



UNIVERSITÀ DEGLI STUDI DI TORINO

SCUOLA DI DOTTORATO



DOTTORATO IN
SCIENZE AGRARIE, FORESTALI E ALIMENTARI

CICLO: XXXVII

Advancements in symbiotic control of stink bug pests of
hazelnut from the local to the global scale

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YEARS

2021-2022; 2022-2023; 2023-2024

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Chapter I – Introduction

1.1 Hazelnut: global cultivation and general threats

The hazelnut tree (*Corylus avellana* L.) is a deciduous shrub, monoecious and dichogamous, whose fruits are widely used in food industry and appreciated for their nutritive properties (Guiné and Correia, 2020; Hicks, 2022; Zhao et al., 2023). Hazelnut kernels contain around 60% of fat, rich in monounsaturated fatty acids, and phenolic compounds with antioxidant activity such as Vitamin E (Alasalvar et al., 2003; Ferrão et al., 2021). Globally, 1.06 million ha are planted with hazelnut groves and the world's hazelnut production reached 1.20 million tons in 2022 (<http://www.faostat.org>) (last accessed 11/04/2024). Turkish yield covers 64.0% of the global production (Fig. 1a) and 70.1% of harvested area (Fig. 1b). Italy is the second producer (Fideghelli and De Salvador, 2008; Zinnanti et al., 2019; Nera et al., 2020), with 8.3% of the total production (Fig. 1a) and 8.0% of surface (Fig. 1b). Both Azerbaijan and USA produce around 6.0% of total hazelnuts (Fig. 1a), but USA shows the highest productivity per hectare (Fig. 1c) since it covers 2.6% of global area while Azerbaijan the 4.9% (Fig. 1b) (www.faostat.org). Hazelnut cultivation is still increasing worldwide, especially in the Southern Hemisphere with new plantations in Chile, South Africa and Australia (Ellena et al., 2012; Ascari et al., 2017; Silvestri et al., 2021; Illán et al., 2022).

Despite being so widespread, hazelnut cultivation must face several threats (e.g., pests and diseases). Among the most threatening bacterial pathogens, particularly dangerous are *Xanthomonas arboricola* pv. *corylina* (formerly *Xanthomonas campestris* pv. *corylina*; Vauterin et al., 1995), the causing agent of bacterial blight, and *Pseudomonas syringae* pv.

144 *avellanae*, the causing agent of bacterial canker (Lamichhane and Varvaro,
145 2014; Jamwal et al., 2022; Nicoletti et al., 2022). Also fungal pathogens
146 can cause severe diseases. Canker causing agents such as *Cytospora*
147 *corylicola* (Martino et al., 2024), the anthracnose causing agent *Piggotia*
148 *coryli* (Drais et al., 2023), powdery mildew agents of genus *Erysiphe*
149 (Bradshaw et al., 2021), and root collar rot agents like *Ganoderma*
150 *adspersum* (Türkkan et al., 2024) are among the most dangerous
151 pathogens.

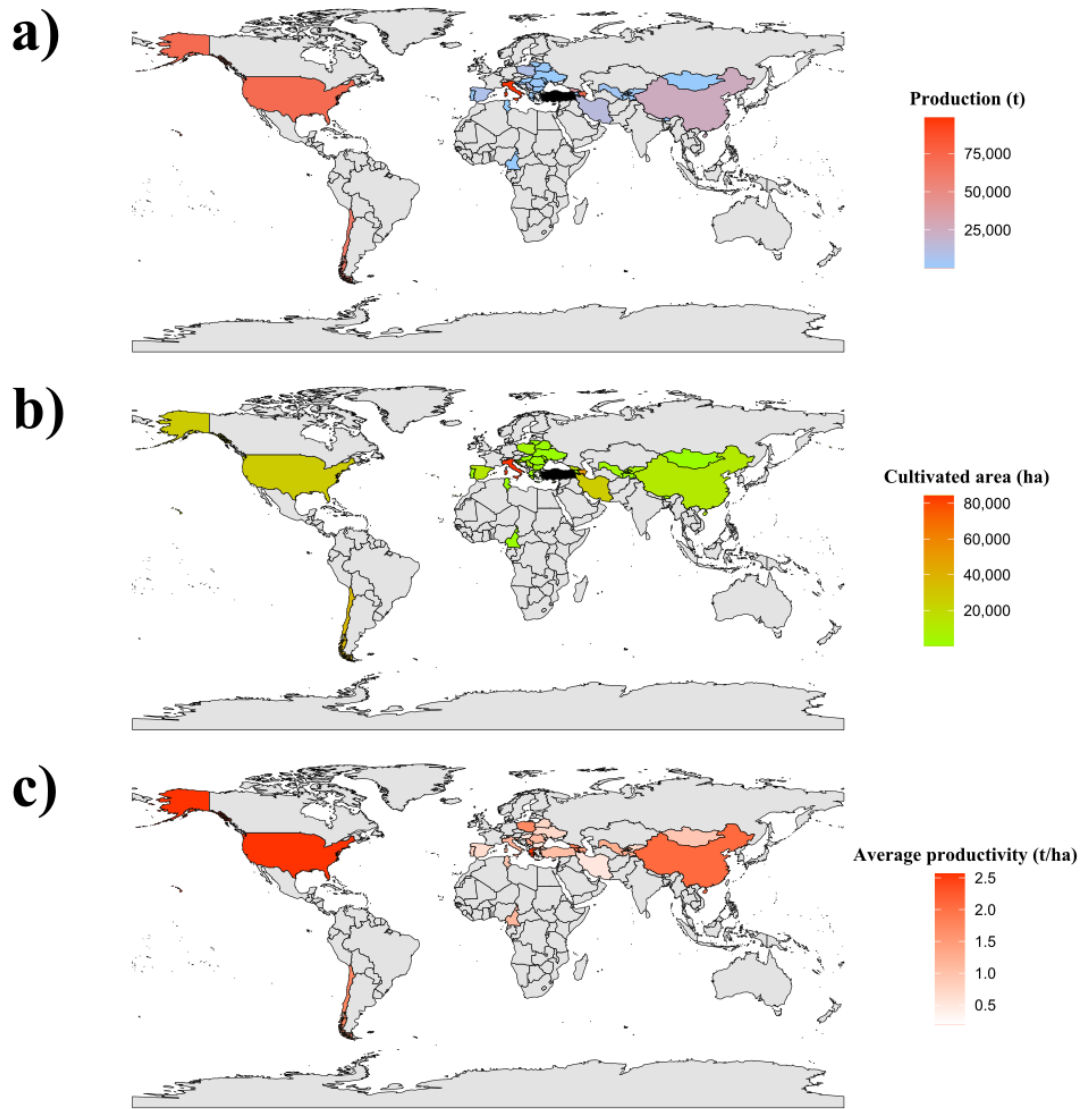


Figure 1. Worldwide map of hazelnut cultivation in 2022. **a** Total national production, Turkey is shown in black due to the high production compared to other countries (765000 t). **b** Total cultivated area, Turkey is shown in black due to the high extension of hazelnut orchards compared to other countries (744047 ha). **c** Average national productivity, calculated as the ratio between hazelnut production and cultivated area. Data retrieved from FAOSTAT database.

1.2 Stink bugs of hazelnuts and “cimiciato” damage

Among insect pests, stink bugs are particularly dangerous since they pierce directly the fruits for feeding, resulting in economic losses (Bosco et al., 2018; Hamidi et al., 2022). Damage entity depends on the developmental stage of the pierced kernels. Stink bug feeding activity in the early stage of fruit development arrests kernel development resulting in either shrivelled fruits or abortion. Feeding during kernel expansion or maturation stages results in “cimiciato” damage, characterized by a corky texture of the areas around the punctures. Besides directly damaging the kernels, stink bug piercing may facilitate the entrance of fungal pathogens resulting in moldy or rotten kernels (Arzone et al., 2000; Hedstrom et al., 2014; Bosco et al., 2018; Hamidi et al., 2022; De Benedetta et al., 2023; Hassan and Sharef, 2023). The “cimiciato” damage changes drastically the taste of hazelnuts with the production of bitter compounds, such as asadanin, and astringent compounds, making the product unsuitable for food transformation industry (Singldinger et al., 2017, 2018). Average “cimiciato” damage varies among years and cultivars in the most productive areas, in Turkey it ranged from 3.16% to 20% between 2014 and 2016 (Saruhan et al., 2023), while trials in Southern Italy showed around 40% of damaged hazelnuts in San Giovanni and 23% in Tonda Romana cultivars in 2022 in plots where no insecticides were sprayed (De Benedetta et al., 2023). In Piedmont (North-Western Italy) Bosco et al. (2018) reported “cimiciato” levels varying from a minimum of 7.8% in 2015 to a maximum of 19.4% in 2016 in Tonda Gentile delle Langhe cultivar, while in Georgia high differences were reported in Tonda di Giffoni (1.4% in 2015, 26.6% in 2016) and Anakliuri (4.3% in 2015, 51.3% in 2016) cultivars in two consecutive years. Several stink bug

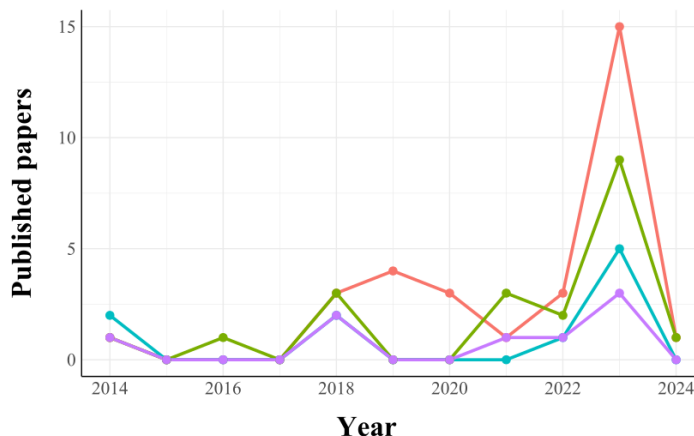
species, native to the major hazelnut-growing countries, are well documented pests for hazelnuts, such as *Palomena prasina* (L.) (Hemiptera: Pentatomidae), *Gonocerus acuteangulatus* (Goeze) (Hemiptera: Coreidae) and *Nezara viridula* (L.) (Hemiptera: Pentatomidae) (Tavella et al., 1996; Bosco et al., 2018; Aymamí et al., 2022; Hamidi et al., 2022; De Benedetta et al., 2023; Driss et al., 2024).

1.3 *Halyomorpha halys*, damage and research interest

Recently, research on stink bug pests showed increasing interest to an invasive species native to Asia, namely the brown marmorated stink bug *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae). After its establishment in most of the major hazelnut-growing countries, the studies regarding this species clearly surpassed those on native pests (Fig 2). The great attention paid on *H. halys* arises from its extremely broad host range (Leskey et al., 2012; Lee et al., 2013; Haye et al., 2015b; Zobel et al., 2016). In both native and invaded regions *H. halys* outbreaks can severely impact apple, peach and pear productions (Leskey et al., 2012; Lee et al., 2013; Bariselli et al., 2016). In Europe economic losses have been reported also on plums, persimmons, peaches, kiwifruit, grapevine, peppers and soybean (Bariselli et al., 2016; Bernardinelli et al., 2017; Vetek and Koranyi, 2017; Andreadis et al., 2018; Scaccini et al., 2024; Rolando et al., 2025). Recently Damos et al. (2019) highlighted the risk for olive production in Greece. As previously mentioned for other species, the damage can be either direct, so imputable to feeding activity, or indirect, so related the development of other pathogens after feeding (Rice et al., 2014; Vetek and Koranyi, 2017; Bosco et al., 2018; Di Serio et al., 2024; Scaccini et al., 2024). Hazelnuts are particularly threatened by *H. halys* in the invaded areas where it already is or is expected to become the main

213 stink bug pest (Bosco et al., 2018; Murvanidze et al., 2018; Ak et al., 2019;
 214 De Benedetta et al., 2023; Demir, 2024).

215



Species ◆ *H. halys* ◆ *P. prasina* ◆ *G. acuteangulatus* ◆ *N. viridula*

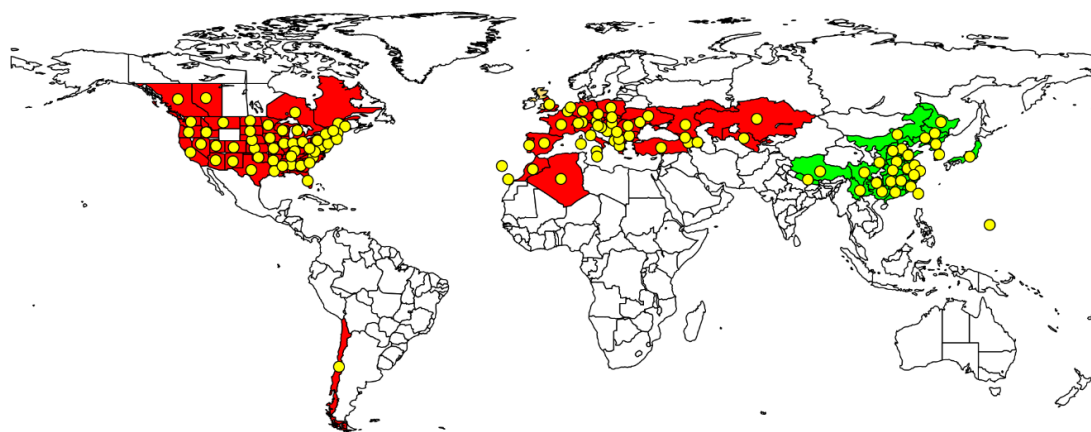
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217 **Figure 2.** Yearly published papers for *Halyomorpha halys*, *Palomena prasina*,
 218 *Gonocerus acuteangulatus* and *Nezara viridula* related to hazelnut cultivation in
 219 the range 2014 – 2024 according to Scopus database. Research queries used on
 220 Scopus: ("Halyomorpha halys" OR "brown marmorated stink bug" OR "BMSB")
 221 AND ("hazelnut" OR "Corylus"); ("Gonocerus acuteangulatus" OR "Gonocerus"
 222 OR "box bug") AND ("hazelnut" OR "Corylus"); ("Palomena prasina" OR
 223 "Palomena" OR "green shield bug") AND ("hazelnut" OR "Corylus"); ("Nezara
 224 viridula" OR "Nezara" OR "southern green stink bug") AND ("hazelnut" OR
 225 "Corylus").

226

1.4 *Halyomorpha halys*, distribution and invasion pattern reconstruction

Halyomorpha halys is native to Eastern China, Korea and Japan (Hoebeke and Carter, 2003; Lee et al., 2013; Xu et al., 2014; Leskey and Nielsen, 2018) and displays an exceptional adaptability to new environments (Reznik et al., 2023; Tortorici et al., 2023). The first description outside its native range dates back to 1996 in the United States of America (USA) in Pennsylvania (Hoebeke and Carter, 2003), now it has been detected in all the States within USA except for Wyoming and South Dakota (Morey et al., 2022), with the Mid-Atlantic and the Eastern Coast regions predicted to be the most favourable areas (Illán et al., 2022). In Europe it was intercepted in Liechtenstein in 2004 (Arnold, 2009), but clearly identified for the first time in Swiss canton of Zurich in 2007 (Wermelinger et al., 2007). From that moment on, *H. halys* steadily spread throughout Europe, being detected in all the major hazelnut producing Countries, such as Italy and France in 2012 (Callot and Brua, 2013; Maistrello et al., 2014), Serbia in 2015 (Šeat, 2015), Spain in 2016 (Dioli et al., 2016) and more recently in Poland (Claerebout et al., 2018). A comprehensive list of first detections in Europe can be found in Table 1. Invasion in Turkey is more recent than other hazelnut producing countries, the first detections probably date back to 2016 but the first confirmation was in 2019 (Çerçi and Koçak, 2017; Güncan and Gümüş, 2019). In Georgia, the 6th world producer, it was detected in 2016 (Gapon, 2016). In Azerbaijan, world's third producer, it was firstly detected in 2017 (Gariepy et al., 2021). A representation of current distribution with the regions in which *H. halys* has been detected can be found in Fig. 3.



● Present
 ■ Invaded area
 ■ Native area
 (c) EPPO <https://gd.eppo.int>

Figure 3. *Halyomorpha halys* global distribution. Native and invaded regions in which the insect was detected are coloured. Modified from EPPO Global Database (<https://gd.eppo.int/> last access 2024/11/11)

Table 1. Timeline of first detection of *Halyomorpha halys* in European Countries.

Year	Country	Reference
2004	Liechtenstein	(Arnold, 2009)
2007	Switzerland	(Wermelinger et al., 2007)
2011	Germany	(Heckmann, 2012)
	Greece	(Milonas and Partsinevelos, 2014)
	Belgium	(Claerebout et al., 2018)
2012	Italy	(Maistrello et al., 2014)
	France	(Callot and Brua, 2013)
2014	Hungary	(Vétek et al., 2014)
2015	Romania	(Macavei et al., 2015)
	Austria	(Rabitsch and Friebe, 2015)
	Serbia	(Šeat, 2015)

2016	Spain (mainland)	(Dioli et al., 2016)
	Bulgaria	(Simov, 2016)
	Ukraine	(Uzhevskaya, 2017)
2017	Croatia	(Šapina and Jelaska, 2018)
	Slovakia	(Hemala and Kment, 2017)
	Slovenia	(Rot et al., 2018)
2018	Albania	(Claerebout et al., 2018)
	Bosnia and Herzegovina	(Zovko et al., 2019)
	Poland	(Claerebout et al., 2018)
	Czech Republic	(Kment and Březíková, 2018)
	Netherlands	(Aukema et al., 2019)
	United Kingdom	(Powell et al., 2021)
	Portugal (mainland)	(Grosso-Silva et al., 2020)
2019	Moldova	(Derjanschi and Chimişliu, 2019)
	North Macedonia	(Konjevic, 2020)
2021	Portugal (Madeira)	(Petrovan et al., 2022)
2022	Spain (Canary)	(Gaspar et al., 2023)

259

260 Preventing *H. halys* invasions is therefore important for the safeguard of
261 agricultural economy in uninvaded areas. For instance, much attention is
262 paid in controlling imported goods in Australia and New Zealand, both
263 predicted as suitable regions for *H. halys* establishment (Kriticos et al.,
264 2017; Horwood et al., 2019). To better prevent outspreads, locating the
265 source of adventive individuals is crucial (Xu et al., 2014; Gariepy et al.,
266 2015; Haye et al., 2015b). On this regard, molecular analyses proved to be
267 a powerful tool for invasion pathway reconstruction, specifically the
268 haplotype identification of target regions. Most of the works dealt with
269 amplifying and sequencing the portion of COI mitochondrial gene

described by Folmer et al. (1994) (Xu et al., 2014; Gariepy et al., 2014; Cesari et al., 2015; Gariepy et al., 2015; Lee et al., 2018; Martinez-Sañudo et al., 2020; Schuler et al., 2021; Gariepy et al., 2021; Gaspar et al., 2023). The use of COI takes advantage of the high number of sequences in databases such as NCBI or BOLD but has the drawback of not being variable enough to clearly unravel the movement of such a widely distributed insect (Gariepy et al., 2021; Horwood et al., 2019; Yan et al., 2021a). To increase the informativity of their analysis, several authors followed an MLST approach by haplotyping COII mitochondrial gene along with COI (Lee et al., 2018; Yan et al., 2021a; Berteloot et al., 2024). More informative approaches taking advantage of NGS sequencing techniques, such as ddRAD sequencing, have been proposed (Yan et al., 2021b), while few studies dealt with the use of nuclear markers (Zhu et al., 2016; Kapantaidaki et al., 2019). A further and yet poorly exploited possibility is the use of *H. halys* primary endosymbiont ‘*Candidatus Pantoea carbekii*’ as an additional source of molecular markers (Otero-Bravo and Sabree, 2018; Martinez-Sañudo et al., 2020; Laterza et al., 2023).

1.5 *Halyomorpha halys* biological cycle

Halyomorpha halys voltinism depends on the photoperiod and thermal conditions, and normally it is either univoltine in colder regions, such as in Pennsylvania, Switzerland or in Northern and Western Japan (Nielsen et al., 2008; Nielsen and Hamilton, 2009; Lee et al., 2013; Haye et al., 2014), or bivoltine in warmer areas such as in Southern China, Italy, Slovenia and California (Haye et al., 2014; Costi et al., 2017; Rot et al., 2022). *H. halys* overwinters in the adult stage and starts its dispersal when daily maximum temperature exceeds 10 °C (Lee et al., 2013; Costi et al.,

2017). Ovarian development depends on mean temperature, diet and photoperiod, with at least 14 h of light required and temperatures higher than 16.3 °C (Lee et al., 2013). In Northern Italy in 2015 the oviposition of overwintering adults ranged from late May to mid August, matching what has been described in China and Japan (Lee et al., 2013; Costi et al., 2017), while the eggs of second generation were laid from the end of July to the beginning of September (Costi et al., 2017).

Eggs are laid in clusters of generally 28 eggs on the abaxial side of leaves (Rice et al., 2014) and hatch after five or six days (Taylor et al., 2014). In laboratory conditions, thermal range for egg hatching spanned from 15 °C to 30 °C with 22 and 3 days required for hatching, respectively (Nielsen et al., 2008). *H. halys* develops through a total of five nymphal stages before reaching the adult stage (Nielsen et al., 2016). The first nymphal stage feeds on the egg masses to acquire both nourishment and gut symbionts (Lee et al., 2013; Taylor et al., 2014; Gonella et al., 2020). After approximately five days the insects molt into second instar and move away from the egg masses to feed on plants (Rice et al., 2014). Subsequent molts, from second up to fifth instar, occur at intervals of around seven or eight days (Rice et al., 2014), while two weeks are required before molting from the fifth instar to the adult stage at 25 °C (Medal et al., 2013). Required temperatures for nymphal development span from a minimum of 14 to a maximum of 35 °C (Nielsen et al., 2008), with the exposition of fifth instar to short photoperiod and low temperature resulting in diapausing adults (Niva and Takeda, 2003). Adults migration to overwintering shelters depends on temperature and photoperiod, but generally migration peaks occur from September to mid-October (Cullum et al., 2020).

324 **1.6 *Halyomorpha halys* gut microbiota**

325 Despite a generally lower complexity compared to what described in
 326 mammals (Douglas, 2011; Engel and Moran, 2013), insects gut microbiota
 327 importance is well documented and spans from nutritional functions, such
 328 as the digestion of complex substrates (Ni and Tokuda, 2013; Handique et
 329 al., 2017) and even plastic polymers (Brandon et al., 2018), to defence
 330 against pathogenic microorganisms (Kaltenpoth and Engl, 2014; Koch and
 331 Schmid-Hempel, 2011) or even modulation of host fecundity (Zytynska et
 332 al., 2021). Gut symbionts can either be facultative (also referred as
 333 secondary), so beneficial but not essential for host life and generally
 334 cultivable (Pontes and Dale, 2006; Guo et al., 2017; Zytynska et al., 2021),
 335 or obligate (also referred as primary), thus essential for host life, incapable
 336 of free-living and often so much coevolved with the host to bear reduced
 337 size genomes compared to their relatives (Shigenobu et al., 2000;
 338 Baumann, 2005; Moran et al., 2008; Douglas, 2013).

339 Also *H. halys* gut microbiota shows a relatively low complexity, in the
 340 crypts of the terminal part of the midgut, named V4 region, it hosts almost
 341 only the primary endosymbiont '*Candidatus Pantoea carbekii*' (Bansal et
 342 al., 2014). A recent and comprehensive analysis of whole gut microbiome
 343 composition based on 16s rRNA sequencing showed the prevalence of
 344 *Pantoea* genus, followed by *Commensalibacter* (Fluch et al., 2024). In
 345 species with an imbalanced diet, gut microorganisms are fundamental to
 346 synthesize lacking amino acids and vitamins (Bennett and Moran, 2015;
 347 Kenyon et al., 2015). Sequencing of the *P. carbekii* genome showed intact
 348 pathways for the biosynthesis of all the 20 amino acids and many vitamins
 349 fundamental for host development (Taylor et al., 2014; Kenyon et al.,
 350 2015). Moreover, like other primary endosymbionts, *P. carbekii* has a

reduced size genome (van Ham et al., 2003; Nakabachi et al., 2006; Chong et al., 2019) of just 1.2 Mb (Kenyon et al., 2015).

In the Pentatomidae family, primary endosymbionts are transmitted from mothers to the nymphs without transovarial transfer. Transmission is achieved through the smearing of secretions containing bacteria on the egg chorion by adult females and newly hatched nymphs feed on the egg masses to acquire the primary endosymbionts (Prado et al., 2006; Tada et al., 2011; Bansal et al., 2014; Gonella et al., 2020). This specific pathway deprives the endosymbionts of the protection given by the host during egg stage.

1.7 *Halyomorpha halys* control strategies

Given the threat that *H. halys* represents for agriculture and the very low efficacy displayed by natural enemies in invaded areas in controlling its population levels (Abram et al., 2017; Costi et al., 2019; Francati et al., 2021), developing effective pest management strategies is needed. Chemical control relies on broad spectrum insecticides (Lee et al., 2013; Kuhar and Kamminga, 2017) and practices to limit its environmental impact have been proposed, such as treating only perimetral areas, and attract-and-kill or trap-based treatments (Bosco et al., 2018; Morrison III et al., 2019; Leskey et al., 2020). Another possibility relies on preventing insects to reach the crop, either with exclusion nets (Candian et al., 2018, 2021; Fornasiero et al., 2023; Marshall and Beers, 2024) or repellent compounds (Bulgarini et al., 2021b).

One of the most interesting and environmentally sustainable practice to control exotic species is biological control (Hoddle and Van Driesche, 2009; Parra, 2014). Even though *H. halys* predators have been studied

377 (Pote and Nielsen, 2017; Bulgarini et al., 2021a), egg parasitoids are
 378 considered the most promising biological control agents (Lee, 2015; Costi
 379 et al., 2019; Moraglio et al., 2020). Generalist egg parasitoids naturally
 380 occurring in the invaded area, such as *Anastatus bifasciatus* (Geoffroy)
 381 (Hymenoptera: Eupelmidae) and *Ooencyrtus telenomicida* (Vassiliev)
 382 (Hymenoptera: Encyrtidae) in Europe (Haye et al., 2015a; Moraglio et al.,
 383 2020; Andreadis et al., 2021; Iacovone et al., 2022) and *Telenomus podisi*
 384 (Ashmead) (Hymenoptera: Scelionidae) or *Anastatus reduvii* (Howard)
 385 (Hymenoptera: Eupelmidae) (Herlihy et al., 2016; Jones et al., 2017) in the
 386 USA, proved to be able to exploit *H. halys* egg masses for their
 387 development, although not at the required levels to achieve control.
 388 However, coevolved egg parasitoids such as *Trissolcus japonicus*
 389 (Hymenoptera: Scelionidae) and *Trissolcus mitsukurii* (Hymenoptera:
 390 Scelionidae) have been reported to be more effective than the
 391 abovementioned parasitoid species (Zhang et al., 2017; Lara et al., 2019;
 392 Sabbatini-Peverieri et al., 2021; Zapponi et al., 2021; Tortorici et al.,
 393 2023). Augmentative biological control programs with *T. japonicus* have
 394 been recently conducted in Italy (Bittau et al., 2021; Costi et al., 2022).

395 Another promising control strategy, known as symbiotic control, takes
 396 advantage of the vertical transmission of *H. halys* primary endosymbiont.
 397 During host egg stage, *P. carbekii* is exposed to external stressors and can
 398 be targeted with bactericidal products (Gonella et al., 2019; Gonella and
 399 Alma, 2023). It has been shown that dysbiosis caused by preventing *P.*
 400 *carbekii* acquisition sensibly increases *H. halys* mortality within the young
 401 stages and reduces the fitness of surviving adults (Taylor et al., 2014;
 402 Gonella et al., 2020). On this regard, several compounds have been tested
 403 to control *H. halys* by means of symbiont-targeted methods, such as insect

growth regulators, surfactants, liquid copper and the more sustainable compost teas (Mathews and Barry, 2014; Taylor et al., 2017). Among these substances, a copper, zinc and citric acid based biocomplex suitable for organic farming stood out for the performances in laboratory conditions (Gonella et al., 2019, 2022). Not only this biocomplex resulted in a nymphal mortality above 90%, but also it did not impair *T. japonicus* parasitism in no choice and paired-choice tests (Orrù et al., 2023). This strategy is highly effective against *H. halys* and theoretically valid for other pentatomids due to their conserved symbiont transmission route; however, efficacy varies depending on how much the host relies on the endosymbiont for its development. As an example, under laboratory conditions mortality was lower in *N. viridula* than that observed in *H. halys* (Prieto et al., 2023).

1.8 Objectives and thesis structure

H. halys is a severe global agricultural threat for several crops and specifically for hazelnuts. The chemical control relies on broad spectrum insecticides, potentially harmful for the arthropods biodiversity in the fields (Lee et al., 2013; Kuhar and Kamminga, 2017; Bosco et al., 2018). Even though the use of insecticides can be limited by optimizing their applications (Morrison III et al., 2019; Leskey et al., 2020), the number of active substances that are authorized for field use is continuously reducing, hence there still is the need to develop sustainable alternatives. On this purpose symbiotic control and biological control are probably the most promising options (Gonella and Alma, 2023; Orrù et al., 2023; Tortorici et al., 2023). However, symbiont-targeted control efficacy needs to be evaluated also in open field conditions. Besides studying control strategies for this pest, preventing further invasions and pinpointing the source of

431 adventive individuals is necessary in still uninvaded or poorly invaded
432 areas. Due to the ongoing primary (= from native countries) and secondary
433 (= from already invaded countries) invasions, the use of only COI or both
434 COI and COII molecular markers have proved to be insufficient for
435 invasion pattern reconstructions (Horwood et al., 2019; Gariepy et al.,
436 2021). Exploiting the host-symbiont hologenome to design additional
437 markers may be helpful not only to better track invasion routes, but also to
438 identify possible genetic variants that are related with enhanced invasive
439 capability and are differently affected by symbiotic control. Moreover, a
440 first laboratory trial opened to the possibility of a joint use of symbiotic
441 and biological control (Orrù et al., 2023), but it is still unknown if the
442 treatments with the anti-symbiont biocomplex that is commonly used for
443 symbiont-targeted treatments can affect *T. japonicus* capability to localize
444 the egg masses when deprived of visual stimuli. Also, it is still not known
445 if the different *H. halys* and *P. carbekii* haplotypes have a biological
446 influence, such as displaying different attractivity for the parasitoids.

447 Chapter I (this chapter) introduces the global importance of hazelnut
448 cultivation and the threat that the brown marmorated stink bug represents
449 for this and many other crops, but also its broad distribution. Moreover,
450 the most promising control strategies are reported with a special focus on
451 the role of gut microbiota and how dysbiosis can be used for a sustainable
452 control strategy.

453 Chapter II (Dho et al., 2025) aims to demonstrate the feasibility and
454 effectiveness of symbiotic control strategy under typical hazelnut grove
455 conditions for one of the most important Italian areas for hazelnut
456 production, i.e. Piedmont region. The effect of egg masses position in the
457 field and on the canopy is considered to evaluate proper spraying and

nymphal mortality. The results show a significant mortality due to field treatment (although lower if compared to experimental evidence under laboratory conditions) and an effect of egg masses position on spraying level, moreover a significant reduction of “cimiciato” damage confirmed the efficacy of this strategy.

Chapter III deals with the identification of novel *H. halys* and *P. carbekii* molecular markers with the aim of developing a more informative MLST approach suitable for either population genetics or invasion routes reconstruction studies. Nuclear markers proved to be highly informative and not redundant with either mitochondrial or symbiotic genetic variability. *P. carbekii* was shown much more variable than what previously described. Moreover, a perfect specialization between host mitochondrial and symbiont haplotypes was unravelled for the first time in invasive populations, with some host-symbiont combinations showing a higher frequency, thus suggesting that some holobiont haplotypes could have enhanced invasive capability.

Chapter IV explores the effect of symbiont targeted treatments, egg masses age and *H. halys* and *P. carbekii* haplotypes on *T. japonicus* preference in olfactometer bioassays, conducted in collaboration with the Entomology and Nematology laboratories of the Plant Protection Strategic Division of Agroscope in Changins (Switzerland). Surprisingly, naïve parasitoid females displayed a preference for old egg masses compared to young ones. The treatments increased the attractivity of young egg masses compared to untreated ones. No effect of either host or symbiont haplotype was seen on *T. japonicus* preference. Overall, the results confirm no negative effect of symbiotic control treatment on biological control outcome.

485 Chapter V draws the conclusions of this thesis, opening to future research
486 perspectives. This study not only confirmed the efficacy of one of the most
487 promising control strategies, but also generally highlighted the possibility
488 of using insect-associated microorganisms as an additional source of
489 informativity or solutions in agricultural entomology.

Chapter II – Symbiotic control in field conditions

Published paper (Dho et al., 2025).

<https://doi.org/10.1016/j.cropro.2024.106952>

Field evaluation of symbiont-targeted control of *Halyomorpha halys* in hazelnut crop

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Abstract

Halyomorpha halys has emerged as one of the most damaging pests for hazelnut production. Due to the continuous reduction of available options for chemical control and the threat this strategy represents for the environment and non-target organisms such as BioControl Agents, alternative methods are required. A promising and sustainable alternative is the use of symbiont-targeted control. Treatment of the egg surface with a biocomplex containing copper, zinc, and citric acid has been shown to effectively prevent the acquisition of the endosymbiont ‘*Candidatus Pantoea carbekii*’ by *H. halys* nymphs, resulting in high nymph mortality under laboratory conditions. The aim of this work was to assess the field efficacy treatments targeting the *H. halys* symbiont in hazelnut orchards to optimize spray applications. Treatments with the anti-symbiont biocomplex were performed in an orchard infested with *H. halys* during 2022 and 2023. To assess product efficacy, *H. halys* egg masses were glued on cardboard tags and hung on different positions of plants canopy before the treatments. The wetting percentage of egg masses according to the

position of the egg on the plant canopy was measured; eggs were then reared to evaluate nymphal mortality after hatching. Symbiont acquisition was measured by quantitative PCR. A preliminary test to set the spraying conditions revealed that a higher fan speed induced higher mortality of the nymphs emerged from treated egg masses, although it did not affect the wetting rate of cardboards. In addition, cardboard tags hung in the central part of the canopy and those closer to the sprayer showed the highest egg coverage, whereas those in the peripheral row were less covered. However, nymphal mortality was not correlated with the percentage of cardboard tags coverage, although it was correlated with symbiont acquisition. A significant reduction in the percentage of damaged hazelnuts was observed in the treated plot compared to an untreated control, confirming the efficacy of symbiont-targeted control in hazelnut orchards.

Highlights

- Symbiont-targeted control was proven effective against *Halyomorpha halys* in laboratory
- Field validation was conducted in a hazelnut orchard
- Cardboard tag coverage and nymphal mortality were measured after anti-symbiont sprays
- Eggs in the central part of the plant canopy and those closer to the sprayer showed higher coverage
- Increased nymphal mortality and lower damage rates than untreated control were obtained

1. Introduction

Halyomorpha halys (Stål) (Hemiptera: Pentatomidae) is an invasive stink bug pest, well known for its broad host spectrum (Lee et al., 2013; Leskey and Nielsen, 2018; Morrison III et al., 2018) and native to eastern China, Korea and Japan (Xu et al., 2014). First reported outside its native range in Pennsylvania (Hoebeke and Carter, 2003), this insect is now widely distributed in North America (Abram et al., 2017; Leskey and Nielsen, 2018) and Europe (Cesari et al., 2018; Garipey et al., 2021). In the recent years, the distribution has continued to expand, with populations detected for the first time in South America (Chile) (Faúndez and Rider, 2017), North Africa (Morocco) (Nouere et al., 2020) and in Central Asia (Uzbekistan, Kazakhstan) (Temreshev et al., 2018; Gandjaeva et al., 2022), showing a high degree of adaptation to local environmental conditions (Reznik et al., 2023).

Crop damage can be a direct consequence of the piercing-sucking feeding behaviour of *H. halys* (Bariselli et al., 2016; Vetek and Koranyi, 2017; Bosco et al., 2018; Daher et al., 2023; Ozdemir et al., 2023; Di Serio et al., 2024), or indirect due to the development of pathogens after feeding (Mitchell, 2004; Rice et al., 2014; Opoku et al., 2019; Scaccini et al., 2024a). In native regions, occasional outbreaks are reported with yield losses in apple, pear and peach among other crops (Lee et al., 2013). In invaded areas, high economic losses have been reported in several crops over the years. In 2010, apple and peach production was severely affected in the Mid-Atlantic states of the USA (Leskey et al., 2012). In Italy, mainly pears, but also persimmons, tomatoes, peaches and plums, have been severely damaged (Bariselli et al., 2016). Damos et al. (2019) recorded up to 87% of damaged olives in Greece in 2018. Significant damage has also

566 been reported on kiwifruit, dry beans and green hot peppers in Europe
567 (Bernardinelli et al., 2017; Vetek and Koranyi, 2017; Andreadis et al.,
568 2018). Moreover, among the feeding hosts, hazelnuts are one of the most
569 threatened in highly productive regions such as Italy, Turkey or Georgia
570 (Bosco et al., 2018; Ak et al., 2019; de Benedetta et al., 2023). Damage
571 varies according to the phenological stage of hazelnut development.
572 Feeding before kernel expansion results in higher abortion rate, while
573 feeding soon after kernel expansion results in shrivelled kernels. Piercing
574 activity in late stages of kernel development results in corking damage
575 (Hedstrom et al., 2014).

576 Chemical control of *H. halys* relies mainly on broad-spectrum insecticides,
577 mostly belonging to the pyrethroid, neonicotinoid, carbamate and
578 organophosphate classes (Lee et al., 2013; Kuhar and Kamminga, 2017).
579 However, the better performing insecticides are not compatible with IPM
580 strategies, and most of them are currently not allowed in some of the
581 invaded areas (e.g., Europe). Furthermore, the tendency of *H. halys* adults
582 to fly away after disturbance makes open field treatments harmful for non-
583 target organisms and potentially less effective (Lee et al., 2013; Kuhar and
584 Kamminga, 2017). Several authors have suggested improvements to
585 reduce the environmental impact of chemical control, such as using
586 pheromones or attractive plants for attract-and-kill strategy or treating only
587 the perimeter plants of orchards (Lee et al., 2013; Blaauw et al., 2015;
588 Bosco et al., 2018; Kuhar and Kamminga, 2017). Sustainable alternatives
589 to chemical control include physical exclusion (Chouinard et al., 2016;
590 Candian et al., 2018, 2020, 2021; Fornasiero et al., 2023; Vergnani et al.,
591 2023; Marshall and Beers, 2024), application of repellents such as sulphur
592 (Scaccini et al., 2024b), treatments with plant extracts (Bulgarini et al.,

2021), and biological control with egg parasitoids introduced from the native region of *H. halys* (Haye et al., 2020; Konopka et al., 2017; Scaccini et al., 2020; Falagiarda et al., 2023; Tortorici et al., 2023). Another low-impact control strategy that is constantly gaining interest is symbiotic control by means of symbiont-targeted methods. This strategy is based on preventing the acquisition of the primary endosymbiont by newborn nymphs, resulting in death of newborn nymphs (Gonella and Alma, 2023). Indeed, the primary endosymbiont of *H. halys*, ‘*Candidatus* Pantoea carbekii’ (hereafter *P. carbekii*), is hosted in crypts of the midgut caeca and it is released on the egg surface during oviposition as part of a maternal secretion. Newly hatched nymphs acquire the endosymbiont by feeding on the eggs (Bansal et al., 2014; Kenyon et al., 2015). Preventing *P. carbekii* acquisition by *H. halys* nymphs results in high mortality rates in the first instars, while the few surviving insects suffer severe developmental impairment, reduced fertility and high mortality in the sibling generation (Taylor et al., 2014; Kenyon et al., 2015; Gonella et al., 2019). Several compounds have been tested for symbiont-targeted control of *H. halys* (Mathews and Barry, 2014; Taylor et al., 2017; Gonella et al., 2019, 2022), among which a micronutrient biocomplex containing copper, zinc and citric acid showed promising potential, as it completely prevented symbiont acquisition by newly hatched nymphs and resulted in high nymph mortality under laboratory conditions (Gonella et al., 2019, 2022).

Given the high potential of symbiont-targeted interventions, and since scaling up from the laboratory to the field can result in different outcomes (Gonella et al., 2020), the aim of this work is to evaluate the anti-symbiont efficacy of field applications using the micronutrient biocomplex that has previously shown high symbiont-targeted containment of *H. halys*. The

620 efficacy of spraying was evaluated on *H. halys* egg masses glued on
 621 cardboard tags with water-sensitive paper, placed on different positions,
 622 both in the field and on the plant. Our evaluation took into account: i) The
 623 colour variation of water sensitive paper to calculate the percentage of
 624 cardboard coverage, ii) the effect on nymphal mortality, iii) the effect on
 625 *P. carbekii* acquisition rates, and iv) the resulting percentage of damaged
 626 hazelnuts.

627 **2. Materials and Methods**

628 **2.1 Insect material, egg mass collection, and cardboard tags** 629 **preparations**

630 Laboratory rearings of *H. halys* were established in 2022 and 2023 from
 631 field-collected adults obtained via plant beating starting from late April to
 632 four days before the last treatment. Insects were sampled from *Prunus*
 633 *avium* L. and *Morus alba* L. until the second half of July, and from *Corylus*
 634 *avellana* L. and *Fraxinus excelsior* L. from the second half of July onward.
 635 Insects were reared in 93.0 x 47.5 x 47.5 cm net cages (BugDorm®,
 636 NHBS Ltd, Devon, UK) in climatic chambers at 25 ± 1 °C, 16:8 h L:D
 637 photoperiod. Adults were fed with *Vicia faba* L. and *Glycine max* (L.)
 638 Merr. plants, as well as apples, green beans, and hazelnuts. Egg masses
 639 were collected daily; only egg masses less than 4 days old were used for
 640 field treatments to avoid the risk of hatching before the end of the trials.

641 Each egg mass collected from the rearing was glued in the centre of a
 642 5.0 x 10.0 cm red cardboard tag either directly or, in case the egg mass was
 643 laid on a leaf, with a portion of the same leaf on which the egg mass was
 644 retrieved. The egg mass was surrounded by 4 pieces of 0.2 x 0.9 cm water-
 645 sensitive paper, which were glued together to form a frame. On the
 646 diagonal of each cardboard tag, another 2 pieces of 1.1 x 0.9 cm water-

sensitive paper were glued. The top of the tag was reinforced with duct tape and a hole was made to insert a string of iron wire to attach it to the hazelnut plant (Fig. S1b).

2.2 Symbiont-targeted field treatments

Field treatments were performed in the summers of 2022 and 2023 in a commercial hazelnut orchard, using a commercial biocomplex (Dentamet®, Diachem SpA, Italy) whose efficacy against the *H. halys* symbiont was previously demonstrated (Gonella et al., 2019). The hazelnut orchard was located in Guarene (Cuneo Province, Italy, 44°44'03.9"N, 8°02'19.9"E). The site was selected based on monitoring data obtained the year before the start of trials, which showed high pest pressure (> 2 *H. halys* nymphs per plant; $>40\%$ kernel damage). The planting plan was 5 x 5 m, with a single-trunk training system. The orchard was divided into two equal plots of 2,400 m² each, one with symbiont-targeted treatments and the other untreated as control. The initial occurrence of *H. halys* in the orchard was monitored from the beginning of June in both years by plant beating technique and the presence of egg masses was checked by visual inspection of the plant canopy. Soon after observing the onset of *H. halys* oviposition in the orchard, the anti-symbiont treatments were started in the treated plot. All the treatments were performed in the morning. The first application was made on June 27th, 2022 and June 8th, 2023, from which treatments were performed approximatively every 10 days in 2022 (four treatments) and every 15 days in 2023 (three treatments), until late July. Each treatment was applied with an Athos trailed round sprayer with a tank capacity of 1500 L (Dragone, Italy). Each application was made with 1000 L/ha of 0.4% v/v Dentamet®; driving speed during spraying was 4.7 km/h. A preliminary

674 trial was conducted within the first two applications in 2022 to determine
675 the most suitable operating conditions for the treatments. Specifically, two
676 airflow velocities were used by setting the fan speed to 1890 rpm and 2700
677 rpm, respectively. All subsequent treatments were conducted at 2700 rpm.
678 No additional insecticide treatments were applied in the orchard.

679 Immediately before each treatment, cardboard tags were attached to seven
680 plants; six plants were in two adjacent rows within the plot where the anti-
681 symbiont treatment was applied, whereas the last one was in a separate
682 row in the control plot. The first plant in the treated plot with the cardboard
683 tags was 15 m away from the untreated plot; the same distance was
684 maintained between the untreated plant with cardboard tags and the treated
685 plot. Three out of the six plants in the treated plot were selected in the first
686 row of the orchard, with one side of the canopy facing the orchard border,
687 and the remaining three were in the second row, completely embedded in
688 the field. Each plant had 10 cardboards on; cardboard tags were placed in
689 different positions: along the row, facing the inter-row spacing, and in the
690 centre of the plant (Fig. S1a). Each position was covered by 2 cardboard
691 tags at different heights, one at about 1.5 m and the other at about 3.0 m.
692 Thus, 70 tags were used for each experiment, 60 of which were treated and
693 10 were untreated controls. Cardboard tags were recollected 2 hours after
694 treatment (Fig S1c) and returned to the laboratory.

695 **2.3 Wetting percentage assessment**

696 Egg masses collected in the field after spray were cut from the cardboard
697 tags to assess the mortality induced in newborn nymphs. Cardboard tags
698 without egg masses were scanned in groups of six. The scan resolution was
699 600×600 DPI, with maximum sharpness. Each cardboard tag was then
700 separated from the others and the background was removed using the R

package magick version 2.8.3, to keep only the water-sensitive paper image. The resulting images were manually adjusted using the GIMP software to remove any residual background. Mask thresholds to identify yellow pixels in images with different average brightness were defined using ImageJ software (Schneider et al., 2012). Once the thresholds were defined, the wetting percentage of each cardboard tag was automatically calculated using the Python packages NumPy (Harris et al., 2020) and OpenCV (Bradski, 2000). Wetting percentage was calculated as: $\left(1 - \frac{N_y}{N_t}\right) * 100$, where N_y is the number of yellow pixels and N_t is the total number of pixels. Results were manually checked on randomly selected cardboard tags by re-running the analysis with the same mask threshold in ImageJ software and comparing the results before proceeding with further analyses.

2.4 Nymphal rearing and mortality assessment

After removal from the cardboard tags, the egg masses were reared into 9 mm diameter Petri dishes with filter paper at the bottom. Rearings were performed in a climatic chamber at 25 ± 1 °C, 16:8 h L:D photoperiod and green beans were provided as food. Egg hatching and nymphal mortality were checked daily. Egg masses were maintained for up to three days after the last individual molted to the second instar. All nymphs were collected and stored at -80 °C in RNAlater® for further RNA extraction, separating those that were dead from those that were alive at the end of the experiment.

2.5 Symbiont quantification in nymphs

The acquisition of living *P. carbekii* cells by dead and live nymphs coming from treated and control egg masses was quantified. Absolute quantification was performed by RNA extraction, retrotranscription and

quantitative Real-time PCR (qPCR) as described by Gonella et al. (2019), using 6 serial dilutions of an external standard. Briefly, total RNA was extracted from dead and live nymphs using the SV Total RNA Isolation System (Promega, WI, USA) and retrotranscription was performed using the Reverse Transcription System (Promega, WI, USA) according to the manufacturer's instructions. qPCR reactions were performed on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, CA, USA). The mix consisted of 12.5 µL of SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad), 2.5 µL of 3 mM PcarQF and PcarQR primers, 6.5 µL of sterile water, and 1 µL of cDNA. The thermal protocol consisted of an initial denaturation at 94 °C for 3 minutes, followed by 50 cycles of 94 °C for 15 seconds and 53 °C for 30 seconds, and final melting curve generation from 53 to 95 °C measuring fluorescence every 0.5 seconds. To check for the presence of false negative samples due to inefficient cDNA preparation, a second Real-Time PCR was performed using a portion of the housekeeping insect's 18S rRNA gene as a target, as described by Marzachi and Bosco (2005). Briefly, primer pair was MqFw and MqRv, the mix was prepared as described above, and the thermal protocol consisted of an initial denaturation at 94 °C for 3 minutes, then 36 cycles of 94 °C for 45 seconds and 65 °C for 1 minute, followed by melting curve generation from 53 to 95 °C measuring fluorescence every 0.5 seconds. Only samples showing amplification with the reference gene were used for symbiont quantification. The concentration of *P. carbekii* cells was calculated as the number of expressed copies of 16S rRNA gene per pg of insect 18S rRNA gene.

2.6 Evaluation of stink bug-related damage on hazelnuts

To estimate the impact of symbiont-targeted control on production damage, 300 harvested hazelnuts were randomly selected each year from both treated and untreated plots to measure the incidence of stink bug-related damage. Hazelnuts were collected at least after the third plant from the border between treated and untreated plots, with a minimum distance of 30 m between the closer collection sites. Hazelnuts were shelled in the laboratory and inspected for the presence of evident kernel alterations due to stink bug feeding, defined as “cimiciato”, resulting in darker color, tissue depression, and necrosis (Memoli et al., 2017). Kernels appearing undamaged after shelling were cut into 4 parts to check for internal symptoms. Finally, kernels were divided into two groups: those showing external or internal symptoms (cimiciato-damaged) and those showing no symptoms (healthy).

2.7 Statistical analysis

Statistical analyses were performed using R (version 4.2.3). Preliminary comparison between cardboard tags wetting percentage and nymphal mortality as a function of applied sprayer fan speed was performed using Mann-Whitney U test (R package stats). The effect of the position of cardboard tags in the field or on the canopy on the wetting percentage was evaluated using the generalized linear model (glm) function with quasibinomial distribution and logit link function, the means were then separated by Bonferroni test (R packages emmeans and effectsize). Mortality of nymphs hatched from treated and untreated egg masses was compared using Mann-Whitney U test (R package agricolae). Regression analysis to describe nymphal mortality in function of wetting percentage was performed using the generalized linear model (glm) function with

780 quasibinomial distribution and logit link function. The correlation between
 781 nymphal mortality at different wetting percentages and according to the
 782 position of the cardboard tag was analysed using glm with quasibinomial
 783 distribution and logit link function, means were then separated by
 784 Bonferroni test (R package emmeans). Differences in *P. carbekii*
 785 acquisition rate between dead and live nymphs in treated and untreated
 786 samples were evaluated by Mann-Whitney U test. Evaluation of damaged
 787 hazelnuts was performed as described by Bosco et al. (2018). In brief,
 788 treated plot was considered as experimental unit and glm with binomial
 789 distribution and logit link function was performed.

790 **3. Results**

791 **3.1 Effect of spraying conditions on treatment efficiency**

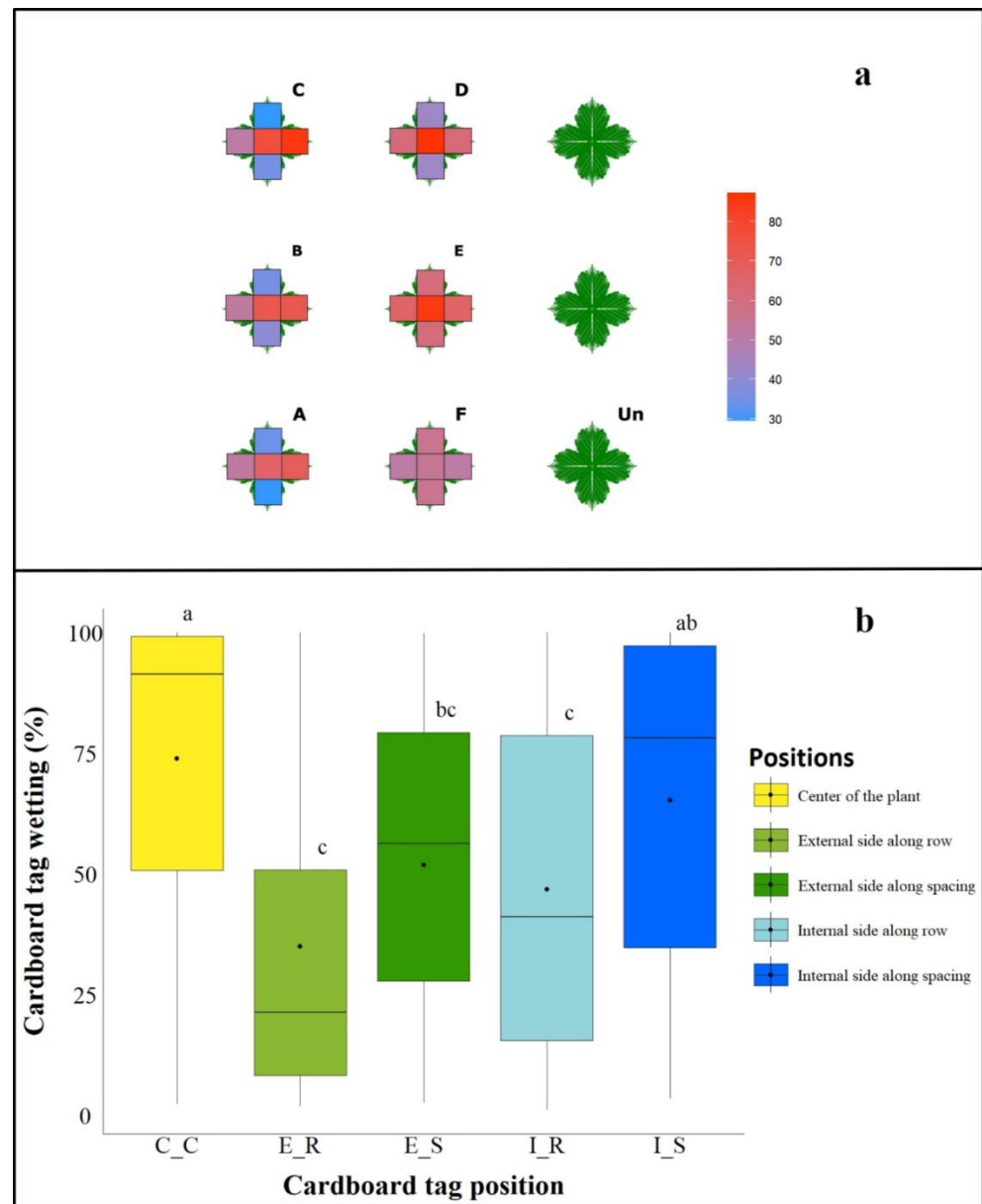
792 A preliminary observation was made to assess the influence of airflow
 793 speed during spraying on the efficacy of the anti-symbiont treatment, i.e.
 794 on the wetting percentage of cardboard tags as well as on the mortality
 795 induced on newborn nymphs from treated egg masses. Cardboard wetting
 796 percentage resulted not significantly different between low ($Mdn = 37.18$)
 797 and high fan speed ($Mdn = 38.04$), $W = 1778$, $p = 0.910$. Wetting
 798 percentage of water sensitive paper surrounding the egg masses was not
 799 significantly different between low ($Mdn = 23.4$) and high fan speed (Mdn
 800 $= 36.33$), $W = 49$, $p = 0.188$. Nymphal mortality showed significant
 801 difference between low ($Mdn = 0$) and high fan speed ($Mdn = 47.43$), $W =$
 802 65 , $p = 0.003$. Based on these results, the higher sprayer fan speed was
 803 selected for the subsequent treatments.

804 **3.2 Effect of cardboard tags position on wetting percentage**

805 The mean wetting percentage for cardboards placed in the treated rows
 806 was 57.02%. The model showed that cardboard tags wetting percentage was

significantly affected by the interaction between positions in the orchard (border or inner row) and in the canopy (centre of the plant canopy, along the row, facing the inter-row spacing) ($F = 0.390$, $\eta^2 = 0.13$, $df = 359$) (Fig. 1a). Since the lower nozzle of the sprayer sprayed under the first branches, the treatment easily reached the central part of the plants, both in the border and in the inner rows. For this reason, all the data generated from the cardboard tags in the central part of the plants were not divided into outer and inner rows. All the cardboard tags placed on the untreated control plant for all the treatments showed no change in the water-sensitive paper colour, confirming that the wetting percentages recorded were due to the treatments and not to environmental moisture.

Overall, the highest cardboard coverage was obtained for tags placed in the central part of the hazelnut canopy, followed by those facing the inter-row spacing in the inner row, with no significant difference between them ($p = 0.356$, $Z = 1.831$). Cardboards facing the inter-row spacing in the orchard border were sprayed with a similar coverage as those in the inner row, both facing the inter-row spacing ($p = 0.194$, $Z = -2.163$) or being along the row ($p = 0.973$, $Z = 0.616$), but they were significantly less wet than those in the central part of the plants ($p < 0.050$, $Z = 3.403$). The least covered cardboards were those in the border along the row, significantly less wet than those in the central part of the plant ($p < 0.050$, $Z = 5.557$) and those in the inner side facing the inter-row spacing ($p < 0.050$, $Z = -4.558$) (Fig. 1b). However, the wetting percentage records showed high variability among cardboards, especially those along the row, where the wetting percentage ranged from a minimum of 1.12% to a maximum of 99.99%.



833

834 **Figure 1: a** Heatmap of average wetting percentage of cardboard tags on treated
 835 plants. Mean values were calculated between all the treatments performed in 2022
 836 and 2023 with the sprayer fan speed set at 2700 r/min. A, B, C represent plants in
 837 the perimetral row, whereas D, E, F represent plants in an inner row. “Un”
 838 corresponds to the selected plant within an untreated row, where no change in

water sensitive paper color has been recorded. **b** Cardboard tags wetting percentage according to the position in field and on the canopy. Black dots represent the mean value. Internal lines represent the median value. Different letters indicate significantly different values according to glm with quasibinomial distribution and logit link function and means separation by Bonferroni test ($p < 0.05$).

3.3 Effect of cardboard wetting percentage on nymphal mortality

Nymphal mortality resulted significantly higher in treated egg masses ($Mdn = 33.33$) compared to untreated ones ($Mdn = 10.00$) ($W = 4018.5$, $p < 0.001$). However, although a slightly higher mortality rate was observed in nymphs hatched from egg masses with more than 30% wetness (Table 1), the regression analysis showed no significant effect of the cardboard wetness on nymphal mortality ($F = 0.083$, $\eta^2 < 0.01$, $p = 0.164$, $R^2 = 0.006$, $df = 283$). In confirmation, there was no correlation between nymphal mortality and the position of the cardboard tags ($F = 0.094$, $\eta^2 < 0.01$, $p > 0.050$ for each combination, $df = 284$) although a moderate increase in nymphal mortality was obtained for egg masses placed facing the inter-row spacing compared to those placed along the row (42.57 ± 7.20 vs $35.45 \pm 5.77\%$ in the orchard border and 48.34 ± 3.83 vs $40.27 \pm 3.37\%$ in the inner row). Egg masses in the central part of the plants, despite being the most widely covered by the treatments, showed an intermediate percentage of nymphal mortality (42.41 ± 4.65). Interestingly, about half of the egg masses (regardless of their position) showed more than the 90% coverage (Table 1).

Table 1: Mean mortality of nymphs from treated egg masses, according to the wetting percentage recorded in the corresponding cardboard. Nymphs are grouped according to cardboard tag wetting percentage rates.

Wetting percentage	Percentage Mortality (mean $\pm$ SE)	No. egg masses
$x \leq 10$	38.95 ± 5.40	36
$10 < x \leq 20$	44.32 ± 5.58	25
$20 < x \leq 30$	31.38 ± 6.91	17
$30 < x \leq 40$	46.48 ± 11.45	11
$40 < x \leq 50$	43.51 ± 10.88	11
$50 < x \leq 60$	39.28 ± 11.37	11
$60 < x \leq 70$	60.34 ± 12.37	10
$70 < x \leq 80$	41.04 ± 9.00	13
$80 < x \leq 90$	33.76 ± 7.58	12
$90 < x \leq 100$	44.60 ± 3.01	139

866

867 3.4 Acquisition of '*Candidatus Pantoea carbekii*' by newborn 868 nymphs

869 A total of 230 nymphs were used to quantify the infection with live *P.*
870 *carbekii* cells; specifically, 110 nymphs from treated egg masses and 120
871 nymphs from untreated egg masses were randomly selected. In the case of
872 treated egg masses, *P. carbekii* acquisition was significantly lower in dead
873 nymphs ($Mdn = 3.36 \times 10^3$ expressed copies of 16S rRNA gene / insect,
874 $IQR = 1.87 \times 10^4$) compared to alive nymphs ($Mdn = 5.46 \times 10^4$ expressed
875 copies of 16S rRNA gene / insect, $IQR = 1.11 \times 10^5$), $W = 1628$, $p < 0.001$
876 (Fig. 2). However, such a difference was not observed for control nymphs,
877 whose *P. carbekii* acquisition level did not show a significant difference
878 between dead ($Mdn = 1.55 \times 10^4$ expressed copies of 16S rRNA gene /
879 insect, $IQR = 1.19 \times 10^5$) and alive specimens ($Mdn = 2.50 \times 10^4$
880 expressed copies of 16S rRNA gene / insect, $IQR = 1.89 \times 10^5$), $W = 34.50$,
881 $p = 0.270$. Complete prevention of *P. carbekii* acquisition was achieved for
882 tested dead (10 out of 53) and live nymphs (3 out of 57) from treated egg
883 masses. All nymphs from control egg masses showed successful *P.*
884 *carbekii* acquisition.

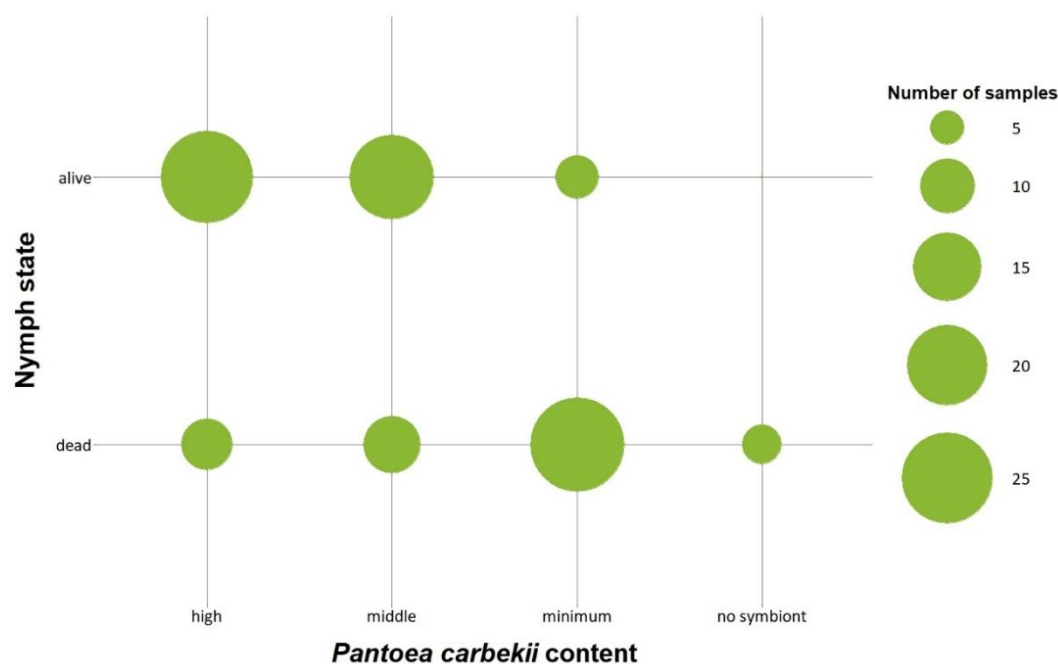


Figure 2: Concentration of alive *P. carbekii* cells in dead and alive *H. halys* nymphs hatched from treated egg masses, expressed as number of expressed copies of *P. carbekii* 16S rRNA gene per pg of insect DNA. “Minimum” *P. carbekii* content: symbiont concentration within the 33rd percentile. “Middle” *P. carbekii* content: symbiont concentration between 33rd and 66th percentile. “High” *P. carbekii* content: symbiont concentration above 66th percentile. The size of dots indicates the number of samples showing a given concentration.

3.5 Damage evaluation

Similar percentages of damaged hazelnuts were recorded in 2022 and 2023 in the same plot (either treated or control). Cimiciato damage was significantly lower in hazelnuts from the treated plot compared to the untreated one in both years ($F = 0.236$, $p < 0.001$, $\eta^2 = 0.05$, $df = 1$) (Table 2).

Table 2: Comparison of cimiciato damage observed in collected hazelnuts from treated and control plot in two consecutive years. Different letters indicate

901 statistically different values in the same year (glm with binomial distribution and
 902 logit link function, Bonferroni post hoc correction, $p < 0.05$).

Year	Plot	Cimiciato (% $\pm$ SE)	Sample size
2022	Control	22.4 $\pm$ 2.4 a	N = 300
	Treated	6.6 $\pm$ 1.4 b	N = 300
2023	Control	24.1 $\pm$ 2.4 a	N = 300
	Treated	7.4 $\pm$ 1.4 b	N = 300

903

904 4. Discussion

905 This study provides the first evaluation of the field efficacy of symbiont-
 906 targeted control of *H. halys*. As the efficacy of any active substance,
 907 including those targeting symbionts, can vary widely when scaled up from
 908 the laboratory to the field (Taylor et al., 2017), the aim of this work was to
 909 understand how the spraying conditions affect the treatment outcome in
 910 terms of ability to reach egg masses in different positions, and
 911 consequently to induce nymphal mortality. The most obvious result of this
 912 work was the lack of a clear correlation between the wetting percentage of
 913 the egg masses and nymphal mortality after egg hatching. This was noted
 914 already within the preliminary evaluation conducted to determine how
 915 sprayer fan speed might affect the egg mass coverage and the consequent
 916 nymphal mortality. There was no significant difference in the wetting
 917 percentage of cardboard tags when the fan speed was increased from 1890
 918 rpm to 2700 rpm. This result may be due to the only partial reliability of
 919 results of the software analysis results on the scanned water-sensitive paper
 920 due to the irregular distribution and size of wet drops (Cerruto et al., 2019).
 921 However, changing the fan speed was not irrelevant to the outcome of
 922 symbiont-targeted control: increasing the fan speed resulted in augmented

mortality of insects within the second instar. A possible explanation could be that the increased pressure made it easier for the anti-symbiont biocomplex to dilute or penetrate deeper into the secretion containing *P. carbekii* on the egg surface. Indeed, not only the amount of a substance reaching the egg mass is important for a proper control strategy, but also the ability of this substance to reach *P. carbekii* cells embedded in the maternal secretion can affect the outcome (Kenyon et al., 2015; Taylor et al., 2017).

When the effect of egg mass position was tested, the result was opposite to the effect of spraying conditions, as the position affected wetting percentage but not mortality induction. The greatest egg mass coverage was obtained when the cardboards were placed in the central part of the plant. This was an expected result: given the single-trunk training system used in the orchard tested and the height of the first branch, the lower nozzles could easily spray the central part of the plants. This outcome could although vary among different training systems or crops. Cardboard tags that were hung facing the inter-row spacing and thus closer to the sprayer were easily reached by the product, whereas the most difficult position to reach was the one along the row. Plants at the edge of the orchard were generally less efficiently wetted than those in the inner row; this result was expected since leaf density tended to be higher in the edge row, making it more likely that some leaves would prevent the treatment from reaching the cardboard tags.

Despite the differences observed in product coverage, no correlation was found between the percentage of wetting of cardboards (and consequently their position) and nymphal mortality. This result suggests that even a low coverage of the egg surface allows the antibacterial compounds contained

950 in the applied biocomplex to reduce the amount of symbiotic cells
951 available for nymphal acquisition. Considering that *H. halys* sampling
952 usually indicates the highest insect abundance at the field borders (Bosco
953 et al., 2018), where product spraying is more difficult, the possibility to
954 achieve significant mortality induction even with low egg coverage
955 supports the success of symbiont-targeted methods. However, to maximize
956 the outcome of symbiont-targeted treatments, the use of appropriate
957 management practices (e.g.: pruning to reduce the canopy vigor) can be
958 suggested to increase the chance of effectively reaching egg masses.

959 It must be pointed out that the average nymphal mortality obtained under
960 field conditions was lower than the mortality recorded in laboratory
961 experiments with the same formulation (Gonella et al., 2019). This
962 difference could be due to different product concentrations (0.4% in this
963 study, 1.0% in Gonella et al. (2019)) and to differences between laboratory
964 and field conditions. The lower mortality observed here is related to the
965 incomplete prevention of *P. carbekii* acquisition that was achieved, at least
966 for most of the samples; in contrast, laboratory trials showed the complete
967 absence of live symbiotic cells in nymphs from egg masses treated with
968 the same biocomplex (Gonella et al., 2019, 2022). Also Taylor et al. (2017)
969 showed a lower efficacy in preventing *P. carbekii* acquisition in field trials
970 compared to laboratory conditions using different formulations.
971 Nevertheless, we have shown that the reduction in symbiont acquisition is
972 sufficient to induce significant mortality even considering the unavoidable
973 limitations of field conditions. The more than 3-fold reduction in damaged
974 hazelnuts in the treated plots strengthens the hypothesis that, under field
975 conditions, it is not strictly necessary to completely prevent symbiont
976 acquisition to achieve an acceptable reduction in economic damage by

applying four anti-symbiont treatments during the summer season. A further confirmation of the efficacy of this strategy in hazelnut orchards would benefit of repeating trials in geographically separated areas.

The limit of copper admitted in organic farming is 28 kg/ha in seven years (Reg. CE 834/07). Since the biocomplex copper content is 25 g/L, a total of 400 g/ha of copper are used following this approach in each season, resulting compatible with current regulation. From three to seven treatments are also indicated for other crops, such as apple or pears. Given the broad *H. halys* host spectrum (Lee et al., 2013; Morrison III et al., 2018) and since all these crops present different canopies, further optimization and validation of symbiotic control field treatments is needed for other cultivations. Even though no perfect coverage is needed to enhance nymphal mortality, operating parameters during sprayings and agronomical practices are likely the key elements to optimize for proper symbiotic control results in other crops.

An additional advantage of symbiont-targeted control is its compatibility with the augmentative biological control programs underway in Europe using the egg parasitoid wasp *Trissolcus japonicus* (Ashmead) (Falagiarda et al., 2023). Field releases of *T. japonicus* are predicted to provide stable parasitoid establishment and successful containment of *H. halys* in Europe (Haye et al., 2020; Tortorici et al., 2023). The introduction of a second allochthonous egg parasitoid, *Trissolcus mitsukurii* (Ashmead), is expected to increase *H. halys* mortality, so the possibility of using the better performing species or biotypes depending on the area has been suggested (Gutierrez et al., 2023). However, parasitoid occurrence in cultivated areas may be severely hampered by the use of chemical insecticides against their host. The application of symbiont-targeted

control, relying on low-impact antimicrobials rather than on insecticidal molecules, has been shown to be harmless to *T. japonicus*, and no alteration of parasitism by this wasp has been observed (Orrù et al., 2023).

Conclusion

This work represents, to the best of our knowledge, the most thorough investigation of the efficacy of symbiont-targeted control of *H. halys* under field conditions and opens up future perspectives for further implementation of this strategy in hazelnut orchards. Future studies could focus on increasing spatial and temporal replication to definitively confirm the efficacy of this methodology. Also, field application of anti-symbiont treatments on different host plants remain to be assessed. Furthermore, the performance of low-impact strategies combining biological control and symbiont-targeted control deserves to be investigated under a realistic scenario in field conditions. However, our results support the effectiveness of the proposed approach in keeping *H. halys* populations low enough to reduce the associated economic damage.

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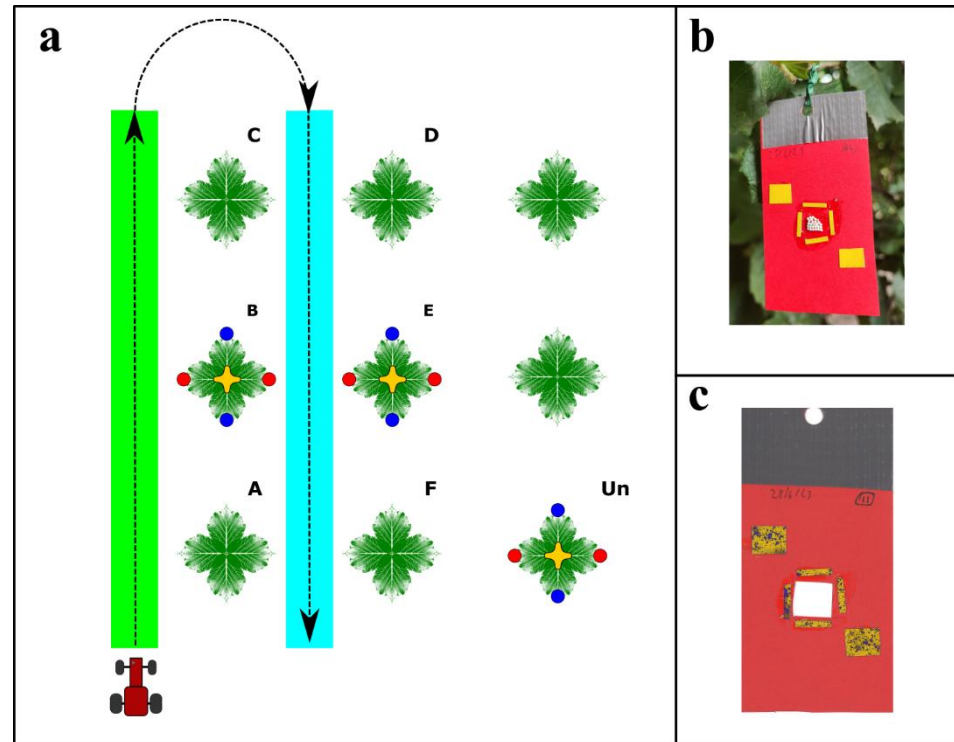
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1283

1284 SUPPLEMENTARY INFORMATION



1285

1286 **Figure S1:** Experimental plan of the field evaluation of anti-symbiont treatments.
 1287 **a** Scheme of plant selection for cardboard tags applications in the grove. Plants
 1288 A, B, C represent the perimetral row, whereas plants D, E, F represent an internal
 1289 row. Accordingly, the green and light blue stripes represent the passages of
 1290 tractor, in the orchard border (green stripe) or in an internal row (light blue stripe).
 1291 Plants B and E represent selected plants of the treated plot. “Un” corresponds to
 1292 the selected plant within an untreated row. In selected plants, blue spots indicate
 1293 cardboards placed along the row, red spots indicate cardboards facing the inter-
 1294 row spacing, and yellow crosses indicate cardboards in the centre of the plant
 1295 canopy. An example of newly-placed cardboard is shown in **b**; two cardboards
 1296 were placed in each position, at two heights on the canopy. Cardboards were
 1297 removed soon after treatments; an example of removed cardboard is given in **c**.

The egg mass was removed from the tag, and blue spots on the water sensitive paper indicate treatment coverage.

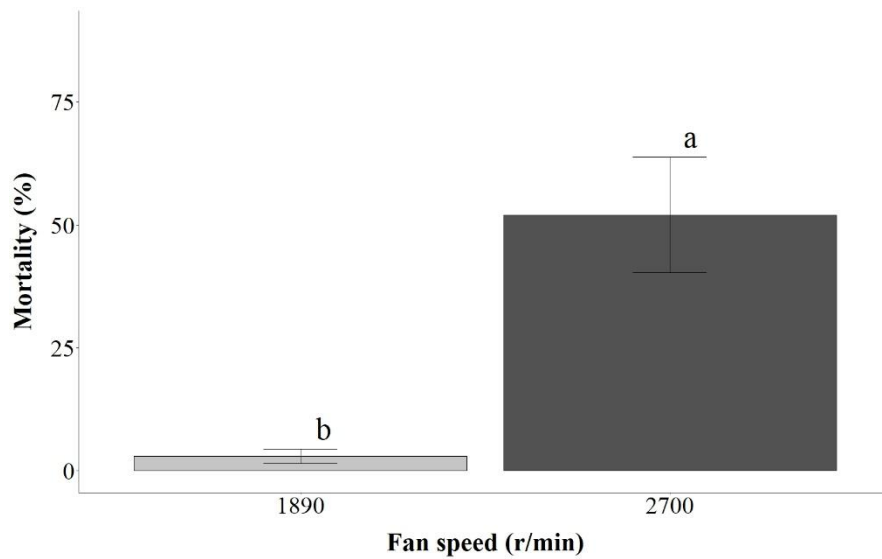


Figure S2: Nymphal mortality rate comparison between nymphs emerging from egg masses treated using different sprayer fan speeds. Different letters indicate significantly different values according to Mann-Whitney U test ($p < 0.050$, $df = 1$).

1307 **Chapter III – Host and symbiont MLST approach**

1308 Article submitted

1309 **Multi locus sequence typing of the invasive pest**
1310 ***Halyomorpha halys* (Hemiptera: Pentatomidae) and**
1311 **associated endosymbiont reveals unexplored**
1312 **diversity**

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1324 **Abstract**

1325 *Halyomorpha halys*, a highly polyphagous stink bug, is an important pest
1326 affecting numerous agricultural productions and has become invasive in
1327 many regions worldwide. Rapid and cost-effective methods for
1328 reconstructing the invasion routes of this species are crucial for
1329 implementing effective management strategies to prevent further spread.

1330 In this context, analysing variable short DNA molecular markers provides
1331 practical alternative to more resource-intensive genome-based approaches.
1332 So far, the mitochondrial markers COI and COII, as well as the pseudogene
1333 $\Delta ybgF$ of the insect primary symbiont '*Candidatus Pantoea carbekii*', have
1334 been used to trace the spread of *H. halys*. However, these markers do not
1335 provide sufficient resolution to fully elucidate its invasion routes. Given
1336 the ongoing primary and secondary invasions in multiple regions, there is
1337 a need for more informative markers, particularly those targeting the
1338 nuclear genome. In this study, 72 individuals from seven populations of *H.*
1339 *halys* (five from invaded areas and two from the native range) were
1340 analysed to identify new DNA markers and evaluate their effectiveness in
1341 a multi-locus sequence typing (MLST) framework. Three novel nuclear
1342 markers for *H. halys* (Hh_KsPi, Hh_UP1 and Hh_D3PDh) were identified,
1343 along with three new markers for *P. carbekii* (Pc_TamA, Pc_SucA and
1344 Pc_SurA). Among these, Hh_D3PDh was the most informative nuclear
1345 marker for *H. halys*, revealing 13 haplotypes, two more than those
1346 identified using COI. For *P. carbekii*, Pc_SucA resulted the most
1347 informative marker, with five haplotypes described. Notably, when all four
1348 *P. carbekii* markers were combined in the MLST analysis, ten distinct
1349 symbiont haplotypes were identified. By integrating the newly developed
1350 nuclear marker Hh_D3PDh with previously used mitochondrial markers,
1351 30 distinct haplotypes were identified, with each of the *H. halys* population
1352 analysed exhibiting multiple haplotypes. The low network specialization
1353 between the newly identified nuclear markers and both mitochondrial and
1354 symbiont markers underscored the higher informativeness of the nuclear
1355 markers. Interestingly, in populations from invaded areas, a perfect
1356 network specialization was found between *H. halys* COI and its symbiont

1357 markers, suggesting that certain host-symbiont variants may contribute to
 1358 enhanced invasive capability. In conclusion, a possible workflow for the
 1359 MLST approach is proposed for analysing population genetics and
 1360 reconstructing the invasion routes of *H. halys*.

1361 **1. Introduction**

1362 *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) commonly known
 1363 as the brown marmorated stink bug, is a highly polyphagous and invasive
 1364 phytophagous insect native to Eastern Asia (Hoebeke and Carter, 2003;
 1365 Lee et al., 2013; Leskey and Nielsen, 2018). This species is harmful to a
 1366 wide range of agricultural crops, such as apples, pears, peaches, tomatoes,
 1367 olives and hazelnuts (Bariselli et al., 2016; Bosco et al., 2018; Damos et
 1368 al., 2019; Leskey et al., 2012). In addition to its agricultural impact, *H.*
 1369 *halys* becomes a domestic nuisance during its mass dispersal from fields
 1370 to buildings for overwintering (Hancock et al., 2019).

1371 First detected outside of the native range in 1996 in Pennsylvania
 1372 (Hoebeke and Carter, 2003), *H. halys* is now widespread across North
 1373 America (Abram et al., 2017; Garipey et al., 2014; Morey et al., 2022; Rice
 1374 et al., 2014). In Europe it was firstly found in 2004 in Liechtenstein (Arnold,
 1375 2009) and in 2007 in Zurich, Switzerland (Wermelinger et al., 2007). Its
 1376 expansion steadily increased with specimens found in 2011 in Germany
 1377 (Heckmann, 2012), Greece (Milonas and Partsinevelos, 2014) and
 1378 Belgium (Claerebout et al., 2018), in 2012 in Italy (Maistrello et al., 2014)
 1379 and France (Callot and Brua, 2013), in 2014 in Hungary (Vétek et al.,
 1380 2014), in 2015 in Romania (Macavei et al., 2015), Austria (Rabitsch and
 1381 Friebe, 2015) and Serbia (Šeat, 2015), in 2016 in Spain (Dioli et al., 2016),
 1382 Bulgaria (Simov, 2016) and Ukraine (Uzhevskaya, 2017), in 2017 in

1383 Croatia (Šapina and Jelaska, 2018), Slovakia (Hemala and Kment, 2017)
 1384 and Slovenia (Rot et al., 2018), in 2018 in Albania (Claerebout et al.,
 1385 2018), Bosnia ed Herzegovina (Zovko et al., 2019), Poland (Claerebout et
 1386 al., 2018), Czech Republic (Kment and Březíková, 2018), Netherlands
 1387 (Aukema et al., 2019), United Kingdom (Powell et al., 2021) and Portugal
 1388 (Grosso-Silva et al., 2020), in 2019 in Moldova (Derjanschi and Chimişliu,
 1389 2019) and in North Macedonia (Konjevic, 2020). *Halyomorpha halys* is
 1390 considered particularly dangerous in invaded regions of western Asia with
 1391 high hazelnut production, such as Turkey where it was likely spotted in
 1392 2017 but confirmed in 2019 (Çerçi and Koçak, 2017; Güncan and Gümüş,
 1393 2019) or Georgia where it was found in 2016 (Gapon, 2016). It is also
 1394 established in Armenia, where it was confirmed in 2020 and further
 1395 expansion are expected (Kalashian et al., 2022), whereas in Central Asia it
 1396 is present in Kazakhstan since 2016 (Temreshev et al., 2018) and in
 1397 Uzbekistan since 2020 (Gandjaeva et al., 2022).

1398 Despite the ongoing efforts to contain its spread, new regions are
 1399 continually being invaded. In North-Africa, in 2019 it has been reported in
 1400 Morocco's Loukkos and Gharb regions (Nouere et al., 2020), both
 1401 considered suitable for its establishment (Tortorici et al., 2023), and a
 1402 single adult was found in Algeria in 2021 (van der Heyden et al., 2021).
 1403 Some individuals were reported also in the two archipelagos of the Canary
 1404 and Madeira, in 2021 and 2022 respectively (Gaspar et al., 2023; Petrovan
 1405 et al., 2022). In the Southern Hemisphere, *H. halys* has been recorded in
 1406 Chile in 2017 (Faúndez and Rider, 2017), but regions such as Australia and
 1407 New Zealand are deemed suitable regions for its establishment (Kriticos
 1408 et al., 2017), and several adults have been intercepted in both countries in
 1409 imported goods (Horwood et al., 2019).

1410 *Halyomorpha halys* hosts its primary endosymbiont, ‘*Candidatus Pantoea*
 1411 *carbekii*’, in the caeca of the V4 midgut region (Bansal et al., 2014). The
 1412 genome of *P. carbekii* revealed functional pathways for synthesizing
 1413 essential amino acids and vitamins that are absent from the diet of a
 1414 phytophagous insect, which may explain *H. halys* broad host plants range
 1415 (Kenyon et al., 2015). *Pantoea carbekii* can be considered a primary
 1416 endosymbiont since deprived insects show significantly decreased
 1417 survival and fitness; interruption of symbiont infection is currently
 1418 considered a promising control strategy (Dho et al., 2025; Gonella and
 1419 Alma, 2023).

1420 Reconstructing the invasion patterns of *H. halys* is crucial for identifying
 1421 pathways of introduction and preventing future invasions (Cesari et al.,
 1422 2018; Gariépy et al., 2015; Xu et al., 2014). For this purpose, molecular
 1423 markers have been proven to be powerful tools for assessing genetic
 1424 diversity among populations, facilitating the tracking of invasions
 1425 (Ficetola et al., 2008; Xu et al., 2014). For *H. halys*, to this aim many
 1426 studies have focused on amplifying and sequencing portions of the COI
 1427 mitochondrial region (Xu et al., 2014; Gariépy et al., 2015; Lee et al.,
 1428 2018; Schuler et al., 2021; Yan et al., 2021a). The COI marker is
 1429 advantageous due to the extensive availability of sequences in public
 1430 databases, allowing for the construction of population-specific profiles
 1431 (Gariépy et al., 2021). However, with both primary and secondary
 1432 invasions occurring rapidly in new areas (Abram et al., 2017; Cesari et al.,
 1433 2018; Schuler et al., 2021) relying solely on a single marker, such as COI,
 1434 has become insufficient to trace the origin of invasive populations
 1435 (Gariépy et al., 2021; Horwood et al., 2019; Yan et al., 2021a). The use of
 1436 few markers can be insufficient to clearly reconstruct the population

genetics, especially for species with high genetic variability (Medina et al., 2006). More in depth analysis based on NGS techniques are possible for biological invasion studies (Rius et al., 2015) and ddRAD Sequencing has already been used for population genetics studies in *H. halys* (Parvizi et al., 2023; Yan et al., 2021b). On the other hand, the selection of few highly informative markers can be a faster, cost-effective alternative that does not demand great computational resources (Pistone et al., 2016), all characteristics that fit well for developing a fast and widely applicable tool.

Additional molecular markers to complement COI have been sought, with COII being one of the most commonly used (Berteloot et al., 2024; Cesari et al., 2018; Yan et al., 2021a). Among nuclear markers, ITS1 has been employed in few studies (Kapantaidaki et al., 2019; Zhu et al., 2016). In addition, molecular markers derived from the primary symbiont have been identified and successfully used to pinpoint the source population of new invasions (Laterza et al., 2023; Otero-Bravo and Sabree, 2018). The pseudogene $\Delta ybgF$ of *P. carbekii* resulted as the best marker so far for reconstructing *H. halys* invasions through symbiont genetic variability analysis, though it has not proven as effective as the host's COI (Otero-Bravo and Sabree, 2018), with only seven distinct haplotypes identified (Martinez-Sañudo et al., 2020). Nevertheless, by considering the primary symbiont marker not as a proxy for the host genetic variability, but as an additional layer of information, coupling $\Delta ybgF$ with host COI increases the resolution of host-symbiont haplotypes and improves discrimination of invasive population origins (Laterza et al., 2023; Martinez-Sañudo et al., 2020).

Despite significant efforts, the current markers are insufficient, and the need for novel, more informative markers has become increasingly

1464 apparent to better defining *H. halys* invasion patterns (Cesari et al., 2018;
 1465 Laterza et al., 2023; Valentin et al., 2017). The aim of this study is to
 1466 develop a cost-effective MLST approach, suitable for population genetics
 1467 analyses and invasion pattern reconstruction by identifying novel
 1468 molecular markers, focusing on *H. halys* nuclear and *P. carbekii* DNA, to
 1469 improve current haplotyping procedures.

1470 **2. Materials and Methods**

1471 **2.1 Insect collection**

1472 Seven distinct sampling sites were selected for the collection of adult *H.*
 1473 *halys* specimens, five from invaded areas (three in Italy and two in Turkey)
 1474 and two from the insect native range (China). The Italian (IT) populations
 1475 were sampled in the provinces of Cuneo (44°40'53.9"N, 7°51'06.6"E) (ten
 1476 individuals), Cremona (45°15'12.9"N, 10°05'20.4"E) (ten individuals),
 1477 and Reggio Emilia (44°37'02.0"N, 10°42'16.6"E) (eleven individuals).
 1478 Turkish (TR) populations were collected from the provinces of Samsun
 1479 (41°10'31.8"N, 36°30'39.8"E) (eight individuals) and Ordu (40°59'54.9"N,
 1480 37°33'59.6"E) (13 individuals). One field-collected population from China
 1481 (CN) was sampled in Henan province (34°24'27.6"N, 110°54'45.6"E) (ten
 1482 individuals), and an additional laboratory-reared population was obtained
 1483 from the Sino-American Biological Control Laboratory in Beijing
 1484 (40°07'44.4"N, 116°09'50.0"E) (ten individuals). Laboratory reared
 1485 colony (Beijing – CN) was maintained for over 35 generations in
 1486 controlled conditions and insects were originally collected in Sanming
 1487 City (Fujian Province, CN) (26°15'40.0"N, 117°38'37.3"E). Adults were
 1488 collected using the plant beating technique, transported alive to the
 1489 laboratory in 25 × 15 × 13 cm plastic boxes, killed at -20 °C, and stored
 1490 in pure ethanol at -20 °C until DNA extraction.

2.2 *Halyomorpha halys* genetic analysis and nuclear marker identification

DNA was extracted from the legs of *H. halys* adults with DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany), following manufacturer's instructions. The extracted DNA was used as a template for the amplification of selected genes mitochondrial genes, COI and COII, and newly identified nuclear markers. All the individuals were tested for each marker under analysis. Mitochondrial genes included COI and COII. For COI, new *H. halys*-specific primers were designed using Primer BLAST function of the NCBI Genome Data Viewer. The target region ranged from position 1,366 to 2,139 of the reference mitochondrial region (GenBank Acc. No. NC_013272). Details of primer sequences and annealing temperature are indicated in Table S1. Thermal cycler conditions were 34 cycles of: 95 °C for 30", annealing for 30" and 72 °C for 1', followed by 10' at 72 °C for final extension. For COII, the chosen primer pair and the thermal cycler conditions were those described in Cesari et al. (2018).

To identify possible nuclear markers, all the 20 available Hemiptera genomes with annotated CDS were downloaded from NCBI (last check 05/27/2022). All the CDS were translated and orthologous genes were inferred with Orthofinder (Emms and Kelly, 2019). Orthogroups found in *H. halys* and in at least six other species were selected. Chosen orthogroups were aligned as proteins with MUSCLE algorithm and back-translated into nucleotides using pal2nal (Suyama et al., 2006). Orthogroups were ranked according to the substitution rates with the corresponding *H. halys* gene. In total, 20 genes were chosen as possible fast-evolving molecular markers. For each gene, primer pairs were designed on two exons flanking an intron, to increase the chance of finding variable sites. Primers were

designed using Primer BLAST function of the NCBI Genome Data Viewer. Optimal annealing temperature for each primer pair was selected via gradient PCR and each primer pair was tested on 10 *H. halys* individuals having at least 2 different COI haplotypes. The three best-performing markers in terms of variability and quality of the sequences were selected. Chosen markers were named Hh_KsPi, Hh_D3PDh and Hh_UP1. Details of primer sequences, target gene, position in the reference genome, and annealing temperature for each marker are indicated in Table S1. Thermal cycler conditions for each nuclear marker were the same as described for novel COI, except for annealing temperature.

Amplicon size was visualized via electrophoresis with 1% agarose gel stained with GelRed® (MilliporeSigma, Temecula, CA). PCR products were purified with QIAquick PCR purification kit® (Qiagen) and Sanger sequenced by an external company (Eurofins Genomics, Köln, Germany).

2.3 ‘*Candidatus Pantoea carbekii*’ markers identification

To isolate DNA from the primary endosymbiont of *H. halys*, the V4 ventricle region of adult insect midgut was dissected in a Petri dish with 500 µl of a sterile NaCl solution 0.9%, under a dissection microscope using fine forceps. DNA was extracted from the V4 ventricles using the DNeasy® Blood and Tissue kit (Qiagen) for all the *H. halys* adults under analysis. High quality DNA was obtained for 66 out of 72 individuals. All these 66 individuals were used for subsequent molecular analyses for all the molecular markers. The $\Delta ybgF$ marker amplified using previously described primers and PCR conditions (Otero-Bravo and Sabree, 2018). To identify novel markers, *P. carbekii* annotated genome was downloaded from GenBank (Acc. No. NZ_CP010907) and each coding sequence

(CDS) was blasted on the other available *P. carbekii* genome (Acc. No. AP012554). All genes having at least three SNPs and/or one indel with their homolog on the second genome were kept for further analyses. From these, ten genes with the highest number of SNPs or indels within an 800 bp range were chosen. Primers for amplifying portions of these target genes were designed using Primer BLAST function of the NCBI Genome Data Viewer. Optimal annealing temperature for each primer pair was determined using gradient PCR, and each primer pair was tested on 10 *P. carbekii* individuals having at least 2 different $\Delta ybgF$ haplotypes. The three best performing markers, based on sequence variability and quality, were selected. Chosen markers were named Pc_SucA, Pc_SurA, Pc_TamA. Details of primer sequences, target gene name, position in reference genome and annealing temperature for each marker are provided in Table S1. The thermal cycler conditions for amplification of each target were 34 cycles of: 95 °C for 30", annealing phase for 30" and 72 °C for 1', followed by 10' at 72 °C. Amplicons size visualization and sequencing were performed as described above.

2.4 Haplotype identification and statistical analyses

All the electropherograms were checked for low quality bases and flanking regions were manually trimmed before generating consensus sequences. Only for *H. halys* nuclear markers, in case electropherograms presented evidence of possible heterozygosis, such as double peaks in a specific point or consecutive double peaks starting after high quality bases, consensus sequences were generated after allele inference with the web application Indigo of Tracy (Rausch et al., 2020). To limit the risk of artifact generation, electropherograms with clear inconsistencies in electrophoretic mobility were excluded from the analysis (Flot et al.,

2011). Each singleton generated by Indigo from heterozygous samples presenting indels or a combination of indels and SNPs was excluded from the analysis to reduce potential artifacts. Each sequence presenting indels and/or SNPs was manually curated by checking the original electropherogram before keeping it for the analysis. All singletons from sequences containing only SNPs were carefully checked.

Sequence translation of novel *P. carbekii* haplotypes was performed with ExPASy (Artimo et al., 2012). To avoid frameshift errors during translation, any missing 5' and 3' regions of novel haplotypes were replaced with the corresponding regions from the reference genome. Structural damage in resulting protein structures due to amino acids missense between haplotypes was predicted using Missense3D (Ittisoponpisan et al., 2019).

Newly identified marker sequences were compared with reference genomes using NCBI BLAST (*H. halys*: GCF_000696795; *P. carbekii*: NZ_CP010907). Orthologous sequences were aligned with sequences retrieved from literature using MUSCLE algorithm implemented in MEGA version 11 (Tamura et al., 2021). COI and COII reference databases were created based on datasets published by Cesari et al. (2018), supplemented with sequences from additional studies (Kapantaidaki et al., 2019; Martinez-Sañudo et al., 2020; Schuler et al., 2021; Yan et al., 2021a) (Tables S2 and S3). In cases where different haplotype names had been assigned to the same sequence, the nomenclature from Cesari et al. (2018) was used. Haplotype identification was performed with DnaSP 6 (Rozas et al., 2017). To check for the presence of haplotypes described by Martinez-Sañudo et al. (2020), the COI haplotype inference was preliminary performed on a 478 bp long region. Since no individual

belonged to H63/H65/H66/H67/H79 haplotypes, these sequences were removed from the database to limit the risk of collapsing different haplotypes and thus reducing the informativity. The definitive COI haplotype inference was redone on a 592 bp long region. $\Delta YbgF$ reference database for haplotype inference was created with the sequences published by Otero-Bravo and Sabree (2018) and Martinez-Sañudo et al. (2020) (Table S4).

For all newly identified *H. halys* markers, haplotype name “_h1” was given to the corresponding sequence deposited in the reference genome (GCF_000696795). For all newly identified *P. carbekii* markers haplotype name “_h1” was given to the corresponding sequence deposited in the genome NZ_CP010907, whilst “_h2” to the corresponding sequence deposited in the genome AP012554.

Number of haplotypes (H), haplotype diversity (h) and nucleotide diversity (π) were calculated with R package pegas version 1.3 (Paradis, 2010). Haplotype networks were generated with TCS version 1.23 (Clement et al., 2000) and graphically displayed with tcsBU (Múrias dos Santos et al., 2016). The association between host and symbiont molecular markers was assessed using the specialization index H_2' (Blüthgen et al., 2006) and visualized as bipartite network using R package bipartite (Dormann et al., 2008) as described by Martinez-Sañudo et al. (2020).

3. Results

3.1 *Hayomorpha halys* mitochondrial markers

Amplicons were obtained for all 72 individuals for COI and for 71 individuals for COII. A total of 11 different COI haplotypes (Fig. S1a; Figs S2–S4) were detected by analysing sequences from 71 individuals, with

three of them never recorded before (individuals from China). The novel haplotypes were named H168, H169 and H170 (Fig. S1a). Except for populations from Reggio Emilia (IT) and Samsun (TR), all the other populations showed at least two different COI haplotypes. The most abundant haplotype was H1 (40 sequences), followed by H3 and H43 (6 sequences). Haplotypes H169 and H170 were present in a single individual (Table S5). The π values varied from a minimum of 0 to a maximum of 0.00409, while h values from a minimum of 0 to a maximum of 0.844 ± 0.068 . In both cases, the lowest values belonged to the invasive populations of Reggio Emilia (IT) and Samsun (TR), while the highest values to the native population of Henan (CN) (Table 1). By grouping the populations for their Country, the greater number of different haplotypes found was displayed by Chinese samples ($H = 8$) (Table 1).

Five different haplotypes were identified for COII (473 bp) (Fig. S1b; Figs S2–S4) out from 67 high quality sequences obtained, with a newly described one named h22 from Henan (CN) (one sequence; Table S5). Similarly to COI, no haplotype diversity was found in Reggio Emilia (IT) and Samsun (TR) populations. In general, the most abundant haplotype was h1 (41 sequences), followed by h3 (23 sequences). All the other haplotypes (h5, n15, h22) were found in single individuals. As for COI, the less variable populations resulted the invasive ones of Reggio Emilia (IT) and Samsun (TR) ($\pi = 0$; $h = 0$), while the other Chinese population of Beijing showed the greatest π (0.00183) and h (0.711 ± 0.111) values (Table 1).

1649

1650 **Table 1.** Summary statistics for each population under analysis using markers available in literature. COI and COII: *H. halys*
 1651 mitochondrial markers; $\Delta ybgF$: *P. carbekii* marker. Results are given separately for the regions where samples were collected and for
 1652 the countries under investigation: Italy includes samples from Cuneo, Reggio Emilia and Cremona; Turkey includes samples from
 1653 Samsun and Ordu; China includes samples from Beijing and Henan. n: number of sequences; H: number of haplotypes; π : nucleotide
 1654 diversity; h: haplotype diversity.

Province - Country	COI				COII				$\Delta ybgF$			
	n	H	π	$h \pm SD$	n	H	π	$h \pm SD$	n	H	Π	$h \pm SD$
Cuneo - IT	9	5	0.00347	0.806 $\pm$ 0.113	10	2	0.00042	0.200 $\pm$ 0.157	7	2	0.00139	0.286 $\pm$ 0.200
Reggio Emilia - IT	11	1	0	0 $\pm$ 0	8	1	0	0 $\pm$ 0	4	1	0	0 $\pm$ 0
Cremona - IT	10	5	0.00379	0.867 $\pm$ 0.058	8	2	0.00091	0.429 $\pm$ 0.166	8	2	0.00208	0.428 $\pm$ 0.166
Samsun - TR	8	1	0	0 $\pm$ 0	8	1	0	0 $\pm$ 0	7	1	0	0 $\pm$ 0
Ordu - TR	13	2	0.00048	0.282 $\pm$ 0.143	13	2	0.00060	0.282 $\pm$ 0.143	11	2	0.00088	0.182 $\pm$ 0.146
Beijing - CN	10	4	0.00154	0.644 $\pm$ 0.150	10	4	0.00183	0.711 $\pm$ 0.111	9	2	0.00270	0.556 $\pm$ 0.070
Henan - CN	10	5	0.00409	0.844 $\pm$ 0.068	10	3	0.00141	0.600 $\pm$ 0.125	10	3	0.00194	0.378 $\pm$ 0.184
North Italy	30	5	0.00388	0.729 $\pm$ 0.062	26	2	0.00107	0.508 $\pm$ 0.036	19	2	0.00238	0.491 $\pm$ 0.064
Turkey	21	2	0.00031	0.181 $\pm$ 0.105	21	2	0.00038	0.181 $\pm$ 0.105	18	2	0.00054	0.111 $\pm$ 0.098
China	20	8	0.00336	0.847 $\pm$ 0.057	20	5	0.00157	0.632 $\pm$ 0.086	19	3	0.00250	0.485 $\pm$ 0.103

1655

1656 **3.2 *Halyomorpha halys* nuclear markers**

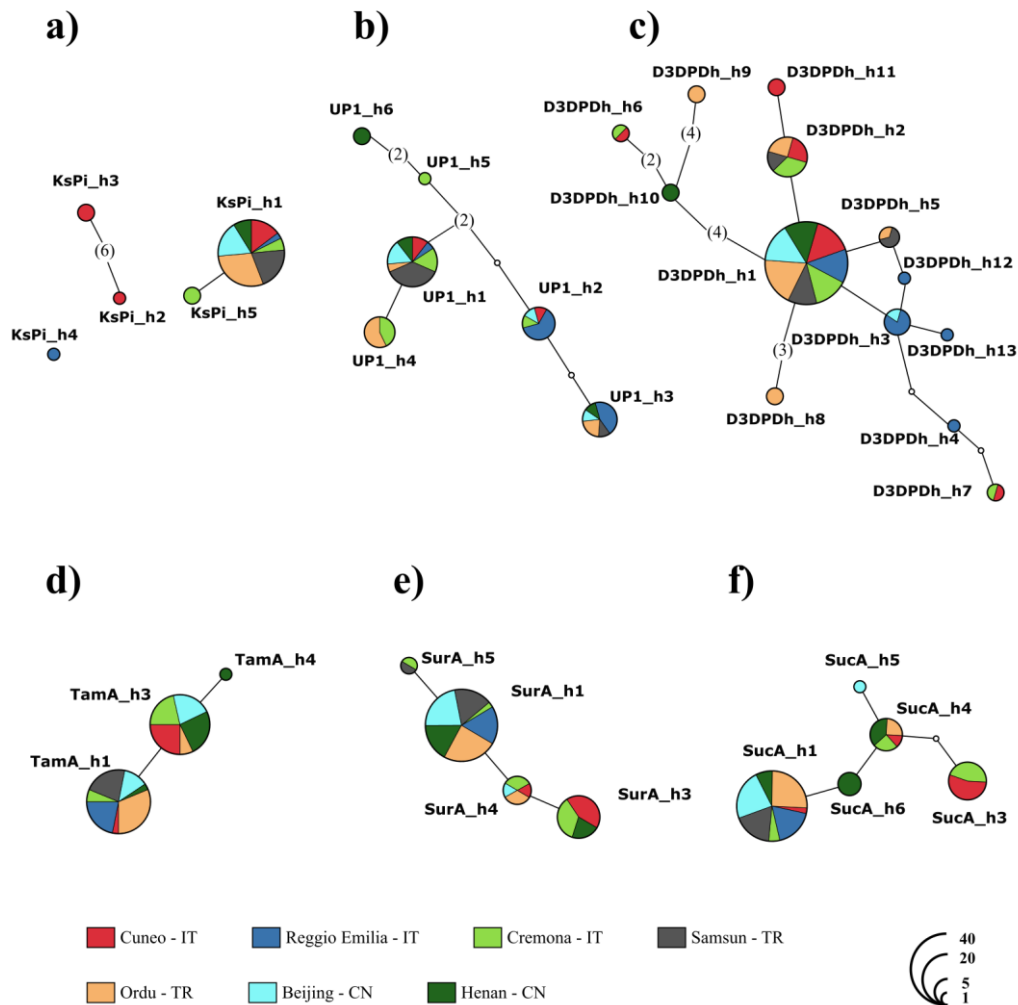
1657 Haplotype inference for Hh_KsPi was performed on a 950 bp region, and
 1658 amplicons were obtained for all the individuals. A total of 40 sequences
 1659 were obtained out of 72 individuals analysed. From the remaining 32
 1660 individuals, amplicons were obtained but the resulting sequences showed
 1661 overlapping peaks after a few high-quality bases, indicating
 1662 heterozygosity. In most of the cases, a difference in the electrophoretic
 1663 mobility was clear, with not perfectly overlapping double peaks. When
 1664 allele inference was performed on remaining electropherograms, many
 1665 indels and SNPs resulted between generated and reference sequences, thus
 1666 indicating a high risk of artifact generation. For these reasons, sequences
 1667 generated from heterozygous individuals were discarded from the analysis.
 1668 A total of five distinct haplotypes were identified (Fig. 1a). Polymorphism
 1669 with reference genome sequence ranged from 1 SNP of KsPi_h5 to 36
 1670 SNPs and six indels of KsPi_h4. Details on number and type of
 1671 polymorphism detected among new haplotypes and reference genome can
 1672 be found in Table S6. All the haplotypes different from Hh_KsPi_h1
 1673 (which was found in all tested populations) were detected from Italian
 1674 samples and all the Italian populations exhibited at least two different
 1675 haplotypes (Fig. 1a; Table 2; Figs S2–S4). Chinese and Turkish
 1676 populations only showed KsPi_h1 haplotype, thus resulting in π and h
 1677 values equal to 0 (Fig. 1a; Table 2; Figs S2–S4).

1678 Haplotype inference for Hh_UP1 was performed on a 392 bp region,
 1679 resulting in 41 sequences from 72 individuals analysed. Nine of them were
 1680 obtained as one allele inferred with Indigo. Given the presence of both
 1681 SNPs and indels in this marker (Table S6), other inferred alleles that
 1682 resulted as singleton were discarded from the analysis to minimise the risk

of generating artefactual sequences. All the individuals showed proper amplification and multiple haplotypes were identified in each population analysed, for a total of six different haplotypes (Fig. 1b; Figs S2–S4). The most prevalent haplotype was UP1_h1 (19 sequences) followed by UP1_h3 (9 sequences) and UP1_h2 (8 sequences) (Fig. 1b; Figs S2–S4). Only UP1_h5 was present as a single copy in a homozygous individual (Fig. 1b). Polymorphisms with reference genome sequence ranged from one SNP of UP1_h4 to five SNPs and one base deletion of UP1_h6. Both π and h were higher in populations from invaded areas compared to what described by mitochondrial markers (Table 2).

Also for Hh_D3PDh amplicons were obtained for all the 72 *H. halys* individuals. Haplotype inference was performed on a 411 bp region, yielding 88 sequences, 48 of which were inferred. Since most of the polymorphisms between the alleles were SNPs, and only a one bp indel was present, allele inference was more successful for this marker (Table S6). A total of 13 haplotypes were identified, with multiple haplotypes detected in each analysed population (Fig. 1c; Figs S2–S4). Populations of invaded areas showed overall higher haplotype diversity compared to native ones (Table 2). The dominant haplotype was D3PDh_h1 haplotype (53 sequences), followed by D3PDh_h2 (12 sequences), and three haplotypes were identified as a single copy (D3PDh_h4, D3PDh_h12 and D3PDh_h13). Indel polymorphisms involved a single base deletion in haplotypes D3PDh_h4, D3PDh_h7 and D3PDh_h8 compared to reference genome. SNP polymorphisms ranged from one SNP (haplotypes D3PDh_h2, D3PDh_h3 and D3PDh_h5) to a maximum of 12 SNPs (haplotype D3PDh_h9). Italian populations generally showed higher π values than using mitochondrial markers. In Turkish populations, both π

1710 and h were generally higher compared to mitochondrial markers. Chinese
1711 populations, instead, showed low π and h values (Table 2).



1712
1713 **Figure 1.** Haplotype distribution according to *H. halys* nuclear markers and
1714 ‘*Candidatus Pantoea carbekii*’ newly identified markers along the sampled
1715 populations. Circle size increases with the number of samples belonging to each
1716 haplotype. Small white circles represent putative or missing haplotypes
1717 forecasted by the analysis. Number of consecutive putative/missing haplotypes

1718 present in the graph are reported in brackets. **a** Hh_KsPi haplotypes, **b** Hh_UP1
1719 haplotypes, **c** Hh_D3PDh haplotypes, **d** Pc_TamA haplotypes, **e** Pc_SurA
1720 haplotypes, **f** Pc_SucA haplotypes.

Table 2. Summary statistics for each population under analysis according to the newly identified markers. Hh_KsPi, Hh_UP1, Hh_D3PDh: *H. halys* nuclear markers; Pc_TamA, Pc_SurA, Pc_SucA: *P. carbekii* markers. Results are given separately for the provinces where samples were collected and for the countries under investigation: Italy includes samples from Cuneo, Reggio Emilia and Cremona; Turkey includes samples from Samsun and Ordu; China includes samples from Beijing and Henan. n: number of sequences; H: number of haplotypes; π : nucleotide diversity; h: haplotype diversity.

Province - Country	n	H	π	h $\pm$ SD	n	H	π	h $\pm$ SD	n	H	π	h $\pm$ SD
Hh_KsPi				Hh_UP1				Hh_D3PDh				
Cuneo - IT	8	3	0.01045	0.607 $\pm$ 0.160	3	2	0.00343	0.667 $\pm$ 0.272	15	5	0.00555	0.695 $\pm$ 0.108
Reggio Emilia - IT	2	2	0.04255	1	10	3	0.00377	0.644 $\pm$ 0.090	14	5	0.00236	0.703 $\pm$ 0.098
Cremona - IT	4	2	0.00075	0.667 $\pm$ 0.144	8	4	0.00376	0.786 $\pm$ 0.098	13	4	0.00550	0.654 $\pm$ 0.102
Samsun - TR	7	1	0	0 $\pm$ 0	8	2	0.00257	0.250 $\pm$ 0.184	10	3	0.00173	0.622 $\pm$ 0.135
Ordu - TR	10	1	0	0 $\pm$ 0	7	3	0.00637	0.667 $\pm$ 0.150	18	5	0.00864	0.673 $\pm$ 0.105
Beijing - CN	6	1	0	0 $\pm$ 0	5	3	0.00514	0.700 $\pm$ 0.208	9	2	0.00054	0.222 $\pm$ 0.170
Henan - CN	3	1	0	0 $\pm$ 0	5	3	0.01080	0.800 $\pm$ 0.129	9	2	0.00473	0.389 $\pm$ 0.164
Italy	14	5	0.01269	0.670 $\pm$ 0.126	21	5	0.00524	0.786 $\pm$ 0.042	42	9	0.00469	0.697 $\pm$ 0.068
Turkey	17	1	0	0 $\pm$ 0	15	3	0.00460	0.648 $\pm$ 0.084	28	5	0.00626	0.643 $\pm$ 0.086
China	9	1	0	0 $\pm$ 0	10	4	0.00811	0.733 $\pm$ 0.114	18	3	0.00281	0.307 $\pm$ 0.133
Pc_TamA				Pc_SurA				Pc_SucA				
Cuneo - IT	8	2	0.00038	0.250 $\pm$ 0.184	7	2	0.00054	0.286 $\pm$ 0.200	8	3	0.00224	0.464 $\pm$ 0.202
Reggio Emilia - IT	7	1	0	0 $\pm$ 0	7	1	0	0 $\pm$ 0	7	1	0	0 $\pm$ 0
Cremona - IT	8	2	0.00064	0.429 $\pm$ 0.167	9	4	0.00221	0.694 $\pm$ 0.143	9	3	0.00312	0.667 $\pm$ 0.125
Samsun - TR	7	1	0	0 $\pm$ 0	8	2	0.00047	0.250 $\pm$ 0.184	7	1	0	0 $\pm$ 0
Ordu - TR	12	2	0.00046	0.303 $\pm$ 0.149	12	2	0.00057	0.303 $\pm$ 0.149	12	2	0.00100	0.303 $\pm$ 0.149
Beijing - CN	10	2	0.00080	0.533 $\pm$ 0.081	10	2	0.00038	0.200 $\pm$ 0.157	10	2	0.00099	0.200 $\pm$ 0.157

Henan - CN	9	3	0.00067	0.417±0.193	10	2	0.00177	0.467±0.127	10	3	0.00154	0.733±0.059
Italy	23	2	0.00077	0.514±0.035	23	4	0.00210	0.660±0.060	24	3	0.00338	0.627±0.048
Turkey	19	2	0.00030	0.199±0.113	20	3	0.00055	0.279±0.125	19	2	0.00066	0.199±0.113
China	19	3	0.00077	0.485±0.103	20	3	0.00115	0.353±0.124	20	4	0.00155	0.605±0.100

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1727

1728 3.3 ‘*Candidatus Pantoea carbekii*’ markers

1729 Amplicons of $\Delta ybgF$ were obtained for 64 out of 66 individuals. Three
 1730 different haplotypes were identified by 56 analysing $\Delta ybgF$ sequences
 1731 (Fig. S1c; Figs S2–S4; Table S5). Two of them had been previously
 1732 described, namely P1 and P2; these haplotypes were nearly equally
 1733 distributed, with 29 and 26 sequences, respectively. The third one was a
 1734 novel haplotype, named P8, found in a single individual from the Henan
 1735 population (CN) (Fig. S4). The highest haplotype diversity (three different
 1736 haplotypes) was identified in the Henan population, while only one
 1737 haplotype was found in the invasive populations of Reggio Emilia (IT) and
 1738 Samsun (TR). In the other populations, both P1 and P2 were present.

1739 Among the newly developed markers, amplicons were obtained for 64 out
 1740 of 66 individuals for Pc_TamA marker. Haplotype inference for Pc_TamA
 1741 was performed on a 665 bp region, revealing three different haplotypes
 1742 (Fig. 1d; Figs S2–S4). Information obtained by this marker was redundant
 1743 with $\Delta ybgF$ in invasive populations (IT – TR), with all the individuals
 1744 having P1 haplotype displaying TamA_h1 and individuals having P2
 1745 haplotype displaying TamA_h3. This was not true for all the native
 1746 individuals (CN), where one individual having P8 haplotype displayed
 1747 TamA_h3 and one individual having P2 haplotype displayed TamA_h4
 1748 (Table S5). No indel with reference genome corresponding sequence was
 1749 found in this or in any other newly identified marker (Table S6). The most
 1750 common haplotype was TamA_h1 (32 sequences), followed by TamA_h3
 1751 (28 sequences) (Fig. 1d). Haplotype TamA_h3 differed by one SNP from
 1752 the corresponding sequence in reference genome, while TamA_h4 by two
 1753 SNPs (Table S6). DNA mutations resulted in amino acids missense with
 1754 TamA_h1 haplotype (Table S7). For both TamA_h3 and TamA_h4 the first

1755 non-synonymous mutation led to the substitution of a Lysine with an
 1756 Asparagine, while the second TamA_h4 missense to the substitution of a
 1757 Methionine with an Isoleucine. The h values were similar to the ones for
 1758 $\Delta ybgF$, whilst π values were generally lower due to the greater length of
 1759 TamA marker compared to $\Delta ybgF$ (Table 2).

1760 Four different Pc_SurA haplotypes have been identified in a 528 bp region
 1761 (Fig. 1e; Figs S2–S4). Proper amplification was obtained for all the
 1762 individuals. All four haplotypes were identified in Italian individuals,
 1763 while three haplotypes were found in Chinese and Turkish individuals
 1764 (Fig. 1e; Figs S2–S4). SurA_h3 haplotype showed two SNPs with
 1765 reference genome sequence, while SurA_h4 and SurA_h5 had a SNP, with
 1766 all these SNPs resulting in amino substitutions (Table S7). The first
 1767 SurA_h3 SNP was shared with SurA_h4 haplotype, resulting in the
 1768 substitution of a Leucine with a Proline, while the second SurA_h3 SNP
 1769 resulted in the substitution of an Asparagine with an Aspartic Acid. The
 1770 SNP of SurA_h5 haplotype was not shared with any other haplotype and
 1771 resulted in the substitution of a Valine with a Glycine (Table S7). Unlike
 1772 for the other *P. carbekii* markers, two haplotypes of Pc_SurA were
 1773 identified in Samsun (TR) population. Higher h values were described for
 1774 this marker in invaded areas compared to $\Delta ybgF$ (Table 2).

1775 A total of 64 amplicons were obtained for Pc_SucA. This marker was the
 1776 most variable for *P. carbekii*, with five different haplotypes identified (Fig.
 1777 1f; Figs S2–S4). Interestingly, only three of them namely SucA_h1,
 1778 SucA_h3 and SucA_h4 were found in invasive populations (IT and TR),
 1779 making this marker the most informative only for the native populations
 1780 (Henan-CN and Beijing-CN) (Fig. S4). The most abundant haplotype was
 1781 SucA_h1, present in 39 individuals along all the populations under

analysis. SucA_h3 and SucA_h4 were almost equally represented, with respectively eleven and eight individuals, but SucA_h3 was only present in Cuneo (IT) and Cremona (IT) populations, whilst SucA_h4 was found also in Ordu (TR) and Henan (CN) populations. SucA_h6 was found only in four samples of the Henan (CN) population, while SucA_h5 in a single individual from Beijing (CN) population. Details of haplotypes distribution can be found in Table S5. SucA_h6/h4/h5/h3 showed 1, 2, 3 and 4 SNPs, respectively, compared to the reference genome sequence (Table S6). Not all the SNPs led to amino acids missense, with just two missenses in total (Table S7). The only SNP of SucA_h6 led to the substitution of an Isoleucine with a Valine, shared with all the other haplotypes. SucA_h3, SucA_h4 and SucA_h5 SNPs resulted in the substitution of Glutamic acid with a Lysine. Also for this marker, higher h values were observed in invaded areas compared to $\Delta ybgF$ (Table 2).

By comparing putative protein structures, even in the cases where mutations led to amino acids with different properties (Table S7), no structural damage was showed by inferring protein 3D structure for any of the markers studied.

3.4 MLST analysis

The combination of COI and COII sequences led to the identification of 13 different haplotypes from 66 individuals (Fig. 2a). Compared with COI, only in the native populations of Beijing (CN) and Henan (CN) the combination with COII increased the number of described haplotypes. This is confirmed by h value, which increased only for Chinese populations (Table 3). In Reggio Emilia (IT) and Samsun (TR) populations, mitochondrial genes identified only a single haplotype (Fig. 2a; Table 3).

Using all four *P. carbekii* markers, the total number of symbiont haplotypes identified out of 48 samples raised from three (obtained using only $\Delta ybgF$) to 10 (Fig. 2b). In line with the host MLST, all populations exhibited at least two different haplotypes, except for that from Reggio Emilia (IT). The higher variability was described for Henan (CN) and Cremona (IT) populations, with six and four haplotypes, respectively. For each population analysed, h values increased compared to those obtained with $\Delta ybgF$ alone (Table 3).

An MLST approach incorporating both mitochondrial and the best-performing nuclear marker (i.e., Hh_D3PDh) was also tested, yielding a total of 78 sequences (Fig. 2c). Compared to the use of COI + COII, the number of described *H. halys* haplotypes increased from 13 to 30 (Fig. 2a and c). Not only native populations, but also invasive populations displayed a higher number of haplotypes. Italian and Turkish populations increased from five and two COI + COII haplotypes to 17 and six, respectively. For all the populations analysed, the h value sensibly increased (Table 3).

A holobiont approach, combining the best performing *H. halys* makers with those from *P. carbekii*, was also tested. By using 52 sequences coming from COI + COII + Hh_D3PDh + the four *P. carbekii* markers, 28 joint *H. halys* + *P. carbekii* haplotypes were identified (Fig. S5). For Henan (CN) and Cremona (IT) populations, h value reached 1 (Table 3).

Finally, the use of the second-best performing *H. halys* nuclear marker (i.e., Hh_UP1) along with mitochondrial and with both mitochondrial and symbiont sequences was assessed (Fig. S6, Table S8). For the combination of mitochondrial markers and Hh_UP1, 41 sequences were used.

1835 Compared to the use of COI + COII the number of identified haplotypes
1836 increased from 13 to 17 (Fig. S6). Despite a general increase in the number
1837 of scored haplotypes, in Cuneo (IT) population the number of haplotypes
1838 decreased from five to three due to the loss of individuals from the analysis
1839 (Table S8). This reduction resulted from the lack of UP1 sequences for
1840 seven individuals of Cuneo (IT) population (Table S5). By combining COI
1841 + COII + Hh_UP1 + the four *P. carbekii* markers only 25 sequences were
1842 available, describing 17 different haplotypes (Fig. S6, Table S8). Except
1843 for Cuneo (IT) and the two Turkish populations of Samsun and Ordu, h
1844 value reached 1 (Table S8).

1845



1848

1849 circles represent putative or missing haplotypes forecasted by the analysis.
 1850 Numbers in round brackets represent the number of consecutive putative/missing
 1851 haplotypes present in the graph. **a** COI+COII markers, **b** $\Delta ybgF$ + Pc_SucA +
 1852 Pc_SurA + Pc_TamA markers, **c** COI+COII+Hh_D3PDh markers

1853

1854 **Table 3.** Summary statistics for each population under analysis using different
 1855 MLST approaches. Results displayed for the provinces where samples were
 1856 collected and for the countries under investigation: Italy comprehends Cuneo,
 1857 Reggio Emilia and Cremona; Turkey comprehends Samsun and Ordu; China
 1858 comprehends Beijing and Henan. *P. carbekii* = $\Delta ybgF$ + Pc_TamA + Pc_SucA +
 1859 Pc_SurA markers. D3PDh = Hh_D3PDh marker. n: number of sequences; H:
 1860 number of haplotypes; π : nucleotide diversity; h: haplotype diversity.

Chapter III – Host and symbiont MLST approach

Province - Country	n	H	π	$h \pm SD$	n	H	π	$h \pm SD$
COI + COII					<i>P. carbekii</i>			
Cuneo - IT	9	5	0.00214	0.806±0.113	5	2	0.00060	0.400±0.239
Reggio Emilia - IT	8	1	0	0	4	1	0	0
Cremona - IT	8	5	0.00252	0.893±0.067	7	4	0.00219	0.714±0.177
Samsun - TR	8	1	0	0	6	2	0.00017	0.333±0.219
Ordu - TR	13	2	0.00053	0.282±0.143	8	2	0.00062	0.250±0.184
Beijing - CN	10	5	0.00148	0.756±0.126	9	3	0.00010	0.667±0.091
Henan - CN	10	6	0.00290	0.889±0.065	9	6	0.00144	0.917±0.058
Italy	25	5	0.00267	0.753±0.062	16	4	0.00211	0.675±0.082
Turkey	21	2	0.00034	0.181±0.105	14	3	0.00043	0.275±0.151
China	20	10	0.00247	0.890±0.048	18	7	0.00135	0.824±0.058
COI + COII + D3PDh					COI + COII + D3PDh + <i>P. carbekii</i>			
Cuneo - IT	13	9	0.00332	0.910±0.064	6	3	0.00086	0.600±0.213
Reggio Emilia - IT	10	4	0.00065	0.711±0.111	4	3	0.00034	0.833±0.188
Cremona - IT	9	7	0.00384	0.944±0.057	8	8	0.00297	1
Samsun - TR	10	3	0.00048	0.622±0.135	8	4	0.00032	0.821±0.084
Ordu - TR	18	6	0.00269	0.778±0.080	12	6	0.00119	0.758±0.120
Beijing - CN	9	4	0.00117	0.694±0.143	8	5	0.00116	0.857±0.096
Henan - CN	9	6	0.00343	0.917±0.058	8	8	0.00219	1
Italy	32	17	0.00342	0.917±0.032	18	12	0.00260	0.935±0.037
Turkey	28	6	0.00193	0.717±0.075	20	7	0.00084	0.763±0.078
China	18	10	0.00254	0.909±0.046	16	13	0.00190	0.967±0.031

1861

1862 3.5 Host and symbiont haplotypes associations

1863 The specialization index H2' was calculated for the association between
1864 COI and COII, COI and the two most informative *P. carbekii* markers
1865 (Pc_SurA and Pc_SucA), COI and the two most informative nuclear
1866 markers (Hh_D3PDh and Hh_UP1), and between the most informative

1867 nuclear and *P. carbekii* markers. The highest network specializations were
 1868 shown between the two mitochondrial markers (COI–COII $H_2' = 0.850$)
 1869 and between COI and the *P. carbekii* markers (COI–Pc_SucA $H_2' = 0.799$;
 1870 COI–Pc_SurA $H_2' = 0.662$). In contrast, Hh_D3PDh showed the lowest
 1871 H_2' values, both in relation to COI (COI–Hh_D3PDh $H_2' = 0.047$) and *P.*
 1872 *cabekii* markers (Hh_D3PDh–Pc_SucA $H_2' = 0.163$; Hh_D3PDh–
 1873 Pc_SurA $H_2' = 0.081$). Moreover, Hh_UP1 demonstrated low network
 1874 specialization, both with COI (COI–Hh_UP1 $H_2' = 0.275$) and with the *P.*
 1875 *cabekii* markers (Hh_UP1–Pc_SucA $H_2' = 0.210$; Hh_UP1–Pc_SurA H_2'
 1876 $= 0.097$). Differences in network specialization between COI with
 1877 Hh_D3PDh and COI with Pc_SurA are shown in Fig. 3a. Bipartite
 1878 interaction matrices for other combinations of markers described in this
 1879 paragraph are shown in Supplementary material (Fig. S7 and S8).
 1880 Interestingly, by considering only the invasive populations (Cuneo-IT,
 1881 Reggio Emilia-IT, Cremona-IT, Samsun-TR and Ordu-TR), a perfect
 1882 network specialization was found between COI and the most informative
 1883 *P. carbekii* markers (COI–Pc_SucA $H_2' = 1$; COI–Pc_SurA $H_2' = 1$) (Fig.
 1884 3 b and c).

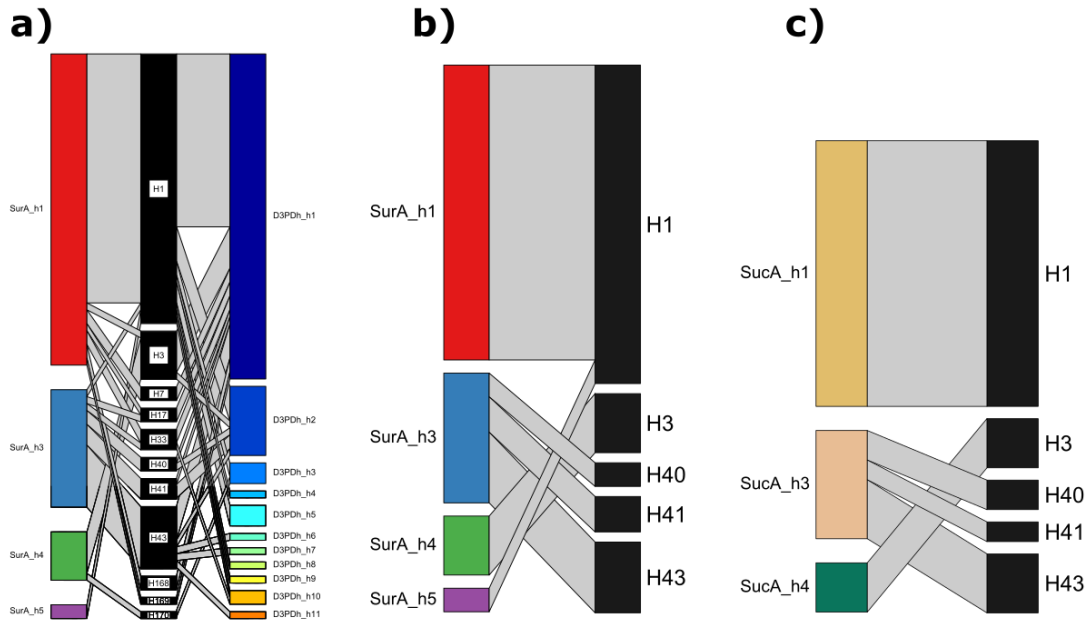


Figure 3. Bipartite networks of interaction between *H. halys* COI haplotypes and novel nuclear and ‘*Candidatus Pantoea carbekii*’ markers haplotypes. The width of the conjunctions represents the frequency with which two haplotypes were found in a same individual. Different colours of the rectangles represent different haplotypes of the used marker. **a** Comparison between Pc_SurA – COI and Hh_D3PDh – COI bipartite interactions, **b** Bipartite network between Pc_SurA and COI in invasive populations, **c** Bipartite network between Pc_SucA and COI in invasive populations.

1895 **4. Discussion**

1896 **4.1 Mitochondrial markers**

1897 The use of molecular markers to reconstruct *H. halys* invasion patterns has
 1898 been widely used over the last decade (Berteloot et al., 2024; Cesari et al.,
 1899 2018, 2015; Gariepy et al., 2021; Kapantaidaki et al., 2019; Laterza et al.,
 1900 2023; Valentin et al., 2017; Xu et al., 2014; Zhu et al., 2016). However,
 1901 defining invasion routes by using one or a few genetic markers has become
 1902 progressively more challenging due to the occurrence of both primary and
 1903 secondary invasions, as well as the growing number of different
 1904 haplotypes in invaded areas over time (Berteloot et al., 2024; Cesari et al.,
 1905 2018; Schuler et al., 2021; Valentin et al., 2017).

1906 In this study, by sequencing a 592 bp region of the COI gene of 71
 1907 individuals, 11 different haplotypes were identified. From Beijing (CN)
 1908 population, two novel haplotypes, H168 and H170, were discovered.
 1909 Haplotype network analysis revealed that H168 is a variant of the
 1910 widespread H1 haplotype, while H170 is a variant of H3, each differing
 1911 from their known counterparts by a SNP. Both H1 and H3 are well
 1912 documented and abundant in Chinese populations (Gariepy et al., 2015;
 1913 Lee et al., 2018; Martinez-Sañudo et al., 2020; Valentin et al., 2017), and
 1914 many haplotypes differing by a SNP from H1 (e.g., H12, H15, H18, H20)
 1915 or from H3 (e.g., H9, H11, H21, H22) have been described before (Cesari
 1916 et al., 2015; Gariepy et al., 2014; Valentin et al., 2017). By increasing the
 1917 number of analysed samples in native areas it is thus expected to identify
 1918 more variants of the most abundantly represented haplotypes. A new
 1919 haplotype, H169, was also identified in an individual from Henan (CN)
 1920 population, differing for one SNP from the less common H7 haplotype,
 1921 previously detected both in China and California (Lee et al., 2018; Valentin

et al., 2017). Interestingly, two individuals of the H3 haplotype were detected in Ordu (TR) population, making the first instance of a haplotype other than H1 being recorded in Turkey. This result suggests that either two separate invasion events occurred in the country, or that this haplotype was not previously detected due to its lower abundance (Yan et al., 2021a). The origin of Turkish populations suggested by COI could be either primary (i.e. from China) or secondary, with Italy being a likely source. Additionally, other haplotypes were identified for the first time in a certain invaded region. For instance, haplotype H40 was newly detected in Cremona (IT) although it had already been present in Cuneo province (IT), suggesting a secondary invasion event (Cesari et al., 2018). These novel haplotype detections may result from the rarity of these haplotypes, which previous studies may have missed due to limited sample size, or they may indicate actual novel invasions. Five haplotypes were identified from COII sequences, including the newly described h22 haplotype, which differs from the common h1 for one SNP, found in a single individual from Henan (CN) population. The presence in Ordu (TR) population of h1 and h3 haplotypes found in both Italian and Chinese populations suggests these two areas as putative sources. Interestingly, the combined use of COI with the less variable COII, commonly employed to increase haplotype resolution (Berteloot et al., 2024; Cesari et al., 2018; Yan et al., 2021a), was more informative than COI alone for the native populations only (Fig. 2a). This finding aligns with a previous study demonstrating that the combined use of these two markers did not outperform COI alone in tracking the origin of *H. halys* populations invasive in Belgium (Berteloot et al., 2024). Moreover, the high network specialization between these two markers ($H_2' = 0.850$) confirms that COII and COI haplotypes tend to be

1949 associated. The reduced number of individuals retained for this combined
 1950 analysis (66 out of 72) resulted from the fact that high-quality sequences
 1951 for both markers were obtained for every stink bug (Table S5). As the
 1952 number of sequenced loci increases, the risk of not obtaining high-quality
 1953 sequences for each marker in every individual also increases. Since even
 1954 single miscalled SNP can result in different haplotype attributions, unclear
 1955 sequences were discarded, leading to a reduced number of individuals
 1956 retained for MLST approaches. Increasing informativeness by adding
 1957 molecular markers comes with a higher risk of reducing the number of
 1958 samples retained for the analysis (Cesari *et al.*, 2018; Berteloot *et al.*,
 1959 2024). These results suggest that markers other than COII might be more
 1960 effective to further explore *H. halys* genetic diversity, also according to the
 1961 occurrence of other, more variable, mitochondrial regions, reported by
 1962 Gogniashvili *et al.* (2021).

1963 **4.2 Symbiont markers**

1964 Since vertically transmitted endosymbionts genomes tend to coevolve with
 1965 the host (Funk *et al.*, 2000), symbiont markers have been proposed to
 1966 reconstruct the movement of invasive insect species (El Sheikha and
 1967 Menozzi, 2019). However, the *P. carbekii* $\Delta ybgF$ marker, so far known in
 1968 seven haplotypes, has shown to be not variable enough to be used for
 1969 properly reconstructing the phylogeography of the host (Martinez-Sañudo
 1970 *et al.*, 2020; Otero-Bravo and Sabree, 2018). In this study, the novel P8
 1971 haplotype was identified in a single individual from Henan population, but
 1972 the overall low variability of the marker was reaffirmed. To address this
 1973 insufficiency, three novel markers were developed in this study by
 1974 exploiting *P. carbekii* annotated genome. Except for Pc_TamA, these
 1975 markers exhibited greater variability than $\Delta ybgF$. Interestingly, even

1976 though five different haplotypes were identified for Pc_SucA, only three
 1977 were found in invaded areas (SucA_h1/h3/h4). On the other hand,
 1978 Pc_SurA revealed four haplotypes, all of which were present in invasive
 1979 populations (Fig. 1f). Notably, in the case of Samsun (TR) population,
 1980 Pc_SurA was more informative than COI, identifying two haplotypes
 1981 instead of only one. The presence in this population of a higher genetic
 1982 variability than the one described by COI only is confirmed by analyzing
 1983 *H. halys* nuclear markers Hh_UP1 and Hh_D3PDh (Fig. 1b and c). Only
 1984 with all four symbiont markers an optimal characterization of the symbiont
 1985 is achieved since each one explains part of the genetic variability
 1986 unrevealed by the others. This approach led to the identification of 10 *P.*
 1987 *carbekii* haplotypes in 48 individuals, two more than those previously
 1988 identified using $\Delta ybgF$ alone (Fig. 2b). Sample size reduction occurs
 1989 because increasing the number of markers also increases the risk of
 1990 obtaining unclear sequences in at least one of them, which may result in
 1991 discarding the individual from the analysis. The presence of two shared
 1992 haplotypes between Italian and Turkish populations (P2 – SucA_h4 –
 1993 SurA_h4 – TamA_h3; P1 – SucA_h1 – SurA_h5 – TamA_h1) and only
 1994 one common to all the countries (P1 – SucA_h1 – SurA_h1 – TamA_h1)
 1995 (Fig. 2b) suggests that a secondary invasion event, from Italy to Turkey,
 1996 could be the source of Turkish populations. To confirm this hypothesis,
 1997 however, detailed studies with larger sampling size and using these newly
 1998 described markers are needed. No indels were observed in the haplotypes
 1999 of the newly identified *P. carbekii* markers compared to the reference
 2000 genome, which is expected given that all these markers were derived from
 2001 coding regions. Interestingly, in several cases, SNPs were related to
 2002 missense mutation, even with the presence of amino acids characterized

by different properties (Table S7). However, protein structure predictions showed no structural damage, suggesting that these coding gene variations are not detrimental to *P. carbekii*; to confirm this, the full sequence of the gene is required. These findings suggest that *P. carbekii* exhibits far greater genetic variability than previously assumed, which may enhance its potential as a tool for tracing the origin of *H. halys* invasive populations. The functional implication of the recorded variability is still to be determined, but we cannot exclude that symbiont variants are involved in different insect performances, with additional consequences on invasive capability.

4.3 MLST: Host mitochondrial and symbiont markers

In a previous study, seven *P. carbekii* haplotypes associated with 25 COI *H. halys* haplotypes were identified (Martinez-Sañudo et al. 2020). In the current study, by combining the four *P. carbekii* markers mentioned above, 10 bacterial haplotypes were found to be associated with 11 *H. halys* COI haplotypes. In accordance with the findings of Martinez-Sañudo et al. (2020), the H2' value highlights a strong network specialization between *H. halys* COI haplotypes and those of the symbiont, even when considering more informative markers such as Pc_SucA and Pc_SurA. As expected for two separate genomes, the specialization between *H. halys* COI and *P. carbekii* markers is lower than the one between COI and COII. This suggests that using COI in combination with *P. carbekii* markers may yield more informative results than using COI and COII. Interestingly, the result changes drastically when only invasive populations are considered. In these populations, the perfect network specialization between COI and the symbiont markers strengthens the hypothesis that certain host-symbiont variants may possess enhanced invasive capabilities (Martinez-Sañudo et

al., 2020) (Fig. 3 b and c). However, further studies are required to understand if this perfect specialization is just the result of the genetic bottleneck, that invasive populations normally undergo (Croft et al., 2024; Puillandre et al., 2008; Tsutsui et al., 2000), or if these host-symbiont variants have an advantage in invading new areas compared to other haplotype combinations. Furthermore, it would be valuable to investigate whether specific functional variants in the genome of *P. carbekii* can contribute to increased host adaptability.

4.4 Nuclear markers

The variability of one or few mitochondrial markers can be insufficient to clearly define the invasion routes. Alternative strategies such as the use of the whole mitochondrial sequence (Morón-López et al., 2022) or the joint use of mitochondrial and nuclear markers to overcome the limit of only matrilineal inheritance (Li et al., 2015) proved to be more effective in population genetics studies and invasion events reconstruction. Thus, several authors suggested to expand the use of *H. halys* nuclear markers to overcome the lack of clear genetic discontinuity between areas identified by mitochondrial markers (Cesari et al., 2018; Valentin et al., 2017). For this purpose, in this study, three novel nuclear markers have been identified. The best performing nuclear marker was Hh_D3PDh. Since only one indel of one nucleotide was present between the haplotypes, this marker suits well for allele inference process with bioinformatic tools such as Tracy (Rausch et al., 2020). The lower π and h values observed in native populations compared to invasive ones could result from continuous inbreeding occurring in the laboratory-reared colony (Beijing – CN). Another reason could be related to the ongoing primary and secondary invasion events occurring in invaded areas, as described in Italy (Cesari et

2057 *al.*, 2018; Schuler *et al.*, 2021). The concomitant presence of haplotypes
 2058 normally separated in native areas can lead to genetic admixture (Parvizi
 2059 *et al.*, 2023). Both Hh_D3PDh and Hh_UP1 markers showed one shared
 2060 haplotype between Italian and Turkish populations (D3PDh_h2 and
 2061 UP1_h4) (Fig. 1b and c), thus corroborating the hypothesis of a secondary
 2062 invasion event in Turkey. Broader studies on invasion pathway
 2063 reconstruction are needed to confirm the source of Turkish populations.

2064 Interestingly, the low H2' value obtained for both Hh_D3PDh and
 2065 Hh_UP1 indicates very low network specialization between nuclear
 2066 markers and both mitochondrial and symbiont markers. This result
 2067 confirms the great potential of nuclear markers in adding informativity to
 2068 the analyses (Cesari *et al.*, 2018; Valentin *et al.*, 2017). However, for almost
 2069 half of the individuals tested (32 for Hh_KsPi; 32 for Hh_D3PDh; 38 for
 2070 Hh_UP1), heterozygosity in these markers' gene regions was observed and
 2071 for Hh_KsPi the discrimination between alleles was unreliable due to the
 2072 presence of multiple SNPs and indels. An NGS-based approach applied to
 2073 the proposed nuclear targets could allow surpassing heterozygosity issues.
 2074 The relatively short regions corresponding to both Hh_D3PDh and
 2075 Hh_UP1 markers make them suitable for high-throughput short-read
 2076 sequencing, thus eliminating the need for allele inference, while Hh_KsPi
 2077 marker, corresponding to a longer gene region, could potentially better
 2078 describe individual variability due to the concurrent presence of multiple
 2079 indels and SNPs.

2080 **Conclusions**

2081 The aim of this study was to identify and validate novel *H. halys* nuclear
 2082 and *P. carbekii* markers, for this reason seven populations were considered,

either European or Chinese. These markers are expected to be valuable tools for population genetics studies and shed light on the invasion sources of *H. halys* populations, helping in preventing or limiting outbreaks. Future studies on invasion pathways reconstruction should be performed, involving populations sampled in more regions, from recently invaded, such as North Africa or Central Asia, to firstly invaded ones such as North America. Since the use of multiple markers tends to increase the risk of losing some individuals from the analysis (Berteloot et al., 2024; Cesari et al., 2018; Yan et al., 2021a), the selection of a limited number of reliable and variable markers is pivotal to increase the informativity of the analysis, keeping it cost-effective and not time consuming. For these reasons a sort of “top-down MLST” approach could be preferable. A first screening with the widely used COI would help in defining putative source regions. A further discrimination with *P. carbekii* markers such as Pc_SurA or Pc_SucA could help narrowing the range of possible sources. The addition of nuclear markers could eventually help in further limiting the possible spectrum when previous markers give an ambiguous answer. Hh_D3PDh is preferable when Sanger sequencing methods are used, while Hh_UP1 and Hh_KsPi suit better high throughput sequencing. Future efforts to strengthen this approach should be conducted to create larger databases with sequences for the here proposed markers from both native and invaded areas.

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2473

2474 **SUPPLEMENTARY INFORMATION**

2475 **Table S1.** Details of target sequences, primer used for amplification, and annealing temperature of markers used for *Halyomorpha*
2476 *halys* and ‘*Candidatus* Pantoea carbekii ’ haplotyping. Asterisks indicate markers found in literature, specifically * = Cesari et al.,
2477 2018. **= Otero-Bravo and Sabree, 2018.

2478

Organism	Target	Primer names	Primer sequence (5'-3')	Annealing Temp.	Amplicon position (bp)	Corresponding gene	
<i>Haltomorpha halys</i> (mitochondrion)	COI	Hh_Alt-COI-F	CTAAAAATTTTCAGCCACTTTA	52.0°C	1366-2159	Cytochrome c oxidase subunit I	2480
		Hh_Alt-COI-R	TATGGGAAATTAAGCCAAATC				2481
	COII	HhalysCO2altF	TTTTAACCCAAGATGCAAATTC	48.0°C	*	Cytochrome c oxidase subunit II	2482
		HhalysCO2altR	TTGTTCTTTCAATTACGATTGG				2483
<i>Haltomorpha halys</i> (nucleus)	D3PDh	Hh_D3PDh-F	TAGGGCAGTCAACATTCGGC	59.5 °C	36173-36666	D-3-phosphoglycerate dehydrogenase	2484
		Hh_D3PDh-R	AAAACGTCCGTTTGTGCTGG				2485
	UP1	Hh_UP1-F	CGCCATGTAGGAAGGTCCTC	60.0°C	307699-308198	Uncharacterized prot LOC106692253	2486
		Hh_UP1-R	TCTTTCGTGGGTGACTCTGC				2487
	KsPi	KsPi_intron_2-F	GGCAACGCTTCCACAATCTG	58.5°C	336427-337443	Kazal-type serine protease inhibitor domain-containing	2488
		KsPi_intron_2-R	TAGACTGCGAAGCCAAAGGG				2489
' <i>Candidatus Pantoea carbekii</i> '	Δ YbgF	Δ Ybg-F	TAGTGCAAGCAGTCGT	50.0°C	**	Pseudogene	2490
		dYbg-R	GAGTATCTTGCTTATGTTGC				2491
	SucA	Pc_SucA-F	AAGCTAGGTTTTCTGCGCCT	57.2°C	543436-544123	2-oxoglutarate dehydrogenase E1 component	2492
		Pc_SucA-R	ATGTCACAAGCTCGCGGTTA				2493
	TamA	Pc_TamA-F	CTCTTGTTACAAAGGGCTCAACA	58.6°C	399277-400025	Autotransporter assembly complex prot TamA	2494
		Pc_TamA-R	GACGCGGTGATACAAAAGCA				2495
	SurA	Pc_SurA-F	GAAGCATTACAAGATGGCAGCA	58.6°C	666772-667372	Peptidylprolyl isomerase	2496
		Pc_SurA-R	TCACATAAGCCATGCCTCGTT				2497

Table S2. List of accession numbers of *Halyomorpha halys* COI haplotypes used for haplotype inference. The list does not include the abundance of sequences present in literature.

Accession_number	Haplotype	Reference
KF273380	H1	Gariepy et al., 2014
KF273381	H2	Gariepy et al., 2014
KF273382	H3	Gariepy et al., 2014
KF273383	H4	Gariepy et al., 2014
KF273384	H5	Gariepy et al., 2014
KF273385	H6	Gariepy et al., 2014
KF273386	H7	Gariepy et al., 2014
KF273387	H8	Gariepy et al., 2014
KF273388	H9	Gariepy et al., 2014
KF273389	H10	Gariepy et al., 2014
KF273390	H11	Gariepy et al., 2014
KF273391	H12	Gariepy et al., 2014
KF273392	H13	Gariepy et al., 2014
KF273393	H14	Gariepy et al., 2014
KF273394	H15	Gariepy et al., 2014
KF273395	H16	Gariepy et al., 2014
KF273396	H17	Gariepy et al., 2014
KF273397	H18	Gariepy et al., 2014
KF273398	H19	Gariepy et al., 2014
KF273399	H20	Gariepy et al., 2014
KF273400	H21	Gariepy et al., 2014
KF273401	H22	Gariepy et al., 2014
KF273402	H23	Gariepy et al., 2014
KX017333	H24	Lee et al., 2018
JX548483	H25	Tillman et al., 2015

KF112039	H26	Xu et al., 2014
MF537223	H28	Valentin et al., 2017
KR070749	H30	Gariepy et al., 2015
KR070748	H31	Gariepy et al., 2015
KR070750	H32	Gariepy et al., 2015
KR070751	H33	Gariepy et al., 2015
KX017331	H34	Lee et al., 2018
KX017376	H35	Lee et al., 2018
KX017360	H36	Lee et al., 2018
KX017355	H37	Lee et al., 2018
KX017337	H38	Lee et al., 2018
KX017357	H39	Lee et al., 2018
KY710346	H40	Cesari et al., 2018
KX017346	H41	Lee et al., 2018
KX017354	H42	Lee et al., 2018
KX017332	H43	Lee et al., 2018
KX017348	H44	Lee et al., 2018
KX017373	H45	Lee et al., 2018
KX017367	H46	Lee et al., 2018
KX017383	H47	Lee et al., 2018
KX017370	H48	Lee et al., 2018
KX017334	H49	Lee et al., 2018
KX017388	H50	Lee et al., 2018
KX017394	H51	Lee et al., 2018
MF537243	H52	Valentin et al., 2017
MF537247	H56	Valentin et al., 2017
MT765144	H63	Martinez-Sañudo et al., 2020
MT765146	H65	Martinez-Sañudo et al., 2020
MT765147	H66	Martinez-Sañudo et al., 2020
MT765148	H67	Martinez-Sañudo et al., 2020

MT765149	H79	Martinez-Sañudo et al., 2020
MK779996	H162	Kapantaidaki et al., 2019
MK779997	H163	Kapantaidaki et al., 2019
MT199091	H164	Schuler et al., 2020
MT199092	H165	Schuler et al., 2020
MT199093	H166	Schuler et al., 2020
MT199094	H167	Schuler et al., 2020
KU601519	H1017	Dhami et al., 2016
MT517231	N4	Yan et al., 2021
MT517234	N7	Yan et al., 2021
MT517236	N9	Yan et al., 2021
MT517239	N12	Yan et al., 2021
MT517240	N13	Yan et al., 2021
MT517241	N14	Yan et al., 2021
MT517248	N21	Yan et al., 2021
MT517249	N22	Yan et al., 2021
MT517251	N24	Yan et al., 2021
MT517252	N25	Yan et al., 2021
MT517253	N26	Yan et al., 2021
MT517254	N27	Yan et al., 2021
MT517255	N28	Yan et al., 2021
MT517256	N29	Yan et al., 2021
MT517257	N30	Yan et al., 2021
MT517259	N32	Yan et al., 2021
MT517260	N33	Yan et al., 2021
MT517261	N34	Yan et al., 2021
MT517262	N35	Yan et al., 2021
MT517263	N36	Yan et al., 2021
MT517264	N37	Yan et al., 2021
MT517265	N38	Yan et al., 2021

MT517266	N39	Yan et al., 2021
MT517267	N40	Yan et al., 2021
MT517268	N41	Yan et al., 2021
MT517270	N43	Yan et al., 2021
MT517272	N45	Yan et al., 2021
MT517273	N46	Yan et al., 2021
MT517274	N47	Yan et al., 2021

2494

2495 **Table S3.** List of accession numbers of *Halyomorpha halys* COII haplotypes used
 2496 for haplotype inference. The list does not include the abundance of sequences
 2497 present in literature.

Accession_number	Haplotype	Reference
KF112000	h1	Xu et al., 2014
KF112001	h2	Xu et al., 2014
KF112002	h3	Xu et al., 2014
KF112003	h4	Xu et al., 2014
KF112004	h5	Xu et al., 2014
KF112005	h6	Xu et al., 2014
KF112006	h7	Xu et al., 2014
KF112007	h8	Xu et al., 2014
KF112008	h9	Xu et al., 2014
KF112009	h10	Xu et al., 2014
KF112010	h11	Xu et al., 2014
KF112011	h12	Xu et al., 2014
KF112012	h13	Xu et al., 2014
KF112013	h14	Xu et al., 2014
KF112014	h15	Xu et al., 2014
KF112015	h16	Xu et al., 2014

KF112016	h17	Xu et al., 2014
KF112017	h18	Xu et al., 2014
KY710617	h20	Cesari et al., 2018
KY710595	h21	Cesari et al., 2018
MT490838	n1	Yan et al., 2021
MT490839	n2	Yan et al., 2021
MT490840	n3	Yan et al., 2021
MT490841	n4	Yan et al., 2021
MT490842	n5	Yan et al., 2021
MT490843	n6	Yan et al., 2021
MT490845	n8	Yan et al., 2021
MT490847	n10	Yan et al., 2021
MT490848	n11	Yan et al., 2021
MT490849	n12	Yan et al., 2021
MT490850	n13	Yan et al., 2021
MT490851	n14	Yan et al., 2021
MT490852	n15	Yan et al., 2021
MT490853	n16	Yan et al., 2021
MT490854	n17	Yan et al., 2021
MT490855	n18	Yan et al., 2021
MT490856	n19	Yan et al., 2021
MT490857	n20	Yan et al., 2021
MT490859	n22	Yan et al., 2021
MT490860	n23	Yan et al., 2021

2498

2499 **Table S4.** List of accession numbers of ‘*Candidatus Pantoea carbekii*’ $\Delta YbgF$
2500 haplotypes used for haplotype inference. The list does not include the abundance
2501 of sequences present in literature.

2502

Accession_number	Haplotype	Reference
KY379175	P1	Otero-Bravo and Sabree, 2018
KY379176	P2	Otero-Bravo and Sabree, 2018
MT773137	P3	Martinez-Sañudo et al., 2020
MT773138	P4	Martinez-Sañudo et al., 2020
MT773139	P5	Martinez-Sañudo et al., 2020
MT773140	P6	Martinez-Sañudo et al., 2020
MT773141	P7	Martinez-Sañudo et al., 2020

2503

Table S5. Haplotypes identified in each individual for each marker under analysis. COI and COII are mitochondrial markers of *H. halys*. All the markers starting with "Hh_" are novel insect nuclear markers. $\Delta ybgF$ is the commonly used pseudogene of *P. carbekii*. All the markers starting with "Pc_" are novel symbiont markers. Haplotypes written in bold are newly described haplotypes of previously known markers. R.E = Reggio Emilia Province.

Sample name	COI	COII	Hh_D3Ph allele1	Hh_D3PDh allele2	Hh_UP1	Hh_KsPi	$\Delta ybgF$	Pc_SucA	Pc_SurA	Pc_TamA	Province
Hh_208	H43	h3	D3PDh_h1			KsPi_h2	P2	SucA_h3	SurA_h3	TamA_h3	Cuneo
Hh_209	H43	h3	D3PDh_h1	D3PDh_h11		KsPi_h1	P2	SucA_h3	SurA_h3	TamA_h3	Cuneo
Hh_210	H41	h3	D3PDh_h1	D3PDh_h2	UP1_h1	KsPi_h1	P2		SurA_h3	TamA_h3	Cuneo
Hh_211	H40	h3	D3PDh_h6	D3PDh_h7	UP1_h1	KsPi_h3		SucA_h3			Cuneo
Hh_212		h3	D3PDh_h1	D3PDh_h2		KsPi_h1					Cuneo
Hh_213	H3	h3	D3PDh_h1				P2	SucA_h4	SurA_h4	TamA_h3	Cuneo
Hh_214	H1	h1	D3PDh_h1	D3PDh_h11	UP1_h2	KsPi_h1	P1	SucA_h1		TamA_h1	Cuneo
Hh_215	H41	h3	D3PDh_h2			KsPi_h1		SucA_h3	SurA_h3	TamA_h3	Cuneo
Hh_216	H43	h3	D3PDh_h1			KsPi_h2	P2	SucA_h3	SurA_h3	TamA_h3	Cuneo
Hh_217	H43	h3	D3PDh_h1				P2	SucA_h3	SurA_h3	TamA_h3	Cuneo
Hh_224	H1	h1	D3PDh_h1	D3PDh_h3	UP1_h2			SucA_h1	SurA_h1	TamA_h1	R. E.
Hh_225	H1	h1	D3PDh_h1	D3PDh_h12	UP1_h2	KsPi_h4					R. E.
Hh_226	H1	h1	D3PDh_h3		UP1_h2						R. E.

Hh_227	H1	h1	D3PDh_h1	D3PDh_h3	UP1_h3		P1	SucA_h1	SurA_h1	TamA_h1	R. E.
Hh_228	H1		D3PDh_h1	D3PDh_h3	UP1_h3						R. E.
Hh_229	H1		D3PDh_h1	D3PDh_h13	UP1_h3						R. E.
Hh_230	H1	h1	D3PDh_h1		UP1_h3			SucA_h1	SurA_h1	TamA_h1	R. E.
Hh_231	H1				UP1_h1		P1	SucA_h1	SurA_h1	TamA_h1	R. E.
Hh_232	H1	h1	D3PDh_h1			KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	R. E.
Hh_233	H1	h1	D3PDh_h4		UP1_h2		P1	SucA_h1	SurA_h1	TamA_h1	R. E.
Hh_234	H1	h1			UP1_h2			SucA_h1	SurA_h1	TamA_h1	R. E.
Hh_235	H43		D3PDh_h1	D3PDh_h2	UP1_h1	KsPi_h1	P2	SucA_h3	SurA_h3		Cremona
Hh_236	H1	h1	D3PDh_h1		UP1_h4		P1	SucA_h1	SurA_h1	TamA_h1	Cremona
Hh_237	H43	h3	D3PDh_h6	D3PDh_h7			P2	SucA_h3	SurA_h3	TamA_h3	Cremona
Hh_238	H40	h3	D3PDh_h1	D3PDh_h2	UP1_h1	KsPi_h1	P2	SucA_h3	SurA_h3	TamA_h3	Cremona
Hh_239	H3	h3	D3PDh_h1	D3PDh_h2			P2	SucA_h4	SurA_h4	TamA_h3	Cremona
Hh_240	H41	h3			UP1_h1	KsPi_h5	P2	SucA_h3	SurA_h3	TamA_h3	Cremona
Hh_241	H1	h1	D3PDh_h1			KsPi_h5	P1	SucA_h1	SurA_h5	TamA_h1	Cremona
Hh_242	H40		D3PDh_h1	D3PDh_h2	UP1_h1						Cremona
Hh_243	H40	h3			UP1_h5		P2	SucA_h3	SurA_h3	TamA_h3	Cremona
Hh_244	H3	h3	D3PDh_h1		UP1_h4			SucA_h4	SurA_h4	TamA_h3	Cremona
Hh_245	H1	h1	D3PDh_h1	D3PDh_h5	UP1_h3	KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Samsun
Hh_246	H1	h1	D3PDh_h1		UP1_h1	KsPi_h1		SucA_h1	SurA_h1	TamA_h1	Samsun

Hh_247	H1	h1	D3PDh_h1		UP1_h1	KsPi_h1	P1	SucA_h1	SurA_h5	TamA_h1	Samsun
Hh_248	H1	h1	D3PDh_h1	D3PDh_h2	UP1_h1	KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Samsun
Hh_249	H1	h1	D3PDh_h1		UP1_h1	KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Samsun
Hh_250	H1	h1	D3PDh_h2		UP1_h1	KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Samsun
Hh_251	H1	h1	D3PDh_h5		UP1_h1		P1	SucA_h1	SurA_h1	TamA_h1	Samsun
Hh_252	H1	h1	D3PDh_h1		UP1_h1	KsPi_h1	P1		SurA_h1		Samsun
Hh_253	H1	h1			UP1_h3	KsPi_h1	P1		SurA_h1	TamA_h1	Ordu
Hh_254	H1	h1	D3PDh_h1		UP1_h3	KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Ordu
Hh_255	H1	h1	D3PDh_h1			KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Ordu
Hh_256	H1	h1	D3PDh_h1	D3PDh_h2	UP1_h4	KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Ordu
Hh_257	H1	h1	D3PDh_h1	D3PDh_h2	UP1_h4	KsPi_h1	P1	SucA_h1	SurA_h1		Ordu
Hh_258	H1	h1	D3PDh_h1	D3PDh_h5	UP1_h4	KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Ordu
Hh_259	H1	h1	D3PDh_h8	D3PDh_h9		KsPi_h1	P1	SucA_h1		TamA_h1	Ordu
Hh_260	H1	h1	D3PDh_h8	D3PDh_h9		KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Ordu
Hh_261	H1	h1	D3PDh_h1					SucA_h1	SurA_h1	TamA_h1	Ordu
Hh_262	H1	h1	D3PDh_h1	D3PDh_h2		KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Ordu
Hh_263	H3	h3	D3PDh_h1			KsPi_h1	P2	SucA_h4	SurA_h4	TamA_h3	Ordu
Hh_264	H1	h1	D3PDh_h1		UP1_h4		P1	SucA_h1	SurA_h1	TamA_h1	Ordu
Hh_265	H3	h3	D3PDh_h1					SucA_h4	SurA_h4	TamA_h3	Ordu
Hh_Beijing_1	H3	h5	D3PDh_h1			KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Beijing

Hh_Beijing_2	H170	h3	D3PDh_h3	UP1_h1	KsPi_h1	P2	SucA_h5	SurA_h4	TamA_h3	Beijing
Hh_Beijing_3	H168	h3	D3PDh_h1	UP1_h2	KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Beijing
Hh_Beijing_4	H168	h3	D3PDh_h1			P1	SucA_h1	SurA_h1	TamA_h1	Beijing
Hh_Beijing_5	H1	h1	D3PDh_h1			P2	SucA_h1	SurA_h1	TamA_h3	Beijing
Hh_Beijing_6	H1	h1	D3PDh_h1			P1	SucA_h1	SurA_h1	TamA_h1	Beijing
Hh_Beijing_7	H1	h1	D3PDh_h1	UP1_h1	KsPi_h1	P2	SucA_h1	SurA_h1	TamA_h3	Beijing
Hh_Beijing_8	H1	n15			KsPi_h1	P2	SucA_h1	SurA_h1	TamA_h3	Beijing
Hh_Beijing_9	H1	h1	D3PDh_h1	UP1_h3			SucA_h1	SurA_h1	TamA_h3	Beijing
Hh_Beijing_10	H1	h1	D3PDh_h1		KsPi_h1	P2	SucA_h1	SurA_h1	TamA_h3	Beijing
Hh_Henan_1	H1	h1	D3PDh_h10	UP1_h3		P8	SucA_h4	SurA_h3	TamA_h3	Henan
Hh_Henan_2	H7	h1	D3PDh_h1			P2	SucA_h6	SurA_h1	TamA_h4	Henan
Hh_Henan_3	H33	h3	D3PDh_h1			P2	SucA_h6	SurA_h1		Henan
Hh_Henan_4	H33	h3	D3PDh_h1			P2	SucA_h4	SurA_h3	TamA_h3	Henan
Hh_Henan_5	H7	h1	D3PDh_h1			P2	SucA_h1	SurA_h1	TamA_h3	Henan
Hh_Henan_6	H33	h3	D3PDh_h10			P2	SucA_h6	SurA_h1	TamA_h3	Henan
Hh_Henan_7	H17	h1	D3PDh_h1		KsPi_h1	P2	SucA_h1	SurA_h1	TamA_h3	Henan
Hh_Henan_8	H17	h1	D3PDh_h1	UP1_h6	KsPi_h1	P2	SucA_h4	SurA_h3	TamA_h3	Henan
Hh_Henan_9	H169	h1	D3PDh_h1	UP1_h6		P2	SucA_h6	SurA_h1	TamA_h3	Henan
Hh_Henan_10	H7	h22			KsPi_h1	P1	SucA_h1	SurA_h1	TamA_h1	Henan

Table S6. Summary of polymorphisms among different haplotypes and reference genomes for the newly identified molecular markers

Target organism	Marker	haplotype	SNPs	insertions	deletions
<i>Halyomorpha halys</i> Ref: GCF_000696795	KsPi	h2	16	2	1
		h3	15	2	1
		h4	36	4	2
		h5	1	0	0
	UP1	h2	2	0	1
		h3	4	0	2
		h4	1	0	0
		h5	2	0	1
		h6	5	0	1
	D3PDh	h2	1	0	0
		h3	1	0	0
		h4	1	0	1
		h5	1	0	0
		h6	8	0	0
		h7	4	0	1
		h8	3	0	1
		h9	12	0	0
		h10	5	0	0
		h11	2	0	0
		h12	2	0	0
		h13	2	0	0
<i>Candidatus Pantoea carbekii</i> Ref: NZ_CP010907	TamA	h3	1	0	0
		h4	2	0	0
	SucA	h3	4	0	0
		h4	2	0	0
		h5	3	0	0
		h6	1	0	0
	SurA	h3	2	0	0
		h4	1	0	0
		h5	1	0	0

2512

2513 **Table S7.** Amino acid missenses resulting from the comparison between newly
 2514 identified '*Candidatus* Pantoea carbekii' haplotypes and h1 (= sequence in
 2515 reference genome, Acc. Num.: NZ_CP010907.1). To avoid frameshift, the
 2516 flanking 5' and 3' regions were substituted with the sequence present in the
 2517 reference genome before translation.

Marker	Haplotype	Amino acid change	Position in primary structure	Amino acid property
Pc_TamA	TamA_h3/h4	Lys → Asn	85	+ charged → polar aa
	TamA_h4	Met → Ile	131	Both non-polar
Pc_SurA	SurA_h5	Val → Gly	282	Both non-polar
	SurA_h3/h4	Leu → Pro	293	Both non-polar
	SurA_h3	Asn → Asp	384	Polar → - charged aa
Pc_SucA	SucA_h3/h4/h5/h6	Ile → Val	436	Both non-polar
	SucA_h3/h4/h5	Glu → Lys	520	- charged → + charged aa

2518

2519 **Table S8.** Summary statistics for each population under analysis with different
 2520 MLST approaches using Hh_UP1 as nuclear marker. Results are given separately
 2521 for the regions where samples were collected and for the countries under
 2522 investigation: Italy comprehends samples from Cuneo, Reggio Emilia and
 2523 Cremona; Turkey samples are from Samsun and Ordu; China samples from
 2524 Beijing and Henan. *P. carbekii* = $\Delta ybgF$ + Pc_TamA + Pc_SucA + Pc_SurA
 2525 markers. n: number of sequences; H: number of haplotypes; π : nucleotide
 2526 diversity; h: haplotype diversity.

Province - Country	n	H	π	$h \pm SD$	n	H	π	$h \pm SD$
COI + COII + UP1					COI + COII + UP1 + <i>P. carbekii</i>			
Cuneo – IT	3	3	0.00321	1	0	0	0	0
Reggio Emilia – IT	7	2	0.00066	0.476±0.165	2	2	0.00058	1
Cremona – IT	6	6	0.00275	1	5	5	0.00237	1
Samsun – TR	8	2	0.00069	0.250±0.184	6	3	0.00048	0.600±0.213
Ordu – TR	7	3	0.00210	0.667±0.150	4	2	0.00072	0.500±0.258
Beijing – CN	5	5	0.00248	1	4	4	0.00174	1
Henan – CN	5	5	0.00523	1	4	4	0.00313	1
Italy	16	9	0.00285	0.858±0.075	7	7	0.00278	1
Turkey	15	4	0.00142	0.714±0.076	10	4	0.00060	0.778±0.080
China	10	9	0.00404	0.978±0.043	8	8	0.00267	1

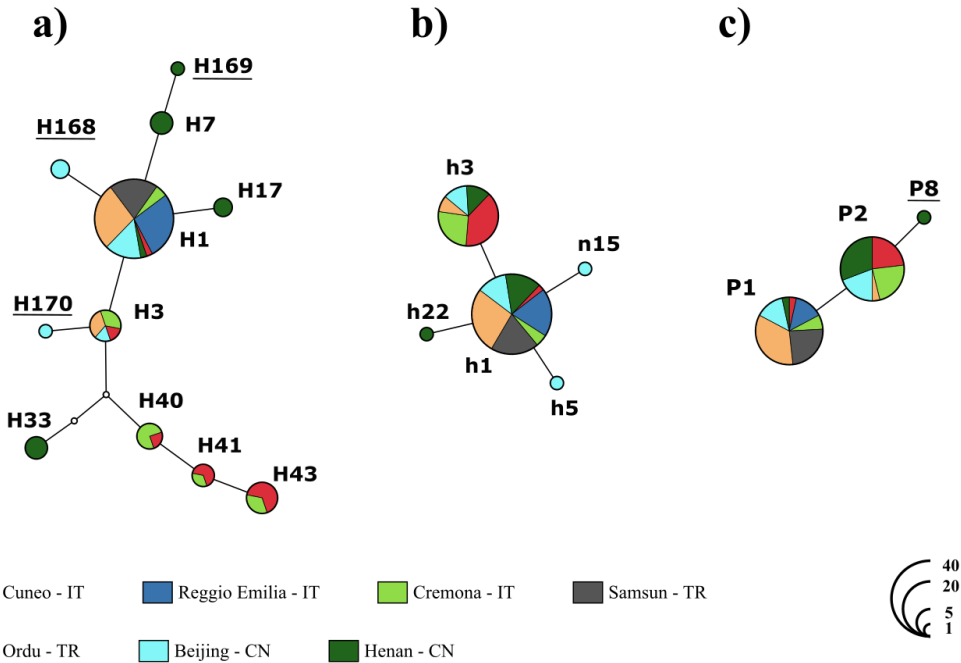


Figure S1. Haplotype distribution according to *H. halys* mitochondrial markers and ‘*Ca. Pantoea carbekii*’ $\Delta ybgF$ along the sampled populations. Circle size increases with the number of samples belonging to each haplotype. Small white

2532 circles represent putative or missing haplotypes forecasted by the analysis.
2533 Underlined haplotypes are described for the first time. **a** COI haplotypes **b** COII
2534 haplotypes **c** $\Delta ybgF$ haplotypes.

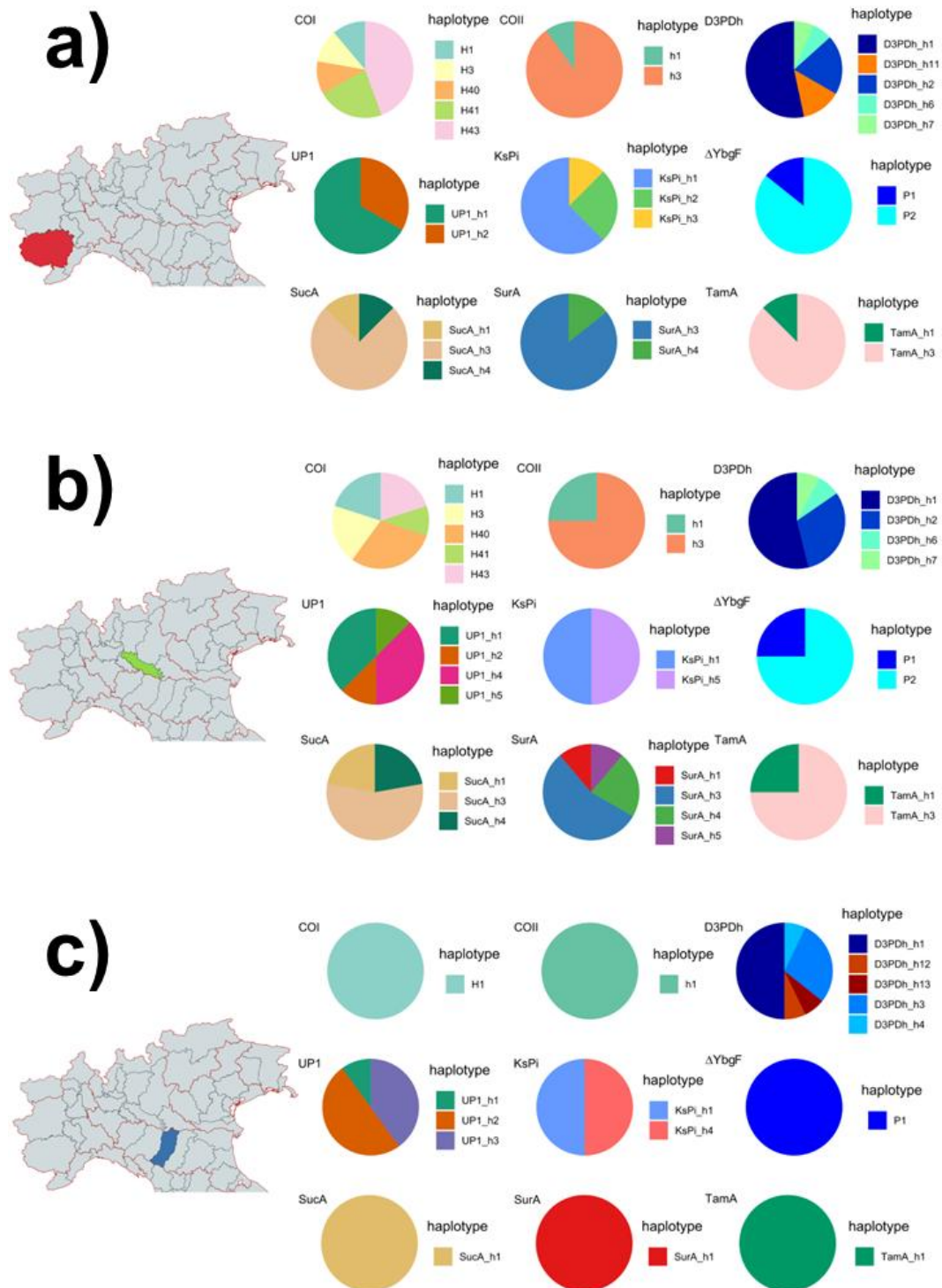


Figure S2. Haplotypes found for each marker under analysis in Italian populations. **a** Cuneo population **b** Cremona population **c** Reggio Emilia population. COI – COII = *H. halys* mitochondrial markers. D3PDh – UP1 – KsPi = *H. halys* nuclear markers. Δ YbgF – SucA – SurA – TamA = *P. carbekii* markers.

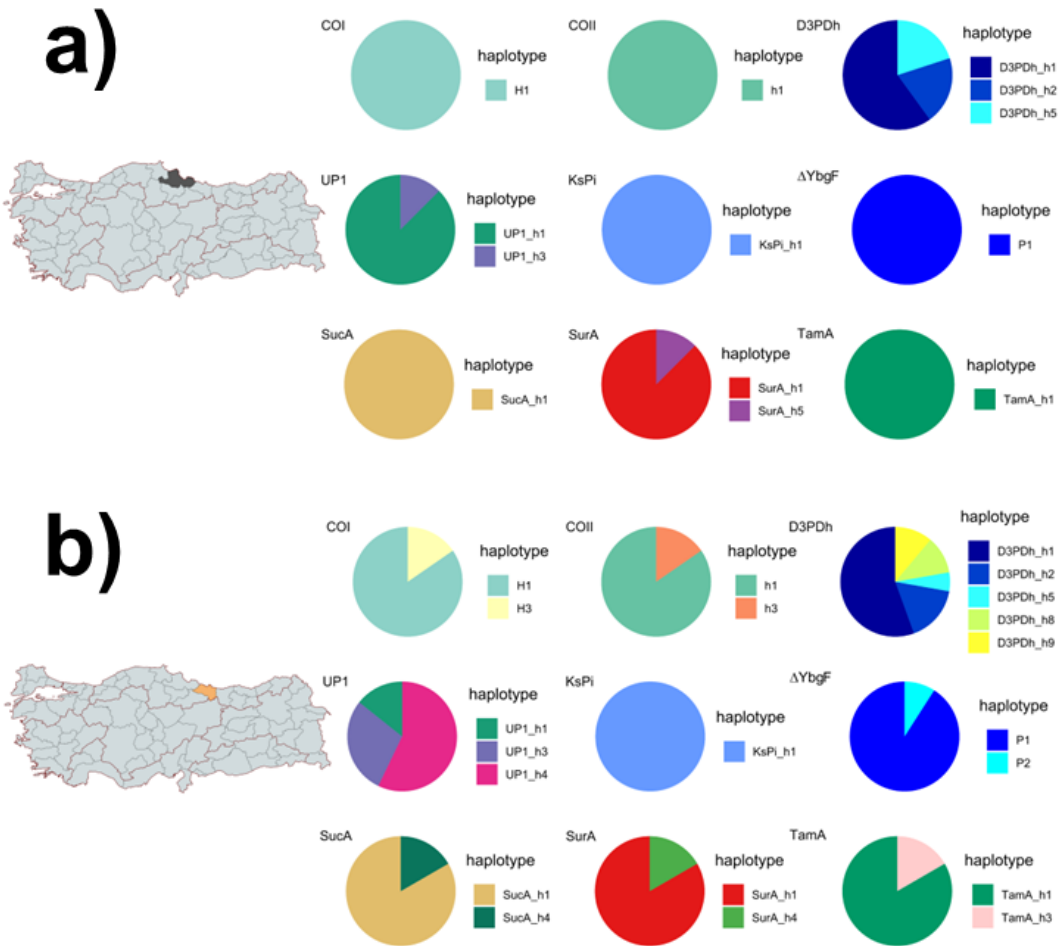
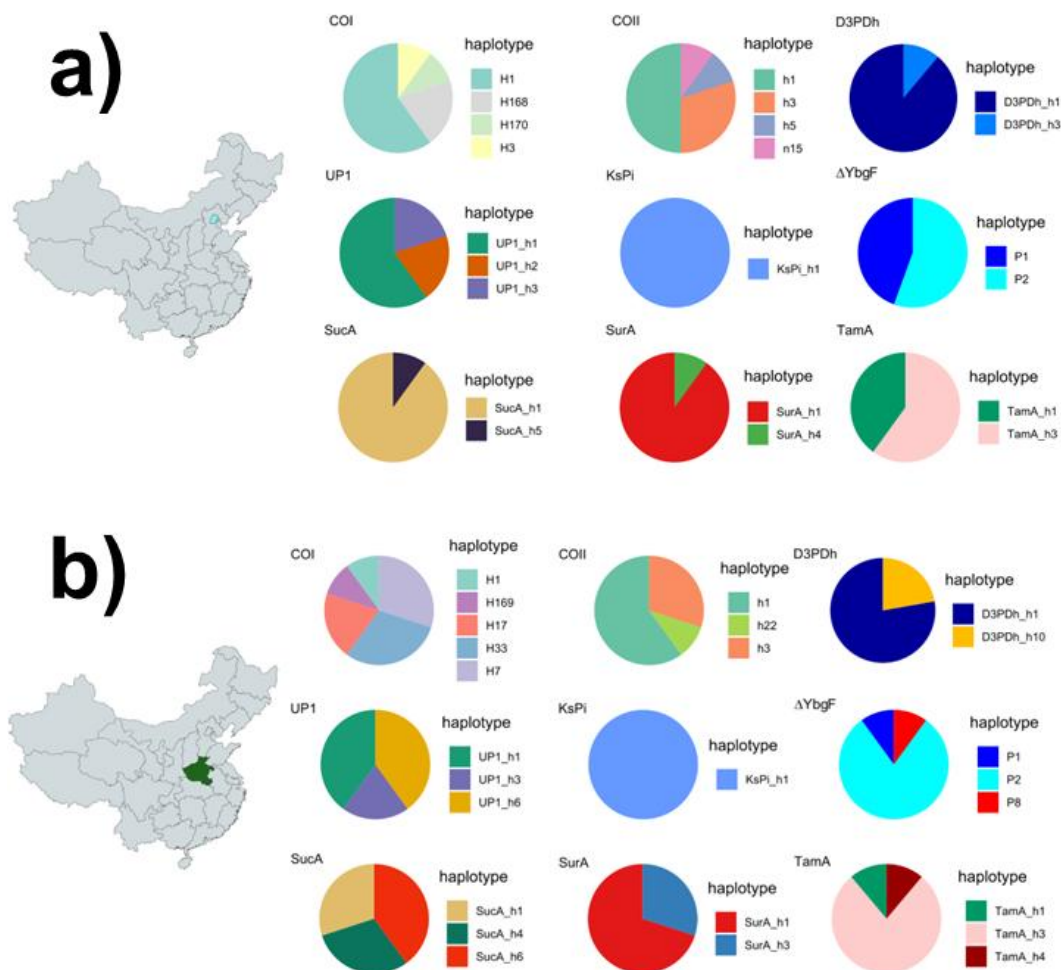


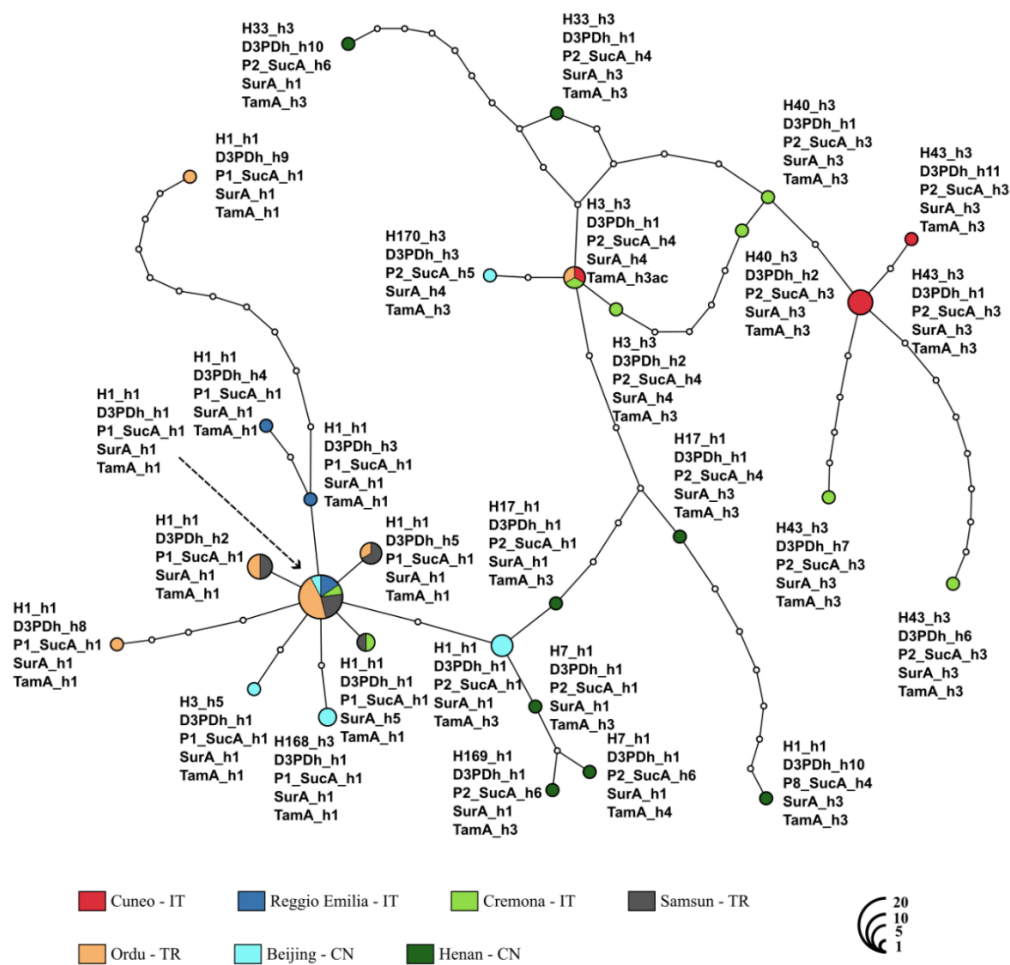
Figure S3. Haplotypes found for each marker under analysis in Turkish populations. **a** Samsun population **b** Ordu population. COI – COII = *H. halys*

2544 mitochondrial markers. D3PDh – UP1 – KsPi = *H. halys* nuclear markers. $\Delta YbgF$
 2545 – SucA – SurA – TamA = *P. carbekii* markers.



2546

2547 **Figure S4.** Haplotypes found for each marker under analysis in Chinese
 2548 populations. **a** Beijing population **b** Henan population. COI – COII = *H. halys*
 2549 mitochondrial markers. D3PDh – UP1 – KsPi = *H. halys* nuclear markers. $\Delta YbgF$
 2550 – SucA – SurA – TamA = *P. carbekii* markers.



2551
2552 **Figure S5.** Haplotype distribution obtained combining the most informative
2553 markers from *Halyomorpha halys* and ‘*Candidatus Pantoea carbekii*’ in the
2554 populations under analysis. Circle size increases with the number of samples
2555 belonging to each haplotype. Small white circles represent putative or missing
2556 haplotypes forecasted by the analysis. Insect markers used: COI, COII and
2557 Hh_D3PDh. Symbiont markers used: $\Delta ybgF$, Pc_TamA, Pc_SucA and Pc_SurA.
2558

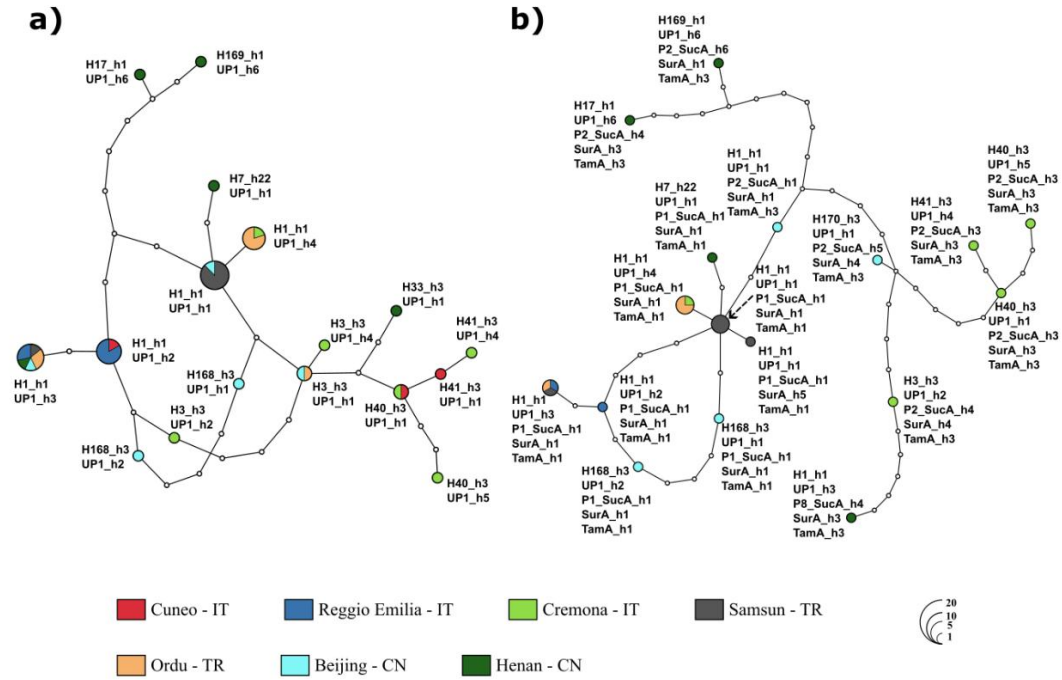


Figure S6. Haplotype distribution obtained using different MLST approaches. Circle size increases with the number of samples belonging to each haplotype. Small white circles represent putative or missing haplotypes forecasted by the analysis. **a** *Halyomorpha halys* haplotyping using COI + COII + Hh_UP1 markers **b** insect + symbiont haplotyping using *H. halys* markers COI + COII + Hh_UP1 and ‘*Candidatus Pantoea carbekii*’ markers $\Delta ybgF$ + Pc_TamA + Pc_SucA + Pc_SurA.

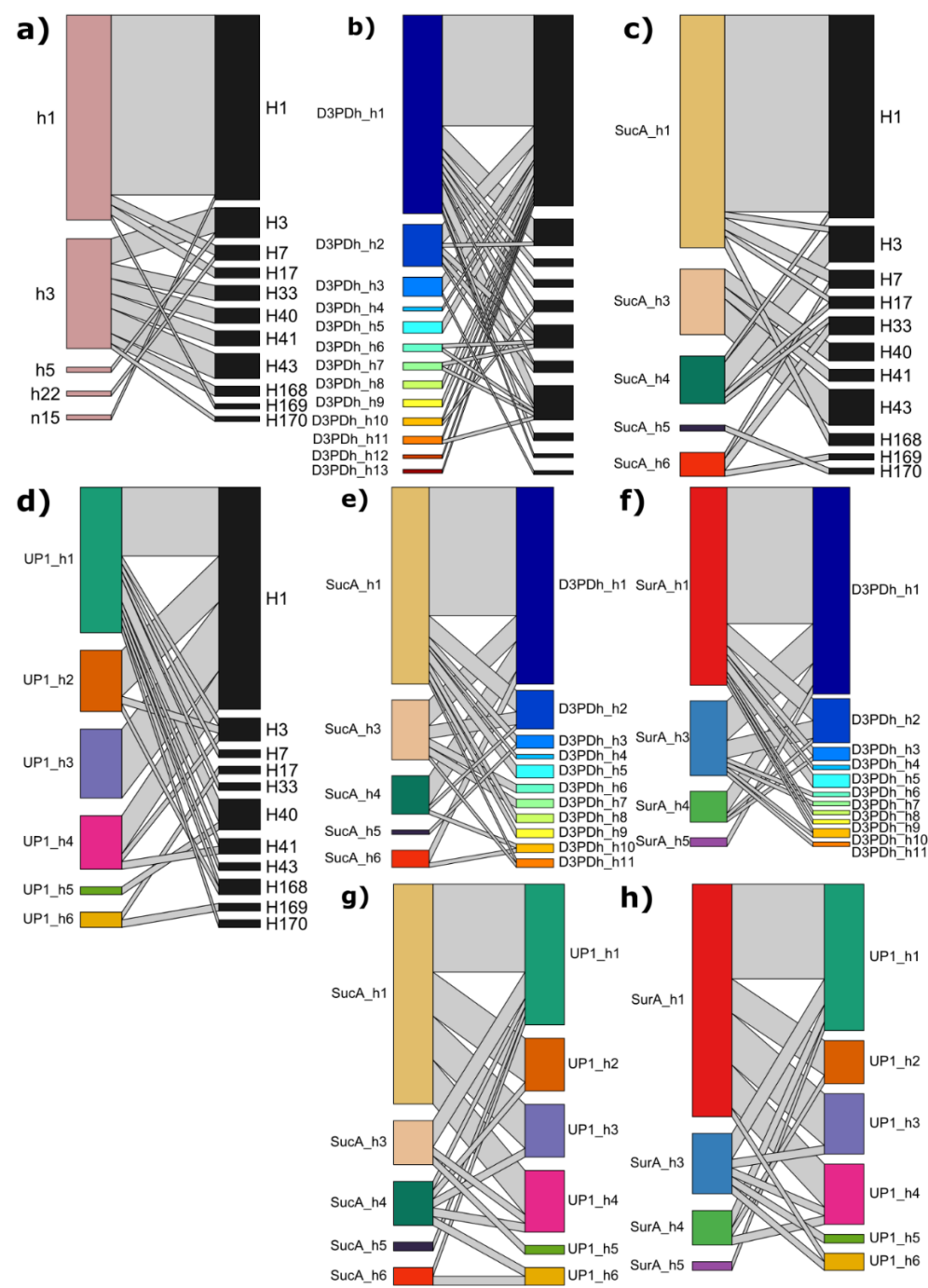
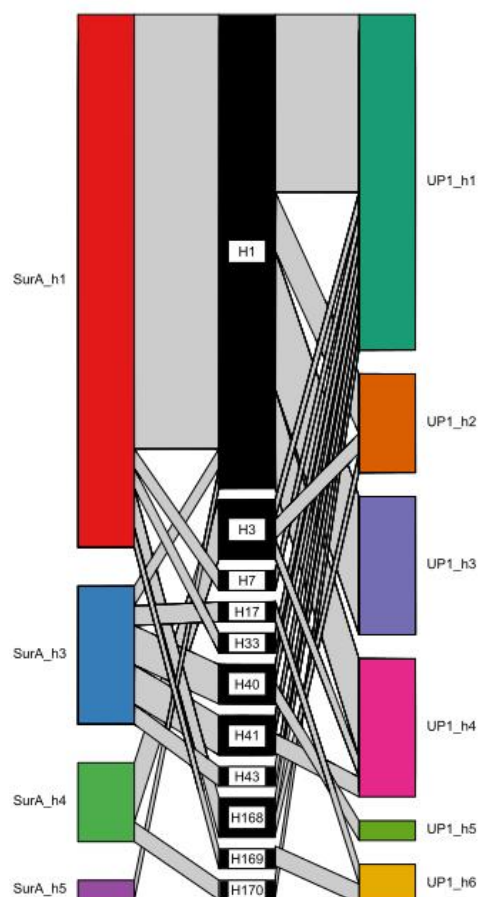


Figure S7. Bipartite interaction matrices between *Halyomorpha halys* and
‘*Candidatus Pantoea carbekii*’ haplotypes for all the populations under analysis.
The width of the conjunctions represents the frequency with which two
haplotypes were found in a same individual. Different colours of the rectangles
represent different haplotypes of the used marker. **a** *H. halys* COI with COII **b** *H.*
halys COI with Hh_D3PDh **c** *H. halys* COI with *P. carbekii* Pc_SucA **d** *H. halys*
COI with UP1 **e** *H. halys* Hh_D3PDh with *P. carbekii* Pc_SucA **f** *H. halys*
Hh_D3PDh with *P. carbekii* Pc_SurA **g** *H. halys* Hh_UP1 with *P. carbekii*
Pc_SucA **h** *H. halys* Hh_UP1 with *P. carbekii* Pc_SurA.

2578



2579

2580 **Figure S8.** Bipartite networks of interaction between haplotypes of the
 2581 '*Candidatus Pantoea carbekii*' Pc_SurA marker and *Halyomorpha halys* COI
 2582 marker (left side), and between *H. halys* COI and Hh_UP1 markers (right side).
 2583 The width of the conjunctions represents the frequency with which two
 2584 haplotypes were found in a same holobiont. Different colours of the rectangles
 2585 represent different haplotypes of the used marker

Chapter IV – Joint use of symbiotic and biological control

Abroad research period activity at Agroscope (Changins, Switzerland)

Preliminary study on the impact of anti-symbiont treatment, genetic variability and *Halyomorpha halys* egg masses age on *Trissolcus japonicus* preference

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Abstract

Halyomorpha halys is a major agricultural threat in invaded areas. To reduce chemical input in agriculture due the use of broad-spectrum insecticides for control programs, more sustainable strategies are under study. The deployment of classical biological control with egg parasitoids and the symbiotic control by means of symbiont-targeted strategies are arguably among the most promising alternatives. A recent investigation showed no negative impact of symbiont-targeted approach on *Trissolcus japonicus* parasitisation in no-choice and paired-choice tests, opening to the possibility of a joint use of those control strategies. This study aimed to preliminarily assess the preference of *T. japonicus* for *H. halys* egg masses of different ages, either treated or untreated with the biocomplex.

2610 The host and primary endosymbiont haplotypes were identified to evaluate
 2611 a possible correlation with *T. japonicus* preference. Treatments suggest no
 2612 negative effect on parasitoid choice. Unexpectedly, old untreated egg
 2613 masses were chosen slightly more frequently than young ones. The low
 2614 variability and imbalanced distribution of the haplotypes made not
 2615 possible to identify a correlation with parasitoid choices, yet an almost
 2616 perfect network specialization confirmed the tendency of *H. halys*
 2617 mitochondrial and symbiont haplotypes to be associated. The optimization
 2618 of an efficient VOCs collection method was not achieved, but other
 2619 strategies for further exploring volatiles bouquet evolution have been
 2620 proposed. These results open to future research activities to validate the
 2621 possibility of a joint use of symbiotic and biological control strategies.

2622 **1. Introduction**

2623 Phytophagous stink bugs are important agricultural pests (Sivri et al.,
 2624 1999; Leskey and Hogmire, 2005; Molina and Trumper, 2012; Ohtani et
 2625 al., 2017) and among them *Halyomorpha halys* (Stål) (Hemiptera:
 2626 Pentatomidae) emerged as one of the most threatening in invaded areas
 2627 (Leskey et al., 2012; Bosco et al., 2018). This Eastern Asian stink bug
 2628 species (Hoebeke and Carter, 2003; Leskey et al., 2012; Lee et al., 2013)
 2629 proved an exceptional adaptability to different environments (Illán et al.,
 2630 2022; Reznik et al., 2023; Tortorici et al., 2023), and is currently
 2631 widespread along Europe (Valentin et al., 2017; Cesari et al., 2018;
 2632 Garipey et al., 2021; Hartung et al., 2022) and North America (Garipey et
 2633 al., 2014; Abram et al., 2017; Lara et al., 2019; Morey et al., 2022).
 2634 Recently it has been detected also in Central Asia (Temreshev et al., 2018;
 2635 Gandjaeva et al., 2022), South America (Faúndez and Rider, 2017) and
 2636 North Africa (Nouere et al., 2020). In invaded countries the increasing

number of described haplotypes of both *H. halys* and the primary endosymbiont '*Candidatus Pantoea carbekii*' reveals a complex pattern of steadily ongoing invasions (Valentin et al., 2017; Cesari et al., 2018; Martinez-Sañudo et al., 2020; Garipey et al., 2021). Recently, highly informative markers for both host and symbiont haplotyping have been identified, revealing a higher haplotype diversity compared to what previously described (Otero-Bravo and Sabree, 2018; Martinez-Sañudo et al., 2020; Laterza et al., 2023; Chapter III).

Characterized by an exceptionally broad host range (Lee et al., 2013; Haye et al., 2015), *H. halys* has become a serious pest of hazelnuts (Bosco et al., 2018; Ak et al., 2019; De Benedetta et al., 2023; Dho et al., 2025) and several other fruit crops like apples, peaches, pears or plums (Leskey et al., 2012; Lee et al., 2013; Acebes-Doria et al., 2016; Bariselli et al., 2016; Candian et al., 2018; Stoeckli et al., 2020). Fruit damage and yield losses have also been reported in olives, dry beans, tomatoes, peppers and kiwifruit (Vetek and Koranyi, 2017; Bernardinelli et al., 2017; Andreadis et al., 2018; Damos et al., 2019).

The chemical control relies mainly on broad spectrum insecticides, potentially harmful for the agroecosystem biodiversity and not compatible with organic farming practices (Lee et al., 2013; Kuhar and Kamminga, 2017). To limit the use of these products, strategies like attract-and-kill, perimeter-based spraying and threshold-triggered treatments have been deployed (Short et al., 2017; Leskey et al., 2020). However, sustainable alternatives are currently under analysis, like the use of essential oils with either insecticidal or repellent effect (Bulgarini et al., 2021; Gokturk, 2021). An emerging approach is symbiont targeted control (Gonella et al., 2020; Gonella and Alma, 2023). Recently, the use of copper, zinc and

2664 citric acid biocomplex proved to cause high mortality of *H. halys* under
 2665 laboratory (Gonella et al., 2019, 2022) and field (Dho et al., 2025)
 2666 conditions.

2667 One of the most promising and widely studied control strategies against *H.*
 2668 *halys* is the use of egg parasitoids, generally considered a better alternative
 2669 to predators since they can kill the host before hatching, preventing early
 2670 damages to the plants (Conti and Colazza, 2012; Haye et al., 2015; Costi
 2671 et al., 2019; Haye et al., 2020). Several monitoring programs of egg masses
 2672 parasitism by resident and exotic egg parasitoids have been conducted in
 2673 the invaded areas (Jones et al., 2014; Costi et al., 2019; Holthouse et al.,
 2674 2020; Moraglio et al., 2020; Zapponi et al., 2021). Overall, exotic egg
 2675 parasitoids like *Trissolcus japonicus* (Ashmead) (Hymenoptera:
 2676 Scelionidae) and *Trissolcus mitsukurii* (Ashmead) (Hymenoptera:
 2677 Scelionidae) are deemed the best candidates for classical biological control
 2678 programs against *H. halys* (Zhang et al., 2017; Sabbatini-Peverieri et al.,
 2679 2021; Falagiarda et al., 2023; Tortorici et al., 2023), and *T. japonicus*
 2680 augmentative biocontrol programs have been conducted in some countries
 2681 (Charles et al., 2019; Bittau et al., 2021; Costi et al., 2022; Simaz et al.,
 2682 2023). To locate and parasitise stink bug egg masses in a complex
 2683 environment, egg parasitoids have developed the ability of recognising
 2684 chemical cues present in the environment (Vet and Dicke, 1992; Vinson,
 2685 1998). Firstly, long-distance cues are used to find the target host, such as
 2686 herbivore induced plant volatiles (HIPVs) or kairomones emitted by the
 2687 target species, even in host stages not exploited by the parasitoid (Vet and
 2688 Dicke, 1992; Howard et al., 1998; Conti et al., 2003; Zhong et al., 2017).
 2689 To finally locate the egg masses, short-distance cues emitted by egg masses
 2690 or either plant synomones induced by oviposition are crucial (Vet and

2691 Dicke, 1992; Fatouros et al., 2008; Salerno et al., 2013; Tognon et al.,
 2692 2016). Egg masses age also plays an important role in *T. japonicus*
 2693 parasitism outcomes. Under laboratory conditions and by using one mated
 2694 egg parasitoid female, it was shown that one and three days old fertilized
 2695 *H. halys* egg masses promoted emergence rate compared to five days old
 2696 ones (Yang et al., 2018). Also, *T. japonicus* parasitism rates were lower on
 2697 four to five days old egg masses of *Glaucias subpunctatus* (Walker)
 2698 (Hemiptera: Pentatomidae) compared to one to three days old ones
 2699 (Tsunashima et al., 2023).

2700 Egg masses microbiota can play a defensive role against fungal pathogens
 2701 or produce anti-nutritional compounds against predators (Kador et al.,
 2702 2011; Flórez et al., 2018; Nyholm, 2020), but no effect on egg parasitoids
 2703 activity has been described to the best of our knowledge. Recently, the
 2704 effect of the copper, zinc and citric acid based biocomplex on *T. japonicus*
 2705 parasitisation in no choice and paired choice tests has been assessed (Orrù
 2706 et al., 2023). Interestingly, the symbiont-targeted treatment did not
 2707 negatively affect substrate acceptance and parasitism, opening to the
 2708 possibility of a joint application of symbiont-targeted and biological
 2709 control strategies (Orrù et al., 2023).

2710 Studying the volatile organic compounds (VOCs) composition of the
 2711 odours emitted by a target host is mandatory to understand which
 2712 compounds are attractant for parasitoids (Li et al., 2015; Fors et al., 2018).
 2713 VOCs composition of *H. halys* egg masses has been studied either by using
 2714 solvents (Tognon et al., 2016), solid phase microextraction (SPME) after
 2715 crushing the egg masses (la Forgia et al, unpublished) or by a conservative
 2716 method with a newly developed Headspace Collecting Device (Karimi and
 2717 Gross, 2024). However, each technique has limitations and could lead to

different outcomes (Diez-Simon et al., 2020). Among the conservative methods, the VOCs collection with Super Q fibers permits to store the eluted volatiles and preserve the samples for further tests (Alborn et al., 2021).

Currently, many open questions remain about the interactions between biological and symbiotic control. The egg mass is not considered as a major stimulus for host recognition by *T. japonicus* (Bertoldi et al., 2019); however, it is unknown if anti-symbiont treatments can modify this condition. This may affect in turn both long and short-distance chemical cues recognition. In this preliminary study, we are aiming at understanding if the combined effect of egg masses age, host or symbiont haplotype and symbiotic control treatment, may affect the egg parasitoid preference. No study has been conducted on if and how the volatiles bouquet changes with the aging of the egg masses. Also, the effect of host and primary endosymbiont genetic variability on egg masses VOCs emission is unknown. Moreover, the effect of symbiotic control treatments on *H. halys* VOCs composition has not been explored yet. According to these observations, the aim of this work was to take a first step in a new set of studies on the interactions between biological and symbiotic control under a behavioural, molecular, and ecochemical point of view.

2. Materials and Methods

2.1 *Halyomorpha halys* and *Trissolcus japonicus* rearing

Halyomorpha halys adults were reared in plastic boxes (32.5 x 21.5 x 9.0 cm) in climate chambers with controlled conditions (24 ± 1 °C, 65% RH, 16L:8D photoperiod) at the Agroscope laboratory (Changins, Switzerland). The diet was composed of a mixture of sunflower seeds, peanuts and green beans. Water was provided by dipping cotton wool in a

2745 small rectangular plastic box (4.0 x 4.0 x 2.0 cm) filled with water. To
2746 encourage oviposition and facilitate egg masses retrieval, slices of black
2747 tissue were folded to form a fan and inserted in the rearings. Egg masses
2748 were carefully removed from the tissues by using clean soft-grip tweezers
2749 to avoid breaking.

2750 *Trissolcus japonicus* specimens were reared in plastic cages (61 x 33 x 41
2751 cm. Nhbs, Bonn, Germany) in climate chambers under controlled
2752 conditions (24 ± 1 °C, 70% RH, 16L:8D photoperiod). Water was provided
2753 as described for *H. halys* rearing. For feed supply, slices of blotting paper
2754 were bent in a triangular shape, the internal side was coated with honey
2755 and crushed pollen. To ensure colony maintenance, *H. halys* egg masses
2756 were placed in the middle of the cage. Fresh egg masses were normally
2757 used for rearing and, only in case of shortage, frozen egg masses were
2758 supplemented as described by McIntosh et al. (2019).

2759 Two different cages of *T. japonicus* rearings were kept in the climatic
2760 chamber, one for rearing maintenance and the second for naïve females
2761 with no oviposition experience. Once a week, a subset of parasitized egg
2762 masses coming from *T. japonicus* maintenance rearing were moved to the
2763 second cage. In this second rearing, only feed and water were provided but
2764 no egg masses to prevent females from ovipositing. Naïve females at most
2765 seven days old were used for the following experiments.

2766 **2.2 Egg masses collection and treatments**

2767 Collected egg masses were classified based on the number of days after
2768 laying. Egg masses from zero to three days old were categorized as
2769 “young”, while from four to five days old as “old”.

2770 Symbiont-targeted treatments were performed on randomly chosen egg
 2771 masses using a copper, zinc and citric acid based biocomplex (Dentamet®,
 2772 Diachem SpA, Italy) that was previously shown effective for symbiont-
 2773 targeted control under laboratory and field conditions (Gonella et al., 2019,
 2774 2022; Orrù et al., 2023; Dho et al., 2025). Each egg mass was placed in a
 2775 plastic Petri dish ($\varnothing = 9$ cm). Treatment was performed with a 200 mL hand
 2776 sprayer filled with 1% v/v solution of the biocomplex diluted in ultrapure
 2777 water. Each egg mass was sprayed once under chemical hood from
 2778 approximately 20 cm, treatment volume was 651 ± 7.42 μ l (mean $\pm$ SD).
 2779 The egg masses were let dry and occasionally moved with clean soft-grip
 2780 tweezers to prevent them from gluing to the Petri dish. Soon after drying,
 2781 egg masses were moved to a clean Petri dish and taken out from the hood.
 2782 For the analysis of the biocomplex effect alone, slices of filter paper of a
 2783 comparable size with the egg masses (1.1 x 0.9 cm) were treated as
 2784 described above. For the following analyses egg masses were divided into
 2785 four categories, young-treated egg masses (yT), young untreated egg
 2786 masses (young), old-treated egg masses (oT), old untreated egg masses
 2787 (old).

2788 **2.3 Olfactometer assays**

2789 Olfactometer assays were performed to study if and how the symbiotic
 2790 control treatment affects the egg parasitoid preference for young and old
 2791 egg masses. One *H. halys* egg mass was placed in clean borosilicate glass
 2792 adapters (Total size = 12 x 8 x 3.5 cm, Chamber containing egg masses =
 2793 5 x 3.5 x 3.5 cm) connected to a Y-type olfactometer (stem length: 100
 2794 mm, arm length: 100 mm, internal diameter: 20 mm, external diameter: 22
 2795 mm, arm angle 30°) inclined at a 40° angle. Since the effect of host and
 2796 symbiont haplotype on *T. japonicus* preference was analysed, only single

egg masses were used in the olfactometer assays to better discriminate the putative impact of different haplotypes. 0.25 L/min of medical grade clean air were pushed in each one of the glass adapters with an air delivery system (Clean Air Supply System, CASS6, VAS, NY, USA). One 18 W neon lamp was placed 65 cm over the olfactometer. To provide uniform light distribution, white plexiglass was placed under the neon lamp. One naïve female of *T. japonicus* was put at the bottom of the stem and parasitoid choice was assessed. Choice was defined when the insect reached the end of the arm and entered the glass adapter. Each trial was performed for a maximum of 60 minutes. *T. japonicus* preference was tested with 18 replicates for “young Vs oT”, 20 replicates for “young Vs old” and “yT Vs oT”, 21 replicates for “oT Vs old” and 25 replicates for “yT Vs young” and “yT Vs old”. The position of each egg mass class was randomized between the trials to avoid directional bias. Parasitoid female that made no choice were discarded from the analysis. To validate the methodology, 15 replicates of young and old egg masses against an empty arm were also performed randomizing the position of the egg masses in the two arms. To check for possible attractive or repellent effect of the biocomplex, 22 replicates of biocomplex treated filter paper against empty arm were performed. Each parasitoid and each egg mass were used for only one replicate, each part of the olfactometer was cleaned with acetone and pentane and oven-baked at 200 °C for 2 hours after no more than three replicates.

2.4 Egg Volatiles collection and analysis

VOCs collection from egg masses was performed by absorption on Super-Q fiber (30mg, VAS Rensselaer, NY, USA) activated with pure dichloromethane (Merck, Darmstadt, Germany). Two different setups for

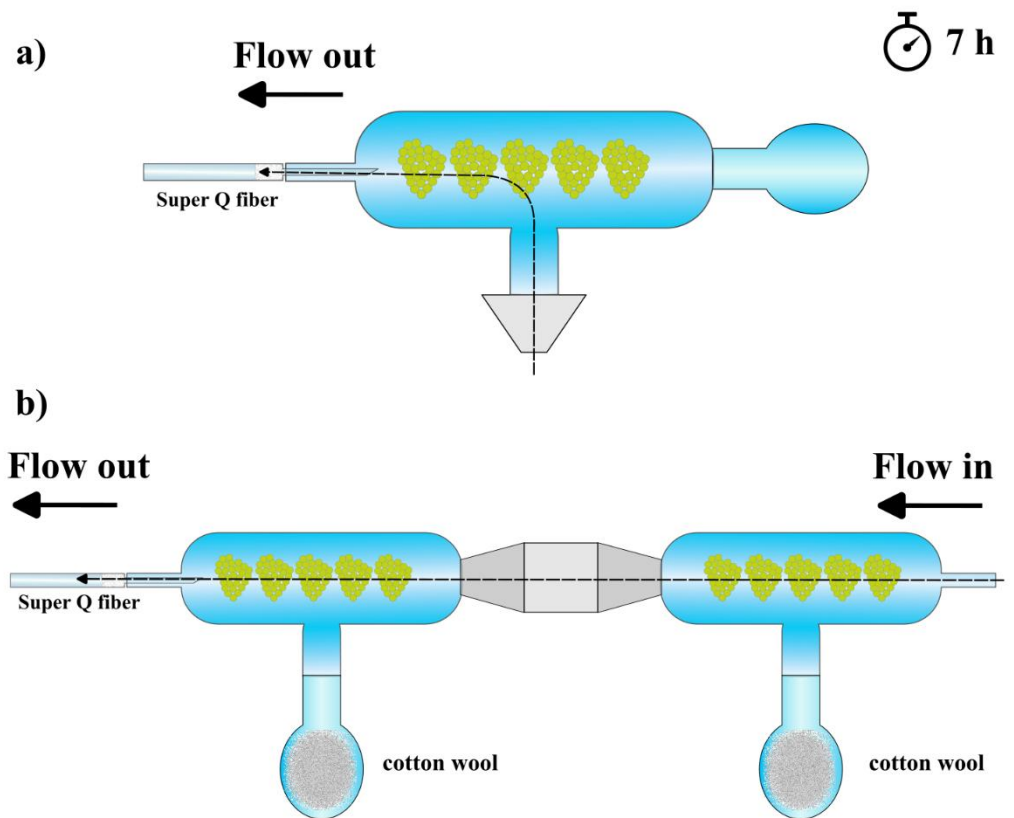
VOCs collection were assessed. In both cases, collection was carried out on a selected number of egg masses rather than a predefined weight. This option was preferred since host and symbiont haplotyping could be performed and the same number of haplotypes could be compared among the different replicates. For the same reason, the use of a conservative and solvent-free method was preferred to avoid stress that could impair either nymphal eclosion or symbiont DNA collection from the eggs.

A first trial with five egg masses placed in a borosilicate glass adapter (Total size = 12 x 8 x 3.5 cm, Chamber containing egg masses size = 5 x 3.5 x 3.5 cm), closed for one side with a glass ampoule was assessed (Fig 1a). Collection was performed for 7 consecutive hours with a constant flow of 0.5 L/min, only environmental air was provided. Collected VOCs were eluted in 150 µL of dichloromethane at room temperature.

In a second setup (Fig. 1b), two glass adapters were connected with a borosilicate junction, closed with glass wool filled ampoules and a constant flow of 0.5 L/min of medical grade clean air was provided during the collection. Collection was performed from ten egg masses, airflow and collection time were the same described above. Two elution strategies were followed, either at room temperature or by keeping the collection vials in ice to limit the evaporation. In both cases, dichloromethane volume was 150 µL. After each collection, all the glassware was cleaned with acetone and pentane and then baked for two hours at 200 °C. Four repetitions were performed for each egg mass category (young, old, yT and oT) in each of the setups. Three negative controls were performed with empty glass adapters for each collection setup.

Samples were analysed with a 7890B gas chromatograph (Agilent Technologies, USA) coupled with a 7010 triple quadrupole mass

spectrometer (Agilent Technologies, USA) (GC–MS). An aliquote of 2 μ L of dichloromethane eluate was injected in a DB-5 capillary column (30 m x 0.25 mm I-D, 0.25 μ m film thickness, Agilent J&W, Santa Clara, CA, USA). Helium was used as carrier gas at a flow rate of 1.2 mL/min. Initial oven temperature was 50 °C, held for three minutes, then ramped up to 180 °C at a rate of 5 °C/min, with a final ramp up to 250 °C at a rate of 8 °C /min. Final temperature was hold for three minutes. For the MS analysis, electron impact ionization was set at 70 eV with the ion source set at 230 °C. Data acquisition was conducted in scan mode; mass range was set as 35–350 amu. 1,6-Heptadien-4-ol was used as internal standard (Merck, Darmstadt, Germany). For volatile molecule recognition, mass spectra were compared with the Nist14.L library. Resulting chromatograms were analysed with Agilent Masshunter Qualitative analysis Version 10.0.



2866

2867 **Figure 1.** Schematisation of VOCs collection setups, in both cases collection time
 2868 was 7 h. **a** Collection from five egg masses in single glass adapter, no medical
 2869 grade clean air was provided and ambient air entered from the side. **b** Collection
 2870 from ten egg masses, two glass adapters were connected with a borosilicate
 2871 junction, all the side openings were closed with a glass ampoule filled with cotton
 2872 wool to reduce the volume and medical grade clean air was provided as a carrier
 2873 for VOCs.

2874

2.5 *Halyomorpha halys* and ‘*Candidatus Pantoea carbekii*’ haplotyping

After olfactometry trials, each egg mass was reared in a Petri dish ($\varnothing = 9$ cm) in a climate chamber under controlled conditions (24 ± 1 °C, 65% RH, 16L:8D photoperiod). Nymphs and eggs coming from yT Vs young, yT Vs old and young Vs old trials were used for molecular analysis. As soon as eclosed nymphs left the egg chorion after nourishing, nymphs and egg mass were stored separately in a sterile 2 mL plastic tube at -20 °C. Insect DNA was extracted by a whole I instar with DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) following manufacturer’s instruction. A portion of COI gene was used as the target for *H. halys* haplotyping, amplification was performed with primer pair Hh_Alt-COI-F/R as described in Chapter III. Amplicon size was visualized via electrophoresis with 1% agarose gel stained with GelRed® (MilliporeSigma, Temecula, CA). PCR products were purified with QIAquick PCR purification kit® (Qiagen) and Sanger sequenced by an external company (Eurofins Genomics, Köln, Germany).

Symbiont DNA was extracted from hatched egg masses with DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany). The protocol was an adaptation of the one described by Storer et al. (2019). Hatched egg masses were put in 180 µL of buffer ATL and vortexed for 1 min as described by Geerinck et al. (2022), then kept at 4 °C overnight. Before adding Proteinase K, egg masses were again vortexed for 1 min and kept into the mix up to the end of step 3 of the manufacturer’s protocol. Successive steps were performed following manufacturer’s instruction. Final elution was performed in 35 µL of AE buffer. A portion of *P. carbekii* SurA gene was used as the target for symbiont haplotyping, amplification was performed

2902 with primer pair Pc_SurA-F/R as described in Chapter III. Amplicon
 2903 visualization, purification and sequencing were performed as described
 2904 above. The association between host and symbiont molecular markers was
 2905 assessed using the specialization index H2' (Blüthgen et al., 2006) and
 2906 using R package bipartite (Dormann et al., 2008).

2907 **2.6 Statistical analysis**

2908 Statistical analysis was performed using R (Version 4.2.3). Parasitoid
 2909 choice in olfactometer assays was analysed using Chi-square goodness of
 2910 fit test, considering 50% of choice in each arm as null hypothesis (R
 2911 package stats).

2912 **3. Results**

2913 **3.1 Olfactometer assays**

2914 The olfactometer methodology was validated by testing *T. japonicus*
 2915 preference for young or old egg masses against an empty glass adapter. In
 2916 both cases, the parasitoid chose the egg mass significantly more than the
 2917 empty glass adapter (young vs empty: $\chi^2 (1, 15) = 5.40, p = 0.020$; old vs
 2918 empty: $\chi^2 (1, 15) = 8.07, p = 0.005$) (Fig. 2a). In all the combinations of
 2919 different classes of egg masses, no statistically significant difference was
 2920 obtained. Assessing the preference between old vs young egg masses, 14
 2921 *T. japonicus* out from 20 chose old over young egg masses ($\chi^2 (1, 20) =$
 2922 $3.20, p = 0.074$). In the yT vs young trials, treated egg masses were chosen
 2923 16 times out from 25 trials ($\chi^2 (1, 20) = 1.96, p = 0.162$) (Fig. 2a). For all
 2924 the other egg masses combinations (oT vs old, young vs oT, yT vs old), no
 2925 tendency in *T. japonicus* choice was suggested by the results (Fig. 2a).

In the olfactometer trials with the biocomplex against empty glass adapter, most of the parasitoid females made no choice (13 out from 22). Five and four parasitoids chose, respectively, biocomplex and empty arm (Fig. 2b).

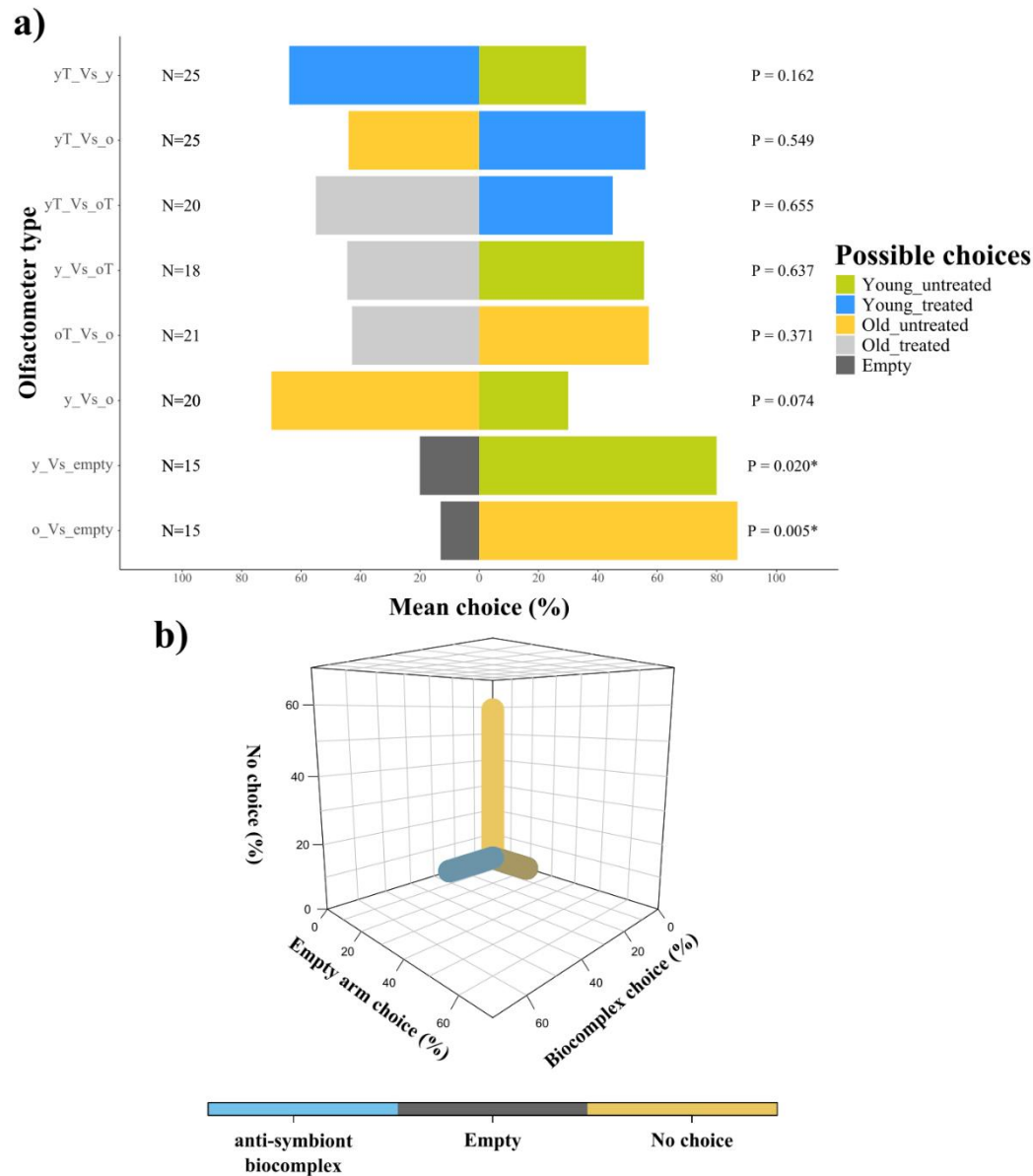


Figure 2. *Trissolcus japonicus* choices in Y-tube olfactometer assays, preference

2932 data were analysed with Chi squared goodness of fit test, with 50% choice for
2933 either combination as null hypothesis. **a** *T. japonicus* choice between different
2934 classes of *H. halys* egg masses. **b** *T. japonicus* choice between biocomplex and
2935 empty arm.

2936 **3.2 Egg Volatiles collection and analysis**

2937 No different peaks were found between negative controls and VOCs
2938 collection from egg masses in the setup with five egg masses. Minimal
2939 differences between negative control and the samples were found in the
2940 setup with ten egg masses (Fig. 3). In all the egg mass classes under
2941 analysis, only a minimal peak at RT = 15.21 min was present only in the
2942 egg masses collection and not in the control. This peak was identified as
2943 decanal according to Nist14.L library. Due to the very low concentration
2944 of VOCs collected no further analysis, such as quantification or the use of
2945 synthetic chemical standards for molecule identification, were performed.

2946

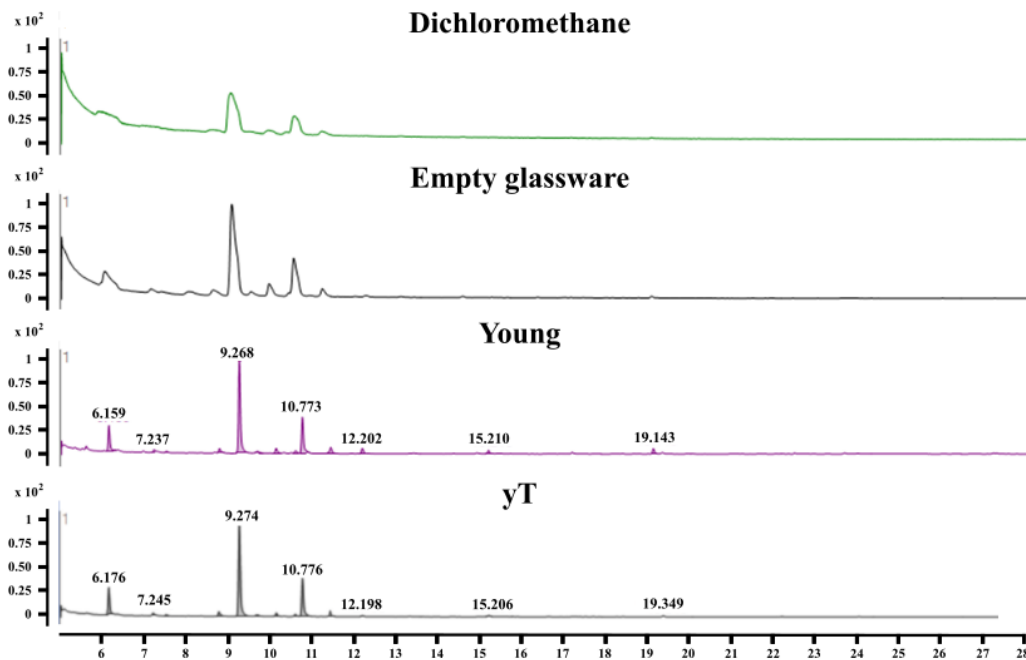


Figure 3. Examples of GC-MS results for VOCs collection from young egg masses. The first two chromatograms display, respectively, negative control with only dichloromethane and negative control with collection from empty glass adapters. Third and fourth chromatograms display, respectively, results from ten young-untreated and ten young-treated egg masses. Similar outcomes were obtained for the two other classes of egg masses (old, oT, data not shown).

3.3 *Halyomorpha halys* and ‘*Candidatus Pantoea carbekii*’ haplotyping

The effect of *H. halys* and *P. carbekii* haplotype on *T. japonicus* choice was assessed for the egg masses combinations where a tendency in choices was seen (young Vs old and yT Vs young) and for the combination yT Vs old. A total of six different COI haplotypes were identified in analysed nymphs. Two haplotypes were the most abundant (H1 for 49 samples, H44 for 38 samples), with four others only rarely found (H3, H40 and H41 one

sample, H43 three samples). In the case of untreated egg masses (young Vs old) a total of three *H. halys* haplotypes were identified (H1, H43 and H44) out of 30 individuals. For the combination of yT against young egg masses four different COI haplotypes were identified (H1, H3, H41, H44). When considering yT Vs old egg masses, three COI haplotypes were retrieved (H1, H40, H43). Of the rare haplotypes, H43 belonged to the chosen egg mass in all three cases (yT Vs old: one egg mass yT and one old; young Vs old: young egg mass).

The variability in terms of *P. carbekii* Pc_SurA haplotypes was lower than *H. halys* COI. Four different SurA haplotypes were identified, but the majority of the samples belonged to SurA_h1 (62), and all the other haplotypes were seldom represented (SurA_h3 five samples, SurA_h4 two samples, SurA_h5 one sample). The specialization index H2' value for COI and Pc_SurA association was 0.91 and all the SurA_h1 haplotypes were associated with either H1 or H44.

Given the low and imbalanced genetic variability retrieved, no statistical analysis was performed regarding the possible impact of host and symbiont haplotypes on parasitoid preference. Since no differences in VOCs composition were observed among the different classes with the methods assessed, no haplotyping was performed on the egg masses used for this step.

4. Discussion

In this study, the biocomplex treatments displayed no negative impact on *T. japonicus* preference in olfactometer assays (Fig. 2a). These results seem to confirm the absence of negative effect on parasitoid preference for either treated or untreated egg masses, similarly to what described by Orrù

et al. (2023) for substrate acceptance in no choice and paired choice tests. Interestingly, older untreated egg masses were chosen in moderately more replicates compared to younger ones (Fig. 2a). Even though further replicates are needed to assess its significance, this is an unexpected result since parasitisation rates tend to be higher in young egg masses over older ones (Bari et al., 2016; Sabbatini-Peverieri et al., 2020; Tayat and Özder, 2022; Priyanka et al., 2023). However, in the above-cited studies, preference was not assessed in olfactometer assays but either in no choice or multiple-choice tests, mainly focusing on parasitoid emergence. Moreover, the parasitoid exhibits other behaviours before accepting a substrate, such as visual inspection, walking on the egg mass and antennal drumming (Botch and Delfosse, 2018).

Despite more trials are needed to confirm egg parasitoid behaviours, the biocomplex treatments resulted in very close number of choices in all the egg masses ages tested (Fig. 2a). Since in the trials with the biocomplex against an empty arm most of the insects did not choose either arm (Fig. 2b), the biocomplex alone can be considered leaving *T. japonicus* indifferent and the distinct behaviour could be ascribed to variation in egg VOCs emission mediated by the treatment. To confirm this hypothesis, description of the volatolome variation of the different classes of egg masses is needed. Unfortunately, the VOCs collection tested here with Super-Q fibers did not permit to discriminate differences (Fig. 3). Only one compound identified as decanal, known to be the most abundant volatile compound emitted by *H. halys* egg masses (Karimi and Gross, 2024), was collected but confirmation with synthetic standards was not performed. *H. halys* eggs are known to have low emission of VOCs, high number of egg masses were required either for headspace collection

3015 (Karimi and Gross, 2024) or solvent based extractions (Tognon et al.,
3016 2016). The development of efficient, thus requiring a low amount of egg
3017 masses, and conservative protocols for headspace collection would indeed
3018 pave the way for more comprehensive studies on volatiles bouquet
3019 evolution with aging or after treatments. Knowing the volatolome
3020 composition of each day-old egg masses or the variations after symbiont-
3021 targeted sprayings or even the variations related to different stink bug
3022 genetic variants could help shedding light on the different behaviours
3023 observed in this study. A still unexplored possibility to increase efficiency
3024 of VOCs detection could be the use of In Tube Extraction (ITEX) of
3025 Dynamic Headspace (DHS) coupled with vacuum (Fuchsmann et al.,
3026 2019; Fuchsmann, 2021).

3027 To better investigate possible correlations between *H. halys* or *P. carbekii*
3028 genetic variants and *T. japonicus* preference or VOCs spectra, future
3029 studies could start from homohaplotypic rearings. Setting up rearings of
3030 individuals with already known haplotypes would overcome the issue of
3031 haplotypes imbalance found in this preliminary study. Moreover, this
3032 would prevent the need for assessing host and symbiont haplotype after
3033 VOCs collection. This option would overcome the limitation of using low
3034 number of egg masses, yet the development of more sensitive
3035 methodologies is preferable especially when many classes of egg masses
3036 should be analysed. Given the great *H. halys* genetic variability described
3037 (Cesari et al., 2018; Schuler et al., 2020; Chapter III), North Italy could be
3038 ideal for setting up this type of rearings. Since it remains unknown whether
3039 *H. halys* COI variability has a functional implication (Cesari et al., 2018;
3040 Gariépy et al., 2021; Yan et al., 2021), it would be interesting to further
3041 investigate if egg masses of H43 haplotype are related to *T. japonicus*

preference. The presence of this variant is documented in Italy (Cesari et al., 2018; Chapter III) where augmentative biological control programs have already been deployed (Bittau et al., 2021; Costi et al., 2022). Moreover, no study has been conducted so far on if and how *P. carbekii* affects the VOCs emission of *H. halys* egg masses. Given the extracellular location of the symbiont on the egg chorion (Kenyon et al., 2015), an effect of different symbiont variants over the volatiles bouquet composition emitted by the egg masses cannot be discarded.

The H2' index value of 0.91 confirmed the tendency of *H. halys* and *P. carbekii* haplotypes to be associated (Chapter III). Since all the insects used for the continuous rearing came from invaded areas, the high network specialization found in this study confirms that possibly only some host and symbiont associations have enhanced invasive capability (Chapter III). The fact that H2' value was not 1, like that described in Chapter III from field collected individuals, can be related to the specific conditions of a continuous rearing. A possible explanation can be the cannibalistic oophagy displayed by *H. halys* (Iverson et al., 2016), so multiple *P. carbekii* haplotypes could be acquired at the same time given its specific vertical transmission (Gonella et al., 2020). The controlled conditions of the rearing, less challenging compared to natural ones, could allow even less adaptable host-symbiont combinations to survive.

Since the focus of this thesis project was the study of *H. halys* – *P. carbekii* model, *T. japonicus* preference was tested for short distance cues emitted by the egg masses. Nevertheless, other stimuli are followed by egg parasitoids to locate the target. It is well documented that insect oviposition triggers the emission of plant synomones capable of attracting parasitoids (Hilker et al., 2002; Colazza et al., 2004; Hilker and Meiners, 2006; Hilker

and Fatouros, 2015), parasitoids tend to be more attracted by other life stages of their host rather than the eggs (Conti et al., 2003; Silva et al., 2006) and that other short-distance cues such as target species footprints are used for substrate location (Zhong et al., 2017; Scala et al., 2022). This study confirmed that anti-symbiont spray does not alter the limited role of egg-related cues, as we did not observe any significant variation of parasitoid choice, further supporting the absence of interference between symbiotic control and egg parasitism. However, the impact of symbiotic control on the other cues remains completely unstudied. More efforts should be made to confirm the impact of anti-symbiont treatments on egg masses location, by exploring the effect on the most attractive cues. Arrestment arena trials, as described by Horrocks et al. (2024) on *H. halys* adults footprints with or without treatment, should be performed regarding short distance cues. Accordingly, the effect of spraying on the other above-mentioned cues deserves further investigations to confirm the compatibility of a joint application of symbiotic and biological control strategies.

Conclusion

This preliminary study aimed to investigate the influence that *H. halys* egg masses age, symbiont-targeted treatment and stink bug and primary endosymbiont haplotypes have on *T. japonicus* preference in Y-tube olfactometer assays and VOCs emission. These initial results suggest no negative impact of symbiotic control treatments on *T. japonicus* preference in Y-tube olfactometer assays. The higher number of older egg masses chosen over young ones deserves further attention, since a confirmed preference could give new insights about short-distance cues orienting *T. japonicus* parasitism. The imbalanced host and symbiont haplotype

distribution did not lead to significant results, even though a COI haplotype worth of further analysis has been identified. A possible future experimental design has been proposed to overcome low haplotype variability and uneven distributions. Even though the development of an efficient and conservative method for VOC collection failed, another more sensitive approach has been identified. This study opens to future research perspectives in the field of *H. halys* and *T. japonicus* chemical ecology and the development of conservative methods for VOCs collection requiring fewer egg masses will be pivotal for in depth analyses of odours bouquet evolution in multiple classes of egg masses.

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Chapter V – Conclusions

Hazelnut cultivation is growing worldwide and stink bug pests represent one of the main threats for the production of high-quality kernels (Bosco et al., 2018; Hamidi et al., 2022; Saruhan et al., 2023). The application of pest control measures against pests such as *H. halys* is seriously hampered by several factors. First, in the future years the environmental impact of agriculture must decrease along with the use of chemicals, while productions need to be increased (Hunter et al., 2017; Brunelle et al., 2024). Moreover, hazelnut productivity is expected to shrink due to climate change (An et al., 2020), while the suitability for *H. halys* of highly productive areas such as Oregon (USA), Northern Italy, France or Poland is expected to remain the same or even increase (Kistner, 2017; Illán et al., 2022). Given these observations, limiting *H. halys* damage on hazelnuts in a sustainable yet effective way is one of the challenges that should be faced. On this purpose two strategies stood up as the most promising, the classical biological control with egg parasitoids and the innovative symbiotic control through symbiont-targeted treatments.

Since dysbiosis given by the prevention of *P. cabekii* acquisition resulted in high mortality of *H. halys* nymphs and very limited fitness of surviving adults (Taylor et al., 2014), researchers worked to identify sustainable products to target the symbiont during host egg stage (Mathews and Barry, 2014; Gonella et al., 2019). One copper, zinc and citric acid biocomplex proved to be highly effective under laboratory conditions (Gonella et al., 2019) so its activity was assessed in open field conditions in a hazelnut orchard (Dho et al., 2025) (Chapter II). The importance of agronomical practices such as pruning to increase treatments efficacy was indicated by

a thorough evaluation of the spraying efficacy in field conditions. Even though nymphal mortality was lower than what found in laboratory, it was significantly higher after treatments compared to untreated controls. *P. carbekii* acquisition was generally lowered but not prevented. “Cimiciato” damage significantly decreased confirming the efficacy of the biocomplex in the field.

Preventing *H. halys* invasion in new areas, such as Australia or New Zealand (Kriticos et al., 2017; Horwood et al., 2019), is for sure the best way of dealing with this pest. To do so, reconstructing the global movements of the stink bug is an invaluable tool to locate the source of invasive individuals. Moreover, deciphering possible relations between genetic variants and invasivity potential could be helpful for a fine-tuning of applied control, including biological control and symbiont-targeted control. To this end, haplotype identification of *H. halys* populations has been attempted using several molecular markers (Cesari et al., 2018; Lee et al., 2018), but not always with satisfactory results. The identification of new highly informative molecular markers for both *H. halys* and *P. carbekii* discussed in Chapter III could help in shedding light on the pest movements when multiple invasion events occur in the same area. Apart from this practical applicability, interestingly *P. carbekii* was shown to be much more variable than what described so far, re-questioning its use as source of information. Moreover, a total specialization between host and symbiont haplotypes in the invaded areas has been reported for the first time. It remains still unclear if this outcome is due to normally occurring phenomena in the case of invasive species, such as bottleneck effect or genetic drift (Xu et al., 2014; Croft et al., 2024). However, since multiple primary invasion events from distinct native areas have been described

3607 especially in Italy (Cesari et al., 2018; Gariepy et al., 2021), this result
 3608 could suggest that these host-symbiont combinations have enhanced
 3609 adaptability to new environments.

3610 Given the potential of *T. japonicus* in controlling *H. halys* and the positive
 3611 results displayed by symbiotic control treatments, confirming the
 3612 possibility of a joint use of the two strategies would be highly beneficial
 3613 for the agricultural ecosystem (Orrù et al., 2023). The preliminary results
 3614 on the symbiont-targeted treatments do not suggest a negative effect on *T.*
 3615 *japonicus* preference for young or old egg masses in olfactometer assays.
 3616 Unexpectedly old untreated egg masses were chosen more frequently than
 3617 young untreated ones, yet further work is needed to confirm a preference.
 3618 Given the low genetic variability with imbalanced haplotypes distribution,
 3619 the identification of a correlation between different haplotypes and *T.*
 3620 *japonicus* choices was not possible. Also, the development of a protocol to
 3621 optimize VOCs collection from low number of egg masses failed.
 3622 Nevertheless, further possibilities to continue and implement the work
 3623 started with this thesis have been identified and discussed. Similar studies
 3624 could take advantage of *H. halys* rearings based on host and symbiont
 3625 haplotypes. Also, a more sensitive strategy for VOCs headspace collection
 3626 from poorly emitting substrates has been identified. This preliminary study
 3627 is a first step for better elucidating the impact of symbiotic control
 3628 treatments on *T. japonicus* capability to locate *H. halys* egg masses, with
 3629 the final aim to confirm the feasibility of joint application of sustainable
 3630 control strategies such as symbiotic and biological control.

3631 In conclusion, the potential of symbiotic control treatments in controlling
 3632 *H. halys* on reproductive host plants such as hazelnut has been confirmed.
 3633 Also, more informative molecular markers for *H. halys* and *P. carbekii*

haplotyping have been identified, highlighting a previously undescribed symbiont genetic variability and host-symbiont haplotype specialization in invaded areas. Moreover, preliminary results suggest that no negative effect is displayed on *T. japonicus* capability of locating the egg masses after symbiotic control treatments.

This thesis project opens to new research perspectives, addressing scientific questions that remain to be answered. The optimization of symbiotic control treatments on other crops remains an unexplored research field. Also, economical comparisons among symbiotic control and other strategies are still missing. Invasion pathways reconstruction studies using the new markers, expected to untangle the complex *H. halys* movements, should be performed. The functional meaning of host and symbiont haplotypes needs to be further investigated. Studies on if and how symbiotic control treatments affects *T. japonicus* capability to recognize other chemical cues remain to be performed.

References (Thesis introduction and conclusions)

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Acknowledgments

I want to start this final chapter with my sincere thanks to my Supervisor, Professor Elena Gonella, whose guide and help were invaluable during this formative, exciting yet challenging period. I would also like to thank Professor Alberto Alma, his scientific approach to the difficulties of this work and his skills as a leader gifted me with many lessons to learn from while building my future career. Both Prof. Alma and Gonella never limited my fantasy and curiosity, but wisely guided me in shaping the experiments and the subsequent data analysis. I have been lucky to become part of their research group and thanks to them I am ending this chapter of my life with more curiosity, hunger and willing to begin new adventures then before. A special thanks to both for giving me the motivation I needed when the bad moments came. I will personally never forget your help and the flexibility you gave me when I most needed it.

I want also to thank the Entomology and Nematology Division of the Agroscope Center of Changins for hosting me during my research period abroad. I would like to thank especially the supervisor I had during this period, Doctor Diana la Forgia (with lowercase “l”, this is really important to her) and the chief of the research group Doctor Ivan Hiltpold. Exactly like for my Supervisor and the Chief of Department in Turin, they never stopped my ideas and made me feel part of their team. More personal thanks will follow.

Lastly, but not least, I would like to thank all the friends I made at the University of Turin during this beautiful experience. We shared so many moments together, so many laughs and even when the bad moments arrived you managed to cheer me up. Among them all, I want to spend few

4372 words to thank Andrea Arpellino. Andre was there since day one, when I
4373 was sad, he had so many lunches alone with me just to make me feel better,
4374 we laughed thousands of times, we played so many pranks to each other
4375 and yet worked hard. I consider you as a friend. Do not get used to this
4376 kind of words, more jokes are coming!

4377 Now I want to move out from the University and start thanking all the
4378 people that were there for me and with me in my personal life.

4379 First of all, I want to thank my family. My mother and my father always
4380 believed in me, gave me the best advice of my life and thought me the
4381 importance of the hard work. Mamma, I want to dedicate my University
4382 career to you, you are the person that most believed in my studies, even
4383 when I took different decisions, I hope you are proud of me. Papà, when I
4384 had tough moments, you don't even imagine how many times I said to
4385 myself "non posso lamentarmi, mio papà lavora molto più di così e non si
4386 lamenta" (= I cannot complain, my dad works much more than that and he
4387 doesn't complain). You taught me the importance of smiling and helping
4388 the others, I hope to become like you one day. My dear little sister, Gabry,
4389 you are a source of energy for me. When I see you, my brain automatically
4390 switches to "fun mode". I bet no one else has a better brother-sister
4391 relationship then us. We are number one, not much to say. I passed my
4392 weeks waiting for the Saturday morning to come back home, just to speak
4393 with you, laugh, and forget about all the problems. I love you to the moon
4394 and back, thanks for being the beautiful person you are.

4395 A special thanks to Sara, the person I love, who has been by my side during
4396 almost all the Master's Degree, every day of this PhD, and I hope every
4397 other day yet to come. Having the chance to share this experience with you

4398 helped me immensely. Returning home with you after the long days was
4399 all I needed to be happy. You support me, you believe in me, and you know
4400 me like nobody else does. I am grateful for having you and I hope we will
4401 realize all our dreams together.

4402 Thanks to all my friends from Beinette, especially Kappa, Emilio, Dani,
4403 Bertu and Francesco. You have been there in each step of my life, I know
4404 I can count on you. I owe you many, guys.

4405 “Mens sana in corpore sano”, I wouldn’t have finished this project without
4406 the gym Dimagrimondo. That gym has been my happy place in Grugliasco,
4407 I made so many friends there to whom I am grateful and recorded so many
4408 videos that are part of my best memories. Dimagrimondo really made my
4409 days.

4410 A few words also for Diana, not as a supervisor but as a friend. You have
4411 been a sister to me during my months in Switzerland. I would have never
4412 imagined spending so much time with you, Miki, Alexandre, Gaël e
4413 Raphaël. You made me feel part of your family and I will never forget it.
4414 When I arrived in Switzerland you helped me dealing with a bad moment,
4415 thank you. I miss so much playing with Alexandre and hearing him say
4416 “cammello!!”.

4417 In this last section I must thank two people who are not with us anymore,
4418 but from whom I learnt a lot.

4419 First of all, I want to thank Sergio Patrucco, Sara’s grandfather. Sergio, you
4420 taught me the importance of thinking outside the box. I spent much time
4421 listening to the incredible stories you lived, trying to pick from your words
4422 part of your knowledge. I wish I had the chance to learn from you much

4423 more. The way you saw the world was simply unique, and you made a
 4424 stubborn like me understand that the limits we have are mostly in our mind.
 4425 Please know that you were important to me, I respected you as a friend and
 4426 as a mentor. I miss you Sergio, thanks for everything.

4427 I want to conclude this section and this PhD thesis remembering my
 4428 grandmother, who passed away right before my abroad period. Nonna
 4429 Jolanda, you raised me, Gabriella and basically all our friends. We had the
 4430 best childhood ever for a simple reason, you were there. All the Sundays,
 4431 before returning to Grugliasco, me and Sara came to your place to have a
 4432 coffee with you. Staying that hour with you always made me feel better.
 4433 You were the supporting column of our family, thanks for everything you
 4434 did for us. This is not the place to remember all the memories I have with
 4435 you, but please know that you helped me a lot during the difficult times.

4436 Ti voglio bene nonna, grazie!

4437 Matte

4438

4439 **Published papers and conference presentations**

4440 **Publications**

4441 **Dho, M.**, Gonella, E., Alma, A., 2025. Field evaluation of symbiont-
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4452 **Conference presentations**

4453 **Dho, M.**; 11/08/2023. Development of a novel MLST approach for
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4456 Science”, Florence, Italy. Oral presentation.

4457 **Dho, M.**; 11/16/2022. Advancements in symbiotic control of stink bug
4458 pests of hazelnut from the local to the global scale. XIII European PhD
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