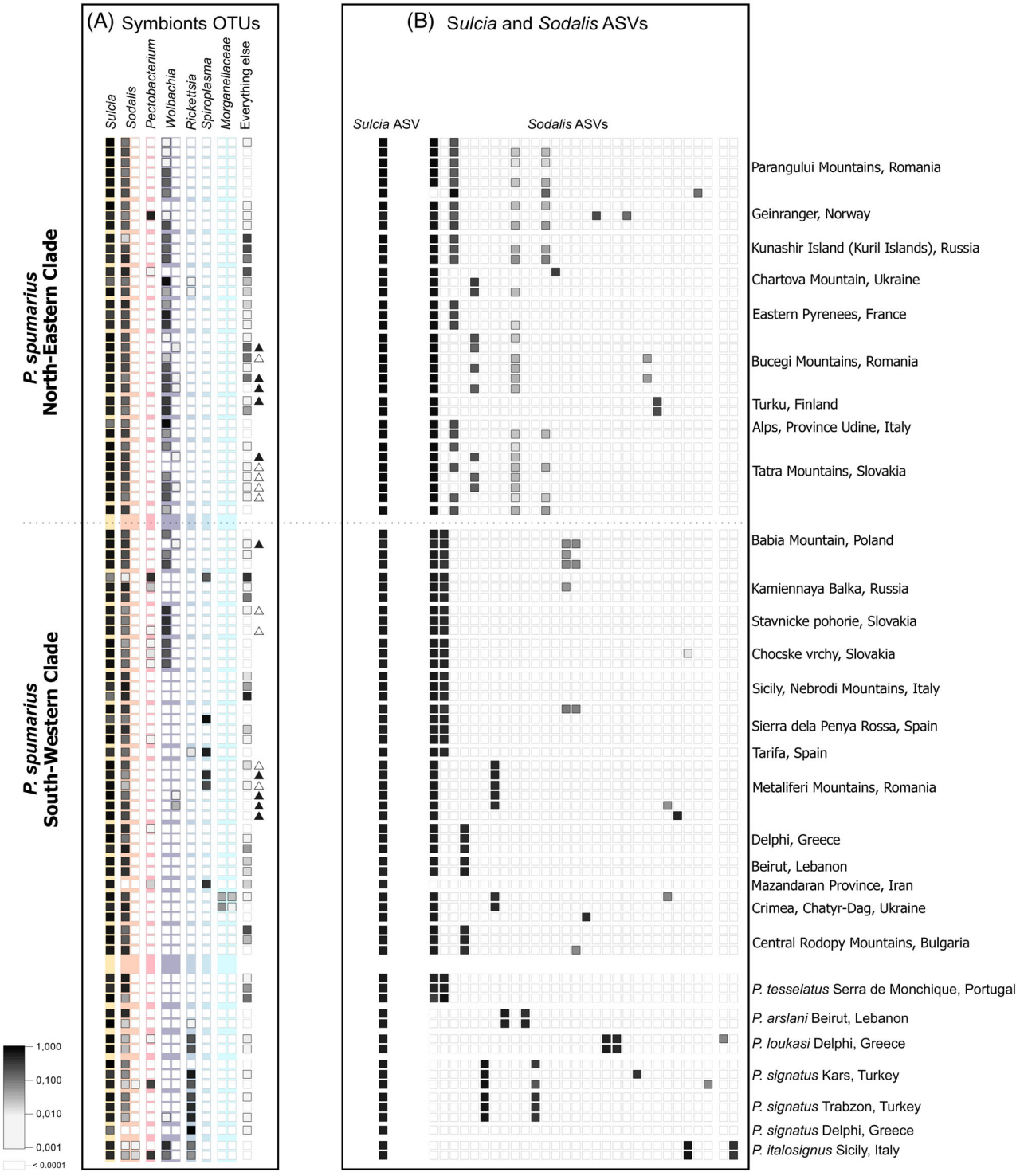

FIGURE 3 The distribution of dominant *Philaenus* symbionts across experimental insect populations and species. (A) The relative abundance of dominant bacterial OTUs in insect bacterial communities. Triangles next to the heatmap rows indicate the presence of *V. aucta* COI reads in the library: black ones relative abundance >1%, white ones relative abundance <1%. (B) The relative abundance of dominant ASVs within the two most abundant symbiont OTUs. The shade of grey represents the relative abundance of the given ASV in the *Sulcia* or *Sodalis* OTU, respectively.

assigned to taxa known to be of environmental origins, including *Pseudomonas*, *Sphingomonas*, *Staphylococcus* or members of Rhizobiaceae family. It is possible that these bacteria colonize insect digestive tracts or other body habitats and can sometimes play endosymbionts' reads. At the same time, we did not detect any known pathogens of plants spread by *Philaenus* spittlebugs such as *Phytoplasma* or *Xylella* (Maejima et al., 2014; Sicard et al., 2018).

Genotype-resolution 16S rRNA data highlight host–symbiont interaction dynamics

The single-nucleotide-resolution data for these dominant symbionts revealed that all species and individuals harbour the same ASV of the slow-evolving symbiont *Sulcia* (Figure 3B). Other symbionts were more variable.

In the case of *Sodalis*, across all individuals, we identified 33 ASVs, of which 12 represented at least 5% of all *Sodalis* reads in at least one sample and were inspected more closely. We found that all specimens contained between two and four of these more abundant variants of *Sodalis* 16S rRNA gene. Specifically, *P. spumarius* individuals contained a shared abundant ASV, accompanied by others that varied among individuals. Generally, most specimens from the same population hosted identical *Sodalis* ASV combinations, but in many populations, there were individuals with different combinations (Figure 3). *Sodalis* genomes have been shown to contain multiple rRNA operons that vary in nucleotide sequences (Koga et al., 2013), with the fully sequenced *Sodalis praecaptivus* (genome GB ref: PRJNA199998) containing seven distinct 16S-V4 sequence variants. This suggested that these ASV combinations may often point at different operons in a single genome rather than at different, co-infecting lineages of *Sodalis*.

Also, in multiple cases, some individuals hosted additional ASVs besides the combination found in other individuals from the same population (Figure 3B).

In specimens of *P. tesselatus*, the Portuguese species unidentifiable from *P. spumarius* at the COI gene, we observed identical *Sodalis* sequence variants as in *P. spumarius* from Spain (Figure 3B). However, all remaining species in our collection were characterized by clearly different *Sodalis* ASV sets than *P. spumarius*, differing also among each other.

In the case of *Philaenus*-specific *Wolbachia* OTUs, we observed between one and five ASVs per individual. The ASV combinations frequently vary among individuals within a population, some ASVs are unique to certain populations, and a single infected *P. signatus* from Turkey harbours a *Wolbachia* genotype absent anywhere else (Figure 4). These data suggest

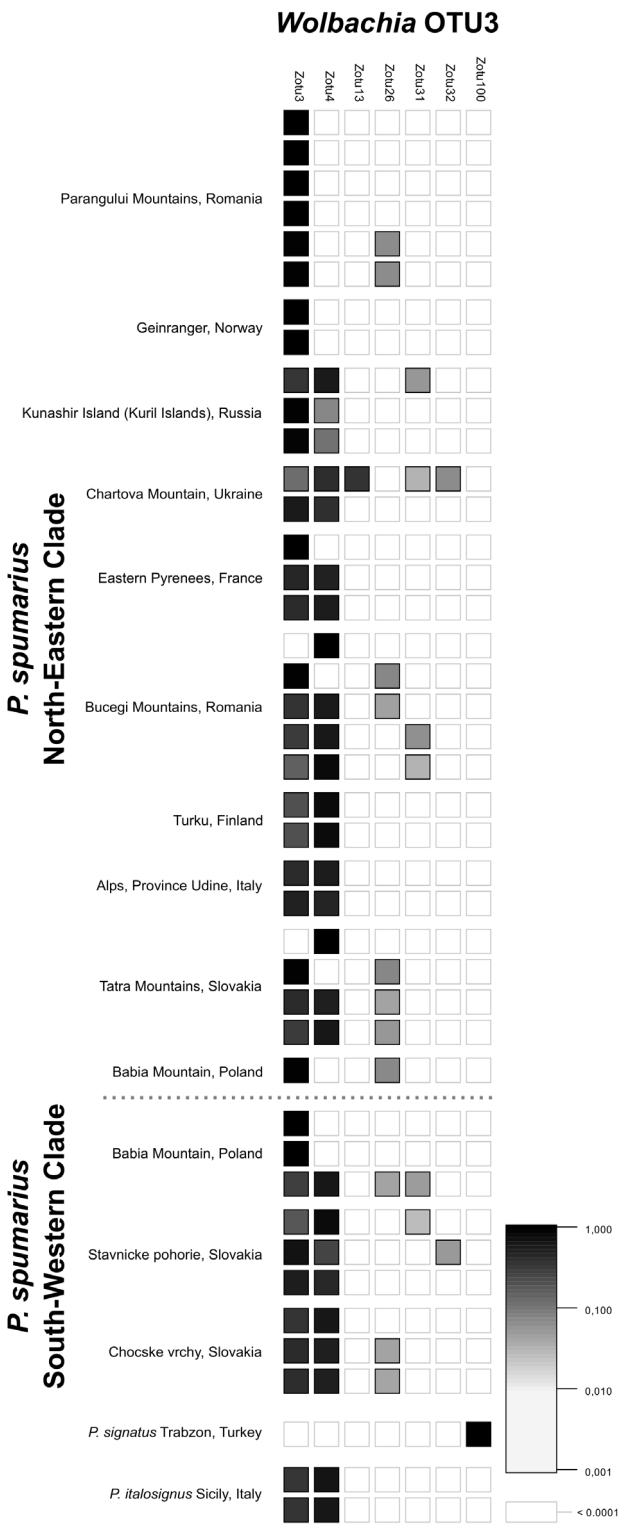

FIGURE 4 Distribution of *Wolbachia* OTU3 genotypes (ASVs) among *Philaenus* infected specimens. *Wolbachia* OTU25 of possible parasitoid origin is not shown here.

Wolbachia infection dynamics and polymorphism within and across host populations.

We also observed variation in ASV-level associations with *Spiroplasma* (Figure S1) and *Rickettsia*

(Figure S2). In the case of both symbionts, the majority of specimens hosted the same combination of two genotypes (*Spiroplasma*) or a single symbiont genotype (*Rickettsia*); however, for both these microbes, we identified specimens with much higher sequence diversity—four and five ASVs, respectively.

Obligate symbionts of *P. spumarius* show conserved bacteriocyte-limited location

Histological and ultrastructural observations revealed that obligate symbionts of the spittlebug *P. spumarius* collected from the Polish population reside in the cytoplasm of dedicated insect cells - bacteriocytes (Figure 5). Bacteriocytes harbouring *Sulcia* are mononucleated and create large bacteriomes surrounded by a thick monolayered bacteriome sheath (Figure 5A–C). The cytoplasm of these bacteriocytes is tightly packed with large, pleomorphic *Sulcia* cells (Figure 5A–C). Additionally, in the cytoplasm of some *Sulcia* bacteriocytes, we observed tiny bacteria, which may represent *Wolbachia*, common in the northern clade of the species (Figure 5D). *Sodalis* symbiont cells are spherical and localized in the cytoplasm of separate large bacteriocytes. However, compared to bacteriocytes with

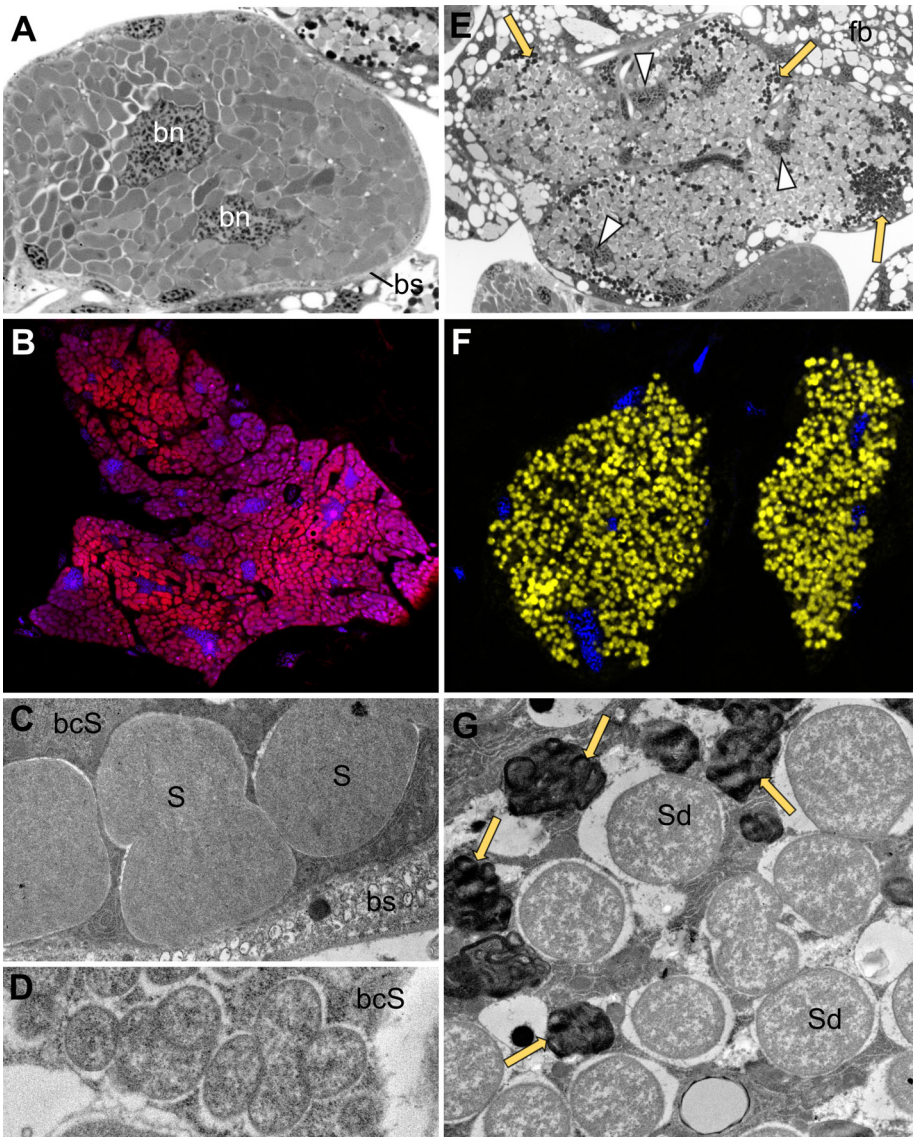

FIGURE 5 Localization and morphology of symbionts in a population of *P. spumarius* from Kraków, Poland. (A) Organization of the bacteriome with *Sulcia* symbiont, bn, bacteriocyte nucleus; bs, bacteriome sheath; LM, scale bar = 10 μm. (B) FISH detection of *Sulcia* symbiont (red); blue represents DAPI staining, FM, scale bar = 10 μm. (C) Ultrastructure of *Sulcia* (S) cells. bcS, bacteriocyte with *Sulcia* symbiont; bs, bacteriome sheath; TEM, scale bar = 1 μm (D) Putative *Wolbachia* cells in the cytoplasm of the bacteriocyte harbouring *Sulcia* (bcS), TEM, scale bar = 1 μm. (E) Tissue localization of *Sodalis* symbiont, arrowhead—nucleus, yellow arrow—lamellar structures, scale bar = 10 μm. (F) *Sodalis* (yellow) visualization using FISH, blue represents DAPI staining, FM, scale bar = 10 μm. (G) Ultrastructure of *Sodalis* (Sd) symbiont, yellow arrow-degenerated *Sodalis* cells, TEM, scale bar = 1 μm.

Sulcia, they are multi-nucleated and do not form compact bacteriomes. Bacteriocytes with *Sodalis* are grouped into larger clusters, but some are separated by fat body cells (Figure 5E,F). Ultrastructural analyses have shown numerous lamellar structures in the cytoplasm of each bacteriocyte, which we interpret as *Sodalis* cells undergoing degeneration (Figure 5G). Our observations correspond well with previous microscopy-based studies of *P. spumarius* symbionts conducted by Buchner (1965) and Koga et al. (2013). Although, despite looking at tissues of over 10 individuals, we could not observe additional symbionts in the studied specimens. Unfortunately, because of the limited material availability, we were unable to conduct microscopy work on any of the populations that we identified as potentially undergoing the symbiont replacement.

DISCUSSION

COI amplicon data as the essential framework for the microbiota characterization across diverse wild insects

The COI amplicon-based characterization of eight morphologically delimited species confirmed the identifications of five (Maryańska-Nadachowska et al., 2010). The sixth species, *P. tesselatus*, closely related to *P. spumarius* and distinguishable by the morphology of male genitalia, is known not to be indistinguishable at mitochondrial markers (Maryańska-Nadachowska et al., 2012; Rodrigues et al., 2014; Seabra et al., 2021). The mismatch between morphological and COI-based identification of two other species (*P. tarifa* and *P. maghresignus*), both previously found to be clearly distinct at mitochondrial markers from *P. spumarius*, (Maryańska-Nadachowska et al., 2010), could point to biological processes, such as introgression or laboratory problems such as misidentification or specimen confusion. Nevertheless, their microbial communities are indistinguishable from those of unambiguously identified *P. spumarius*, supporting our decision to reclassify them as this species.

In *P. spumarius*, COI amplicon data provided additional information on the genetic structure below the species level. Our patterns agreed with the prior identifications of two major clades: the South-Western clade ranging from Western Europe, through the Mediterranean, to the Middle East, and the North-Eastern clade ranging from Eastern Asia to Central and Northern Europe (Maryańska-Nadachowska et al., 2012; Rodrigues et al., 2014; Seabra et al., 2021) with the contact zones in the Carpathians, the Alps, the Pyrenees, and the Caucasus (Lis et al., 2014; Maryańska-Nadachowska et al., 2015). These clades have been known to vary in *Wolbachia* infection prevalence (Lis et al., 2015), and this study revealed further genetic differences across the sampled geographic regions. However, using only a relatively short (418 bp) mitochondrial gene fragment we were unable to capture a subdivision of the southeastern clade (Rodrigues et al., 2014).

We argue that in the future, microbiome surveys in species of broad interest such as *P. spumarius*, co-sequencing host marker genes, combined with genomics datasets for some specimens (Maryańska-Nadachowska et al., 2012; Seabra et al., 2021), will be essential for the reconstruction of the symbiont distribution, transmission, co-diversification and replacement patterns, as well as description of their biological roles.

Of particular interest here was the signal of the specialized dipteran parasitoid, *V. aucta*, in 24.7% of *P. spumarius* individuals studied. This ratio is comparable to the 17.5% infection prevalence estimates for *P. spumarius* populations from northern Italy (Molinatto et al., 2020), based on diagnostic PCRs. Then, the COI amplicon sequencing-based screens of individual insects, besides confirming their identity, can provide reliable information on some of their biotic associations. Information on the presence and distribution of parasitoid haplotypes within and across *P. spumarius* populations serves as a foundation for a more comprehensive investigation into host-parasitoid-symbiont interaction dynamics.

Not-so-stable nutritional endosymbioses in *Philaenus* spp.

Sulcia presence in all studied individuals, and its identity at the targeted rRNA region across all individuals, confirmed its status as a stable nutritional symbiont of spittlebugs (Koga & Moran, 2014). The observation of the lack of *Sulcia* genetic variation in our collection is consistent with its stability across other Cicadomorpha (McCutcheon et al., 2009). Importantly, observing only one genotype of *Sulcia* in all studied individuals also serves as a strong indication that the diversity of other symbionts at the ASV level, discussed below, is not an artefact resulting from PCR or sequencing errors, and represents true biological patterns.

The relationship of the symbiont *Sodalis* with *Philaenus* spittlebugs appears less stable than *Sulcia*. It was almost universally prevalent and highly abundant in most individuals, but there were exceptions. Specifically, in two individuals, it seems to have been replaced by other bacteria: either *Pectobacterium* or *Rickettsia*. In other individuals, it was present at low abundance, much lower than that of *Pectobacterium*, *Rickettsia* or *Wolbachia*, suggestive of complementation or perhaps an ongoing symbiont replacement process. *Symbiopectobacterium* has recently been identified as a

versatile microbial clade that has established heritable mutualistic relationships with multiple invertebrate taxa, similar to *Sodalis* (Martinson et al., 2020). For example, Nadal-Jimenez et al. (2022) demonstrated that a *Pectobacterium* strain identical within the 16S-V4 region to one of *Philaenus* associates is an apparent nutritional endosymbiont in a leafhopper *Empoasca decipiens*, encoding pathways for the biosynthesis of various vitamins and amino acids. Likewise, *Pectobacterium* has been pointed out as a potential nutritional symbiont in the parasitic nematode *Howardula aoronymphium* (Martinson et al., 2020). It seems plausible that in the Iranian *P. spumarius* lineage, *Pectobacterium* has taken over its nutritional responsibilities and replaced *Sodalis*. It would be interesting to investigate more systematically whether this and other *Pectobacterium* infections in our dataset may represent stages of stable associations, cases of vectoring plant pathogens similar to *P. carotovorum* (Mansfield et al., 2012), pathogenic infections, or perhaps sporadic, temporary infections with environmentally sourced bacteria.

Interestingly, another case of potential *Sodalis* loss is a specimen of *P. signatus* from Greece, where *Sulcia* is accompanied by abundant *Rickettsia*. This is surprising as there is no direct evidence that *Rickettsia* can be an obligatory nutritional symbiont in sap-feeding insects, despite the presence of genomic evidence that in some blood-feeders, they may contribute vitamins (Hunter et al., 2015). Although there is no particular *Rickettsia* clade correlated with feeding habits, phloem-feeding insects have been considered as an infection hotspot for this microbe (Pilgrim et al., 2021).

Recent genomics-enabled data from mealybugs and planthoppers suggest relatively high turnover rates of gammaproteobacterial symbionts such as *Sodalis* during the host evolution (Husnik & McCutcheon, 2016; McCutcheon et al., 2019; Michalik et al., 2021). Our data suggest substantial rRNA sequence variation among, but also within populations of *P. spumarius*, as well as within individuals. *Sodalis* has been shown to have several rRNA operons in its genome, with sequence differences among rRNA copies, which would translate to the presence of several distinct sequence variants per individual. The conservative analytical procedure that we used, including the data denoising step (Edgar, 2016) and the application of a high within-individual relative abundance threshold until a given sequence variant was scored as 'true', should have eliminated sequencing error-derived artefacts. Patterns such as the usual presence of the same set of variants in replicate individuals from a population, and differences across populations, especially between N/S clades or *Philaenus* species, are consistent with biological expectations for a multi-rRNA-operon and rapidly evolving symbiont known to regularly establish 'de novo' within insect lineages (McCutcheon et al., 2019). We thus argue that the set of *Sodalis* ASVs can serve as a sort of 'barcode', highlighting cases when the symbiosis has likely changed. Specifically, differences in *Sodalis* ASV set among two insects suggest either divergence in their symbiont genome sequences, infection with independently derived strains, or perhaps different combinations of strains in case of multiple infections. We are unable to distinguish among these scenarios based on the available data. However, the variation in ASV sets that we observe among *Philaenus* species, populations and especially among individuals within a population, indicate either rapid sequence evolution of this symbiont, frequent co-infections with additional strains that result in multiple infections, common symbiont replacements or a combination of all three processes (McCutcheon et al., 2019). On the other hand, the high similarity of studied *Sodalis* ASVs to those of *Sodalis praecaptivus* (GB ref: PRJNA199998)—a versatile, culturable bacterium originally isolated from a human wound, seemingly close to the ancestral state of many insect symbionts (McCutcheon et al., 2019)—could also indicate, in some cases, not a stable, heritable co-infection/replacement, but a short-lived, non-heritable infection, or parasitism by another organism bearing additional *Sodalis* strains. *Sodalis* seem to be a diverse group of bacteria inhabiting different environments (such as soil and decomposing wood), as shown in the study conducted by Tláskal et al. (2021). Furthermore, it has been proposed that *Sodalis* can help cope with stress caused by pesticide exposure (Hubert et al., 2022), making the wild *Sodalis* population a potential genetic reservoir for hosts' adaptation. Future genomics-enabled work will allow us to distinguish among these scenarios in what appears as a surprisingly dynamic system.

The diversity and distribution of *Philaenus* facultative endosymbioses

We observed clear patterns in the distribution of dominant facultative endosymbionts, *Wolbachia* (two OTUs), *Rickettsia* and *Spiroplasma*, across species and populations. Neither symbiont was present in all populations of the studied species, contrary to the results by Kapantaidaki et al. (2021) for Greek populations.

Wolbachia, the most broadly distributed insect facultative endosymbiont, is common in *P. spumarius* but its distribution shows clear geographic stratification. The frequencies of *Wolbachia* infection that we estimated in the North-Eastern and South-Western clades of *P. spumarius* are nearly identical to values reported by Lis et al. (2015) on a much larger sampling (c. 70%, and c. 20%, respectively, the latter almost exclusively in the contact zone). This proves that *Wolbachia* diagnostic screens and characterization using Multilocus

Sequence Typing (MLST) (Baldo et al., 2006) provide concordant results to 16S rRNA amplicon sequencing. At the same time, our simultaneous sequencing of insect COI and bacterial 16S rRNA gene amplicons may explain some of Lis et al. (2015) observations. We suspect that the previously undescribed *Wolbachia* MLST profiles that they reported in populations in which only single individuals were infected represent *Wolbachia* of parasitoid origin, corresponding to our OTU 25. With parasitoids regarded as a vector for the horizontal transmission of *Wolbachia*, it would be interesting to investigate the nature of these infections further (Ahmed et al., 2015). Our findings are also partly congruent with a screening on a much higher scale conducted in Italy by Formisano et al. (2022). They described *Wolbachia* as lacking in southern populations with endosymbiont present only in northern Italy, with an average infection rate of 40.5%, and suggested climate as a driving force of the infection rate.

Although Kapantaidaki et al. (2021) reported a high infection rate of *Rickettsia* in populations of *P. spumarius*, we found it rare in this species. However, our findings are congruent in terms of *Rickettsia* that reaches high abundance within infected *Philaenus* individuals. Furthermore, we may be the first to report *Spiroplasma* infection in the *Philaenus* group. The fourth widespread arthropod reproductive manipulator—*Cardinium*—was absent in our dataset as well as all datasets published previously, adding to evidence on the absence or rarity of this symbiont in the *Philaenus* species.

The patterns of the distribution of facultative endosymbionts, especially *Wolbachia* and *Rickettsia*, may be linked to their fitness effects, potentially including influencing their hosts' thermal susceptibility. According to Hague et al. (2020), some strains of *Wolbachia* supergroup A may shift host preferences toward cooler temperatures, and strains belonging to supergroup B may do the opposite. However, those claims do not seem to be broadly supported by data from insects other than *Drosophila* spp. In *P. spumarius*, however, we find the opposite patterns: *Wolbachia* prevalent in the North-Eastern clade (distributed in colder areas) belongs to the supergroup B. The explanation proposed by Formisano et al. (2022) that the hot, dry climate can negatively affect *Wolbachia* transmission, may thus better explain its low prevalence in southern *P. spumarius* populations. On the other hand, *Rickettsia* may confer protection against heat shock, as demonstrated in whitefly *Bemisia tabaci* (Brumin et al., 2011) and, to an extent, in aphids (Montllor et al., 2002). Such adaptive properties could explain patterns of infection observed in the *Philaenus* species, namely the presence of *Rickettsia* in specimens from southern regions. However, both these symbionts have multiple other fitness effects that could have contributed to their observed distribution. For example, the combination of reproductive manipulation and positive effects on multiple life-history traits seems to explain the rapid spread of *Wolbachia* across Australian and North American populations of *Drosophila simulans* (Kriesner et al., 2013) and of *Rickettsia* in American populations of the whitefly *Bemisia tabaci* (Himler et al., 2011). In aphids, *Rickettsia* can also confer protection against pathogenic fungi, perhaps justifying its prevalence in populations when entomopathogenic pressure is high (Łukasik, van Asch, et al., 2013), with its negative effects on fecundity and longevity ameliorated by co-infections with other microbes (Łukasik, Guo, et al., 2013). Unfortunately, we know little about facultative symbiont roles or fitness effects in Auchenorrhyncha. They are likely to vary substantially among host species and symbiont clades and genotypes, perhaps explaining different *Rickettsia* genotype associations among the North-Eastern and South-Western clades.

Noteworthy, we did not observe the occurrence of some of the facultative endosymbionts described in Greek populations of *P. spumarius* described by Kapantaidaki et al. (2021), such as *Arsenophonus* and *Hamiltonella*. Since specimens used in both studies were collected in different sites almost a decade apart, there is a possibility that some of the Greek populations of *P. spumarius* were colonized by these new endosymbionts (Smith et al., 2015). Another explanation is that only some populations of *P. spumarius* in Greece are infected by these bacteria, and our sampling (just three individuals) omitted these sites.

On the other hand, since the authors provide information only about a signal and not about the abundance of *Hamiltonella* and *Arsenophonus*, we cannot entirely exclude the environmental origin of that signal. The detection of moderately abundant reads representing *Buchnera*, a strictly heritable nutritional endosymbiont of aphids that seems exceedingly unlikely to successfully colonize divergent hosts such as *P. spumarius* and *P. tesselatus* where we observed it, almost certainly represents some sort of contamination rather than any lasting association. Contamination with *Buchnera*-containing honeydew produced by aphids feeding on the same plant is a possibility, laboratory contamination is another. The knowledge of the biology of diverse insect-associated bacteria helps identify such suspicious cases and is critical for interpreting the data on symbiont distributions.

Parasitoid effects on host-associated microbial community profiles associations

To our knowledge, we may be the first to show the additional signal of parasitoids in the COI amplicon sequencing data for wild insects and at the same time, its contribution to the overall endosymbiotic signal in

microbial community profiles. Although there were studies suggesting additional *Wolbachia* strain as parasitoid origin combining obtained molecular data with previous findings of parasitism (Štarhová Serbina et al., 2022) we are firmly confident that we provided a 'smoking gun' evidence for such phenomenon. We obtained information about the parasitoid's presence, abundance and mitochondrial genotype alongside information on the mitochondrial diversity of the host. It is clear that molecular methods have opened up new avenues for studying host-parasitoid interactions, with tools such as diagnostic PCR and qPCR assays and sequencing parasitoid marker genes unravelling interaction dynamics and cryptic diversity (Hrček & Godfray, 2015). We argue that COI gene amplicon sequencing shows particular promise as a way of simultaneously obtaining information about the presence of parasitoids alongside their genetic diversity (Šigut et al., 2017; Sow et al., 2019).

Being able to use a single pair of broad-spectrum primers to simultaneously acquire host genotype information, parasitoid presence, species and genotype and host-parasitoid read number ratio as a proxy of parasitoid size does open up exciting avenues for high-throughput study of multitrophic interactions in diverse natural communities. In particular, parasitoid infections may be an important explanatory variable for determining microbial composition, whether by preferential parasitism of hosts that harbour-specific combinations of microbes, a change to the host microbiota as a result of parasitism, or by parasitoids contributing their microbes to the community profile (Fredensborg et al., 2020; Vorburger, 2022). At the same time, symbiotic bacteria are increasingly regarded as shaping host-parasitoid interaction dynamics (Vorburger & Perlman, 2018).

Across these three types of information—host identity confirmation, host genotype information and the dissection of parasitoid association—COI amplicon data for individual insects are emerging as a groundbreaking tool for the study of microbiota in natural communities.

CONCLUSIONS

The combination of broad specimen sampling with amplicon data for two marker genes proved to be a powerful way of reconstructing host–symbiont relationships, substantially expanding our understanding of a widespread ecologically and economically significant insect clade. COI amplicon data validated morphology-based species identification and reconstructed parasitoid infections, contributing to the host microbiota signal. OTU-level characterization of microbial communities allowed the reconstruction of broader patterns related to symbiont distribution, whereas genotype-resolution data provided more specific insights into patterns and processes. These complementary approaches have confirmed the stability of the ancient nutritional endosymbiosis with *Sulcia*, the more versatile and dynamic nature of its co-symbiont *Sodalis*, and the geographic and host-phylogenetic structuring of their facultative endosymbioses. Parasitoid infections and likely environmental contamination help explain more of the observed variation. And while genomics or experimental approaches are necessary to fully comprehend the biological significance of the observed patterns, the multi-target amplicon sequencing has proven to be a highly effective way or characterizing broader patterns.

AUTHOR CONTRIBUTIONS

Michał Robert Kolasa: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (equal); software (equal); validation (equal); visualization (lead); writing – original draft (equal). **Łukasz Kajtoch:** Conceptualization (supporting); resources (supporting); writing – original draft (supporting). **Anna Michalik:** Formal analysis (equal); investigation (equal); methodology (equal); resources (supporting); visualization (equal); writing – original draft (equal). **Anna Maryańska-Nadachowska:** Resources (supporting). **Piotr Łukasik:** Conceptualization (equal); formal analysis (equal); funding acquisition (lead); investigation (equal); methodology (equal); project administration (lead); resources (lead); software (equal); supervision (lead); validation (equal); visualization (equal); writing – original draft (equal).

ACKNOWLEDGEMENTS

The project was supported by the Polish National Agency for Academic Exchange grant PPN/PPO/2018/1/00015, and Polish National Science Centre grants 2018/31/B/NZ8/01158 and 2018/30/E/NZ8/00880.

CONFLICT OF INTEREST STATEMENT

The authors declare that no conflict of interests.

DATA AVAILABILITY STATEMENT

The raw sequence has been deposited in the Sequence Read Archive of the National Centre for Biotechnology Information with the accession number: PRJNA832912.

ORCID

Michał Kolasa https://orcid.org/0000-0003-2143-429X
Łukasz Kajtoch https://orcid.org/0000-0001-7345-9400
Anna Michalik https://orcid.org/0000-0003-2952-9867
Anna Maryańska-Nadachowska https://orcid.org/0000-0003-3270-825X
Piotr Łukasik https://orcid.org/0000-0002-4164-6487

REFERENCES

Ahmed, M.Z., Li, S.-J., Xue, X., Yin, X.-J., Ren, S.-X., Jiggins, F.M. et al. (2015) The intracellular bacterium *Wolbachia* uses parasitoid wasps as Phoretic vectors for efficient horizontal transmission. *PLoS Pathogens*, 11, e1004672.

Amiri, N., Keady, M.M. & Lim, H.C. (2023) Honey bees and bumble bees occupying the same landscape have distinct gut microbiomes and amplicon sequence variant-level responses to infections. *PeerJ*, 11, e15501.

Apprill, A., McNally, S., Parsons, R. & Weber, L. (2015) Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic Microbial Ecology*, 75, 129–137.

Baldo, L., Dunning Hotopp, J.C., Jolley, K.A., Bordenstein, S.R., Biber, S.A., Choudhury, R.R. et al. (2006) Multilocus sequence typing system for the endosymbiont *Wolbachia* pipientis. *Applied and Environmental Microbiology*, 72, 7098–7110.

Bennett, G.M. & Moran, N.A. (2015) Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole. *Proceedings of the National Academy of Sciences*, 112, 10169–10176.

Brumin, M., Kontsedalov, S. & Ghanim, M. (2011) *Rickettsia* influences thermotolerance in the whitefly *Bemisia tabaci* B biotype. *Insect Sci.*, 18, 57–66.

Buchner, P. (1965) *Endosymbiosis of animals with plant microorganisms*. New York: Interscience Publishers.

Douglas, A.E. (2009) The microbial dimension in insect nutritional ecology. *Functional Ecology*, 23, 38–47.

Douglas, A.E. (2016) How multi-partner endosymbioses function. *Nature Reviews. Microbiology*, 14, 731–743.

Driscoll, T.P., Verhoeve, V.I., Guillotte, M.L., Lehman, S.S., Rennoll, S.A., Beier-Sexton, M. et al. (2017) Wholly *rickettsia*! Reconstructed metabolic profile of the quintessential bacterial parasite of eukaryotic cells. *MBio*, 8, e00859-17.

Edgar, R.C. (2016) UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *bioRxiv*, 081257.

Elbrecht, V., Braukmann, T.W.A., Ivanova, N.V., Prosser, S.W.J., Hajibabaei, M., Wright, M. et al. (2019) Validation of COI metabarcoding primers for terrestrial arthropods. *PeerJ*, 7, e7745.

Feldhaar, H. (2011) Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecological Entomology*, 36, 533–543.

Formisano, G., Iodice, L., Cascone, P., Sacco, A., Quarto, R., Cavalieri, V. et al. (2022) *Wolbachia* infection and genetic diversity of Italian populations of *Philaenus spumarius*, the main vector of *Xylella fastidiosa* in Europe. *PLoS One*, 17, e0272028.

Fredensborg, B.L., Fossdal í Kálvalíð, I., Johannesen, T.B., Stensvold, C.R., Nielsen, H.V. & Kapel, C.M. (2020) Parasites modulate the gut-microbiome in insects: a proof-of-concept study. *PLoS One*, 15, e0227561.

Gerardo, N.M. & Parker, B.J. (2014) Mechanisms of symbiont-conferred protection against natural enemies: an ecological and evolutionary framework. *Current Opinion in Insect Science*, 4, 8–14.

Glenn, T.C. (2011) Field guide to next-generation DNA sequencers. *Molecular Ecology Resources*, 11, 759–769.

Godefroid, M., Morente, M., Schartel, T., Cornara, D., Purcell, A., Gallego, D. et al. (2021) Climate tolerances of *Philaenus spumarius* should be considered in risk assessment of disease outbreaks related to *Xylella fastidiosa*. *Journal of Pest Science*, 95, 855–868.

Hague, M.T.J., Caldwell, C.N. & Cooper, B.S. (2020) Pervasive effects of *Wolbachia* on host temperature preference. *MBio*, 11, e01768-20.

Himler, A.G., Adachi-Hagimori, T., Bergen, J.E., Kozuch, A., Kelly, S.E., Tabashnik, B.E. et al. (2011) Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science*, 332, 254–256.

Hrček, J. & Godfray, H.C.J. (2015) What do molecular methods bring to host-parasitoid food webs? *Trends in Parasitology*, 31, 30–35.

Hubert, J., Navratilova, B., Sopko, B., Nesvorna, M. & Phillips, T.W. (2022) Pesticide residue exposure provides different responses of the microbiomes of distinct cultures of the stored product pest mite *Acarus siro*. *BMC Microbiology*, 22, 1–15.

Hunter, D.J., Torkelson, J.L., Bodnar, J., Mortazavi, B., Laurent, T., Deason, J. et al. (2015) The *Rickettsia* endosymbiont of *Ixodes pacificus* contains all the genes of De novo folate biosynthesis. *PLoS One*, 10, e0144552.

Husnik, F. & McCutcheon, J.P. (2016) Repeated replacement of an intrabacterial symbiont in the tripartite nested mealybug symbiosis. *PNAS*, 113, E5416–E5424.

Jaenike, J., Unckless, R., Cockburn, S.N., Boelio, L.M. & Perlman, S.J. (2010) Adaptation via Symbiosis: recent spread of a *drosophila* defensive symbiont. *Science*, 329, 212–215.

Ju, J.-F., Bing, X.-L., Zhao, D.-S., Guo, Y., Xi, Z., Hoffmann, A. A. et al. (2019). Wolbachia supplement biotin and riboflavin to enhance reproduction in planthoppers. *The ISME Journal*, 14, 676–687.

Kapantaidaki, D.E., Antonatos, S., Evangelou, V., Papachristos, D.P. & Milonas, P. (2021) Genetic and endosymbiotic diversity of Greek populations of *Philaenus spumarius*, *Philaenus signatus* and *Neophilaenus campestris*, vectors of *Xylella fastidiosa*. *Scientific Reports*, 11, 3752.

Kircher, M., Heyn, P. & Kelso, J. (2011) Addressing challenges in the production and analysis of Illumina sequencing data. *BMC Genomics*, 12, 1–14.

Knight, R., Vrbanac, A., Taylor, B.C., Aksenov, A., Callewaert, C., Debelius, J. et al. (2018) Best practices for analysing microbiomes. *Nature Reviews Microbiology*, 16, 410–422.

Koga, R. & Moran, N.A. (2014) Swapping symbionts in spittlebugs: evolutionary replacement of a reduced genome symbiont. *The ISME Journal*, 8, 1237–1246.

Koga, R., Bennett, G.M., Cryan, J.R. & Moran, N.A. (2013) Evolutionary replacement of obligate symbionts in an ancient and diverse insect lineage. *Environmental Microbiology*, 15, 2073–2081.

Kriesner, P., Hoffmann, A.A., Lee, S.F., Turelli, M. & Weeks, A.R. (2013) Rapid sequential spread of two *Wolbachia* variants in *Drosophila simulans*. *PLoS Pathogens*, 9, e1003607.

Kwong, W.K., Medina, L.A., Koch, H., Sing, K.W., Soh, E.J.Y., Ascher, J.S. et al. (2017) Dynamic microbiome evolution in social bees. *Science Advances*, 3, e1600513.

Leigh, J.W. & Bryant, D. (2015) POPART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 6, 1110–1116.

Leray, M., Ho, S.-L., Lin, I.-J. & Machida, R.J. (2018) MIDORI server: a webserver for taxonomic assignment of unknown metazoan mitochondrial-encoded sequences using a curated database. *Bioinformatics*, 34, 3753–3754.

Lis, A., Maryańska-Nadachowska, A. & Kajtoch, Ł. (2015) Relations of *Wolbachia* infection with Phylogeography of *Philaenus spumarius* (Hemiptera: Aphrophoridae) populations within and beyond the Carpathian contact zone. *Microbial Ecology*, 70, 509–521.

Lis, A., Maryańska-Nadachowska, A., Lachowska-Cierlik, D. & Kajtoch, Ł. (2014) The secondary contact zone of phylogenetic lineages of the *Philaenus spumarius* (Hemiptera: Aphrophoridae): an example of incomplete allopatric speciation. *Journal of Insect Science*, 14, 1–10.

Maejima, K., Oshima, K. & Namba, S. (2014) Exploring the phytoplasmas, plant pathogenic bacteria. *Journal of General Plant Pathology*, 80, 210–221.

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P. et al. (2012) Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology*, 13, 614–629.

Martinson, V.G., Gawryluk, R.M.R., Gowen, B.E., Curtis, C.I., Jaenike, J. & Perlman, S.J. (2020) Multiple origins of obligate nematode and insect symbionts by a clade of bacteria closely related to plant pathogens. *PNAS*, 117, 31979–31986.

Maryańska-Nadachowska, A., Drosopoulos, S., Lachowska, D., Kajtoch, Ł. & Kuznetsova, V.G. (2010) Molecular phylogeny of the Mediterranean species of *Philaenus* (Hemiptera: Auchenorrhyncha: Aphrophoridae) using mitochondrial and nuclear DNA sequences. *Systematic Entomology*, 35, 318–328.

Maryańska-Nadachowska, A., Kajtoch, Ł. & Lachowska, D. (2012) Genetic diversity of Philaenus spumarius and P. tesselatus (Hemiptera, Aphrophoridae): implications for evolution and taxonomy. *Systematic Entomology*, 37, 55–64.

Maryańska-Nadachowska, A., Sanaieb, E. & Kajtocha, Ł. (2015) High genetic diversity in southwest Asian populations of *Philaenus spumarius* (Hemiptera: Auchenorrhyncha). *Zoology in the Middle East*, 61, 264–272.

McCutcheon, J.P., Boyd, B.M. & Dale, C. (2019) The life of an insect endosymbiont from the cradle to the grave. *Current Biology*, 29, R485–R495.

McCutcheon, J.P., McDonald, B.R. & Moran, N.A. (2009) Convergent evolution of metabolic roles in bacterial co-symbionts of insects. *PNAS*, 106, 15394–15399.

Michalik, A., Castillo Franco, D., Kobiałka, M., Szklarzewicz, T., Stroiński, A. & Łukasik, P. (2021) Alternative transmission patterns in independently acquired nutritional cosymbionts of *Dictyopharidae planthoppers*. *mBio*, 12, e01228-21.

Molinatto, G., Demichelis, S., Bodino, N., Giorgini, M., Mori, N. & Bosco, D. (2020) Biology and prevalence in northern Italy of *Verrallia aucta* (Diptera, Pipunculidae), a parasitoid of *Philaenus spumarius* (Hemiptera, Aphrophoridae), the Main vector of *Xylella fastidiosa* in Europe. *Insects*, 11, 607.

Montllor, C.B., Maxmen, A. & Purcell, A.H. (2002) Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. *Ecological Entomology*, 27, 189–195.

Moran, N.A., McCutcheon, J.P. & Nakabachi, A. (2008) Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics*, 42, 165–190.

Nadal-Jimenez, P., Siozios, S., Halliday, N., Cámara, M. & Hurst, G.D. (2022) *Symbiopectobacterium purcellii, gen. Nov., sp. nov., isolated from the leafhopper Empoasca decipiens*, Vol. 72. United Kingdom: *International Journal of Systematic and Evolutionary Microbiology*, 005440.

Paddock, K.J., Finke, D.L., Kim, K.S., Sappington, T.W. & Hibbard, B.E. (2022) Patterns of microbiome composition vary across spatial scales in a specialist insect. *Frontiers in Microbiology*, 13, 898744.

Parada, A.E., Needham, D.M. & Fuhrman, J.A. (2016) Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental Microbiology*, 18, 1403–1414.

Perreau, J. & Moran, N.A. (2022) Genetic innovations in animal–microbe symbioses. *Nature Reviews Genetics*, 23, 23–39.

Pilgrim, J., Thongprem, P., Davison, H.R., Siozios, S., Baylis, M., Zakharov, E.V. et al. (2021) Torix *Rickettsia* are widespread in arthropods and reflect a neglected symbiosis. *GigaScience*, 10, giab021.

Prodan, A., Tremaroli, V., Brolin, H., Zwinderman, A.H., Nieuwdorp, M. & Levin, E. (2020) Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. *PLoS One*, 15, e0227434.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P. et al. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, 41, D590–D596.

Rodrigues, A.S.B., Silva, S.E., Marabuto, E., Silva, D.N., Wilson, M.R., Thompson, V. et al. (2014) New mitochondrial and nuclear evidences support recent demographic expansion and an atypical phylogeographic pattern in the spittlebug Philaenus spumarius (Hemiptera, Aphrophoridae). *PLoS One*, 9, e98375.

Rognes, T., Flouri, T., Nichols, B., Quince, C. & Mahé, F. (2016) VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, 4, e2584.

Salter, S.J., Cox, M.J., Turek, E.M., Calus, S.T., Cookson, W.O., Moffatt, M.F. et al. (2014) Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biology*, 12, 87.

Saponari, M., Loconsole, G., Cornara, D., Yokomi, R.K., de Stradis, A., Boscia, D. et al. (2014) Infectivity and transmission of *Xylella fastidiosa* by *Philaenus spumarius* (Hemiptera: Aphrophoridae) in Apulia, Italy. *Journal of Economic Entomology*, 107, 1316–1319.

SAS Institute Inc. (2013) *SAS® 9.4 statements: reference*. Cary, NC: SAS Institute Inc.

Seabra, S.G., Rodrigues, A.S.B., Silva, S.E., Neto, A.C., Pina-Martins, F., Marabuto, E. et al. (2021) Population structure, adaptation and divergence of the meadow spittlebug, *Philaenus spumarius* (Hemiptera, Aphrophoridae), revealed by genomic and morphological data. *PeerJ*, 9, e11425.

Sicard, A., Zeilinger, A.R., Vanhove, M., Schartel, T.E., Beal, D.J., Daugherty, M.P. et al. (2018) *Xylella fastidiosa*: insights into an emerging plant pathogen. *Annual Reviews.*, 56, 181–202.

Smith, A.H., O'Connor, M.P., Deal, B., Kotzer, C., Lee, A., Wagner, B. et al. (2021) Does getting defensive get you anywhere?—Seasonal balancing selection, temperature, and parasitoids shape real-world, protective endosymbiont dynamics in the pea aphid. *Molecular Ecology*, 30, 2449–2472.

Smith, A.H., Łukasik, P., O'Connor, M.P., Lee, A., Mayo, G., Drott, M.T. et al. (2015) Patterns, causes and consequences of defensive microbiome dynamics across multiple scales. *Molecular Ecology*, 24, 1135–1149.

Song, H., Buhay, J.E., Whiting, M.F. & Crandall, K.A. (2008) Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences*, 105, 13486–13491.

Sow, A., Brévault, T., Benoit, L., Chapuis, M.-P., Galan, M., Coeur d'acier, A. et al. (2019) Deciphering host-parasitoid interactions and parasitism rates of crop pests using DNA metabarcoding. *Scientific Reports*, 9, 3646.

Tinker, K.A. & Ottesen, E.A. (2021) Differences in gut microbiome composition between sympatric wild and allopatric laboratory populations of omnivorous cockroaches. *Frontiers in Microbiology*, 12, 703785.

Tláskal, V., Pylro, V.S., Žifčáková, L. & Baldrian, P. (2021) Ecological divergence within the enterobacterial genus *Sodalis*: from insect symbionts to inhabitants of decomposing deadwood. *Frontiers in Microbiology*, 12, 668644.

Truitt, A.M., Kapun, M., Kaur, R. & Miller, W.J. (2019) *Wolbachia* modifies thermal preference in Drosophila melanogaster. *Environmental Microbiology*, 21, 3259–3268.

Vorburger, C. & Perlman, S.J. (2018) The role of defensive symbionts in host–parasite coevolution. *Biological Reviews*, 93, 1747–1764.

Vorburger, C. (2022) Defensive symbionts and the evolution of parasitoid host specialization. *Annual Review of Entomology*, 67, 329–346.

Zhang, J., Kobert, K., Flouri, T. & Stamatakis, A. (2014) PEAR: a fast and accurate Illumina paired-end reAd mergeR. *Bioinformatics*, 30, 614–620.

Łukasik, P., Guo, H., van Asch, M., Ferrari, J. & Godfray, H.C.J. (2013) Protection against a fungal pathogen conferred by the aphid facultative endosymbionts *rickettsia* and *Spiroplasma* is expressed in multiple host genotypes and species and is not

influenced by co-infection with another symbiont. *Journal of Evolutionary Biology*, 26, 2654–2661.

Łukasik, P., Newton, J.A., Sanders, J.G., Hu, Y., Moreau, C.S., Kronauer, D.J.C. et al. (2017) The structured diversity of specialized gut symbionts of the New World army ants. *Molecular Ecology*, 26, 3808–3825.

Łukasik, P., van Asch, M., Guo, H., Ferrari, J. & Godfray, H.C.J. (2013) Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecology Letters*, 16, 214–218.

Šigut, M., Kostovčík, M., Šigutová, H., Hulcr, J., Drozd, P. & Hrček, J. (2017) Performance of DNA metabarcoding, standard barcoding, and morphological approach in the identification of host–parasitoid interactions. *PLoS One*, 12, e0187803.

Štarhová Serbina, L., Gajski, D., Malenovský, I., Corretto, E., Schuler, H. & Dittmer, J. (2022) *Wolbachia* infection dynamics in a natural population of the pear psyllid *Cacopsylla pyri* (Hemiptera: Psylloidea) across its seasonal generations. *Scientific Reports*, 12, 16502.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Kolasa, M., Kajtoch, Ł., Michalik, A., Maryańska-Nadachowska, A. & Łukasik, P. (2023) Till evolution do us part: The diversity of symbiotic associations across populations of *Philaenus* spittlebugs. *Environmental Microbiology*, 25(11), 2431–2446. Available from: https://doi.org/10.1111/1462-2920.16473