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AQUATIC MICROBIOMES

Biotic and abiotic factors affecting the microbiota of Chrysomelidae inhabiting wetland vegetation

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Abstract A subject of particular interest concerns the understanding of which biotic and/or abiotic factors shape the insect's microbiota composition and diversity. In this study, we focus our attention on the microbiota associated with leaf beetles (Coleoptera: Chrysomelidae) inhabiting a freshwater wetland in Northern Italy for investigating which factors shape it in this peculiar environment. A DNA metabarcoding approach targeting the 16S rRNA has been used to characterize the bacteria associated with seven leaf beetles species living in different zones of the wetland (trees/shrubs, meadow and emergent vegetation zones). The obtained results suggest that three factors potentially affecting microbiota diversity and composition are species membership in the first place, then living zone in the wetland and sex. Within the studied species, males were found possibly more prone to acquire bacteria from the surrounding environment, while females' microbiota of most of the species resulted dominated by symbiotic bacteria (reproductive manipulators, e.g. *Wolbachia*, or other symbionts with functional roles in the species, e.g. *Candidatus* Macropleicola in *Donacia* genus). The present study, even if based on a limited sample size, contributed to exploring the factors affecting the composition and diversity of bacteria associated with Chrysomelidae.

Keywords Leaf beetles · Insect symbionts · *Wolbachia* and *Rickettsia* · Sex impact on microbiota

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Introduction

Insects are one of the most diverse animal groups on Earth, playing a central ecological role in many terrestrial and freshwater ecosystems. For many reasons, such as their abundance, ubiquity and their ecological

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relevance, insects are one of the major focus of studies on the microbiota (Charroux & Royet, 2012; Engel & Moran, 2013; Pernice et al., 2014). A subject of particular interest in these studies concerns the understanding of the biotic and abiotic factors shaping the insect's microbiota composition and diversity (Colman et al., 2012; Yun et al., 2014). Many questions are still open on this topic. Since various major drivers of microbial diversity and composition were identified in different study systems, up to now no general conclusion has been drawn (e.g., Yun et al., 2014; Kolasa et al., 2019; Malacrinò, 2022). Among the main abiotic factors that can shape insect microbiota are geographic gradients (latitude and altitude) that influence the global distribution of microbes (Adams et al., 2010; Roe et al., 2011; Montagna et al., 2015a; Hernández-García et al., 2018), and local environmental factors, shaping local distribution in specific environments (e.g., mean annual temperature or soil properties; Huang & Zhang, 2013; Tiede et al., 2017; Muturi et al., 2018). Among the interactions that occur between insects and the surrounding environment, those related to alimentation are known to greatly influence the insect associated microbial communities, especially those of the gut (the main reservoir of insect microbiota, Engel & Moran, 2013). The influence of the diet on insects' microbiota has been deeply studied in herbivores (Montagna et al., 2015b; Xu et al., 2016; Zhang et al., 2018; Leite-Mondin et al., 2021) but also in species with different trophic attitudes like omnivores (Ben Guerrero et al., 2016; Bruno et al., 2019; Luo et al., 2021) and predators (Tiede et al., 2017). The influence of the surrounding environment in shaping insect microbiota is particularly evident in many holometabolous species where larvae and adults occupy different ecological niches. In these cases, drastic changes in the associated microbial community commonly occur, sometimes also related to the diet change (*e.g.,* Vasanthakumar et al., 2008; Morales-Jiménez et al., 2012; Huang and Zhang, 2013; Shukla et al., 2016; Briones-Roblero et al., 2017; Kim et al., 2017; Zhang et al., 2018; Ali et al., 2019; Chouaia et al., 2019). Among biotic factors that can greatly affect insects' microbiota one is sex, even though studies on this topic show contrasting results possibly suggesting that its influence is species specific. As an example, differences in the microbiota composition and diversity between sexes have been reported for *Spodoptera littoralis* (Boisduval) (Chen et al., 2016), while no significative differences were found for the co-generic *Spodoptera exigua* (Hübner, 1808) (Gao et al., 2019; Martínez-Solís et al., 2020). Similar contrasting patterns have been found also within Coleoptera, with few studies recording differences between sexes in Curculionidae (Xu et al., 2016) and *Scarabaeidae* (Shukla et al., 2016), but not in Chrysomelidae (Ali et al., 2019). When males and females have different trophic attitudes, such as in mosquitos where only females feed on vertebrate blood, differences in the microbiota of the two sexes are instead a secondary effect of divergent diets (Minard et al., 2013, 2018). In other cases, these differences may be related to the vertical transmission mechanisms of symbionts, especially of primary symbionts or reproductive manipulators, that usually lead to a higher abundance of such microbes in females.

In this study, we focus our attention on the microbiota associated with leaf beetles (Coleoptera: Chrysomelidae) inhabiting freshwater wetlands. Wetlands are ecosystems whose soil is saturated and sometimes also covered by water. The persistent presence of water strongly influences the vegetation of wetlands, and consequently the communities of phytophagous present in this environment (Cowardin et al., 1976), including insects. Among them leaf beetles, a species-rich family within Coleoptera, composed of typically phytophagous species feeding on both herbaceous plants and shrubs of many different environments. Within this family, there are species considered serious pests of crops (Ulrich et al., 2004; Toepfer & Kuhlmann, 2006; Coral Shain et al., 2018; Schoville et al., 2018) but also some of conservation interest (e.g., Biondi et al., 2013; Brunetti et al., 2019). Few Chrysomelidae species are found on the typical vegetation of West Palearctic freshwater wetlands. Different species are associated with plants of distinct wetland zones from shrubs to emergent vegetation. Among the Chrysomelidae living in wetlands of Northern Italy, the (semi-) aquatic species of the subfamily Donacinae are the most typical: adults usually feed on leaves or pollen of emergent macrophytes, while larvae live underwater and feed on roots. Interestingly, some of these larvae have specific structures allowing to breathe underwater by exploiting plant aerenchyma (Kleinschmidt et al., 2011; Montagna et al., 2012; Buczynski et al., 2019). The emergent plants are usually colonized also

by Chrysomelinae (e.g., genera *Phaedon* and *Prasocuris*; Buczynski et al., 2019). Some Donacinae species live also in wetland meadows (e.g., *Plateumaris*) together with Cassidinae (*Cassida* genus). Moreover, scrub vegetation and shrubs are usually inhabited by Galerucinae (such as *Agelastica alni* (Linnaeus, 1758) on *Alnus*; Ramsay, 2009) and Alticinae. Less common in such a wet environment are instead Cryptocephalinae.

In this study, the diversity and structure of the microbiota (bacterial component only) of seven selected leaf beetle species collected in the same freshwater wetland environment in Northern Italy were analysed. The main aim was to investigate the factors shaping Chrysomelidae microbiota structure and diversity in this peculiar environment where the regular presence of water could enhance bacteria spread. Specifically, nonetheless considering that the limited sampling prevents drawing general conclusions, we aim at investigating which factors among the living environment, species membership and sex mainly affect the microbiota of Chrysomelidae in our study case.

Materials and methods

Sampling

Alserio lake area in North Italy (45°47′46″ N–9°12′59″ E, 261 m a.s.l.) was identified as sampling area. Before performing specimens' collection, the wetland area was investigated and the zones characterized according to the different vegetation types identified, from trees/shrubs zone to submerged plants one. Nineteen adult insects from seven Chrysomelidae species were collected from three different zones of the wetland (i.e., emergent vegetation, meadow, trees/shrubs zones). After the collection, the specimens were preserved in absolute ethanol and stored at −20 °C. Specimens associated metadata are reported in Table 1.

Table 1 Information on the analysed specimens

ID	Species	Subfamily	Sex	Coll. Date[a]	Host plant	Zone[b]
P733_3	*Agelastica alni*	Galerucinae	female	10.05.2014	*Alnus glutinosa*	Trees/shrubs
P733_2	*Agelastica alni*	Galerucinae	male	10.05.2014	*Alnus glutinosa*	Trees/shrubs
P1068_10	*Cassida rubiginosa*	Cassidinae	female	13.04.2021	*Tanacetum vulgare*	Meadow
P1070_2	*Cassida rubiginosa*	Cassidinae	male	02.05.2021	*Tanacetum vulgare*	Meadow
P1068_11	*Chaetocnema conducta*	Alticinae	female	13.04.2021	n.a.[c]	Trees/shrubs
P1068_12	*Chaetocnema conducta*	Alticinae	male	13.04.2021	n.a.[c]	Trees/shrubs
P1068_13	*Chaetocnema conducta*	Alticinae	male	13.04.2021	n.a.[c]	Trees/shrubs
P1070_4	*Donacia simplex*	Donacinae	female	02.05.2021	*Thypa* sp.	Emergent
P1070_5	*Donacia simplex*	Donacinae	female	02.05.2021	*Thypa* sp.	Emergent
P1070_3	*Donacia simplex*	Donacinae	male	02.05.2021	*Phragmites australis*	Emergent
P1068_16	*Phaedon cochleariae*	Chrysomelinae	female	13.04.2021	*Nasturtium officinale*	Emergent
P1068_2	*Phaedon cochleariae*	Chrysomelinae	female	13.04.2021	*Nasturtium officinale*	Emergent
P1068_4	*Phaedon cochleariae*	Chrysomelinae	male	13.04.2021	*Nasturtium officinale*	Emergent
P1068_1	*Plateumaris consimilis*	Donacinae	male	13.04.2021	*Carex* sp.	Meadow
P1068_8	*Plateumaris consimilis*	Donacinae	male	13.04.2021	*Carex* sp.	Meadow
P1068_9	*Plateumaris consimilis*	Donacinae	male	13.04.2021	*Carex* sp.	Meadow
P733_4	*Prasocuris phellandrii*	Chrysomelinae	female	10.05.2014	*Caltha palustris*	Emergent
P733_5	*Prasocuris phellandrii*	Chrysomelinae	female	10.05.2014	*Caltha palustris*	Emergent
P733_6	*Prasocuris phellandrii*	Chrysomelinae	male	10.05.2014	*Caltha palustris*	Emergent

[a]Coll. Date = Collection date

[b]Zone = living zone in the wetland

[c]Collection host plant not available, the specimens were collected from grasses under the shrubs

DNA isolation and sequencing of 16S rRNA bacterial region

The outer surface of collected specimens was sterilized using the following protocol: one wash of 2 min in 0.1% SDS, two washes of 1 min in 1% PBS, followed by two washes of 1 min in sterile water. The DNA was extracted from the whole insect body using the phenol–chloroform method by Doyle & Doyle, 1990, tissues were disrupted using glass beads (ø 0.1 mm) and sterile pestles. For each DNA extraction batch, a blank replicate (negative control) was performed to monitor environmental and reagents contaminations. The purity of the extracted DNA was determined by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and Qubit 4.0 fluorometer (Thermo Fisher Scientific) was used to determine the DNA concentration. Libraries were prepared by following Illumina 16S metagenomic sequencing library preparation protocol in two amplification steps: an initial PCR amplification using locus specific PCR primers (V3-V4 region of the bacterial 16S rRNA, 341F 5'-CCTACGGGNBGCASCAG-3' and 805R 5'-GACTACNVGGGTATCTAATCC-3') and a subsequent amplification that integrates relevant flow-cell binding domains and unique indices (NexteraXT Index Kit, FC-131–1001/FC-131–1002). The libraries were then sequenced on NovaSeq instruments (Illumina, San Diego, CA) using 250 bp paired end mode. Generated raw data were submitted to NCBI SRA (Leinonen et al., 2011) under the project PRJNA858425 (Accession numbers SAMN29715000—SAMN29715018).

DNA extraction blanks were checked for bacterial contamination through PCR amplification of the V3-V4 regions of the 16S rRNA (using the same primer pairs mentioned before). No amplicons were obtained for any of them (the PCR amplification results were visualized by 1.5% agarose gel electrophoresis).

Species level identification of specimens

Species level identification of the specimens used in this study was obtained through two approaches: morphological identification and molecular identification. The morphological identifications to species level were achieved using dichotomous keys for Chrysomelidae (Muller, 1953; Warchalowski 2003, Bedzek & Mlejnek, 2016) and specimen manipulation and dissection for genitalia observation (when necessary) were completed with the stereomicroscope Leica MS5. For confirming morphological identification, one or more specimens for each morphotype (depending on the difficulty level related to species morphological identification; Magoga et al. 2021) were also molecularly identified. An aliquot of the DNA extracted from each sample (as described in the previous paragraph) was used as template for the amplification through PCR of COI 5' region (using Folmer, 1994 primers pair). PCR conditions and protocol used follow previously published works (Montagna et al., 2013; Magoga et al., 2018). Positive amplicons were sequenced on one-strand using Sanger sequencing (Microsynth, Balgach, Switzerland). The open reading frame of each of the obtained sequences was verified using the on-line tool EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). The sequences were then compared with those available on the BOLD COI database (Ratnasingham & Hebert, 2007) and the species level identification was assigned when a sequence similarity > 99% was obtained in the comparison between the query and the reference sequence. The sequences were deposited in BOLD (IDs: MEDLB951-22 – MEDLB960-22).

Bioinformatic and statistical analyses

The bioinformatic analyses were performed using the QIIME2 platform (Bolyen et al., 2019) and the R software (R Core Team, 2020). The obtained raw reads for the 16S rRNA V3-V4 hypervariable region were denoised using the DADA2 algorithm (Callahan et al., 2016), to remove errors and obtain the actual biological sequences present (ASVs, Amplicon Sequence Variants). The obtained ASVs have been taxonomically annotated with the fit-classifier-sklearn method (Pedregosa et al., 2011; Bokulich et al., 2018) using the release 138 of the SILVA database (Quast et al., 2012) as reference for sequences and taxonomy. The naïve Bayes classifier was trained on the reference sequences trimmed to correspond to the amplified region. To increase the taxonomic classification accuracy, environment-specific taxonomic abundance information was incorporated using the q2-clawback plugin (Kaehler et al., 2019). Since 16S rRNA sequences from the specific bacterial symbionts of

Chrysomelidae ("*Candidatus* Stammera capleta" and "*Candidatus* Macropleicola") are not present in the SILVA database used for taxonomic annotations, the presence of sequences from these symbionts has been double-checked with a blast search (Altschul et al., 1990) on the NCBI database. The SEPP technique (SATé-enabled phylogenetic placement; Janssen et al., 2018) was applied to place the ASVs on a reference phylogeny inferred using the full 16S rRNA and based on the release 138 of the SILVA database (Quast et al., 2012), the obtained tree was then used in the computation of phylogenetically informed diversity metrics. The ASVs table was normalized by scaling with ranked subsampling (Beule & Karlovsky, 2020; Heidrich et al., 2021) at the same depth (15,440 sequences per sample) to be used in the diversity analyses. The diversity analyses were performed using the Hill number family of diversity indices (Hill, 1973; Alberdi & Gilbert, 2019; Roswell et al., 2021), computed using the hilldiv R package (Alberdi & Gilbert, 2020) for three increasing values of the order parameter q, corresponding to increasing weight on the species relative abundance (number of reads per ASV over the total number of reads detected) and also to different well-known alpha-diversity indices: q = 0, counting mainly the rare species (those with low relative abundances), corresponds to richness (McIntosh, 1967); $q = 1$, counting mainly the common species (those with medium–high relative abundances), corresponds to the exponential of Shannon index (Shannon, 1948); $q = 2$, counting mainly the dominant species (those with very high relative abundances), corresponds to the inverse of Simpson index (Simpson, 1949). This explicit parametrization is particularly useful in microbiota studies to test for differences between the diversity of the most abundant species (possible symbionts) and the diversity of lower abundant bacteria (usually acquired from the environment and possibly without functional role). The complement of the Sørensen-type overlap (1-CqN) applied to the beta-diversity computed within the Hill numbers framework was used to measure dissimilarities between samples (Chao et al., 2012). Alpha-diversity comparisons between groups of samples were also performed on the full ASV table using a sample size and coverage-based integrations of interpolation (rarefaction) and extrapolation (prediction) of the Hill numbers with the R packages iNEXT (Chao et al., 2014). The Principal Coordinate Analysis (PCoA) was performed on beta diversity dissimilarities to graphically represent differences across samples (Hotelling, 1933, 1936). Differences among groups of samples (i.e., grouping samples by sex, species and living zone in the wetland, Table 1) were tested using the Kruskal–Wallis test (Kruskal & Wallis, 1952) for alpha-diversity estimates, and the Wilcoxon test (Wilcoxon, 1992), ANOSIM (Analysis of similarities; Clarke, 1993), ADONIS (Permutational multivariate analysis of variance) and PERMDISP (Permutational analysis of multivariate dispersion) (Excoffier et al., 1992; Anderson, 2001; Warton et al., 2012) for the beta-diversity dissimilarities.

Results

A total of 3,984,644 paired end reads (209,718 reads per sample on average, min = 73,201, max = 301,897) have been obtained from sequencing. After the denoising and chimera filtering steps 2,589,822 sequences were retained (136,306 reads per sample on average, ~ 64% of the raw sequences) corresponding to 1238 unique sequences (ASVs). All the ASVs assigned to mitochondria (9 ASVs, 0.04% of the sequences) or chloroplast (20 ASVs, 4.72% of the sequences) have been excluded from further analyses, leaving 1209 bacterial ASVs.

Microbiota composition

The most abundant bacterial class found associated with the analysed specimens was Alphaproteobacteria (on average ~ 80% of the bacterial sequences in each species; Fig. 1). Specifically, the microbiota of the majority of the analysed species was dominated by *Wolbachia* (18 ASVs) and *Rickettsia* (3 ASVs belonging to endosymbiotic *Rickettsia*) (Table 2). In detail, the microbiota of *Agelastica alni*, *Prasocuris phellandrii* (Linnaeus, 1758) and *Plateumaris consimilis* (Schrank, 1781) was dominated by *Wolbachia*, with relative abundances between 75 and 99%; while *Chaetocnema conducta* (Motschulsky, 1838) and *Phaedon cochleariae* (Fabricius, 1792) harboured both *Wolbachia* and *Rickettsia* (Table 2). These bacteria were found to be almost absent in *Cassida rubiginosa* (Muller, 1776) and *Donacia simplex* (Fabricius 1775). In both last species the microbiota of males and

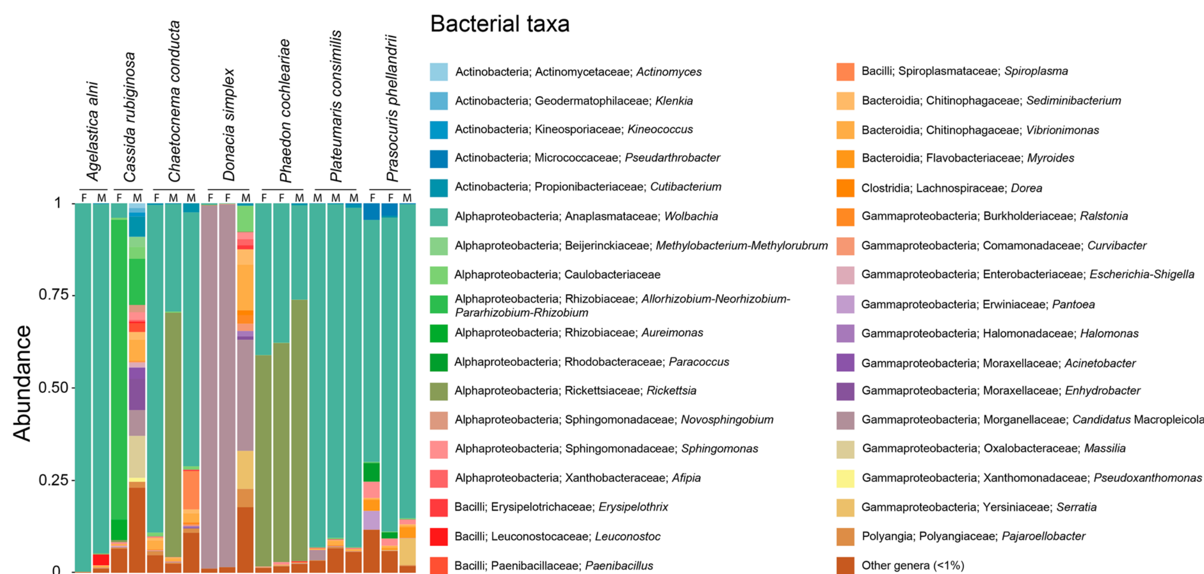

Fig. 1 Taxonomic composition of the bacterial microbiota of the Chrysomelidae species selected for the study. Species level identification of the analysed specimens is reported over the bars, M = male individual, F = female individual. Colours represent different bacterial taxa and the height of each box corresponds to the relative abundance of each bacterial taxon. Bacteria genus level identification is reported (in some cases only higher taxonomic levels are reported since the identification at genus was not reached). Bacterial taxa representing at least 1% of the reads in one sample are shown, less abundant bacteria are included in the group "Other genera"

Table 2 Average relative abundance of sequences assigned to *Wolbachia* and *Rickettsia* in females and males of each species

Insect species	Relative abundance of *Wolbachia*		Relative abundance of *rickettsia*	
	Females	Males	Females	Males
Agelastica alni	99.7%	94.5%	< 0.2%	< 0.2%
Cassida rubiginosa	3.9%	< 0.2%	0.6%	< 0.2%
Chaetocnema conducta	88.6%	46.2%	< 0.2%	37.4%
Donacia simplex	< 0.2%	< 0.2%	< 0.2%	< 0.2%
Phaedon cochleariae	39.0%	25.6%	58.6%	70.9%
Plateumaris consimilis	n.a.[a]	91.6%	n.a. [a]	< 0.2%
Prasocuris phellandrii	75.7%	85.0%	< 0.2%	< 0.2%

[a]for *P. consimilis* no data are available on females since only males were analysed

females resulted quite different: female's microbiota was dominated by a single bacterium with relative abundance higher than 80%, while male's microbiota resulted more evenly composed, without any dominant bacterium (Fig. 1). In the microbiota of *C. rubuginosa* female, the dominant bacterium (82% relative abundance) has 100% sequence identity with the *Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium* complex of soil nitrogen fixing bacteria and the plant pathogenic genus *Agrobacterium* (16S rRNA sequences do not allow to distinguish among these bacterial taxa) (Fig. 1). The microbiota of *Donacia simplex* was mainly dominated by the already known Donacinae symbiont ("*Candidatus* Macropleicola"), having ~ 98% relative abundance in females and ~ 30% in males (Fig. 1). One individual of *P. consimilis*, for which only males were sampled, was infected by "*Candidatus* Macropleicola" too but at a lower relative abundance than *Donacia* (~ 2.6%).

Three ASVs only were shared among all the specimens analysed in this study. One of them (average relative abundance per sample 1.50%, *sd* = 2.98%) was assigned to *Vibrionimonas magnilacihabitans* a bacterium previously isolated from lake water (Albert et al., 2014), another to the genus *Pajaroellobacter* (average relative abundance per sample 0.57%, *sd* = 1.15%), which includes soil bacteria but also the etiologic agent of epizootic bovine abortion (*P. abortibovis,* Brooks et al., 2016). The last ASV (average relative abundance per sample 0.74%, *sd* = 1.67%)

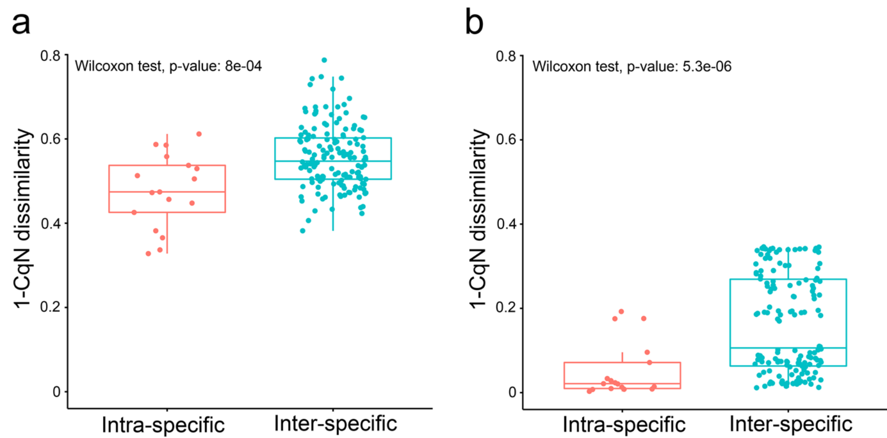

Fig. 2 Comparison of intra- and inter-specific dissimilarities. Boxplots represent the distribution of intra- and inter-specific pairwise beta-diversity dissimilarities (1-CqN) between samples computed for two values of the q parameter: **a** q = 0; **b** q = 1. The p-values of the Wilcoxon test for testing the hypothesis that intraspecific dissimilarities are lower than interspecific ones are also reported

was assigned to Caulobacteraceae (it was not possible to obtain a high confidence assignment at lower taxonomic levels).

Microbiota diversity

Lower dissimilarity values were found within species ($q=0$ median 0.47, $q=1$ median 0.02) than between ($q=0$ median 0.55, $q=1$ median 0.11). Dissimilarity estimates for intraspecific and interspecific level, regardless of the parameter q value considered, were significantly different ($q=0$ Wilcoxon test, $W=697$, p-value = 0.0008; Fig. 2). The statistical significance of the difference was greater for higher value of q ($q=1$ Wilcoxon test $W=455$, p-value = 0.000005; $q=2$ Wilcoxon test $W=484$, P-value = 0.00001; Fig. 2, Supplementary Fig. 1). Alpha diversity of males and females resulted significantly different when $q=0$, both using the classic (Kruskal–Wallis $H=4.86$, P-value = 0.027) and the phylogenetically informed version (Kruskal–Wallis $H=3.84$, P-value = 0.05) of Hill numbers (Table 3). Specifically, the microbiota of males' was found to be richer than females one. While for values of q higher than zero (when the metric is influenced by the presence of the most abundant ASVs), no significant difference between sexes was recorded (Table 3). Similar results were obtained when a coverage-based integration of interpolation and extrapolation of Hill numbers was used to compare alpha-diversity estimates among sexes. Also in this case, the males' microbiota resulted richer, regardless of the considered q parameter value, but the difference between sexes is emphasized for lower q values (Fig. 3): when comparing sexes at the same sample coverage (*e.g.,* 0.5), diversity estimates for males are almost four times ($q=0$), three times ($q=1$) and two times ($q=2$) higher than females ones (Fig. 3; Supplementary Fig. 2).

Clear differences in the composition of the microbiota of the two sexes have been observed mainly when using presence-absence metrics, that emphasize the effect of rare species ($q=0$ ANOSIM on Hill numbers $R=0.40$, P-value = 0.001; $q=0$ ANOSIM on phylogenetic Hill numbers $R=0.25$, P-value = 0.002). While sex is not particularly determinant when using metrics influenced by the taxon relative abundance and thus emphasizing the effect of the most abundant bacteria ($q=1$ ANOSIM on Hill numbers $R=0.12$, P-value = 0.049; $q=1$ ANOSIM on phylogenetic Hill numbers $R=-0.01$, p-value = 0.48; $q=2$ ANOSIM on hill numbers $R=0.04$, P-value = 0.22; $q=2$ ANOSIM on phylogenetic Hill numbers $R=-0.005$, P-value = 0.42) (Table 3).

Since the multivariate dispersion did not vary between sexes (PERMDISP P-value > 0.24), the significant ANOSIM results represent differences in the centroid of the distributions (Table 3, Fig. 4).

The PCoA performed on the 1-CqN dissimilarity matrix with $q=0$ (Fig. 4a) showed that species membership is the main factor influencing the microbiota structure (ADONIS $R^2=0.279$, P-value = 0.004), followed by the living zone in the wetland (ADONIS $R^2=0.153$, P-value = 0.003), and sex that is still determinant in partitioning the variability of the microbiota (ADONIS $R^2=0.127$, P-value = 0.001). In fact, males are associated with high values on the first PCoA axis while females to

Table 3 Results of Kruskal–Wallis test (alpha-diversity) and ANOSIM (beta-diversity dissimilarities) for males and females microbiota diversity comparison

		q parameter	Statistic	p-value
Kruskal–Wallis	Hill numbers	q = 0	4.86	0.027
		q = 1	0.43	0.51
		q = 2	0.006	0.93
	Phylogenetic Hill numbers	q = 0	3.84	0.05
		q = 1	1.93	0.16
		q = 2	0.81	0.34
ANOSIM	Hill numbers	q = 0	0.40	0.001
		q = 1	0.12	0.049
		q = 2	0.04	0.22
	Phylogenetic Hill numbers	q = 0	0.25	0.002
		q = 1	−0.01	0.48
		q = 2	−0.005	0.42

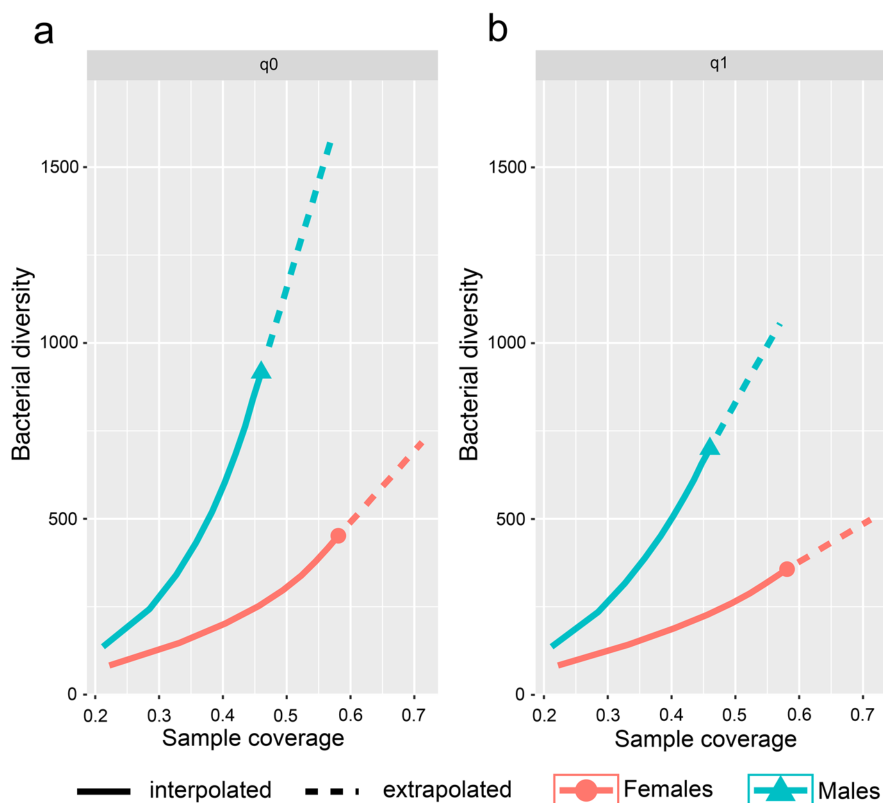

Fig. 3 Microbiota alpha-diversity comparison between males and females. Coverage based interpolation/extrapolation curves of the Hill's numbers estimated for two values of the q parameter: **a** q = 0; **b** q = 1. The x-axis represents the coverage (that estimates the completeness of the sampling) and the y-axis represents the Hill's number estimates. Colours correspond to the two sexes (females in red, males in light blue) and line type to the methodological approach adopted (solid line = interpolation, dashed line = extrapolation)

the lowest (Fig. 4a). From the same analysis performed with $q = 1$ the effect of the sex is much less noticeable (ADONIS $R^2 = 0.039$, P-value = 0.002), the one of the living zone in the wetland is constant (ADONIS $R^2 = 0.144$, P-value = 0.002), while most of the variation is explained by the species membership (ADONIS $R^2 = 0.707$, P-value = 0.001) (Fig. 4b).

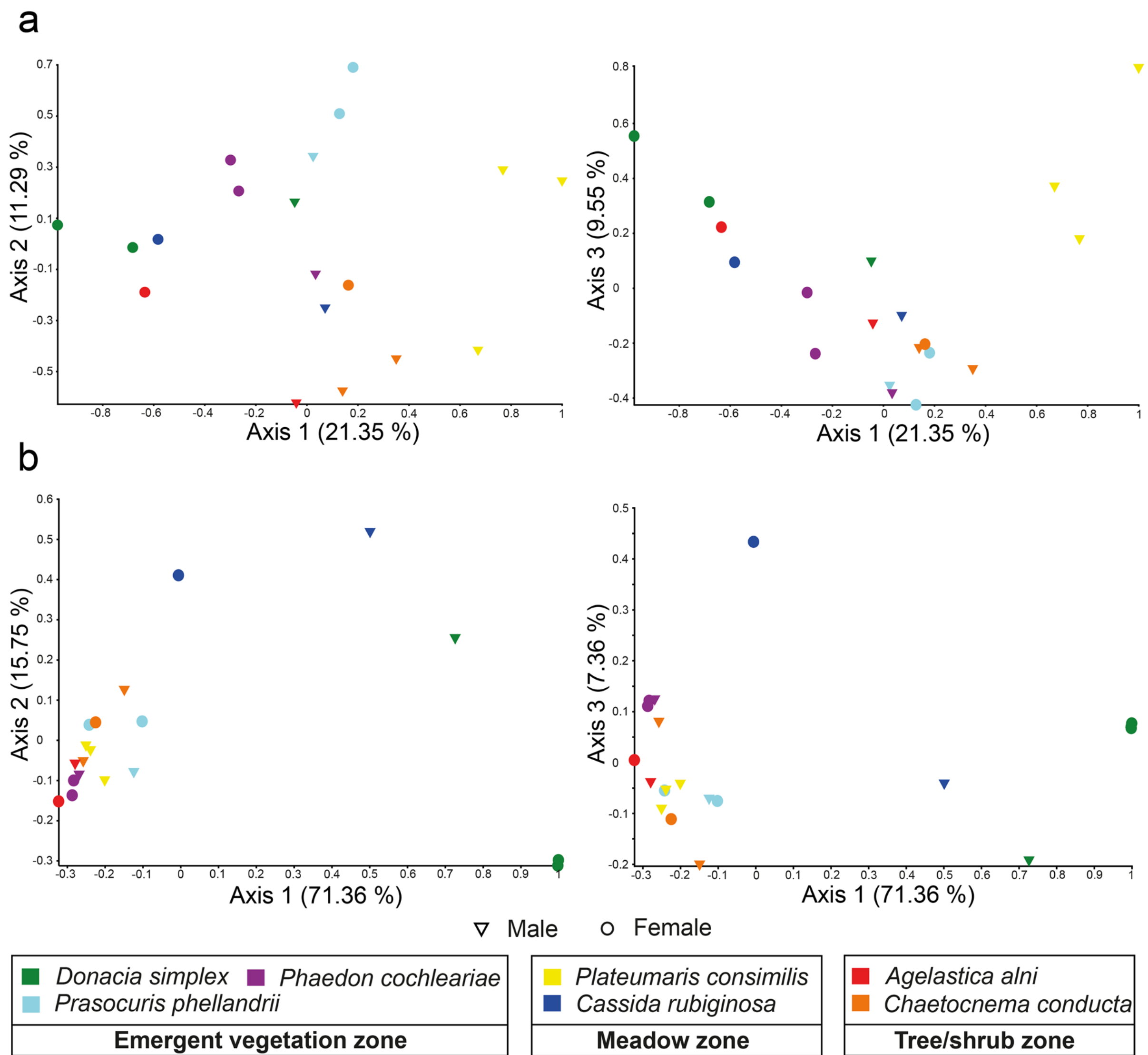

Fig. 4 Principal Coordinates Analysis (PCoA) plots. Plots are based on pairwise beta-diversity dissimilarities (1-CqN) between samples for two values of the order parameter q: **a** q = 0; **b** q = 1. The first two axes of each PCoA are shown on the left side of the figure, first and third axes are on the right side. Colours correspond to the Chrysomelidae species and shapes to the sex, as reported in the legend. Chrysomelidae living zone in the wetland is also reported within boxes in the legend

Discussion

In the present study, the microbiota of seven Chrysomelidae species, including (semi-) aquatic and terrestrial species, inhabiting the vegetation of different zones of a freshwater wetland was characterized. The main aim was to define the drivers of diversity and composition in this peculiar environment where the regular presence of water within and/or on the soil and vegetation can be a source of bacteria proliferation and migration (Taketani et al., 2017). This condition can potentially influence and enhance the environmental acquisition of bacteria in Chrysomelidae inhabiting the wetland vegetation. In general, the obtained results suggest that the microbiota associated with the analysed Chrysomelidae species is shaped by both biotic and abiotic factors. The most abundant bacterial taxa associated with the

analysed Chrysomelidae species were *Wolbachia* and *Rickettsia*, two bacterial genera including the widespread reproduction manipulators that in most insects' species are vertically transmitted from female to the progeny (Giorgini et al, 2010). These bacteria are frequently associated with Chrysomelidae and occur in species with a very different ecology (Montagna et al., 2014; Kolasa et al., 2017; Gómez-Zurita, 2019; Brunetti et al., 2021). *Wolbachia* and *Rickettsia* represented the dominant bacteria in five over seven of the analysed species, this pattern is similar to the one observed in a very different environment, i.e. dry grassland, for other phytophagous species (both Chrysomelidae and Curculionidae, Kolasa et al., 2019). Many different *Wolbachia* haplotypes of the considered 16S rRNA region (18 ASVs were assigned to this genus) were found to be associated with the analysed species, only a few of them widely shared among species. While only a few *Rickettsia* (three ASVs) were shared among the majority of the species letting hypothesize a stronger role of the living environment in spreading the latter among the Chrysomelidae inhabiting the wetland. This scenario is concordant with what was found in previous studies that demonstrated how phytophagous insects can acquire *Rickettsia* via horizontal transmission from the living environment, e.g., from the host plants (Caspi-Fluger et al., 2012) but, as regards the present study case, even a role of water in its diffusion can be postulated. Interestingly, *Wolbachia* and *Rickettsia* were less abundant within the microbiota of the two species *Donacia simplex* (Fabricius, 1775) and *Cassida rubiginosa* (Muller, 1776). As expected, within the microbiota of *D. simplex*, the most abundant bacterium was "*Candidatus* Macropleicola", the well-known Donacinae endosymbiont (Kölsch et al., 2009; Kleinschmidt & Kölsch, 2011; Brunetti et al., 2021). By contrast, in *C. rubiginosa* specimens of this study, the most widespread bacterial symbiont of Cassidinae that was firstly described exactly from this species, namely "*Candidatus* Stammera capleta" (Salem et al., 2017, 2020), was not detected. Further symbionts were found associated also with other analysed species but in a lower relative abundance, as in the case of "*Candidatus* Macropleicola" in *Plateumaris consimilis* (Schrank, 1781) and *Spiroplasma* in *Chaetocnema conducta* (Motschulsky, 1838). Interestingly, the dominant bacterium in *C. rubiginosa* female microbiota (present also in males but in a lower relative abundance) is typically found in soil (*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* complex or *Agrobacterium,* not distinguishable between each other using 16S rRNA sequences; Mousavi et al., 2014). Likely, *C. rubiginosa* could have acquired it while overwintering, in fact adults take refuge in the soil during the coldest months (Kutcherov et al., 2019) (the collection period of this study specimens is compatible with overwintering adults' emergence from litter).

A core of bacteria shared among all the analysed Chrysomelidae was found to be almost absent. Only three ASVs are shared by all samples with a mean relative abundance per specimen $< 1.5\%$ (assigned to *Vibrionimonas magnilacihabitans, Pajaroellobacter* sp., and an unidentified species of the family *Caulobacteraceae*). All of them are bacteria that potentially could have been acquired from the living environment, from wetland water and soil in particular (Albert et al., 2014; Foysal1 et al., 2019; Friedrich et al., 2021). Other bacteria, potentially environmentally acquired and present in the Chrysomelidae microbiota in low relative abundance, were found to be shared between groups of specimens. This finding is concordant with another result of this study, i.e. the living zone in the wetland (emergent vegetation, meadow or tree/shrubs zones) was found to be a factor significantly influencing the microbiota of the analysed Chrysomelidae. A slightly higher relative abundance of potential environmentally acquired bacteria was detected in males, that on the other hand hosted also a lower relative abundance of symbionts than females. This situation is more evident in *C. rubiginosa* and *D. simplex* where the composition of the microbiota drastically changes between sexes. This result, together with the significant difference found in alpha diversity between the two sexes (males have in general a richer microbiota than females, especially in the low abundance component potentially acquired from the environment; Fig. 3), lets hypothesize that males are more prone than females to acquire bacteria from the environment. Whereas sex did not result the main biotic factor shaping the composition and diversity of wetland Chrysomelidae microbiota, in fact a significant difference in the bacterial composition among species was found (Fig. 2, Fig. 4). This result is compatible with the idea that a species-specific microbiota in Chrysomelidae exists. In our study case, the species core microbiota was composed in

a higher abundance of well-known insect endosymbionts (i.e., *Wolbachia* and *Rickettsia*) or symbionts with a functional role in the species (especially in females), and lower by other bacterial taxa potentially acquired from each species in its specific living environment (e.g., the host plant or the wetland living zone in general). Considering that many oligophagous and polyphagous Chrysomelidae exist and that host plant use can vary within the species geographical range, and also that the global distribution of bacteria is influenced by many other factors, the last component of the core could be more population-related than species-related. Anyway, in general, the results concerning the core microbiota presence and the influence of the species membership on the microbiota structure and composition could be biased by the limited intraspecific sampling of this study, including the fact that the specimens were collected from a single plant within the species' host plant range (except for *Donacia simplex*) thus preventing the evaluation of the influence of the host plant use on the intraspecific microbiota variability.

In conclusion, the present study, even if based on a limited sample size (19 individuals of seven species), contributed to examining the factors potentially affecting the composition and diversity of bacteria associated with Chrysomelidae, with a special focus on those living in wetlands of West Palearctic.

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Author contributions GM, MB, and MM contributed to the study conception and design. GM and MM collected and performed morphological identification of the insects. AS participated in the insect sampling activities and identified the insect's host plants. GM performed the molecular biology wet lab work. MB, GM, and LK performed the bioinformatic analyses on the 16S rRNA and COI raw data and the statistical analyses. The first draft of the manuscript was written by MB and GM. All authors contributed to the first draft of the manuscript. All authors read and approved the final manuscript.

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Data availability 16S rRNA gene raw data generated for Chrysomelidae microbiota characterization are available in NCBI SRA under the project PRJNA858425 (Accession numbers: SAMN29715000—SAMN29715018). COI gene sequences used for Chrysomelidae molecular identification are available on BOLD (BOLD IDs: MEDLB951-22 – MEDLB960-22).

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval The authors declare that this manuscript complies with the Publishing Ethics for this journal. The presented research complies with ethical principles and relevant National legislation, both for invertebrate collection and use of DNA resources in DNA taxonomy studies.

Informed consent The manuscript has been read and approved by all named authors.

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