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Identification of predatory arthropods of the invasive *Halyomorpha halys* through molecular gut content analysis

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Abstract

1. *Halyomorpha halys* (Stål, 1855) is an invasive agricultural pest in North America and Europe.
2. Most of the information on *H. halys* predators in invaded areas comes from North America. This work focused on the molecular identification of arthropod predator species capable of feeding on *H. halys* in northern Italy. Predatory arthropods were collected in the field in four urban parks using the tree-beating technique. A real-time PCR workflow was applied to detect *H. halys* DNA from the gut content of predators.
3. Of the 190 predator individuals analysed, 46 were positive for *H. halys* DNA and belonged to 10 insect taxa (1 Dermaptera, 3 Coleoptera, 2 Hemiptera and 4 Orthoptera) and six arachnid taxa (2 Opiliones and 6 Araneae).
4. The integration of gut content analysis with laboratory bioassays and field observations allows the identification of a greater number of predators and therefore a better understanding of how the invaded ecosystem is responding to the introduction of a new species, given that samples are taken from the invaded environment itself. Therefore, the gut content analysis provides essential elements for conservation biocontrol in integrated pest management programmes.

KEYWORDS
Brown Marmorated Stink Bug, conservation biocontrol, generalist predators, invasive species, molecular detection, Pentatomidae, real-time PCR

INTRODUCTION

Halyomorpha halys (Stål, 1855) (Heteroptera, Pentatomidae) is a severe crop pest native to East Asia (Lee et al., 2013). It has a high invasive capacity facilitated by human activities and trade (Maistrello et al., 2018), which has led to the rapid colonization of other continents. The invaded regions include North America, where it is present in 46 US states and 4 provinces of Canada (Stopbmsb, 2021), South America (Chile; Faúndez & Rider, 2017) and Europe, where it is reported in 28 countries and around the Black Sea (Claerebout et al., 2018; Inaturalist, 2021). In Italy, *H. halys* was first detected in 2012 in northern regions (Maistrello et al., 2016), although it was likely introduced as early as 2009 (Maistrello et al., 2018). Established populations are currently found in all Italian regions (Inaturalist, 2021; Maistrello et al., 2018) and are the result of multiple invasions from native and already invaded countries (Cesari et al., 2015; Cesari et al., 2017). In Italy, *H. halys* has rapidly become the most important key pest in fruit orchards (Maistrello et al., 2017) and hazelnut groves

(Bosco et al., 2018). Feeding on fruits and seeds causes damage that makes products unmarketable. Losses in fruit production in northern Italy in 2019 were estimated at € 588 million (CSO Italy, 2020).

Halyomorpha halys is known for its high polyphagy (Rice et al., 2014), high mobility of all instars (Lee et al., 2014; Lee & Leskey, 2015; Wiman et al., 2015) and high reproductive potential (Costi et al., 2017), and proved to be extremely difficult to manage on crops. To try to tackle *H. halys* invasions of the fields, farmers have increased the use of broad-spectrum insecticides, seriously disrupting previous integrated pest management (IPM) strategies and causing negative economic and environmental impacts in the invaded countries (Leskey et al., 2012; Maistrello et al., 2017).

When dealing with an invasive pest, knowledge of the natural enemy community in the introduced regions is a key element to developing a sustainable management programme. In the case of stink bugs, approaches to obtain information on potential natural enemies of invasive species include field monitoring to collect the naturally laid eggs (Rot et al., 2021; Zapponi et al., 2021) and field exposure of sentinel eggs (fresh or frozen) of the target species for some time (Conti et al., 2020; Rot et al., 2021). In both cases, the eggs are subsequently analysed in the laboratory to check for parasitization and/or signs of predation. The sentinel egg approach has been used both in the United States (Abram et al., 2017; Cornelius et al., 2016; Ogburn et al., 2016; Shanovich et al., 2020) and in Europe (Costi et al., 2019; Haye, Fischer, et al., 2015; Moraglio et al., 2020). Both approaches have proved very useful in obtaining information on parasitoids that use *H. halys* eggs as hosts. However, unless video cameras are associated with exposed eggs, these approaches can only provide clues to the mouthparts used to pierce or chew eggs and are not useful for identifying predator species. Furthermore, these methods can only provide information on biocontrol agents that use eggs as substrates/food items and exclude all those consuming the other development stages.

One approach to obtain these data is to perform laboratory bioassays in which the eggs and/or other instars of the target species are exposed to potential biocontrol agents (possibly fasted before testing) and are subsequently checked for the outcome of predation/parasitization. This approach was used to identify some generalist predator species that can effectively feed on specific instars of *H. halys*. For example, in the United States, eggs were eaten by some Orthoptera (Tettigoniidae, Gryllidae, Acrididae), Neuroptera (Chrysopidae), Dermaptera (Forficulidae), Coleoptera (Coccinellidae, Carabidae) and Araneae (Salticidae) (Abram et al., 2014; Morrison et al., 2016; Poley et al., 2018; Pote & Nielsen, 2017); young nymphs (N1-N2) were consumed by Hemiptera (Nabidae, Reduviidae, Pentatomidae) (Arellano et al., 2019; Pote & Nielsen, 2017); older nymphs (N3-N5) were consumed by Hemiptera (Pentatomidae) and Hymenoptera (Crabonidae) (Arellano et al., 2019; Biddinger et al., 2017), and adults were consumed by Hemiptera (Pentatomidae) and Aranea (Agelenidae, Pholcidae, Theridiidae) (Arellano et al., 2019; Morrison et al., 2017). The outcome of similar studies carried out in Europe, and precisely in Italy, is that the eggs were consumed by Coleoptera (Coccinellidae), Orthoptera (Tettigoniidae) and Hemiptera (Reduviidae) (Bulgarini et al., 2020), young nymphs were consumed by Orthoptera (Tettigoniidae), Neuroptera (Chrysopidae), Hemiptera (Nabidae, Reduviidae) (Bulgarini et al., 2020) and by the ants *Crematogaster scutellaris* (Olivier, 1792) (Castracani et al., 2017) and *Lasius niger* (Linnaeus, 1758) (Hymenoptera: Formicidae) (Bulgarini et al., 2021), older nymphs were consumed by *C. scutellaris* (Castracani et al., 2017), and adults were consumed by *Rhynocoris iracundus* (Poda, 1761) (Hemiptera: Reduviidae) (Bulgarini et al., 2020).

An alternative approach for predatory species is based on the molecular analysis of their gut content and/or excrements to detect traces of DNA of the prey of interest through different techniques (PCR, real-time PCR, NGS) (Casey et al., 2019; Dhami et al., 2016; Greenstone et al., 2010; Siegenthaler et al., 2019; Symondson, 2002; Unruh et al., 2016). This approach is considered more effective than field observations, especially for species of small size, with hidden and/or nocturnal habits or for predation events that occur in difficult to access places (i.e., canopy, burrows) or in case of fluid feeder predators like spiders or hemipterans (Birkhofer et al., 2017). With this approach, it was possible to identify 13 different predators of stink bug pests of soybean and cotton (Tillman et al., 2015), the predators of the vineyard pest *Homalodisca vitripennis* (Germar, 1821) (Rhynchota: Cicadellidae) (Fournier et al., 2008), and the predators of the orchard pest *Conotrachelus nenuphar* (Herbst, 1797) (Coleoptera: Curculionidae) (Schmidt et al., 2016). It was also possible to confirm *Orius insidiosus* (Say, 1832) (Hemiptera: Anthocoridae) as a predator of the corn pest *Helicoverpa zea* (Boddie, 1850) (Lepidoptera: Noctuidae) (Peterson et al., 2018).

Among the techniques for molecular analysis of gut content, real-time PCR allows the rapid and specific detection and amplification of a gene or a portion of it from the undigested DNA of a target species. Therefore, many protocols rely on dyes combined with species-specific primers or probes designed to initiate replication only in case of a complete match with the target DNA, and the results are immediately displayed on the real-time PCR machine. A similar approach was used to detect the bat *Eptesicus fuscus* (Beauvois, 1796) (Chiroptera: Vespertilionidae) as a predator of *H. halys*, by searching for DNA of the target species in bat droppings (Valentin et al., 2016). So far in Italy, only laboratory tests have been carried out to identify potential predators of *H. halys*, so the aim of this study was to identify species capable of feeding on *H. halys* in northern Italy using molecular analysis of the gut content of arthropod predators occupying the same habitat as the invasive pest. Moreover, we also assessed the occurrence of the predators over-time on the different host trees.

MATERIAL AND METHODS

Sampling of predators

Potential predators were field-sampled using the tree-beating technique during summer in 2017 and 2018 in four urban parks of Reggio

Emilia (northern Italy): Mauriziano (44.683747, 10.674409), Rodano (44.677606, 10.664297), Fucini (44.673567, 10.615283), Baragalla (44.666831, 10.601160). Each selected park was at least 500 square metres, close (30–50 m) to a water source and close (30–50 m) to an agro-ecosystem (small fields of wheat and alfalfa, vegetable gardens). Sampling was performed in each park every 15 days between 8 and 11 AM from the beginning of May to the beginning of October. In total, each park was visited 11 times in 2017 and 11 times in 2018.

In each urban park, 20 trees were selected. They belonged to the genera *Fraxinus*, *Acer*, *Prunus*, *Morus*, *Cornus*, *Corylus*, *Quercus* and *Robinia*, all known to be host plants of *H. halys* (Haye, Gariepy, et al., 2015; Rice et al., 2014). These plant species typically occur in the hedges around the cultivated fields and in the groves of the farmers' houses. A tree-beating session consisted of three strong beats in three points at a height between 1 and 3 m, using a stick and a white tray to collect all the dislodged arthropods. For each sampled tree, the total number of *H. halys* individuals (adults and nymphs) was also recorded. Sampling took place on the same trees in the same parks in both years, therefore each tree was sampled 22 times in 2 years. Captured specimens were individually collected in 50-ml tubes, which were adequately labelled and placed inside thermally insulated bags to prevent further DNA degradation/digestion during transport to the laboratory. In the laboratory, the tubes were placed at −20°C to kill the specimens and were then filled with ethanol 100%. The captured specimens were accurately identified to the level of genus and possibly species, using specific taxonomic keys. All the sampled arthropods were recognized as non-endangered and unprotected species.

TABLE 1 Total number of tested and positive predator individuals per order, family, genus and species in alphabetical order, with the first four orders being insects (Coleoptera–Orthoptera) and the last two orders being arachnids (Araneae, Opiliones)

Order	Family	Genus/species	Total	Positive
Coleoptera	Coccinellidae	*Coccinella septempunctata* (Linnaeus, 1758)	2	0
		Harmonia axyridis (Pallas, 1773)	19	4
		Hippodamia variegata (Goeze, 1777)	4	1
		Oenopia conglobata (Linnaeus, 1758)	2	1
		Propylea quatuordecimpunctata (Linnaeus, 1758)	1	0
Dermaptera	Forficulidae	*Forficula auricularia* (Linnaeus, 1758)	70	14
Hemiptera	Nabidae	*Himacerus mirmicoides* (Costa, 1834)	2	2
	Reduviidae	*Nagusta goedelii* (Kolenati, 1857)	1	1
Orthoptera	Gryllidae	*Oecanthus pellucens* (Scopoli, 1763)	1	0
	Mogoplistidae	*Arachnocephalus vestitus* (Costa, 1855)	25	9
	Tettigoniidae	*Phaneroptera falcata* (Poda, 1761)	3	1
		Tylopsis liliifolia (Fabricius, 1793)	1	1
		Yersinella raymondi (Yersin, 1860)	1	1
Araneae	Anyphaenidae	*Anyphaena* sp. (Sundevall, 1833)	26	1
	Araneidae	*Araneus* sp. (Clerick, 1757)	2	1
	Dictynidae	*Dictyna* sp. (Sundevall, 1833)	1	0
	Philodromidae	*Philodromus* sp. (Walckenaer, 1826)	3	3
	Pisauridae	*Pisaura* sp. (Simon, 1885)	1	0
	Salticidae	*Calositticus* sp. (Lohmander, 1944)	2	2
		Europhrys gambosa (Simon, 1868)	1	0
		Icius congener (Simon, 1871)	1	0
		Icius hamatus (C.L. Koch, 1846)	1	0
		Phintella castriesiana (Grube, 1861)	1	0
		Phlegra cinereofasciata (Simon, 1868)	1	0
		Talavera aequipes (O.P.-Cambridge, 1871)	1	0
	Tetragnathidae	*Tetragnatha* sp. (Latreille, 1804)	2	0
	Theridiidae	*Platnickina tincta* (Walckenaer, 1802)	2	0
	Thomisidae	*Pistius truncatus* (Pallas, 1772)	4	0
Opiliones	Phalangiidae	*Mitopus morio* (Fabricius, 1799)	4	2
		Opilio canestrinii (Thorell, 1876)	4	2
Total			190	46

TABLE 2 List of samples resulted positive to *Halyomorpha halys* DNA divided by species, with relative mean Ct (mean of each triplicate) and ± standard error

Species	Sample type	Mean Ct and ± standard error
Halyomorpha halys	Positive control	18.85 ± 1.27
Eupholidoptera chabrieri	Positive control	21.97 ± 1.08
Nezara viridula	Negative control	37.87 ± 2.30
Raphigaster nebulosa	Negative control	37.45 ± 2.07
Harmonia axyridis	Unknown (positive)	34.46 ± 1.43
Harmonia axyridis	Unknown (positive)	31.88 ± 1.44
Harmonia axyridis	Unknown (positive)	31.50 ± 1.18
Harmonia axyridis	Unknown (positive)	30.11 ± 0.09
Hippodamia variegata	Unknown (positive)	33.63 ± 0.70
Oenopia conglobata	Unknown (positive)	32.14 ± 1.44
Forficula auricularia	Unknown (positive)	31.41 ± 0.06
Forficula auricularia	Unknown (positive)	29.37 ± 0.39
Forficula auricularia	Unknown (positive)	30.23 ± 0.59
Forficula auricularia	Unknown (positive)	29.49 ± 0.48
Forficula auricularia	Unknown (positive)	33.93 ± 1.29
Forficula auricularia	Unknown (positive)	33.72 ± 1.53
Forficula auricularia	Unknown (positive)	22.17 ± 0.06
Forficula auricularia	Unknown (positive)	30.93 ± 0.52
Forficula auricularia	Unknown (positive)	31.08 ± 1.44
Forficula auricularia	Unknown (positive)	30.97 ± 0.15
Forficula auricularia	Unknown (positive)	31.38 ± 1.39
Forficula auricularia	Unknown (positive)	34.29 ± 1.39
Forficula auricularia	Unknown (positive)	33.38 ± 0.14
Forficula auricularia	Unknown (positive)	37.19 ± 0.14
Himacerus mirmicoides	Unknown (positive)	34.23 ± 0.03
Himacerus mirmicoides	Unknown (positive)	30.47 ± 1.40
Nagusta goedelii	Unknown (positive)	32.35 ± 1.39
Arachnocephalus vestitus	Unknown (positive)	25.28 ± 0.06
Arachnocephalus vestitus	Unknown (positive)	25.73 ± 0.61
Arachnocephalus vestitus	Unknown (positive)	23.77 ± 0.03
Arachnocephalus vestitus	Unknown (positive)	22.32 ± 0.45
Arachnocephalus vestitus	Unknown (positive)	31.08 ± 0.65
Arachnocephalus vestitus	Unknown (positive)	33.77 ± 1.39
Arachnocephalus vestitus	Unknown (positive)	31.58 ± 0.86
Arachnocephalus vestitus	Unknown (positive)	32.76 ± 1.05
Arachnocephalus vestitus	Unknown (positive)	30.99 ± 0.49
Phaneroptera falcata	Unknown (positive)	32.48 ± 0.83
Tylopsis liliifolia	Unknown (positive)	30.95 ± 0.44
Yersinella raymondi	Unknown (positive)	31.71 ± 0.70
Anyphaena sp	Unknown (positive)	34.16 ± 0.64
Araneus sp.	Unknown (positive)	32.29 ± 0.04
Philodromus sp.	Unknown (positive)	25.28 ± 0.11
Philodromus sp.	Unknown (positive)	29.32 ± 1.39
Philodromus sp.	Unknown (positive)	33.37 ± 0.04
Calositticus sp	Unknown (positive)	34.08 ± 0.06
Calositticus sp	Unknown (positive)	34.25 ± 0.04
Mitopus morio	Unknown (positive)	28.44 ± 1.43
Mitopus morio	Unknown (positive)	31.91 ± 1.34
Opilio canestrinii	Unknown (positive)	32.49 ± 0.82
Opilio canestrinii	Unknown (positive)	25.65 ± 2.37

Arthropod preparation and dissection

For the gut content analysis, only the predators found on the trees where *H. halys* was detected on the same sampling session were selected. Before dissection, each specimen was placed in a 1.5 ml tube and washed in a solution containing 0.001 Triton X-100 by inverting the tube for 1 min. This process was implemented to remove impurities from the samples and reduce the risk of DNA contamination. The sample was then transferred to a second 1.5 ml tube and washed with ultra-distilled water for 1 min. If individuals were too large to fit inside the 1.5 ml tube, their legs and/or wings were removed with a flame sterilized cutter. Subsequently, each predator was positioned supine and blocked with the use of entomological needles sterilized with alcohol and flame. With the use of microsurgical scissors and entomological tweezers, also sterilized with alcohol and flame, the abdomen of the predator was opened and its gut removed and transferred to a 1.5 ml tube and placed on ice. Because spiders and opilionids carry out extraoral pre-digestion and the digestion of food takes place in different parts of the body (midgut diverticula extend throughout the prosoma and legs), the separation of the gut content is more difficult (Macías-Hernández et al., 2018). For this reason, the arachnid individuals were used as a whole.

DNA extraction and genetic analysis

Only predators found on trees where *H. halys* was detected in the same sampling session were selected for the analyses. Two positive and two negative controls were used for these analyses. Positive controls were represented by (i) genomic DNA (gDNA) extracted from the head of *H. halys* raised in the laboratory and (ii) gDNA extracted from the gut content of laboratory-reared *Eupholidoptera chabrieri* (Charpentier, 1825) (Orthoptera: Tettigoniidae) that were freeze-killed (at −20°C) 30 min after feeding on adults of *H. halys*. The negative controls were represented by the gDNA extracted from the legs and heads of *Nezara viridula* (Linnaeus, 1758) (Hemiptera: Pentatomidae) and *Rhaphigaster nebulosa* (Poda, 1761) (Hemiptera: Pentatomidae), two very common stinkbugs in Italy. The used pentatomids were collected in Modena (Italy) and stored in 100% ethanol at −20°C before dissection. In addition to the negative controls, a No Template Control

(NTC) was also used for each real-time PCR, consisting of 18 μl reaction, but without the presence of the 2 μl of gDNA from the samples. The NTCs were used to check that there were no contaminations, even minimal.

Total DNA extraction was performed on predators and controls using the DNeasy Blood & Tissue kit (Qiagen Sciences, Germantown, MD, USA), following the protocol 'Total DNA from Animal Tissue' (Dneasy Blood & Tissue Handbook, July 2006). The extracted gDNA was re-suspended in 200 μl of Qiagen buffer ATE and then measured with a NanoDrop® spectrophotometer (Life Technologies) to estimate the amount of detectable DNA in a sample. The negative and positive controls underwent the same extraction protocol.

A very sensitive real-time PCR assay (BMITS1 protocol) was used to detect traces of *H. halys* DNA in the extracted DNAs. The assay was described in Valentin et al. (2016) and was specific to 96 bp of the conserved region of the rDNA internal transcribed spacer

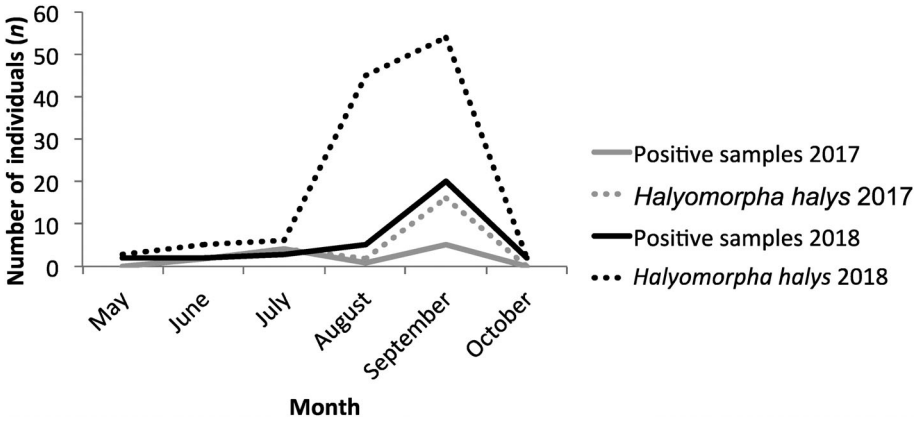

FIGURE 1 Trends over time (sample months) for the abundance of predators that tested positive for *Halyomorpha halys* DNA and of the abundance of *H. halys* individuals collected during sampling sessions in 2017 and 2018

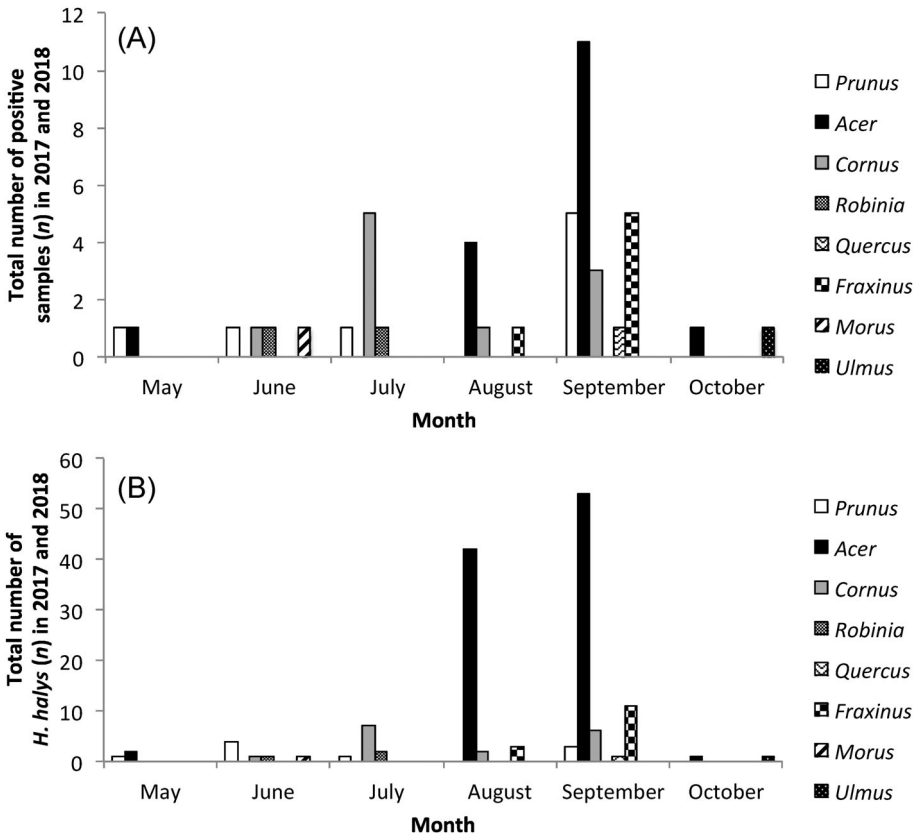

FIGURE 2 (a) Number of positive samples for *Halyomorpha halys* DNA over time (sample months) on each tree genus (sum of individuals in 2017 and 2018) and (b) abundance of *H. halys* over time (sample months) on each tree genus (sum of individuals in 2017 and 2018)

1 (ITS1, 211 bp) of *H. halys*. Three replicates were performed for each sample in 20 μl reactions using 500 nM of each primer (BMITS1F: 5′-CGA GGC CGC CGA TGA-3′; BMITS1R: 5′- CCC ACG AGC CGA GTG ATC-3′), 1× TaqManTM Fast Advanced Master Mix with Uracil-N glycosylase (UNG), 250 nM of the TaqManTM fluorescent probe (BMITS1TM 5′- CAG GCA ATG AAG CAC A-3′) with a dye label (VIC) on the 5′ end and a minor groove binder (MGB) and non-fluorescent quencher (NFQ) on the 3′ end, and 2 μl of undiluted gDNA from the samples. The optimized real-time PCR protocol consists of an initial step of 2 min at 50°C to activate the UNG, a denaturation phase of 20 s at 95°C followed by 40 denaturation cycles of 10 s at 95°C, annealing at 67°C for 20 s, 30 s at 72°C for extension and a final extension of 7 min at 72°C.

All reactions were carried out using a BioRad CFX96 Real-time PCR system (Bio-Rad Laboratories, Inc.) and results were analysed using CFX MaestroTM Software version 1.1 (Bio-Rad). The software compared the final relative fluorescence units (RFUs) of each positive control and unknown content wells to the RFUs of the negative control wells. A positive sample was identified when its RFU value was greater than the mean RFU values of the negative controls plus the default cut-off value (Ct = 18.85 ± 1.27). A sample was considered positive for *H. halys* DNA if at least two replicates were positive.

Statistical analysis

A generalized linear model (GLM) with a binomial error structure (logit link function) was performed to compare the proportion of positive samples between the 2 years (2017, 2018) and to compare the proportion of positive samples considering the month of capture, the tree on which they were captured and their interaction as variables. To assess the general significance of year, month, tree and their interaction, an analysis of deviance of the fitted model with Wald statistics χ^2 was performed. All statistical analyses were performed using R version 3.6.3 (R Core Team, 2019).

RESULTS

Among the 190 individuals analysed in total, the gut content was positive for *H. halys* DNA in 46 samples (24%). Table 1 shows the samples analysed with a relative abundance and the proportion of conspecifics of each taxon that resulted in a positive out of the total of those collected for that species, and Table 2 shows the mean Ct values for each positive sample. Among insects, the species with positive readings for *H. halys* DNA in their guts are the coccinellids *Harmonia axyridis*, *Hippodamia variegata*, *Oenopia conglobata*, the dermapteran *Forficula auricularia*, the nabid *Himacerus mirmicoides*, the orthopterans *Arachnocephalus vestitus*, *Phaneroptera falcata*, *Tylopsis liliifolia*, *Yersinella raymondi* and the reduvid *Nagusta goedelii*. As for the arachnids, those positive to *H. halys* DNA were the opilionids *Mitopus morio* and *Opilio canestrinii*, and the spiders belonging to the genera *Anyphaena*, *Araneus*, *Philodromus* and *Calossitticus*.

The proportion of positive samples was significantly higher in 2018 (34) than in 2017 (12) ($\chi^2 = 5.56$; d.f. = 1; $p = 0.01$). According to the GLM results, no significant differences were detected considering the month of sampling ($\chi^2 = 0.69$; d.f. = 5; $p = 0.98$), the tree species ($\chi^2 = 10.37$; d.f. = 8; $p = 0.23$) and their interaction ($\chi^2 = 7.71$; d.f. = 15; $p = 0.93$). However, from Figures 1 and 2, it is possible to observe a trend in the number of predators that resulted positive over time, which showed a peak in September in both years. The greatest number of predators that resulted positive was found mainly on *Acer*, *Prunus*, *Fraxinus* and *Cornus*, particularly in September and July, respectively.

DISCUSSION

Compared with the information obtained from laboratory bioassays, where the potential candidates are chosen by the experimenters, this study allowed us to obtain a broader view of the potential of biocontrol agents that can consume the invasive pest *H. halys* in northern Italy. The chosen approach was to perform molecular analysis on the gut content of the field-collected predatory arthropod species that share the same habitat of *H. halys*. The plant species present in the urban parks where the sampling was performed are the same ones that typically occur in the hedges around the cultivated fields and in the groves of the farmers' houses. Due to their greater biodiversity, urban parks, hedges and small woods represent optimal refuge areas where the polyphagous *H. halys* can find a variety of optimal food sources as well as oviposition sites. Thus, the sampling sites represented a reliable model of the potential biodiversity of the predators in the agro-ecosystems. All the predators found in the study had been previously observed in the mentioned refuge areas during *H. halys* monitoring sessions with tree beating.

The results showed that about 25% of the collected predators contained *H. halys* DNA in their gut. This is a considerable result, taking into account that *H. halys* is an invasive species, which may not be recognized as typical prey by any of the predatory species residing in the invaded areas. All the sampled specimens are generalist species with a more or less broad range of prey items, which have proven to be able to exploit the invasive stink bug as a food source. Importantly, the time elapsed between the actual consumption of the prey and the moment the predator was captured is unknown. It is possible that a greater number of predators ate this stinkbug, but too much time had elapsed between predation and capture of the predator, and therefore the prey's DNA had already been digested by the predator's enzymes, becoming undetectable (Dhami et al., 2016; Symondson, 2002). Other hypotheses that could explain this seemingly low percentage of positive samples include:

i. predatory species can exploit *H. halys* as a food resource, but captured individuals of that species did not consume it on sampling days;
ii. a predator can only feed on a specific instar of *H. halys*, which was absent at the date of capture of the sampled specimen. According to Costi et al. (2017), in Italy, *H. halys* completes two

generations/year, with overwintered adults moving from shelter sites to surrounding vegetation from mid-March. They mate and oviposit between mid-May and late July-early August, when they die. The new generation of adults emerges at the beginning of July and lay their eggs until the beginning of September. Young nymphs can be found between the end of May and mid-September, whereas the older nymphs can be detected between mid-June and mid-October;

iii. a predator cannot exploit *H. halys* as prey for physical or physiological reasons, that is, inadequacy of the mouthparts, absence of cues used to detect potential prey, presence of cues that repel/have a feeding deterrent effect, etc.;

iv. the prey DNA was too degraded to be detected.

Looking at the taxa of predators that resulted positive, it emerged that 100% of the analysed Hemiptera and 50% of the Opiliones were positive, although the samples were few (three and eight, respectively). The order showing the highest ratio of positive samples was Orthoptera, with 38% (12 out of 31), followed by Coleoptera with 21% (6 out of 28) and Dermaptera with 20% (14 out of 70). Last was the order Araneae, in which only 14% of samples were positive (7 out of 31). Focusing on the identity of the predators, the nabid *H. mirmicoides* and the reduvid *N. goedelii* were positive (2 of 2 samples and 1 of 1 sample, respectively). In general, nabids and reduvids proved to be able to consume the eggs and young nymphs of *H. halys* (Jones, 2013; Lee et al., 2013; Morrison et al., 2016; Pote & Nielsen, 2017). More specifically, *H. mirmicoides* successfully preyed upon the first instar nymphs, whereas *N. goedelii* consumed both the eggs and the first instar nymphs (Bulgarini et al., 2020).

Among the analysed coccinellids, the positive ones were *Ha. axyridis* (4 samples out of 19), *H. variegata* (1 sample out of 4) and *O. conglobata* (1 sample out of 2). Coccinellidae, where both larvae and adults are efficient predators, are notorious biocontrol agents and many species are often used to suppress pest populations, especially aphids (Rutledge et al., 2004). Some coccinellids were laboratory tested as potential predators on the eggs and young nymphs of *H. halys*. Adults of *C. septempunctata*, *Adalia bipunctata* (Linnaeus, 1758), *Ha. axyridis* and adult and larvae of *Coleomegilla maculata* (De Geer, 1775) have occasionally been shown to feed on the eggs, but none of them had consumed nymphs (Abram et al., 2014; Bulgarini et al., 2020; Morrison et al., 2016; Poley et al., 2018; Pote & Nielsen, 2017). It is thus possible that the positive specimens detected in the analysis had fed on *H. halys* eggs before capture.

The dermapteran *F. auricularia* was positive in almost half of the samples (33 out of 70). Some Forficulidae are known as important biocontrol agents (Suckling et al., 2006) and some of them have been recognized as the most effective predators of *H. halys* sentinel eggs in a field survey (Poley, 2017). However, in laboratory studies, *F. auricularia* has shown very poor predatory performance on *H. halys* eggs (Poley et al., 2018) or no predation at all, although it may damage them, and it has never been able to consume young nymphs (Bulgarini et al., 2020). Therefore, it is likely that the samples of *F. auricularia* positive to *H. halys* DNA had fed on the eggs. The differences between field and laboratory situations could be explained by the fact that the eggs used for the laboratory tests were freshly laid ones (<24 hours). At this stage, the eggs may have some physical or semiochemical features that protect them from being eaten by many predators. These characteristics are eventually lost over time, allowing a greater number of predators to exploit them. Another possibility is that the individuals tested in the laboratory and those found in the field are not the same species. Indeed, recent studies indicate that the taxon *Forficula auricularia* is, in fact, a complex of cryptic species that retain the same external morphology but are genetically differentiated, and the only way to correctly identify these species is to perform specific genetic analyses using mtDNA and nuclear sequence data (González-Miguéns et al., 2020).

Among the orthopterans, *A. vestitus* (9 samples out of 25), *P. falcata* (1 sample out of 3), *T. liliifolia* (1 sample out of 1) and *Y. raymondi* (1 sample out of 1) resulted positive. In previous investigations, orthopterans always showed good predation on *H. halys* eggs (Bulgarini et al., 2020; Morrison et al., 2016; Poley et al., 2018; Pote & Nielsen, 2017), and *Eupholidoptera chabrieri* (Charpentier, 1825) can consume both the first and second instar nymphs of the pest (Bulgarini et al., 2020). Overall, Orthoptera is medium–large-sized insects equipped with big and strong chewing mouthparts that can be effective generalist predators even as immatures.

As for the arachnids, the opilionids *M. morio* and *O. canestrinii* were positive (2 samples out of 4 each). No studies on these animals have been published so far, but opilionids are generally known as predators of a large number of insects, including the Hemiptera nymphs (Acosta & Machado, 2007; Phillipson, 1960). It is therefore likely that the sampled opilionids fed on the nymphs of *H. halys*. Considering spiders, four genera tested positive for *H. halys* DNA: *Anyphaena* (1out of 26 samples), *Araneus* (1 out of 2), *Philodromus* (3 out of 3) and *Calossitticus* (2 out of 2). Among the spiders tested in the laboratory, Salticidae such as *Phidippus audax* (Hentz, 1845) are the ones that show the greatest predation on eggs (Morrison et al., 2016; Poley et al., 2018). From the studies carried out on the spider webs found in the overwintering sites of the invasive stinkbug, it emerged that Agelenidae, Pholcidae and Theridiidae fed on *H. halys* (Morrison et al., 2017). The low number of positive samples among spiders may be due to the small size of the collected specimens. In fact, spiders usually consume smaller prey than themselves, as the optimal prey size is 50–80% of the spider's body (Nentwig & Wissel, 1986). Therefore, positive specimens are likely to have fed on the smaller juvenile instars of *H. halys*, excluding adults and larger nymphs.

There was a significant difference between the 2 years of sampling, as the number of *H. halys* DNA positive samples was almost three times higher in 2018 than in 2017. This difference is probably due to specific climatic conditions, as summer 2017 was exceptionally hot and dry in northern Italy, with very little rainfall, and this may have negatively impacted the survival of many arthropods, including both *H. halys* and generalist predators. Many farmers, as well as phytosanitary staff, said that *H. halys* populations and related damage in fruit orchards were considerably lower in 2017 than in other years

(Maistrello, pers. comm.). With fewer prey items available, predators were less likely to feed on *H. halys* in 2017 than in 2018.

Despite the absence of significant differences, trends were observed considering the number of *H. halys* and predators positive to its DNA over time, as well as for the tree species on which predators and prey were detected. The number of stink bugs began to increase in July, mainly on *Cornus*, and peaked in September, when most of the specimens were found on *Acer*, followed by *Fraxinus*. The number of *H. halys*-positive predators followed a very similar pattern, peaking in September, especially on *Acer*, followed by *Prunus* and *Fraxinus*. In July, *Cornus* berries begin to ripen, becoming more attractive to *H. halys* than fruits of other species. In August and especially September, *H. halys* is found mainly on *Acer* and *Fraxinus*, probably because their very nutritious fruits (the samaras) attract them, whereas the fruits on other trees are no longer present. Therefore, the increased detection of positive samples is likely related to the increased availability of this prey on those trees. Other hypotheses to explain why September is the peak month for positive samples are: (i) in September most of the predators have reached the adult and/or the latest stages of development, so they are larger in size and are probably more efficient in predation; (ii) September is the pre-overwintering period for *H. halys*, when adults tend to aggregate on the last available host plants before reaching recovery sites to overwinter, and predators are probably taking advantage of the favourable situation; (iii) September corresponds to one of the population peaks for *H. halys* (Nielsen, 2008), therefore the chances of predators intercepting the pest increase.

Thanks to the gut content analysis, it was possible to increase the knowledge on the number of species that can use *H. halys* as a suitable food. However, this analysis is not necessarily exhaustive, as there are limitations specifically related to sampling techniques. Tree-beating sessions were limited to a maximum of three metres above the ground, thus excluding all the predators that occupy the upper parts of the trees, where *H. halys* also tends to stay. Additionally, ant species were excluded from this study, as some worker ants may have caught *H. halys* and brought it to the nest without eating it. This would result in a negative sample, giving erroneous results, although it has been shown that some species of ants can successfully exploit *H. halys* as prey in laboratory tests (Bulgarini et al., 2021; Castracani et al., 2017).

Molecular analysis of the gut content of generalist predators has proved to be a quick and effective tool to get a better overview of the potential of biocontrol agents on the invasive *H. halys*, thus expanding our knowledge on the number of species that can contribute to the control of this pest. However, real-time PCR analysis is qualitative and cannot provide information on the number or size of prey eaten, the stage of development of the prey, whether the predator obtained DNA by scavenging or by secondary transfer, and whether the predator fed all the prey or only a part of it (Greenstone et al., 2010). Therefore, integrating this technique with laboratory bioassays and field observations is a better way to provide the information needed to understand how the invaded ecosystem is responding to the introduction of a new species. In the case of a severe pest, the combination of different approaches can provide crucial elements for the development of a sustainable management programme.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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