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Multilevel interactions between plants, microbes and insects: ecological and evolutionary constraints underlying interactions

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1. Introduction

The term microbiota includes all the commensal, symbiotic and pathogenic microbes (bacteria, archaea, protists, fungi, viruses) that can be found associated to a multicellular organism or present in a specific habitat (Lederberg and McCray, 2001). Similarly, the term microbiome describes the collective genomes of these microbes in their environmental context, thus considering how the metabolic potential (e.g., structural proteins, enzymes, lipids, signalling molecules, toxins) can be expressed in a specific environmental context (e.g., organs or tissues of a multicellular organism) (Whipps *et al.*, 1988; Rohwer *et al.*, 2002; Berg *et al.*, 2020). In the context of host associated microbial communities this holistic approach led also to two other definitions, with a meaning similar to the previous two, but also including the host organism: holobiont and holobiome (Rosenberg *et al.*, 2007; Zilber-Rosenberg and Rosenberg, 2008). Indeed, in recent years we are increasingly realizing the importance that the influence of the microbiota can have on the physiology and ecology of multicellular organisms. The magnitude of these effects can be impressive and, when the host fitness is involved, the eco-evolutionary dynamics of whole biological populations are affected. In fact, according to the hologenome concept of evolution, the holobiont is more and more often considered a unit of evolutionary selection (Guerrrero *et al.*, 2013; Richardson, 2017) and mathematical models to describe this kind of evolution are being developed (Roughgarden *et al.*, 2018; Roughgarden, 2020). Most of the microbiome studies have been performed on humans, where the gut microbiota has a particular importance since it is involved in several fundamental processes (e.g., nutrient metabolism, xenobiotic and drug metabolism, structural integrity of the gut mucosal barrier, immunomodulation, protection against pathogens), so a healthy gut flora is largely responsible for overall health of the host (Flint *et al.*, 2012; Jandhyala *et al.*, 2015; Valdes *et al.*, 2018). Besides humans the microbiota can have a huge importance also in a lot of other multicellular organisms, like insects.

1.1 The microbiota of insects

Insects are another major focus of microbiota studies, mainly because insects are the most diverse animal class on earth and play a central role in many terrestrial and fresh-water ecosystems, both in term of biomass and ecological role (*i.e.*, they are present at all the levels in terrestrial trophic networks, obviously besides producers, and occupy a huge varieties of ecological niches). Moreover, insects include several model species with well-developed laboratory protocols for breeding and

manipulations, thus allowing an in-depth investigation of the intimate connections that occur between the microbial communities and the eukaryotic host. As example, the gut microbiota of *Drosophila melanogaster* is the main model for studies on the interaction of microbial communities with the host immune system and for all the implications related to aging and neurodegenerative processes (*e.g.*, Fan *et al.*, 2018; Kitani-Morii *et al.*, 2021; Kong *et al.*, 2021; Salim *et al.*, 2021). Several insect species harbour a more or less stable microbiota, including mainly commensal species not affecting the host, but in many cases beneficial effects for the host have been reported (Douglas, 2009, 2015; Engel & Moran, 2013; Clay, 2014; Hurst & Frost, 2015; Wang *et al.*, 2020). The presence of microbes conferring a fitness advantage to the host generates a selective pressure led to maintain the symbiosis through time. The degree of host-symbiont interconnection can range from obligate symbioses (primary or p-symbionts) to facultative symbioses (secondary or s-symbionts), where the symbiont can be experimentally removed without severely affecting the fitness of the host. In the former case, the level of host-microbe interaction developed into a strict mutualism, where the disruption of the partnership can seriously affect the development and survival of the host as well. Another difference among p- and s-symbionts is that the advantages provided by s-symbionts generally is more environment-dependant, so that the benefits may vary over time and space. This factor contributes to the maintenance of variable prevalence in different populations. Obligate symbionts can be located inside specialized cells (endosymbionts), called bacteriocytes that can also aggregate in large number creating bacteria containing structures or organs named bacteriome. P-symbionts are vertically transmitted, and often show drastic genome reduction, usually maintaining only few metabolic pathways involved in the basal metabolism or in providing ecological and functional traits to the host (McCutcheon and Moran, 2012; Lo *et al.*, 2016); such biological and physiological adaptations to intracellular environment are incompatible with independent life outside the host (Boscaro et al., 2017; Latorre & Manzano-Marín, 2017; Ankrah et al., 2018). In general, the most important insect symbionts, tightly associated with the metabolic and physiological activity of the host, can frequently be found in gut associated structures, or hosted in specialized abdominal organs or bacteriomes, or in some tissues and cells in the female genitalia, where they can more easily guarantee their vertical transmission (Stammer, 1935, 1936; Mann & Crowson, 1983; Becker, 1994). Moreover, several microorganisms can colonize the outer surfaces of the insect exoskeleton. Symbiotic fungi are often found in such location where invagination of the integument lined with secretory glands (mycangia) are dedicated to the acquisition and transport of the fungal symbiont, this is often the case of insects with xylophagous larval stages that relies on fungal symbionts to efficiently extract nutrients from

wood (Klepzig and Six, 2004). Several other transmission mechanisms evolved in insects to vertically spread the symbionts to offspring and maintain the infection through generations (Luan *et al.*, 2016; Szklarzewicz and Michalik 2017; Russel, 2019). Symbionts vertical transmission often generate a strict co-speciation between the members of the partnership over evolutionary times and speciation processes (Kölsch and Pedersen 2010; Xu *et al.*, 2018; Bolaños *et al.*, 2019, Salem *et al.*, 2020). Another important phenomenon, reported in different insect taxa, is the horizontal transmission of symbionts, demonstrated by the presence of phylogenetically identical or highly similar bacteria in distantly related hosts or on the host plants (Pistone *et al.*, 2014; Kolasa *et al.*, 2017; Cardoso and Gómez-Zurita, 2020). A particular group of this highly specialized bacteria are the so-called 'reproductive manipulators' (e.g., *Wolbachia*, *Rickettsia*, *Cardinium*, *Spiroplasma*) that should be perhaps grouped into a special category of sexual parasites or reproduction parasites. These bacteria exhibit a wide range of behaviours and exert different effects on the hosts, but in general they share the capacity to interfere with the insect reproductive processes. One of the main characteristics is to be able to maintain their infection across insect generations, often increasing the number of infected females; a mechanism contributing and favouring their spreading in the host population (*e.g.*, Harris *et al.*, 2010; Correa and Ballard, 2016; Larracuente and Meller, 2016). These bacteria can secure their evolutionary success with a variety of different strategies including cytoplasmic incompatibility (CI), male-killing, induction of parthenogenesis, and feminization of genetic males (Fialho and Stevens, 2000; Narita *et al.*, 2007; Takano *et al.*, 2017).

Herbivorous insects are particularly interesting in the context of holobiome studies (Hansen & Moran, 2014; Giron *et al.*, 2017; Mason *et al.*, 2019, 2020; Frago *et al.*, 2020), because of their ecological and economic importance, but also because plants can be a very challenging food source. In fact, plant tissues are usually poor in nutrients, difficult to digest (rich in complex macromolecules like cellulose and lignin) and are often well defended by chemical barriers (e.g., toxic compounds of the plant secondary metabolism). So, the presence of prokaryotic symbionts, providing a complex biochemical machinery with different origins, highly expand the insect metabolic potential and can also improve the nutritional efficiency and extend the range of possible food sources. Microbial symbionts are able to confer several physiological traits that make herbivorous insects able to use plants as a food source: e.g., providing essential nutrients that are not present in the plant-based diet (*e.g.*, Buchner, 1965; McCutcheon & Moran, 2007; Russel *et al.*, 2013; Hansen *et al.*, 2020), producing enzymes able to digest the complex plant macromolecules (*e.g.*, Anand *et al.*, 2009; Salem *et al.*, 2017; Luo *et al.*, 2019; Reis *et al.*, 2020) and/or detoxifying toxic compounds (*e.g.*, Kikuchi *et*

al., 2012; Adams *et al.*, 2013, van den Bosch & Welte, 2017; Itoh *et al.*, 2018) present in the plant tissues. The insects can become extremely dependent on its symbionts, experiencing a drastic fitness reduction when the symbionts are removed (Berasategui *et al.*, 2017; Cai *et al.*, 2018; Li *et al.*, 2019).

Due to the huge taxonomic diversity of insects and the resulting even bigger complexity of the associated microbial communities here I will focus only on the microbiota of species belonging to Coleoptera, the order that includes also the family Chrysomelidae (leaf beetles), which is the main focus of this thesis.

1.2 Taxonomic composition of beetles' microbiota

The major component of the microbiota in the order Coleoptera, at least in terms of diversity, is represented by bacteria and most studies are focused on this component of the microbiota. Four bacterial phyla (Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes) cover most of the bacterial diversity encountered in beetles. Among these phyla some families or genera are rather widespread and ubiquitous in different beetle taxa such as Gammaproteobacteria, Alphaproteobacteria, Bacteroidetes, Firmicutes, Lactobacillus, Bacillus, Clostridium, Actinomycetes, Spirochetes, Verrucomicrobia, and Actinobacteria (Colman *et al.*, 2012). Regarding Fungi, three phyla (Ascomycota, Zygomycota, and Basidiomycota) are widely represented in beetles, but such communities can be deeply influenced either by the diet (e.g wood feeding beetles) or environmental fungi derived by host plant and tissue type (Ziganshina *et al.*, 2018). Moreover, archaea species are rarely reported in beetles' microbiota, but the phyla Euryarchaeota and Crenarchaeota have been detected in the hindgut of the scarab beetles *Oryctes nasicornis* and *Amphimallon solstitiale* (Ziganshina *et al.*, 2018). Viruses are also often reported in beetles, especially in herbivorous species that can act as vectors of plant pathogens infecting also economically important crops. As example, adults and larval stages of Chrysomelidae can regurgitate during feeding, thus allowing the transmission to the plant of viruses belonging to the genera *Tymovirus*, *Comovirus*, *Bromovirus* and *Sobemovirus* (Bhat and Rao, 2020).

1.3 Factors shaping beetles' microbiota composition and diversity

The composition and diversity of the insects' microbiota can exhibit large variability among individuals of the same host species depending on several biotic and abiotic factors (Colman *et al.*, 2012; Yun *et al.*, 2014). The geographic location is probably one of the main factors shaping the beetles' microbiota, especially the transient part that is acquired from the environment (mainly through the diet). In fact, the composition of the environmental microbial community changes in different environments and varies along geographic gradients (*e.g.*, latitude, altitude), thus also influencing the microbiota associated to multicellular organisms. As example, two *Dendroctonus* species (*D. valens* and *D. mexicanus*) sampled in different locations in Mexico show variation in the microbiota composition, but mainly in the rarer taxa and no correlation between differences in the microbiota composition and the geographic distance was found (Hernández-García et al., 2018). On the contrary, different populations of *D. valens*, sampled in the USA from Wisconsin to Oregon, showed significant differences in the bacterial microbiota and the strength of these differences was positively correlated with the distance between sites (Adams *et al.*, 2010). The fungal component of the microbiota can be also affected by geographic factors. A study on the outbreak of *D. ponderosae* in Canada identified significant spatial patterns in fungal species abundances, indicating symmetrical replacement along a latitudinal gradient and little variation in response to altitude (Roe *et al.*, 2011). On the other hand, altitude was identified as the main factor shaping the bacterial microbiota of beetle species in the *Chryptocephalus marginellus* complex (Montagna *et al*., 2015a). Besides macroscopic geographical gradients beetles' microbiota is affected also by local environmental conditions. For example, bacterial diversity and community structure of the hindgut microbiota of *Holotrichia parallela* larvae (Scarabeidae) vary across populations occupying different geographic locations, and the observed variation can be explained by environmental factors related to soil (pH, organic carbon, total nitrogen), and climate (e.g., mean annual temperature) (Huang and Zhang, 2013). In other species the microbiota resulted more stable across different populations of the same species, such as the case of *Diabrotica virgifera* in the USA (Ludwick *et al.*, 2019) and two weevil species from the Negev desert of Israel (Meng *et al.*, 2019).

The most important interactions between the insect and the environmental context occur through the alimentation. In fact, the differences among the gut microbiota of beetle populations occupying different environments can be often related to the different trophic sources present. Such as the case of populations of different species of lady beetle occupying two different environments (soy fields and prairies), insects from soy fields have richer gut bacteria and lower fat content than those from

prairies, suggesting that the different composition of the microbiota is related to the diet, specifically to the better feeding conditions offered by prairies (Tiede *et al.*, 2017). The importance of the diet in shaping the gut microbiota has been demonstrated in beetles with different trophic attitudes: herbivores (Montagna *et al.*, 2015b; Xu *et al.*, 2016; Zhang *et al.*, 2018), omnivores (Ben Guerrero *et al.*, 2016) and predators (Tiede *et al.*, 2017). The influence of the diet on insect gut microbiota is particularly evident in phytophagous species, especially those feeding on toxic plants. For example, three weevils (Coleoptera) and two lycaenid butterflies (Lepidoptera) species, feeding on the same toxic plants (cycads), share a core set of bacteria, not present in their non-cycad-feeding relatives, that probably help the insects in the detoxification process (Salzman *et al.*, 2018). Similarly, also the microbiota of *Hylobius abieti*, the large pine weevil, is distinct from that of closely related weevils feeding on non-conifer plants, while is very similar to that of bark beetles that also exploit conifers as a food source (Berasategui *et al.*, 2016).

Besides environmental factors, beetles' microbiota composition and diversity are also highly influenced by characteristics of the insect host. Since Coleoptera are holometabolous insects, with larvae often occupying a different ecological niche from that of adults, the microbiota of different developmental stages can vary a lot. Various studies carried on Cerambycidae (Vasanthakumar *et al.*, 2008; Kim *et al.*, 2017; Zhang *et al.* 2018), Chrysomelidae (Ali *et al.*, 2019), Curculionidae (Morales-Jiménez *et al.*, 2012; Briones-Roblero *et al.*, 2017) and Scarabeidae (Huang and Zhang, 2013; Shukla *et al.*, 2016; Chouaia *et al.*, 2019) identified a core gut microbiota shared across different life stages (egg, larva, pupa, imago) but also fluctuations in the presence and abundance of several bacterial taxa, possibly related to changes in the host's ecological and physiological needs. The same group of studies identified different patterns regarding changes in gut microbiota alpha-diversity during insect development. The microbiota of Cerambycidae shows the highest level of diversity in the pupal stage, while larvae and adults have lower diversity levels, (usually the larval microbiota is slightly richer than that of adults). On the other hand, species of Chrysomelidae and Scolytinae shows a trend of increasing richness of the microbiota during the development, with the adults showing the highest microbial diversity. Conversely, Scarabeidae have the highest microbiota richness during the larval stages while adults show a drop in microbial diversity. These differences probably reflect the different physiological and ecological needs experienced during the development by beetles with diverse ecologies, and underlies the importance of the pupal stage for the necessary rearrangement of the microbiota of holometabolous insects that after the metamorphosis often switch to a new ecological niche, different from that of the larval stages (Hammer and Moran, 2019).

Another characteristic of the host often supposed to influence the beetles' microbiota, but less studied, is the sex. Few studies, on *Dendroctonus valens* (Curculionidae) in China (Xu *et al.*, 2016) and two *Euoniticellus* species (Scarabeidae) in South Africa (Shukla *et al.*, 2016), found differences among the microbiota of male and female beetles, both in term of composition and diversity, with the females showing a richer microbiota. Conversely, a study on the microbiota of *Octodonta nipae* (Chrysomelidae) found no clear difference between the microbiota of the two sexes (Ali *et al.*, 2019).

Microbial colonization in beetles' gut is dependent on the physicochemical conditions, so different microbial communities can be hosted in different gut compartments (Engel and Moran, 2013). Most of the studies comparing the microbiota of different gut region have been performed on Cerambycidae (Kim *et al.*, 2017) and Scarabeidae (Egert *et al.*, 2003, 2005; Chouaia *et al.*, 2019), that are characterized by a pattern of increasing microbial diversity along the gut, with the hindgut having the richer microbiota and, in most cases, by significant differences among the microbiota composition of different gut regions.

1.4 Symbioses in beetles and their effect on the insects' fitness

Symbiotic microbes can leave a profound evolutionary mark on their hosts, allowing to access novel nutritional resources, to occupy highly constrained niche or even to promote speciation. One of the most important aspects of insect biology that is affected by the presence of symbionts is nutrition. Most of the symbionts of beetles are somehow involved in the nutrition process, especially in the case of phytophagous insects. Species in the Phytophaga clade (Chrysomeloidea and Curculionoidea), share a conserved set of cellulases, xylanases, and pectinases that have been acquired with a series of horizontal gene transfer events from bacterial and fungal donors (McKenna *et al.*, 2019). However, several independent losses of these genes occurred along the Phytophaga phylogeny but were often offset through the acquisition of heritable symbionts providing the lost enzymes (Salem *et al.*, 2017, 2020; Reis *et al*., 2020; Berasategui and Salem, 2020, 2021). In reed beetles (Chrysomelidae, Donaciinae) bacterial species of the genus '*Candidatus* Macropleicola' are harboured in specialized organs associated to the gut and provide life stage-specific benefits to larvae and adult beetles. In the plant sap-feeding larvae, the symbionts are inferred to synthesize most of the essential amino acids as well as the B vitamin riboflavin lacking in their diet, while in adults, symbiont-encoded pectinases complement the host-encoded set of cellulases (Reis *et al*., 2020). Similarly in another subfamily of

Chrysomelidae (Cassidinae), the bacteria '*Candidatus* Stammera capleta' is hosted in specialized organs associated with the foregut-midgut junction of several species and is involved in pectinase production (Salem *et al.*, 2017, 2020). As already seen in the case of reed beetle's larvae (Reis *et al.*, 2020), symbiotic nutritional advantages often include the provisioning of essential nutrients lacking in an unbalanced diet based on poor food sources, like plant sap, wood, or vertebrate blood (Douglas *et al.*, 2015). The gut of wood feeding species of longhorn beetles (Cerambycidae) harbours bacterial symbionts that are involved in the production of essential amino acids and in nitrogen fixation and recycling (Ayayee *et al.*, 2014; Scully *et al.*, 2014). A role of the gut microbiota in nitrogen fixation has been hypothesized also in Passalidae (Ceja-Navarro *et al.*, 2014), Scarabeidae (Alonso-Pernas *et al.*, 2017) and Curculionidae (Morales-Jiménez *et al.*, 2009; Bar-Shmuel *et al.*, 2020). A particularly important biosynthetic pathway for Coleoptera is the one for the biosynthesis of tyrosine. Indeed, tyrosine is the precursor for the biosynthesis of melanin and catecholamines, that are fundamental molecules for the sclerotization and tanning of the insect cuticle, extremely important in beetles due to their huge investment in the development of highly sclerotized front wings, the elytra (Noh *et al.*, 2016). Since the pathway for the production of aromatic compounds is usually lacking in insects, beetles feeding on diet with a limited amount of molecular precursors for tyrosine biosynthesis can benefit from the presence of bacteria providing those compounds (Lemoine *et al.*, 2020). This phenomenon has been well studied in Curculionidae (Kuriwada *et al.*, 2010; Anbutsu *et al.*, 2017; Hirota *et al.*, 2017; Engl *et al.*, 2018), but it has been inferred also for other beetle groups. The functional role of beetles' symbionts in host nutrition has not been always clarified. As in the case of pollen-feeding beetles in the genus *Dasytes* (Dasytidae), where intracellular symbionts ('*Candidatus* Dasytiphilus stammeri') were detected in bacteriomes associated with the mid- to hind-gut transition in adult male and female beetles. Given the specialized pollen-feeding habits of the adults (larvae are carnivorous), the symbionts may provide essential amino acids or vitamins, or they might produce digestive enzymes that break up the fastidious pollen walls and thereby contribute to the host's nutrition (Weiss and Kaltenpoth, 2016).

Besides providing digestive enzymes and essential nutrients, the bacterial symbionts of beetles can also confer resistance to toxic compound, that is often fundamental for phytophagous beetles feeding on poisonous plants. As example, the coffee berry borer, *Hypothenemus hampei* (Curculionidae), is able to complete its life cycle within the seed of the coffee plant, where the plant accumulates caffeine, an alkaloid that inhibiting the phosphodiesterase activity causes intoxication and paralysis in insects (Nathanson, 1984; Guerreiro and Mazzafera, 2003). If *H. hampei* larvae are treated with antibiotics

the detoxifying function is compromised and none is able to complete pupation (Ceja-Navarro *et al.*, 2015). The demethylase-encoding bacteria *Pseudomonas fulva* is the most plausible candidate for the detoxification process of caffeine, since it is consistently isolated from the gut of *H. hampei* but is absent in the microbiota of close species in the same genus (Ceja-Navarro *et al.*, 2015). A similar problem is experienced by beetles feeding on conifers, that accumulates flavonoids and terpenoids in bark and cambium (Keeling and Bohlmann, 2006). Terpene-degrading bacteria have been isolated in vitro from bark beetles and metagenomic sequencing of their microbiota revealed the enrichment in genes involved in terpene degradation (Adams *et al.*, 2013; Berasategui *et al.*, 2017). Also the detoxification of conifer's flavonoids, in bark beetles, involves the activity of bacterial gut symbionts (Cheng *et al.*, 2018). Beetles can exploit their microbiota also to control plant defences, like the case of the Colorado potato beetle, *Leptinotarsa decemlineata* (Chrysomelidae), that secreting its oral microbiota on the plant induces the upregulation of antimicrobial defences and consequently the downregulation of the signalling pathway dedicated to herbivores (Chung *et al.*, 2013, 2017).

The nutritional aspects of beetles-microbiota interaction have been studied in depth in the case of phytophagous species but also carnivores can take advantage of their microbiota for feeding purposes. The most studied case is probably represented by burying beetles (Silphidae, Necrophorinae) whose diet is composed by carcasses of small vertebrates that adults bury in the soil to nourish the offspring (Scott, 1998). Fresh carcasses represent a better-quality food source respect to decaying carcasses, so the beetles are able to manipulate their food source to prevent decaying (Rozen *et al.*, 2008). It is possible thanks to the antimicrobial secretion released by the beetle on the carrion (Hall *et al.*, 2011, Arce *et al.*, 2012) but also to the insect gut microbial community that includes both bacteria (Enterobacteriales, Xanthomonadales, Neisseriales, Lactobacillales, Clostridiales) and yeasts (*Yarrowia* sp.) (Kaltenpoth and Steiger, 2014; Vogel *et al.*, 2017). These microbes grow on the carcass in a biofilm like matrix preventing the growth of antagonistic bacteria that would lead to putrescence and produce digestive enzymes (mainly lipases) that may facilitate larval nutrition (Duarte *et al.*, 2018; Shukla *et al.*, 2018a; Wang and Rozen, 2018). This mechanism also ensures the vertical transmission of these fundamental gut symbionts to the offspring (Shukla *et al.*, 2018b).

Besides facilitating the nutrition process, the microbiota of beetles is often involved also in the protection from pathogens and natural enemies. Several microbial isolates obtained from beetles' microbiota show inhibitory activity against pathogens (Blackburn *et al.*, 2008; Heise *et al.*, 2019; Skowronek *et al.*, 2020). The gut microbiota of the red palm weevil *Rhynchophorus ferrugineus*

(Curculionidae) have a strong immunostimulatory effects. Aposymbiotic larvae show a compromised immune response and an increased susceptibility to pathogens (Muhammad *et al.*, 2019). Another interesting case of defence from pathogens is represented by the symbioses between the bacteria *Burkholderia gladioli* and tenebrionid beetles in the Lagrinae subfamily. Multiple strains of *B. gladioli* produce various bioactive compounds (*e.g.*, lagriamide) that protect the eggs from the fungal pathogens present in the soil where eggs are laid (Flórez and Kaltenpoth, 2017; Flórez *et al.*, 2017, 2018). Beside protection from pathogens the beetles' microbiota is also involved in the interaction with predators. As example, the most vulnerable stages (*i.e.*, eggs and first instar larvae) of beetles in the genus *Paederus* (Staphylinidae) are chemically defended by predation (*e.g.*, by wolf spiders) due to the accumulation of pederin in their tissues (Kellner, 2002; Piel *et al.*, 2004). This toxic amide is produced by a bacterial symbiont in the genus *Pseudomonas* and not by the insect itself (Kellner and Dettner, 1996).

1.5 Aims of the thesis

The main aim of this thesis is to investigate the principal factors shaping microbiota composition and diversity of phytophagous beetles, using leaf beetles (Coleoptera: Chrysomelidae; Figure 1) as case study. The family Chrysomelidae includes ~40,000 species worldwide, all of them feed on leaves or other plant organs at least at the adult stage showing a highly variable degree of trophic specialization (Leschen and Beutel, 2014). Moreover, the microbiota of leaf beetles, as mentioned above, is characterized by the presence of several vertically transmitted bacterial symbionts that are also involved in facilitating host nutrition (*e.g.*, *Stammera* in the Cassidinae, *Macropleicola* in the Donacinae). This makes Chrysomelidae a perfect model for investigating the ecological (*e.g.*, diet) and physiological factors (*e.g.*, sex) affecting the composition and structure of the microbiota of phytophagous beetles, and to further investigate the presence of vertically transmitted symbionts. In details, this thesis is composed of three main studies. The first one (section 2.1) characterize the bacterial microbiota of a selection of Euro Mediterranean species of Chrysomelidae and investigate the effects of the breadth of the diet spectrum in shaping its composition and diversity. The results of this study have been already published in a peer reviewed journal (Environmental microbiology). The second study (section 2.2) explores the bacterial microbiota of seven species of Chrysomelidae sampled in the same environment, with a focus on the differences between the microbiota of male and female insects. The manuscript reporting this result is still in preparation. The third study (section

2.3) is derived from the results of the first study, where two possible bacterial symbionts have been identified in three species of the Eumolpinae subfamily of Chrysomelidae. So, a genomic approach was applied to further characterize these possible symbioses, but the data analysis is still ongoing so I will report only the preliminary results of this study.

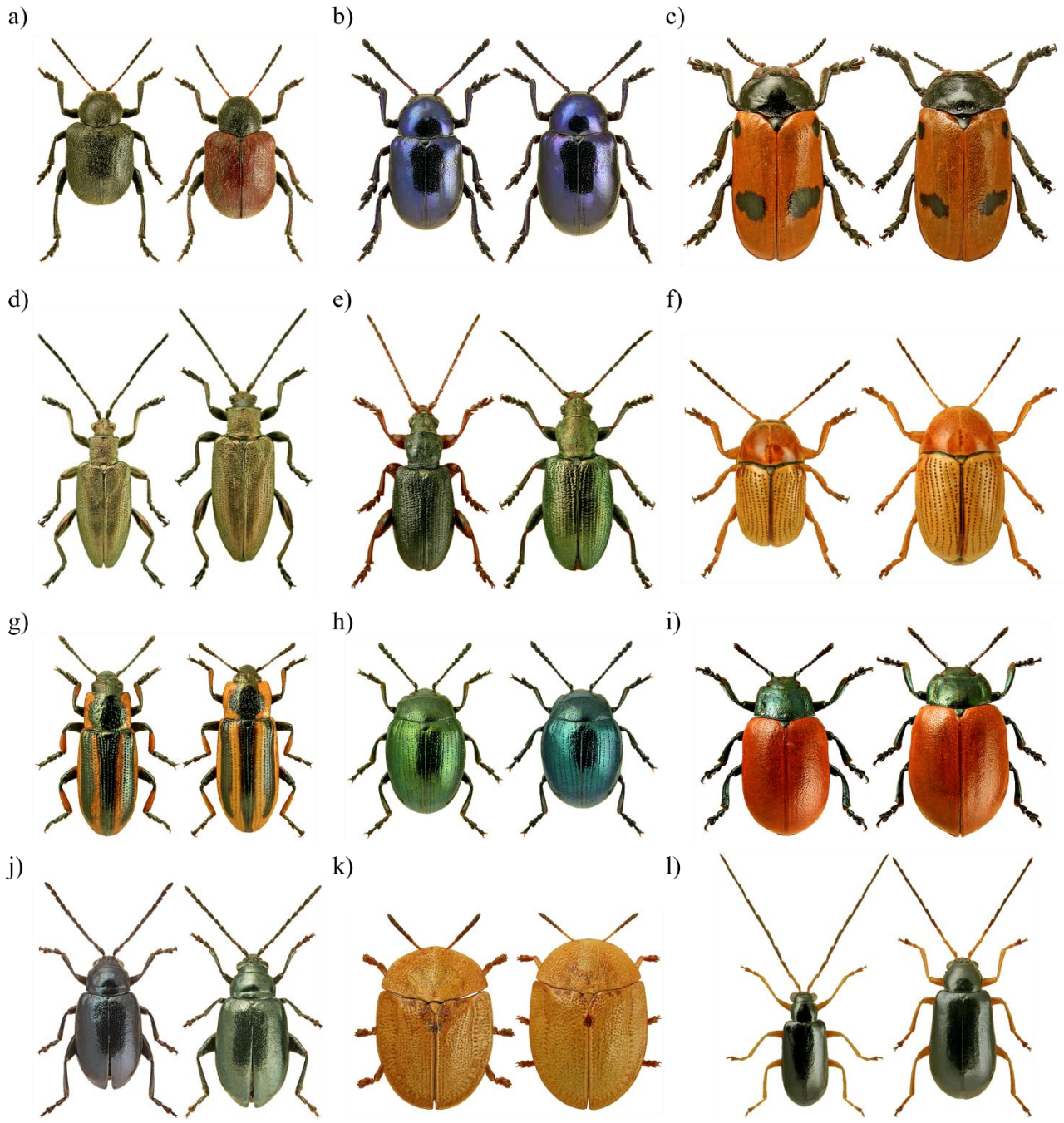

Figure 1. Pictures representing some of the Chrysomelidae species studied in this thesis. In each picture males are on the left and females on the right. a) *Bromius obscurus*, b) *Chrysochus asclepiadeus*, c) *Clytra quadripunctata*, d) *Donacia obscura*, e) *Plateumaris consimilis*, f) *Cryptocephalus fulvus*, g) *Prasocuris phellandrii*, h) *Phaedon cochleariae*, i) *Chrysomela saliceti*, j) *Altica oleracea*, k) *Cassida rubiginosa*, l) *Luperus longicornis*. Images courtesy of Dr. Lech Borowiec (http://www.cassidae.uni.wroc.pl/Colpolon/chrysomelidae.htm).

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2. Results

2.1 Research article

Brunetti, M., Magoga, G., Gionechetti, F., De Biase, A., & Montagna, M. (2021). Does diet breadth affect the complexity of the phytophagous insect microbiota? The case study of Chrysomelidae. *Environmental Microbiology.* doi:10.1111/1462-2920.15847

2.1.1 Summary

Chrysomelidae is a family of phytophagous insects with a highly variable degree of trophic specialization. The aim of this study is to test whether species feeding on different plants (generalists) harbour more complex microbiotas than those feeding on a few or a single plant species (specialists). The microbiota of representative leaf beetle species was characterized with a metabarcoding approach targeting V1–V2 and V4 regions of the bacterial 16S rRNA. Almost all the analysed species harbour at least one reproductive manipulator bacteria (e.g., *Wolbachia*, *Rickettsia*). Two putative primary symbionts, previously isolated only from a single species (*Bromius obscurus*), have been detected in two species of the same subfamily, suggesting a widespread symbiosis in Eumolpinae. Surprisingly, the well-known aphid symbiont *Buchnera* is well represented in the microbiota of *Orsodacne humeralis*. Moreover, in this study, using Hill numbers to dissect the components of the microbiota diversity (abundant and rare bacteria), it has been demonstrated that generalist insect species harbour a more diversified microbiota than specialists. The higher microbiota diversity associated with a wider host-plant spectrum could be seen as an adaptive trait, conferring new metabolic potential useful to expand the diet breath, or as a result of environmental stochastic acquisition conveyed by diet.

2.1.2 Manuscript

Introduction

Insects are colonized by a variety of microorganisms, prevalently living as commensals, but which in many cases can confer either beneficial or detrimental effects to their host (e.g., Douglas, 2009; Kikuchi *et al.*, 2012; Engel and Moran, 2013; Clay, 2014; Douglas, 2015; Hurst and Frost, 2015; Wang *et al.*, 2020). Since symbiont-mediated traits highly influence the host nutrition, in herbivorous

insects this influence is often crucial in the interaction with the host plant (Hansen and Moran, 2014; Giron *et al.*, 2017; Mason *et al.*, 2019; Frago *et al.*, 2020; Mason, 2020). Most of the microorganisms that can be found within the insect body colonize the gut lumen, but they can be also hosted in specialized organs often connected to female genitalia, especially when the vertical transmission of the symbiont is required (Stammer, 1935, 1936; Mann and Crowson, 1983; Becker, 1994). The most specialized bacterial symbionts live inside the insect cells are vertically transmitted, and show drastic genome reduction (e.g., *Blattabacterium*, *Buchnera*), usually maintaining only the metabolic pathways involved in providing functional traits to the host (Boscaro *et al.*, 2017; Latorre and Manzano-Marín, 2017; Ankrah *et al.*, 2018). Other bacteria are able to colonize insect cells, such as the reproductive manipulators belonging to the so-called male-killing group (e.g., bacteria of the genera *Wolbachia* and *Rickettsia*) that can manipulate the host reproduction to maintain their infection across generations and spread within the population (Harris *et al.*, 2010; Correa and Ballard, 2016; Larracuente and Meller, 2016). Most studies investigating the relationship between bacterial symbionts and the insect host have been conducted on model species, mainly focusing on single interactions. More recently the advent of next-generation sequencing techniques coupled with the 16S rRNAbased approach for the bacterial taxonomy has greatly facilitated the characterization of the full microbiota associated with non-model organisms, allowing to expand the experimental scale (e.g., Montagna *et al.*, 2015a; Mohammed *et al.*, 2018; Ziganshina *et al.*, 2018; Kolasa *et al.*, 2019). This innovation opened the possibility to characterize the microbiota associated with several wild species and so to investigate the correlations between the composition of microbial communities and several ecological or physiological traits of the insect host, such as the breadth of the insect diet (Colman *et al.*, 2012; Yun *et al.*, 2014). Indeed, insects feeding on several plant species may be expected to harbour more complex microbial communities. The gut microbiota composition can be influenced by the diet, directly since food may inoculate bacteria able to colonize the insect gut or indirectly by promoting the growth of specific bacteria (Pérez-Cobas *et al.*, 2015; Montagna *et al.*, 2015b; Chouaia *et al.*, 2019; Muturi *et al.*, 2019). So, insects with a wider food source spectrum are expected to be colonized by a higher diversity of microbial taxa. Anyway, the higher diversity in the microbiota of generalist species could also be due to the wider metabolic potential, conferred by the presence of a more variegated microbial community, which makes those insects able to exploit several different food sources. The covariation of microbiota diversity and breadth of the animal diet has been investigated also in non-insect taxa, usually achieving inconclusive results that do not support the hypothesis of a higher diversity in the microbiota of generalist species (e.g., Kartzinel *et al.*, 2019;

Chen *et al.*, 2021). Leaf beetles (Coleoptera: Chrysomelidae), including ~40 000 species worldwide, constitute one of the most diverse insect groups in the world (Leschen and Beutel, 2014). This Coleoptera family includes almost only phytophagous species, feeding on leaves or other plant organs at least at the adult stage. The degree of trophic specialization is highly variable, since some leaf beetle species can exploit only one or few specific plant species as a food source, while others can feed on hundreds of plant species belonging to several different families. This makes Chrysomelidae a perfect model to investigate the relationship between the level of microbiota complexity and the breadth of the host plant spectrum. Furthermore, leaf beetles are of great interest for the presence of vertically transmitted symbionts (i.e., in Donacinae, in Cassidinae and in Eumolpinae), which are harboured in specialized host organs associated with gut and genitalia (Stammer, 1935, 1936; Tayade *et al.*, 1975; Mann and Crowson, 1983; Becker, 1994). Bacteria of the genus '*Candidatus* Macropleicola' (Enterobacteriaceae) are hosted in specialized organs at the midgut–hindgut junction of Donacinae. These bacteria show a tight co-speciation with the insect host and are involved in supporting its nutrition providing essential nutrients during the larval stage (essential amino acids, riboflavin) and digestive enzymes (pectinases) to the adult insects (Kölsch *et al.*, 2009; Kölsch and Pedersen, 2010; Kleinschmidt and Kölsch, 2011; Reis *et al.*, 2020). Similarly, '*Candidatus* Stammera capleta' (Enterobacteriaceae), hosted in specialized organs associated with the foregut of several Cassidinae species, is involved in pectinase production (Salem *et al.*, 2017, 2020). Within Eumolpinae only a single species is known to host symbionts in specialized organs, *Bromius obscurus* (Stammer, 1936). This symbiosis has been less studied, but two different symbionts have been isolated from it (Kölsch and Synefiaridou, 2012). The first one (henceforth *B. obscurus* symbiont A) is hosted intracellularly in blind sacs at the foregut–midgut junction and extracellularly in female specific genital accessory organs, suggesting the presence of vertical transmission. The second one (henceforth *B. obscurus* symbiont B) is hosted in small crypts at the end of the midgut and is phylogenetically related to bacteria species living in the gut lumen, not tightly associated with the host (Kölsch and Synefiaridou, 2012; Fukumori *et al.*, 2017). Previous studies on the bacteria associated to leaf beetles were mainly focused on reproductive manipulators (Clark *et al.*, 2001; Keller *et al.*, 2004; Kondo *et al.*, 2011; Roehrdanz and Wichmann, 2013; Montagna *et al.*, 2014; Krawczyk *et al.*, 2015; Kolasa *et al.*, 2017; Takano *et al.*, 2017; Gómez-Zurita, 2019), single species of economic importance (Muratoglu *et al.*, 2011; Chung *et al.*, 2013; Ali *et al.*, 2019; Ludwick *et al.*, 2019; Wang *et al.*, 2019; Shukla and Beran, 2020) or few strictly related species (Kelley and Dobler, 2011; Montagna *et al.*, 2015a; Blankenchip *et al.*, 2018; Wei *et al.*, 2020). The present study aims to

characterize the microbiota associated with a selection of leaf beetle species, representative of the taxonomic diversity and of the various degrees of trophic specialization. In detail, it aims: (i) to determine the principal bacterial taxa that characterize the microbiota of the selected leaf beetle species, also detecting the presence of important insect symbionts (e.g., *Wolbachia*) and symbionts typically present in specific Chrysomelidae subfamilies (e.g., Donacinae, Cassidinae); (ii) to test the hypothesis that the microbiota of generalist phytophagous species is more complex than the microbiota of more specialist species.

Results

Efficiency and taxonomic resolution of the 16S rRNA gene V1–V2 and V4 regions

From the 30 Chrysomelidae species analysed a total of 841 822 (mean per sample = 28 060.7) and 1 711 075 (mean per sample = 57 035.8) raw reads have been obtained from the sequencing of the V1–V2 and V4 regions of the bacterial 16S rRNA respectively. Raw sequences have been deposited on the NCBI SRA database under the project accession number PRJNA729224. After the denoising and filtering steps, the V1–V2 dataset consisted of 1080 amplicon sequence variants (ASVs) (total reads = 449 368; mean per sample = 14 978.9) and the V4 dataset consisted of 1572 ASVs (total reads = 1 047 226; mean per sample = 34 907.5). All the ASVs assigned to mitochondria (<0.01% of the V1–V2 reads, 0.74% of the V4 reads) or chloroplast (45.3% of the V1–V2 reads, 17.8% of the V4 reads) have been excluded from further analyses. Regarding the taxonomic identification of the ASVs, 119 bacterial genera have been identified by both regions while 162 genera are present only in the V4 dataset and 35 genera only in the V1–V2 dataset. Comparing the results of the taxonomic assignment of the two regions, the V4 region results the marker almost always more efficient in detecting bacterial taxa (Supplementary Fig. 1), with few exceptions in which those for the V1–V2 region slightly outperform the others (e.g., the genera *Brevundimonas* and *Aeromonas*). The estimated diversity using the two regions separately (Supplementary Fig. 2) is identical when putting much weight on the most abundant species (q = 2), while the V4 region provides slightly higher estimates when increasing the weight of rare species (q = 1, q = 0).

Microbiota composition

The most represented bacterial classes associated to the analysed Chrysomelidae species (Figs 1A and 2) are Alphaproteobacteria (~39%), Gammaproteobacteria (~45%) and Bacilli (~14%). Several genera belonging to Bacteroidia are also present in the microbiota of the selected species (Fig. 2) but this class constitutes only ~1% of the total dataset. Within Alphaproteobacteria the most abundant genera recorded are *Wolbachia*, *Rickettsia* and *Sphingomonas*. *Wolbachia* is the most represented genus in the dataset (~30% of the total reads) and sequences belonging to this genus have been found in all the species except *Chrysomela saliceti* and *Timarcha tenebricosa* (Fig. 1B, Supplementary Table 1). In most cases *Wolbachia* sequences represent a low percentage of the sample reads (<1%), while in eight species it comprises the most abundant ASVs. *Rickettsia* is another well-represented genus (Fig. 1B, Supplementary Table 1). Reads assigned to *Rickettsia* have been found in nine species and represents a high percentage of the reads from *Hispa atra* (~31%) and from all the sampled species belonging to Clytrini tribe (*Labidostomis longimana* ~18%, *Clytra quadripunctata* ~44%, *Smaragdina affinis* ~92%). *Sphingomonas* is also quite common in the dataset (Fig. 1B). It is present in all the sampled species, with the only exception of *Plateumaris consimilis*, and it reaches the highest densities in *Timarcha tenebricosa* (~10%) and *Cryptocephalus transcaucasicus* (~30%). Within Gammaproteobacteria the most abundant genus is *Pseudomonas* (Fig. 1A). *Pseudomonas* is the second most abundant genus in the dataset (~12% of the total reads) and it is the only bacterial genus that is present in all the sampled species, with relative abundances that varies from 0.3% to 82%. In total 141 ASVs (corresponding to 15 97%-similarity OTUs) are assigned to *Pseudomonas*, 69 of them are present only in one species so that 21 species have at least one unique ASV assigned to this genus. Another surprisingly well-represented bacterial genus belonging to Gammaproteobacteria is *Buchnera* (Fig. 1A). It has been found in 21 species, mostly at low abundance but representing a quite high proportion of the reads obtained from *Orsodacne humeralis* (~22%). The most abundant genus belonging to Bacilli is *Spiroplasma*, followed by *Paenibacillus* and *Brevibacillus* (Fig. 1A). *Spiroplasma* is the dominant genus in *Crioceris paracentesis* (~98%) and it has been found also in 18 other species, but with low densities (Fig. 1B). While in the microbiota of *Lilioceris merdigera* the dominant genus is *Paenibacillus* (~58%), that is also present in few other species but with low abundances. The results of the NCBI blast search (Supplementary Table 2) and phylogenetic tree inference (Supplementary Fig. 3) shed further light on the bacterial taxa that characterize the microbiota of Chrysomelidae. Two ASVs, which have been found only in *Cryptocephalus fulvus* (53.6%), have been assigned by the naïve Bayes classifier (confidence >0.95)

only to the domain level and the top hits of the blast search (query coverage 100%, identity >80%) correspond to uncultured bacteria isolated from acidic biofilm in caves (DQ499258). A huge number of sequences obtained from Donacinae (*Donacia obscura* 55.3%, *Plateumaris consimilis* 93.8%) belong, with high confidence (CP046230; query coverage 100%, identity 99.7%), to the vertically transmitted endosymbiont widespread in this subfamily (Kölsch et al., 2009). In both the maximum likelihood (ML) trees those sequences cluster with sequences obtained from the bacterial symbiont of Donacinae with quite high confidence (bootstrap values >70). Similarly, most of the sequences obtained from Cassidinae (*Cassida inopinata* 74.7%, *Hypocassida subferruginea* 93.1%) resulted to belong to '*Candidatus* Stammera capleta' (CP024013; query coverage 100%, identity 98.8%) and cluster with sequences of this species in the ML trees with high confidence (bootstrap value =100). Three ASVs from the V1–V2 region assigned to Enterobacterales are present only in *Macrocoma henoni* (23.9% of the reads). Blast search top hits (query coverage 100%, identity >78%) correspond to endosymbionts of weevils (AP018159, KX067892) while in the ML tree they cluster with a sequence from the *B. obscurus* symbiont A (LC273302) with high confidence (bootstrap value =99). Also, two ASVs from the V4 region from *M. henoni* (that represent almost all the reads previously assigned to *Buchnera* in this species) clustered together with sequences of the *B. obscurus* symbiont A (bootstrap value =88; Supplementary Fig. 3); the blast search confirms this taxonomic annotation (query coverage 100%, identity 93.9% and 93.5% with LC273302 and JQ805030 respectively). Six ASVs are present only in *Chrysochus asclepiadeus* and represent 93.3% of the reads from this species, one of them can be assigned to the *B. obscurus* symbiont A (LC273302, JQ805030; query coverage 99%, identity >87%, bootstrap value =97). Among the remaining ASVs blast search top hits assigned two ASVs to *Lelliottia amnigena* (LR134135; query coverage 100%, identity >98%) and the other three ASVs to *Klebsiella* sp. (MN860163, LR134475, MT279983, MT255043; query coverage 100%, identity >98%). In the ML trees (Supplementary Fig. 3) those sequences are part of a clade that includes *Klebsiella* and *Lelliottia*, but also other bacterial genera including symbionts of Hemiptera (JQ322760, HM156667, AB650515, AY620432) and the *B. obscurus* symbiont B (JQ805033).

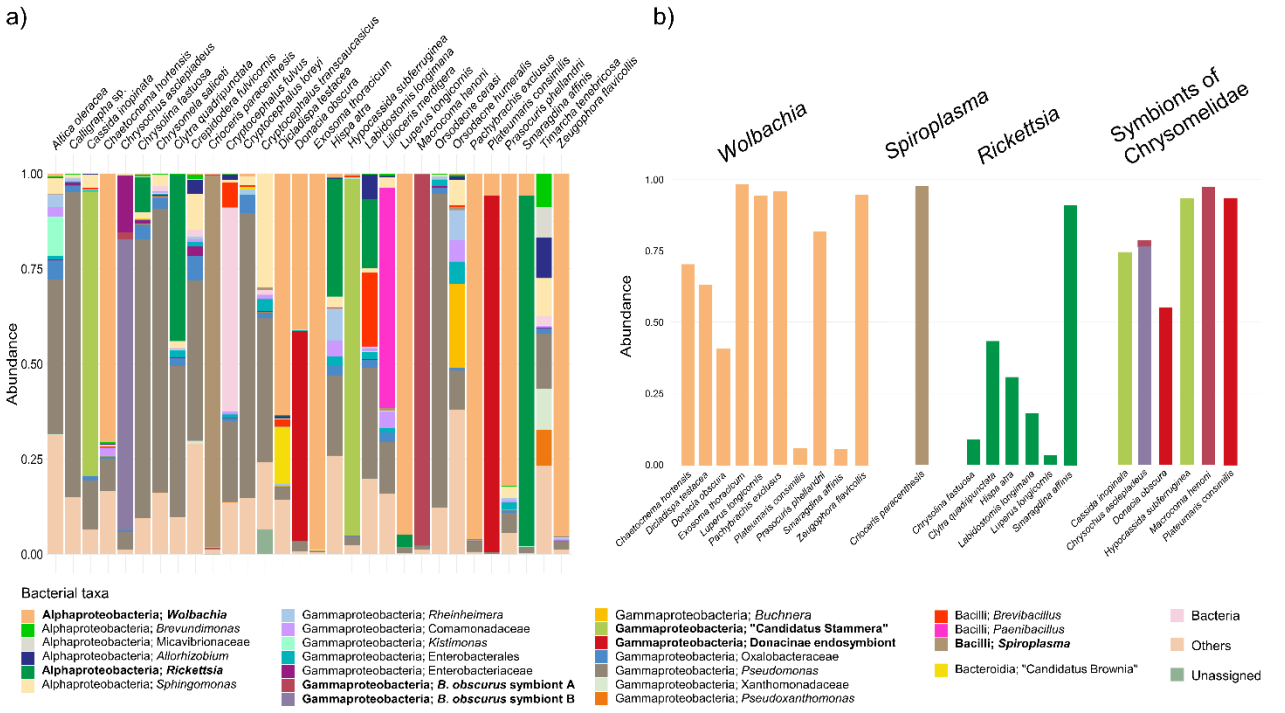

Figure 1. Taxonomic composition of the bacterial microbiota of Chrysomelidae. a) Barplot representing the composition of the microbiota of each Chrysomelidae species at the genus level (or any higher taxonomic level when the identification at the genus level was not possible). Colours represent different bacterial ranks, as reported in the legend, and the height of each box corresponds to the average relative abundance of each bacterial rank. Only bacterial ranks representing on average at least 5% of the reads in one species are shown, less abundant bacteria are included in the group "Others". b) Relative abundance of reproductive manipulators (*Wolbachia*, *Rickettsia*, *Spiroplasma*) and bacterial symbionts present only in Chrysomelidae. Colours representing different bacterial genera are reported in the legend (in bold). Only insect species with a relative abundance of these bacteria of at least 3% are shown.

Microbiota diversity

Diversity estimates for the group of generalist species, identified as those feeding on several plant families, are always higher than estimates for specialist species (Fig. 3; Supplementary Fig. 2). Similar results have been obtained also defining the two trophic groups (i.e., generalists, specialists) by working at the level of plant genera (Supplementary Fig. 4). As an example, with q = 2 (i.e., counting mainly the dominant taxa) the diversity estimated for generalist species is almost twice the diversity estimated for specialist species (coverage >0.3). Also, the diversity partitioning analysis in the framework of Hill numbers confirms this pattern (Table 2). The α-diversity component (average diversity of single species microbiotas) is always higher in generalist species, regardless of the value

of the order parameter (q). Also, the γ-diversity (diversity of the microbiota of all the species together) is higher in generalist species, except in the case of q = 0 (i.e., counting mainly the rare species). When using an intermediate weight (q = 1; counting mainly the common species) the gamma diversity estimated for the entire dataset (5.5) has an intermediate value between specialist species (4.9) and generalist species (6.4), as expected, while using other values for the order parameter there are no clear differences. The β-diversity estimates (γ-diversity/ α-diversity; corresponding to differences between samples) are similar in generalists and specialists, except for q = 0. The ancestral state reconstructions of the microbiota diversity estimates along the Chrysomelidae phylogenetic tree show no clear pattern (Fig. 2), in fact, no phylogenetic signal has been recorded. Some phylogenetic clades share low levels of microbial diversity (e.g., Donacinae, Cassidinae) but it is probably related to the presence of primary symbionts at high abundances (Fig. 2). In fact, most of the species hosting primary symbionts (e.g., '*Candidatus* Stammera capleta') or reproductive manipulators (e.g., *Wolbachia*) have low microbial diversity estimates (especially for q < 2). In any case, there are still several species hosting reproductive manipulators that have highly diverse microbiota (e.g., *Labidostomis longimana*, *Chaetocnema hortensis*).

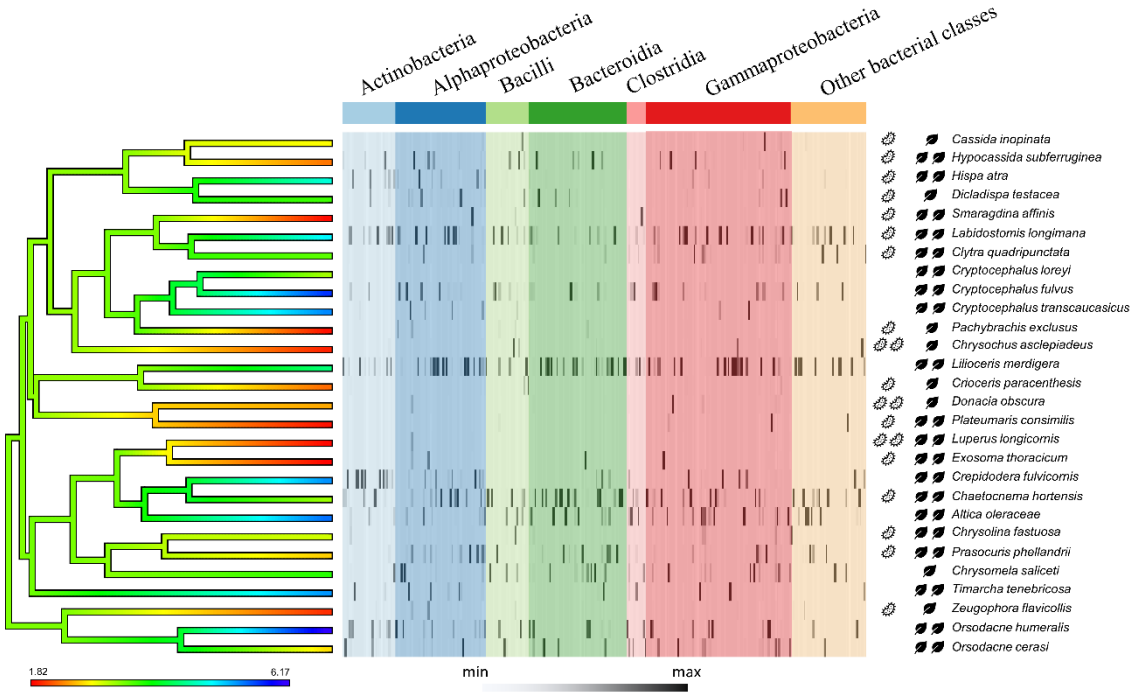

Figure 2. Diversity and composition of the Chrysomelidae microbiota. On the left side the ancestral state reconstruction of the microbiota diversity (Hill numbers, q = 1) is plotted as a colour gradient on the ML phylogenetic tree of the selected Chrysomelidae species. The heatmap represents the relative abundance of each genus with colours corresponding to the bacterial class (only classes with at least five different bacterial genera are reported, other classes are included in the category "Other bacterial classes"). On the right side, together with the insect species names, the presence of one or more reproductive manipulators or Chrysomelidae specific symbionts (one or two bacterium icons) and the trophic classification of the insect (specialists with one leaf icon; generalists with two leaf icons) are reported.

Discussion

Efficiency and taxonomic resolution of the 16S rRNA gene V1–V2 and V4 regions

The V4 region of the 16S rRNA, one of the two markers used in this study to characterize the microbiota associated to Chrysomelidae, allowed to obtain a higher number of ASVs and seems less prone to chloroplast contamination in respect to the second marker adopted, the V1–V2 region. Moreover, several bacterial genera were identified only through the examination of V4 region reads (Supplementary Fig. 1) and the diversity analyses suggest that this region better recovers rare taxa (Supplementary Fig. 2). These results are in accordance with what was found in previous studies supporting the use of the V4 region of the 16S rRNA gene in metabarcoding studies on bacteria (Zhang et al., 2018; Chen et al., 2019). Nevertheless, the V4 region missed the amplification of some bacterial taxa (e.g., *Brevundimonas* and *Aeromonas*) and the values of the diversity indices obtained combining the two regions resulted higher than those obtained from a single region (Supplementary Fig. 2). These results support the use of multiple marker regions to increase the resolution of metabarcoding studies targeting bacterial communities.

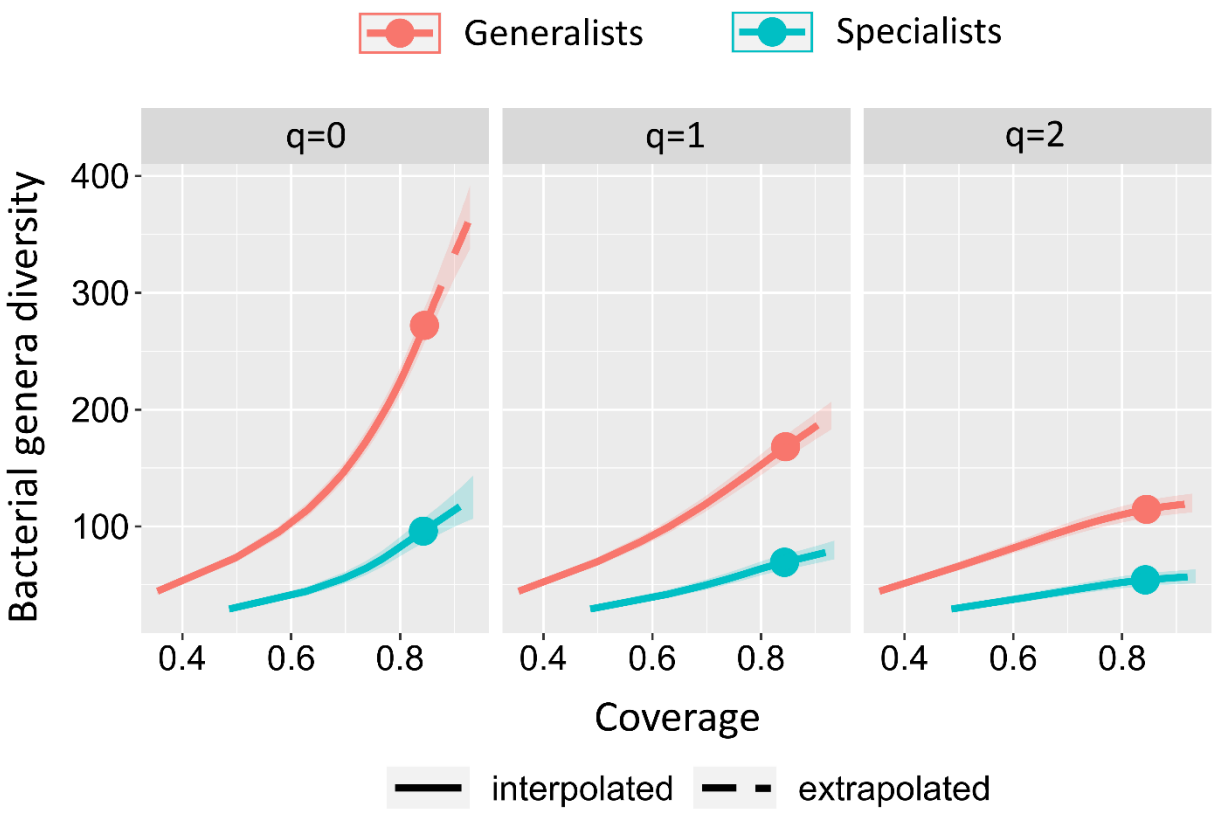

Figure 3. Microbiota diversity of specialist and generalist Chrysomelidae defined using the plant taxonomic level of family (specialists feed on plants all belonging to the same family, generalists feed on plants belonging to different families). Coverage based rarefaction/extrapolation curves of the Hill numbers estimated for three values of the order parameter (q = 0, q = 1, q = 2). The x-axis represents the coverage (that estimates the completeness of the sampling) and the y-axis represents the Hill number estimates, 95% confidence interval is also reported. As reported in the legend, colours correspond to the trophic category (specialist or generalist) and line type to the methodological approach (interpolation or extrapolation).

Microbiota composition

Within the microbiota associated with the 30 species of Chrysomelidae analysed in this study, three endosymbiotic bacterial genera belonging to the so-called male-killing group (Engelstädter and Hurst, 2009) have been identified: *Wolbachia* (in 28 species), *Spiroplasma* (in 17 species) and *Rickettsia* (in nine species) (Supplementary Table 1). For the majority of the analysed Chrysomelidae, the association with these bacteria is reported in this study for the first time. The presence of reproductive manipulators, such as *Wolbachia*, in Chrysomelidae is well known (Montagna et al., 2014; Kajtoch and Kotásková, 2018; Gómez-Zurita, 2019). These endosymbiotic bacteria are usually abundant in

infected species, tending to dominate the community, as observed in this study for four Chrysomelidae species, where *Wolbachia* represent 94%–99% of the reads. Endosymbionts can also represent only a minimum fraction of the bacterial community, e.g., *Wolbachia* represent less than 0.05% of the reads in nine species analysed in this study. The latter cases could be signs of horizontal acquisition that did not lead to an infection (Rasgon et al., 2006; Pietri et al., 2016; Chrostek et al., 2017; Kolasa et al., 2017; Cardoso and Gómez-Zurita, 2020) rather than real infections able to produce effects on the host even with a low bacterial titre (Richardson et al., 2019). Surprisingly, *Orsodacne humeralis* was found to host *Buchnera* (Gammaproteobacteria: Enterobacteraceae) representing the 22.1% of bacterial reads obtained for this species. This bacterium is a well-known endosymbiont that is strictly associated with aphids (Buchner, 1965; Shigenobu and Wilson, 2011) and to our knowledge infections caused by it in a non-aphid host have never been reported. For this reason, it is also hard to determine the relationship between *Buchnera* and *O. humeralis* microbiota (e.g., acquisition from the environment, commensality, symbiosis). *Pseudomonas* is the second most abundant bacterial genus found to be associated with the Chrysomelidae species of this study and it is the only one detected in all analysed species. Species of this genus can live under diverse environmental conditions; they are ubiquitous in soil, water and are important pathogens of plants and animals (Moore et al., 2006). *Pseudomonas* species are also known to play a functional role in insect symbiosis (e.g., providing digestive enzymes) (Piel et al., 2004; Huang et al., 2012; Ceja-Navarro et al., 2015; Zhang et al., 2020a,b). The high variety of *Pseudomonas* species makes it difficult to distinguish among environmental contamination (presumably from the food source), facultative association and functional symbiosis. Nevertheless, the high prevalence and uniqueness of *Pseudomonas* ASVs in some samples allows to suppose that, at least in these cases, it could represent a symbiont potentially playing a functional role for some Chrysomelidae species. Three Chrysomelidae subfamilies (Donacinae, Cassidinae, Eumolpinae) are known to host specific bacterial symbionts in specialized organs associated with the gut. Symbionts of Donacinae and Cassidinae have been intensively studied in the last years and are known to support host nutrition supplying digestive enzymes and/or providing essential nutrients lacking in the insect diet (Kleinschmidt and Kölsch, 2011; Salem et al., 2017, 2020; Reis et al., 2020). While for Eumoplinae those kinds of symbiosis have been less studied and are known only in *B. obscurus*. In both the species of Donacinae included in this study the microbiota is dominated by an endosymbiont already known to be widespread within the species of the subfamily (Fig. 1B, Supplementary Table 1). Specifically, in *Plateumaris consimilis* ~94% of the sequences are assigned to the symbiont isolated from that same

species in Reis et al. (2020), while in *Donacia obscura* ~55% of the sequences have been assigned to the symbiont isolated in the same study from *Donacia cinerea* and *Donacia marginata*, since no reference sequences are available for *Donacia obscura* symbiont. Most of the reads obtained from both the species of Cassidinae included in this study, *Cassida inopinata* (74.7%) and *Hypocassida subferruginea* (93.1%), have been assigned to symbiont of Cassidinae '*Candidatus* Stammera capleta' (Stammer, 1936; Salem et al., 2017) (Fig. 1B, Supplementary Table 1). This result is the first report of '*Candidatus* Stammera capleta' in these two Cassidinae species. The two analysed species of Eumolpinae (*M. henoni* and *Chrysochus asclepiadeus*) host bacterial taxa previously reported only from *B. obscurus* (*B. obscurus* symbiont A and B). The microbiota of *M. henoni* is dominated by the *B. obscurus* symbiont A. Since in *B. obscurus* this intracellular bacterium is present in specialized gut organs and in the female genitalia, it is possible to hypothesize a similar localization and a vertical transmission mechanism also in *M. henoni* but further studies are needed to investigate this symbiotic relationship and confirm this hypothesis. The symbiont A of *B. obscurus* is also present in *Chrysochus asclepiadeus*, but with a low abundance (~2%). The microbiota of *Chrysochus asclepiadeus* is dominated by a group of closely related bacterial taxa including the *B. obscurus* symbiont B together with the two bacterial genera *Lelliottia* and *Klebsiella*. *Lelliottia* spp. are usually isolated from plants, water and clinical samples (Brady et al., 2013). This genus has been also found in insect microbiota but usually at low abundances (e.g., Wang et al., 2019; Xu et al., 2019). *Klebsiella* spp. are often isolated from a variety of environmental sources such as soil, vegetation, water and animals (Brisse et al., 2006), but some species are also known to play functional roles in insect symbiosis (e.g., providing enzymes and antibiotics) (Dillon et al., 2002; Dantur et al., 2015; Miyashita et al., 2015). The high prevalence of ASVs closely related to the *B. obscurus* symbiont B in *Chrysochus asclepiadeus* suggests the presence of similar symbioses in closely related Eumolpinae species, which should be further investigated. Interestingly, in the 16S rRNA phylogenetic analyses (Supplementary Fig. 3) '*Candidatus* Stammera capleta', the endosymbiont of Donacinae and the *B. obscurus* symbiont A (together with five *M. henoni* ASVs and one *Chrysochus asclepiadeus* ASV) clustered in a clade with the most specialized Enterobacteriaceae symbionts (e.g., *Buchnera, Blochmannia, Nasonia, Baumannia*). However, the symbiont B of *B. obscurus* is placed in a separate clade with more generalist bacteria (e.g., *Escherichia, Enterobacter, Serratia, Klebsiella, Lelliottia*). This supports the hypothesis that the *B. obscurus* symbiont B, also present in *Chrysochus asclepiadeus*, is related to quite generalist gut bacteria, suggesting a loose association with the host. While the *B. obscurus* symbiont A is in the same clade with the other subfamily specific Chrysomelidae symbionts, and

since it has been detected also in both the Eumolpinae species included in this study, it is probably widespread in the subfamily.

Microbiota diversity

The comparison of α-diversity metrics between the specialist and the generalist species included in this study confirms that those usually feeding on several plant families harbour a more diversified microbiota (Figure 3). The higher microbiota richness of generalist insects has been previously observed comparing different insect orders and broad diet categories (*e.g.*, detritivores *vs* herbivores/carnivores) (Colman *et al*., 2012; Yun *et al*., 2014). Also, a study performed on Tephritidae supports this hypothesis (Ventura *et al*., 2018), while other studies on different taxonomic groups do not confirm this pattern (Blankenchip *et al*., 2018; Rothman *et al*., 2020). The higher microbiota diversity observed in generalist insects can be the result of bacteria randomly acquired from the environment, without any specific functional role in the host physiology, or due to the establishment of a diversified microbiota that provides adaptive advantages to the host (*i.e.*, a wider metabolic potential that allows the exploitation of diversified food sources). An exemplar case can be identified in detritivorous insects that harbour one of the richest microbiotas among insects (Colman *et al.*, 2012). Detritivorous insects' food source is composed by substrates of different origins that are colonized by a high variety of bacterial taxa, potentially contributing to insect microbiota diversity. On the other hand, detritus includes some of the most difficult molecules to digest (*e.g.*, lignocellulose), thus insects could benefit from the amplified metabolic capabilities supplied by a richer microbiota. Also in the case of phytophagous insects, the higher microbiota richness observed in generalist species can be easily related to the acquisition of different bacteria that are part of the environmental microbial communities (Montagna *et al*., 2015b; Hannula *et al*., 2019; Jones *et al*., 2019; Chouaia *et al*., 2019). The host plant-soil system is one of the major drivers of the phytophagous insect microbiota (Hannula *et al*., 2019), thus feeding on more than one plant species can highly influence insect's microbiota diversity and composition (Jones *et al.*, 2019). Secondary metabolites (Zhang *et al.*, 2020) and plant defences (Chung *et al.*, 2017) often playing a fundamental role in these plant-insect-microbiota interactions. Moreover, bacteria colonising plant surfaces and tissues can be able to degrade the toxic compounds produced by the plant itself (*e.g.*, Shukla and Beran, 2020; Leite-Mondin *et al.*, 2021). So, insects feeding on plants can acquire bacteria that, if established in their microbiota, can provide adaptative advantages. In fact, a richer microbiota determines a wider range of metabolic capabilities that can help the phytophagous insect to overcome the defences of different

host plants (e.g., Martinez *et al*., 2019; Santos-Garcia *et al*., 2020), thus also allowing the expansion of the trophic spectrum. Distinguishing between the processes that shape the microbiota diversity of generalist insects, especially if phytophagous, is quite difficult. Indeed, our results suggest that probably both the random acquisition from the environment and the adaptive advantage of an amplified metabolic potential participate to increase the diversity of the microbiota of generalist species. In this study, the estimated diversity of the microbiota is always higher in generalist species. This is observed both when the diversity value is estimated considering all bacteria (the weight is mostly on rare and low-abundance species, more likely acquired from the environment; q = 0), as well as when the weight is on more common species (considering mainly medium-high abundance bacteria having a possible functional role; q = 1, q = 2) (Figure 3). The importance of the food source in influencing the composition of the microbiota is also highlighted by the higher β-diversity observed in specialist insects when focusing on rare bacteria (q = 0) (Table 2). In fact, the microbiota of each specialist species results simpler than that of each generalist species (lower α-diversity) but considering together all the species in each of the two groups the overall diversity reaches similar levels (same γ-diversity). Based on previous results, the overall microbiota diversity of the specialists is mainly due to the high amount of not-shared bacterial taxa among species (reflected by a high β-diversity). This can be explained by the acquisition of phylogenetically distant bacteria from the host plant exploited by each insect species, supporting the importance of the food source in influencing the microbiota of phytophagous insects. The higher α-diversity of the microbiota harbored by generalist species (approximately twice higher than that of specialists) is confirmed also when focusing on dominant bacteria (q = 2; Figure 3). In this last case the difference is probably related to non-transient bacteria that may have a functional role in insect physiology. These results support the hypothesis that the high bacterial diversity hosted by generalist insects can expand the host metabolic potential enabling the exploitation of different food sources. Further studies, with an increased sample size or focusing on other phytophagous insect groups, are needed to confirm the pattern here observed and to better clarify the most influential causes of this phenomenon.

Experimental procedures

Species selection and host plant information

Thirty adult insects, collected from vegetation by sweep net and identified as belonging to thirty different species of Chrysomelidae, have been selected for this study (Table 1). The selection was performed to maximize the taxonomic coverage and the representativeness of the variability in the

trophic specialization. The sampling includes representatives of the ten main subfamilies of Chrysomelidae: Alticinae, Chrysomelinae, Galerucinae, Donacinae, Criocerinae, Cassidinae (including Hispini), Cryptocephalinae (including Clitrini), Eumolpinae, Orsodacninae, Zeugophorinae. The list of plants included in the diet of each Chrysomelidae species was compiled from a database of the host plants of Euro-Mediterranean Chrysomelidae (Magoga *et al*. in preparation). The trophic spectrum of the selected species ranges from exclusively monophagous species, restricted to feed on a single plant species (*e.g.*, *Chrysocus asclepiadeus* feeds only on

Table 1. Information on the analysed samples.

Species	Diet	Subfamily	Date	Country	Latitude	Longitude
Altica oleracea	Generalist	Alticinae	08/02/2010	Italy	45.794 N	9.250 E
Chaetocnema hortensis	Generalist	Alticinae	08/02/2010	Italy	45.794 N	9.250 E
Crepidodera fulvicornis	Generalist	Alticinae	08/02/2010	Italy	45.794 N	9.250 E
Cassida inopinata	Specialist	Cassidinae	28/06/2009	Italy	44.517 N	8.819 E
Hypocassida subferruginea	Generalist	Cassidinae	14/07/2008	Italy	42.796 N	11.242 E
Dicladispa testacea	Specialist	Casssidinae	03/06/2011	Italy	44.195 N	8.281 E
Hispa atra	Generalist	Casssidinae	26/06/2011	France	42.512 N	2.124 E
Clytra quadripunctata	Generalist	Chryptocephalinae	17/07/2010	Italy	45.941 N	9.416 E
Cryptocephalus fulvus	Generalist	Chryptocephalinae	06/04/2010	Italy	40.855 N	12.956 E
Cryptocephalus loreyi	Generalist	Chryptocephalinae	17/05/2009	Italy	45.857 N	9.253 E
Cryptocephalus transcaucasicus	Generalist	Chryptocephalinae	25/07/2009	Italy	44.702 N	7.142 E
Labidostomis longimana	Generalist	Chryptocephalinae	13/07/2010	Italy	45.824 N	9.279 E
Pachybrachis exclusus	Specialist	Chryptocephalinae	21/06/2008	Italy	44.056 N	9.832 E
Smaragdina affinis	Generalist	Chryptocephalinae	17/05/2009	Italy	45.857 N	9.253 E
Calligrapha sp.	Unknown	Chrysomelinae	01/04/2017	USA	32.268 N	110.808 W
Chrysolina fastuosa	Generalist	Chrysomelinae	17/05/2009	Italy	45.857 N	9.253 E
Chrysomela saliceti	Specialist	Chrysomelinae	21/06/2011	Italy	44.456 N	9.823 E
Prasocuris phellandrii	Generalist	Chrysomelinae	24/04/2010	Italy	45.795 N	9.216 E
Timarcha tenebricosa	Generalist	Chrysomelinae	04/06/2012	France	43.847 N	6.518 E
Crioceris paracenthesis	Specialist	Criocerinae	04/06/2012	Italy	45.894 N	13.551 E
Lilioceris merdigera	Generalist	Criocerinae	21/05/2010	Italy	45.794 N	9.250 E
Donacia obscura	Specialist	Donacinae	11/05/2011	Italy	44.625 N	9.542 E
Plateumaris consimilis	Generalist	Donacinae	07/04/2017	Italy	45.794 N	9.250 E
Chrysocus asclepiadeus	Specialist	Eumolpinae	28/06/2010	Italy	45.831 N	9.286 E
Macrocoma henoni	Unknown	Eumolpinae	23/05/2013	Morocco	31.150 N	5.393 W
Exosoma thoracicum	Generalist	Galerucinae	11/06/2011	Turkey	37.232 N	27.611 E
Luperus longicornis	Generalist	Galerucinae	28/06/2010	Italy	45.831 N	9.286 E
Orsodacne cerasi	Generalist	Orsodacninae	28/05/2009	Italy	45.893 N	9.281 E
Orsodacne humeralis	Generalist	Orsodacninae	21/05/2011	Turkey	39.761 N	27.571 E
Zeugophora flavicollis	Specialist	Zeugophorinae	08/07/2011	Italy	45.943 N	9.410 E

Taxonomic (species, families), ecological (diet spectrum width, i.e. specialist or generalist) and collection (date, country, latitude, longitude) information are reported.

Vincetoxicum hirundinaria, Apocynaceae), to extremely polyphagous species able to exploit several different food sources (*e.g.*, *Cryptocephalus fulvus* feeds on several plant species belonging to at least 14 different families). To compare the structure and diversity of the microbiota of Chrysomelidae with different breadth of the trophic spectrum, the selected species were divided in trophic classes (generalist and specialist) depending on the number of host plant families: *i)* specialist includes species feeding on a single plant family; *ii)* generalist includes species exploiting more plant families.

Table 2. Diversity partitioning.

Group	q	α-diversity	γ-diversity	β-diversity
Total dataset	0	35.2	318.4	9.0
	1	1.9	5.5	2.9
	2	1.2	3.0	2.5
Specialists	0	26.8	318.4	11.9
	1	2.7	4.9	1.8
	2	2.0	2.9	1.4
Generalists	0	39.1	318.4	8.1
	1	3.5	6.4	1.8
	2	2.3	3.0	1.3

The three diversity components (*α*-diversity, within sample diversity; *γ*-diversity, whole group diversity; *β*-diversity, among samples diversity) are reported for the whole dataset (total dataset) and for each of the two trophic categories considered (specialists and generalists).

DNA extraction

DNA was extracted from the whole insect body using the classical phenol–chloroform methods (Doyle & Doyle, 1990) with the following modifications. First, 500 µL of 2% CTAB (2% CTAB, 0.2% ascorbic acid, 1.5% PVP, 1.4 mmol/L NaCl, 20 mmol/L EDTA and 100 mmol/L Tris-HCl, pH 8.0) was added to each sample. Tissues were then disrupted using glass beads (ø 0.1 mm) with the Precellys®24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) and incubated at 65° C for 15 min to inactivate nucleases. After centrifugation, the supernatant was incubated overnight with 20 µL of proteinase K (20 mg/mL) at 56° C. To purify the DNA, two phenol–chloroform washes (phenol/chloroform/isoamyl alcohol, 25:24:1, pH 8.0) were performed. DNA was, then, precipitated after addition of 500 µL of isopropanol and incubation for 1 h. Pellet was washed twice with 70% ethanol and eluted in 40 µL of Ultrapure Water (Sigma-Aldrich, Saint Louis,

Missouri, USA). Qubit 4.0 fluorometer (Thermo Fisher Scientific) was used to determine the DNA concentrations. A DNA extraction blank, using the same extraction protocol and molecular biology grade water, was performed as control to monitor environmental contamination.

Library preparation and sequencing

Two regions of 16S rRNA gene (V1-V2 and V4) were sequenced by means of Ion Torrent platform (Life Technologies). PCR primers 27FYM (Frank *et al*., 2008) and 338R (Amann *et al*., 1990) were used to amplify V1-V2 region, while primers 515F (Caporaso *et al*., 2011), 802R (Claesson *et al*., 2009) and 806R (Caporaso *et al*., 2011) were used for V4 region, in two separated reactions. PCR primers were tailed with two different GC rich sequences enabling barcoding in a second amplification. The first PCR amplification of the V4 region has been performed as reported in Chouaia *et al*. (2019). The first V1-V2 PCR was performed in the same conditions as V4 following 34 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 15 s and a final extension of 72°C for 2 min. The second PCR amplification was performed in 25 µL volume containing 10 µL HotMasterMix 5Prime 2.5 X (Quanta Bio), 1.25 µL EvaGreen™ 20X (Biotium), 1.5 µL barcoded primer (10 µM), 1 µL of the first PCR amplification with the following conditions: 8 cycles of 94°C for 10 s, 60°C for 10 s, 65°C for 40 s and a final extension of 72°C for 3 min. To control for bacterial contaminations, PCR amplifications of the V1-V2 and V4 regions were performed, as previously reported, using as template the DNA extraction blank (*i.e.*, reagents of the used DNA extraction kit) and the PCR reagents. No amplicons were obtained by visualisation on 1.5% agarose gel electrophoresis. Furthermore, real-time PCRs were performed on the DNA extraction blank in order to monitor for contamination and select the appropriate number of the first PCR cycles to avoid the raise of the negative control curves. All the amplicons were checked for their quality and size by agarose gel electrophoresis, quantified with the Qubit™ dsDNA BR Assay Kit in the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and pooled together in equimolar amounts. The library was purified running it in a precasted E-Gel® SizeSelect™ (Invitrogen) agarose gel 2% and finally quality checked and quantified with High Sensitivity DNA reagents in the Agilent 2100 Bioanalyzer (Agilent Technologies). For sequencing the library was first subjected to emulsion PCR on the Ion OneTouch™ 2 system using the Ion PGM™ Template Hi-Q OT2 View (Life Technologies) according to the manufacturer's instructions. Then Ion sphere particles (ISP) were enriched using the E/S module. Resultant live ISPs were loaded and sequenced on an Ion 316 chip (Life Technologies) in the Ion Torrent PGM System.

Bioinformatic analyses

The bioinformatic analyses were performed using the QIIME2 platform (Bolyen *et al*., 2019). The obtained raw reads for the two 16S rRNA gene regions (V1-V2 and V4) were denoised and taxonomically annotated separately. The DADA2 algorithm (Callahan *et al*., 2016) was used for denoising to obtain an estimation of the actual ASVs present (Amplicon Sequence Variants) using default parameters (*e.g.*, trunQ = 2, maxEE = 2). 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 classifiers were trained on the reference sequences trimmed to correspond to the amplified region. To obtain a common phylogeny for the ASVs from the two 16S rRNA regions, the SEPP technique (SATé-enabled phylogenetic placement; Janssen *et al*., 2018) was applied to place the ASVs on a reference phylogeny based on the release 138 of the SILVA database (Quast *et al*., 2012). In specific cases (possible Enterobacteriaceae primary symbionts) the taxonomic annotation of the ASVs have been checked using the BLAST algorithm (Altschul *et al*., 1990) on the NCBI nt database and confirmed using phylogenetic tree inference.

To infer maximum likelihood trees for the phylogenetic placement of putative primary symbionts, 16S rRNA reference sequences for selected genera representative of the Enterobacteriaceae were downloaded from the NCBI nt database. Sequences were aligned using the mafft algorithm v.7.471 (Katoh & Standley, 2013) considering information on the secondary structures of the rRNA. The Maximum Likelihood (ML) trees have been inferred with iq-tree v.2.0.3 (Minh *et al*., 2020) using ModelFinder (Kalyaanamoorthy *et al*., 2017) to select the substitution model according with AIC (Akaike, 1973). Ten trees for each amplified region have been inferred to check for concordance of different runs. The same phylogenetic tree inference pipeline has been applied to manually aligned COI sequences of the 30 selected Chrysomelidae species (Magoga *et al.* 2018) (Supplementary table 3) with the topology constrained to the one published in Nie *et al*. (2020).

The microbiota diversity analyses were performed with a sample size and coverage-based integrations of interpolation (rarefaction) and extrapolation (prediction) of the Hill numbers (Hill, 1973; Alberdi and Gilbert, 2019; Roswell *et al*., 2021) using the R packages iNEXT and iNextPD (Chao *et al*., 2014, 2015; Hsieh and Chao, 2016). The computation of Hill numbers was performed for three increasing values of the order parameter q, corresponding to increasing weight on the species abundance (or any other taxonomic level considered) and also to different well-known diversity and phylogenetic

diversity indices: q = 0, counting mainly the rare species (those with low abundances), corresponds to richness (McIntosh, 1967) and Faith's Phylogenetic Diversity (Faith, 1992); q = 1, counting mainly the common species (those with medium-high abundances), corresponds to the exponential of Shannon index (Shannon, 1948) and Allen's Phylogenetic entropy (Allen *et al*., 2009); q = 2, counting mainly the dominant species (those with very high abundances), corresponds to the inverse of Simpson index (Simpson, 1949) and Rao's quadratic entropy (Rao, 1982). This explicit parametrization is particularly useful to test our hypothesis, since we can assume that symbionts with a functional role are present at high abundances (q = 1, q = 2) while the bacteria acquired from the environment have usually low abundances, so can be considered rare species (q = 0). The hilldiv R package (Alberdi & Gilbert, 2021) was used to partition the diversity in its components: α-diversity (diversity at the sample level), γ-diversity (total diversity in the selected group) and β-diversity (γ-diversity/ α-diversity, corresponding to the among sample component of the total diversity). Comparing diversity partitioning between the two trophic groups considered in this study (specialists and generalists) allows us to understand which component is most influential in determining the different diversity estimates. The R package phytools (Revell, 2012) was used for ML ancestral state reconstruction (Revell, 2013) of the microbiota diversity estimates along the insect phylogenetic tree and to compute phylogenetic signals (Blomberg *et al*., 2003; Ives *et al*., 2007).

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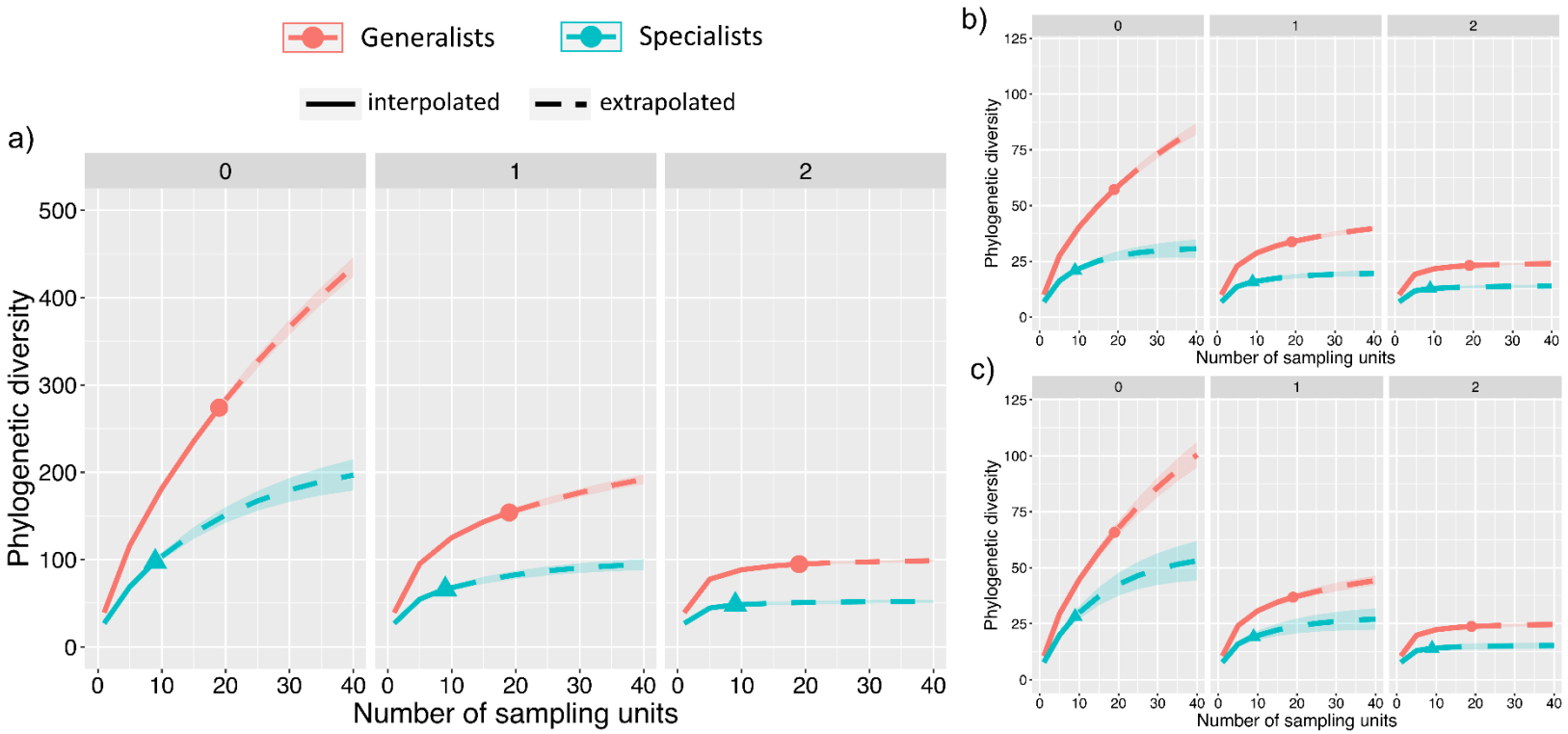
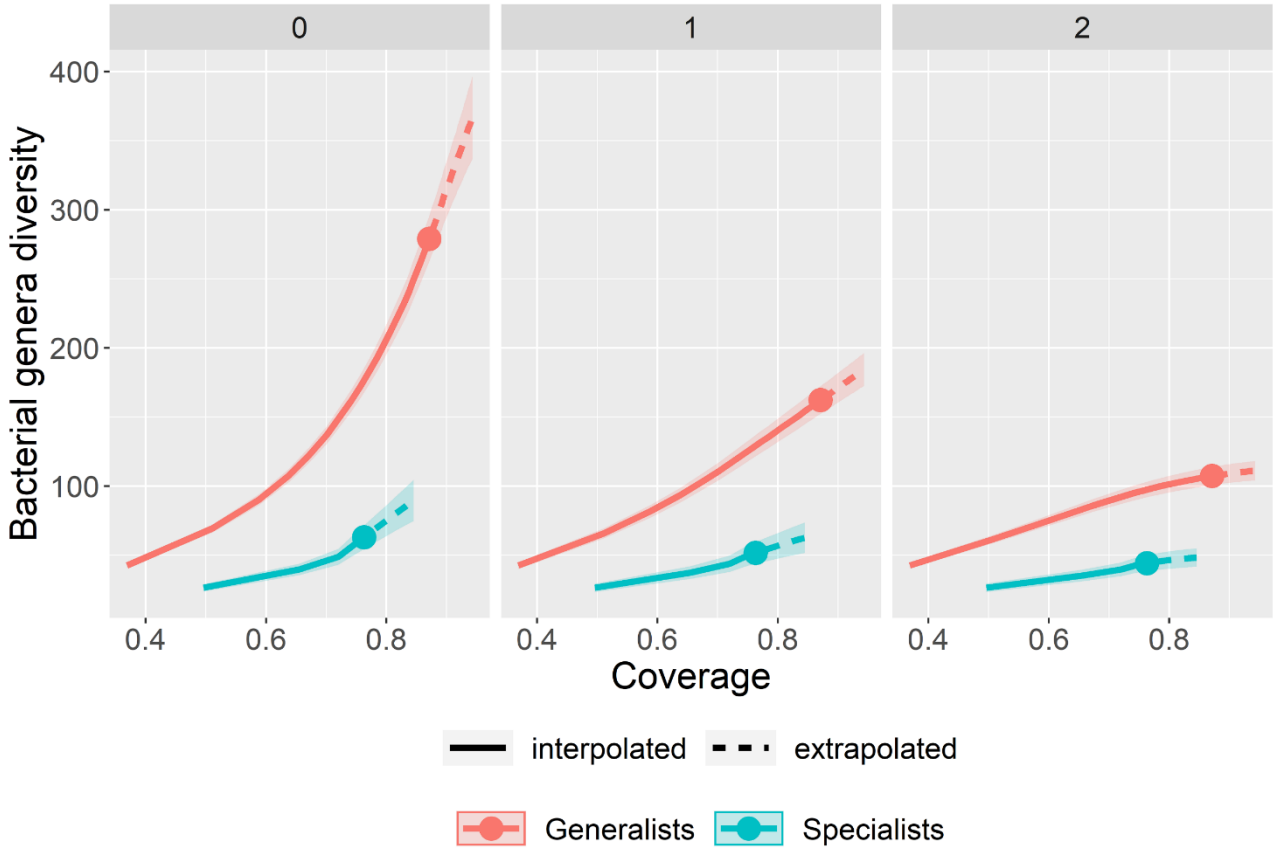
2.1.4 Supplementary material

Supplementary Figure 1. Bacteria abundance in single marker datasets. Heatmap representing the abundance of bacterial taxa (classes, families, genera) present in the single marker datasets (V4 and V1-V2). In the genera heatmap only the 50 most abundant genera are shown. Colour intensity is proportional to the normalized relative abundance of the bacterial taxa.



Supplementary Figure 2. Microbiota diversity estimates inferred on the total dataset (V1-V2 and V4), V1-V2 and V4 regions of the 16S rRNA. Sample-based rarefaction/extrapolation curves of the Hill numbers estimated for three values of the order parameter (q = 0, q = 1, q = 2). The x-axis represents increasing sampling and the y-axis represents the Hill number estimates, 95% confidence interval is also reported. As reported in the legend, colours correspond to the trophic category (specialist or generalist) and line type to the methodological approach (interpolation or extrapolation). a) Global dataset (V1-V2 and V4 regions of the16S rRNA). b) V1-V2 region of the16S rRNA. c) V4 region of the 16S rRNA.

Supplementary Figure 3. Maximum likelihood phylogenetic trees. Sequences obtained from the NCBI database report the accession numbers while sequences produced in this study are highlighted in bold. a) Tree obtained from sequences of the V1-V2 region of the 16S rRNA. b) Tree obtained from sequences of the V4 region of the 16S rRNA.



Supplementary Figure 4. Microbiota diversity of specialist and generalist Chrysomelidae defined using the plant taxonomic level of genus (specialists feed on plants all belonging to the same genus, generalists feed on plants belonging to different genera). Coverage based rarefaction/extrapolation curves of the Hill numbers estimated for three values of the order parameter (q = 0, q = 1, q = 2). The x-axis represents the coverage (that estimates the completeness of the sampling) and the y-axis represents the Hill number estimates, 95% confidence interval is also reported. As reported in the legend, colours correspond to the trophic category (specialist or generalist) and line type to the methodological approach (interpolation or extrapolation).

Supplementary table 1. Primary symbionts relative abundance.

	Wolbachia	*Rickettsia*	*Spiroplasma*	*Buchnera*	*Ca.* Stammera capleta	Donacinae endosymbiont
Cassida inopinata	<0.1%				74.7%	
Luperus longicornis	94.8%	3.3%	<0.1%	<0.1%		
Chrysolina fastuosa	0.15%	9.2%	<0.1%	<0.1%		
Smaragdina afffinis	5.8%	92.1%		<0.1%		
Cryptocephalus loreyi	0.65%					
Cryptocephalus transcaucasicus	0.11%		<0.1%	<0.1%		
Crepidodera fulvicornis	0.34%	<0.1%		<0.1%		
Labidostomis longimana	<0.1%	18.1%	<0.1%	<0.1%		
Altica oleracea	0.75%	0.35%	<0.1%	<0.1%		
Chaetocnema hortensis	70.6%		<0.1%	<0.1%		
Pachybrachis exclusus	96%		<0.1%			
Cryptocephalus fulvus	0.11%		<0.1%			
Clytra quadripunctata	<0.1%	43.8%	<0.1%	<0.1%		
Lilioceris merdigera	<0.1%	<0.1%	0.65%			
Hispa atra	1.0%	31%				
Dicladispa testacea	63.5%			<0.1%		
Donacia obscura	41%			<0.1%		55.3%
Chrysomela saliceti			<0.1%	<0.1%		
Exosoma thoracicum	98.9%			<0.1%		
Zeugophora flavicollis	95.2%		<0.1%	<0.1%		
Timarcha tenebricosa						
Orsodacne humeralis	0.18%		0.74%	22.1%		
Plateumaris consimilis	5.7%			<0.1%		93.8%
Calligrapha sp.	<0.1%					
Macrocoma henoni	<0.1%		<0.1%			
Prasocuris phellandrii	82%			<0.1%		
Crioceris paracenthesis	<0.1%		97.63%	0.12%		
Chrysochus asclepiadeus	<0.1%		<0.1%	<0.1%		
Orsodacne cerasi	<0.1%					
Hypocassida subferruginea	<0.1%		<0.1%	<0.1%	93.1%	

Supplementary table 2. Blast search results on NCBI nt database.

Species	16S region	ASV ID	BLAST top hits	Coverage	Identity	Accession numbers
D. marginata	V1V2	ccd07d986f55fb44711d72e11f09b659	Enterobacteriaceae endosymbiont of *Donacia marginata* isolate DmarSym chromosome	100%	97.72%	CP046184.1
D. marginata	V4	2e6cb2d446162b484ddb1f893e2b5d56	Enterobacteriaceae endosymbiont of *Donacia marginata* isolate DmarSym chromosome	100%	99.24%	CP046184.1
D. marginata	V4	faba20b5149aa5026652812559c9d699	Enterobacteriaceae endosymbiont of *Donacia marginata* isolate DmarSym chromosome	100%	98.09%	CP046184.1
D. marginata	V4	bbf8a9b157a911b7cce73163733899e6	Enterobacteriaceae endosymbiont of *Donacia marginata* isolate DmarSym chromosome	100%	98.47%	CP046184.1
P. consimilis	V1V2	a2de34e4ef8c20f055df5bb781665d19	Enterobacteriaceae endosymbiont of *Plateumaris consimilis* isolate PconSym chromosome	100%	99.67%	CP046230.1
P. consimilis	V1V2	49727f65c846f8bbec0f84c3d508acb9	Enterobacteriaceae endosymbiont of *Plateumaris consimilis* isolate PconSym chromosome	100%	99.35%	CP046230.1
P. consimilis	V1V2	3a5761441618ca08bdb566cb1ba88566	Enterobacteriaceae endosymbiont of *Plateumaris consimilis* isolate PconSym chromosome	100%	99.67%	CP046230.1
P. consimilis	V4	f62f09560221682e1a625fcca0d44ee8	Enterobacteriaceae endosymbiont of *Plateumaris consimilis* isolate PconSym chromosome	100%	99.62%	CP046230.1
P. consimilis	V4	e0507ff236b3798330e5dcf7e28877ad	Enterobacteriaceae endosymbiont of *Plateumaris consimilis* isolate PconSym chromosome	100%	100.00%	CP046230.1
C. inopinata	V4	ef667290ff98855b92b443b361312ec3	*Candidatus* Stammera capleta isolate NZ1215 chromosome, complete genome	100%	98.47%	CP024013.1
C. inopinata	V4	646181e0c79acd878f4869c66a5599e2	*Candidatus* Stammera capleta isolate NZ1215 chromosome, complete genome	100%	98.85%	CP024013.1
H. subferruginea	V4	0d34bfff399e23325eb998a4ac1c7f8a	*Candidatus* Stammera capleta isolate NZ1215 chromosome, complete genome	100%	95.42%	CP024013.1
H. subferruginea	V4	47a0e256723619721dd47ccaa0b92a6b	*Candidatus* Stammera capleta isolate NZ1215 chromosome, complete genome	100%	95.80%	CP024013.1
C. asclepiadeus	V1V2	3626b529c9ad0df417998386ba71f7ad	*Lelliottia amnigena* strain NCTC12124 genome assembly, chromosome: 1	100%	98.37%	LR134135.1
C. asclepiadeus	V1V2	40863afc9f2945b3c2fe538f5837ae08	*Lelliottia amnigena* strain NCTC12124 genome assembly, chromosome: 1	100%	98.70%	LR134135.1
C. asclepiadeus	V4	7edeb5fcb2c2156b60abafe5e9312712	*Klebsiella aerogenes* strain K64 16S ribosomal RNA gene, partial sequence	100%	98.47%	MN860163.1
C. asclepiadeus	V4	97a084dda531bad3cc838682fb00f3aa	*Klebsiella aerogenes* strain K64 16S ribosomal RNA gene, partial sequence	100%	98.85%	MN860163.1
			Klebsiella aerogenes strain NCTC9735 genome assembly, chromosome: 1	100%	98.85%	LR134475.1
C. asclepiadeus	V4	d83b3cad29505ce0ca43e4a1edb23b7a	*Klebsiella variicola* strain EM09 16S ribosomal RNA gene, partial sequence	100%	98.09%	MT279983.1
			Klebsiella pneumoniae strain KlPn 3 16S ribosomal RNA gene, partial sequence	100%	98.09%	MT255043.1
C. asclepiadeus	V4	c5ea5f98290ae954d06c089e4e0ade41	Gamma proteobacterium Manza-kogen gene for 16S ribosomal RNA, partial sequence	99%	87.36%	LC273302.1
			Uncultured bacterium clone *Bromius*_blind_sacs_symbiont 16S ribosomal RNA gene, partial sequence	99%	87.36%	JQ805030.1
M. henoni	V1V2	d67d5f62a09a10a23a4408455b697d3d	Endosymbiont of *Euscepes postfasciatus* DNA, complete genome, isolate: NAREPO1	100%	78.50%	AP018159.1
			Blochmannia endosymbiont of *Rhabdoscelus similis* clone NAN-4 16S ribosomal RNA gene, partial sequence	100%	78.30%	KX067892.1
M. henoni	V1V2	f325955f01e756636321611130aba6c4	Endosymbiont of *Euscepes postfasciatus* DNA, complete genome, isolate: NAREPO1	100%	79.15%	AP018159.1
			Blochmannia endosymbiont of Rhabdoscelus similis clone NAN-4 16S ribosomal RNA gene, partial sequence	100%	78.93%	KX067892.1
M. henoni	V1V2	2735ad9a9a0bc786c815421cfe4d4e7b	Endosymbiont of *Euscepes postfasciatus* DNA, complete genome, isolate: NAREPO1	100%	78.83%	AP018159.1
			Blochmannia endosymbiont of *Rhabdoscelus similis* clone NAN-4 16S ribosomal RNA gene, partial sequence	100%	78.62%	KX067892.1
M. henoni	V4	ab830d2707e1b450fc38852198a07928	Gamma proteobacterium Manza-kogen gene for 16S ribosomal RNA, partial sequence	100%	93.89%	LC273302.1
			Uncultured bacterium clone *Bromius*_blind_sacs_symbiont 16S ribosomal RNA gene, partial sequence	100%	93.89%	JQ805030.1
M. henoni	V4	62d583fccf82bb73caecf0acade0a797	Gamma proteobacterium Manza-kogen gene for 16S ribosomal RNA, partial sequence	100%	93.51%	LC273302.1
			Uncultured bacterium clone *Bromius*_blind_sacs_symbiont 16S ribosomal RNA gene, partial sequence	100%	93.51%	JQ805030.1

Supplementary table 3. Accession numbers of COI sequences.

Species	Accession Number
Altica oleracea	JF890683
Cassida inopinata	JF890687
Chaetocnema hortensis	JF890767
Chrysochus asclepiadeus	JF890698
Chrysolina fastuosa	JF890727
Chrysomela saliceti	MH322815
Clytra quadripunctata	JF890821
Crepidodera fulvicornis	JF890763
Crioceris paracenthesis	MH322856
Cryptocephalus fulvus	MH322918
Cryptocephalus loreyi	JF890726
Cryptocephalus transcaucasicus	LS973870
Dicladispa testacea	MH323090
Donacia obscura	MH323097
Exosoma thoracicum	MH323108
Hispa atra	MH323146
Hypocassida subferruginea	JF890707
Labidostomis longimana	MH323150
Lilioceris merdigera	JF890824
Luperus longicornis	JF890701
Macrocoma henoni	MH323229
Orsodacne cerasi	JF890673
Orsodacne humeralis	MH323283
Pachybrachis exclusus	JF890775
Plateumaris consimilis	KM450130
Prasocuris phellandrii	JF890801
Smaragdina afffinis	MH323362
Timarcha tenebricosa	MH323399
Zeugophora flavicollis	MH323403

2.1.5 Personal contribution to the work

Conceiving the study, performing bioinformatic/statistical analyses and writing the manuscript in collaboration with M.M. and G.M.

2.2 Unpublished results: "How sex influence the microbiota of wetland leaf beetles"

2.2.1 Summary

Insects are great models to investigate factors affecting the diversity and composition of the microbiota associated to eukaryotic organisms. In fact, several factors are known to influence insects' microbiota (*e.g.*, latitude, altitude, local climate, diet, development). Among the characteristics of the insect host able to influence its microbiota, sex is one of the most interesting, but the few studies performed on this topic show contrasting results. In this study, the microbiota of seven species of leaf beetles (Coleoptera: Chrysomelidae), collected in the same wetland environment, have been characterized with a metabarcoding approach targeting the V3-V4 region of the bacterial 16S rRNA. The microbiota of most of the selected species is dominated by reproductive manipulator bacteria (*Wolbachia, Rickettsia*) or by primary symbionts (*e.g.,* "*Candidatus* Macropleicola"). Surprisingly the primary symbiont associated to Cassidinae ("*Candidatus* Stammera capleta") was not recorded in *Cassida rubiginosa*, while the dominant bacterium in this species belongs to Rhizobiaceae. The two sexes in leaf beetles show a slightly different microbiota composition with males having richer microbiotas. These differences are emphasized in the low-abundance transient component of the microbiota, that is mainly acquired from the environment, while are less evident in the high-abundance more stable component possibly involved in vertical transmission mechanisms. This support the hypothesis that differences in the diversity and composition of different components of the insect microbiota can be related to constrains on the vertical transmission mechanisms acting differently in the two sexes.

2.2.2 Manuscript

Introduction

Insects, being one of the most diverse animal groups on earth, playing a central ecological role in most terrestrial ecosystems and including several model species with well-developed laboratory protocols, are one of the major focus of studies on the microbiota associated to eukaryotic organisms. Most insect species harbour a stable microbiota that includes both commensal species, not directly affecting host biology, and more or less specialized symbionts that can greatly affect host's fitness (Douglas, 2009, 2015; Engel & Moran, 2013; Clay, 2014; Hurst & Frost, 2015; Wang *et al.*, 2020). A particularly interesting field of insect microbiota studies regards the biotic and abiotic factors

shaping insect's microbiota composition and diversity (Colman *et al.*, 2012; Yun *et al.*, 2014). In fact, several factors are known to influence the composition of the insect's microbiota. The main abiotic factors shaping insect microbiota are probably geographic gradients, like 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 their local distribution in specific environments, such as mean annual temperature or soil properties (Huang and Zhang, 2013; Tiede *et al.*, 2017; Muturi *et al.*, 2018). Since the main reservoir of insect microbiota is in the gut, the alimentation represents the most important interaction with the environment, able to generate a direct link between the insect microbiota and all the microbial communities present in the environment. In fact, the influence of the diet on insect's 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 it has been demonstrated 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). Insect microbiota composition and diversity are also highly influenced by characteristics of the insect host, such as the development. In fact, the microbiota can drastically change during insect development, especially in the case of holometabolous insects where larvae and adults often occupy different ecological niches and show different trophic attitudes (*e.g.*, Vasanthakumar *et al.*, 2008; Kim *et al.*, 2017; Zhang *et al.* 2018; Ali *et al.*, 2019; Morales-Jiménez *et al.*, 2012; Briones-Roblero *et al.*, 2017; Huang and Zhang, 2013; Shukla *et al.*, 2016; Chouaia *et al.*, 2019). In most of these cases, changes in the microbiota composition can be related to the different ecological needs experienced by larvae and adults and/or to the influence of the microbial communities present in the different microenvironment inhabited. Another characteristic of the host that could greatly influence insects' microbiota is the sex, but studies on this topic show contrasting results. As example, differences in the microbiota composition and diversity of the two sexes have been reported for *Spodoptera littoralis* (Chen *et al.*, 2016), while other studies on a species in the same genus, *S. exigua*, found no significative differences between the microbiota of males and females (Gao *et al.*, 2019; Martínez-Solís *et al.*, 2020). Similar contrasting patterns have been found also in Coleoptera, with few studies recording differences between sexes in Curculionidae (Xu *et al.*, 2016) and Scarabeidae (Shukla *et al.*, 2016) but not in Chrysomelidae (Ali *et al.*, 2019). When males and females show different trophic attitudes, such as happen in mosquitos where only females feed on vertebrate blood (Minard *et al.*, 2013, 2018), differences between the microbiota of the two sexes could be a secondary effect related to the different composition of the diet. But in other cases, it may be also related to the need of a maternal vertical transmission mechanisms, especially in the case of primary symbionts or

reproductive manipulators, that lead to a higher abundance of such microbes in females and so to a different structure of the microbial communities in the two sexes.

In this study a selection of individuals from seven species of leaf beetles (Coleoptera: Chrysomelidae), collected in the same wetland environment in Italy, was used to characterize the composition of the bacterial microbiota of taxonomically related species inhabiting the same environment. The main aim is to investigate differences in the composition and diversity of the bacterial communities associated to male and female leaf beetles. Trying to isolate the effect due to the transient part of the microbiota, mostly represented by bacteria with low relative abundances acquired from the environment, from the effect due to the more stable component of the microbiota, mainly represented by relatively high abundance bacteria with a more intimate relationship with the insect host (*e.g.*, primary and secondary symbionts, reproductive manipulators). To test the hypothesis that differences in the structure and composition of the microbiota can be related to different constrains on the vertical transmission mechanisms acting in the two sexes. In details, since females are the main responsible of the vertical transmission of bacteria to the offspring, their microbiota is expected to be biased toward this stable component, while in males this component of the microbiota should be less abundant and the transient part acquired from the environment more evident.

Materials and methods

Sampling

Nineteen adult insects from seven Chrysomelidae species (Table 1) have been collected from vegetation by sweep net in the same environment near Alserio lake, in Italy (45°47’46” N - 9°12’59” E, 261 m a.s.l.) and preserved in 100% ethanol. The sampling includes representatives of species often associated to wetlands from five Chrysomelidae subfamilies: Alticinae (*Chaetocnema conducta*), Cassidinae (*Cassida rubiginosa*), Chrysomelinae (*Phaedon cochleariae, Prasocuris phellandrii*), Donacinae (*Donacia vulgaris, Plateumaris consimilis*), Galerucinae (*Agelastica alni*).

Table 1. Information on the analysed samples.

ID	Species	Subfamily	Sex	Collection date
P733_3	*Agelastica alni*	Galerucinae	female	2014
P733_2	*Agelastica alni*	Galerucinae	male	2014
P1068_10	*Cassida rubiginosa*	Cassidinae	female	13.04.2021

P1070_2	*Cassida rubiginosa*	Cassidinae	male	02.05.2021
P1068_11	*Chaetocnema conducta*	Alticinae	female	13.04.2021
P1068_12	*Chaetocnema conducta*	Alticinae	male	13.04.2021
P1068_13	*Chaetocnema conducta*	Alticinae	male	13.04.2021
P1070_4	*Donacia vulgaris*	Donacinae	female	02.05.2021
P1070_5	*Donacia vulgaris*	Donacinae	female	02.05.2021
P1070_3	*Donacia vulgaris*	Donacinae	male	02.05.2021
P1068_16	*Phaedon cochleariae*	Chrysomelinae	female	13.04.2021
P1068_2	*Phaedon cochleariae*	Chrysomelinae	female	13.04.2021
P1068_4	*Phaedon cochleariae*	Chrysomelinae	male	13.04.2021
P1068_1	*Plateumaris consimilis*	Donacinae	male	13.04.2021
P1068_8	*Plateumaris consimilis*	Donacinae	male	13.04.2021
P1068_9	*Plateumaris consimilis*	Donacinae	male	13.04.2021
P733_4	*Prasocuris phellandrii*	Chrysomelinae	female	2014
P733_5	*Prasocuris phellandrii*	Chrysomelinae	female	2014
P733_6	*Prasocuris phellandrii*	Chrysomelinae	male	2014

DNA isolation and sequencing

DNA was extracted from the whole insect body, after sterilization of the outer surface, using the classical phenol–chloroform methods (Doyle & Doyle, 1990) with the following modifications. First, 500 µL of 2% CTAB (2% CTAB, 1.5% PVP, 1.4 mmol/L NaCl, 20 mmol/L EDTA and 100 mmol/L Tris-HCl, pH 8.0) was added to each sample. Tissues were then disrupted using glass beads (ø 0.1 mm) with the Precellys®24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) and incubated at 65° C for 15 min to inactivate nucleases. After centrifugation, the supernatant was incubated overnight with 20 µL of proteinase K (20 mg/mL) at 56° C. To purify the DNA, two phenol–chloroform washes (phenol/chloroform/isoamyl alcohol, 25:24:1, pH 8.0) were performed. DNA was, then, precipitated after addition of 500 µL of isopropanol and incubation for 1 h. Pellet was washed twice with 70% ethanol and eluted in 40 µL of Ultrapure Water (Sigma-Aldrich, Saint Louis, Missouri, USA). Purity of the extracted DNA were 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.

Bioinformatic and statistical analyses

The bioinformatic analyses were performed using the QIIME2 platform (Bolyen *et al*., 2019). 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 have 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 metrices (*e.g.*, Faith's phylogenetic diversity, unifrac distances). After random subsampling the data at the same depth per sample (15,440 sequences), the following alpha and beta diversity metrices were computed: ASVs richness (McIntosh, 1967), Shannon index (Shannon, 1948), Simpson index (Simpson, 1949), Pielou's evenness (Pielou, 1966), Faith's phylogenetic diversity (Faith, 1992), Jaccard index (Jaccard, 1908), Bray-Curtis dissimilarity (Sorenson, 1948), unweighted and weighted unifrac (Lozupone and Knight, 2005; Lozupone *et al.*, 2007). Principal Coordinate Analysis (PCoA) was performed on beta diversity indices to graphically represent dissimilarities across samples (Hotelling, 1933, 1936). The microbiota alpha-diversity analyses were also performed with a sample size and coverage-based integrations of interpolation (rarefaction) and extrapolation (prediction) of the Hill numbers (Hill, 1973; Alberdi and Gilbert, 2019; Roswell *et al*., 2021) using the R packages iNEXT (Chao *et al*., 2014). The computation of Hill numbers was performed for three increasing values of the order

parameter q, corresponding to increasing weight on the species abundance and also to different well-known diversity indices: q = 0, counting mainly the rare species (those with low abundances), corresponds to richness; q = 1, counting mainly the common species (those with medium-high abundances), corresponds to the exponential of Shannon index; q = 2, counting mainly the dominant species (those with very high abundances), corresponds to the inverse of Simpson index. 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 low abundance bacteria (mainly acquired from the environment and with no functional role).

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 1,238 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 1,209 bacterial ASVs.

In most of the investigated leaf beetle species the microbiota is dominated by the class Alphaproteobacteria, that represents ~80% of the sequences per species on average (Figure 1). This class includes the most important manipulators of insect reproduction (*e.g.*, *Wolbachia*, *Rickettsia*) that, in fact, dominate the microbiota of the majority of the analysed species (Table 2). Specifically, the microbiota of *Agelastica alni*, *Prasocuris phellandri* and *Plateumaris consimilis* is dominated by *Wolbachia*, with relative abundances of 75-99%; while *Chaetocnema conducta* and *Phaedon cochleariae* harbour both *Wolbachia* and *Rickettsia* (Table 2). Instead, sequences assigned to reproductive manipulators are almost absent (very low relative abundance, compatible with cross-sample contamination) in *Cassida rubiginosa* and *Donacia vulgaris*. In both these species the microbiota of males and females is quite different. Female's microbiota is dominated by a single bacterium with relative abundance higher than 80%, while male's microbiota is more evenly composed, with no dominant bacterium (Figure 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) (Figure 1). It is a quite strange result since these bacteria are usually found in soil and, moreover, *C. rubiginosa* is known to harbour the bacterial symbiont "*Candidatus* Stammera capleta", that was expected to be the dominant taxon, while no sequences of this symbiont have been identified in this case. Also, species in the Donacinae subfamily of Chrysomelidae are known to harbour a specific bacterial symbiont ("*Candidatus* Macropleicola"). In fact, in the microbiota of *Donacia vulgaris* most of the sequences can be assigned to this bacterium (~98% in females and ~30% in males, Figure 1) and the same bacterium was expected to dominate the microbiota of *P. consimilis* (for which only male individuals were sampled), but in this species *Wolbachia* infections resulted prevalent (>90%, Table 2) and only one individual was infected by "*Candidatus* Macropleicola", at low relative abundance (~2.6%).

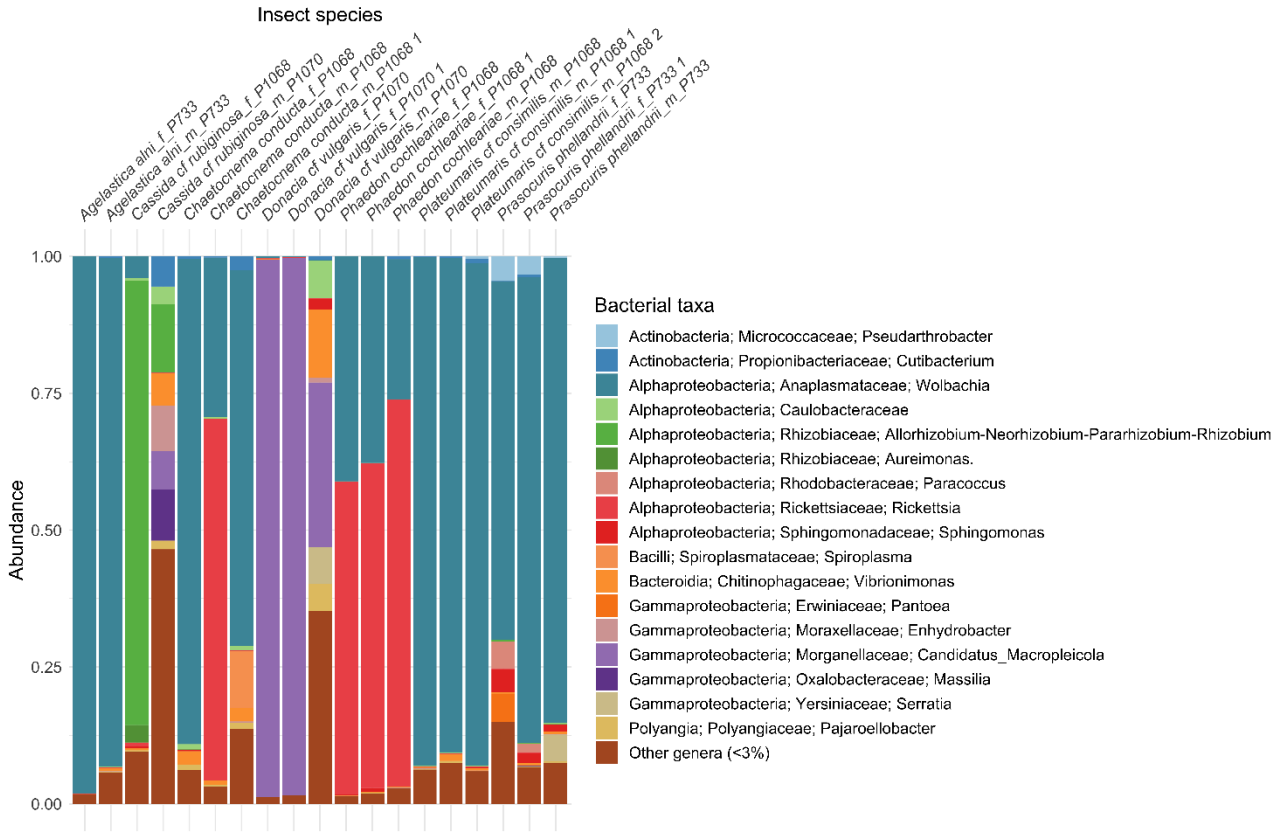


Figure 1. Taxonomic composition of the bacterial microbiota of the selected Chrysomelidae species. The barplot represents the composition of the microbiota of each Chrysomelidae species at the genus level (or any higher taxonomic level when the identification at the genus level was not possible). Colours represent different bacterial ranks, as reported in the legend, and the height of each box corresponds to the relative abundance of each bacterial rank. Only bacterial ranks representing at least

3% 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 insect reproductive manipulators (*Wolbachia, Rickettsia*) in females and males of each species. In the case of *P. consimilis*, only male individuals were analysed, so no data are available for females.

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 vulgaris	<0.2%	<0.2%	<0.2%	<0.2%
Phaedon cochleariae	39.0%	25.6%	58.6%	70.9%
Plateumaris consimilis	n.a.	91.6%	n.a.	<0.2%
Prasocuris phellandrii	75.7%	85.0%	<0.2%	<0.2%

The diversity analyses of the microbiota of the selected leaf beetle species show clear differences between sexes. Males' microbiota is significantly richer than that of females, at least for metrics that are not influenced by the relative abundance (*i.e.*, number of distinct ASVs and Faith's phylogenetic diversity), so counting mainly the rare taxa (Table 3). While metrics more influenced by the presence of abundant ASVs (*i.e.*, Simpson, Shannon and Pielou indices) show no significant difference among sexes (Table 3). A similar pattern was recorded using a coverage-based integrations of interpolation and extrapolation of Hill numbers to compare alpha-diversity estimates among sexes. Males have a richer microbiota than females, regardless of the value used for the q parameter, but the difference is emphasized for low values of q (Figure 2). In fact, when comparing sexes at the same sample coverage (*e.g.*, 0.5), diversity estimates for males are almost four times, thrice and twice higher than females for q=0, q=1 and q=2, respectively (Figure 2). So, most of the diversity of males' microbiota is due to rare bacteria (q=0), while the difference between sexes is reduced for the most abundant bacteria (q=1, q=2). Beta-diversity analyses show clear differences also in the composition of the microbiota of the two sexes only when using presence-absence metrics, that emphasize the effect of rare species (Jaccard index and unweighted unifrac distance, Figure 3a, 3b). While sex is not determinant when using metrics influenced by the taxon relative abundance and thus emphasizing the effect of the most abundant bacteria (Bray-Curtis dissimilarity and weighted unifrac distance, Figure 3c 3d). Specifically, in the PCoA (Principal Coordinates Analysis) performed on the Jaccard distance matrix

sex is more determinant than species in partitioning the variability of the microbiota. In fact, males tend to cluster on the lower left of the PCoA plot, so sharing most of the ASVs, while females occupy the top right portion but do not cluster together (Figure 3a). The effect of the sex is also evident in the PCoA performed on the unweighted unifrac distance matrix (Figure 3b), also in this case males tend to be in the lower left part of the plot and females in the top right, but since here the phylogenetic distance among bacterial ASVs is considered the effect of the species is more evident, probably due to the presence of phylogenetically related taxa in both sexes of the same species. The composition of the microbiota resulted to be significantly different in the two sexes, both comparing directly the ASVs (Jaccard index; PERMANOVA: pseudo-F = 1.61, p-value = 0.001) or using a phylogenetically informed metrics (unweighted unifrac distance; PERMANOVA: pseudo-F = 1.89, p-value = 0.001).

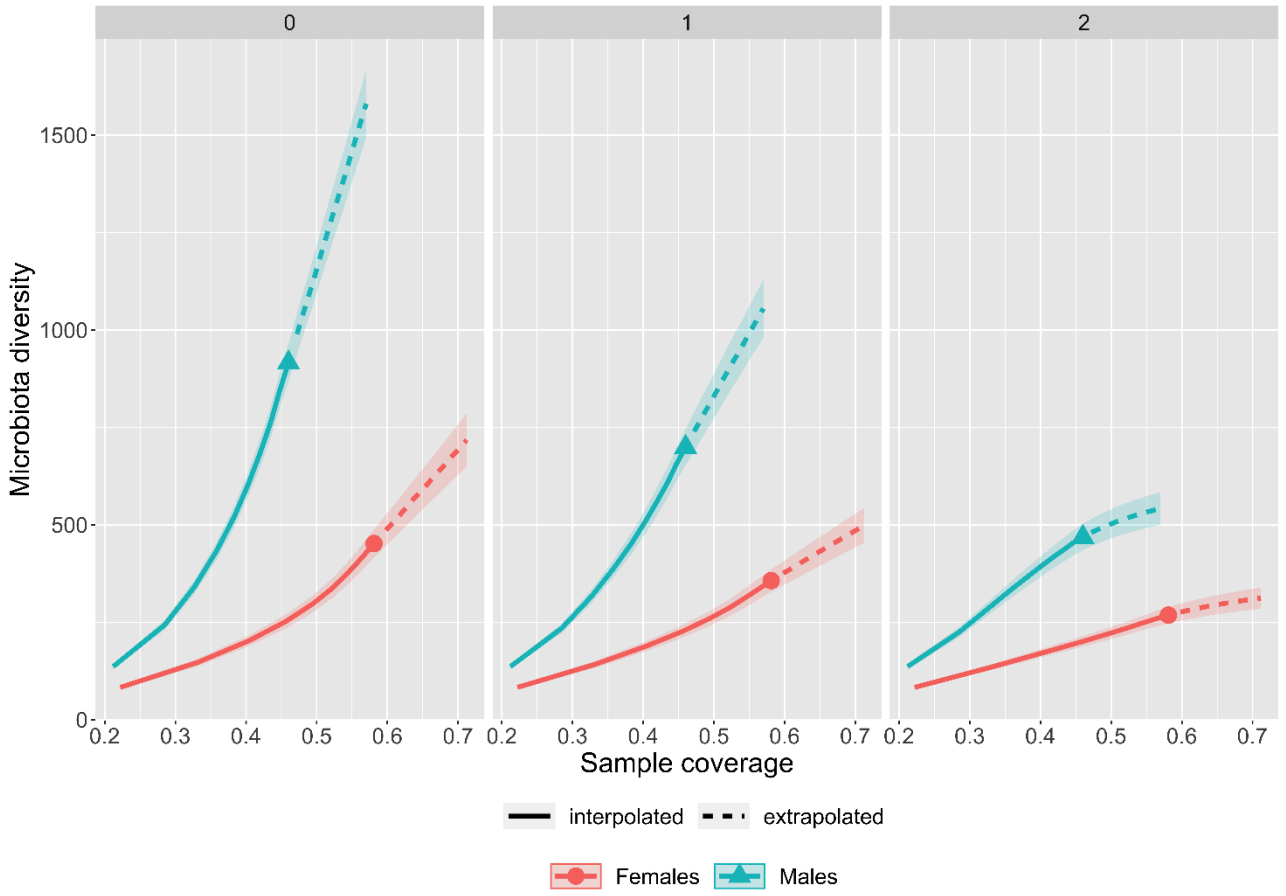


Figure 2. Microbiota alpha-diversity comparison of male and female leaf beetles. Coverage based rarefaction/extrapolation curves of the Hill numbers estimated for three values of the order parameter (q = 0, q = 1, q = 2). The x-axis represents the coverage (that estimates the completeness of the sampling) and the y-axis represents the Hill number estimates (95% confidence interval is also

shown). As reported in the legend, colours correspond to the two sexes (females or males) and line type to the methodological approach (interpolation or extrapolation).

Table 3. Results of the Kruskal-Wallis test for differences among alpha-diversity of the microbiota of males and females.

Alpha-diversity metric	Reference	H statistic	p-value
Number of distinct ASVs	McIntosh, 1967	4.86	0.027
Shannon index	Shannon, 1948	0.42	0.514
Simpson index	Simpson, 1949	0.03	0.870
Faith's phylogenetic diversity	Faith, 1992	4.51	0.034
Pielou's evenness	Pielou, 1966	0.03	0.871

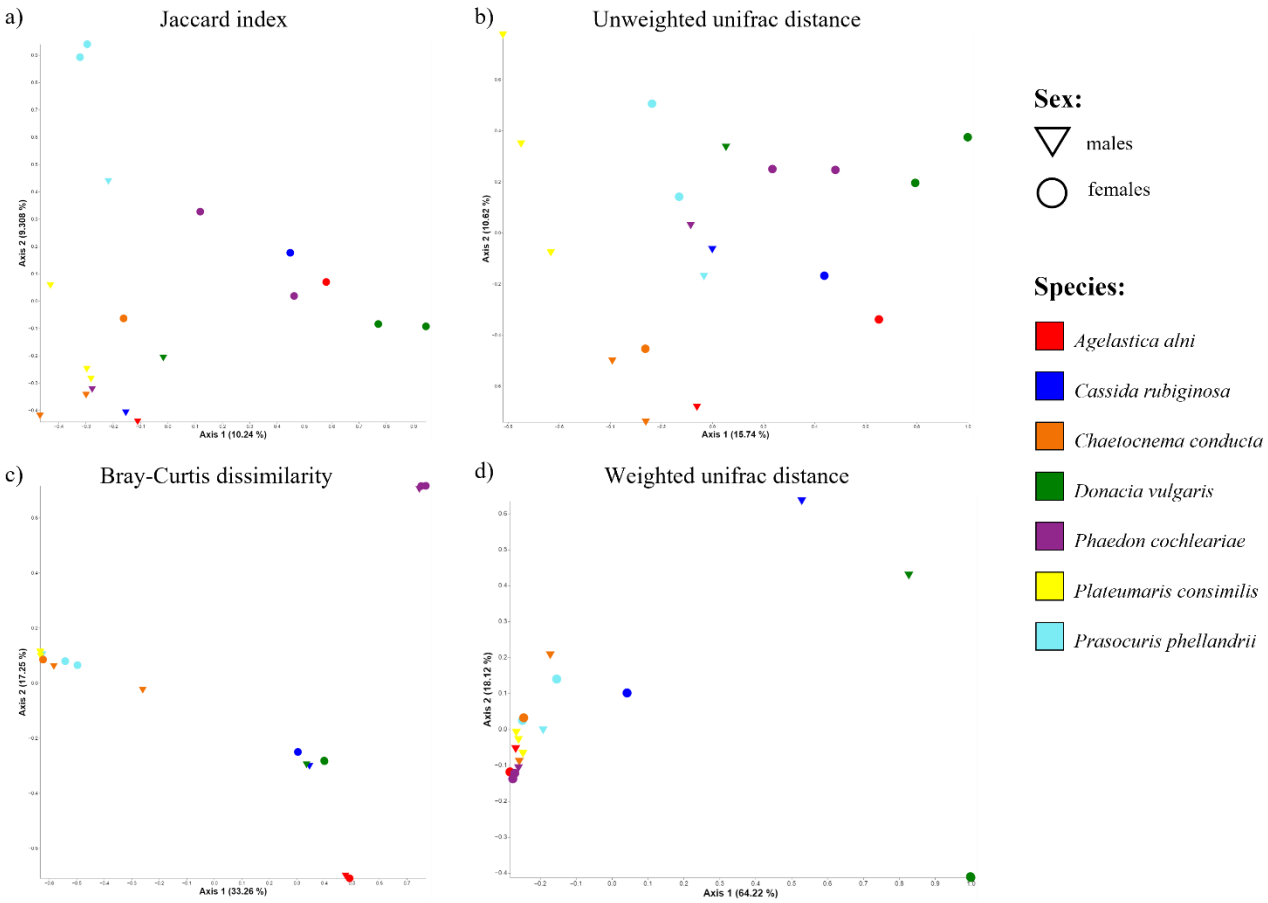


Figure 3. Principal coordinates analysis (PCoA) plots. First two axes of each PCoA are shown. As reported in the legend colours correspond to the insect species and shapes to the sex (triangles for

males and circles for females). Plots are based on different beta-diversity metrices: a) Jaccard index, b) unweighted unifrac distance, c) Bray-Curtis dissimilarity, d) weighted unifrac distance.

Discussion

In the present study the bacterial composition of the microbiota of seven species of Chrysomelidae associated to wetland environments have been characterized. Five of the seven species resulted to be infected by *Wolbachia* and/or *Rickettsia*, two well-known parasites of the insect reproduction that have been often recorded in the microbiota of Chrysomelidae (Montagna et al., 2014; Kajtoch and Kotásková, 2018; Gómez-Zurita, 2019, Brunetti *et al.*, 2021). In most of the cases *Wolbachia* and *Rickettsia* represent the dominant bacteria of leaf beetle microbiota, showing the highest relative abundance, while when other important insect symbionts are present reproductive manipulators can be almost absent (*e.g.*, "*Candidatus* Macropleicola" in *Donacia vulgaris*). Even if, in other cases, they can be prevalent also when other insect symbionts are present, such as the case of *Plateumaris consimilis*, where the primary symbiont "*Candidatus* Macropleicola" have very low relative abundance while the microbiota is dominated by *Wolbachia* (91.6%). Surprisingly "*Candidatus* Stammera capleta", the bacterial symbiont widespread in the Cassidinae subfamily of Chrysomelidae, resulted absent from the microbiota of *Cassida rubiginosa*, while this bacterium was firstly individuated precisely in this species (Salem *et al.*, 2017, 2020). The dominant bacterium (~82%) in *C. rubiginosa* female microbiota resulted to be in the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* complex, a group of soil bacteria that includes nitrogen fixing species but also some plant pathogens (the pathogenic genus *Agrobacterium* cannot be distinguished from the others using 16S rRNA sequences, Mousavi *et al.*, 2014). This bacterium is present also in males of *C. rubiginosa*, but with lower relative abundances (~14%) that are comparable to those of other bacteria present. Moreover, in *C. rubiginosa* and *D. vulgaris* the composition of the microbiota drastically changes between sexes. In both cases males are characterized by more evenly composed communities, where no dominant bacteria can be identified; while in females, as already noticed, a single bacterium is by far the most represented. Differences between the composition and diversity of the microbiota of males and females have been identified also in the global dataset. In fact, males have a richer microbiota, especially in the transient component of the less abundant bacteria (Table 3, Figure 2), that are more probably acquired from the environment. While when the focus is on the most abundant bacteria, those that more probably influence insects' biology, the difference between sexes is less

evident (Table 3, Figure 2). Also the composition of the microbial communities of the two sexes is significantly different only when considering the low abundance bacteria (Figure 3a, 3b).

The achieved results show that the microbiota of male and female leaf beetles is different both in term of diversity and composition. Also supporting the hypothesis that differences in the structure and composition of the microbiota in the two sexes can be related to different constrains on the vertical transmission mechanisms. In fact, bacteria establishing a stable relationship with the insect host are often maternally transmitted to the offspring, so they usually reach higher abundances in females (*e.g.*, they are harboured also in organs associated to female genitalia that allow the vertical transmission). While the composition and diversity of males' microbiota, where these stable bacteria have lower abundances, can be more prone to the influence of transient bacteria acquired from the environment, usually present at low relative abundances and that can be hidden by the presence of highly abundant bacteria.

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2.2.4 Personal contribution to the work

Conceiving the study, performing bioinformatic/statistical analyses and writing the manuscript.

2.3 Preliminary results: "Genomics of two putative bacterial symbionts in three Eumolpinae species"

2.3.1 Summary

Leaf beetles (Coleoptera: Chrysomelidae) harbour two genera of vertically transmitted Enterobacteriaceae symbionts widespread in specific subfamilies, "*Candidatus* Stammera" in the Cassidinae and "*Candidatus* Macropleicola" in the Donacinae. These bacteria are hosted in specialized organs associated to the gut and the female genitalia. Similar organs have been identified also in the Eumolpinae subfamily and two bacterial symbionts associated to these organs have been recorded in a single Eumolpinae species (*Bromius obscurus*). Recently these symbionts have been identified also in the microbiota of two other Eumolpinae species, C*hrysochus asclepiadeus* and *Macrocoma henoni*. The aim of this study is to genomically characterize these two symbionts in all the three Eumolpinae species harbouring them (*B. obscurus*, *C. asclepiadeus*, *M. henoni*) and in the specific insect organs harbouring them (only for *B. obscurus* and *C. asclepiadeus*): blind sacs associated to the foregut-midgut junction, small crypts at the midgut-hindgut junction and female genitalia. The preliminary results support the presence of two bacterial symbionts in these three Eumolpinae species, the first one is closely related to "*Candidatus* Stammera capleta" (the primary symbiont of Cassidinae) while the second to bacteria in the genera *Lelliottia* and *Klebsiella*. Preliminary assemblies suggest that we have been probably able to recover the full genome of the second symbiont, but not of the first one. The genetic distance within the same bacteria in different insect species support the hypothesis of a widespread symbioses in Eumolpinae possibly also characterized by coevolutionary processes.

2.3.2 Manuscript

Introduction

Leaf beetles, besides being a perfect model to study the microbiota of phytophagous insects, are of great interest also for the presence of two genera of vertically transmitted bacterial symbionts widespread in specific subfamilies, "*Candidatus* Stammera" in the Cassidinae (Stammer, 1936; Salem *et al*., 2017, 2020; Bauer *et al.*, 2020) and "*Candidatus* Macropleicola" in the Donacinae (Stammer, 1935; Kölsch *et al*., 2009; Kölsch & Pedersen, 2010; Kleinschmidt & Kölsch, 2011; Reis *et al*., 2020). Both these symbionts are harboured in specialized organs associated to the gut (small

evaginations at the foregut-midgut junction in the Cassidinae and modified malpighian tubules in the Donacinae) and in the female genitalia. Moreover, both symbionts are involved in pectinase production that complements the host-encoded set of digestive enzymes and their phylogenetic histories are tightly related with that of the insect hosts. In fact, the phylogeny of the symbionts and that of the insect hosts show similar branching patterns, suggesting a coevolutive process or reciprocal coadaptation. Similar organs associated to the gut and hosting bacterial symbionts have been identified also in other species of Chrysomelidae belonging to the Sagrinae (Tayade *et al.*, 1975) and Eumolpinae subfamilies (Mann and Crowson, 1983; Becker, 1994). Within Eumolpinae the presence of such organs has been identified in 31 species, but the bacterial symbionts hosted have been characterized only in one species, *Bromius obscurus* (Kölsch & Synefiaridou, 2012; Fukumori *et al*., 2017). The symbiosis in *B. obscurus* has not been studied in depth such as the symbioses of Cassidinae and Donacinae, but two different bacterial symbionts (Gammaproteobacteria: Enterobacteriaceae) have been identified. The first one (henceforth Sym-A) is hosted intracellularly in blind sacs associated to the foregut-midgut junction and extracellularly in genital accessory organs present only in the female, thus suggesting the presence of maternal vertical transmission. The second one (henceforth Sym-B) is hosted in small crypts at the midgut-hindgut junction and seems less strictly related to the host, since it is not reported from the genitalia and is phylogenetically related to other commensal bacteria species inhabiting in the gut lumen (Kölsch & Synefiaridou, 2012; Fukumori *et al*., 2017). In a previous study (Brunetti *et al.*, 2021), aimed to characterize the microbiota of several Chrysomelidae species with a metabarcoding approach, these bacterial symbionts have been identified also in two other species of Eumolpinae, *Macrocoma henoni* (only Sym-A) and *Chrysochus asclepiadeus* (both Sym-A and Sym-B). The presence of bacterial symbionts in *C. asclepiadeus* is particularly interesting since this insect feed almost exclusively on leaves of the toxic plant *Vincetoxicum hirundinaria* (Apocynaceae), so these symbionts could provide enzymes involved in the detoxification process. Given the widespread distribution of specialized symbiont-hosting organs in Eumolpinae species (Mann and Crowson, 1983) and the identification of the *B. obscurus* symbionts also in two other species of the subfamily, it is possible to hypothesize a widespread symbiosis, within Eumolipinae, like the ones in Cassidinae and Donacinae.

The aim of this study is to provide a first genomic characterization of the two bacterial symbionts harboured in specialized gut organs and female genitalia of three Eumolpinae species (*B. obscurus*, *C. asclepiadeus* and *M. henoni*).

Materials and methods

Insect specimens of *B. obscurus*, *C. asclepiadeus* and *M. henoni*, preserved in the ethanol (Table 1), were used for DNA extractions from the whole insect body. To also investigate the localization of the bacterial symbionts within the insect body, fresh individuals of *B. obscurus* and *C. asclepiadeus* were field collected in summer 2021 (Table 1) and dissected to isolate the symbiont-hosting organs. Unfortunately, it was not possible to collect new individuals of *M. henoni* for a fresh dissection. Insects bodies (3 females and 2 males of *B. obscurus*; 4 females and 7 males *C. asclepiadeus*) were surface sterilized and then dissected, under a hood and using sterilized instruments, to isolate the three tissues supposed to host bacterial symbionts: the foregut-midgut junction with the blind sacs (Figure 1a); the last part of the midgut, before the junction with the hindgut, with the small crypts (Figure 1b); and the female genitalia (Figure 1c). The obtained material was pooled by insect species and type of tissue. Total genomic DNA was isolated from whole insect body (for the ethanol preserved specimens) and from dissected organs (for the insects collected alive). DNA was extracted using the classical phenol–chloroform methods (Doyle & Doyle, 1990) with the following modifications. First, 500 µL of 2% CTAB (2% CTAB, 1.5% PVP, 1.4 mmol/L NaCl, 20 mmol/L EDTA and 100 mmol/L Tris-HCl, pH 8.0) was added to each sample. Tissues were then disrupted using glass beads (ø 0.1 mm) with the Precellys®24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) and incubated at 65° C for 15 min to inactivate nucleases. After centrifugation, the supernatant was incubated for 2 h 30 min with 20 µL of proteinase K (20 mg/mL) at 56° C. To purify the DNA, three phenol–chloroform washes (phenol/chloroform/isoamyl alcohol, 25:24:1, pH 8.0) were performed. DNA was, then, precipitated after addition of 500 µL of isopropanol and incubation for 1 h. Pellet was washed twice with 70% ethanol and eluted in 50 µL of Ultrapure Water (Sigma-Aldrich, Saint Louis, Missouri, USA). Purity of the extracted DNA were determined by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and by agarose gel electrophoresis. Qubit 4.0 fluorometer (Thermo Fisher Scientific) was used to determine the DNA concentration. Libraries prepared using Nextera XT DNA library preparation kit (Illumina, San Diego, CA) were sequenced with the Illumina MiSeq (2 x 150 bp) at a depth of ~100 millions and ~60 millions of paired end reads per sample for the whole insect body and the dissected tissues, respectively. The reads were assembled using SPAdes and subjected to a modified version of the blobology pipeline (Kumar et al. 2013), in order to select only the symbiont sequences. Briefly, we selected contigs for their coverage and GC content, extracted and reassembled separately the reads mapping on those contigs, and extensively revised manually the results. All the ribosomal DNA sequences with high

coverage and not assigned to plants have been subjected to a search on the NCBI nt database using the BLAST algorithm (Altschul *et al*., 1990). The MEGA X software (Kumar *et al.*, 2018) was used to compute pairwise distances among 16S rRNA sequences and to infer a Maximum Likelihood phylogeny with Kimura two parameters model (Kimura, 1980).

Table 1. Collection information on the analysed insect individuals. The status column report if the insect individuals were preserved in ethanol (EtOH) or collected and processed alive (living).

Species	N. individuals	Date	Country	Latitude	Longitude	Status
B. obscurus	1	29.06.2020	Italy	44.074 N	7.399 E	EtOH
C. asclepiadeus	1	06.06.2020	Italy	45.855 N	9.350 E	EtOH
M. henoni	1	23.05.2013	Morocco	31.150 N	5.393 W	EtOH
B. obscurus	3 ♀; 2 ♂	05.07.2021	Italy	44.151	7.582	living
C. asclepiadeus	4 ♀; 7 ♂	06.07.2021	Italy	45.855 N	9.350 E	living

Results

Only a low proportion of the reads obtained for the DNA isolated from the entire insect body (*B. obscurus*, *C. asclepiadeus*, *M. henoni*) can be assigned to bacteria, so we decided to first process the reads from specific insect tissues (only *B. obscurus* and *C. asclepiadeus*), to obtain draft genomes that may be used to process data for the entire insect bodies. Nonetheless, 16S rRNA assigned to Sym-A and Sym-B can be identified in the data for the whole insect body (Table 2). Specifically, in *B. obscurus* both symbionts have been identified, while in *C. asclepiadeus* and *M. henoni* only sequences belonging to SymB and SymA, respectively, have been found. The 16S rRNA sequences of Sym-A and Sym-B obtained from the full insect body of *B. obscurus* (S5, S6a, S6b) and *C. asclepiadeus* (S2, S3) are identical to sequences of those symbionts isolated from specific tissues of the same insect species (Table 3). While the Sym-A 16S rRNA sequence obtained from *M. henoni* has a p-distance of ~0.2 and ~0.08 to sequences of the same symbiont in *C. asclepiadeus* and *B. obscurus*, respectively (S7 in Table 3). Blast search top hits on NCBI database show that this sequence has ~90% identity with *B. obscurus* Sym-A sequences, suggesting a close relationship with this symbiont.

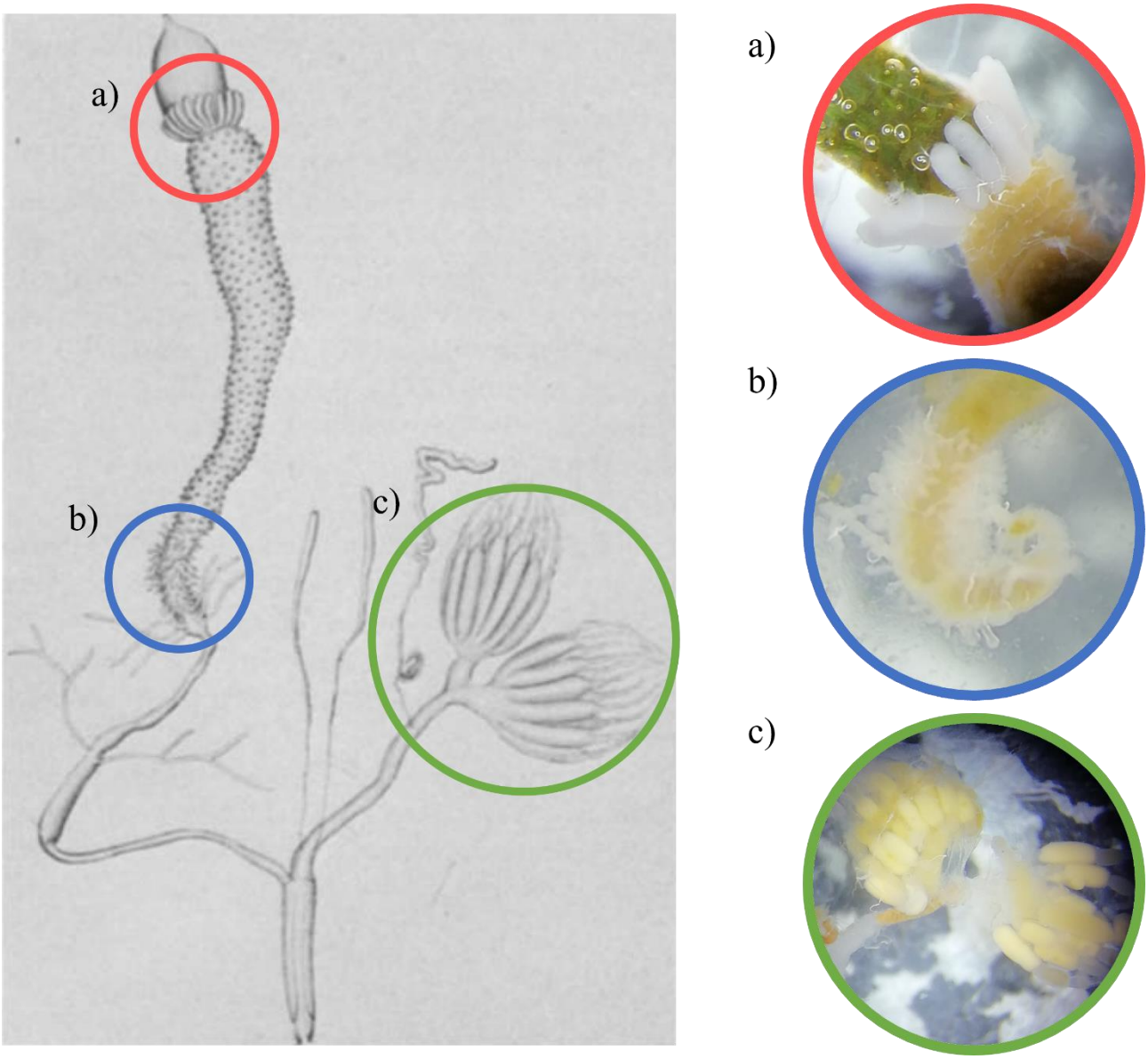

Figure 1. Different insect tissues isolated during the dissection. The drawing represents the gut and female genitalia of *B. obscurus* (taken from Stammer, 1936). Pictures represent the different portion of the insect body isolated during the dissection of *B. obscurus* individuals: (a) the foregut-midgut junction with the blind sacs harbouring the intracellular form of *B. obscurus* symbiont A, (b) the last part of the midgut, before the junction with the hindgut, with the small crypts harbouring *B. obscurus* symbiont B, and (c) the female genitalia harbouring the extracellular form of *B. obscurus* symbiont A.

Table 2. Blast search (NCBI-nt database) top hits for each of the high coverage 16S rRNA bacterial sequences obtained from different insect tissues and species.

Seq.	Species	Tissue	Acc. Num.	Q. cov.	identity	description
S1	*C. asclepiadeus*	foregut-midgut	LC273302	86%	85.09%	Sym-A
		junction	CP043989	99%	84.53%	*Candidatus* Stammera capleta
S2	*C. asclepiadeus*	midgut-hindgut	MG916974	100%	98.50%	*Lelliottia aquatilis*
		junction, full body	CP018628	100%	98.50%	*Lelliottia jeotgali*
S3	*C. asclepiadeus*	genitalia, full body	MG916974	100%	98.50%	*Lelliottia aquatilis*
			CP018628	100%	98.50%	*Lelliottia jeotgali*
S4	*B. obscurus*	foregut-midgut	LC273302	89%	99.72%	Sym-A
		junction	JQ805030	69%	99.91%	Sym-A
S5	*B. obscurus*	midgut-hindgut	MG916974	100%	99.09%	*Lelliottia aquatilis*
		junction, full body	CP018628	100%	99.09%	*Lelliottia jeotgali*
S6a	*B. obscurus*	genitalia, full body	LC273302	89%	99.86%	Sym-A
			JQ805030	69%	100%	Sym-A
S6b	*B. obscurus*	genitalia, full body	CP026715	100%	99.50%	*Klebsiella oxytoca*
			CP071383	100%	99.40%	*Leclercia* sp.
S7	*M. henoni*	Full body	LC273302	89%	90.12%	Sym-A
			CP043985	100%	85.49%	*Candidatus* Stammera capleta

Table 3. Pairwise p-distances calculated for each of the high coverage 16S rRNA bacterial sequences.

	S1	S2	S3	S4	S5	S6a	S6b
S2	0.196						
S3	0.196	0.000					
S4	0.148	0.175	0.175				
S5	0.196	0.015	0.015	0.175			
S6a	0.147	0.174	0.174	0.001	0.174		
S6b	0.198	0.015	0.015	0.172	0.001	0.171	
S7	0.163	0.151	0.151	0.077	0.142	0.078	0.142

The blobology approach allow to distinguish DNA sequences of the bacterial symbionts from sequences of the insect host and other associated organisms (*e.g.*, Nematoda, Platyhelminthes, Viruses) or contaminants (*e.g.*, plant DNA ingested with the diet, human DNA due to sample manipulation or laboratory contamination). The taxon-annotated GC-coverage plots relative to

specific insect tissues (Figure 2) show two groups of bacteria characterized by a different GC content. The first one, with a GC content of about 0.2-0.3, can be easily identified as a distinct cloud of point in the case of the foregut-midgut junction of both *B. obscurus* and *C. asclepiadeus*, but is also present in the female genitalia of *B. obscurus*, even if with a lower coverage. The localization of this bacteria in the insect body suggest that it is the Sym-A. The blast search top hits for the 16S rRNA sequences isolated for this bacterium from *B. obscurus* (S4, S6a) confirm this taxonomic assignment with an identity higher than 99.70% to sequences of the Sym-A present in the NCBI database (Table 2). Also the blast top hits for the 16S sequence obtained from *C. asclepiadeus* (S1) include the Sym-A, but with a sequence identity of only 85.09% (Table 2), suggesting that probably it is not the same species present in *B. obscurus*, but a related one. In fact, the 16S sequence S1 (*C. asclepiadeus*, foregut-midgut junction) have a p-distance of ~0.15 from the 16S sequences S4 and S6a (*B. obscurus* foregut-midgut junction and genitalia, respectively) (Table 3). While the two sequences from *B. obscurus* (S4 and S6a) have a low p-distance (0.001), confirming that the same bacterium is present in the foregut-midgut junction and associated to the female genitalia (Table 3). A second bacteria, with a GC content of about 0.5-0.6 (Figure 2), is present in the midgut-hindgut junction and female genitalia of both *B. obscurus* and *C. asclepiadeus*, suggesting that it represent the Sym-B. Unfortunately, no 16S rRNA sequence of the Sym-B is available in the NCBI database. Blast search top hits for the 16S sequences of this bacterium, both from *B. obscurus* (S5, S6b) and *C. asclepiadeus* (S2, S3), includes bacteria in the genera *Lelliottia*, *Klebsiella* and *Leclercia* with sequence identity higher than 98.50% (Table 2). The two 16S sequences from *C. asclepiadeus* (S2 and S3, midgut-hindgut junction and genitalia, respectively) are identical (p-distance = 0) and the two sequences from *B. obscurus* (S5 and S6b, midgut-hindgut junction and genitalia, respectively) have a p-distance of 0.001 (Table 3), thus suggesting that in both insect species the same bacterium in present in the midgut-hindgut junction and in the female genitalia. However, the 16S sequences of this bacterium in the two insect species are not identical (p-distance = 0.015) supporting the hypothesis that *B. obscurus* and *C. asclepiadeus* harbour different species or bacterial strains also for the Sym-B. Those differences among the symbionts hosted in the two insect species are supported also by the results from the preliminary assemblies. Specifically, the genome of the Sym-B in *B. obscurus* is characterized by the presence of repetitive mobile genetic elements, that are not present in the Sym-B from *C. asclepiadeus*. The size of the preliminary assembly of the Sym-B genome (~5,000,000 bp) is similar to the genome size of closely related species (*e.g.*, *Klebsiella* sp.) and the contigs are connected, so we are quite confident to have obtained almost all the genome of this bacteria. While in the case of Sym-A the genome coverage is probably lower, in fact in the preliminary assemblies obtained are of 50,000-150,000 bp,

but the estimated size of the genome is ~270,000 bp (the genome size of "*Candidatus* Stammera capleta" the closest species with a published genome).

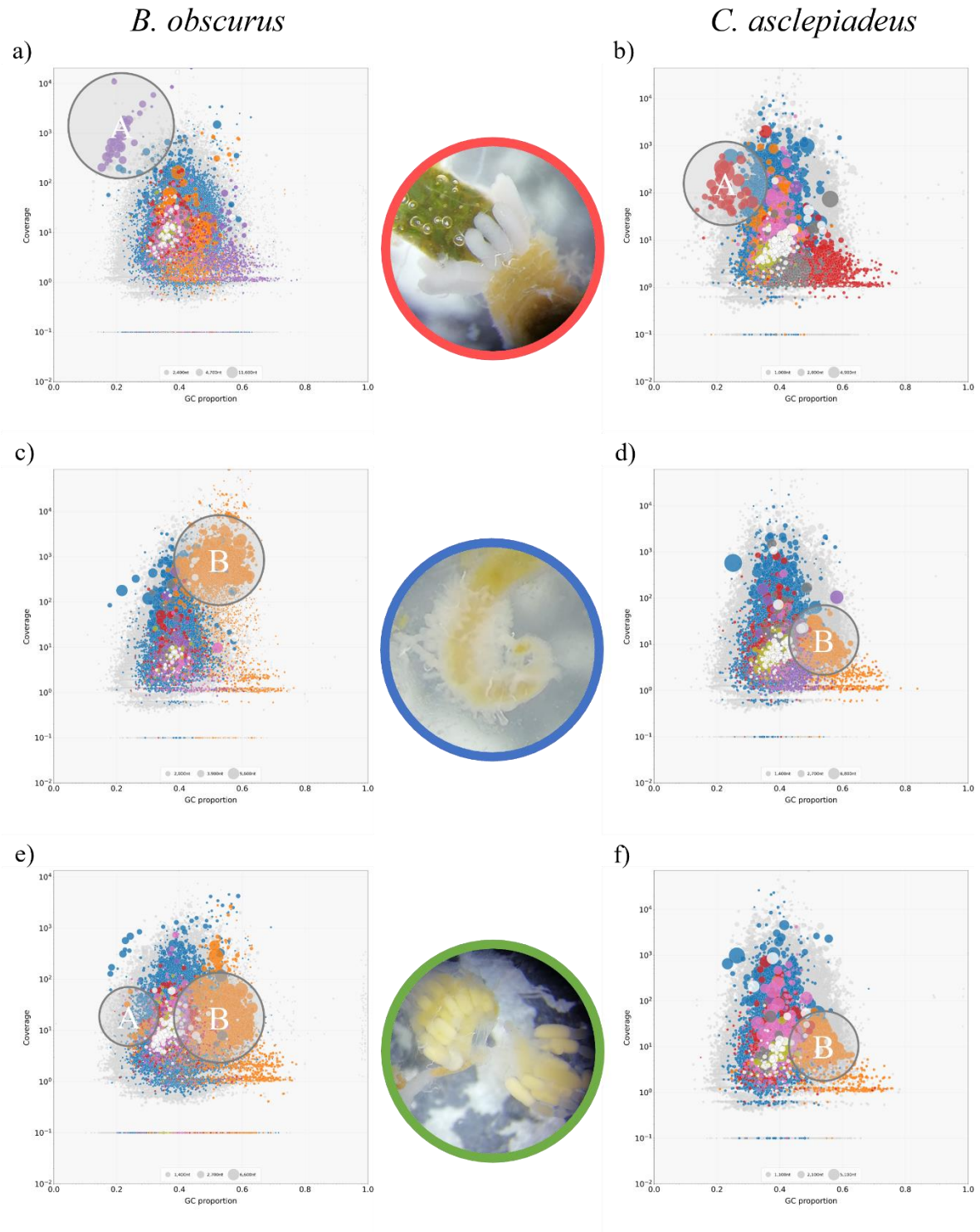

Figure 2. GC-coverage plots coloured according to taxonomic information. Plots are ordered in rows according to the insect tissue: foregut-midgut junction, (a-b); midgut-hindgut junction, (c-d); female genitalia, (e-f). And in columns according to the insect species: *B. obscurus* (a,c,e) and *C.*

asclepiadeus (b,d,f). Sequences assigned to bacteria are coloured in purple in the plot (a), in red in the plot (b) and in orange in the remaining plots (c-f). The blobs assigned to the two bacterial symbionts (Sym-A = A and Sym-B = B) are circled. Sequences assigned to the insect host are always coloured in blue.

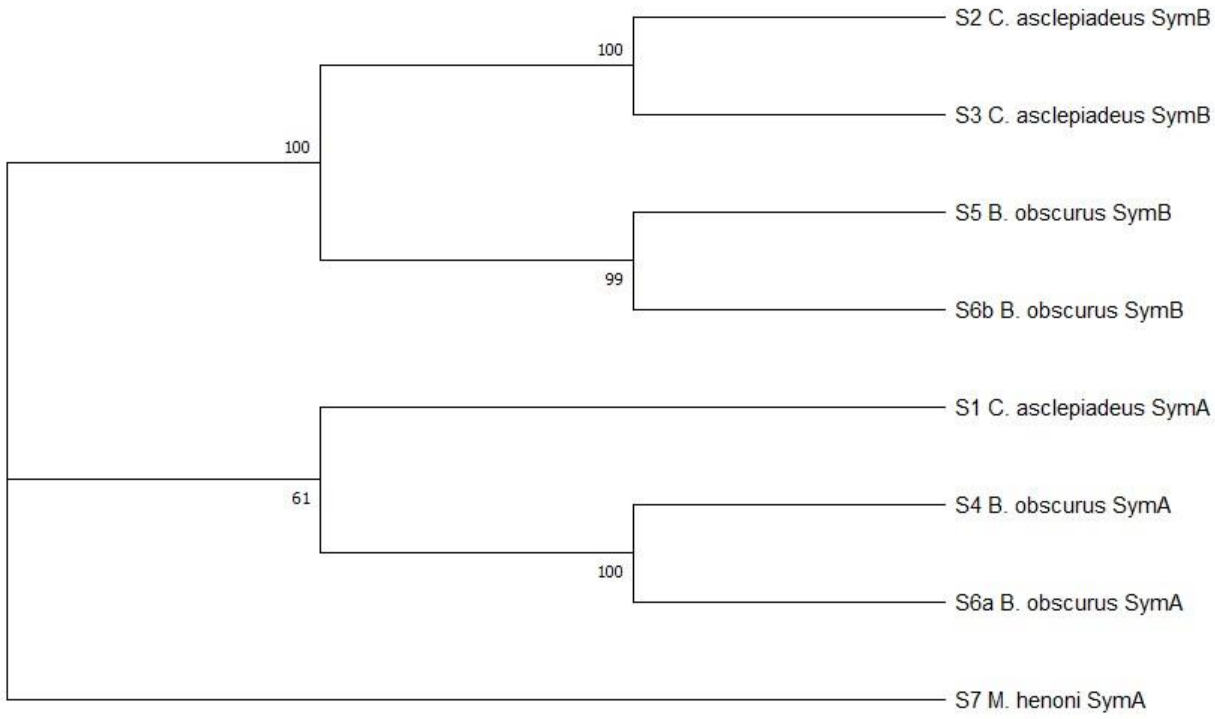

Figure 3. Bootstrap consensus ML tree (K2P model) of the 16S rRNA high coverage sequences. Bootstrap values are shown at the nodes (number of bootstrap replications = 500).

Discussion

The achieved preliminary results confirm the presence of two different bacterial symbionts in *B. obscurus*. The first one (Sym-A) is harboured in the blind sacs associated to the foregut-midgut junction and associated to the genitalia of females, as previously reported (Fukumori *et al*., 2017). The second one (Sym-B) is harboured in small crypts of the midgut-hindgut junction, but in this study, it has been identified also in the female genitalia, suggesting a vertical transmission strategy that include the sexual organs of the female, possibly similar to the one hypothesized for Sym-A. Two similar symbionts have been identified also in *C. asclepiadeus*. The preliminary assemblies and results from 16S rRNA sequences suggests that they belong to bacterial species or strains other than

those isolated from *B. obscurus*. Furthermore, the Sym-A from *C. asclepiadeus* is present only in the foregut-midgut junction and not in the genitalia of females. While Sym-B is present both in the midgut-hindgut junction and in the female genitalia, confirming the possibility of a vertical transmission strategy including female sex organs. Moreover, the abundance of mobile genetic elements in the genome of the Sym-B in *B. obscurus* suggests that this bacterium has started a genome degradation process, often occurring during the development of a strict symbiotic relationship. While the genome of Sym-B in *C. asclepiadeus* do not show this pattern of genome degradation and seems more similar to free-living bacteria. A possible scenario is that the acquisition of Sym-B in *B. obscurus* is older than in *C. asclepiadeus*, so it had time to develop in a more intimate symbiosis. Another possibility is that a common ancestor of *B. obscurus* and *C. asclepiadeus*, potentially the ancestor of all Eumolpinae, acquired the free-living ancestor of Sym-B, then the symbiosis developed separately in different species, starting the genome degradation process only when local selective pressures, maybe driven by the biology of specific insect species, pushed for it. Also *M. henoni* resulted to be infected by Sym-A, but no data on specific tissues are available. The obtained results support the presence of two bacterial symbionts in three different Eumolpinae species, opening to the possibility of widespread symbioses in this subfamily. The genetic distance among 16S rRNA sequences of the two symbionts in different Eumolpinae species suggest that these symbioses could be also characterized by co-speciation patterns, such as those reported from Cassidinae (Salem *et al*., 2017, 2020; Bauer *et al*., 2020) and Donacinae (Kölsch *et al*., 2009; Kölsch & Pedersen, 2010; Kleinschmidt & Kölsch, 2011; Reis *et al*., 2020).

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2.3.4 Personal contribution to the work

Conceiving the study, performing part of the laboratory procedure (dissections, DNA isolation) and bioinformatic analyses, writing the manuscript.

3. Conclusions

In this thesis a selection of Euro Mediterranean leaf beetles (Coleoptera: Chrysomelidae) has been used as case study to investigate two host related factors possibly affecting insects' microbiota, diet and sex. In chapter 2.1 the effect of the breadth of the trophic spectrum of phytophagous insects in shaping composition and diversity of their microbiota have been studied. It has been demonstrated that generalist species harbour a more diversified microbiota than specialists, and evidences that this phenomenon can be interpreted both as an adaptive trait and as a result of environmental stochastic acquisition conveyed by diet, are provided. In chapter 2.2, where the effect of the sex is considered, it has been demonstrated that male leaf beetles have a richer microbiota than females, especially in the low-abundance transient component, and differences in the distribution of bacterial primary symbionts and reproductive manipulators have been identified. This phenomenon may be related to constrains on the vertical transmission mechanisms acting differently in the two sexes. During the work for the first study (chapter 2.1) two putative bacterial symbionts have been identified in different species of the same subfamily, Eumolpinae. So, in chapter 2.3 preliminary results of a genomic characterization of these two bacteria isolated from different tissues of three Eumolpinae species are provided. These results support the presence of widespread symbioses in Eumolpinae, possibly similar to the well-known symbioses of two other Chrysomelidae subfamilies, Cassidinae and Donacinae.

Further relevant studies to which I contributed (attached to Annex A)

- Brunetti, M., Magoga, G., Iannella, M., Biondi, M., & Montagna, M. (2019). Phylogeography and species distribution modelling of *Cryptocephalus barii* (Coleoptera: Chrysomelidae): is this alpine endemic species close to extinction? *ZooKeys*, *856*: 3-25.

  Summary

  The alternation of glacial and interglacial cycles of the Quaternary period contributed in shaping the current species distribution. Cold-adapted organisms experienced range expansion and contraction in response to the temperature decrease and increase, respectively. In this study, a fragment of the mitochondrial marker COI was used to investigate the phylogeography of *Cryptocephalus barii*, a cold-adapted alpine leaf beetle species endemic of Orobie Alps, northern Italy. The relationships among populations, their divergence time, and the most probable migration model were estimated and are discussed in light of the

Pleistocene climate oscillations. Through a species distribution modelling analysis, the current habitat suitability was assessed and the distribution in a future global warming scenario predicted. The main divergence events that led to the actual population structure took place from ~750,000 to ~150,000 years ago, almost following the pattern of the climate oscillations that led to the increase of the connections between the populations during cold periods and the isolation on massifs in warm periods. The most supported migration model suggests that the species survived to past adverse climatic conditions within refugia inside and at the limit of the actual range. The species distribution modelling analysis showed that is extremely sensitive to air temperature variations, thus the increase of temperature caused by global warming will reduce the suitable areas within the species range, leading to its possible extinction in the next 50 years. *Cryptocephalus barii* is a representative case of how cold adapted and limited distributed species have been and could be affected by climate change, that highlights the implementation of conservation actions.

Personal contribution

Performed phylogenetic analyses and gene flow modelling, written the manuscript in collaboration with MM and GM.

- Goda, N., Mirzaei, M., & Brunetti, M. (2020). Potentially entomopathogenic nematode isolated from *Popillia japonica*: bioassay, molecular characterization and the associated microbiota. *Bulletin of Insectology 73*(2): 295-301.

Summary

The Japanese beetle, *Popillia japonica* Newman (Coleoptera Scarabaeidae), is a highly invasive pest recently introduced in Europe. In the current study a nematode is isolated from the third larvae instar of *P. japonica* collected in northern Italy. Both BLAST search and the phylogenetic maximum likelihood tree inferred from 18S rRNA sequences confirm the attribution of the isolated nematode to the genus *Oscheius* (Nematoda Rhabditidae). The entomopathogenicity of the isolated nematode was tested on larvae of the model organism *Galleria mellonella* L. (Lepidoptera Pyralidae). The mortality of the host after five days varied from 54% to 60%, depending on nematodes concentration. Furthermore, the microbiota associated with the isolated nematode was characterized using a metabarcoding approach. Our results suggest that the bacterial community of the isolated nematode is dominated by bacteria belonging to the genus *Ochrobactrum*, that includes entomopathogenic

species. Further studies are needed to test the possibility of using this nematode as a biocontrol agent of *P. japonica* in Europe.

Personal contribution

Conceived the study, performed metabarcoding and statistical analyses, written the manuscript in collaboration with NG.

- Brunetti, M., Capasso, V., Montagna, M., & Venturino, E. (2020). A mathematical model for *Xylella fastidiosa* epidemics in the Mediterranean regions. Promoting good agronomic practices for their effective control. *Ecological Modelling*, *432*: 109204.

  Summary

  Mathematical models represent essential tools allowing a quantitative analysis of an epidemic system with the consequent identification of possible strategies to control a disease outbreak or even to prevent it. However, to be used in decision-making, they must be carefully parametrized and validated with epidemiological data as well as biological information on the relevant players. Here, benefitting of the Olive Quick Decline Syndrome (OQDS) outbreak, which has occurred in Southern Italy since 2013, an epidemiological model describing this epidemic is presented. Beside the bacterium *Xylella fastidiosa*, the OQDS main players considered in the model are its insect vectors, *Philaenus spumarius*, and the host plants (olive trees and weeds) of the insects and of the bacterium. The model is based on a system of ordinary differential equations, the analysis of which have provided interesting results about possible equilibria of the epidemic system and guidelines for its numerical simulations. These, under a variety of parameter scenarios, have led to the model sensitivity analysis, hence to understanding the parameters relative importance in the transmission of the disease. Although the model presented here is mathematically rather simplified, its analysis has highlighted threshold parameters that could be the target of control strategies within the integrated pest management framework, not requiring the removal of the productive resource represented by the olive trees. Indeed numerical simulations support the outcomes of the mathematical analysis according to which a removal of a suitable amount of weeds biomass (reservoir of *Xylella fastidiosa*) from olive orchards and surrounding areas resulted the most efficient strategy to control the spread of the OQDS. In addition, as expected, the adoption of more resistant olive tree cultivars has been shown to be a good strategy, though less cost-effective, in controlling the pathogen.

Personal contribution

Collected biological data to inform model parameters and written the manuscript in collaboration with VC and MM.