

1 | ***Halyomorpha halys* in Mediterranean areas: ~~invasion~~ local and landscape**  
2 | **drivers predictors, genetic diversity, and potential biological control**  
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19 **Abstract**

20 The brown marmorated stink bug *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae),  
21 native to eastern Asia, is an invasive species currently established in several non-native  
22 regions, where it can cause severe economic losses for growers. The factors driving *H. halys*  
23 spread are however poorly understood. In the current study, we ~~investigated the invasion~~  
24 ~~drivers studied~~ *H. halys* populations in southern Italy, where the pest is present but not  
25 established. We monitored pest occurrence in three different habitats potentially important for  
26 the spread and the establishment of *H. halys* (i.e., crop fields, urban gardens, fruit  
27 warehouses) selected along a gradient in the cover of urban habitats in the landscape. We also  
28 measured the potential biological control by natural enemies with sentinel egg masses in crop  
29 fields and urban gardens. Finally, we carried out a genetic characterization of southern Italian  
30 populations using multiple markers to identify the potential pathways of entry in the region.  
31 We found that high proportions of urban habitats in the landscape generally supported a  
32 higher occurrence and abundance of *H. halys* in crop fields, whereas the high cover of semi-  
33 natural habitats increased parasitism and predation rates. Moreover, the parasitoids *Anastatus*  
34 *bifasciatus* and *Ooencyrtus* sp., important control agents of *H. halys* in areas where the pest is  
35 already established, were the most common species observed. Our findings suggest that the  
36 composition of agricultural landscapes affects both the occurrence and the biological control  
37 of *H. halys* in newly invaded areas, potentially influencing the establishment probability of  
38 local populations. Finally, the finding of several host and symbiont haplotypes within the  
39 populations of *H. halys* in southern Italy, and the high similarity with the northern Italian  
40 populations, suggested continuous events of introduction probably occurred from the northern  
41 part of the Peninsula. Future monitoring programs focused on both urban and cultivated areas  
42 will be fundamental to track the spread of brown marmorated stink bug.

43 **Keywords:** alien invasive pest, brown marmorated stink bug, landscape composition, stink  
44 bug, egg parasitism, egg predation

Commento [A1]: Nuovi risultati?

## 45 **Introduction**

46 Invasive phytophagous insects have spread worldwide during the last 200 years, often  
47 impacting the communities of native organisms, altering ecosystem processes and causing  
48 severe economic damages (Kenis et al. 2009; Marbuah et al. 2014; Pyšek et al. 2020).  
49 International trade of goods is a major contributor to the exponential increase of new  
50 introductions around the globe (Early et al. 2016; Roques 2010). After introduction, the  
51 establishment and persistence of alien insect populations in newly invaded territories depend  
52 on numerous interacting abiotic and biotic factors, such as climate suitability, host plant  
53 availability, and presence of effective natural enemies (Bebber et al. 2014; Messing and  
54 Wright 2006). Urban habitats often favor the spread and establishment of invasive insect  
55 populations, being characterized by high transportation activity and by a heterogeneous  
56 mosaic of anthropogenic structures (e.g., houses, gardens, warehouses, parks) that may  
57 provide alternative food and shelter (Borden and Flory 2021; McIntyre 2000). Understanding  
58 the factors potentially influencing the establishment and spread of important invasive insect  
59 species is crucial to plan efficient monitoring programs and pest control strategies able to  
60 prevent pest outbreaks.

61 The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) is a  
62 phytophagous stink bug native to East Asia, first introduced in the United States in the 1990s  
63 (Leskey et al. 2012). The distribution of *H. halys* has grown exponentially worldwide in the  
64 coming decades, with abundant populations currently established in several non-native  
65 countries including Italy (Haye et al. 2015b; Maistrello et al. 2018). The species is highly  
66 polyphagous, capable to feed on more than 170 host species including crops, ornamentals, and  
67 wild plants (Leskey and Nielsen 2018). Moreover, it is considered as nuisance pest since it  
68 overwinters in large groups in residential dwellings. Adults and nymphs are highly mobile,  
69 easily dispersing across the landscape matrix, utilizing different habitats (Lee et al. 2014;  
70 Venugopal et al. 2015). Nevertheless, the factors driving *H. halys* spread are still unclear.

71 Although previous studies explored the habitat preference of *H. halys* (Bergh et al. 2021;  
72 Grabarczyk et al. 2022; Venugopal et al. 2014), little is known regarding how landscape  
73 composition may affect its abundance. Urban areas have been shown to facilitate the spread of  
74 *H. halys*, especially at the initial invasion stage (Maistrello et al. 2018, Wallner et al. 2014).  
75 Additionally, where the pest is already well established, semi-natural habitats have been  
76 observed to support both *H. halys* populations and the biological control provided by  
77 specialized natural enemies (Mele et al. 2022), whereas urban habitat seems to not affect the  
78 seems to not play an population dynamics of the pest in those area important when the pest is  
79 widely spread in the invaded area (Tamburini et al. 2023). Although current control strategies  
80 rely mainly on broad-spectrum insecticides, the control provided by parasitoid wasps might  
81 limit the growth and spread of brown marmorated stink bug populations. Several native  
82 parasitoid species have been in fact observed to adapt and to effectively control the non-  
83 coevolved host *H. halys* in North America and Europe (Abram et al. 2017; Conti et al. 2021,  
84 Ogburn et al. 2016; Roversi et al. 2016). These egg parasitoids include mainly species  
85 belonging to Hymenopteran families: Scelionidae (*Telenomus* spp., *Trissolcus* spp., *Gryon*  
86 spp.), Eupelmidae (*Anastatus* spp.) and Encyrtidae (*Ooencyrtus* spp.). Moreover, two  
87 adventive exotic Scelionid wasp species, *Trissolcus japonicus* (Ashmead) (Hymenoptera:  
88 Scelionidae) and *Trissolcus mitsukurii* (Ashmead) (Hymenoptera: Scelionidae), considered  
89 the predominant egg parasitoids in the native range (Yang et al. 2009), have been recently  
90 reported in several newly invaded countries (Conti et al. 2021; Moraglio et al. 2020;  
91 Sabbatini-Peverieri et al. 2018; Scaccini et al. 2020). Studies exploring the impacts of  
92 landscape characteristics on *H. halys* populations and its biological control agents in newly  
93 invaded areas are hence urgently needed.

94 In Italy, field populations of *H. halys* were recorded for the first time in 2012 (Modena  
95 district, Emilia-Romagna Region, northern Italy; Maistrello et al. 2016), and then this species  
96 quickly spread to neighboring areas becoming a key pest for local fruit production (Haye et al.

97 2015b; Leskey et al. 2012; Maistrello et al. 2018; Moore et al. 2019; Rice et al. 2014) and  
98 causing serious economic damage, e.g., of € 588 million as observed in northern Italy in 2019  
99 (CSO 2020). Information regarding its occurrence in the southern part of the country remains  
100 instead scarce. Here the pest was first recorded in 2016 (Apulia region, southern Italy;  
101 Cianferoni et al. 2018), and, despite the abundance of hosts and the potentially suitable  
102 climate (Zhu et al. 2012), no serious damages to crops have been reported so far (Laterza et  
103 al. 2022). Moreover, contrary to the northern populations for which an intensive genetic  
104 characterization of the pest and of its endosymbiont “*Candidatus Pantoea carbekii*”  
105 (henceforth called *P. carbekii*) has been carried out (Cesari et al. 2018; Martinez-Sañudo et al.  
106 2020), little information is available regarding the invasion pathways of *H. halys* in southern  
107 Italy.

108 In the current study, we investigated the factors potentially driving the invasion dynamics of  
109 *H. halys* in southern part of Italian peninsula, where this stink bug is present but whose  
110 distribution is not yet investigated. We hence monitored pest occurrence in three different  
111 habitats potentially important for the spread and the establishment of *H. halys* (i.e., crop  
112 fields, urban gardens, fruit warehouses) selected along a gradient in the cover of urban  
113 habitats in the landscape. While crop fields and urban gardens might potentially provide food  
114 resources and shelter opportunities, fruit warehouses (i.e., storage centers that commercialize  
115 fruits and vegetables with both national and international partners) can represent important  
116 points of entry of this pest. We also measured the potential biological control of brown  
117 marmorated stink bug with sentinel egg masses. ~~Finally, we investigated the genetic diversity~~  
118 ~~of this pest and its endosymbiont “*Candidatus Pantoea carbekii*” (henceforth called *P.*~~  
119 ~~*carbekii*) using multiple genetic markers, to better understand the origins of the southern~~  
120 ~~Italian populations. Finally, we carried out a preliminar genetic characterization of the~~  
121 ~~specimens using two mitochondrial *H. halys* markers (COI and COII) to track the origins of~~  
122 ~~the pest in the investigated area. Moreover, we included the haplotype characterization of~~

123 endosymbiont “*Candidatus Pantoea carbekii*” (henceforth called *P. carbekii*) recently used to  
124 improve the understanding of the origins of the southern-Italian populations (Martinez-  
125 Sanudo et al. 2020).

126 We hypothesized that (i) urban habitats are important in supporting abundant pest populations  
127 in the region, (ii) fruit warehouses are important points of entry, (iii) semi-natural habitats in  
128 the landscape promote the biological control of *H. halys*, and that (iv) the pest arrived in  
129 southern Italy from multiple introduction pathways and source populations.

130

## 131 **Materials and methods**

### 132 *Sampling design*

133 The monitoring campaign was conducted between May and November 2021 across the  
134 agricultural landscapes of Bari and Foggia districts (Apulia region, southern Italy) (Fig. 1). A  
135 total of ten non-overlapping circular landscapes with a 2 km radius were selected in the study  
136 area along a gradient of cover in urban habitats ranging from 1.2 to 81.6%. This scale has  
137 been shown appropriate to study the effects of landscape on this mobile pest and its potential  
138 natural enemies (Tscharntke et al. 2007; Wallner et al. 2014; Mele et al. 2022). Landscape  
139 centroids were separated by at least 6 km, except for two located at shorter distance (3.5 km).  
140 We measured for each landscape the cover of annual crops (e.g., wheat, barley, lentils,  
141 vegetables), perennial crops (e.g., olive groves, stone-fruit orchards, vineyards) and semi-  
142 natural habitats (forests and grasslands) using high-resolution satellite images in Google Earth  
143 Pro (Google Inc.© 2017) both at 1 and 2 km scale (correlations between landscape variables  
144 are presented in Table S1). Within each landscape we selected three sites expected to favour  
145 the establishment and the spread of *H. halys* (crop habitats, urban gardens, fruit warehouses),  
146 except for one landscape characterized by 0% of urban habitat gradient and for which only  
147 crop habitat and fruit warehouses were included. The missing urban garden was hence  
148 selected outside the 2 km radius area. Within each landscape, the monitored sites (30 in total)

149 were selected to minimize the distance from centroid. Monitored crop habitats were olive (n =  
150 3) and stone-fruit (n = 5) groves and vegetable gardens (n = 2). Urban gardens were  
151 characterized by both ornamental plants and vegetables. Finally, fruit warehouses (i.e.,  
152 storage centers receiving fruits and vegetables) were selected among those having commercial  
153 trade with international and northern Italian partners.

154

#### 155 *Halyomorpha halys* occurrence and abundance

156 We measured the occurrence of *H. halys* in all 30 sites with pheromone baited traps (one trap  
157 per site). Traps were based on the Suckling et al. (2019) prototype which was observed to  
158 increase monitoring efficiency and allows to capture alive individuals (Fig. S1). An  
159 aggregation pheromone lure (Trécé, Adair, OK, USA) was inserted into the trap, and replaced  
160 every two months, following the manufacturer's instructions. In each site, all traps were  
161 deployed on local vegetation at ~1.5 m from the ground, preferably on cultivated or wild host  
162 plant of *H. halys*. Since *H. halys* showed a resting period within 2.5 m around the traps when  
163 monitored with aggregation pheromone (Morrison et al. 2016a), ~~At each sampling round~~  
164 ~~(every 14 days),~~ we also collected *H. halys* individuals present on the local vegetation (three  
165 plants per site) within a 10 m radius around the trap. Thus, at each sampling round (every 14  
166 days), ~~trees~~ canopies and bushes were sampled via beating technique (i.e., beating the  
167 branches from the lower and upper part of the canopies and collecting insects on a 1 x 1 m  
168 white cloth placed underneath). Adults and nymphs of *H. halys* collected alive, active and in  
169 good shape were used for laboratory rearing (see below).

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170

#### 171 *Potential biological control*

172 We studied the potential biological control of *H. halys* provided by both specialist and  
173 generalist natural enemies exposing *H. halys* sentinel egg masses in the field, measuring both  
174 parasitism and predation rates, and identifying egg parasitoids. This method is widely used to

175 study the natural enemies of *H. halys* in invaded countries especially when wild egg masses  
176 cannot be easily found in the field because of low pest abundance (Ogburn et al. 2016;  
177 Roversi et al. 2016; Tillman et al. 2020). A laboratory colony of *H. halys* was hence  
178 established from alive specimens collected in field. Insects were reared in metal net cages (30  
179 × 30 × 30 cm) at  $25 \pm 1^\circ\text{C}$ , 65-70% RH, and photoperiod of 16:8 h (L:D). Adults were fed  
180 with carrots, green beans, apple, tomato, sunflower and soybean seeds. Water was supplied ad  
181 libitum using a cotton ball. A paper towel was also introduced on the cage as oviposition  
182 substrate. Cages were inspected daily between late May and late August to collect fresh laid  
183 egg masses. Twenty-four hours old egg masses were collected and stored at  $7 \pm 1^\circ\text{C}$  for at  
184 least one week, following the procedure described in Wong et al. (2020). This protocol allows  
185 to terminate the host development, thus avoiding unintentional introductions of the invasive  
186 pest in the monitored sites, and reduces the potential sublethal effects on parasitoid  
187 development compared to frozen ( $-80^\circ\text{C}$ ) egg masses. Individual egg masses were hence  
188 moved onto paper cardboards (3 cm x 2 cm) by means of a soft brush and attached to the  
189 supports with non-toxic glue (Elmer's Clear Glue). Each egg mass was then coded and the  
190 total number of eggs in each mass was recorded prior to field exposition (mean number of  
191 eggs =  $26.13 \pm 0.18$  SE).

192 We measured the potential biological control in eight landscapes previously selected for  
193 monitoring *H. halys* (Fig. 1) and that showed a relevant gradient in semi-natural habitat (range  
194 = 0.9 – 25.4%). Within each landscape, we surveyed the two habitats where an efficient  
195 biological control is probably fundamental to limit the spread of stink bug populations: crop  
196 fields and urban gardens (16 sites in total). Egg masses were exposed in the field in June,  
197 July, and August 2021 (three rounds). At each round, paper cardboards with 6-7 egg masses  
198 per site were clipped directly on leaves of potential host plants at ~1.5 m from the ground  
199 level (Cornelius et al. 2016). Egg masses were then retrieved after 7 days (Haye et al. 2015a),  
200 transported to the laboratory ( $25 \pm 1^\circ\text{C}$ , 65-70% RH, 16:8 h L:D) and placed into plastic



201 boxes (i.e., 50 mL Falcon tubes) until parasitoid emergence. The number of missing or  
202 damaged eggs was recorded, and the hatched parasitoids were stored in 70% ethanol until  
203 species identification, which was made using specific taxonomic keys (Askew and Nieves-  
204 Aldrey 2004; Talamas et al. 2015, 2017; Tortorici et al. 2019). Finally, all eggs were dissected  
205 to verify the presence of parasitoid larvae or pupae that failed to develop and hatch. We hence  
206 calculated the proportion of parasitized and predated eggs for each site and round. Parasitism  
207 rate was potentially underestimated as parasitized eggs could have been predated.

208

#### 209 *Statistical analysis*

210 We ~~used generalized linear mixed-effects models to explore~~ explored the effects of habitat type and  
211 landscape composition on *H. halys* occurrence (presence/absence data) and abundance  
212 (average number of individuals) per site, and per month ~~(binomial and negative binomial~~  
213 ~~distribution, respectively). We used generalized linear mixed-effects models to account for the~~  
214 ~~nested structure of the data, considering a binomial family for the conditional distribution of~~  
215 ~~the response variable and a logit-link function for occurrence data, and a negative binomial~~  
216 ~~family for the conditional distribution of the response variable and a log-link function for~~  
217 ~~abundance data.~~ In the analysis, brown marmorated stink bugs found in pheromone traps and  
218 beating samplings were merged in their numbers. ~~We considered as landscape predictors the~~  
219 ~~proportion of urban, annual crops, perennial crops, and semi-natural habitats in the landscape.~~  
220 ~~Ten models were run for each response variable, each including a different landscape~~  
221 ~~predictor (five landscape metrics at two scales: 1 km and 2 km of radius). The model~~  
222 ~~displaying the lowest AIC was considered as the best fitting model (Akaike 2011).~~ The  
223 models included the occurrence or the abundance of *H. halys* (both adults and juveniles) as  
224 response variable, and habitat type (categorical; three levels), landscape composition  
225 (continuous; % cover of urban, annual crops, perennial crops or semi-natural habitats in the  
226 landscape), time (continuous; months) and their interactions as predictors (i.e. fixed factors).

227 We did not consider three-way interactions to reduce model complexity. In order to select the  
228 most appropriate ~~We considered as landscape predictors the proportion of urban, annual~~  
229 ~~crops, perennial crops, and semi-natural habitats in the landscape~~ variable and scale to be  
230 included in each model, we run ~~Eighteen alternative models were run for each response~~  
231 variable, each including ~~among~~ besides the other fixed factors considered, a different  
232 landscape predictor (five ~~four~~ landscape metrics at two scales: 1 km and 2 km of radius). The  
233 model displaying the lowest AIC was considered as the best fitting model (Akaike 2011).  
234 Landscape and site ID were included as random factors in all the models. Models analyzing  
235 the effect of landscape composition, time and their interaction, separately for the three  
236 habitats, produced qualitatively similar results.

237 Regarding the analysis of potential biological control, we used general linear mixed-effect  
238 models to explore the effects of habitat type and landscape composition on parasitism and  
239 predation rates considering a normal gaussian family for the conditional distribution of the  
240 response variable and an identity-link function. In the analysis, parasitism and predation were  
241 averaged at the site level to reduce model complexity. The most appropriate landscape  
242 variable was selected as explained before (i.e., comparing the AIC across models with  
243 different landscape variables). The two final models selected included the proportion of  
244 parasitized or predated eggs as response variables, and habitat type (categorical; two levels),  
245 landscape composition (continuous; % cover of urban, annual crops, perennial crops or semi-  
246 natural habitats in the landscape), ~~sampling round (continuous; three levels)~~ and their  
247 interactions as predictors (i.e., fixed factors). We also included local *H. halys* abundance to  
248 account for potential density dependence mechanisms. The proportion of parasitized eggs was  
249 log-transformed to abide by model assumptions.

250 ~~For all the models, H~~ landscape and site ID were ~~was~~ included as random factors ~~and we did~~  
251 ~~not consider three-way interactions to reduce model complexity.~~ We performed the analyses  
252 using the “glmmTMB” package (Brooks et al. 2017) implemented in R (R Core Team 2022).

253 We checked the models for overdispersion and residual distribution using the “DHARMA”  
254 package (Hartig 2017).

255

#### 256 *Genetic analysis*

257 We carried out a global genetic analysis of *H. halys* and its symbiont *P. carbekii* to better  
258 understand the invasion pathways of the pest in southern Italy. Host cytochrome c oxidase  
259 subunit I (*COI*, 782 sequences), subunit II (*COII*, 790 sequences), and the putative  
260 pseudogene of the symbiotic bacteria *P. carbekii* (*ΔybgF*, 277 sequences) were used to  
261 explore genetic diversity among *H. halys* populations. For each genetic marker, 44 sequences  
262 were newly analyzed, while the others were obtained from previous studies (Cesari et al.  
263 2018; Garipey et al. 2014; Lee et al. 2018; Martinez-Sañudo et al. 2020; Otero-Bravo and  
264 Sabree 2018; Xu et al. 2014; Yan et al. 2021a). The final dataset comprised sequences from  
265 17 countries: China, Japan, South Korea, Serbia, Slovenia, Switzerland, Austria, Turkey,  
266 Georgia, Hungary, Romania, Greece, France, Italy, USA, Chile, and Canada (see “Global  
267 genetic analysis of *H. halys* and *P. carbekii*” section of the supplementary material for  
268 details). The new specimens were collected in southern Italy (as described before), northern  
269 Italy, France, and Canada. These regions were considered underrepresented in genomic  
270 libraries. The number of newly collected specimens in southern Italy (in the focus  
271 populations) was limited by the low occurrence of the pest (see Results). All specimens were  
272 collected alive in the fields, stored in 96% ethanol and dissected with sterile forceps to extract  
273 the muscle and gut (V4 region) tissue. Extraction of DNA from each individual sample was  
274 performed using the Qiagen Dneasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA)  
275 following the manufacturer’s instruction. The LCO-1490/HCO-2198 (Folmer et al. 1994),  
276 HhalysCO2F2/HhalysCO2R2 (Xu et al. 2014) primers were used to amplify *H. halys* loci  
277 (*COI* and *COII* regions, respectively), while the putative pseudogene *ΔybgF* was used to  
278 study the genetic diversity of the symbiont *P. carbekii* (Otero-Bravo and Sabree 2018). PCR

279 was performed with GoTaq Green Master Mix from Promega and amplicons were checked  
280 via electrophoresis on 2.0% agarose gel, cleaned with QIAquick PCR purification Kit  
281 (Qiagen, Valencia, CA, USA) and finally sequenced at the National Cancer Institute (NCI) at  
282 the Ohio State University (Columbus, OH, USA).

283 Sequences were edited and aligned using MEGA X (Kumar et al. 2018) and checked on  
284 GenBank to assess the identity of the sequences. Measures of genetic diversity (number of  
285 haplotypes, nucleotide diversity and haplotype diversity) and neutrality test were performed  
286 with DnaSP v5.10.1 (Librado and Rozas 2009). Calculation of population diversity indices of  
287 the new sequences obtained was limited to those populations counting more than five  
288 samples. To place the newly collected sequences in a global context, we generate a TCS  
289 haplotype network (Clement et al. 2002) including data from previous collections in East  
290 Asia, Europe and America using PopART 1.7 (Leigh and Bryant 2015). Haplotype  
291 designation followed that of previous studies (Garipey et al. 2014; Martinez-Sañudo et al.  
292 | 2020; Otero-Bravo and Sabree 2018; Xu et al. 2014; Yan et al. 2021a). Genetic differentiation  
293 among populations of *H. halys* and among those of *P. carbekii* was estimated by the fixation  
294 index ( $F_{ST}$ ) and the overall genetic variance was calculated by AMOVA (Analysis of  
295 molecular variance) via Arlequin 3.5 (Excoffier and Lischer 2010), while pairwise  
296 comparison of  $F_{ST}$  index was performed in R (R Core Team 2022).

297

## 298 **Results**

### 299 *Halyomorpha halys* occurrence and abundance

300 We collected a total of 194 specimens of *H. halys* (135 adults and 59 nymphs) during the  
301 investigated period. Pest occurrence and abundance generally decreased with time and were  
302 influenced by both habitat type and landscape composition (Table 1). Locally, occurrence of  
303 *H. halys* was higher in crop habitats compared to urban gardens and fruit warehouses at the  
304 beginning of the sampling period (Fig. S2). Occurrence in crop habitats decreased faster with

305 time than in the other two habitats (Habitat x Time interaction). The inclusion of the  
306 proportion of urban habitats as landscape predictor always yielded the lowest AIC for the  
307 models on the pest (Table S2). We found that the proportion of urban habitats in the  
308 landscape increased both occurrence and abundance in crop habitats and fruit warehouses, but  
309 it did not influence populations inhabiting urban gardens (Fig. 2, Fig. S3; Urban x Habitat  
310 interaction).

311

### 312 *Potential biological control*

313 We exposed a total of 333 egg masses in three different occasions (June, July and August)  
314 comprising a total of 8702 eggs. The 3.3% of sentinel eggs were found parasitized (9.3% of  
315 sentinel egg masses were found with at least one egg parasitized), and 5 parasitoid species  
316 among the identified ones emerged from *H. halys* eggs (Fig. 3C). *Anastatus bifasciatus*  
317 (Geoffroy) (Hymenoptera: Eupelmidae) and *Ooencyrtus* sp. Ashmead (Hymenoptera:  
318 Encyrtidae) (both present in 26.9% of the parasitized egg masses) were the most abundant,  
319 followed by *Trissolcus basalis* (Wollaston, 1858) (7.7%), *Telenomus* spp. Haliday  
320 (Hymenoptera: Scelionidae) and *Trissolcus belenus* (Walker) (Hymenoptera: Scelionidae)  
321 (3.8%). Moreover, in 30.8% of the parasitized egg masses no adults emerged, but larval or  
322 pupal stages of parasitoid were observed in the egg (Fig. 3D; Table S3). Finally, none of egg  
323 masses were parasitized by more than one (identifiable) species. The 15.6% of the sentinel  
324 eggs presented signs of predator activity on the shell (e.g., incomplete/complete chewing;  
325 25.0% of the sentinel egg masses exposed) (Fig 3A, 3B), while 11 were not found after the  
326 exposure period.

327 We found the potential biological control to be influenced by the studied factors (Table 1).  
328 Including the proportion of semi-natural habitats as landscape predictor, always yielded the  
329 lowest AIC for the models on pest control (Table S2). We found the proportion of semi-  
330 natural habitats in the landscape to be positively related to parasitism rate, ~~but only in crop~~

331 ~~fields (Fig. 4A; Semi-natural x Habitat interaction), whereas at habitatthe local scale .~~  
332 ~~Moreover, parasitism rate decreased over time. p~~Predation rate ~~\_~~ was higher in crop fields than  
333 in urban gardens (Fig. 4B). ~~Finally, and it was positively correlated to the proportion of semi-~~  
334 ~~natural habitats in the landscape, but only during the first sampling round (June), whereas it~~  
335 ~~showed no or slightly negative trend along the landscape gradient in July and August,~~  
336 ~~respectively (Fig. S4). p~~Pest abundance did not influence parasitism and predation rates (Tab.  
337 1).

### 338 339 Genetic analysis

340 The overall genetic analysis was carried out on a total of 1849 sequences from *H. halys*  
341 samples including both mitochondrial *H. halys* loci (*COI* and *COII*) and *ΔybgF* symbiont  
342 pseudogene used as markers to investigate genetic diversity of the pest (see “Global genetic  
343 analysis of *H. halys* and *P. carbekii*” section of the supplementary material for details).  
344 Among all these sequences, 54 high quality sequences (*COI* and *COII* region and of *ΔybgF*  
345 pseudogene) were obtained from specimens collected in several locations across the Apulia  
346 region (current study; Table S8).  
347 All haplotypes detected in southern *H. halys* populations were also detected in northern Italy  
348 (Fig. 5). Moreover, the most abundant haplotypes found in southern Italy were also the most  
349 common haplotypes at the Italian and global scale (Fig. S4~~5~~ and S6; Table S7), named H1 and  
350 H3 for *COI* region, h1, h3 and h11 for *COII*, and P1 and P2 for the endosymbiont. We also  
351 found rare but globally present haplotypes such as H40, H41, H42, H43, and, interestingly,  
352 the haplotype H8 previously recorded only in Europe (Austria, France, Hungary, southern  
353 Italy, Switzerland) and the haplotype P4, previously recorded only in northern Italy. The  
354 genetic diversity of southern Italian populations presented significant differences compared to  
355 populations of several countries such as Japan (*COI*, *COII* and *ΔybgF*), China and USA (*COI*,  
356 *COII*) but not to northern Italian ones (Fig. 6; Table S6).

357

358 **Discussion**

359 This study provides first insights into the population drivers of *H. halys* and its potential  
360 natural enemies in Apulia region (southern Italy), an area of potential expansion for this pest,  
361 and into the introduction history of the pest in the region. We found landscape composition  
362 and local habitat type to influence both the occurrence of this invasive stink bug and the  
363 potential biological control by native natural enemies. In particular, high proportions of urban  
364 habitats in the landscape generally supported a higher occurrence and abundance of *H. halys*  
365 in crop fields, whereas high cover of semi-natural habitats increased parasitism ~~and predation~~  
366 rates ~~ananddwhile higer predation rate were observed in cultivated area.~~ ~~T-Moreover,~~ the  
367 parasitoids *A. bifasciatus* and *Ooencyrtus* sp., important control agents of *H. halys* in areas  
368 where the pest is already established, were the most common species observed. Our findings  
369 suggest that the composition of agricultural landscapes affects both the occurrence and the  
370 biological control of *H. halys* in newly invaded areas, potentially influencing the  
371 establishment probability of local populations. The preliminary genetic analysis presented  
372 here suggests that southern Italian *H. halys* populations might have originated from northern  
373 Italy, although this assessment needs to be deepened.

374 Our data showed that urban areas potentially play an important role in the spread of *H. halys*  
375 populations in newly invaded areas, since the cover of urban habitats in the landscape was  
376 positively correlated with the occurrence and the abundance of the pest. Human structures can  
377 indirectly increase the frequency of introduction or provide important resources required for  
378 colonization (Thomas et al. 2017). Our findings are consistent with previous studies  
379 (Holthouse et al. 2021; McIntyre 2000). Commercial and residential developments are known  
380 to support *H. halys* overwintering survival (Inkley 2012), while human transportation can  
381 facilitate its spread through hitchhiking (Maistrello et al. 2018; Wallner et al. 2014).  
382 However, the cover of urban habitats in the landscape did not affect the occurrence and

383 abundance of *H. halys* in urban gardens, probably because urban areas acted more as source  
384 of introduction than sink, with insect individuals moving to agricultural settings.

385 *Halyomorpha halys* was found in all the surveyed habitats, and strongly responded to  
386 landscape composition measured at 2 km scale. These results confirm the high mobility of *H.*  
387 *halys* (Lee et al. 2014) and its ability to move across habitats ([Tamburini et al. 2023](#);  
388 Venugopal et al. 2015), similarly to other stink bug species (Laterza et al. 2023). Contrary to  
389 our expectation, we did not observe an increase in captures at the end of the surveyed period  
390 in urban areas, where aggregation around overwintering sites are supposed to occur. Similar  
391 seasonal decline in *H. halys* captures has been observed by Ingels and Daane (2018) in urban  
392 areas in California when the temperatures raised up to 35°C. Reproduction of brown  
393 marmorated stink bug is in fact impaired above 30 – 32°C (Haye et al. 2014; Rice et al. 2014).  
394 It is hence possible that the high temperature characterizing the Apulian summer might have  
395 influenced the insect dynamics, limiting its population built up (Fisher et al. 2021; Scaccini et  
396 al. 2019). Similarly, the low relative humidity in the study area may [have affected](#) *H. halys*  
397 population dynamics by decreasing hatching rates and nymphal survival (Fisher et al. 2021;  
398 Khadka et al. 2020; Stahl et al. 2021). Moreover, the low number of *H. halys* individuals  
399 captured in this study seems to indicate that local populations are not well established and  
400 struggle to grow, potentially depending on hitchhikers for maintaining their presence in the  
401 region. Additional evaluations and extensive monitoring programs are needed to better  
402 understand the effect of local environmental conditions on the survival and potential  
403 establishment of the pest. Finally, we did not find more abundant populations in fruit  
404 warehouses compared to the other monitored habitats. We cannot hence conclude that these  
405 structures are particularly important for the pest to spread.

406 We found landscape composition [and habitat type](#) to drive the potential for biological control  
407 of *H. halys*, influencing [respectively both](#) egg parasitism and predation. The proportion of  
408 parasitized eggs increased with the cover of semi-natural habitats around the sampling sites.



409 These results are consistent with previous studies that found unmanaged vegetation to  
410 promote the biological control of this pest (González et al. 2017; Mele et al. 2022), with  
411 grasslands and woodlands providing alternative resources and suitable microclimatic  
412 conditions for the development of natural enemies (Bianchi et al. 2006; Tillman et al. 2020;  
413 Tamburini et al. 2022). ~~Our data also indicate higher parasitism rates in crop habitat compared~~  
414 ~~to urban gardens (P = 0.051), probably because of the higher availability of alternative hosts.~~  
415 Most of the eggs were parasitized by the generalist parasitoids *A. bifasciatus* and *Ooencyrtus*  
416 sp., which were previously addressed as the most promising indigenous parasitoids to control  
417 *H. halys* in the newly invaded areas (Haye et al. 2015a; Roversi et al. 2016; Stahl et al. 2019).  
418 In the coming years, exotic species such as *Trissolcus japonicus* and *T. mitsukurii* might get  
419 established as occurred in northern Italy (Moraglio et al. 2020; Scaccini et al. 2020). We also  
420 found that egg masses exposed in the field were attacked by generalist predators. Most of the  
421 predated eggs indeed presented incomplete/complete chewing signs, attributable to generalist  
422 predators such as carabid beetles, earwigs, and bush crickets as described in Morrison et al.  
423 (2016b). ~~Predation rates were higher in landscape characterized by high cover of semi-natural~~  
424 ~~habitats, but only at the beginning of the sampling. Generalist predators were probably more~~  
425 ~~active in late spring/early summer, when the climate was still relatively cool. As for~~  
426 ~~parasitism~~ Predation rate, predation was higher in crop fields than in urban gardens, where  
427 prey availability is probably higher as well. Moreover, contrary to parasitoids, predators were  
428 not affected by landscape composition, suggesting that the two natural enemy groups in the  
429 study region may use the agricultural matrix (Dominik et al. 2018; Tamburini et al. 2022). We  
430 recorded an overall percentage of parasitized and predated egg masses of 3.3% and 15.6%,  
431 respectively. Similar relatively low levels of parasitization were observed in previous studies  
432 (Cornelius et al. 2016, 2021; Ogburn et al. 2016) and might be explained by the lack of a  
433 shared evolutionary history between local natural enemies and the alien pest. Non-coevolved  
434 parasitoids often fail to successfully identify alien hosts and to complete their development

435 (Abram et al. 2016), as also suggested by our finding of unhatched parasitoids. However, it is  
436 also possible that the use of sentinel egg masses led to an underestimation of parasitoid true  
437 potential for biological control (Jones et al. 2014). In fact, although sentinel egg masses have  
438 been widely used to assess parasitism and predation rate in the field (Abram et al. 2017;  
439 Cornelius et al. 2016; Tillman et al. 2020), the lack of cues released by an active host during  
440 egg laying might have impaired the ability of natural enemies to efficiently locate the egg  
441 masses (Conti et al. 2021; Tognon et al. 2016; Zhong et al. 2017). Further studies are hence  
442 needed to better identify the natural enemy complex of *H. halys* in southern regions,  
443 surveying wild eggs in multiple habitats.

444 Finally, the current study provided first insights into the genetic diversity of brown  
445 marmorated stink bug populations in southern Italy. Our results suggested that multiple and  
446 continuous introductions might have occurred in the region, as we observed different *COI*,  
447 *COII* and *ΔybgF* haplotypes within the same locations (Martinez-Sañudo et al. 2020).  
448 Moreover, the data obtained from pairwise comparison of  $F_{ST}$  value suggested that *H. halys*  
449 population mostly arrived in the study area from northern Italy, which is also confirmed by  
450 the finding of the symbiont haplotype P4 previously recorded only in northern part of the  
451 country (Martinez-Sañudo et al. 2020). Nevertheless, events of entry from Asia and America  
452 cannot be completely excluded. These results are consistent with previous studies which have  
453 observed the pest to spread via secondary invasions from successful established populations  
454 (e.g., “bridgehead effect”, Lombaert et al. 2010; Cesari et al. 2018), mostly following the  
455 commercial routes and acting as hitchhiker (Maistrello et al. 2018). The use of multiple  
456 markers, including the symbiont pseudogene, was useful to clarify the potential introduction  
457 pathways. However, our collections were fairly limited in geographical range and a more  
458 extensive sampling is hence needed. Finally, further studies should also include more variable  
459 genetic marker to detect new haplotypes and clarifying the invasion scenario (Gogniashvili et  
460 al. 2022; Kapantaidaki et al. 2019; -Parvizi et al. 2023; Yan et al. 2021b).

461 In conclusion, our study highlights the crucial role of urban habitat for the population  
462 dynamic of *H. halys*, potentially representing an important driver for its spread in southern  
463 Italy where the pest is not clearly established yet. Landscapes characterized by high urban  
464 cover ~~are-might~~ hence be more susceptible to the pest invasion, probably because of the  
465 higher human activity (e.g., human transportation, commercial trade) and presence of  
466 alternative resources and shelters (e.g., human dwellings). However, the presence of semi-  
467 natural habitats might partially limit *H. halys* spread in southern regions, as they support the  
468 communities of natural enemies, and potentially the biological control of this pest.  
469 Nevertheless, multiple year monitoring programs regularly surveing a higher numer of sites  
470 are need to better understand the factors driving the invasion dynamics of the pest in southern  
471 Italy. Our genetic analysis suggests that southern Italian *H. halys* populations might have  
472 originated from the spread of populations present in northern Italy. Although this invasive  
473 pest has not caused serious damages to crop production in southern Italy so far, it is not clear  
474 whether population built up is limited in this area by climatic conditions or whether the  
475 invasion process is just at an early stage. Monitoring programs focusing on both urban and  
476 cultivated areas will be fundamental to track the future spread of *H. halys* in this region.

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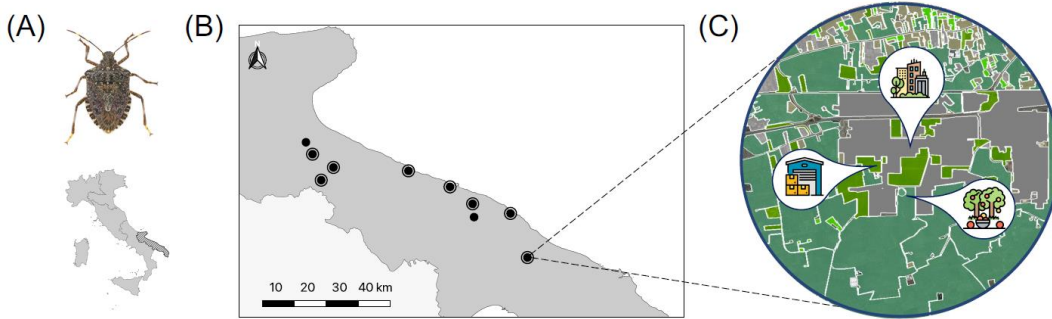
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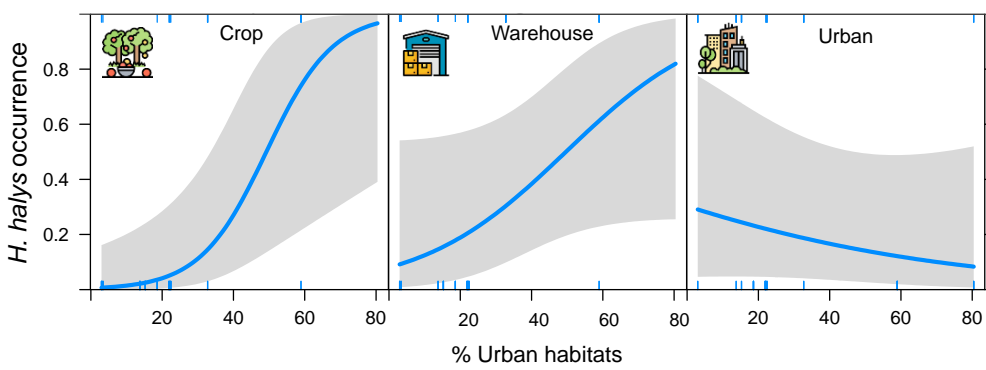
747 **FIGURES**



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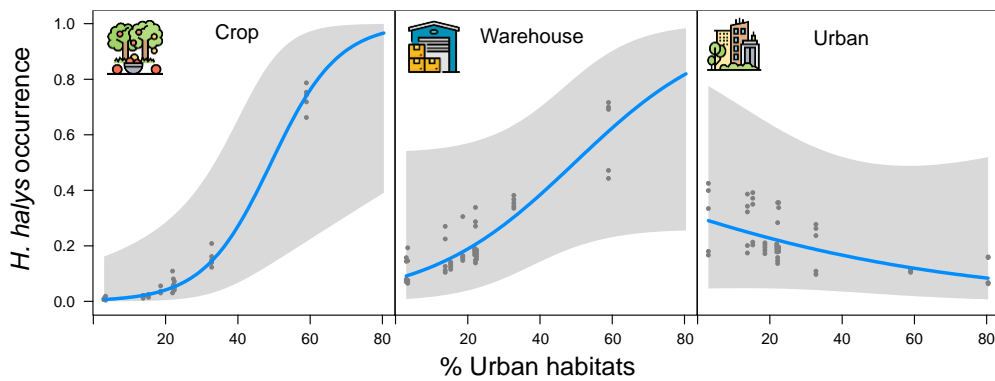
749 **Figure 1** – Study organism *Halyomorpha halys* and map of Italy (A), location of the  
 750 landscapes investigated (B) which were characterized by different composition (C).  
 751 *Halyomorpha halys* individuals were collected in all the indicated locations (black dots),  
 752 while biological control potential was measured only in landscapes indicated with black  
 753 circles. Habitat icon: Flaticon.com.

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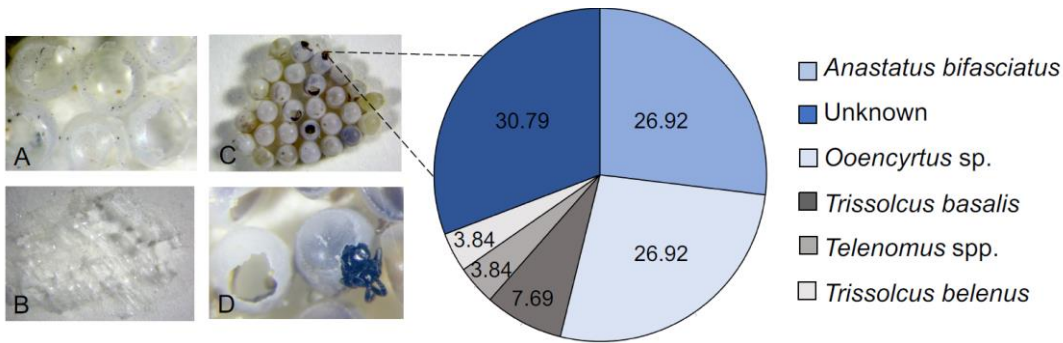
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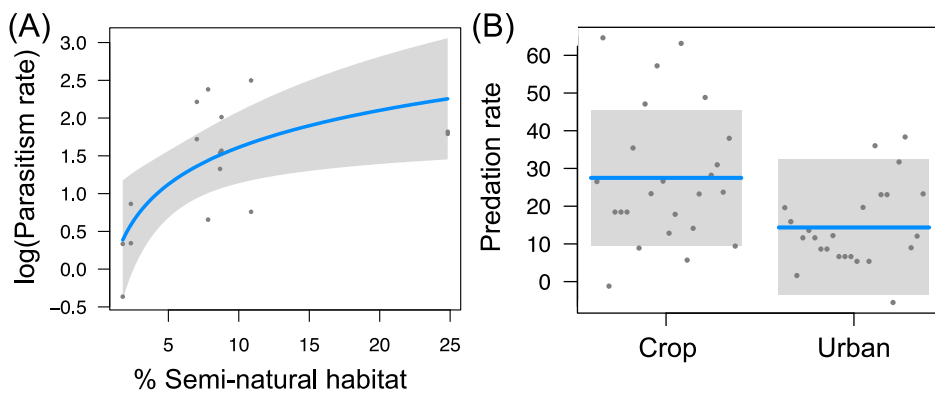
756

757 **Figure 2** – Effects of percentage of urban cover in the landscape (2 km radius scale) on the  
 758 occurrence of *H. halys* in the different habitats investigated. Grey dots represent partial  
 759 residues. Icons: Flaticon.com.  
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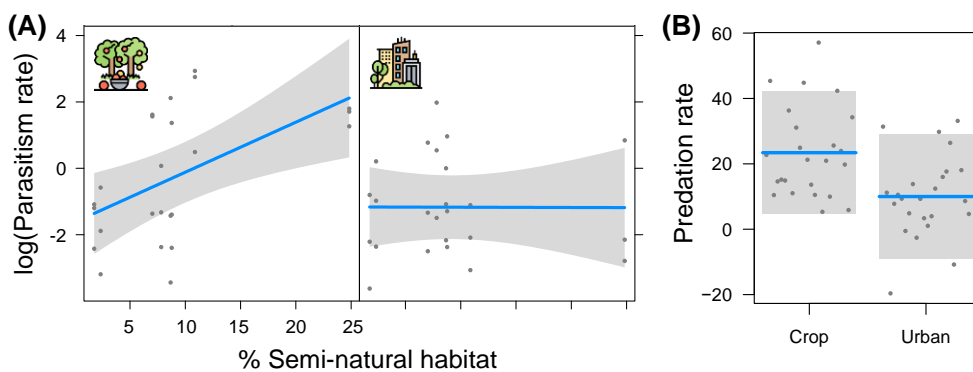
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763 **Figure 3** – Natural enemies damage syndrome on sentinel egg masses exposed in the field:  
 764 incomplete (A) and complete (B) chewing by generalist predators; hatched (C) and unhatched  
 765 (D) parasitoids found in parasitized egg masses. The percentage of egg masses parasitized by  
 766 the different species of parasitoids are reported in the pie chart.  
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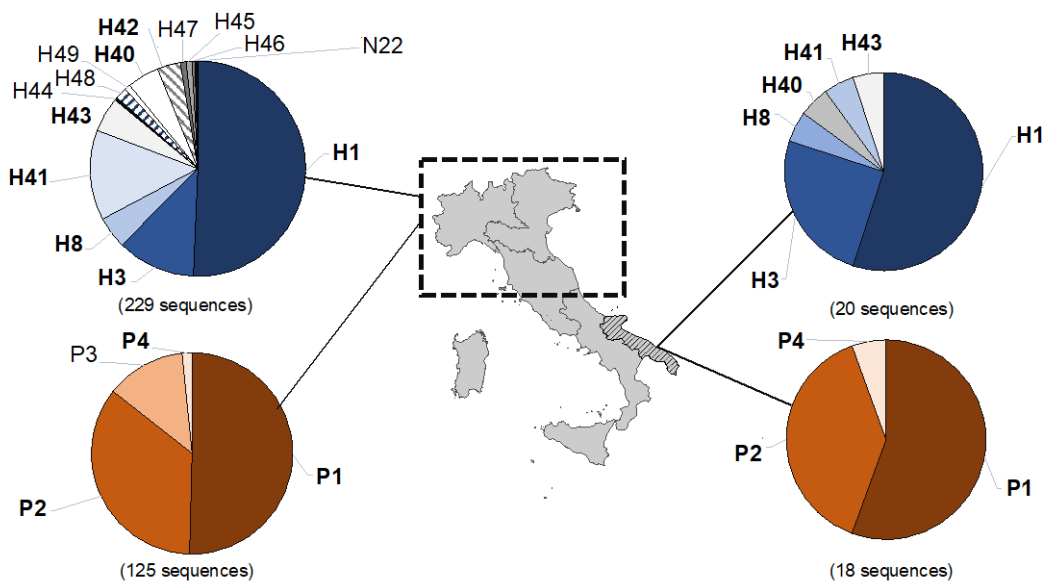
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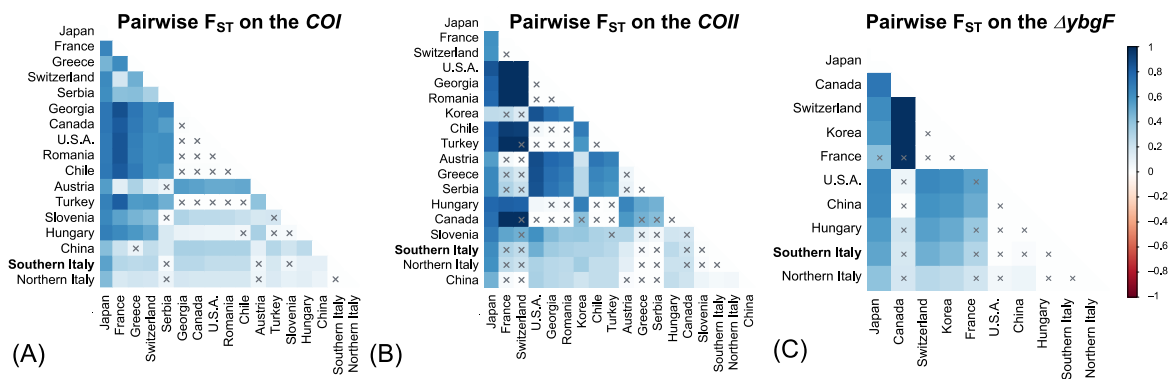
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 770 **Figure 4** – Effects of semi-natural cover in the landscape on the parasitism rate **in different**  
 771 **habitats** (A) and effects of habitat type on predation rate (B). **Grey dots represent partial**  
 772 **residues.** Icons: Flaticon.com.  
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 775 **Figure 5** – Geographical distribution of *H. halys* (above, blue pie charts) and its  
 776 endosymbiont *P. carbekii* (below, orange pie charts) haplotypes across Italian peninsula using  
 777 the *COI* region and *ΔybgF* pseudogene as marker, respectively. Haplotypes in bold were  
 778 found both in southern and northern Italy.

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782 **Figure 6** – Matrix of the pairwise  $F_{ST}$  comparison of *COI* (A), *COII* (B), and  $\Delta ybgF$  (C) data  
 783 from the AMOVA conducted on the different *H. halys* population from global dataset of  
 784 sequences. The figure shows the genetic similarity between each collection location where 1  
 785 (dark blue) indicates no genetic similarity, and 0 (white) indicates identical sequences present  
 786 between the individuals of the collection sites, while crosses (x) indicate not significant  
 787 differences.

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789 **TABLES**

790 **Table 1.** Results of the best fitting generalized linear mixed models (lowest AIC) testing the  
 791 effects of local habitat type (urban gardens, fruit warehouses, crop fields), landscape  
 792 composition (cover of urban or semi-natural habitats in the landscape at 2 km scale) and time  
 793 (months, they correspond to sampling round) on the occurrence and abundance of *H. halys*,  
 794 ~~and on parasitism and predation rates. We also included the P~~pest abundance was included for  
 795 the models concerning biological control potential.

Variable	Chisq	P-value
<i>1) Halyomorpha halys occurrence</i>		
Habitat	0.30	0.859
% Urban	0.21	0.646
<b>Time</b>	<b>7.25</b>	<b>0.007</b>
<b>Habitat x % Urban</b>	<b>9.35</b>	<b>0.009</b>
<b>Habitat x Time</b>	<b>6.35</b>	<b>0.042</b>
% Urban x Time	0.29	0.589
<i>2) Halyomorpha halys abundance</i>		
Habitat	1.41	0.815
% Urban	3.13	0.077
<b>Time</b>	<b>18.08</b>	<b>&lt;0.001</b>
<b>Habitat x % Urban</b>	<b>6.85</b>	<b>0.033</b>
Habitat x Time	5.53	0.063
% Urban x Time	0.24	0.625
<i>3) Parasitism rate</i>		
Habitat	<u>2.58</u>	<u>0.108</u>
<b>% Semi-natural</b>	<b>4.83</b>	<b>0.028</b>
Pest abundance	<u>3.18</u>	<u>0.074</u>
Habitat x % Semi-natural	<u>2.71</u>	<u>0.992</u>
<i>4) Predation rate</i>		
<b>Habitat</b>	<b>8.80</b>	<b>0.003</b>
% Semi-natural	<u>0.14</u>	<u>0.705</u>
Pest abundance	<u>0.43</u>	<u>0.510</u>
Habitat x % Semi-natural	<u>0.01</u>	<u>0.907</u>

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