Doctoral School in

Agrifood and Environmental Sciences

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Exploitation of underexplored physiological, metabarcoding and evolutionary aspects to enhance invasive insect pest management

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XXXV Cycle, 2021/2022

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LIST OF SYMBOLS AND ABBREVIATIONS

ASVs	amplicon sequencing variants
CWL	cuticular water loss
COI	cytochrome c oxidase subunit 1
$msVCO_2$	mass-specific VCO_2
$msVH_2O$	mass-specific VH_2O
MR	metabolic rate
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
RWL	respiratory water loss
SIT	sterile insect technique
VCO_2	volume of carbon dioxide
VH_2O	volume of water
WLR	water loss rate

Abstract

Invasive pests threaten agriculture and ecology of the invaded countries. To contrast and reduce their impact, entomologists develop specific and sustainable control strategies, which should target the pest and should not affect the surrounding environment. Therefore, a profound knowledge of pest biology and ecology is necessary to afford this task.

During my PhD, I delved into three underexplored approaches that hold the potential to unveil crucial biological information and develop more effective pest management strategies tailored to specific species: metabolic rate, microbiota shifts and molecular divergences. Although these three aspects might sound secondary in the context of pest management, they are important descriptors of the insect biological processes as they determine both the internal balance and the external interaction of the organism. I analyzed these three aspects for three case studies on three invasive pests diffused on the Italian territory. None of these investigated topics had been previously examined in relation to the studied species, and the findings I obtained hold promise for advancing species-specific control strategies. The overarching objective of my PhD research was to demonstrate the significance of exploring overlooked aspects of pest species biology, thereby augmenting the wealth of information necessary to enhance control strategies.

The metabolic rate aspect (**Chapter 2)** was addressed considering the spotted wing drosophila (*Drosophila suzukii*), an invasive species from eastern Asia that particularly threatens soft fruit crops. The study focused on measuring metabolic and water loss rates of *D. suzukii* flies captured at different sites located on an altitudinal gradient in Val dei Mocheni (Trentino, Italy) from early summer to fall. Respirometry was used to quantify the emission of carbon dioxide (CO_2) and water (H_2O) from individual flies, with the obtained CO_2 values serving as a proxy for the metabolic rate. The study aimed to compare the metabolic and water loss rates among *Drosophila* populations, aiming to enhance the ecological understanding of this species and

shed light on the metabolic and water management implications of living at different altitudes. Consequently, the results would provide insights into survivability, adaptability, pest distribution, and habitat suitability, offering valuable perspectives for management considerations.

Concerning the microbiota aspect (**Chapter 3**), I analyzed the brown marmorated stink bug (*Halyomorpha halys*), an invasive polyphagous insect from Asia that threatens most crops. Populations of this bug are able to survive severe winters, although during this period of the year they don't feed and they are subject to severe abiotic conditions, such as freezing. It is well known that, in general, bacterial microbiota can shift due to severe and prolonged conditions, but I find out that this is not the case for *H. halys*. Thus, targeting the bacterial microbiota could be a key factor for the management of this species. Moreover, I also searched for the presence of the microsporidian *Nosema maddoxi* and the overwintering consequences on its infection. The role of this microsporidian is still not clear, but it is supposed to be a parasite that negatively affects the ecology of *H. halys*. Findings of this study are also interesting for the sterile insect technique (SIT), since it has been suggested to be performed on overwintering wild males to overcome mass-rearing limitations in laboratory conditions, and therefore it is important to unravel microbial communities that characterize them to avoid accidental spreading of deleterious microbes.

Ultimately, the molecular divergence aspect (**Chapter 4**) was tackled studying the molecular clock of a newly invasive species of leafhoppers, *Arboridia dalmatina*, that was very recently found in Puglia (Apulia, Italy). This species might be a threat for vineyards, since it can feed on grapevines' leaves causing their decay. A phylogenetic and molecular clock analysis on the COI gene was performed to attempt to trace back the origin of this invasion and to understand the threats of this species.

Overall, the three approaches I used unveiled under-explored aspects of the three studied species, pointing out key information on their biological processes that can be exploited to further improve their pest-specific management strategies. The work presented in this thesis further proves the importance of using these approaches to deeply figure out pests' biology that can serve agricultural entomology purposes.

Chapter 1 - Useful biological information for pest management from neglected approaches

1.1. General introduction

Insects are the foremost represented class of animals in the world (Nagarajan et al., 2022). They originated between 400 and 500 million years ago and nowadays they have conquered most of the ecosystems of the Earth (Misof et al., 2014). The very high biological diversity that characterizes insects allowed them to cover every ecological role (Crespo-Pérez et al., 2020; Rosenberg et al., 1986; Scudder, 2017). Regarding their relationship with humans, they play several crucial roles in either positive or negative ways (Hill, 2012). Besides their usage as food, insects can increase crop production, as in the case of pollinators and decomposers, or produce commodities, such as honey or silk. On the other hand, several insect species can have negative effects for human health, as in the case of vector of diseases such as mosquitoes, or for human activities, as in the case of crop and livestock pests (Dent, 2000; Jankielsohn, 2018).

One of the most important branches of agricultural entomology concerns pest management because of the huge economic losses insect pests cause, especially for growers, farmers, and the agricultural industry. Although there are autochthonous pests that have always been problematic for agriculture in specific areas of the world (*i.e. Lobesia botrana* Denis & Schiffermüller, 1775, is native to Italy and it is a significant pest of Italian grapevines since it was discovered; Gutierrez et al., 2018) nowadays main threats come from alien species, which cause significant problems not only to agriculture, but also to the ecology of the invaded countries. The main causes of ever-increasing emerging insect pests' invasions are globalization, climate change and native habitat destruction (Skendžić et al., 2021).

To address the problems caused by invasive insect pests, pest-specific control systems are constantly studied, developed, and improved. In general, agricultural entomologists want to understand the most important aspects involving an invasive pest in order to develop a specific control system, such as: the stage of the life cycle of the pest that represents a threat; behaviors and ecological interactions that can be targeted or used to control the species; and type of crops,

seasons and locations that can be subject to attacks. Pest control systems usually consist in introducing or enhancing the presence of pests' antagonists, such as parasitoids and predators, and/or in directly controlling the crop pests, for example using insecticides or food/pheromone traps. However, especially nowadays, specificity and sustainability are mandatory factors for a control system, since the main objective is to reduce the impact of a pest on a crop without influencing human health and the surrounding environment (Barzman et al., 2015; Bottrell and Schoenly, 2018; Savary et al., 2012). Therefore, a profound knowledge of the pest insect biology and ecology is required, and thus it is necessary to understand all the fundamental aspects of the biology and the ecology of the insect pest (Fitri et al., 2021).

Despite the ever-increasing availability of tools and data to deepen our understanding of the biology and ecology of pest insects, there are some important aspects that are often overlooked, which can conceal a complete picture of action. During my PhD, I explored three areas of agricultural entomology that were underexplored, each with a different case study, in order to address the gaps that characterize them. These areas were:

1. **Metabolic rate** (**Chapter 2**): I characterized the metabolic rate (MR) and water management of *Drosophila suzukii* Matsumura, 1931, in relation to altitude and seasonal changes between summer and fall. This was the first time this information was obtained in *D. suzukii* using the flow-through respirometry technique. The results suggest that metabolic rate does not change in relation to altitude and season, while water management is influenced by altitude. These findings can improve management plans for *D. suzukii* by considering these two aspects, especially in the context of monitoring and invasiveness predictions.
2. **Microbiota** (**Chapter 3**): I investigated the influence of overwintering on the microbiota of *Halyomorpha halys* Stål, 1855. While the importance of certain microbes, such as *Pantoea carbekii*, for *H. halys* is known, it is unclear whether overwintering

had an impact on the overall microbiota. My results support the hypotheses that *H. halys* is highly dependent on its microbiota and its balance and that it is not well-suited to be a pathogen vector. Moreover, the fact that overwintering does not impact the microbiota is helpful for management purposes, especially in the context of the sterilized insect technique (SIT) which involves releasing sterilized male insects into the wild population to reduce reproduction. By selecting males that are free of unwanted microbes before overwintering, the unintended spread of microbes can be avoided during the SIT males' release.

3. **Molecular dating** (**Chapter 4**): I identified and traced the invasion of a new alien leafhopper species (*Arboridia dalmatina* Wagner, 1962) in South Italy. This information is helpful for accurately classifying retrieved specimens, estimating how long the species has been in Italy, assessing the severity of the situation, and applying appropriate prevention or control measures.

1.2. Metabolic rate

1.2.1. Exploiting metabolic information in pest management

Information gathered from insects' physiology are useful in agricultural entomology. Several control strategies have been developed to interfere with sensory processes (*i.e.* chemical compounds such as pheromones) (Howse et al., 2013; Reddy and Guerrero, 2010; Rizvi et al., 2021; Witzgall et al., 2010), with reproduction- or communication-associated processes (*i.e.* sterile insect technique and biotremological techniques based on vibrations) (Eriksson et al., 2012; Hill et al., 2019; Klassen et al., 2021; Lance and McInnis, 2021; Mazzoni et al., 2019; Polajnar et al., 2015; Takanashi et al., 2019), with locomotory processes (*i.e.* glue traps or repellents) (Child, 2015) or with vital processes (*i.e.* insecticides) (Horowitz and Ishaaya, 2004; Perveen, 2012; Rosell et al., 2008). All these physiological processes have one aspect in common: they produce and/or need energy. Therefore, targeting these processes reflects into changing the amount of energy produced and/or consumed.

Metabolism refers to biochemical processes by which cells convert nutrients into energy and other necessary substances that are required for life, and it can be considered a cornerstone of physiology. Processes of metabolism influence and are influenced by the components of the organism itself and the interactions of that organism with external abiotic and biotic factors, thus being the cause and the consequence of several physiological processes (as mentioned above), interactions and behaviors (Brand, 1997; Soyer et al., 2006, Dillon & Frazier, 2006; Frazier et al., 2008). Therefore, metabolic information plays a crucial role in planning control strategies. For example, suppose that a flying insect pest has to be managed in a cold region. The energy availability determines if the insect will be able to fly or not in this environment. In such situation, it is very likely that the flying pest will not have the amount of energy needed to fly, since most of the energy will be rather used for other functions, such as the production

of cryoprotectants or transformed into heat through metabolic heat production (Mołoń et al., 2020; Teets and Denlinger, 2013). Therefore, targeting the locomotory processes to manage that flying pest in a cold environment might not be the smartest choice, since it might be less deterred by repellents and less likely to get stuck in glue traps.

The previous example was referring to the case of low temperature, but there are several other abiotic factors that can influence the metabolism, such as humidity, high temperature, pressure and, in general, weather conditions. Furthermore, biotic factors, such as food availability, presence of predators and presence of competitors, also play a crucial role (Prasad, 2022; Waters and Harrison, 2012). This is particularly true for alien species, which are characterized by several new interactions that might affect the metabolism, such as new food, new competitors, new predators and parasitoids, a new landscape and a new climate.

In addition, monitoring pest insects and predicting their invasiveness play crucial roles in managing pest populations and minimizing crop damage. Traditionally, monitoring has relied on visual observations and trapping methods, which provide valuable information for invasiveness predictions, such as climate preferences, host range and pest behaviors (Dent and Blinks, 2020). These aspects are usually coupled with studies aimed to investigate pest's life history, resistance, survivability and genetic relationships among populations. However, the incorporation of metabolic information, such as metabolic rate (MR) and water loss rate (WLR), into these monitoring practices can greatly enhance outcomes.

MR serves as an indicator of insect activity and energy expenditure, offering insights into population dynamics and behavior. By incorporating MR into monitoring protocols, agricultural entomologists can gain a more comprehensive understanding of pest species, enabling more accurate predictions of their spread and impact on crops. WLR, on the other hand, reflects the insect's water balance and can provide valuable information about its adaptation to different environments and potential invasiveness.

Pairing metabolic information to other essays that evaluate the insect response to some condition unveils what are the strategies adopted by that insect as response (Smit et al., 2021). Therefore, metabolic information can also provide insights into beneficial aspects of agricultural entomology, such as studying the effectiveness of pest control strategies, optimizing integrated pest management practices, and assessing the impact of environmental factors on insect populations.

1.2.2. Metabolic measurements in insects

Adenosine triphosphate (ATP) is the most abundant biological compound that is used as an energy carrier in living organisms. In most eukaryotes this compound is produced through aerobic respiration, which consumes oxygen (O_2) to produce ATP, water (H_2O) and carbon dioxide (CO_2; Wikström et al., 2015). Thus, measuring O_2 consumption and CO_2 production can be used to have a proxy of the metabolic activity of a eukaryote, which is called MR.

Flow-through respirometry is the technique that I used to get a proxy of MR in my study (briefly represented in Fig. 1.1). This technique can be used to measure the concentration of O_2, CO_2 and H_2O in air and therefore quantify the exchange of gasses between an organism and the surrounding environment (Lighton, 2008). In the case of very small animals, such as fruit flies, the amount O_2 consumed by an individual is so small compared to the background O_2 concentration in the air that the O_2 fluctuations logged by the O_2 gas analyzers are not reliable (due to the low signal to noise ratio). In such cases, CO_2 is usually measured and considered as a proxy of MR (Lighton, 2008).

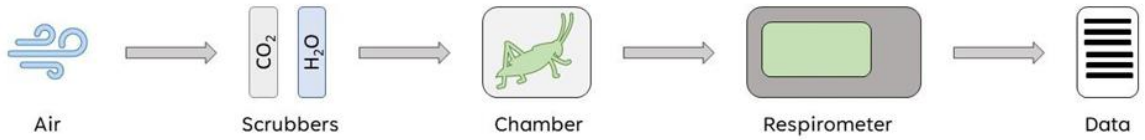

Fig. 1.1. Flow-through respirometry (push system) basic scheme.

1.2.3. Water management implications

Water is the most important molecule on which survival depends. Especially in stress conditions, such as climate extremes, absence of food and drought, reducing the water loss can significantly increase survivability. For example, in insects it was shown that water management is a key factor for desiccation survivability (Benoit, 2010; Gibbs, 1994, 2002).

Besides that, there are different strategies insects can adopt to manage water. In these animals, water can be lost mainly through the oral-fecal route (excretion), the cuticle (cuticular water loss, CWL) and spiracles (respiratory water loss, RWL). However, the way used to manage one or more of the physiological processes in order to save water in stress conditions is usually species-specific (Bradley, 1985; Chown, 2002; Mellanby and Haldane, 1934; Schilman et al., 2005).

Measurements of water exchange, through flow-through respirometry or changes in mass, can be used to define the water balance of an individual, and thus to study how water is managed in response to some factors or conditions, such as temperature, humidity or diet (Ciancio et al., 2021; Gibbs et al., 2003; Gibbs and Johnson, 2004; Lighton and Turner, 2004; Quinlan and Gibbs, 2006). In this way, water management, especially when coupled with MR information, depicts what is the overall physiological state of a pest in some conditions, how much the pest is resistant and thus strengthen the knowledge that uncover what are the aspects that might influence the management of the pest (Ciancio et al., 2021).

1.3. Microbiota

1.3.1. Importance of the microbes-host relationship

The common vision usually categorizes microbes as negatively affecting macro-organisms (called hosts), since they are usually associated with diseases. However, most of the microbes interacting with a host are either beneficial or neutral for its health, as in the case of microbes composing the animal gut flora (Mueller and Macpherson, 2006).

Symbiosis is the term used to describe an interspecific relationship between two living organisms (Martin and Schwab, 2012). If one of the organisms that are in symbiosis is living inside the other one, it is called endosymbiosis. Otherwise, if one of the two organisms is living on the surface of the host, it is called ectosymbiosis (Moran, 2006). The set of all microbes in endosymbiosis or ectosymbiosis with a host is called microbiota, whereas the genetic material of the whole microbiota of a host defines the microbiome of a host (Turnbaugh et al., 2007). It might be the case that the life cycle of one or both organisms interacting with each other depends on that symbiotic interaction, as it is the case of several microbes of the gut microbiota (Kucuk, 2020). In such cases, the symbiosis is called obligated, otherwise it is called facultative (Moran, 2006).

As well as for most organisms, also for insects, symbiosis is essential for survivability, especially endosymbiosis and ectosymbiosis with their microbiota. Each insect has its own microbiota that helps it in several biological processes, such as nutrition, reproduction, immune defense, and intra- and inter-specific communication (Fig. 1.2; Jing et al., 2020; Siddiqui et al., 2022).

Although the microbiota is individual-specific and depends on several biotic and abiotic factors (Li et al., 2022), such as temperature, diet and ecological interactions (Fig. 1.2), a significant

part of the microbiota of an individual is inherited by the parents (*i.e.* 5-13% in humans; Grieneisen et al., 2021). Due to these parental inheritances, part of the microbiota is the result of coevolution between microbes and their hosts, and they became essential for survival and fitness of each other (Kuechler et al., 2013; Yang et al., 2017), therefore there is a set of microbes that are likely species- or genus-specific (Maraci et al., 2021; Yan et al., 2020). Moreover, it is well established that there is a strict relationship between microbiota, physiological state and fitness (Berasategui et al., 2017; Jurkevitch, 2011; Shan et al., 2021). Indeed, factors affecting one of these aspects are likely to have consequences also on the other two.

Obviously, also in the case of insect pests, microbes play a crucial role in their survivability and lifestyle, and this information can be exploited for management purposes (Qadri et al., 2020). For example, comparative studies might be performed in order to uncover the effects of an event or a condition on a pest when a certain microbe is present in its microbiota, in order to understand if that microbe can be manipulated to reduce the pest spreading.

1.3.2. Techniques to unveil the microbiota

The traditional way to identify microbes of the insect microbiota consists in cultivation (Ferone et al., 2020). Despite the very high resolution in the identification of the grown microbial colonies, namely microbes can be recognized at the strain level, this methodology has a very important limitation, relevant from the agricultural perspective: not all microbes are culturable (Figdor and Gulabivala, 2008). Indeed, phytoplasmas, for example, which are one of the most studied genera of bacteria plant pathogens, are non-culturable in vitro yet, although they have been firstly identified in 1967 (Doi et al., 1967). In such cases, molecular methods can be used for a broader investigation, which are also faster to use and more economical than cultivation (Hugenholtz et al., 1998; van Elsas and Boersma, 2011).

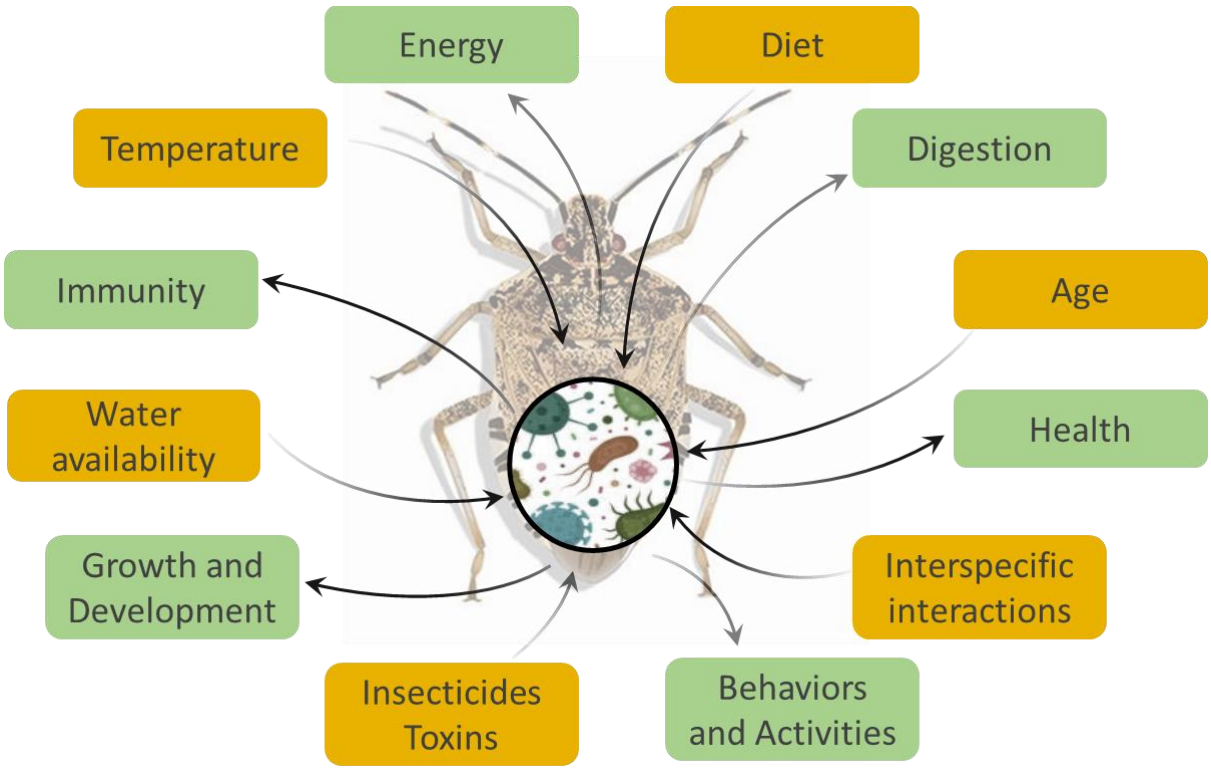

Fig. 1.2. Factors that can influence the microbiota of an insect (orange) and insects aspects that can be affected by microbiota (green).

One of the most used molecular methods consists in using qualitative or quantitative PCR (depending on the question to be answered) to amplify one or more genomic regions of interest in microbes. This approach is highly accurate when targeting one or a few specific bacteria. However, it becomes time-consuming when attempting to analyze the entire microbiota of an individual or a sample (Hospodsky et al., 2010). In such cases, one of the most popular molecular approaches is the amplification and high-throughput sequencing of a region of the 16S ribosomal gene (Kolbert and Persing, 1999). The 16S gene is present in all bacteria and can be divided into nine regions (Fig. 1.3), referred to as V1-V9. This approach is based on the barcoding technique, which is further elaborated in the evolution section and is also known as bacterial metabarcoding. Briefly, barcoding utilizes a molecular marker that enables the rapid identification of living organisms (more details in **1.4.3. DNA barcoding**). Metabarcoding is

a general term to define the barcoding of all the living organisms that are present in a sample, hence bacterial metabarcoding refers to barcoding of all bacteria within a sample (Elbrecht and Leese, 2015). Using the 16S approach, it is possible to identify bacteria at the genus level, and in some rare cases, at the species level. However, the quality of recognition depends on factors such as amplification and sequencing quality, the specific region of the 16S gene that was amplified, and the type of bacteria being identified (Mizrahi-Man et al., 2013). Metabarcoding is also applicable to fungi (Tedersoo et al., 2022), where the commonly amplified genetic region includes the internal transcribed spacers 1 and 2 (ITS1 and ITS2) of the ribosomal DNA (fig. 1.4) and the 5.8S subunit gene (Horisawa et al., 2009). Although fungi can achieve a higher resolution compared to bacteria, a challenge arises as there is no single genetic region that can identify all fungi (Badotti et al., 2017; Lücking et al., 2020; Thines et al., 2018).

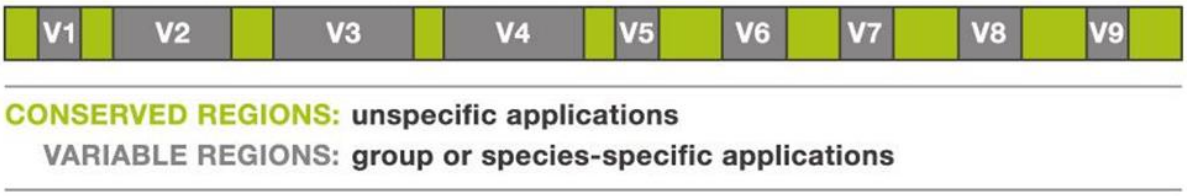

Fig. 1.3. General scheme of the 16S rRNA gene from bacteria (Renvoisé et al., 2013).

Despite the frequent usage of the 16S metabarcoding in defining the microbiota, the recognition at the genus level, also called low resolution, makes this approach not appropriate when researchers are interested in having detailed bacterial information (Elbrecht et al., 2017). An alternative molecular method that overcomes the 16S resolution limitation is shotgun sequencing metagenomics (Thomas et al., 2012). This approach consists in sequencing the whole genetic material extracted from an individual and then isolating the metagenomic sequences, namely the genomes of the microbes, from the host genome (Segata et al., 2013). Afterwards, metagenomic sequences are assembled and the final data are genomic sequences, each of which should be related to a prokaryotic species (or strain in some cases). This approach

is very recent and still under development, but its usage keeps growing in popularity. However, the main limitations of this method are complexity in terms of computation, requiring a powerful computer, and bioinformatic knowledge needed to properly use it.

Moreover, there are several tools based on this approach that can be used to identify bacteria and viruses, but very few were developed for other microorganisms such as fungi (Bell et al., 2021; Donovan et al., 2018).

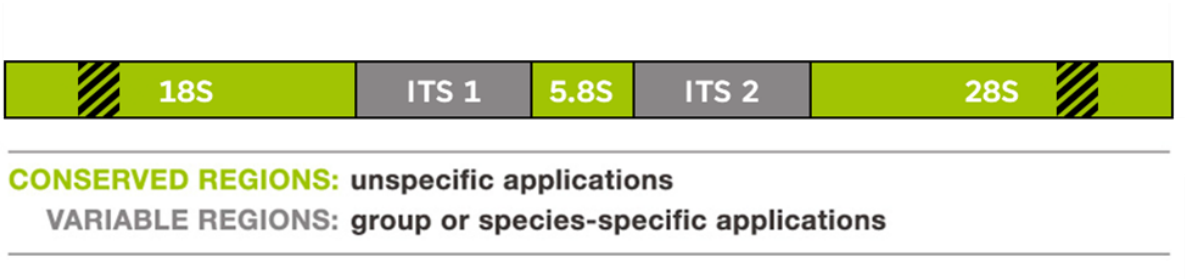

Fig. 1.4. General scheme of the ribosomal DNA genomic region of fungi (picture layout based on Renvoisé et al., 2013).

1.3.3. Potential applications of microbiota information

One of the most famous examples of species-specific bacteria that have a potential usage in control strategies belongs to the *Wolbachia* genus (Taylor et al., 2018). *Wolbachia* are intracellular mutualists or parasites that are harbored in several species of arthropods and nematodes (Werren, 1997; Werren et al., 2008). It has been estimated that 25-70% of all the insect species are potential hosts for *Wolbachia* species (Kozek and Rao, 2007; Oliveira et al., 2015). *Wolbachia* can infect several organs, most notably the ovaries, therefore the main way of transmission of these bacteria is from the mother to its offspring. The most interesting thing about these bacteria is how they maximize their spread. They can induce alterations on the reproductive behavior of their infected hosts in mainly four ways:

- males are killed during the development, thus more infected females grow and can further spread *Wolbachia* (Kozek and Rao, 2007);

- feminization (complete or partial) of males (Fujii et al., 2001);
- parthenogenesis (Knight, 2001);
- cytoplasmic incompatibility, which means that *Wolbachia* infected males can't reproduce with uninfected females or females infected by another *Wolbachia* strain (Breeuwer and Werren, 1990).

If an insect pest can be infected by a *Wolbachia* species, it is also likely to have one of the fitness alterations just mentioned. Thus, there is the possibility to use *Wolbachia* to manage that insect pest, for example introducing sterile females infected with *Wolbachia* in a region where the insect pest causes several problems (Darrington et al., 2017; Klassen et al., 2021; Vreysen et al., 2000; Zhang et al., 2015). In addition to *Wolbachia*, there are a few other genera of bacteria, such as *Spiroplasma* and *Rickettsia* (Sanada-Morimura et al., 2013), that have similar potential as control agents against insect pests and disease vectors. However, the approach of inducing sterilization or incompatibility with these bacteria is limited to a narrow range of species compared to the number of species that harbor these bacteria. It should also be noted that the effectiveness of bacterial SIT or incompatible insect technique (IIT) can vary depending on the specific insect species and the strain of bacteria being used, and scaling up these approaches for large-scale pest or disease control may present challenges. Therefore, in such cases, SIT induced by radiation overcomes the limitations of bacterial SIT or IIT.

Regarding the parental transmission of microbiota, in a lot of hemipterans it has been found a maternal behavior to transfer the mother microbiota to the offspring (Degnan et al., 2011; Hansen and Moran, 2014; Kuechler et al., 2013; Li et al., 2022; Matsuura et al., 2018). In some Hemiptera species it was shown that part of this maternally transferred microbiota is essential for the bug development and survival (Gonella et al., 2020). Thus, in case of pest bugs, there is a high potential for developing control strategies to negatively affect them. Noteworthy, these microbes are usually essential for survivability and development of the host, although in some

cases they are facultative symbiont. This is the case of *Burkholderia* that produce all essential amino acids and B vitamins that are usually not present in the herbivores diet (Kikuchi et al., 2011, 2007; Ohbayashi et al., 2019).

Nevertheless, several studies have shown that the microbiota shifts in response to some conditions (Fig. 1.2). These conditions can be abiotic, such as the change of season or the exposure to extreme temperatures, or biotic, such as the competition with other species for the food or the change of diet. For example, a study on *Hermetia illucens* Linnaeus (1758) has shown that part of the gut microbiota of these insects during the development depends on temperature (Raimondi et al., 2020). Another study on *Spodoptera frugiperda* Smith (1797) and *Helicoverpa zea* Boddie (1850) has shown that diet significantly influences the gut microbiota (Mason et al., 2020).

One of the most interesting consequences of microbiota-insect pest interaction from the agricultural perspective is the immune response improvement. Several studies have shown that there are facultative symbionts that increase the parasitoid resistance, heat tolerance and insecticide resistance (Feldhaar, 2011; Kikuchi et al., 2012; Montllor et al., 2002; Oliver et al., 2010), which are all methods that can be used as management strategies. For example, Kikuchi et al. (2012) showed that *Burkholderia*, can degrade fenitrothion (an insecticide), therefore inducing resistance in *Riptortus pedestris* Fabricius (1775).

It is straightforward that agrarian entomology does not only studies insects that directly cause problems to crop, such as lepidopteran, caterpillars or grasshoppers, but also those insects that are vectors of plant diseases, such as leafhoppers. Although bacteria and viruses that cause plant disease pass only a part of their life in the insect that they use as a vector, they can be considered as part of the insect microbiota during this period of time (Mogren and Shikano, 2021). Nevertheless, managing vector insects is as necessary as managing the herbivores, if not more. Thus, detecting which insect is able to carry and transfer a plant pathogen is essential

before planning a control strategy. For example, one of the most studied groups of bacteria is the group of phytoplasmas. There are several species of phytoplasmas, and they can cause diseases in more than 700 plant species. Several control strategies have been developed to control them, however the most effective and used ones consist in breeding phytoplasmas-resistant plants or managing the insect that vector them (Lee et al., 2000).

1.4. Molecular dating

1.4.1. Molecular phylogenetics

Phylogeny or phylogenetic tree refers to the evolutionary history and relationships among a group of organisms, typically represented in the form of a phylogenetic tree or diagram, which is constructed based on shared characteristics or traits among organisms (Fig. 1.5). It is well known that the evolutionary process of an organism is the result of a set of molecular modifications, called mutations. Therefore, the phylogeny of a group of organisms can be inferred from genetic and genomic sequences, transcripts or proteins (called molecular phylogeny; Allendorf et al., 2012; Nei et al., 2000) as well as using morphological traits and behaviors.

Mutations occur in cells mainly through biological processes such as DNA repairing and errors in DNA replication (Lanfear et al., 2014; Sniegowski et al., 2000). These can happen in germ cells, which are oocytes and sperm cells, or in somatic cells, which are all the other cells of an organism. They are mainly divided into two big categories: large-scale mutations, such as recombination and rearrangements, and small-scale mutations, *i.e.* single base mutations. In particular, the mutations of interest from the evolutive perspective are germ cell mutations: the offspring retains these mutations and in case they are beneficial they will likely be retained in the following generations, and they may eventually become fixed in a species (Allendorf et al., 2012). The mutation rate is the number of retained mutations that occur in a gene, genomic region or genome in a unit of time (generations or years). Of particular interest are the single base mutations (also called substitutions) since they are easier to detect compared to other kinds of mutations and they are usually used to infer molecular phylogenetics. The substitution rate is the number of mutations occurring in a new generation multiplied by the probability that these mutations become fixed in the population. Genetic drift and natural selection are the two

factors that influence the substitution rate (Charlesworth, 2009; Lynch et al., 2016). Genetic drift consists of a change in the frequency of a gene variant due to chance, whereas natural selection consists of the chance an individual has in surviving and reproducing depending on its phenotype.

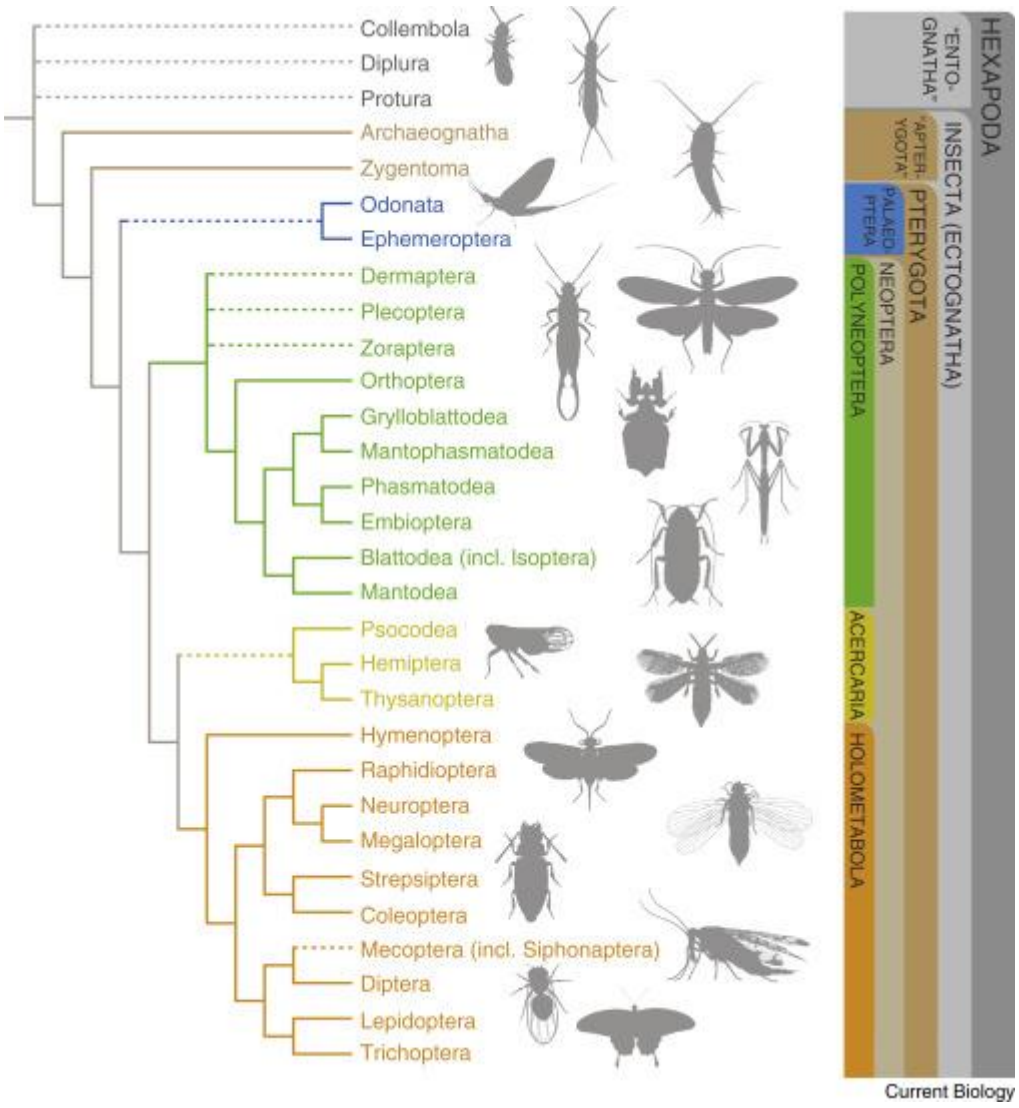

Fig. 1.5. Molecular phylogeny of Hexapoda (from Tihelka et al., 2021).

It has been shown that substitutions are not completely random, but there are certain types of substitution that occur more often compared to others (Lebeuf-Taylor et al., 2019). For example, silent substitutions, which are substitutions that do not change the amino acid

translated, are more likely to occur compared to substitutions that change the amino acid translated (Hughes et al., 2008). This is an important aspect to consider when inferring molecular phylogeny using substitutions, which is afforded through substitution models: statistical models that specify the rules governing how nucleotides or amino acids can change over time. One of the most used substitution models is the general time reversible model (GTR; Tavaré, 1986), which assumes different frequencies and occurrence of nucleotides and different substitution rates for each pair of nucleotides, usually assumed to vary among sites following a Gamma distribution (GTR+Γ model).

1.4.2. Types of data used in molecular phylogenetics

One of the most commonly used types of data for inferring molecular phylogeny is DNA barcoding sequences (Hebert et al., 2003). The DNA barcoding technique involves using a small genetic region to distinguish between species and describe their population structure. This genetic region can be located anywhere in the genome but should be small enough for easy PCR amplification using Sanger techniques. It should also vary sufficiently to distinguish between species but not too much between individuals of the same species.

The most commonly used barcoding sequence for animal identification is a region of the mitochondrial encoded gene cytochrome c oxidase subunit I (COI) (Lobo et al., 2013). However, for some taxa, the usual genetic sequences used for barcoding, such as COI for animals, may not be adequate to differentiate one species from another (Gibbs, 2018). This issue can be visualized by creating a histogram that represents the frequencies of pairwise distances between the barcoding sequences obtained for that taxon, distinguishing between intra- and inter-specific distances (Fig. 1.6). If the histogram reveals a gap between intra- and inter-specific pairwise distances, known as barcoding gap, then the gene can be used to distinguish the species within that taxon. On the other hand, if there is an overlap between the

two distributions, it indicates that the analyzed sequence is not sufficiently informative, and an alternative sequence should be utilized (Meier et al., 2008; Meyer and Paulay, 2005).

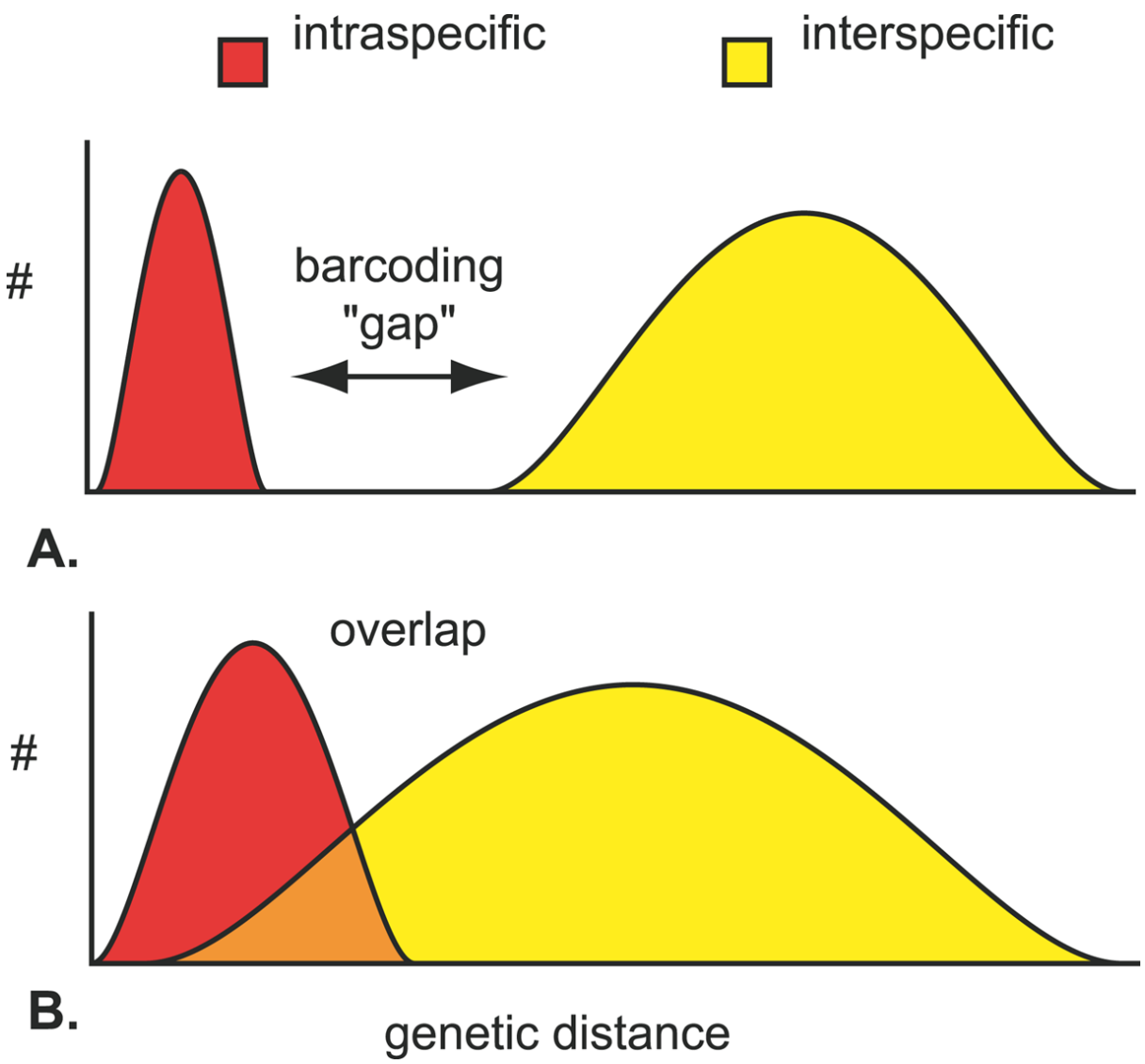

Fig. 1.6. Difference between barcoding gap and overlap (from Meyer & Paulay, 2005).

The main limitation of using only one barcoding sequence to infer phylogenetics is the lack of phylogenetic resolution, which means that the sequence phylogenetic tree is not descriptive enough of the evolution of the analyzed group of organisms (Trewick, 2008; Zamani et al., 2022). In such cases, using more barcoding sequences from different genomic regions might be helpful and more informative. Through this approach, discrepancies between mitochondrial (mtDNA) and nuclear (nDNA) sequences have been identified, remarking the need to consider

both types of information to increase the reliability of molecular phylogenetics when tracing back evolutionary history (Zadra et al., 2021). This issue mainly arises due to the different mutation rates characterizing mtDNA and nDNA, the different functions of mtDNA and nDNA genes, and hence, different selective pressure. Moreover, the inheritance of mtDNA is solely maternal, whereas nDNA is inherited from both parents. Additionally, there can be conflicts between gene trees, such as horizontal gene transfer, incomplete lineage sorting and hybridization (Leaché et al., 2014; Vawter and Brown, 1986).

Recent technological advancements have enabled the application of phylogenetics concepts to genomes, thanks to the availability of large amounts of data, the computational feasibility of managing data, and cost-effective sequencing facilities. This is referred to as phylogenomics (Kumar et al., 2012; Pennisi, 2008). Phylogenomics is still a field under development that requires way more bioinformatics skills and computational complexity compared to traditional molecular phylogenetics, mainly because reconstructing the whole set of genes of an individual is more complex and newer than reconstructing a few gene sequences. Despite this, phylogenomics results are generally considered more reliable because they consider many more genes and thus characteristics of living organisms compared to traditional phylogenetics (Simion et al., 2020).

While molecular phylogenetics and phylogenomics are widely used and accepted, there are instances where a combination of morphological and molecular information is necessary to achieve a well-resolved phylogeny (Moura et al., 2019).

1.4.3. Approaches to infer molecular phylogeny

There are two main approaches to infer molecular phylogenetics: phenetic methods, for example distance methods, and cladistic methods, for example maximum likelihood (ML).

Distance-based methods, such as neighbor-joining (Saitou and Nei, 1987) and UPGMA (Sokal, 1958), are simple, very fast and intuitive but not always accurate in reconstructing evolutionary relationships. They infer phylogeny by performing a clustering of molecular sequences based on a distance matrix, which is a symmetrical matrix where each cell contains the distance of a pair of sequences, computed based on the quantity of mutations that differentiate a sequence from the other one. However, usually phylogenetic trees realized using distance-based approaches are not reliable because they do not provide statistical support for the inferred relationships, and they assume a constant rate of evolution across all branches. Moreover, they do not incorporate substitution models. In such terms, maximum likelihood (ML) and Bayesian methods can be used to overcome all these issues.

ML phylogenetic methods rely on the principle of ML to construct phylogenetic trees (Cavalli-Sforza and Edwards, 1967; Felsenstein, 1981). Through this principle, the aim consists of finding the tree that most likely generated the input sequences. To address this aim, a set of parameters can be specified, such as the substitution model, the molecular rate and the substitution rate. However, the main limitation of ML approaches is the computational complexity. Indeed, increasing the parameter set and the amount of data to be analyzed, significantly increases the amount of time needed for the computation. Moreover, parameters are treated as constants.

Bayesian methods (Rannala and Yang, 1996; Yang and Rannala, 1997) instead calculate the probability of the tree and the model given the sequences, while utilizing ML to guide the tree search. These methods overcome the limitations of fixed parameters by incorporating priors (prior probability distributions assigned to the parameters) and representing estimated parameters as statistical distributions. Additionally, unlike ML, the outcome of Bayesian methods is not a single optimal tree but rather a summary of the observed variability during the search for results. To achieve reasonable computation times, Bayesian methods employ

Markov Chain Monte Carlo (MCMC) to obtain a heuristic approximation of the best result. Consequently, they exhibit faster computational efficiency compared to ML approaches.

1.4.4. Molecular clock

Bayesian inference has the capability of handling many parameters, allowing molecular studies to estimate when evolutionary events happened, particularly speciation and divergence. Techniques used to afford this task are called molecular clock techniques, which exploit the mutation *vs.* time relationship. This relationship was first detected and deepened in the 1960s. Zuckerkandl and Pauling in 1962 noticed that the difference in the amino acid sequences of hemoglobin between lineages was almost linear in time. Margolish in 1963 noticed that the differences in the cytochrome c among species were almost linear, and Sarich and Wilson in 1967 found that the albumin of primates changes at approximately constant rates. Thereafter, it was found that the molecular clock can vary significantly depending on the genomic region studied, as it can be affected by several factors, such as molecular characteristics and functions associated with the studied region (Kim et al., 2006; Wilke et al., 2009). Therefore, models that considered the mutation rate to vary over time (relaxed molecular clock; Drummond et al., 2006) started to be used when the strict molecular clock assuming a constant rate of mutation was not supported by the data.

In general, molecular clocks can encounter issues when applied to very short and very long timescales (Marshall et al., 2016). In the case of very short timescales, the molecular clock may be inflated by allelic polymorphism rather than fixed mutations. On the other hand, in the case of very long timescales, the observed changes might have undergone more than one mutation event, leading to a non-linear relationship between mutation rate and time (known as the saturation problem).

The following priors are required for molecular clock techniques:

- the substitution model;
- the branching model, namely the process that most likely traces back the evolutionary history of the studied species/sequences;
- the type of clock, which is the prior used to describe if and how the substitution rate changes throughout the tree branches;
- the calibrations, such as fossils, biogeographical information and tip dating (sequences date), used as temporal information.

1.4.5. Molecular clock applications in agricultural entomology

Evolutionary studies, particularly those based on population genetics and molecular phylogenetics, are widely used in agricultural entomology because they provide initial insights into the potential agricultural and ecological impact of the studied species. Evolutionary relationships between species can generate hypotheses that guide more specific studies on biological and ecological aspects. In general, molecular evolution studies are uausally faster and more cost-effective than traditional field and laboratory approaches that aim to achieve similar goals.

Although phylogenetics is widely used in agricultural entomology, molecular clocks are still not commonly utilized. This is mainly due to the fact that using molecular clocks requires sophisticated bioinformatics and evolutionary knowledge, which are not typically areas of expertise for agricultural entomologists. However, studying the evolutionary history of a pest species, such as estimating when it radiated, can provide insight into its biology, ecology, and behavior that can be useful for developing more targeted or effective control measures (Leftwich et al., 2016; Thrall et al., 2011). An example of using a molecular clock to obtain information on biology and ecology of a pest is illustrated by *D. suzukii*. This species has successfully invaded Europe and North America, posing a threat to soft fruits (Cini et al., 2012;

Rota-Stabelli et al., 2013). Several comparative genomics and physiological studies have been conducted on *D. suzukii*, particularly due to its close relationships to the well-known *Drosophila melanogaster* Meigen, 1830. One of the first evolutionary studies suggested that *D. suzukii* likely diverged from one of its closest relatives during the Himalayan uplift, and consequently evolved under cooler climate conditions (Ometto et al., 2013). This explains the species' preference for temperate climates during recent invasions.

1.5. Main objectives of the study

The main objective of this thesis was to verify whether underexplored areas of biology and ecology of insect pests may be useful from the pest management perspective. In the first two studies this was afforded by exploring aspects of two widely studied invasive pests that were still unknown. Whereas, in the last study, I used an often-neglected technique in agricultural entomology to obtain information on a new invasive pest that might be helpful for prediction purposes and to guide further research.

The first overlooked element I tackled involved the metabolism of *Drosophila suzukii*. Metabolism is usually studied in insect pests in order to explore how ecological interactions influence their physiology and behaviors. For *D. suzukii*, several of this kind of studies have been conducted to deepen the knowledge on cold resistance in order to find out the strategy adopted by this species to survive during temperate winters (Enriquez et al., 2018; Enriquez and Colinet, 2019, 2017; Everman et al., 2018; Jakobs et al., 2015; Rendon et al., 2016; Rossi-Stacconi et al., 2016; Shearer et al., 2016; Stephens et al., 2015; Terhzaz et al., 2018). Most of them were performed using a metabolomics approach. However, another important and under-explored aspect of metabolism is MR. While through metabolomics researchers are able to unveil processes and metabolic pathways involved in certain conditions, MR gives a proxy of the energy produced and consumed. Thus, MR studies unveils the effects of some factors on the overall physiological state of a species or a population, allowing speculations on their adaptation and/or acclimation (Ciancio et al., 2021), therefore helping to improve scheduling management plans against the pest and better predict its invasiveness. Although *D. suzukii* has been widely studied for the last 20 years, and it can be considered a model organism for pest and alien species (Biondi et al., 2016; Iacovone et al., 2015), to date very little is known about

its MR. The first study shown in this thesis (**Chapter 2**) is on the metabolic rate of *D. suzukii* and how it is affected by altitude and seasons.

The second aspect concerned the microbiota of *Halyomorpha halys* in relation to overwintering (**Chapter 3**). *Halyomorpha halys*, during overwintering, is exposed to severe conditions, such as cold, low humidity and starvation (Ciancio et al., 2021; Cira et al., 2016). Although these aspects are already widely studied in this pest (Ciancio et al., 2021; Grisafi et al., 2021; Nielsen and Hamilton, 2009; Watanabe et al., 1978; Wiman et al., 2015), it is still unclear what are the implications of overwintering on its microbiota. Since microbial communities can shift because of several factors, such as temperature and diet (De Filippo et al., 2017; Gong et al., 2021; Martínez-Solís et al., 2020; Obadia et al., 2018; Raimondi et al., 2020), assessing whether microbes are maintained in the microbiota of an individual in spite of the presence of severe stresses indicates that there is a sort of dependency between the individual and those microbes (Mushegian and Tougeron, 2019; Selosse et al., 2014) and it is important from the management perspective. Indeed, one of the most promising techniques to fight *H. halys* is the sterile insect technique (SIT), where sterility is induced by radiation on overwintered males, therefore defining the microbiota of these males is necessary to unintentionally spread plant pathogens or other problematic microbes (Suckling et al., 2019a).

The last element I considered was molecular dating of a new alien species of leafhoppers (**Chapter 4**). While phylogenetics and population genetics are usually well studied in invasive pests, in order to verify the number of invasion events and identify the origin of the invasive population, molecular dating is often neglected. The evolutive history of an invasive pest suggests the reasons why invasive pests evolved in that way, proposing possible solutions against them and providing helpful insight to predict further problems. Moreover, it can also be used to trace back invasion events and shed light on the reasons why these happened (Ometto et al., 2013). Therefore, it is important to study the divergence, especially when a new invasive

species is detected in a place. For this reason, I performed this kind of study on a new Italian invasive leafhopper (*Arboridia dalmatina*) that was found for the first time in South Italy (Apulia, Italy) in 2021.

Chapter 2 - Measurement of metabolic rates in *Drosophila suzukii* collected along an altitudinal gradient in an alpine valley (Val dei Mocheni, Trentino)

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2.1. Abstract

Drosophila suzukii is a soft fruit pest that successfully invaded temperate countries in the last two decades. Despite being a chill susceptible species, it is able to overwinter in the Alps, where winters are characterized by the severe conditions. One of the key factors for the persistence of *D. suzukii* in these territories is phenotypic plasticity: when the circadian cycle and the temperature decrease, the phenotype of this pest shifts between summer morph and winter morph. Furthermore, it was suggested that migrations alongside an altitudinal gradient might play a crucial role in winter survivability as well.

In this study, we investigated the adaptation to the mountain landscape of *D. suzukii* measuring the metabolic rate and water loss rate of flies caught along an altitudinal gradient in Val dei Mocheni (Trentino, Italy) from July to October. The metabolic rate of the flies did not vary among flies captured from summer to autumn at different altitudes, while water loss rate was lower in flies captured at higher altitudes, but did not depend on the sampling period, indicating that flies at higher altitude are more resistant to desiccation and dehydration stresses. These findings might be useful for predictive purposes on small- and large-scale invasion events of this species. Moreover, this study was the first attempt to use flow-through respirometry on *D. suzukii*, despite this species being studied for 20 years.

Keywords: *Drosophila suzukii*, respirometry, insect ecology, invasive alien species

2.2. Introduction

Insects modulate the oxygen (O_2), needed to produce energy, and the carbon dioxide (CO_2), waste product from the energy usage, through a system consisting of air sacs and capillary tubes called tracheae (Wigglesworth, 1990). Tracheae open out to the external environment as spiracles where the gaseous exchange between O_2 and CO_2 is accomplished through convection and/or diffusion (Woods and Smith, 2010). However, depending on the species, there could be differences in the number and the physical structure of spiracles. For example, mosquitoes have functional terminal spiracles, cockroaches have simple valved spiracles, while most endoparasitic larvae have cutaneous gas exchange (without any spiracles) (Gullan and Cranston, 2014; Oladipupo et al., 2022).

The metabolic rate (MR) of an insect (i.e. the energy expenditure of an individual, Nespolo et al., 2003) can be based on the quantity of O_2 consumed and CO_2 produced, however, the measurement of O_2 in insects is very difficult, especially for insects of small size. This is mainly due to the higher concentration of O_2 in ambient air compared to the very low change of O_2 during insect respiration. On the other hand, CO_2 has very low concentration in the air compared to O_2, which makes CO_2 measurements more accurate, despite the relatively small quantity of CO_2 exhaled by insects (Quinlan and Gibbs, 2006), which however can be accurately quantified if the flow is scrubbed of CO_2.

A consequence of the gas exchange, in particular of the opening of the spiracles, is the loss of an amount of water, called respiratory water loss (Woods and Smith, 2010). Thus, there is a relationship between metabolism and water management in insects. The latter is an important factor, especially in perspective of environmental changes leading to heat waves or periods of high temperature and low precipitation (Chown, 2011). Indeed, previous works have studied the relationship between the water management and the resistance to stresses such as starvation, desiccation, cold and heat (Chown et al., 2004; Colinet and Hoffmann, 2012; Dautel, 1999;

Hadley, 1994; Jakobs et al., 2015; Stephens et al., 2015). In several cases, better water management was related to a higher resistance to those stresses. Moreover, some studies have shown that some insects can trigger a peculiar respiratory pattern, called discontinuous gas exchange (DGC), in certain circumstances that are required to reduce the amount of energy expended. This was observed, for example, in populations of *Drosophila melanogaster* Meigen, 1830, adapted to desiccation. Furthermore, water management can be influenced not only by tuning the respiration (Chown et al., 2006; Lighton, 1996), but also changing the diet and the cuticle hydrocarbons composition (Benoit, 2010; Botella-Cruz et al., 2021; Ferveur et al., 2018). Indeed, water can also be lost in insects through the alimentary canal (excretory water loss) and the cuticle (Mellanby and Haldane, 1934; O'Donnell, 2022). In the former case, the quantity of water introduced through the diet influences the urine production and the reabsorption of the lost water through complex physiological pathways (Benoit, 2010; Bradley, 1985; Chown et al., 2004; Coast et al., 2002; Gäde, 2004; Kastin, 2013; O'Donnell, 2022; Riehle et al., 2002). In the latter case, studies have shown that there are three possible changes in the cuticle to improve the cuticle permeability (Benoit, 2010). The most studied of these consists in a change of the lipidic composition (mainly hydrocarbons) of the wax layer secreted by dermal glands upon the cuticle, whose aim is to increase the waterproofness of the cuticle (Blomquist et al., 1987; de Renobales, 1991; Gibbs, 1994, 2002; Hadley, 1981).

The spotted wing drosophila, *Drosophila suzukii* Matsumura, 1931 (Diptera: Drosophilidae), is an invasive fruit fly originated in eastern Asia, that successfully colonized the European and the American continents from 2008 (Cini et al., 2014; Rota-Stabelli et al., 2013). This species is well studied given its preference for soft fruits, such as strawberries, blueberries and raspberries (Cini et al., 2012). Alongside the agricultural interest, *D. suzukii* is also used as a model organism for invasive species, especially in physiological and molecular studies, due to its phylogenetic closeness to the well-studied *D. melanogaster* and its successful and relatively fast colonization of

temperate countries, as well as the Alps (Mazzotta et al., 2021). The sexual dimorphism of *D. suzukii*, characterized by females with bigger body and wing size and males with black spots on the wings, is paired to physiological differences between genders, such as the survivability and the resistance to stresses, that are higher in females (Panel et al., 2020).

From the physiological perspective, the strategy adopted by this species to survive the temperate winters, called winter morph, is also intriguing. The winter morph consists of morphological changes and physiological adaptations allowing the fly to survive the low temperatures and the decrease of food availability of the winter season. Winter morph *D. suzukii* are generally characterized by darker pigmentation, bigger wings, lower activity and reproductive diapause in both sexes compared to their aestival counterpart (Asplen et al., 2015; Kanzawa, 1939; Mitsui et al., 2010; Ometto et al., 2013; Rossi-Stacconi et al., 2016). Moreover, females were shown to be more suited to survive the overwintering period compared to males (Dalton et al., 2011; Grassi et al., 2018; Panel et al., 2020; Shearer et al., 2016; Stockton et al., 2018; Zerulla et al., 2015). Regardless the sexual difference in survivability, previous works showed that the morphological and physiological traits cited before are also associated with an increase of desiccation and starvation resistances and a longer lifespan of winter morphs (Enriquez et al., 2018; Panel et al., 2020; Shearer et al., 2016; Stockton et al., 2018).

In addition to winter morph, another factor suspected to be relevant for the overwintering survivability of *D. suzukii* is migration. Seasonal migration alongside an altitudinal gradient is recognized as being a strategy adopted by other drosophilids to enhance survivability (Kimura, 2004; Mitsui et al., 2010; Tait et al., 2018). Considering that, despite the phenotypic plasticity, *D. suzukii* is a chill-susceptible species (Dalton et al., 2011; Enriquez et al., 2018; Enriquez and Colinet, 2017; Kimura, 2004; Ryan et al., 2016), during winter this pest might migrate to lower altitudes, in the woods or in human-made shelters, where temperature is usually higher than it would be in other environments during winter, such as grasslands (Grassi et al., 2018; Mitsui et

al., 2010; Rossi-Stacconi et al., 2016; Tait et al., 2018; Tochen et al., 2014; Wiman et al., 2014). Furthermore, migration to higher altitudes in a mountain landscape could also be important during summer, to search oviposition sites and food resources (Evans et al., 2017; Mitsui et al., 2010; Tait et al., 2018; Samantha Tochen et al., 2016; Tochen et al., 2014; Wiman et al., 2014), when temperatures at low altitudes are warm, considering that this species prefers temperatures below 30°C (Eben et al., 2018; Enriquez and Colinet, 2017; Evans et al., 2017; Kinjo et al., 2014; Ryan et al., 2016; Tochen et al., 2014; Winkler et al., 2020).

Although it is known that there are differences between genders in terms of physiology, it is still not known how *D. suzukii* MR changes in relation to altitude and seasonality. Moving to higher altitudes and going from summer towards the winter, the temperatures become colder and the available resources become scarce. These aspects influence both biotic and abiotic factors, which in turn influence *D. suzukii* physiology. Furthermore, although this species has been widely studied in the last 20 years, information on MR is still poorly known, since, to our knowledge, only one other study has directly measured it, in order to assess the efficacy of monoterpenes on this pest (Finetti et al., 2021). Moreover, performing MR measurements we were also able to measure the water loss rate (WLR), and therefore gather some information to be compared with what was already present in literature on the water management of *D. suzukii.*

The aim of this study was to investigate the impact of gender, altitude and seasons on the metabolism and the water management of wild *D. suzukii* flies. Moreover, we investigated whether the preparation to overwinter, thus the presence of the conditions to trigger the winter morph, implies changes on the metabolism and the water management.

2.3. Methods

2.3.1. Study sites

D. suzukii was sampled in three periods ranging from 6 July until 25 October 2021 in three sites located in Val dei Mocheni (Trentino, Italy). The three sampling periods were named as the month in which most of the measured flies were caught: July (acronym: Jul, sampling period: 06/07/2021 - 03/08/2021), September (Sep, 26/08/2021 - 07/09/2021; for Sma it was prolonged until the 30/09/2021 due to absence of catches) and October (Oct, 30/09/2021 - 25/10/2021). The sampling sites are located along an altitudinal gradient ranging from 668 m a.s.l. to 1478 m a.s.l.: Viarago (Acronym: Via, 46°07′ N, 11°25′ E; 668 m a.s.l.; T = 10.7 °C; P = 989 mm), Borti (Bor, 46°12′ N, 11°31′ E; 1161 m a.s.l.; T = 8.3 °C; P = 1016 mm) and Redebus (Red, 46°14′ N, 11°32′ E; 1478 m a.s.l.; T = 6.2 °C; P = 1056 mm). These sites were chosen because of the presence of several soft fruit crops which were heavily attacked by *D. suzukii* in past years, and because previous studies (Rossi-Stacconi et al., 2016; Tait et al., 2018) were performed in the same area. An additional location (San Michele all'Adige; Sma, 46°19′ N, 11°14′ E; 210 m a.s.l; T = 12.9 °C; P = 958 mm) was selected in order to have a comparison with a plain site not belonging to the same valley and, at the same time, less affected by *D. suzukii*, since the majority of the cultivations in the area are vineyards and apple orchards. The climate characteristics of the sites (forty years average values of temperature (T: °C) and precipitation (P: mm) for the period 1980-2020) were derived from Crespi et al. (2021). During the sampling periods, the air temperature (at 2 m height) was monitored at 10 min interval with data loggers (SentritusTM, Laird connectivity, Akron OH, USA) in Via and Red7 , whereas we referred to the data collected from the closest meteorological station for the sites Sma (San Michele all'Adige) and Bor (Sant'Orsola)(Table 2.1).

2.3.2. Sampling

Droso-Traps (Biobest, Westerlo, Belgium) were used for trapping, they were baited with banana, apple and blueberries placed in a container together with a gel composed of water and agar 2% to

reduce fruits' rottenness and to provide a source of water for insects in addition to fruits. Three traps were placed in each site at a distance of at least 15 m from each other in forested areas in proximity of soft fruit orchards (Sites: Sma, Via, Bor) or where wild soft fruit plants were naturally growing (*e.g.* blueberries, strawberries, raspberries; Site: Red).

Right after the capture, flies were left to recover for two hours in BugDorm® (https://shop.bugdorm.com/), plastic net cages with available bananas and water as rearing food. Single individuals were then sampled from each cage and chilled at -4°C for 2-4 minutes to induce coma and to allow for *D. suzukii* identification, using a stereoscope. Afterwards, each fly was placed back in its respective cage with new bananas and water available. The cages were placed in a cell at 25°C, 70% RH and 12-12 hours day-night cycle for 1-7 days, until the day of respiration measurement. The 85% of the flies were measured within the third day of capture.

A total of 200 (96 f, 104 m) different flies were processed, from which only the flies caught in Red in Oct were winter morphs, recognized from the darker pigmentation compared to the counterparts caught in the same period in the other locations. Only the treatments where at least 5 flies were successfully captured, held in the lab and measured were included in the statistical analysis. Due to the low number of captures in Sma Sep (2 females), Via Oct (3 males) and Via Oct (3 females) these flies were only included in the graphical representations but excluded from the statistical analyses.

Table 2.1. Air temperature data of the sampling locations during the 12 days preceding the period of sampling activity.

Site	Temperature	Jul	Sep	Oct
Sma	max	35.4	34	27.3
	min	12.5	8.2	1.2
	mean	22.6	20.7	14.4
Via	max	28.8	25.2	22.2
	min	13.7	12.1	6.6
	mean	20.2	18.9	14.1
Bor	max	29	30.8	23.5
	min	9.4	7.3	0.6
	mean	19	17.5	11.6
Red	max	25.6	25.7	20
	min	9.8	7.6	3.8
	mean	17	15.6	11.2

2.3.3. Respirometry measurements

To assess metabolic rate and water loss rate of populations from different sampling sites, we measured the individual rate of CO_2 and H_2O exchange of adult individuals with flow-through respirometry ("push mode"; Fig. 2.1; J. R. B. Lighton, 2008). Adult flies were weighed before and after each measurement using an AX105 DeltaRange balance at 0.01 mg (Mettler Toledo, Columbus, Ohio, USA) in order to evaluate the weight loss during the trial. The respirometer consisted in a small cylindrical 1.2 ml plastic chamber connected to a LI-COR LI-7000 infrared gas analyser (LI-COR, Lincoln, NE, USA). The air flow was provided by a certified tank of dry and CO_2-free air (80% N_2 and 20% O_2). The air was further scrubbed of CO_2 and H_2O using a Silica gel – Ascarite – Magnesium Perchlorate column. Dry, CO_2-free air was then directed through an Alicat mass-flow meter-controller MC-500SCCM-D/5M (Alicat Scientific, Tucson, Arizona, USA) in order to create a constant flow of 70 ml min^{-1}. The flow was then conveyed to the chamber and finally to the LI-7000 through Bev-A-Line® 3.2 mm internal diameter tubes (1.6 mm wall thickness). The tubing between the mass flow controller and the analyzer was kept as short as possible (approximately 15 cm). With a flow rate of 70 ml min^{-1} and a chamber volume of 1.2 ml, the time constant of the system (*i.e.* the time necessary to reach the 63.2% of the final value of the measured variable after a step change; Lighton 2008) resulted in 1.0 s. The chamber was maintained at a constant temperature of 25 ± 1°C by enclosure in a home-made 560 cm^3 polystyrene insulated box. The box was provided with a digital thermometer connected to a thermistor and controlling two Peltier thermoelectric modules, one for warming and the other for cooling. The air temperature inside the chamber was monitored with a "T-type " 1 mm thermocouple connected to a CR10X data logger (Campbell Scientific, Logan, UT, USA); the temperature of the controlling thermometer was set to a target temperature allowing to maintain 25 ± 1°C inside the measuring chamber. The absence of air leakages in the chamber was assessed by immersion in water with an order of magnitude higher flow rate. To avoid the fly to escape

from the chamber, small pieces of synthetic sponge were positioned on each end of the chamber, these also allowed, at the same time, the disruption of the air laminar flow and favored the creation of a turbulent mixing which is highly recommended for a correct evaluation of the exchanged gasses (Gray and Bradley, 2006). Adult flies were measured singularly; before each measurement, the baseline of the empty chamber (for both CO_2 and H_2O) was recorded for 90 s. Each fly was allowed to acclimate in the chamber for 10 minutes before starting the measurement and afterwards, the concentrations of CO_2 and H_2O were recorded for 45 minutes at 1 Hz sampling frequency using the LI-7000 software. At the end of the measuring period, the chamber was opened and the fly removed; during these operations the logging was stopped for 4 minutes (time evaluated during test trials), in order for the air to return to 0 [CO_2] and [H_2O]. Eventually, the empty chamber baseline was recorded again for 90 seconds to allow for corrections of the gasses concentration in case of drifts during the measurement. During the measurements, the insect chamber was covered by a black sheet in order to reduce the fly activity (Mazzotta et al., 2020; Rieger et al., 2007). Measured flies were placed in a different cage from the original one.

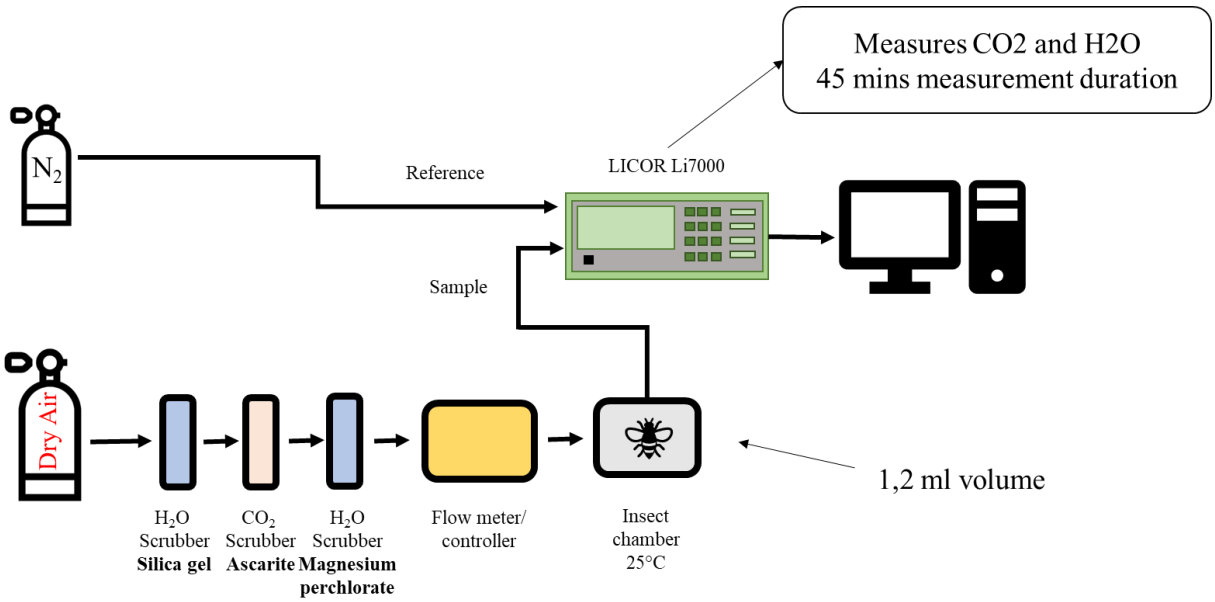

Fig. 2.1. Schematic representation of the experimental setup employed to assess the metabolic rate of *D. suzukii*. The CO_2 and H_2O levels were measured using a Licor LI-7000. The reference air flow was supplied by a nitrogen tank, which served to calibrate the measurement apparatus. The sample air flow originated from a tank containing free-CO_2 and free-H_2O, underwent three subsequent stages of H_2O scrubbing (two steps) and CO_2 scrubbing (one step), and was then regulated to a flow rate of 70 ml/min using a flow meter/controller. Finally, the air passed through the insect chamber, which had a volume of 1.2 ml. The measurements were conducted over a period of 45 minutes.

2.3.4. Data elaboration

The rates of CO_2 emission (VCO_2 µl h^{-1}) and WLR (VH_2O µl h^{-1}) reported below were calculated according to equations 10.4 and 10.9 from Lighton (2008):

$$VCO_2 = FR_i\ (F_eCO_2 - F_iCO_2)$$

$$VH_2O = \frac{FR_i\ (F_eH_2O - F_iH_2O)}{(1 - F_eH_2O)}$$

Where FR_i is the incurrent flow rate of the dry, CO_2 free air (ml min^{-1}), F_e CO_2 and F_i CO_2 are the excurrent and incurrent fractional concentrations of CO_2 respectively; F_e H_2O and F_i H_2O are the excurrent and incurrent fractional concentrations of H_2O. The water loss was also expressed in mg

min^{-1} by multiplying VH_2O by the ratio between molar mass of water and molar volume of water vapor (Lighton, 2008). Before applying the above equations, the values of CO_2 (mmol mol^{-1}) and H_2O (mmol mol^{-1}) obtained from the Li-7000 were corrected according to the measured baselines: for each measurement two arrays of data were built, one for CO_2 and one for H_2O, and each value was scaled linearly from the mean of the initial baseline to the mean of the final baseline. Collected data were analyzed both as such and as per unit body-mass (afterwards referred to as "mass specific values" $msVCO_2$ and $msVH_2O$) in order to eliminate the variation due to body size differences (Lighton, 2008).

2.3.5. Statistical analysis

The collected data were analyzed using generalized linear models (GLM) in the R software (version 4.1.2; R Core Team, 2022). The influence of sex, site and month of sampling on VCO_2 and VH_2O were tested. Four GLMs were built for each dependent variable with Gamma family distribution and log link function, differentiating each other by the interaction terms of the independent variables (Table 2.6). The statistical goodness-of-fit of the models was evaluated with the "performance" package (Lüdecke et al., 2021) and "aictab" function, and validated with "DHARMa" (Hartig and Hartig, 2017). The best performing model was selected based on the Akaike information criterion (AIC). AIC is an estimator of prediction of a model, resuming information on the goodness-of-fit considering overfitting and underfitting of that model. The lower the AIC value, the better the model (Stoica and Selen, 2004). Results of the best models were shown using the "effects" package (Fox, 2003; Fox and Weisberg, 2018). The package "emmeans" was used to perform the post hoc tests on the best GLMs to investigate the pairwise differences between factors and their categories (Lenth et al., 2018).

2.4. Results

2.4.1. Analysis on the CO_2 production

The model without interaction terms between the independent variables resulted in the best fitting of VCO_2, with an AIC of 556.82 (Table 2.2). The model pointed out a significant difference in VCO_2 between males and females, with males having a lower VCO_2 (Table 2.2; $p \simeq 0$). The model also showed that the insects (both males and females) caught in Oct had a statistically significant lower VCO_2 compared to those trapped in Jul (Table 2.2, $p < 0.01$). VCO_2 did not differ among individuals from different sites. The post hoc test confirmed the same result (Table 2.6).

When considering $msVCO_2$, the simple model (Table 2.3; AIC = 308.15), the difference between males and females was reversed compared to VCO_2 model, with males having higher $msVCO_2$ than females (Table 2.3, $p \simeq 0$). Consistently with the VCO_2 results, there were no significant differences among sites, but there were also no differences related to months, whereas VCO_2 GLM detected differences between Jul and Oct.

2.4.2. Analysis on the water loss

Also in the case of WLR, the model selection pointed out the simple GLM as the best model, with an AIC of -968.82 (Table 2.4).

The model pointed out a significant decrease in VH_2O in males (Table 2.4, $p \simeq 0$) and highlighted that insects caught in Sep (Table 2.4; $p < 0.01$) and Oct (Table 2.4; $p < 0.05$) had a lower VH_2O compared to those trapped in Jul and that all flies coming from the high altitude sampling points located in Val dei Mocheni had a lower VH_2O than flies caught in Sma (Table 2.4; Bor $p < 0.01$; Red $p \simeq 0$). The pairwise post-hoc test confirmed the results shown in the model, except for the Sma *vs.* Via difference, without finding further significant differences between the flies sampled in Val dei Mocheni (Via, Bor and Red) and between Sep and Oct.

When considering the simple model of the $msVH_2O$ (Table 2.5; AIC -1128.50), as in the case of VCO_2 *vs.* $msVCO_2$, the difference between males and females was inverted, with males having higher $msVH_2O$ (Table 2.5; $p < 0.01$). In addition, insects caught in Bor and Red had a statistically significant lower $msVH_2O$ compared to insects caught in Sma (Table 2.5; both with $p < 0.01$), which was also confirmed by the post-hoc test (Table 2.6; both with $p < 0.05$). Also in this case, the post-hoc test did not show any significant difference between sites from Val dei Mocheni (Via, Bor and Red). However, differently from the VH_2O model, the $msVH_2O$ model did not show any significant differences between months, although there were not found significant differences in initial mass among sampling locations (Fig. 2.2).

Table 2.2. GLM with Gamma family (log linked) to analyze the effect of month, site and sex on the VCO_2 ($\mu L\ CO_2\ h^{-1}\ insect^{-1}$). The table summarizes the simple model ($VCO_2 \sim$ month + site + sex). All estimates are computed against the base value of that variable (*i.e*. Jul for month, Sma for site and f for sex). Jul = July, Sep = September, Oct = October, Sma = San Michele all'Adige, Via = Viarago, Bor = Borti, Red = Redebus. f = females, m = males.

Category	**Coefficient**	**Estimate**	**SE**	**t-value**	**p-value**
	Intercept	1.650	0.05	34.451	0.000
Site	Via	-0.064	0.05	-1.155	0.250
	Bor	-0.049	0.05	-1.001	0.318
	Red	-0.042	0.05	-0.850	0.396
Month	Sep	-0.040	0.04	-1.000	0.318
	Oct	-0.135	0.05	-2.957	0.003
Sex	m	-0.275	0.03	-7.792	0.000

Table 2.3. GLM with Gamma family (log linked) to analyze the effect of month, site and sex on the $msVCO_2$ ($\mu L\ CO_2\ h^{-1}\ g^{-1}$). The table summarizes the model that does not consider interactions ($msVCO_2 \sim$ month + site + sex). All estimates are computed against the base value of that variable (*i.e.* Jul for month, Sma for site and f for sex). Jul = July, Sep = September, Oct = October, Sma = San Michele all'Adige, Via = Viarago, Bor = Borti, Red = Redebus. f = females, m = males.

Category	**Coefficient**	**Estimate**	**SE**	**t-value**	**p-value**
	Intercept	0.946	0.04	25.290	0.000
Site	Via	0.014	0.04	0.315	0.753
	Bor	-0.074	0.04	-1.934	0.055
	Red	-0.011	0.04	-0.292	0.771
Month	Sep	0.018	0.03	0.581	0.562
	Oct	-0.061	0.04	-1.697	0.091
Sex	m	0.167	0.03	6.066	0.000

Table 2.4. GLM with Gamma family (log linked) to analyze the effect of month, site and sex on the VH_2O (mg H_2O h^{-1} $insect^{-1}$). The table summarizes the simple model (VH_2O ~ month + site + sex). All estimates are computed against the base value of that variable (*i.e.* Jul for month, Sma for site and f for sex). Jul = July, Sep = September, Oct = October, Sma = San Michele all'Adige, Via = Viarago, Bor = Borti, Red = Redebus. f = females, m = males.

Category	**Coefficient**	**Estimate**	**SE**	**t-value**	**p-value**
	Intercept	-2.318	0.06	-41.603	0.000
Site	Via	-0.118	0.06	-1.835	0.068
	Bor	-0.177	0.06	-3.099	0.002
	Red	-0.223	0.06	-3.874	0.000
Month	Sep	-0.130	0.05	-2.763	0.006
	Oct	-0.127	0.05	-2.398	0.017
Sex	m	-0.321	0.04	-7.816	0.000

Table 2.5. GLM with Gamma family (log linked) to analyze the effect of month, site and sex on the $msVH_2O$ (mg H_2O h^{-1} g^{-1}). The table summarizes the model that does not consider interactions ($msVH_2O$ ~ month + site + sex). All estimates are computed against the base value of that variable (*i.e.* Jul for month, Sma for site and f for sex). Jul = July, Sep = September, Oct = October, Sma = San Michele all'Adige, Via = Viarago, Bor = Borti, Red = Redebus. f = females, m = males.

Category	**Coefficient**	**Estimate**	**SE**	**t-value**	**p-value**
	Intercept	-3.014	0.06	-50.558	0.000
Site	Via	-0.039	0.07	-0.569	0.570
	Bor	-0.177	0.06	-2.900	0.004
	Red	-0.176	0.06	-2.849	0.005
Month	Sep	-0.082	0.05	-1.624	0.107
	Oct	-0.062	0.06	-1.087	0.278
Sex	m	0.116	0.04	2.637	0.001

Table 2.6. Statistically significant results ($p < 0.05$) of the post hoc test on GLMs. Jul = July, Sep = September, Oct = October, Sma = San Michele all'Adige, Via = Viarago, Bor = Borti, Red = Redebus. NS = no significant results.

Model	**Contrast**	**Estimate**	**SE**	**p-value**
VCO_2 ~ month + site + sex	Jul - Oct	0.135	0.46	0.01
$msVCO_2$ ~ month + site + sex	NS	NS	NS	NS
VH_2O ~ month + site + sex	Jul - Sep	0.130	0.05	0.02
	Jul - Oct	0.127	0.05	0.05
	Sma - Bor	0.177	0.06	0.01
	Sma - Red	0.223	0.06	0.0008
$msVH_2O$ ~ month + site + sex	Sma - Bor	0.177	0.06	0.02
	Sma - Red	0.176	0.06	0.02

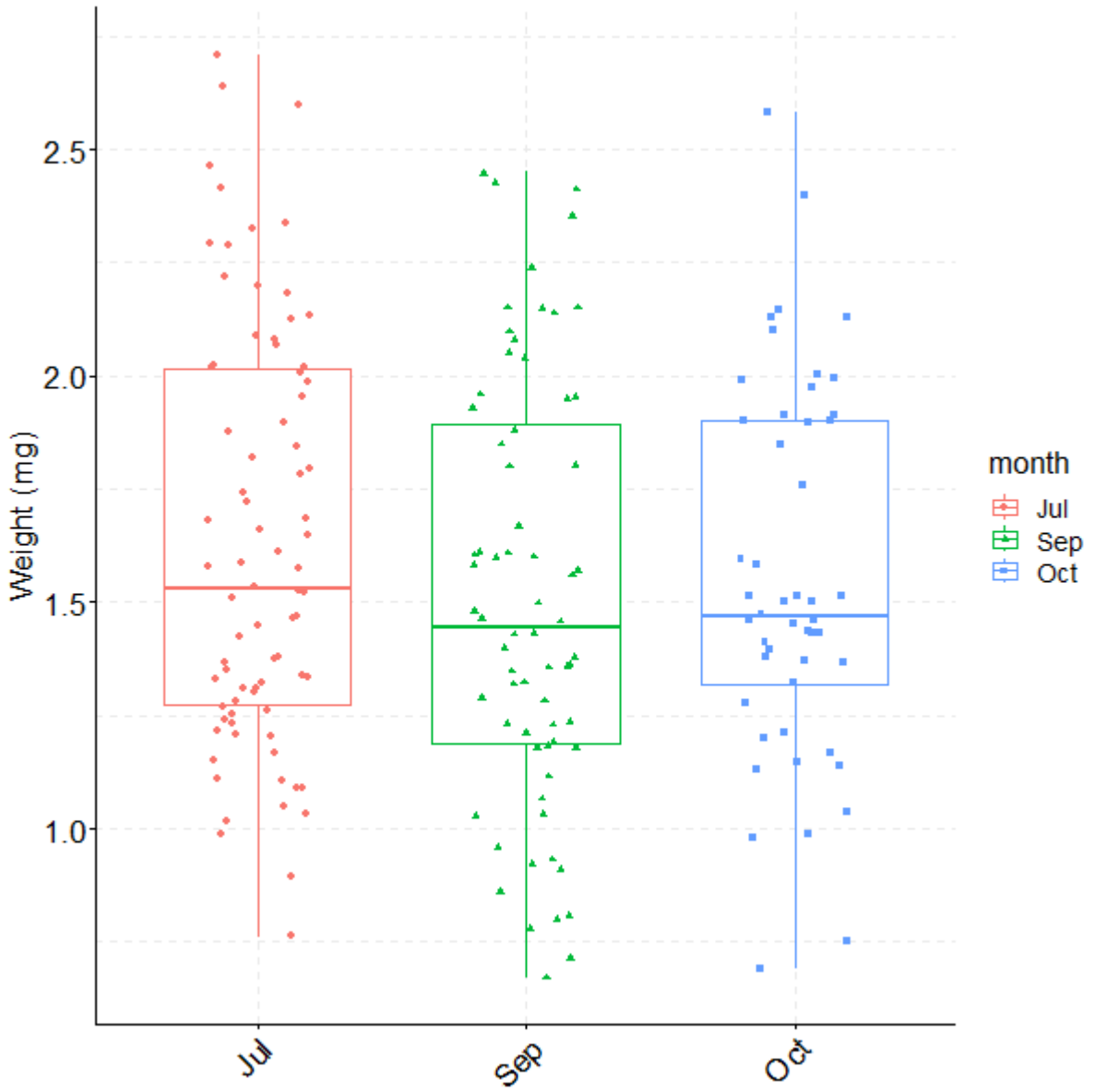

Fig. 2.2. Initial weight (mg) of measured flies in relation to sampling locations. The statistical test applied was a Mann-Whitney U test. No significant differences were found. Jul = July, Sep = September, Oct = October.

2.5. Discussion

To date, most of the studies on *D. suzukii* metabolism examined specific metabolic pathways or metabolomics (Bianchi et al., 2020; Enriquez et al., 2018; Zhai et al., 2019), while only one study has directly measured MR (as VCO2), in relation to the exposure to monoterpenes biopesticides (Finetti et al., 2021). In the present study, we investigated the changes in the metabolism of *D. suzukii* in relation to altitude and season using VCO_2 as a proxy of MR. We also investigated whether different altitudes and seasons influence the water management considering the water loss of the flies during the measurements.

Overall, we did not find the presence of DGC in our metabolic rate measurements (all the obtained data are referable to continuous gas exchange) and we always found significant differences between males and females. Considering the raw VCO_2 measurements, our results showed that females had a higher MR than males. This was expected, since there is a positive relationship between body size and metabolic rate (Chown et al., 2004; Terblanche and Chown, 2007), and in this species females are larger than males (Cini et al., 2012; Rota-Stabelli et al., 2013). Indeed, when normalizing by the initial mass of the insect ($msVCO_2$), females had a lower MR than males, indicating females have a slower metabolism. Considering that individuals characterized by a slower metabolism are reported to have longer lifespan and decreased activity compared to individuals with higher metabolism (Moloń et al., 2020), our findings indicate that females might live longer, which is something known for other drosophilids (Austad and Fischer, 2016), and be more passive in general activities (such as food searching, flying, moving) than males. We also found that males lost less water than females. This might sound controversial, since usually in dipterans the smaller the insect the greater the rate of water loss, due to the higher surface area to volume ratio (Benk et al., 2020; Benoit and Denlinger, 2007; Hadley, 1994; Hull-Sanders et al., 2003). However, the higher VH_2O observed in females might be dependent on the larger size, the higher metabolic rate or a more permeable cuticle (Ciancio et al., 2021; Grisafi et al., 2021; Wang

et al., 2019, 2020; Wiman et al., 2015; Woods and Smith, 2010). Indeed, when considering msVH$_2$O the analysis showed that females had a lower WLR than males. Considering that water management might be directly involved in the desiccation resistance, previous studies on *D. suzukii* (Fanning et al., 2019; Terhzaz et al., 2018; Williams et al., 2016) and other closely related species (Matzkin et al., 2007; Parkash and Ranga, 2013) have shown an increase in desiccation resistance in females compared to males, therefore our msVH$_2$O results are in agreement with them. Moreover, considering that females of *D. suzukii* are more likely to survive throughout the winter as winter morph compared to males and that during this period they are less active and subject to severe stresses (Ometto et al., 2013; Shearer et al., 2016), our MR and WLR results are consistent with previous findings indicating females better manage cold, starvation and desiccation stresses than males (Fanning et al., 2019; Shearer et al., 2016; Wallingford and Loeb, 2016).

Regarding the differences in MR in relation to the altitude, we did not find significant differences in VCO$_2$ and msVCO$_2$ among sites, consistent with the absence of genetic differences observed in a previous study conducted in the same valley (Tait et al., 2018). Considering that we performed all our measurements at 25°C and that within the same sampling period temperatures at different sites were slightly different (Table 2.1), it seems that *D. suzukii* have the plasticity to acclimate in a certain range of temperatures. However, altitude influences VH$_2$O and msVH$_2$O with flies caught at higher altitude that seem to be better at managing water. It is known that biotic and abiotic factors, such as food and water availability and humidity, change according to altitude. Thus, a decrease in water loss might be a strategy adopted to better face the different variety of food, the different amount of water available and the different relative humidity that characterized the different sampling locations (Chown et al., 2004; Gibbs, 2002; Hadley, 1994). Moreover, WLR results pointed out differences between high altitude Val dei Mocheni locations (Bor and Red) and Sma (which is in another valley) and no differences between Val dei Mocheni sites (Via, Bor, Red), suggesting the populations of the two valleys might have adapted differently to the

landscapes and climate conditions characterizing these two territories and that migration across an altitudinal gradient, as shown in Tait et al. (2018), might not be attributable to the need of cooler/warmer (depending on the season) and moister environments. However, to better afford this point, this kind of research must be carried out for more years, to see if the pattern observed in this study is corroborated over time.

Our analyses did not reveal consistent differences between sites in terms of both MR and WLR. Specifically, when comparing the measurements of VCO_2 and H_2O to their mass-specific counterparts, the results did not align, indicating that neither MR nor WLR are significantly affected by seasonal changes from summer to autumn. However, it would be interesting to investigate this further in future studies, particularly when considering winter morphs (WMs). WMs experience more severe conditions during the winter season and exhibit lower activity levels (Fanning et al., 2019; Shearer et al., 2016; Wallingford and Loeb, 2016), which would suggest a lower MR. Furthermore, analyzing WLR may provide supporting evidence for previous studies suggesting that WMs possess increased resistance to desiccation and superior water management compared to their "summer" counterparts (Fanning et al., 2019; Shearer et al., 2016).

2.6. Conclusions

Drosophila suzukii females analyzed in this study exhibited higher MR and WLR compared to males, which can be attributed to their larger size. When normalized by the mass, both MR and WLR were lower in females, suggesting that they actually had a lower MR than males and were more efficient in water management.

Flies from different altitudes, when measured at 25°C, did not show significant differences in MR, indicating a certain degree of plasticity in energy management. However, variations in WLR between Sma and the high-altitude sampling points located in Val dei Mocheni (Bor and Red) suggest the presence of two populations adapted differently to the environmental and climatic characteristics of the respective valleys. The lower WLR observed in flies captured in Val dei Mocheni indicates their greater resistance to desiccation and makes migration less likely as a response to water scarcity or desiccation issues. Nevertheless, further investigation over multiple years is necessary to strengthen and validate these findings, particularly concerning the last point.

In terms of the sampling period, we did not observe significant differences between July and September, suggesting that MR and WLR remain relatively stable during the transition from summer to autumn.

Overall, these results have the potential to inform predictions regarding the small-scale spatial dynamics of *D. suzukii* in persisting territories and contribute to more accurate forecasts of future invasions. However, to gain a better understanding of seasonal implications, future research should include winter morphs specimens and focus on studying the differences in MR and WLR between winter and summer morphs.

Chapter 3 - Overwintering does not affect the microbiota diversity in *Halyomorpha halys*: implications for its ecology and management

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3.1. Abstract

The brown marmorated stink bug, *Halyomorpha halys*, is an invasive agricultural pest that has spread across the globe from East Asia. This species is characterized by an overwintering strategy where individuals aggregate then enter a state of dormancy and starvation. Microbial communities have recently been shown to play a crucial role in the biology of many organisms, including the overwintering process; however, the effects of overwintering on *H. halys* microbiota are still largely unknown. We investigated the microbiota diversity of whole *H. halys* before and after dormancy using 16S amplicon analysis of wild specimens caught in four sampling locations in Trento Province (Italy). We found that bugs sampled in one of the sampling locations had significantly different levels of microbial diversity compared to the other three, but we did not find significant differences in microbial diversity and composition between individuals before and after overwintering. Unlike previously studied insects, *H. halys* maintains a stable host-symbiont relationship with its microbiota despite stressful overwintering conditions, a strategy that may be related to the highly specific associations of stink bugs with some of their symbionts. In addition, with regards to pathogens associated with this stink bug, we did not detect phytoplasmas, but found *Nosema maddoxi* in two sampling sites. Our results are relevant to pest management plans, especially when wild insects are collected for applying the sterile insect technique.

Keywords: *Halyomorpha halys*; metabarcoding; invasive pest; microbiota shifts; overwintering; phytoplasma; *Nosema maddoxi*

3.2. Introduction

The brown marmorated stink bug, *Halyomorpha halys* Stål, 1855 (Hemiptera: Pentatomidae), is an invasive species from eastern Asia. It was accidentally introduced to North America in the late 1990s (Hoebeke and Carter, 2003) and to Europe in 2007 (Wermelinger et al., 2008). This species has been steadily enlarging its distribution in other temperate regions including Chile and Turkey (Bosco et al., 2018; Leskey and Nielsen, 2018; Maistrello et al., 2018), feeding on more than 300 species of plants, comprising many crops (Tassini and Mifsud, 2019). Such behavior, together with high reproductive capacity and mobility, means this insect is a major agricultural pest in most of the countries it has successfully invaded (Leskey and Nielsen, 2018). In northern Italy, the effect of *H. halys* on various crops has been severe (Bosco et al., 2018; Maistrello et al., 2017).

Halyomorpha halys was first described as a chill-intolerant species (Cira et al., 2018), meaning that mortality is high when temperatures fall below 0^{o}C (Cira et al., 2016; Denlinger and Lee, 2010). In temperate conditions, adults minimize the risk of freezing by entering diapause in late autumn (Cira et al., 2018; Nielsen et al., 2017; Nielsen & Hamilton, 2009; Watanabe et al., 1978), during which they also starve (Papa and Negri, 2020). To improve their probability of survival during the winter, *H. halys* aggregate in large groups in natural or artificial refuges, such as hollow trees, wooden sheds, cellars or attics (Cira et al., 2016). In March - April (Bergh et al., 2017), survivors emerge, start feeding again on available host plants (Leskey and Nielsen, 2018), and adults become reproductively active (Musolin et al., 2019; Nielsen et al., 2017; Nielsen and Hamilton, 2009). .

As for other animal species, host-microbiota relationships are likely to be important for *H. halys* development and growth (Gonella et al., 2020); however, the impact of overwintering on its microbiota is still not known. Studying such changes might provide useful information for

planning pest management strategies. For example, microbial diversity is associated with diet and environmental parameters in a wide number of insects (*Gryllus veletis* Alexander & Bigelow: Ferguson et al., 2018; *Spodoptera frugiperda* Smith: Jones et al., 2019; *Trichoplusia ni* Hübner: Leite-Mondin et al., 2021; Chrysomelidae spp.: Magoga et al., 2022; *Drosophila suzukii* Matsumura: Martínez-Solís et al., 2020; Mason et al., 2020; *D. melanogaster* Meigen: Sepulveda and Moeller, 2020), including Pentatomomorpha (Li et al., 2022), an infraorder of insects to which *H. halys* belongs. Furthermore, dietary changes, such as starvation, and environmental stressors, such as low temperature, are known to cause shifts in insect microbiota communities (*Diploptera punctata* Eschscholtz: Ayayee et al., 2020; *G. veletis*: Ferguson et al., 2018; *Hermetia illucens* Linnaeus: Yang et al., 2021). Moreover, one component of *H. halys* midgut microbiota, *Nosema maddoxi* (Microsporidia: Nosematidae), a microsporidian pathogenic for various species of Pentatomids other than *H. halys* (Hajek et al., 2018), was found in overwintered adults (Preston et al., 2020a). This microorganism could be considered a potential biological control candidate, as it has been recently noted that this microsporidian has a negative impact on *H. halys* lifespan, fecundity and survival (Preston et al., 2020b), although further investigation is needed to assess the impact on other species. Therefore, it is important to characterize the microbiota of *H. halys* to understand the prevalence of this pathogen in natural populations, and the risk of microbial diffusion to newly invaded regions.

Here, we investigated changes in the microbiota of whole *H. halys* associated with overwintering in four populations collected in the Province of Trento (Italy) using amplicon sequencing focusing on the V3-V4 region of the prokaryotic 16S ribosomal subunit. We characterized the microbial diversity and confirmed the presence of plant pathogens that could be vectored by *H. halys*, including the presence of *N. maddoxi*. Finally, we discussed how our results could be applied to effective control strategies against this pest.

3.3. Materials and Methods

3.3.1. Sample collection

Halyomorpha halys individuals were collected from single live traps with high doses of aggregation lures (Trécé, Adair, OK, USA; Suckling et al., 2019b) placed in four locations in the Province of Trento, Italy (Fig. 3.1): San Michele all'Adige (acronym: SM, coordinates: 46° 11' 23'' N, 11° 08' 17" E, altitude: 269 m a.s.l.); Denno (DN: 46° 15' 46'' N, 11° 03' 42'' E, 280 m a.s.l.); Trento (TN: 46° 01' 24'' N, 11° 08' 00'' E, 219 m a.s.l.); and Besenello (BS: 45° 56 '12'' N, 11° 06' 39'' E, 210 m a.s.l.) from October to November 2020 during the peak of aggregating behavior.

Immediately after collection, 10 individuals per site (40 bugs in total, hereafter "Not overwintered") were selected using sterile disposables, placed in sterile DNA/DNAse-free 2 ml tubes and stored at -80°C until further processing. The remaining adults were reared in four cloth cages (30 x 30 x 30 cm; BugDorm®, Taichung, Taiwan), one per sampling location. The insects were initially placed in a greenhouse under natural photoperiod conditions at 15-18°C and a relative humidity of 70%, with *ad libitum* access to fresh tomatoes, green beans, carrots, and apples (provided daily), and water provided with a wad of wet cotton, conditions considered to mimic preparation for overwintering. At the end of November, insects were transferred to a wooden shed with outdoor ambient conditions. Cardboard rolls were put in the cloth cages to act as shelter. A data logger (EL-USB-2, Lascar Electronics, Whiteparish, UK) was also placed inside the shed to monitor temperature and relative humidity. Emerging overwintered adults (hereafter: "Overwintered") were collected in March 2020 using sterile disposables; each individual bug was placed in a sterile DNA/DNAse-free 2 ml tube and stored at -80°C until DNA extraction (SM: 4; BS: 5; TN and DN: 6 each).

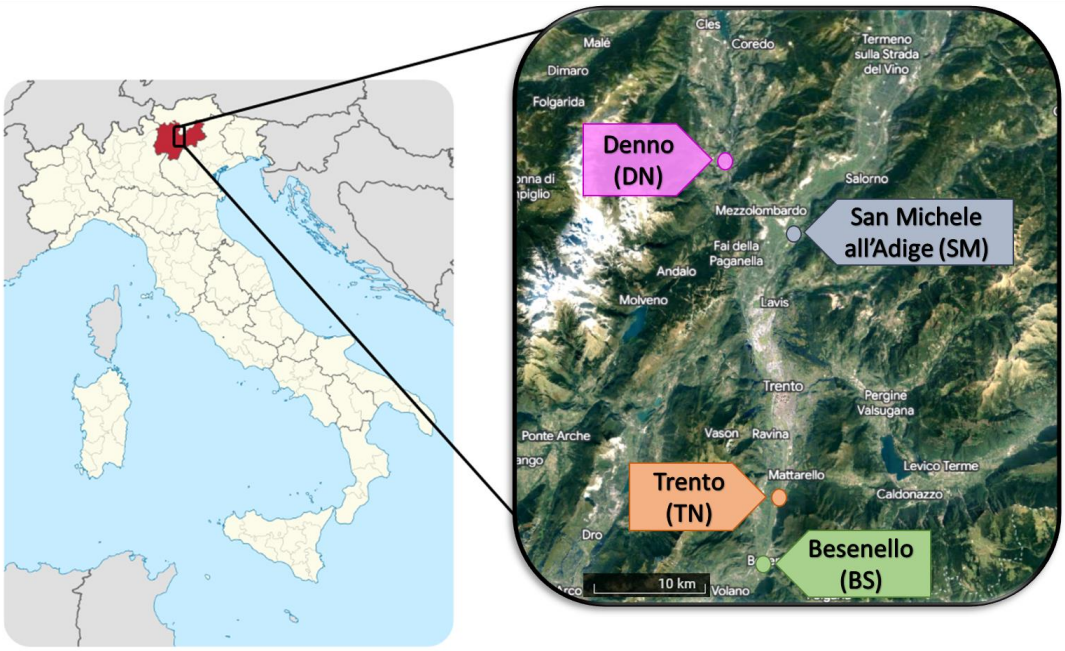

Fig. 3.1. Map showing the four sampling locations for *Halyomorpha halys* in the Province of Trento, Italy.

3.3.2. DNA extraction, amplification and sequencing

Whole animals were placed in sterile 2 ml DNA/DNAse-free tubes, flash frozen in liquid nitrogen for at least 30 seconds, then ground to powder using a Tissuelyser II (QIAGEN) set to 30 Hz for 1 minute. We then added 320 µl of enzymatic lysis buffer (Protocol: Pretreatment for Gram-Positive Bacteria; Composition: 20 mM Tris-Cl, pH 8.0; 2mM sodium EDTA; 1.2% Triton X-100) to each sample. This mixture was vortexed for 20 seconds and divided in two aliquots of approximately 160 µl each. One aliquot was used for DNA extraction while the other aliquot was frozen at -80 for future use. We continued the DNA extraction by adding 18 µl of lysozyme (200 mg/mL) to one aliquot of *H. halys* homogenate. DNA extractions were carried out with the DNeasy Blood and Tissue kit (QIAGEN) by following the instructions provided by the manufacturers using the protocol "Pretreatment for Gram-Positive Bacteria" (full protocol provided in the Supplementary Information). Negative controls for DNA extraction (*i.e.* tubes containing all reagents but no bug) were included to detect kit

contamination. Quantification of DNA extracts as well as quality checks (i.e. 260/280 and 260/230 ratios) were performed using a Spark® multimode microplate reader (Tecan, Switzerland) in UV/VIS mode. All DNAs were diluted in DNA/DNAse/RNAse free water to a final concentration of 50 ng/µl .

For the amplicon sequencing analysis, we amplified the 16S rRNA gene region V3-V4 using the KAPA HiFi HS ReadyMix (Roche). Specifically, PCR reactions were performed in a volume of 25µl, containing 1X KAPA HiFi HS ReadyMix Buffer, the two primers 341F_ILL (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 805R-2_ILL (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTWTCTAATCC-3') (Klindworth et al., 2013; Walters et al., 2015) to a final concentration of 0.3 µM each and 100 ng of DNA (50 ng/µl). Amplification reactions were performed on a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems, USA) using the following parameters: an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles consisting of 30 seconds at 95°C, 30 seconds at 55°C, and 90 seconds at 72°C, and a final step at 72°C for 7 minutes. Negative controls for DNA amplifications (i.e. tubes containing all reagents but no sample DNA) were also included. Amplification success was estimated using the QIAxcel Advanced System (QIAGEN) with default parameters and reagents. Library preparation, quantification and sequencing were performed at the Sequencing and Genotyping Platform, Fondazione E. Mach (San Michele all'Adige, Italy) using Illumina MiSeq 2x300bp with a minimum depth of 100,000 reads per sample.

To evaluate *Nosema maddoxi* infection, we amplified the microsporidian ribosomal SSU 16S (18f 5'-CACCAGGTTGATTCTGCCTGAC-3', 1492r 5'-GGTTACCTTGTTACGACTT-3'), using the protocol provided by (Hajek et al., 2018). Briefly, amplification reactions were performed in a volume of 20 µl, containing 1X Green GoTaq® Flexi Buffer (Promega), 2 µM

$MgCl_2$, forward and reverse primers to a final concentration of 0.25 μM each, 2 U of GoTaq® G2 Hot Start Taq Polymerase (Promega) and 100 ng of sample DNA (50 ng/μl). All reactions were performed on a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems, USA) using the following parameters: an initial denaturation step at 95°C for 3 minutes, followed by 40 cycles consisting of 30 seconds at 95°C, 30 seconds at 55°C, and 90 seconds at 72°C, and a final step at 72°C for 7 minutes. Negative controls for DNA amplifications were also included. Amplification success was estimated as detailed above. Microsporidian 18f-1942r amplicons were then enzymatically purified using ExoI/FastAP (ThermoFisher Scientific) and sequenced using Sanger technology at the Sequencing and Genotyping Platform, Fondazione Edmund Mach, Italy.

3.3.3. Bioinformatic analyses

For the bacterial microbiota analysis, we used CutAdapt (Martin, 2011) to remove adapters from the 16S V3-V4 reads. The remaining analyses were performed in RStudio (Allaire, 2012; R version 4.1.2 R Core Team, 2022). DADA2 (Callahan et al., 2016) was used to filter the reads by quality, remove errors, merge the forward and reverse reads, remove chimeras, and assign taxonomic names to the resulting ASVs using Silva v138 (Quast et al., 2013; Yilmaz et al., 2014). Decontam (Davis et al., 2018) was used to remove contaminant sequences considering the negative controls and using the prevalence method, and phyloseq (McMurdie and Holmes, 2013) to compute microbial abundance, as well as alpha and beta diversity estimates. We used a Mann-Whitney U test (Fay and Proschan, 2010; Mann and Whitney, 1947) to evaluate the significance of differences in alpha diversity between populations and related to overwintering in the same individuals. We used the non-metric multidimensional scaling (NMDS) approach in vegan to perform ordination of samples based on beta diversity estimates (Oksanen et al., 2013). PERMANOVA and pairwise adonis using adonis, adonis2 and permutest from vegan were used to test differences in beta diversity estimates between

populations and seasons (overwintering vs non-overwintering; Oksanen et al., 2013). A Mantel test (Mantel, 1967) was used to investigate population connectivity in relation to the similarity/diversity of microbiota profiles. Finally, DESeq2 (Love et al., 2014) was used to identify amplicon sequencing variants (ASVs) that were differentially abundant in overwintered and not overwintered bugs.

Microsporidian sequences were visualized using Chromas software (version 2.6.6). Forward and reverse reads were assembled using a Biopython script (Cock et al., 2009). Species assignment was performed using NCBI BLAST (Johnson et al., 2008) and the assembled sequences. We used R version 4.1.2 software (R Core Team 2022) to compute standard statistics, and the ggplot2 R package (Wickham, 2009) to draw graphs.

3.4. Results and discussion

3.4.1. Dataset cleaning and preparation

Sequencing of 61 *H. halys* generated a median number of 88,853 sequence reads per library (ranging from 46,234 to 158,761 reads), resulting in the assembly of 2,551 ASVs. A stringent filtering procedure was implemented to ensure the reliability of the data, considering the negative controls, which comprised samples with no target DNA. These negative controls yielded a median number of 5,712 reads. By comparing the ASVs found in the negative controls with the dataset, we were able to identify and remove 28 ASVs that were potentially contaminants (14,380 reads in total), which resulted in a final dataset consisting of 2523 ASVs.

3.4.2. Geographically adjacent populations have diverse microbiota

Overall, our study showed that bug-associated microbiota varied considerably between geographically neighboring sites. Taxonomic classification of microbial communities at the phylum level identified Proteobacteria as the most abundant phylum across all samples and populations (Fig. S3.1A); however, compositional differences between the four populations were found at genus level (Fig. 3.2A). Considering the genera with relative abundance higher than 0.3%, we found that 58% of genera were shared among the four populations (Fig. 3.2B). The majority of the identified ASVs, calculated as the mean percentage of reads in all samples >50%, belong to the *Pantoea* genus, especially in SM (10/14 samples) and BS (13/15 samples). This result is consistent with previous studies, which identified *P. carbekii* as the primary symbiont of *H. halys* (Bansal et al., 2014). Interestingly, *P. carbekii* encodes genes involved in primary metabolic processes (Kenyon et al., 2015, Gonella et al., 2020), whose absence has been shown to negatively affect the development of nymphs and survival of the following generation (Taylor et al., 2014). However, in DN bugs, *Commensalibacter* is the most abundant genus (9/16 samples), while the microbiota of TN bugs is more heterogeneous, with a similar

number of samples having *Pantoea*, *Commensalibacter* or *Yokenella* as the most abundant genus (3, 6, and 5 samples out of 16, respectively). *Commensalibacter* have been found in other insects, such as honey bees (*Apis mellifera* Linnaeus, *Apis florea* Fabricius and *Apis dorsata* Fabricius), fruit fly (*D. melanogaster*), butterflies (*Heliconius spp*. Linnaeus) and firebugs (*Probergrothius angolensis* Distant), where it was suspected to suppress the proliferation of deleterious organisms by competition (Botero et al., 2023). *Yokenella* has been found in other Pentatomidae, such as *Nezara viridula* Linnaeus (Medina et al., 2018); little is known about its function, but it is believed to be involved in detoxification of food resources as in other Enterobacteriaceae (Gonella et al., 2020).

In keeping with the above differences, the PERMANOVA analysis, considering Bray-Curtis dissimilarities at the phylum level, indicated a statistically significant difference in beta diversity among the populations (R2: 0.177, p-value: 0.003). However, the pairwise analysis did not show any significant difference across the four sampling locations (Table 3.1). The NMDS plot representing the phylum-level data (NMDS stress: 0.022) reveals considerable overlap among the four ellipses, which were calculated based on a 95% confidence interval. It also indicates that the TN population is less dispersed compared to the other three populations (Fig. S1B). This finding aligns with the observation that Proteobacteria was the predominant phylum in all samples, therefore it was anticipated that no differences would be detected among populations when assessing phylum-level alpha diversity. In contrast, at the genus level, the ellipses for BS, SM, and DN overlap, while the TN ellipse is slightly shifted (Fig. 3.3A; NMDS stress: 0.108). The sizes of the ellipses differ, suggesting that BS is the least dispersed population, whereas TN is the most dispersed population. This result is further supported by examining the distances of each sample from the centroids (Fig. 3.3B). The PERMANOVA analysis performed using the same Bray-Curtis dissimilarities indicates significant differences in microbiota composition among bug microbiotas from the four sampling locations (R2: 0.3,

$p < 0.001$). In the pairwise analysis conducted to assess significant differences in beta diversity among the four sampling locations (Table 3.1), all contrasts, except for BS vs. SM, showed significant differences. These results indicate that neighboring populations were different in terms of alpha diversity. We did not observe significant covariance between Bray-Curtis dissimilarity estimates and geographic distances (Mantel test, R2: 0.037 P-value: 0.11), a finding that indicates that diversity found among microbiota of the four *H. halys* sampling locations is not related to geographical distances, but might be related to environmental factors, such as food availability (Brunetti et al., 2022) and usage of chemicals (Syromyatnikov et al., 2020).

The BS, DN, and SM samples had similar richness and evenness at both the phylum (Fig. S3.1C-D) and genus levels (Fig. 3.3C-D). However, at the phylum level, TN population had a lower Shannon index compared to the other three sites (Fig. S3.1C), while no significant differences were found for the Chao1 index among the sites (Fig. S3.1D). At the genus level, the TN population exhibited a significantly lower Chao1 (Fig. 3.3D) index compared to the other three populations, but the Shannon index was similar (Fig. 3.3C). As the two diversity indices are characterized by different sensitivity to common and rare species (with Chao1 being more sensitive than Shannon to rare species; Jost, 2006), our results indicated that the TN population had fewer rare prokaryotic genera and fewer abundant prokaryotic phyla compared to the other three populations. Consistently, these alpha diversity estimates are in agreement with the taxonomic classification of bacterial taxa at the phylum level (Fig. S3.1A), which highlighted a strong dominance of bacterial taxa belonging to the phylum Proteobacteria for the population TN.

Possible explanations for the differentiation found in microbial communities hosted by TN bugs can be traced back to at least two different processes: variation in diet and anthropogenic disturbance. Since *H. halys* is a polyphagous species the observed shift in microbial community

composition could be due to differences in the composition or relative abundance of available plant species for feeding (Garcia-Mantrana et al., 2018; Lee et al., 2017; Tang et al., 2012). An analysis of the plant species present in the DNA extracted from the bugs and a survey of the vegetation in the collection area would be useful for examining the possibility that the observed shift in microbial community composition in the TN sampling location of *H. halys* is due to differences in the available plant species for feeding. At the same time we cannot rule out that variation in microbial diversity observed in TN might be due to anthropogenic disturbance, such as the frequency and composition of chemical treatments, that can influence the *H. halys* microbiota and that are not present in the other sampling locations (Giambò et al., 2021; Juma et al., 2020; Syromyatnikov et al., 2020).

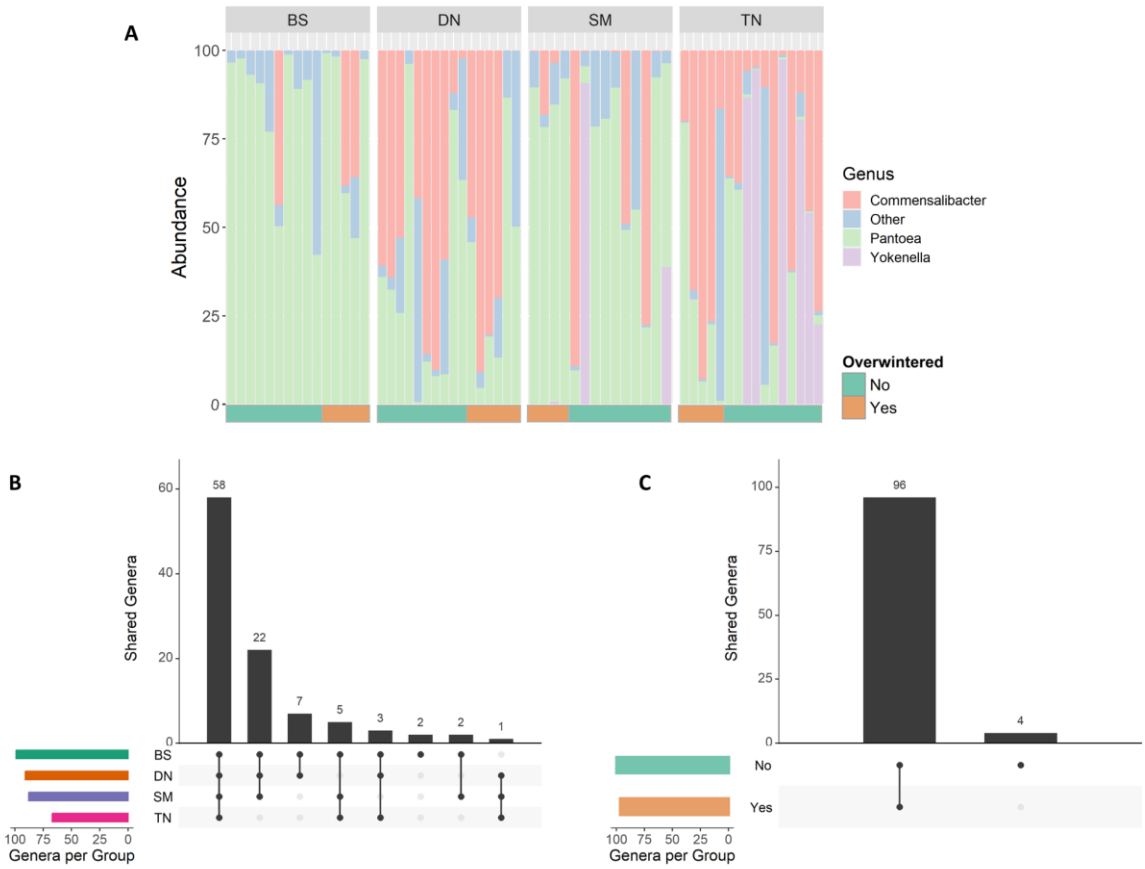

Fig. 3.2. Abundance plots computed considering bacterial genera in *H. halys* microbiota. (A) Relative abundance of the three most abundant genera. Each bar represents one *H. halys* individual. (B) Upset plot depicting shared genera among the four populations, considering genera with a sample relative abundance of at least 0.3% for each bug individually. The x-axis represents the populations, while the y-axis represents the number of shared genera. (C) Upset plot illustrating the shared genera between overwintered and not overwintered bugs, considering genera with a sample relative abundance of at least 0.3% for each bug individually.

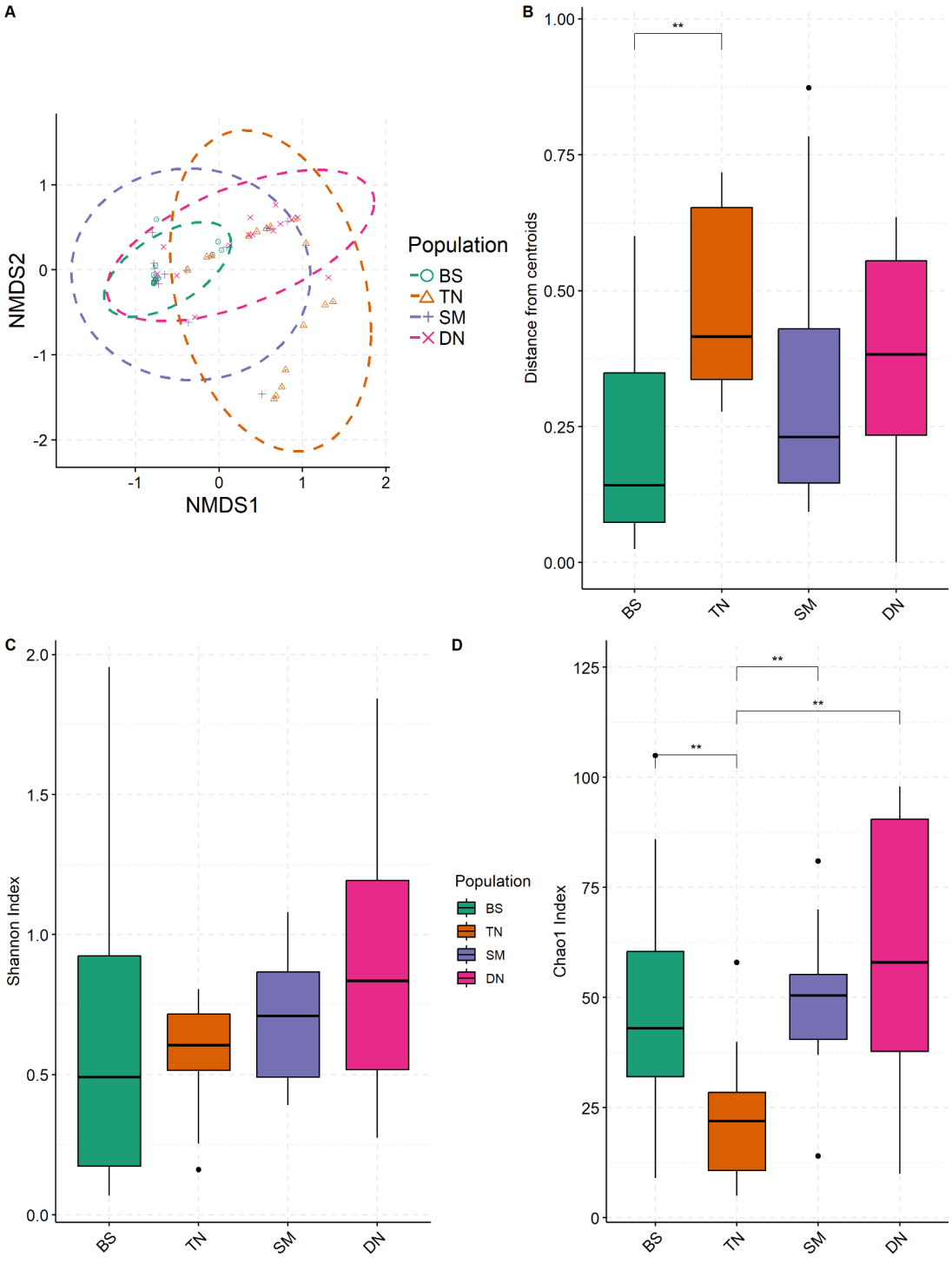

Fig. 3.3. Comparison of whole *H. halys* microbiota diversity and composition between sampling locations at the genus level. (A) Nonmetric multidimensional scaling (NMDS) plot of sampled *H. halys* microbiota. PERMANOVA based on dissimilarities was also estimated (R2 = 0.17745; P = 0.002). (B) Distances from centroids computed using Bray-Curtis method. (C, D) Alpha diversity plots of Shannon (C) and Chao1 (D) indices at the genus level. ** $p < 0.01$.

Table 3.1. Results of the pairwise adonis analysis on the Bray-Curtis dissimilarities.

Type of analysis	Pairs	F-statistic	p-value
Population differences at the Genus level	BS -TN	14.66	0.001
	BS - SM	1.39	0.199
	BS - DN	11.97	0.002
	TN - SM	6.57	0.002
	TN- DN	3.37	0.016
	SM - DN	5.63	0.010
Population differences at the Phylum level	BS -TN	0.96	0.327
	BS - SM	1.07	0.334
	BS - DN	0.53	0.584
	TN - SM	2.66	0.086
	TN- DN	1.55	0.219
	SM - DN	1.64	0.172
Overwintering differences at the Genus level	Yes - No	1.31	0.27
Overwintering differences at the Phylum level	Yes - No	0.67	0.52

3.4.3. Overwintering does not affect *Halyomorpha halys* microbiota

We found no significant differences in alpha or beta microbial diversities between populations that overwintered and those that did not overwinter. Although we observed an increase in Chao1 index at the phylum level (Fig. S3.2D) in overwintered bugs compared to non-overwintered bugs, there was no significant change in the Shannon index at the phylum level (Fig. S3.2C). Furthermore, there were no significant differences in beta diversity estimates across overwintered and non-overwintered bugs (Fig. S3.2A-S2B; NMDS stress: 0.022; PERMANOVA R2: 0.011, p-value: 0.53; Table 3.1). In detail, our analysis at the genus level showed similar values between populations for both seasons regarding the Shannon (Fig. 3.4C) and Chao1 (Fig. 3.4D) indices, indicating that the richness and evenness of the non-overwintered and overwintered populations were comparable. Similarly, there was no variation in beta diversity estimates between overwintered and non-overwintered bugs, as shown in the NMDS plot (Fig. 3.4A; NMDS stress: 0.108) and the corresponding distance from centroids plotted in Fig. 3.4B.

However, a population-wise analysis between overwintered and non-overwintered bugs (Fig. 3.5-S3.3) revealed a significant increase in richness estimates in microbial communities hosted by TN overwintered bugs (Fig. 3.5B-S3.3B), a finding that was not observed in the other three populations. The differential analysis at the genus level (Fig. 3.5C, 3.5F, 3.5I, 3.5N) identified only a few genera whose presence significantly differed between overwintered and non-overwintered bugs (percentage of significant genera among all genera that characterize the population: TN 3.91%, BS 0.66%, DN 0.39%, SM 1.40%), most of which were attributed to the presence of these genera in a few bugs. Interestingly, the *Yokenella* genus, which was one of the most abundant genera in the TN population (Fig. 3.2A), was present in all non-overwintered bugs and none of the overwintered bugs (Fig. 3.5C). Similarly, this genus was present in four BS bugs before overwintering and none of the overwintered bugs (Fig. 3.5F).

The role of *Yokenella* in Pentatomidae is very poorly known, but it was suggested to play an important role in nutrition and in chemical defense in *N. viridula* (Medina et al., 2018). If this bacterium has similar functions in *H. halys*, it is possible that the observations made in TN bugs are linked to a substantial dietary change, leading to the loss of dominant bacteria and allowing other microbial communities to thrive, thereby increasing microbial richness. This study's findings could be attributed to the fact that the bugs were not fed corn during their time in the cage before overwintering, whereas corn was the primary crop available nearby the TN sampling location. Additionally, it is plausible that *Yokenella* played an important role in detoxifying pesticides used near the sampling location. Since these pesticides were absent in the cages, *Yokenella* presence may no longer have been necessary for the overwintered bugs, resulting in its loss.

Despite the differences observed in TN specimens, the overall diversity of the microbiota in both not overwintered and overwintered bugs was similar. When considering genera with a total relative abundance of at least 0.3%, the composition was mostly characterized by shared genera between overwintered and not overwintered bugs (96%; Fig. 3.2C). This result was unexpected, since starvation and other abiotic winter stresses, such as cold and low humidity, have been previously associated with alterations in microbiota composition in various insects species (*Drosophila suzukii* Matsumura: Fountain et al., 2018; *Laodelphax striatellus* Fallén: Liu et al., 2019; *Acrosternum hilare* Say and *Murgantia histrionica* Hahn; Prado et al., 2010). However, our results were consistent with a previous study on cockroaches *Periplaneta americana* (Tinker and Ottesen, 2016), where no statistical differences were found between microbial compositions and alpha diversity of gut microbiota of starved and non-starved individuals, which led the authors to hypothesize that these insects have evolved a mechanism to maintain a stable host-microbiota relationships even during starvation periods. A stable host-microbiota interaction was already hypothesized in *H. halys*. In these bugs part of the

microbiota is vertically transmitted: mothers leave secretions over the egg mass surface that is ingested by newly-emerged nymphs, thus inheriting part of the mothers' microbiota (behavior common to other Pentatomids; Gonella et al., 2020; Kikuchi et al., 2012; Prado and Almeida, 2009). It has been shown that sterilizing the egg mass surface causes severe negative effects on reproduction, growth and development of *H. halys* specimens (Taylor et al., 2014). Therefore, *H. halys* may also have evolved a mechanism that stabilizes the host-microbiota relationship throughout the overwintering period, resulting in the preservation of the host bacterial communities during overwintering, despite starvation and abiotic stressors.

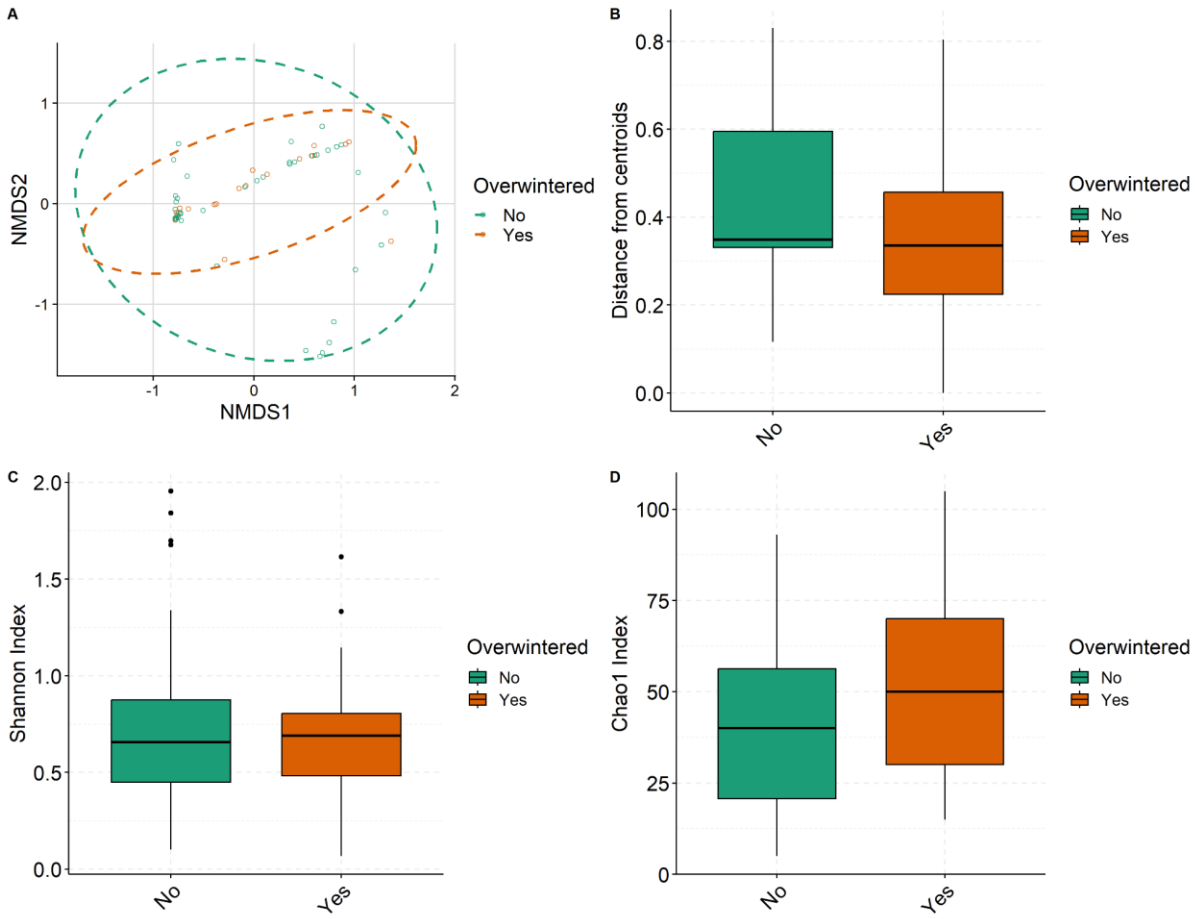

Fig. 3.4. Comparison of microbiota composition and diversity between not overwintered and overwintered *H. halys* at the genus level. (A) NMDS plot of sampled *H. halys*. PERMANOVA based on dissimilarities was also estimated (R2 = 0.0218, p = 0.254). (B) Distances from centroids computed using Bray-Curtis method. (C, D) Alpha diversity plots of Shannon (C) and Chao1 (D) indices at the genus level.

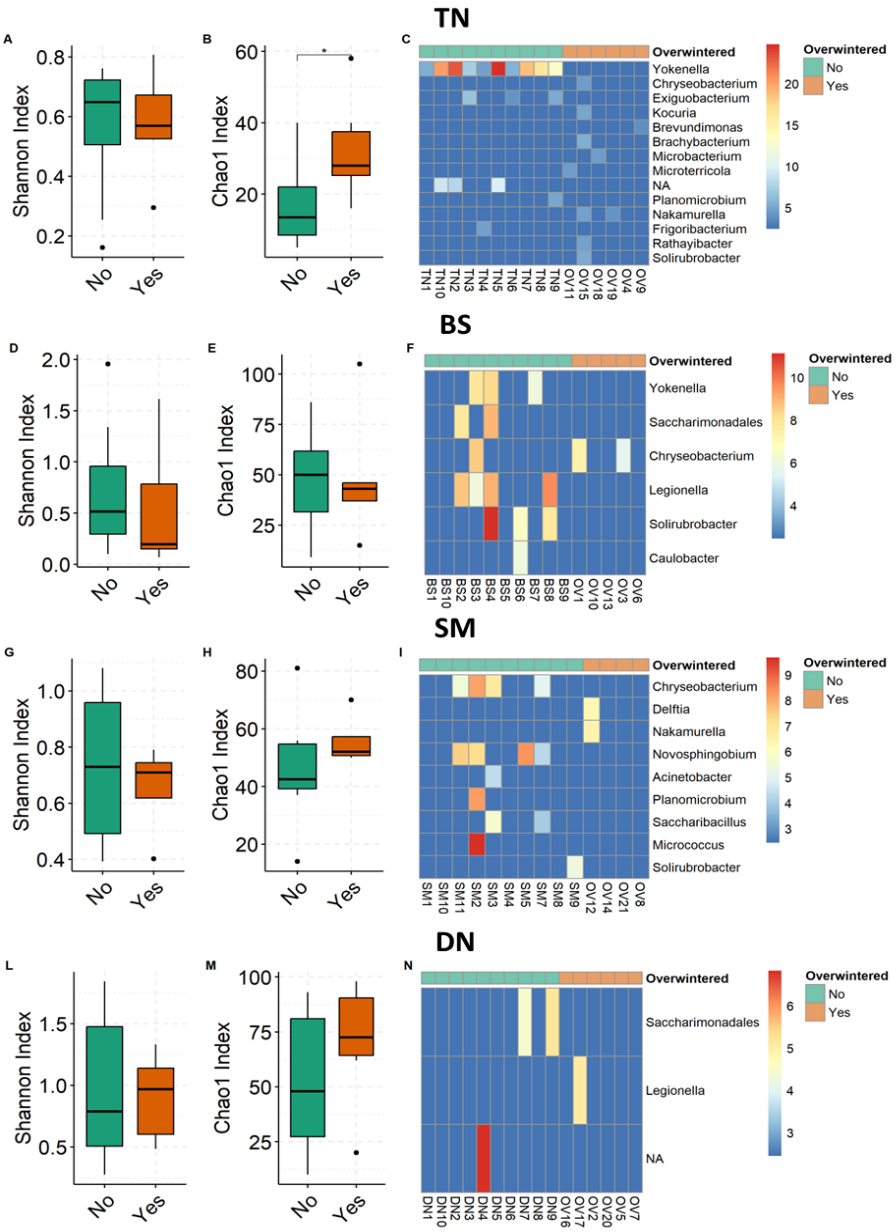

Fig. 3.5. Comparison of alpha diversities of bacterial microbiota in each of four sampled *H. halys* populations at the genus level. (A,B, C) Shannon (A) and Chao1 (B) indices and differentially present ASVs (C) computed for the Trento (TN) population. (D, E, F) Shannon (D) and Chao1 (E) and differentially present ASVs (F) indices computed for the Besenello (BS) population. (G, H, I) Shannon (G) and Chao1 (H) and differentially present ASVs (I) indices computed on the San Michele all'Adige (SM) population. (L, M, N) Shannon (L) and Chao1 (M) indices and differentially present ASVs (N) computed on the Denno (DN) population. In heatmaps (C, F, I, N), the cell coloration varies depending on the z-score value. Blue indicates the absence of the ASV in the sample, red indicates the highest presence in that sample. * $p < 0.05$.

3.4.4. Pathogen screening: absence of Phytoplasmas and presence of *Nosema maddoxi*

We specifically searched public databases for plant and animal pathogens vectored by *H. halys.* This species has been shown to host witches' broom phytoplasma, a threat for princess trees (*Paulownia tomentosa*) and other plants (Gao et al., 2008; Jones and Lambdin, 2009). Insect vectors can transmit multiple types of phytoplasma (Lee et al., 1998); therefore, assessing the presence of phytoplasmas in polyphagous pests such as *H. halys* is important for agricultural pest management. In the Province of Trento, the productive output of apple trees and grapevines is threatened by *H. halys*, and these same crops are subject to diseases caused by phytoplasmas (*i.e.* apple proliferation phytoplasma, grapevine flavescence dorée and grapevine bois noir). Consistently with previous reports, we did not find phytoplasmas carried by the analyzed specimens (Hoebeke and Carter, 2003; Tassini and Mifsud, 2019), suggesting that the types of phytoplasma present in this Province are unlikely to be transmitted by *H. halys*.

We additionally examined the presence of other phytopathogens from the following genera: *Agrobacterium*, *Burkholderia*, *Erwinia*, *Pectobacterium*, *Pseudomonas*, *Ralstonia* and *Xanthomonas*. Among these, we only detected the presence of the *Pseudomonas* genus in 45 bugs, with 17 overwintered bugs and 28 not overwintered. The distribution of *Pseudomonas* in the bugs was as follows: TN (6 bugs), BS (12 bugs), SM (13 bugs), and DN (14 bugs). A total of 52 ASVs were assigned to Pseudomonas. Some of these ASVs were assigned to 20 different species, with two species of particular interest as agricultural pathogens: *P. cichorii*, which has a wide hosts range and causes leaf blighting and spotting (Trantas et al., 2013), was found in one bug (SM; overwintered) with relative abundance of 0.02%; and *P. tolaasii*, which casually causes bacterial blotch on cultivated mushrooms (Liu et al., 2022), was found in six bugs (SM;

one overwintered, five not overwintered) with a total relative abundance of 0.17%. Assuming correct species assignments, even though the 16S analysis provides genus-level resolution, it is noteworthy that all the bugs positive for these two pathogens belonged to the SM sampling location, suggesting that these pathogens might be location-specific. Furthermore, the relatively low abundance makes it unlikely that *H. halys* acts as a vector for these pathogens. However, further studies should be conducted to assess their potential transmission.

Nosema maddoxi was found in TN (1/16 bugs) and in BS (7/15 bugs) (Fig. S3.2). The consistent prevalence of *N. maddoxi* in only BS suggests that the presence of this parasite might be location-specific. Although these results seem to be in contrast with previous findings recorded in the Republic of Georgia and in North America (Kereselidze et al., 2020; Preston et al., 2020a), we sampled the bugs as soon as they emerged from their diapause, and not in the field during spring, like previous authors. Therefore, it might be possible that during spring, the susceptibility of *H. halys* to *N. maddoxi* increases and *N. maddoxi* is propagated by horizontal transfer (Preston et al., 2020b). Moreover, this result comes from analyzing fewer bugs compared to the previous studies, and it also should be considered that ~70% of the bugs in the cages died during the overwintering period. However, in BS, we found infected bugs after the overwintering period. This result confirms that *N. maddoxi* is retained in the population, regardless of the stress exposure that typifies overwintering, therefore retaining the potentiality to spread in the following season.

3.4.5. Implication for the control of *Halyomorpha halys*

The stability of microbiota composition and diversity despite overwintering conditions is relevant to the possible efficacy of biological control, for example, the sterile insect technique (SIT). SIT is a validated, biological pest control strategy based on mass rearing, sterilization, and inundative releases of predominantly male sterile insects; with male sterility induced using

bacteria (Enkerlin et al., 2017; Vreysen et al., 2000) or by irradiation with gamma or X-rays (Klassen and Curtis, 2005). It has been suggested that this technique could be used to eradicate or suppress *H. halys* as soon as they colonize a new area. However, in order to successfully apply this technique, bugs must be mass-reared, in laboratory conditions, an approach which has not been perfected for *H. halys* (Nguyen et al., 2021; Suckling et al., 2019a; Welsh et al., 2017). Therefore, an alternative approach has been suggested, which involves capturing wild bugs from areas where the pest is already established with a high population density, inducing overwintering, and sterilizing them after the reproductive diapause (Roselli et al., 2023; Suckling et al., 2019a) This process requires checking for the presence of potential plant pathogens that may be exported along with the sterilized insects, which, unfortunately, necessitates sacrificing bugs. Conducting this check on overwintered bugs prior to sterilization poses a challenge due to the high mortality rate associated with the overwintering of this pest, further reducing the number of bugs available for SIT. However, our findings indicate that it is possible to perform this check on samples from the population before overwintering since microbial composition and diversity remains unchanged after overwintering. In this way, there is no need to sacrifice overwintered bugs for plant pathogen control purposes.

Regarding *N. maddoxi*, the effects of its infection are not yet fully understood, but it is believed to have a negative impact on the fitness of infected individuals (Preston et al., 2020b). Despite this information, prior to using infected individuals for SIT, it is important to evaluate whether they can transmit pathogens to other insects. It is known that this microsporidian can infect other Pentatomids, such as *C. hilaris* (Hajek et al., 2017). Therefore, it is crucial to assess the potential risks associated with the use of *N. maddoxi*-positive specimens in control strategies, and to carefully consider the ecological implications of introducing them into new environments.

3.5. Conclusions

We observed variations in the composition and diversity of bacterial communities hosted by *H. halys* across neighboring populations. However, we did not observe significant differences in microbial composition and diversity between bugs that underwent overwintering and those that did not, suggesting that this species maintains a stable host-symbiont relationship throughout the winter. It's worth noting that our sample sizes were small, so these conclusions should be interpreted with caution. Despite this limitation, we noticed that the population with the lowest microbial diversity exhibited an increase in richness following the overwintering period. This observation suggests a potential correlation between diet, environmental characteristics of the location, and the microbiota. Of particular interest is the presence of *Yokenella*, which was one of the most abundant genera in bugs from our study sites before overwintering but was absent in overwintered specimens. Further studies could focus on investigating the functions impacted by the presence of this genus.

Our analysis did not detect the presence of phytoplasmas responsible for agricultural plant diseases in any of the specimens we examined. It is worth noting that these pathogens are abundantly found in the host plants of this insect species in the sampled area. While further research is needed to better understand the potential threats posed by *H. halys* as a vector, our results support the notion that this bug is not a significant carrier of phytoplasmas. This finding is beneficial for its use in SIT. However, we identified the presence of two pathogens of agricultural interest (i.e. *Pseudomonas cichorii* and *Pseudomonas tolaasii*), therefore further investigations should be conducted to assess whether *H. halys* can serve as a vector for associated diseases. *N. maddoxi* was identified post-overwintering in one of two populations where it was initially detected before overwintering. This emphasizes the importance of characterizing the microbiota of field *H. halys* populations before establishing rearing colonies for subsequent SIT experiments. This characterization helps prevent the propagation of this

pathogen. *N. maddoxi* is suspected to have a negative impact on *H. halys*, although limited information is currently available. Further studies are needed to assess the ecological effects of *N. maddoxi* on *H. halys* and the surrounding environment, and to determine if it can potentially be used as a control agent.

Supplementary information

DNA extraction protocol

This protocol was optimized according to the manufacturer's instructions.

The first part of the protocol (steps 2-9) was taken from the "Protocol: Pretreatment for Gram-Positive Bacteria" (pp. 45-46 of QIAGEN DNeasy Blood and Tissue kit manual). The last steps (10-13) were taken from the "Animal Tissue (Spin-Column Protocol)" (p. 30).

1. Flash-freeze the sample in liquid nitrogen for a minimum of 30 seconds and then grind it using a Tissuelyser II (QIAGEN) for 1 minute at 30 Hz, or until the bug inside is completely powdered.
2. Add 320 µL of the lysis buffer (20 mM Tris-Cl pH 8, 2mM sodium EDTA, 1.2% Triton® X-100) to the sample.
3. Vortex for 20 seconds to obtain the lysis mixture, then spin for 10 seconds.
4. Pipet 162 µL of the pellet of the lysis mixture in a new 2 ml DNA/DNAse-free tube and store the remaining mixture at -80 for future uses. Depending on the mixture composition, it might be needed to cut the pipette tip with a sterile DNA/DNAse-free scalpel to allow the pipetting of the mixture.
5. Add 18 µL of 200 mg/mL lysozyme to the mixture (to reach the final concentration of lysozyme of 20 mg/ml) and mix thoroughly .
6. Incubate for at least 30 min at 37°C. Then spin for 10 seconds.
7. Add 25 µL proteinase K and 200 µL Buffer AL (without ethanol) and vortex.
8. Incubate at 56°C for 30 minutes. Then spin for 10 seconds.
9. Add 200 µL ethanol (96-100%) to the sample and vortex. Then spin for 10 seconds.

10. Pipet the mixture into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at 8000 rpm for 1 minute. Discard the flow-through and the collection tube.

11. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µL Buffer AW2, centrifuge for 1 minute at 8000 rpm, then centrifuge for 2 minutes at 14000 rpm to dry the DNeasy membrane. Discard the flow-through and the collection tube.

12. Place the DNeasy Mini spin column in a clean 1.5 ml microcentrifuge tube, and pipet 50 µL Buffer AE directly onto the DNeasy membrane.

13. Repeat 12 another time.

Eluted DNA was conserved at -18°C.

Phylum-level population analyses

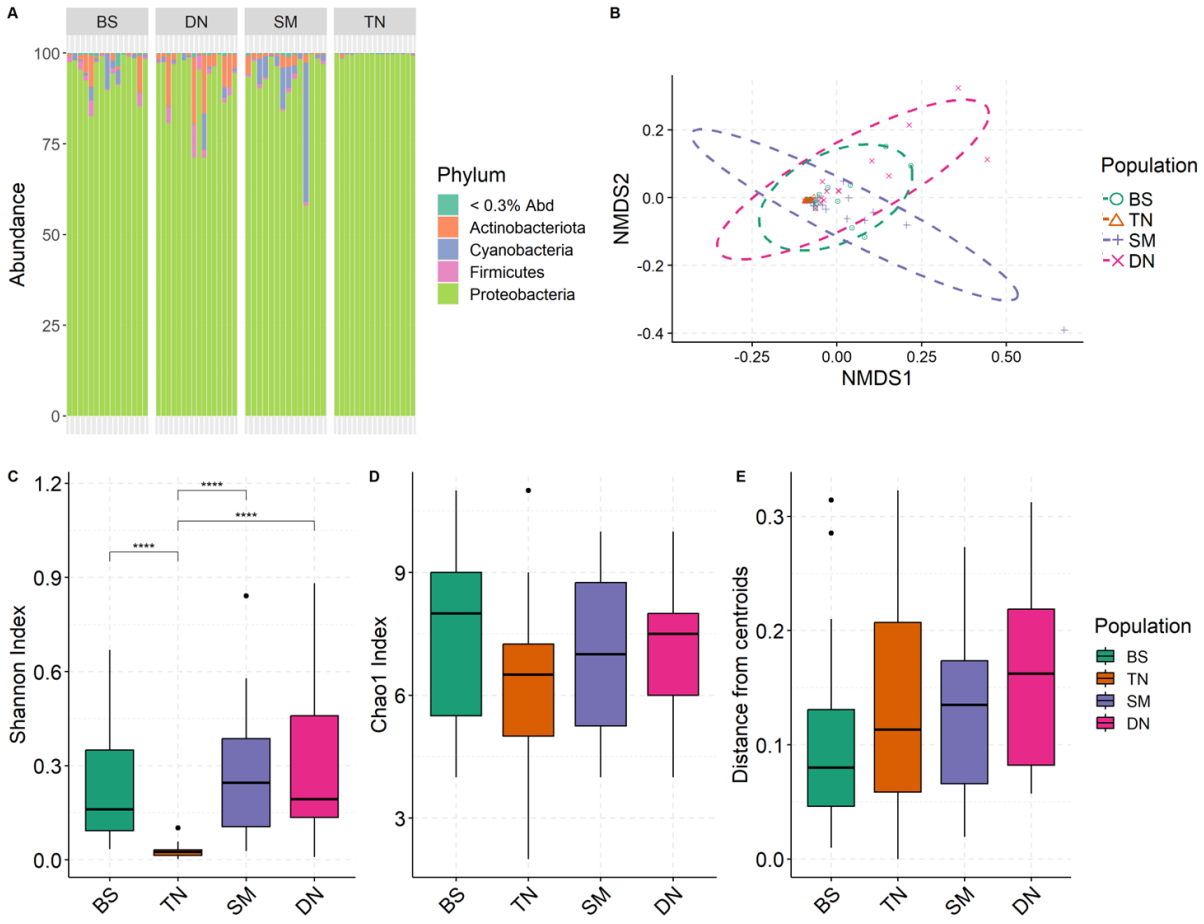

Fig. S3.1. Comparison of whole *H. halys* microbiota diversity and composition between sampling locations at the genus level. (A) Relative abundance plot of the four most present phyla identified (Abd < 0.3%: all phyla with a sample relative abundance < 0.3%). Each bar represents a *H. halys* individual. (B) Nonmetric multidimensional scaling (NMDS) plot of sampled *H. halys* microbiota. PERMANOVA based on dissimilarities was also estimated (R2 = 0.17745; P = 0.002). (C, D) Alpha diversity plots of Shannon (C) and Chao1 (D) indices at the phylum level. (E) Distances from centroids computed using Bray-Curtis method. **** $p < 0.0001$.

Phylum-level overwintering analyses

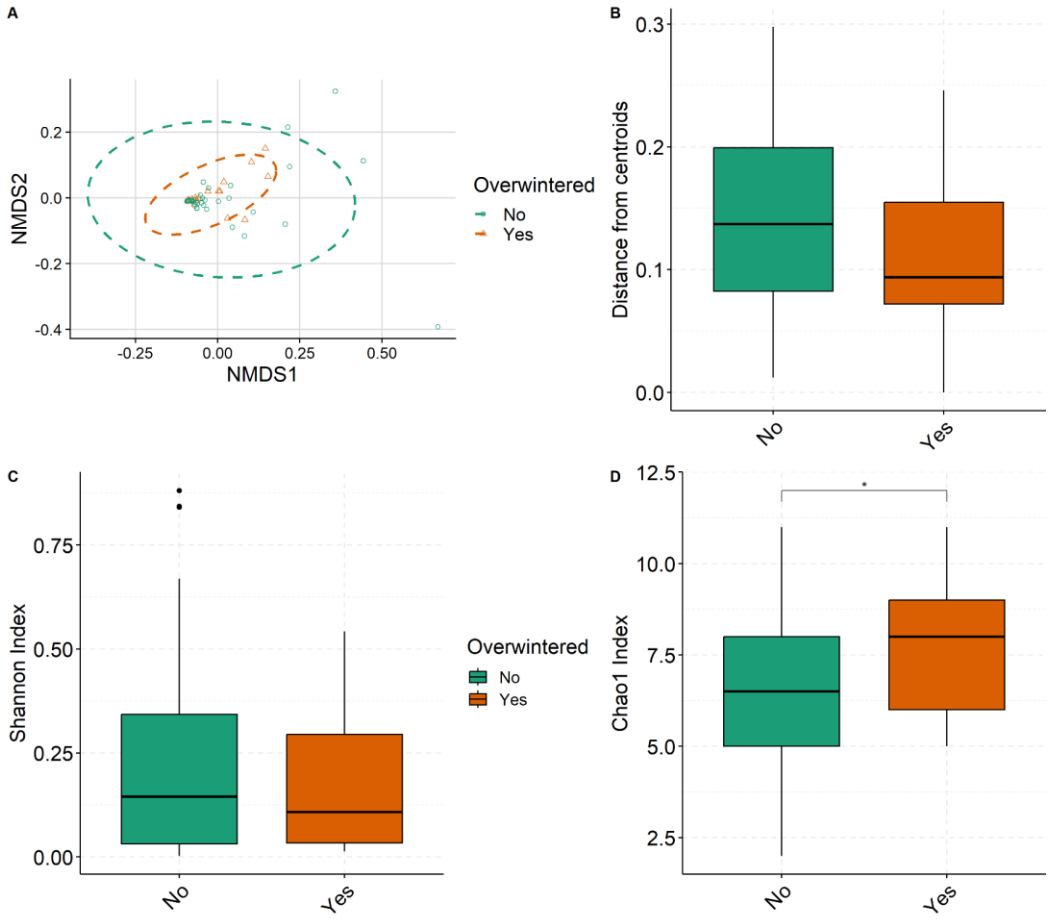

Fig. S3.2. Comparison of microbiota composition and diversity between not overwintered and overwintered *H. halys*. (A) Nonmetric multidimensional scaling (NMDS) plot of sampled *H. halys*. PERMANOVA based on dissimilarities was also conducted (R2 = 0.01128, p = 0.54). (B) Distances from centroids computed using Bray-Curtis method. (C, D) Alpha diversity plots considering Shannon (C) and Chao1 (D) indices at the phylum level. * $p < 0.05$.

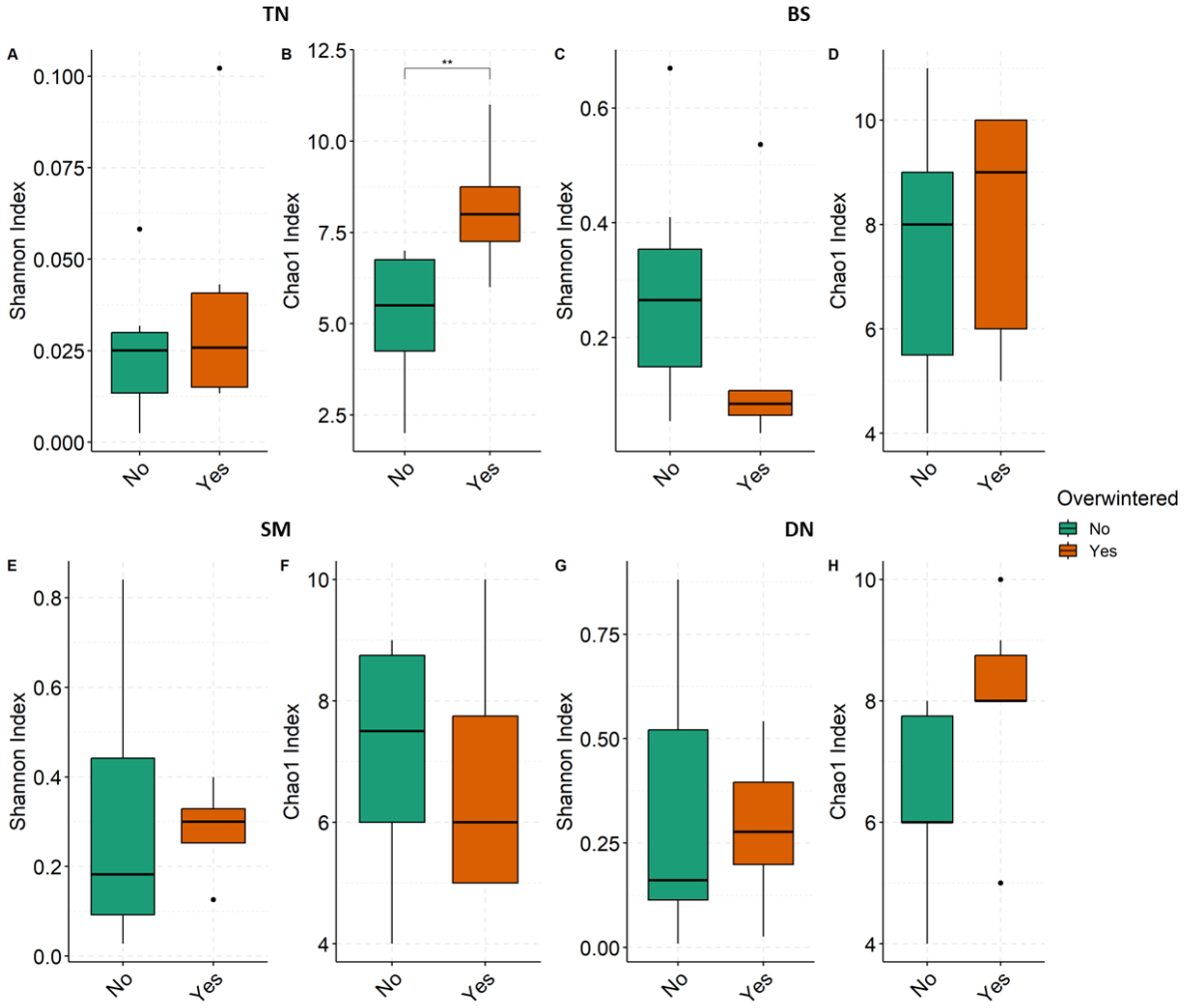

Fig. S3.3. Comparison of alpha diversities population wise at the Phylum level. (A,B) Shannon (A) and Chao1 (B) indices computed on the Trento (TN) population. (C, D) Shannon (C) and Chao1 (D) indices computed on the Besenello (BS) population. (E,F) Shannon (E) and Chao1 (F) indices computed on the San Michele all'Adige (SM) population. (G,H) Shannon (G) and Chao1 (H) indices computed on the Denno (DN) population.

Tables of the differentially present ASVs

Table S3.1. Taxonomy and overall log2 fold change value of the significant ASVs ($p < 0.05$) that were found to be differentially present in the comparison of overwintered vs. not overwintered individuals. A positive value means that the relative ASV presence was significantly higher in the overwintered samples. A negative value means that the relative ASV presence was significantly higher in the not overwintered samples.

Population	ID	Phylum	Class	Order	Family	Genus	Log2fold change (Overwintering: Yes-No)
Besenello (BS)	ASV2228	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Yokenella	-24.73
	ASV321	Patescibacteria	Saccharimonadia	Saccharimonadales	NA	NA	-24.78
	ASV2512	Bacteroidota	Bacteroidia	Flavobacteriales	Weeksellaceae	Chryseobacterium	19.81
	ASV2515	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella	-23.63
	ASV2541	Actinobacteriota	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	Solirubrobacter	-26.51
	ASV2429	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter	-18.30
Trento North (TN)	ASV2228	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Yokenella	-36.72
	ASV2512	Bacteroidota	Bacteroidia	Flavobacteriales	Weeksellaceae	Chryseobacterium	40.86
	ASV1236	Firmicutes	Bacilli	Exiguobacterales	Exiguobacteraceae	Exiguobacterium	-22.13
	ASV1751	Actinobacteriota	Actinobacteria	Micrococcales	Micrococcaceae	Kocuria	18.71
	ASV1719	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	15.40
	ASV1981	Actinobacteriota	Actinobacteria	Micrococcales	Dermabacteraceae	Brachybacterium	19.86
	ASV2063	Actinobacteriota	Actinobacteria	Micrococcales	Microbacteriaceae	Microbacterium	17.46
	ASV88	Actinobacteriota	Actinobacteria	Micrococcales	Microbacteriaceae	Microterricola	17.02
	ASV2201	NA	NA	NA	NA	NA	-27.45
	ASV246	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	-21.03
	ASV796	Actinobacteriota	Actinobacteria	Frankiales	Nakamurellaceae	Nakamurella	18.53
	ASV1632	Actinobacteriota	Actinobacteria	Micrococcales	Microbacteriaceae	Frigoribacterium	-18.67
	ASV2084	Actinobacteriota	Actinobacteria	Micrococcales	Microbacteriaceae	Rathayibacter	18.07
	ASV2541	Actinobacteriota	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	Solirubrobacter	22.59
San Michele all'Adige (SM)	ASV2512	Bacteroidota	Bacteroidia	Flavobacteriales	Weeksellaceae	Chryseobacterium	-36.46
	ASV225	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	Delftia	23.55
	ASV796	Actinobacteriota	Actinobacteria	Frankiales	Nakamurellaceae	Nakamurella	22.90
	ASV2133	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	-23.47
	ASV2319	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	-15.27
	ASV246	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	-21.59
	ASV1391	Firmicutes	Bacilli	Paenibacillales	Paenibacillaceae	Saccharibacillus	-17.31
	ASV1941	Actinobacteriota	Actinobacteria	Micrococcales	Micrococcaceae	Micrococcus	-23.25
	ASV2541	Actinobacteriota	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	Solirubrobacter	-22.26
Denno (DN)	ASV321	Patescibacteria	Saccharimonadia	Saccharimonadales	NA	NA	-18.71
	ASV2515	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella	22.79
	ASV2201	NA	NA	NA	NA	NA	-22.51

Chapter 4 - Molecular phylogeny, evolutionary divergence, and microbiota analysis of *Arboridia dalmatina*, a new invasive species of South Italy

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4.1. Abstract

Arboridia dalmatina is a leafhopper belonging to the Erythroneurini tribe and originally from the Balkans. In 2021, it was detected for the first time in the Apulia region of South Italy. Little is known about this species, it may however represent a threat for vineyards and other crops because of chlorosis due to the heavy stippling on leaves. To confirm the taxonomic status of this species and to reconstruct its demographic history, here we have sequenced the COI marker from 15 *A. dalmatina* individuals sampled from Apulia, Crete and Dalmatia plus 8 individuals of its sister specie *A. adanae* sampled from Turkey. Our molecular phylogenies support the presence of this species also in Turkey, which was not known to date, and identify at least three major haplogroups which are not constrained to the sampling location. Results are compatible with more than one invasive event to Apulia. The radiation of *A. dalmatina* was estimated to have happened during the Pleistocene glaciations, suggesting that this species is ecologically pre-adapted to temperate climate: this hypothesis suggests that this species represents a threat for agriculture and ecology not only for South Italy, but also for other temperate regions. To assess if the invasive individuals carry potentially deleterious microbes and relevant symbionts, we further sequenced the microbiota of the 14 *A. dalmatina* specimens using 16S amplicon sequencing: we found the presence of *Wolbachia* in three Apulian specimens all belonging to the same haplogroup, a finding which is compatible with the hypothesis of multiple invasions. Overall, our results provide the first molecular phylogenetic and metagenomic characterisation of *A. dalmatina* populations, and this knowledge will likely be useful for future studies of this newly invasive pest.

Keywords: *Arboridia dalmatina*, phylogeny, molecular divergence, *Wolbachia*, COI

4.2. Introduction

Arboridia dalmatina Wagner, 1962, is a leafhopper species from the Balkans (Dworakowska, 1970; Nast, 1987, 1972). It belongs to Hemiptera order, Auchenorrhyncha suborder, Cicadellidae family, Typhlocybinae subfamily and Erythroneurini tribe. This species accomplishes its annual cycle on grapevines (*Vitis vinifera*) and overwinters on *Rubus spp.* (Vidano et al., 1987a).

Several species of the Auchenorrhyncha suborder are of viticultural interest because they can be vectors of pathogenic agents such as the golden flavescence and other grapevine mycoplasmoses. In the past, *A. dalmatina* has been suggested to be a potential vector of pathogens for vineyards (Vidano et al., 1987b). Following studies have shown that, in general, the species belonging to the *Arboridia* genus are not phloematic species (Ahn et al., 2005; Chireceanu et al., 2019; Gnezdilov et al., 2008; Health (PLH) et al., 2022): adults and nymphs of these species feed directly on *Vitis* leaves, causing stippling damage due to the piercing and sucking activity on the lower side of leaves. *Arboridia* are therefore unlikely to transmit phytoplasmas (Wilson and Weintraub, 2007), but causes a loss of chlorophyll which eventually leads to chlorosis and related yield loss (Gnezdilov et al., 2008).

Little is known in general about *A. dalmatina* biology, ecology, and evolution. Considering that it was recently found (2021-2022) for the first time in South Italy (Apulia region) and that it might represent a threat for vineyards in this and possibly other Mediterranean regions, further knowledge of this species is required to characterize its ecology and demography, in particular to understand its invasive route. In this study, we performed molecular analyses on individuals of *A. dalmatina* collected in 2021-2022 in Apulia using the cytochrome c oxidase subunit 1 (COI) marker. To ensure the species identification, to investigate the Apulian invasion, and in general to build a first picture of the species genetic diversity, we further amplified COI from *A. dalmatina* specimens from Dalmatia and Crete as well as from the

putative sister species *A. adanae* Dlabola, 1957, sampled from Turkey. A molecular divergence analysis of COI was performed in order to estimate the timing of this species origin and radiation to hypothesize possible palaeoecological scenarios. We further characterize the microbiota of *A. dalmatina* using the 16S V3-V4 amplicons to detect possible microbes of pathogenetic and/or control relevance. Results revealed the first molecular characterization of *A. dalmatina* population structure and related microbiome, a knowledge which is the fundament for next studies of the biology of this newly invasive pest.

4.3. Materials and Methods

4.3.1. Samples collection

A. dalmatina specimens were collected in 2021-2022 in Valenzano (Apulia, Italy), Potomje (Dalmatia, Croatia) and Kappariana (Crete, Greece). Putative *A. adanae* were collected in 2022 in Yurtbaşı (Elazığ, Turkey) and were used as an outgroup species for the downstream analyses. All the analyzed specimens were caught by means of nets in vineyards and stored in 80% alcohol.

4.3.2. DNA extraction and amplification

A total of 23 leafhoppers were sequenced (5 Apulian, 5 Cretan, 5 Dalmatian and 8 Turkish). The total DNA was purified from lyophilized and homogenized individuals with the NucleoSpin Tissue kit (MACHEREY-NAGEL GmbH & Co. KG), according to the manufacturer's instructions. The cytochrome oxidase subunit I (COI) gene was amplified using the universal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994), and the GoTaq® Green Master Mix (Promega Corporation - USA). Polymerase chain reaction (PCR) conditions were: 2 min at 95°C, 5× (45 sec at 95°C, 45 sec at 45°C, 1 min at 72°C), 35× (45 sec at 95°C, 45 sec at 50°C, 1 min at 72°C), 5 min at 72°C (modified from EPPO 2021). PCR products, after purification with illustra ExoProStar1-Step (GE Healthcare, Little Chalfont, UK), were sequenced with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3130 xl Genetic Analyzer (Carlsbad, CA, USA) at the Genomics Platform, Fondazione E. Mach (San Michele all'Adige, Italy).

The DNA extraction for the bacterial microbiota analysis (not performed on the Turkish and one Cretan specimens) was performed using QIAGEN DNeasy Blood and Tissue kit for the

DNA extraction, according to manufacturer's instructions, adding the pretreatment for gram-positive bacteria step, also provided by the manufacturer, to improve bacterial DNA extraction. We added to the analyses DNA extraction negative controls to detect kit contaminations made at the opening and termination of each kit used. The extracted DNA quality and quantity was controlled using a Spark® multimode microplate reader (Tecan, Switzerland). We amplified the 16S V3-V4 region using using the KAPA HiFi HS ReadyMix (Roche), with the two primers 341F_ILL (5'-CCTACGGGNGGCWGCAG-3') and 805R-2_ILL (5'-GACTACNVGGGTWTCTAATCC-3'). The PCR cycle consisted of three steps: 30 sec at 95°C, 30 sec at 55°C, and 90 sec at 72°C. This cycle was repeated 35 times to amplify bacteria, 40 times to amplify microsporidians. Before starting the PCR, the device Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems, USA) was set to reach 95°C in 3 min. The PCR cycle consisted of three steps: 30 sec at 95°C, 30 sec at 55°C, and 90 sec at 72°C. This cycle was repeated 35 times to amplify bacteria, 40 times to amplify microsporidians. At the end of the PCR, the temperature was stable at 72°C for 7 min. At the end, we used the QIAxcel Advanced System (QIAGEN) to assess the success of the amplification and evaluate a repetition of the PCR. Bacterial amplicons were sequenced using Illumina MiSeq 2x300bp with a minimum depth of 100,000 reads per sample, performed at the Sequencing and Genotyping Platform, Fondazione E. Mach (San Michele all'Adige, Italy).

4.3.3. Phylogenetic and clock analyses

COI sequences were visualized using Chromas software (Technelysium Pty Ltd). Forward and reverse reads were assembled using a Biopython script (Cock et al., 2009). A total of 16 *Arboridia* COI sequences were downloaded from the NCBI nucleotide database, 9 of them belonging to *A. kakogawana* Matsumura, 1932, and 7 belonging to *A. maculifrons* Vilbaste, 1968. All sequences (more details inTable 4.S1) were aligned to our sequences using MAFFT version 7 (Katoh et al., 2019; Kuraku et al., 2013). The final dataset comprises 650 aligned

nucleotide positions from 39 samples. We use this dataset to infer phylogenies under a Maximum Likelihood (ML) framework, using RAxML (Stamatakis, 2014) and employing a GTR + Gamma replacement model with branch robustness assessed with 100 bootstrap replicates. FigTree (version 1.4.4) was used for the topology visualization and figures preparation.

We further used this dataset to estimate divergence times between species using BEAST2 (Bouckaert et al., 2014). We employed a birth and death model as a tree prior and set the tree topology according to the ML topology. In order to calibrate the tree, we added 11 COI sequences of a species belonging to the Dikraneurini tribe (*Dikrella cruentata* Gillette, 1898) and 14 COI sequences belonging to *Mileewa sp*. Distant, 1908 (Table 4.S1). We calibrated the tree root using the fossil of a Dekraneurini gen. sp (Dietrich and Vega, 1995), according to (Yan et al., 2022), to calibrate the Dikraneurini-Erythoneurini split at 17.5-90 Million years ago (Mya) using a normal distribution (mean 53.6 Mya, standard deviation 18.4) and the fossil of *Youngeawea bicolorata* (Mileewinae: Mileewini) to set the minimum of the divergence between *Mileewa* and Typhlocybinae at 44 Mya (Gębicki and Szwedo, 2001). The analysis was run for 100 million Markov chain Monte Carlo (MCMC) iterations, or until it reached convergence, sampling every 10,000 steps after a 10% initial burn-in. We used Tracer 1.7.1 (Rambaut et al., 2018) to visualize convergence, and it was considered to be reached when all variables had an Effective Sample Size (ESS) >200 and a bell-shaped posterior distribution. The substitution saturation was checked using DAMBE (Xia, 2017; Xia and Lemey, 2009) considering Xia's observed index of saturation (Xia et al., 2003).

4.3.4. Barcoding gap analyses

To analyze the COI barcoding gap, we used our *Arboridia* sequences and all the Erythroneurini COI sequences available from the NCBI nucleotide database in November 2022. Sequences were aligned using MAFFT version 7 (Katoh et al., 2019; Kuraku et al., 2013) and a custom

python script was used to trim the head and tail of the alignment to avoid gaps. The resulting alignment was composed of 2875 sequences and 442 positions. The newly obtained alignment was used to calculate the pairwise distance matrix using the DistanceCalculator class of the TreeConstruction module of Biopython (Cock et al. 2009) with the "identity" model. To visualize the distribution of genetic distances, the intraspecific and interspecific genetic distances were plotted in a histogram using the matplotlib library, excluding those involving the species *Arboridia dalmatina* and *Arboridia adanae*. The distribution without outliers was assessed using the formula First Quartile - 1.5 * Interquartile Range and Third Quartile + 1,5 * Interquartile Range. Finally, the genetic distances between and among *A. dalmatina* and *A. adanae* were processed by custom python scripts and plotted on the same histogram. The python script uses the mean of the pairwise distance distribution to define if a sample belongs to an intraspecific or an interspecific distribution.

4.3.5. Microbiota analysis

CutAdapt (Martin, 2011) was used to remove adapters from the 16S V3-V4 reads. The rest of the analysis was performed in R version 4.1.2 software (R Core Team 2022). DADA2 package (Callahan et al. 2016) was used to filter the reads by quality, remove errors, merge the forward and reverse reads, remove chimeras, and assign the taxonomy to the resulting ASVs using Silva v138 (Yilmaz et al. 2014; Quast et al. 2013). Decontam (Davis et al. 2018) was used to remove contaminant sequences considering the negative controls. phyloseq (McMurdie and Holmes 2013) was used to compute abundance and richness plots and statistics.

4.4. Results and discussion

4.4.1. First molecular evidence of *A. dalmatina* in Turkey?

We preliminary assigned all the samples from Turkey to *A. adanae*, the sister species of *A. dalmatina*: this is because we followed the indication of turkish colleagues who curated the sampling and because to our knowledge, *A. dalmatina* has not been found in Turkey to date. However, the COI phylogenetic tree (Fig. 4.1) indicated that the specimens *A. adanae* 4 and *A. adanae* 5 (from Turkey) cluster with maximum support within *A. dalmatina*. In particular, these two samples are closely related to two of the *A. dalmatina* sampled from Italy (*A. dalmatina* Apulia 4 and *A. dalmatina* Apulia 5) with a ML support of 73/100 and posterior probability of 0.93. This evidence is reinforced by the barcoding gap analysis which indicates that the distance between *A. adanae* 4-5 and the other *A. adanae* fall outside of the intraspecific distances (blue cross in Fig. 4.2), while the distance between *A. adanae* 4-5 and the other *A. dalmatina* falls within the distribution of intraspecific distance (purple cross). These results indicate that *A. adanae* 4-5 are actually *A. dalmatina*: this is the first (molecular) evidence of the presence of this species in Turkey. The exact taxonomic status of *A. adanae* and *A. dalmatina* is not however clear, because the distance between *A. dalmatina* (including *A. adanae* 4-5) and the *A. adanae* (blue cross) is inside the actual barcoding-gap generated by comparing various species of Erythroneurini, the tribe to which *Arboridia* belong. This reduced interspecific distance is still compatible with separate species because it maintains, although reducing it, the barcoding-gap; alternatively it is possible that these two species form a species complex with the possibility of interbreeding. Morphological characterisation of other individuals sampled from Turkey is ongoing in our lab to confirm the presence of *A. dalmatina* in this country, and/or to define the exact morphological disparity and possibility of mixed characters compatible with a species complex.

4.4.2. Phylogeny of *A. dalmatina* is compatible with multiple invasions in Italy

The COI phylogenetic tree (Fig. 4.1) shows the presence of 3 groups with high node supports: the blue group composed by two Apulian samples (4 and 5) and two Turkish samples (4 and 5); the green group composed of three Cretan samples (3, 4 and 5) and four Dalmatian samples (1, 2, 3 and 5); the orange group composed by three Apulian samples (1,2 and 3), two Cretan samples (1, 2) and one Dalmatian sample (4). The presence of different locations in the three groups indicates that there is not a clear geographic structure. This is compatible with a fragmented history of geographic isolation likely due to a recent spread of this species attributable to human activities related to vineyarding.

The Apulian specimens belong to two different groups (blue and orange): this suggests that there were likely more than one single invasion event in the past. Because *A. adanae* 4 and 5 from Turkey are indeed *A. dalmatina*, one invasion root may have been directly from Turkey. It is not possible to understand precisely where the other invasion originated, since Apulian specimens 1,2,3 are not related to only one indigenous population, but to both Cretan and Dalmatian.

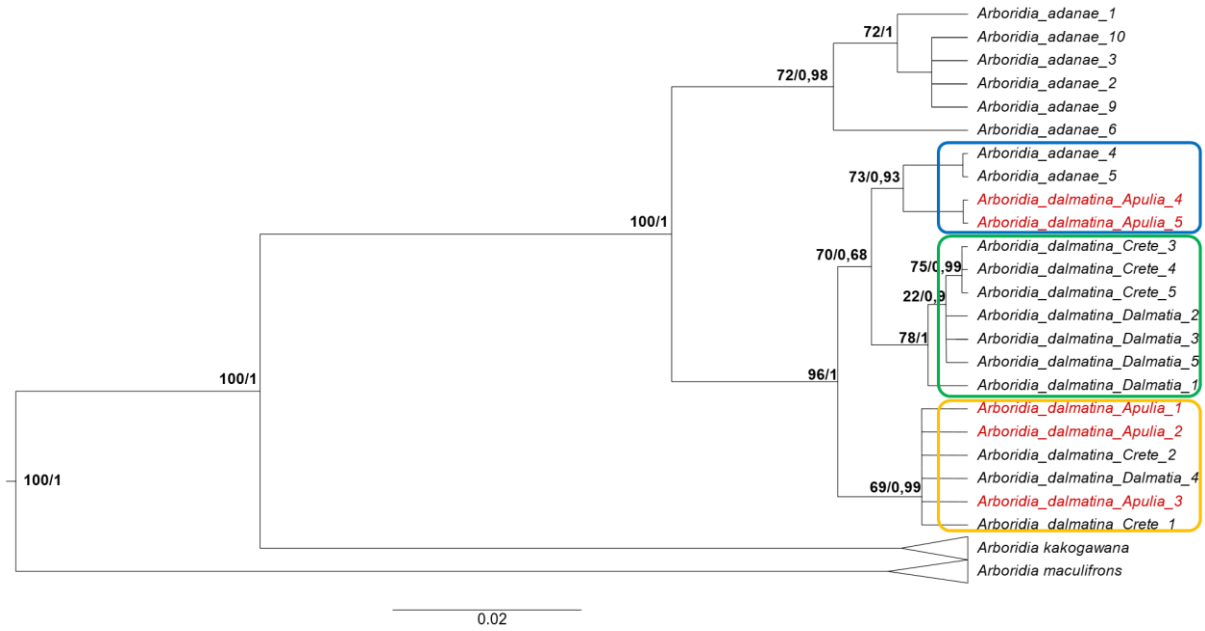

Fig. 4.1. Phylogenetic tree of COI gene. In red sequences from Apulian samples. The two numbers at nodes are the number of bootstrap replicates under a ML framework and the posterior probability in a Bayesian framework respectively.

4.4.3. The recent origin and divergence of *A. dalmatina*

To calibrate the phylogeny, we utilized sequences from highly divergent taxa, specifically *Mileewa sp*. and *Dikrella cruentata*. Therefore, we employed DAMBE to assess the presence of substitution saturation, which revealed a negligible to minimal saturation level (P-invariant = 0.219, Iss = 0.565, Iss.c = 0.717, p-value = 0.001). According to the divergence times estimated by a molecular clock analysis of the COI gene (Fig. 4.3), *A. dalmatina* started its current radiation circa 810.000 years ago (95% high posterior density between 290,000 years ago and 1.81 million of years ago), in the middle of the Pleistocene epochs, which was characterized by an alternation of glaciation events (Elderfield et al., 2012; Pisias and Moore, 1981). During Pleistocene, Turkey, South Italy, Dalmatia, and the southern part of the Balkans were glacial refugia (Jochim, 1987; Salvi et al., 2013), places where species could have survived the glaciation events and afterwards recolonize the northern surrounding areas. This

suggests that *A. dalmatina* might have evolved at the proximity of the ice limit in a paleoecological scenario characterized by climate conditions, which were more likely as temperate as those of its current areal distribution (Balkans). Because of the increasing temperatures due to climate change and the geographical conformation of the Italian peninsula, which aid both natural movement and human related passages through its east coast, this species represents a potential threat to vineyards and, in general, to the temperate ecosystems of the rest of Italy and of other mediterranean regions.

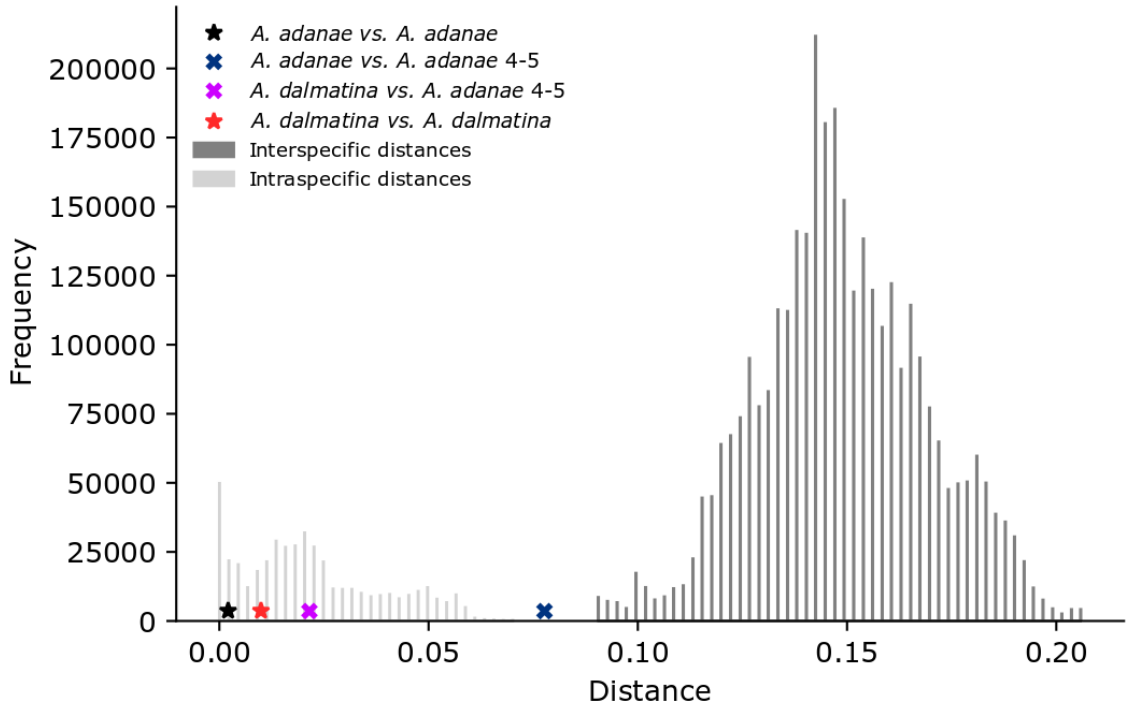

Fig. 4.2. Barcoding gap of COI gene using Erythoneurini sequences. The putative *A. adanae* samples 4 and 5 from Turkey have a distance to the other *A. adanae* (blue cross) that falls outside the intraspecific barcoding distance.

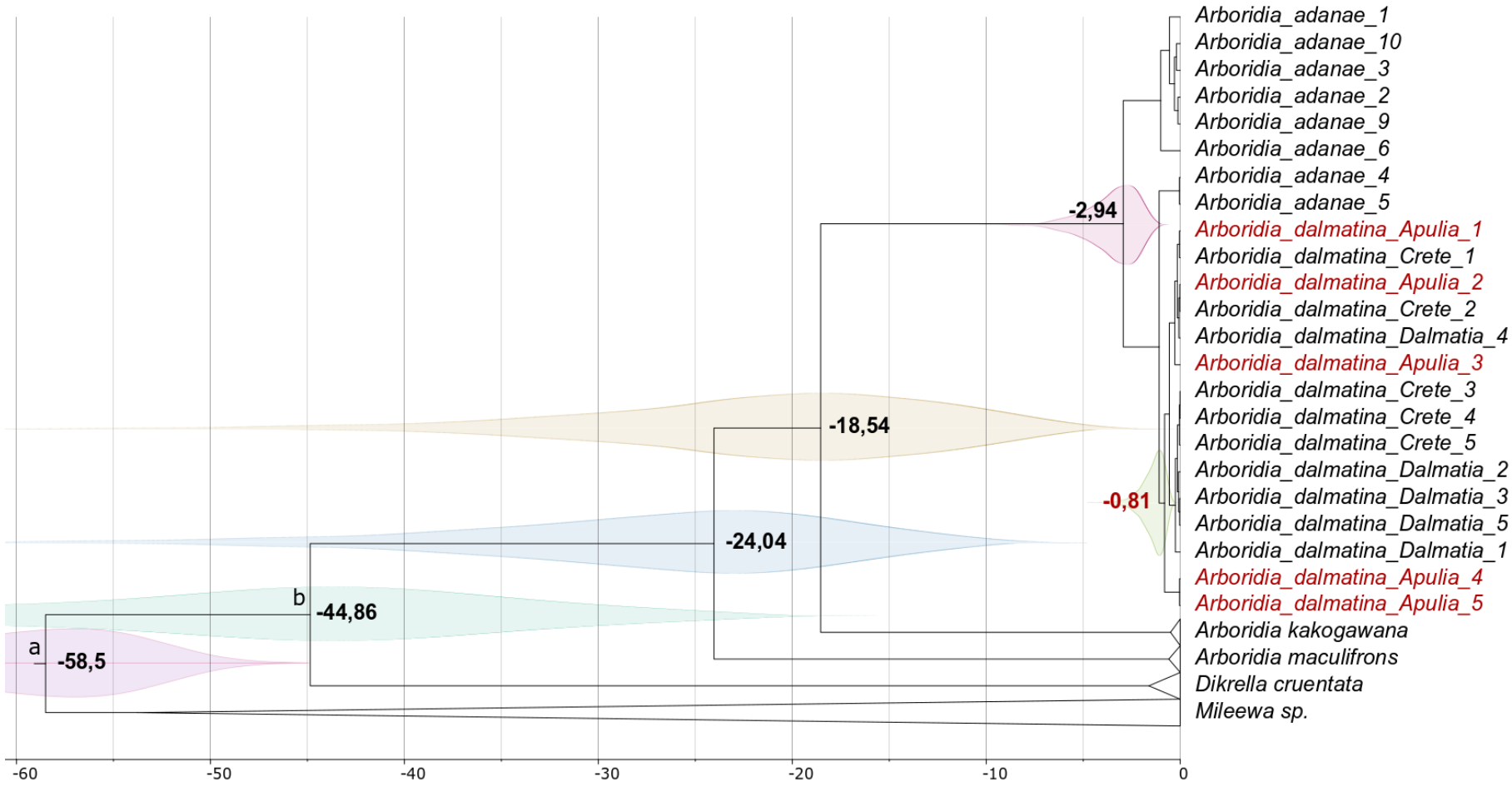

Fig. 4.3. Molecular divergence of *Arboridia dalmatina* COI gene. On each node it is shown the estimated marginal density function. a) fossil calibration minimum 44.4 Mya; b) fossil calibration 17.5-90 Mya.

4.4.4. Different microbial profiles and presence of Wolbachia only in Italian samples

The abundance plot (Fig. 4.4; Table 4.S2) shows the presence of different genus of bacteria whose quantity of reads is higher than 1.5% of the total reads. It is difficult to assess whether these genera have positive or negative implications on *Arboridia* biology and/or on its management, since this aspect is mostly related to species or strains rather than genus. For example, *Pseudomonas fluorescens* positively affects plants (Sah et al., 2021), while *Pseudomonas syringae* causes diseases in a lot of crops (Arnold and Preston, 2019). However, it can be seen that the only genus common among all specimens is *Pseudomonas. Rickettsia* and *Tsukamurella* are present only in the 4th Dalmatian specimen, while the other abundant genera, except for *Wolbachia*, are widespread among all specimens.

Wolbachia was found in three Apulian specimens (1, 2, 3) and slightly present in specimen 4, but it was not found in all the individuals sampled from outside Italy. *Wolbachia* bacteria are known to be maternally inherited intracellular insect parasites that can induce different reproductive phenotypes through cytoplasmic incompatibility and other strategies (Dobson et al., 1999; Kozek and Rao, 2007; O'Neill et al., 1992; Taylor et al., 2018; Werren, 1997; Werren et al., 2008). The presence of *Wolbachia* only in the Italian samples is puzzling. One possibility is that the infected Italian individuals come from infected Dalmatian or Cretan (or even Turkish) populations that we have not sampled. An alternative hypothesis is that *Wolbachia* might have been transferred horizontally by parasitoids only recently, after the introduction in Italy (Ahmed et al., 2015), by local parasitoids: it has been shown that parasitoids from Mymaridae and Trichogrammatidae can affect various *Arboridia* species (Hesami et al., 2004; Hu and Triapitsyn, 2016; Triapitsyn et al., 2020).

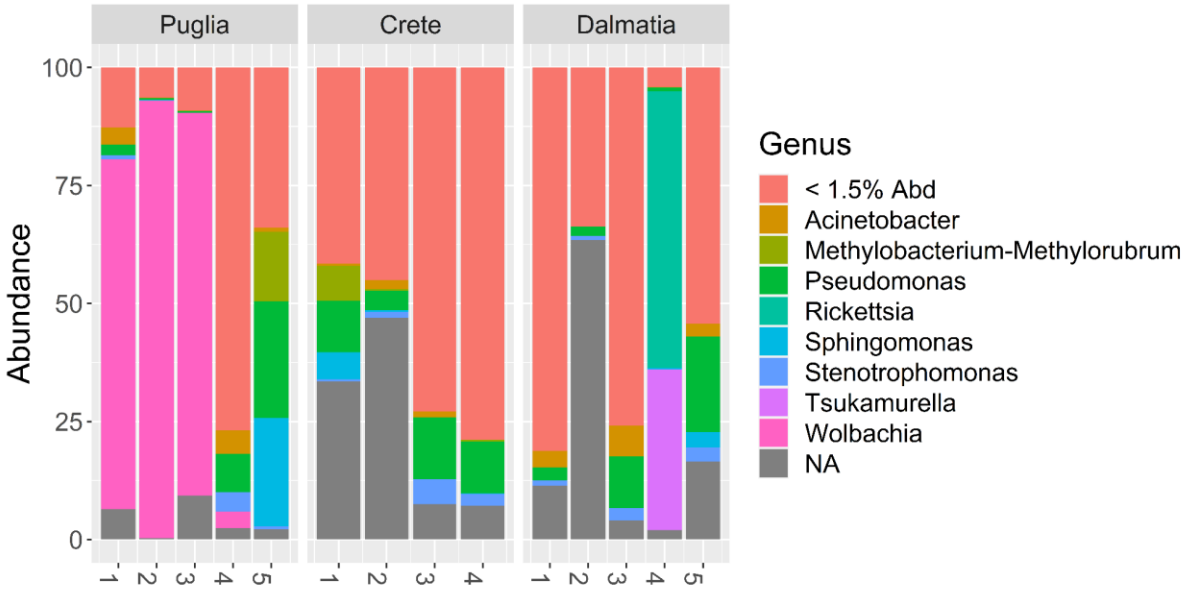

Fig. 4.4. 16S V3-V4 region relative abundance plot of *A. dalmatina* from the three European locations Puglia, Crete and Dalmatia.

4.5. Conclusions and future perspectives

In this study we provided the first evolutionary study of *Arboridia dalmatina* to date by employing both COI phylogenetics and metagenomic survey.

We found the first molecular evidence that *A. dalmatina* is present in Turkey and we are currently carrying out a morphological inspection of Turkish specimens' genitalia to validate this finding and to further clarify the taxonomic status of *A. dalmatina* compared with its sister species *A. adana*e.

The phylogenetic and barcoding analysis showed the presence of three COI genetic groups of *A. dalmatina* in the Mediterranean basin. The (invasive) Italian samples belong to two of these three groups, suggesting the occurrence of more than one invasion event. The groups defined through the phylogenetic analysis are geographically heterogeneous, suggesting that the spreading of this species might be a relatively recent event, possibly attributable to human activities.

Our clock analysis indicates that the *A. dalmatina* radiation happened in Pleistocene refugia, suggesting a pre-adaptation to temperate environments, a good predisposition to the current climatic conditions of Italy. Despite a general high statistical support and the negligible presence of substitution saturation, our phylogenies are based on only one mitochondrial marker, therefore our evolutionary evidence and related implications reflect the evolutionary history of the mitochondria. It is well known that there may be pervasive discrepancies between mitochondrial and nuclear markers for what concern both the genealogy and the divergence estimates (Zadra et al., 2021). In order to have a more general and reliable picture of *Arboridia* evolution, analysis should be in future extended to nuclear genes and more broadly to genome-scaled dataset including both nuclear and mitochondrial information. Future should also

concentrate on performing taxon sampling in more regions of the Balkans and on having an increased number of specimens to analyze.

Overall, our results substantially improve our understanding of *A. dalmatina* biology and shed light on its recent Italian invasion. The data presented here are the bases for future molecular monitoring of this species in both invaded and original range.

Supplementary information

Table 4.S1. Sequences downloaded from NCBI nucleotide to perform the phylogenetic and molecular divergence analysis.

NCBI ID	Species	NCBI ID	Species
MN972661.1	*Arboridia kakogawana*	MN972656.1	*Arboridia maculifrons*
MN972662.1	*Arboridia kakogawana*	MN972655.1	*Arboridia maculifrons*
MN972665.1	*Arboridia kakogawana*	MN972660.1	*Arboridia maculifrons*
MN972667.1	*Arboridia kakogawana*	MF935009.1	*Dikrella cruentata*
MN972663.1	*Arboridia kakogawana*	MF929911.1	*Dikrella cruentata*
MN972668.1	*Arboridia kakogawana*	MF934624.1	*Dikrella cruentata*
MN972664.1	*Arboridia kakogawana*	MF933368.1	*Dikrella cruentata*
MN972666.1	*Arboridia kakogawana*	MG398285.1	*Dikrella cruentata*
MN972669.1	*Arboridia kakogawana*	MF932414.1	*Dikrella cruentata*
MN972659.1	*Arboridia maculifrons*	MF931840.1	*Dikrella cruentata*
MN972658.1	*Arboridia maculifrons*	MF934830.1	*Dikrella cruentata*
MN972657.1	*Arboridia maculifrons*	MF932997.1	*Dikrella cruentata*
MN972654.1	*Arboridia maculifrons*	KT623269.1	*Dikrella cruentata*
KR581114.1	*Dikrella cruentata*	MK603803.1	*Mileewa sp.*
MK603796.1	*Mileewa sp.*	MK603792.1	*Mileewa sp.*
MK603800.1	*Mileewa sp.*	MK603799.1	*Mileewa sp.*
MK603793.1	*Mileewa sp.*	MK603801.1	*Mileewa sp.*

MK603797.1	*Mileewa sp.*	MK603791.1	*Mileewa sp.*
MK603788.1	*Mileewa sp.*	MK603802.1	*Mileewa sp.*
MK603794.1	*Mileewa sp.*	MK603789.1	*Mileewa sp.*
MK603787.1	*Mileewa sp.*	OQ410656	*Arboridia dalmatina*
OQ410659	*Arboridia dalmatina*	OQ410661	*Arboridia dalmatina*
OQ410669	*Arboridia dalmatina*	OQ410670	*Arboridia dalmatina*
OQ410658	*Arboridia dalmatina*	OQ410657	*Arboridia dalmatina*
OQ410662	*Arboridia dalmatina*	OQ410663	*Arboridia dalmatina*
OQ410664	*Arboridia dalmatina*	OQ410668	*Arboridia dalmatina*
OQ410665	*Arboridia dalmatina*	OQ410667	*Arboridia dalmatina*
OQ410660	*Arboridia dalmatina*	OQ410666	*Arboridia dalmatina*
OQ410669	putative *Arboridia dalmatina*	OQ410670	putative *Arboridia dalmatina*
OQ410673	*Arboridia adanae*	OQ410674	*Arboridia adanae*
OQ410675	*Arboridia adanae*	OQ410676	*Arboridia adanae*
OQ410677	*Arboridia adanae*	OQ410678	*Arboridia adanae*

Table 4.S2. 20 most abundant bacterial genus for each specimen.

Population	Specimen	Genus	Population	Specimen	Genus
Apulia	1	Wolbachia	Apulia	2	Staphylococcus
Apulia	1	Acinetobacter	Apulia	2	Corynebacterium
Apulia	1	Pseudomonas	Apulia	2	Stenotrophomonas
Apulia	1	Anaerobacillus	Apulia	2	Bacillus
Apulia	1	Burkholderia-Caballeronia-Paraburkholderia	Apulia	2	Anaerobacillus
Apulia	1	Stenotrophomonas	Apulia	2	Cutibacterium
Apulia	1	Methylobacterium-Methylorubrum	Apulia	2	Craurococcus-Caldovatus
Apulia	1	Bacillus	Apulia	2	Rickettsia
Apulia	1	Cutibacterium	Apulia	2	Tsukamurella
Apulia	1	Staphylococcus	Apulia	2	Methylobacterium-Methylorubrum
Apulia	1	Sphingomonas	Apulia	2	Veillonella
Apulia	1	Craurococcus-Caldovatus	Apulia	2	Mycobacterium
Apulia	1	Rickettsia	Apulia	2	Sphingobacterium
Apulia	1	Tsukamurella	Apulia	2	Tepidimonas
Apulia	1	Corynebacterium	Apulia	2	Blastococcus
Apulia	1	Veillonella	Apulia	3	Wolbachia
Apulia	1	Mycobacterium	Apulia	3	Bacillus
Apulia	1	Sphingobacterium	Apulia	3	Pseudomonas
Apulia	1	Tepidimonas	Apulia	3	Acinetobacter
Apulia	1	Blastococcus	Apulia	3	Anaerobacillus
Apulia	2	Wolbachia	Apulia	3	Stenotrophomonas
Apulia	2	Pseudomonas	Apulia	3	Staphylococcus
Apulia	2	Sphingomonas	Apulia	3	Cutibacterium
Apulia	2	Acinetobacter	Apulia	3	Burkholderia-

					Caballeronia-Paraburkholderia
Apulia	2	Burkholderia-Caballeronia-Paraburkholderia	Apulia	3	Corynebacterium
Apulia	3	Methylobacterium-Methylorubrum	Apulia	4	Veillonella
Apulia	3	Tepidimonas	Apulia	4	Sphingobacterium
Apulia	3	Mycobacterium	Apulia	4	Tepidimonas
Apulia	3	Sphingomonas	Apulia	4	Blastococcus
Apulia	3	Craurococcus-Caldovatus	Apulia	5	Methylobacterium-Methylorubrum
Apulia	3	Rickettsia	Apulia	5	Pseudomonas
Apulia	3	Tsukamurella	Apulia	5	Sphingomonas
Apulia	3	Veillonella	Apulia	5	Acinetobacter
Apulia	3	Sphingobacterium	Apulia	5	Stenotrophomonas
Apulia	3	Blastococcus	Apulia	5	Anaerobacillus
Apulia	4	Acinetobacter	Apulia	5	Corynebacterium
Apulia	4	Pseudomonas	Apulia	5	Staphylococcus
Apulia	4	Bacillus	Apulia	5	Burkholderia-Caballeronia-Paraburkholderia
Apulia	4	Stenotrophomonas	Apulia	5	Bacillus
Apulia	4	Wolbachia	Apulia	5	Cutibacterium
Apulia	4	Cutibacterium	Apulia	5	Tepidimonas
Apulia	4	Anaerobacillus	Apulia	5	Veillonella
Apulia	4	Staphylococcus	Apulia	5	Wolbachia
Apulia	4	Corynebacterium	Apulia	5	Craurococcus-Caldovatus
Apulia	4	Burkholderia-Caballeronia-Paraburkholderia	Apulia	5	Rickettsia
Apulia	4	Mycobacterium	Apulia	5	Tsukamurella
Apulia	4	Methylobacterium-	Apulia	5	Mycobacterium

		Methylorubrum			
Apulia	4	Sphingomonas	Apulia	5	Sphingobacterium
Apulia	4	Craurococcus-Caldovatus	Apulia	5	Blastococcus
Apulia	4	Rickettsia	Crete	1	Methylobacterium-Methylorubrum
Apulia	4	Tsukamurella	Crete	1	Pseudomonas
Crete	1	Sphingomonas	Crete	2	Corynebacterium
Crete	1	Acinetobacter	Crete	2	Sphingomonas
Crete	1	Burkholderia-Caballeronia-Paraburkholderia	Crete	2	Veillonella
Crete	1	Staphylococcus	Crete	2	Staphylococcus
Crete	1	Cutibacterium	Crete	2	Tepidimonas
Crete	1	Bacillus	Crete	2	Blastococcus
Crete	1	Stenotrophomonas	Crete	2	Wolbachia
Crete	1	Anaerobacillus	Crete	2	Craurococcus-Caldovatus
Crete	1	Blastococcus	Crete	2	Rickettsia
Crete	1	Veillonella	Crete	2	Tsukamurella
Crete	1	Corynebacterium	Crete	2	Mycobacterium
Crete	1	Wolbachia	Crete	2	Sphingobacterium
Crete	1	Craurococcus-Caldovatus	Crete	3	Acinetobacter
Crete	1	Rickettsia	Crete	3	Pseudomonas
Crete	1	Tsukamurella	Crete	3	Burkholderia-Caballeronia-Paraburkholderia
Crete	1	Mycobacterium	Crete	3	Stenotrophomonas
Crete	1	Sphingobacterium	Crete	3	Bacillus
Crete	1	Tepidimonas	Crete	3	Anaerobacillus
Crete	2	Pseudomonas	Crete	3	Tepidimonas
Crete	2	Acinetobacter	Crete	3	Cutibacterium

Crete	2	Methylobacterium-Methylorubrum	Crete	3	Corynebacterium
Crete	2	Burkholderia-Caballeronia-Paraburkholderia	Crete	3	Methylobacterium-Methylorubrum
Crete	2	Stenotrophomonas	Crete	3	Staphylococcus
Crete	2	Anaerobacillus	Crete	3	Wolbachia
Crete	2	Bacillus	Crete	3	Sphingomonas
Crete	2	Cutibacterium	Crete	3	Craurococcus-Caldovatus
Crete	3	Rickettsia	Dalmatia	1	Blastococcus
Crete	3	Tsukamurella	Dalmatia	1	Sphingobacterium
Crete	3	Veillonella	Dalmatia	1	Acinetobacter
Crete	3	Mycobacterium	Dalmatia	1	Mycobacterium
Crete	3	Sphingobacterium	Dalmatia	1	Pseudomonas
Crete	3	Blastococcus	Dalmatia	1	Craurococcus-Caldovatus
Crete	4	Pseudomonas	Dalmatia	1	Anaerobacillus
Crete	4	Burkholderia-Caballeronia-Paraburkholderia	Dalmatia	1	Staphylococcus
Crete	4	Bacillus	Dalmatia	1	Stenotrophomonas
Crete	4	Staphylococcus	Dalmatia	1	Sphingomonas
Crete	4	Anaerobacillus	Dalmatia	1	Bacillus
Crete	4	Stenotrophomonas	Dalmatia	1	Burkholderia-Caballeronia-Paraburkholderia
Crete	4	Acinetobacter	Dalmatia	1	Cutibacterium
Crete	4	Corynebacterium	Dalmatia	1	Corynebacterium
Crete	4	Cutibacterium	Dalmatia	1	Veillonella
Crete	4	Sphingomonas	Dalmatia	1	Methylobacterium-Methylorubrum
Crete	4	Methylobacterium-Methylorubrum	Dalmatia	1	Wolbachia

Crete	4	Mycobacterium	Dalmatia	1	Rickettsia
Crete	4	Wolbachia	Dalmatia	1	Tsukamurella
Crete	4	Craurococcus-Caldovatus	Dalmatia	1	Tepidimonas
Crete	4	Rickettsia	Dalmatia	2	Staphylococcus
Crete	4	Tsukamurella	Dalmatia	2	Pseudomonas
Crete	4	Veillonella	Dalmatia	2	Burkholderia-Caballeronia-Paraburkholderia
Crete	4	Sphingobacterium	Dalmatia	2	Anaerobacillus
Crete	4	Tepidimonas	Dalmatia	2	Bacillus
Crete	4	Blastococcus	Dalmatia	2	Cutibacterium
Dalmatia	2	Stenotrophomonas	Dalmatia	3	Methylobacterium-Methylorubrum
Dalmatia	2	Acinetobacter	Dalmatia	3	Wolbachia
Dalmatia	2	Sphingomonas	Dalmatia	3	Rickettsia
Dalmatia	2	Methylobacterium-Methylorubrum	Dalmatia	3	Tsukamurella
Dalmatia	2	Corynebacterium	Dalmatia	3	Bacillus
Dalmatia	2	Blastococcus	Dalmatia	3	Mycobacterium
Dalmatia	2	Wolbachia	Dalmatia	3	Sphingobacterium
Dalmatia	2	Craurococcus-Caldovatus	Dalmatia	3	Blastococcus
Dalmatia	2	Rickettsia	Dalmatia	4	Rickettsia
Dalmatia	2	Tsukamurella	Dalmatia	4	Tsukamurella
Dalmatia	2	Veillonella	Dalmatia	4	Staphylococcus
Dalmatia	2	Mycobacterium	Dalmatia	4	Pseudomonas
Dalmatia	2	Sphingobacterium	Dalmatia	4	Sphingomonas
Dalmatia	2	Tepidimonas	Dalmatia	4	Burkholderia-Caballeronia-Paraburkholderia
Dalmatia	3	Staphylococcus	Dalmatia	4	Acinetobacter
Dalmatia	3	Pseudomonas	Dalmatia	4	Anaerobacillus

Dalmatia	3	Acinetobacter	Dalmatia	4	Bacillus
Dalmatia	3	Veillonella	Dalmatia	4	Stenotrophomonas
Dalmatia	3	Stenotrophomonas	Dalmatia	4	Cutibacterium
Dalmatia	3	Anaerobacillus	Dalmatia	4	Wolbachia
Dalmatia	3	Tepidimonas	Dalmatia	4	Craurococcus-Caldovatus
Dalmatia	3	Burkholderia-Caballeronia-Paraburkholderia	Dalmatia	4	Methylobacterium-Methylorubrum
Dalmatia	3	Craurococcus-Caldovatus	Dalmatia	4	Corynebacterium
Dalmatia	3	Cutibacterium	Dalmatia	4	Veillonella
Dalmatia	3	Sphingomonas	Dalmatia	4	Mycobacterium
Dalmatia	3	Corynebacterium	Dalmatia	4	Sphingobacterium
Dalmatia	4	Tepidimonas	Dalmatia	5	Bacillus
Dalmatia	4	Blastococcus	Dalmatia	5	Methylobacterium-Methylorubrum
Dalmatia	5	Pseudomonas	Dalmatia	5	Tsukamurella
Dalmatia	5	Sphingomonas	Dalmatia	5	Wolbachia
Dalmatia	5	Acinetobacter	Dalmatia	5	Craurococcus-Caldovatus
Dalmatia	5	Staphylococcus	Dalmatia	5	Rickettsia
Dalmatia	5	Stenotrophomonas	Dalmatia	5	Corynebacterium
Dalmatia	5	Anaerobacillus	Dalmatia	5	Veillonella
Dalmatia	5	Burkholderia-Caballeronia-Paraburkholderia	Dalmatia	5	Mycobacterium
Dalmatia	5	Tepidimonas	Dalmatia	5	Sphingobacterium
Dalmatia	5	Cutibacterium	Dalmatia	5	Blastococcus

Conclusions

The battle against pest insects that harm crops has long been a matter of economic and social significance. The rise of globalization and climate change has amplified the threats posed by foreign agricultural pests, primarily because their invasion disrupts agricultural and ecosystem equilibrium. In contrast, native insects that pose harm to agriculture have well-established ecological interactions that have been extensively studied over time, and their biology has also been thoroughly investigated. A comprehensive understanding of these two foundational aspects reveals crucial implications that can be leveraged or mitigated for the benefit of farmers. Conversely, the emergence of invasive agricultural pests engenders various consequences for agriculture, primarily due to the lack of effective countermeasures. These consequences encompass ecological interactions that impede the activity of these species, such as the presence of natural enemies like predators, parasitoids, bacteria and viruses, as well as the capacity of plants to defend themselves, resulting in significant economic losses. Furthermore, these aspects exert a broader ecological impact, as the establishment of invasive species often disrupts ecological chains, potentially leading to the extinction of native species. Hence, conducting thorough studies on the biological and ecological aspects of invasive pests is of utmost importance, enabling us to comprehend the distinctive features of the species and strategize how to manage them.

Depending on the invasive species and the type of threat that an insect pest poses to agriculture, there are several profound aspects that need to be characterized regarding the insect's basic biology. These include understanding the insect's life cycle in the invaded country and investigating ecological interactions that may benefit the plants affected by the damage. Such interactions can involve agents that have a detrimental impact on the invasive insect's life cycle or its interaction with the host plant. Nevertheless, there are other crucial aspects that are often

overlooked in the field of agricultural entomology, despite their potential to provide valuable insights into combating invasive insect pests. This work explores three approaches that address these overlooked aspects.

Studying the physiology of pest insects can provide valuable insights into their adaptability and flexibility. Specifically, investigating how these insects manage energy and water can yield interesting information. From an agricultural perspective, these aspects are crucial for predicting the risk of invasion in neighboring regions and countries. They also help understand the insect's preferences during different seasons and thus fit the management strategy as response. This is particularly important in mountainous areas, where insects adapted to temperate or cold climates, like *Drosophila suzukii*, may exhibit varying temperature, humidity, and food preferences at different altitudes throughout the year. Through the study on the metabolic rate (MR) and water loss rate (WLR) of *D. suzukii* at different altitudes shown in this thesis, valuable insights were gained. It was observed that the plasticity of this species did not impact energy management, as specimens captured from summer to autumn at various altitudes exhibited unchanged MR. However, water management appeared to be influenced by altitude, with insects at higher altitudes losing less water, indicating greater resistance to dehydration and desiccation. It is important to note that these differences were observed between insects from two different valleys, raising the possibility of adaptation within distinct populations. To validate this hypothesis, future research on population genetics would be necessary. Additionally, considering that the results were based on observations from a single year, repeating this analysis over subsequent years and that also consider winter morphs specimens would help to confirm or reject the identified differences and similarities, further validating the hypothesis that water management is influenced by altitude, whereas MR remains unchanged. Overall, this study demonstrates how the use of a physiological technique rarely employed on agricultural pests, such as flow-through respirometry, can provide

intriguing insights into their biology. Such knowledge is essential for effectively managing these species, as it suggests the presence of location and time-dependent differences that could require tailored control strategies. Furthermore, this study can serve as a foundation for future research on other aspects, such as population genetics, which could uncover additional crucial information for pest management.

Molecular studies are rapidly increasing due to advancements in laboratory techniques and bioinformatic approaches for analysis. However, there are still unresolved aspects for many species, despite extensive research. One such aspect is the study of microbiota, which is of utmost importance in understanding pest species. Characterizing the bacteria interacting with a species provides valuable information about the risks associated with the pest and guides further studies on the host's dependence on symbiotic microorganisms. This study focused on the microbiota of *Halyomorpha halys*, despite the abundance of available information, remains poorly understood. Although this pest has been studied since the 1990s, there has been a lack of characterization of microbiota changes during overwintering. The analysis conducted in this thesis revealed that overwintering does not significantly alter *H. halys* microbiota, suggesting a high dependence of this pest on its bacterial symbionts. Furthermore, essential information was provided for the management of the species using the SIT, for which it was recommended the use of overwintering specimens free from potential plant pathogens or harmful microbes that could impact crops and the ecosystem. The analysis performed in this study indicates that sacrificing the already limited number of individuals that survive overwintering and serve as good candidates for SIT is not necessary to characterize the presence of pathogens. Instead, population analysis before overwintering can provide valuable insights on this aspect. It is important to note that the technique used in this analysis, sequencing of the V3-V4 region of 16S rRNA, has limitations, particularly in terms of taxonomic resolution. While this approach allows for reliable identification of bacterial species at the genus level, it only permits

speculation in cases where only certain species within a genus are harmful to agriculture (*e.g.*, *Pseudomonas* spp.). Therefore, it would be appropriate to complement the study by employing metagenomic analysis on the individuals to characterize the specific bacterial species (including pathogens) which are present.

The use of phylogeny in agricultural entomology studies is widespread and, in conjunction with population genetics and morphological studies, provides crucial information on the origin of invasive pests. In contrast, molecular clocking is a rarely employed technique in agricultural entomology due to its unfamiliarity and complexity compared to traditional molecular phylogeny. However, molecular clock analysis yields insights into the evolutionary history of pests and is particularly valuable for speculating on their adaptation, especially during the early stages of invasion, thereby guiding future studies. An example of the application of molecular clocking is seen in the case of *Arboridia dalmatina*, first observed in Italy (Puglia region) in 2020. Limited knowledge existed about this species, but the molecular clock analysis revealed information regarding its speciation, likely occurring during the glaciation period. This information allows for speculations on the pest's adaptation, climatic preferences, and territorial behavior, thereby assessing its invasiveness. These findings provide valuable insights for future research on the species' adaptation, enabling the prediction of adjacent areas susceptible to invasion. Despite the importance of the technique and the robustness of results given buy absence of saturation and high statistical supports, the presented study focused on a single mitochondrial gene (COI), which represents a significant limitation. Using only one mitochondrial gene poses the risk of not capturing incomplete lineage sorting and/or introgressions. Furthermore, mitochondrial genes often evolve at different rates compared to nuclear genes. Therefore, it should be necessary to use multiple nuclear and mitochondrial genes to enhance the reliability and consistency of the results.

In conclusion, despite the limitation of each analysis, this study demonstrates the importance of exploring understudied areas and employing underutilized techniques in agricultural entomology to obtain critical information for invasive species control. Embracing new approaches is essential for facilitating exponential scientific progress, optimizing problem-solving time. Specifically in the field of agricultural entomology, new approaches would enhance our understanding of pests and help develop improved control strategies that are environmentally friendly, highly specific, minimize the impact on non-target species, and promote sustainability.

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