UNIVERSITY OF MODENA AND REGGIO EMILIA

Doctoral School
Models and Methods for Material and Environmental Sciences

Cycle XXXII

Detection and population structure assessment of the invasive alien species *Halyomorpha halys* (Hemiptera, Pentatomidae) using molecular approaches

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CHAPTER 1

Biological invasions and the invasive alien species *Halyomorpha halys* (Stål, 1855) (Hemiptera, Pentatomidae)

1.1 Biological Invasions

1.1.1 Invasive Alien Species

Biological invasions are the outcome of successful establishments of invasive alien species in areas beyond their native range wherein they drove directly or indirectly impacts on biodiversity, environment, economy, and healthcare (Sax et al. 2005; Hulme 2009; Pyšek and Richardson 2010; Keller et al. 2011; Seebens et al. 2017; Pereyra 2019).

All those organisms, from viruses and bacteria to fungi, plants and animals, that occur in geographic ranges outside the spatial and temporal limits of their natural expansion range are referred as alien species. Either unintentional or deliberate, the introduction events are always driven by human-mediated processes (Elton 1958; Simberloff 2015).

The mechanisms through which alien species are introduced in a new area are:

- Intentional: imply the introduction of target species as livestock (i.e. farming, aquaculture), agriculture, ornamental, domestic animal, biological control, and fashion (i.e. for fur);
- Unintentional: imply the introduction of non-target species with a commodity (i.e. parasites, pathogens), with a transport vector as ships, airplanes, trains, and automobiles, through infrastructure corridors (i.e. roads and railway networks).

The transition steps for a species to become invasive imply the survival of the introduced nucleus of individuals in the new environment (colonization), the ability to reproduce without direct human intervention generating large and stable populations (establishment), and the range-expansion through natural (active dispersal) or passive (human-mediated) dynamics (Keller and Taylor 2008; Hulme 2009).

The introduction of alien species in all ecosystems (terrestrial, marine, fresh water) has increased enormously in the last 200 years as a consequence of the globalisation of trade and travels (Levine and D'Antonio 2003; Kobelt and Nentwig 2008; Hulme 2009; Pyšek et al. 2010; Keller et al. 2011; Seebens et al. 2017; Tay and Gordon 2019). The introduction of non-native flora and fauna across continents leads to a biotic homogenisation (Lockwood and McKinney 2001) that decreases the resilience of native habitats increasing the opportunities to become invasive for certain introduced species. The biotic homogenisation, particularly intense in anthropic areas (i.e. agroecosystems, urban areas), promotes the establishment and diffusion of invasive species since disturbed habitats or habitat characterised by a very low biodiversity can offer empty niches for those species and lack the strong ecosystemic structure that could prevent their settlement and expansion (Olden et al. 2011; Baiser et al. 2012; Capinha et al. 2015; Lososová et al. 2016). Thus, invasive species can reduce the diversity of local species or lead to the extinction of many others by directly (out-

competing with them, or feed on them) or indirectly causing ecosystem-level changes as resources exploitation or modifications of nutrient cycles (Mooney and Cleland 2001; Gurevitch and Padilla 2004; Bellard et al. 2016) . The increased movement of goods and commodities also triggered the diffusion of invasive species introduced as contaminants or stowaways.

Among alien organisms, terrestrial plants and invertebrates are far longer the more likely to be introduced and become invasive, generating ecological and economic impacts (Hulme 2009; Roderick and Navajas 2015; Bradshaw et al. 2016; Paini et al. 2016; Tay and Gordon 2019). In particular, insects represent the majority of introduced species in terrestrial environments; on ~2,500 alien invertebrates in Europe around 87% is represented by insects (Roques et al. 2008; Bradshaw et al. 2016), with 923 alien insect species reported for Italy (Inghilesi et al. 2013). These data are not surprising since insects represent the largest class of all animals with 927,346 described species (data updated to 2019 according to the Catalogue of Life; Roskov et al. 2019); however, several biological and behavioural factors contribute to the success of insects as introduced species. Generally, insects have higher rates of survival to the transport conditions and the stress factors related to it, mainly due to the protection offered by the exoskeleton and the form of resistance of many eggshells (Resh and Cardé 2009). In addition to this, insects are characterised by very small dimensions in all the life stages that enable them to be unseen when passively dispersed and to go undetected in the new environments until the established populations reach a large abundance. Reproductive strategies with more generations per year and short developmental stages are another important factor in the success of many invasive insect species (Engelmann 1970; Resh and Cardé 2009; Roderick and Navajas 2015). Invasive insects are strictly associated to a large variety of human activities playing several ecological functions in natural and managed ecosystems. The extreme variety of feeding habits (phytophagy, predation, parasitism, internal and external) determines the insurgence of harmful pests in agricultural systems affecting either plant and animal stocks, forestry and horticulture pests but also vectors of disease for human, animals and plants (Roderick and Navajas 2015; Tay and Gordon 2019).

1.1.2 Molecular strategies for invasive species management

Invasive species management is the integrated and synergic effort to translate the accumulated knowledge from various disciplines into actionable management procedures devoted to mitigating the impacts generated by invasive species (Hulme 2009; Pyšek and Richardson 2010; Dibble et al. 2013). Effective management relies on strategies developed to promptly take action on invasive species in each stage of their invasion process from introduction to dispersal. These strategies are focused on the prevention, the early detection, the containment or long-term reduction of

populations, the mitigation of the impact(s) and, when possible, the eradication (Myers et al. 1998; DiTomaso et al. 2017).

The establishment and even more the impact that an alien species has in introduced areas can occur over large temporal and spatial scales. Furthermore, the introduction can involve multiple propagules from one or more source populations. When the introduced organisms are small or the interaction with native species is difficult to identify, many of these events can be undetected or underestimated (Hulme 2009; Shine et al. 2010; David et al. 2013).

The molecular approach to the study of biological invasions is a common strategy to obtain reliable information on the invasion process, overcoming the high costs and long time required by traditional strategies based on observational surveys (Cristescu 2015).

Molecular techniques can be applied to study every stage of a biological invasion, from early detection to the long-term containment or eradication. Selected mitochondrial and/or nuclear markers or genome-wide single nucleotide polymorphisms (SNPs) can trace the original source population, routes of invasions and patterns of dispersal of the introduced population(s) (Allendorf and Lundquist 2003; Estoup and Guillemaud 2010; Lawson Handley et al. 2011).

Molecular markers like mitochondrial genes (i.e. cytochrome b, cytochrome c oxidase I, and cytochrome c oxidase II) can be used to identify invasive species and its geographic origin (i.e. DNA barcoding) and the genetic diversity of introduced populations (Hebert et al. 2003; Teske et al. 2011; Kress and Erickson 2012; Smith et al. 2012).

Recent methods based on genomic approaches can empower the rapid and reliable characterization of the genetic structure of invasive populations revealing the relationship with native populations, and the human-mediated pathways underwent the invasion success (Metzker 2009; Estoup and Guillemaud 2010; Reitzel et al. 2013; Chown et al. 2015; Goubert et al. 2016; van Boheemen et al. 2017). Genotyping-by-sequencing (GBS) techniques, like Restriction site-associated DNA sequencing (RADseq; Baird et al. 2008) allow the analysis of a large numbers of randomly distributed markers in genomes from non-model organisms and for which low genomic resources are available. The elevated molecular resolution of these methods coupled with a large number of bioinformatic analyses have the ability to solve very complex invasion scenarios (Reitzel et al. 2013; Harvey and Brumfield 2015; Goubert et al. 2016; Trucchi et al. 2016; Jeffery et al. 2017; Xuereb et al. 2018).

Moreover, molecular identification techniques can detect invasive species from biological degraded samples (i.e. environmental DNA, molecular gut content) providing a sensitive approach to early detect an organism and its trophic relationships even when the organism is not detectable with traditional methods (Pompanon et al. 2012; Yoccoz 2012; Clare 2014; Peters et al. 2015; Esnaola et

al. 2018).

In the same context, molecular techniques such as RNA interference (RNAi) (Montgomery et al. 1998; Yu et al. 2013; Wynant et al. 2014; Harvey-Samuel et al. 2017; Rodrigues et al. 2017; Cagliari et al. 2019) and the most recent genome editing technique CRISPR/cas9 (Webber et al. 2015; Kopf et al. 2017; Taning et al. 2017; Moro et al. 2018) can be applied to control the population fitness and thus limit expansion and diffusion of alien species.

1.2 Research objectives

In light of the massive diffusion of *Halyomorpha halys* and the threat that poses, the objectives of this doctoral study were aimed at implementing molecular approaches for the detection and the population structure assessment of this species in Italy.

In particular, the objectives of the research were:

1) Define the population structure and invasion dynamics of the brown marmorated stink bug *Halyomorpha halys* in Italy.

A previous study from Cesari et al. (2018) on the genetic diversity of *H. halys* evidenced the high diffusion and frequency of one mitochondrial haplotype (TH1) across Italy and Europe, likely indicating a single introduction and expansion event from an undefined point of origin.

This dissertation study aimed to deepen the knowledge of the genetics of *H. halys* individuals characterised by the most widespread mitochondrial haplotype (TH1), dissecting local population structure and patterns of introduction/dispersal. The study applied the RADseq method on 30 specimens from seven Italian regions and Greece selected from those previously analysed in Cesari et al. (2018).

2) Identification of arthropod predators of *Halyomorpha halys* in urban environment through their molecular gut content analysis.

The massive occurrence of *H. halys* in urban areas is causing nuisance and concerns, demanding a long-term control of the populations. The identification of native predator species able to affect *H. halys* populations in Italy has been investigated through observational surveys of sentinel egg mass in agroecosystems (Haye et al. 2015; Moraglio et al. 2018; Costi et al. 2019) and laboratory trials (Castracani et al. 2017).

This doctoral study aimed to identify native predator arthropods to use as biological control agents against *H. halys*. This study applied the Real-time PCR protocol from Valentin et al. (2016) to detect *H. halys* DNA in 50 predator arthropods from two urban parks in Reggio Emilia, Italy.

3) Early detection of *Halyomorpha halys* and determination of the predatory potential of

generalist Italian chiropteran through the molecular analysis of bat guano.

A previous study from Maslo et al. (2017) in US agroecosystems identified the predatory potential of the big brown bat (*Eptesicus fuscus* Beauvois, 1796) as an agent of surveillance by analysing the guano content through Real-time PCR. The study indicated also the sensitivity of the method to early detect the presence of *H. halys*.

This doctoral study aimed to evaluate the predatory potential of Italian generalist chiropterans as pest control agents and to early detect the potential presence of new propagule of *H. halys*. The study applied the Real-time PCR protocol from Valentin et al. (2016) to detect *H. halys* DNA in 27 guano samples of 15 Italian chiropteran species from three Italian regions.

1.3 *Halyomorpha halys* (Stål, 1855)

1.3.1 Morphology

Halyomorpha halys (Stål, 1855) (Hemiptera: Heteroptera: Pentatomidae: Pentatominae), commonly known as the brown marmorated stinkbug (BMSB) is a phytophagous and extremely polyphagous invasive species native from East Asia (Lee et al. 2013; Fig. 1.1). Adult of this species range from 12 to 17 mm in length and in humeral width of 7 to 10 mm, albeit a limited degree of variability occurs at an individual level.

The key characters of heteropterans are the forewings, referred as hemelytra that in resting phase are folded on the dorsal part of the abdomen, and the piercing-sucking oral apparatus also called *rostrum*. This apparatus is typical of the phytophagous members of the family and is used to pierce and suck the vegetable fluids (Fig. 1.2; Schaefer and Panizzi 2000).

Like other heteropterans when disturbed, *H. halys* can emit a substance with a stinging and unpleasant smell thanks the presence of scent glands located on ventral surface of the thorax and on the dorsal surface of the abdomen. The coloration can vary from brown or ash-brown, reddish brown, greyish-tan, or brownish- yellow. Hemelytra present dark spots, with the corium reddish. The most distinctive character of BMSB is the alternating white bands on the last two antennal segments, that appears as a single white band in both nymphs and adults. Alternate brown and white bands are also present on the visible lateral margins of the abdomen; the legs are pale brown with white banding. The ventral part is generally pale yellow, with grey or black spots (Hoebeke and Carter 2003).

In Europe and Italy *H. halys* can be erroneously identified with a very common stink bug species, *Rhaphigaster nebulosa* Poda, 1761 (Hemiptera: Heteroptera: Pentatomidae: Pentatominae) (Fig. 1.3). However, *R. nebulosa* can be discriminated from *H. halys* by the presence of an abdominal spine facing towards the rostrum, the presence of dark stripes on the hemelytra, and for

quadrangular juga on the head.

Halyomorpha halys is characterised by a hemimetabolous life cycle with eggs and five instar stages before developing in adult. Eggs are pale green or white with the form of a barrel, usually laid in clusters of about 25 eggs on the undersides of leaves (Fig. 1.4).

All the five instars lack fully developed wings and a tick-like appearance, their size range from 2.4 mm (1st instar) to 12 mm (5th instar). First instars are coloured red, or orange and they can remain near the egg mass until they molt to the 2nd instar stage. The 2nd instar is black, while successive instars (3rd, 4th, and 5th) start to acquire the adult coloration. From the 4th instar the dark stripes on wings start to be visible (Fig. 1.5; Hoebeke and Carter 2003).

1.3.2 Biological cycle

The biological cycle of *H. halys* is strongly influenced by diet, temperature, and photoperiod. In the native areas is described to be multivoltine, with 4-6 generations per year (Lee et al. 2013).

In temperate regions of Central Europe, it has been described to be univoltine, with one generation per year. In April overwintering adults become active, nevertheless the peak of oviposition is not observed before early July. Each female lay 80 eggs on average (maximum of 160). The oviposition period lasts from mid-June to end of September. Eggs laid in August and September do not generate offspring due to the low temperatures. The life cycle from egg to adult takes between 60 and 131 days. The first new generation of adults do not occur before mid-August (Costi et al. 2017; Nielsen et al. 2017)

In Italy *H. halys* is described to be bivoltine, with an overwintering period between October-November and April. Overwintering adults leave the recovery sites in April-May, dispersing on host plants, while the reproductive period lasts until August. In September-October *H. halys* specimens aggregate in overwintering refuges (Costi et al. 2017) the same behaviour is reported also in the mid-Atlantic region of the USA (Nielsen et al. 2017). In natural habitats, adults overwinter in woodlots, while in anthropic habitats overwinter in human structure such as buildings, stores and sheds. After mating, females start laying egg masses at circa weekly intervals, and each female lay approximately 400 eggs in her lifetime. Eggs development require 5-6 days and egg with an eclosion success of nearly 100% in the field (Lee et al. 2013).

1.3.3 Host plants

Halyomorpha halys is a phytophagous and highly polyphagous species. In China, it has been reported to feed on 45 host plants, including economically important crops (Lee et al. 2013), with a majority of these in the subclasses Rosidae and Asteridae.

In Japan, the range host of adults and nymphs encompasses 21 plant families and over 49 species (Toyama et al. 2010).

The host range of *H. halys* includes many economically important crops like tree fruits [i.e. *Malus domestica* (apple), *Pyrus communis* (pear), *Prunus persica* (peach), *P. armeniaca* (apricot), *P. avium* (cherry), *P. domestica* (plum), *Citrus* spp*., Morus* spp.], small fruits [i.e. *Rubus idaeus* (raspberry), *Corylus* spp. (hazelnuts); *Vitis vinifera* (grapevine)], vegetables [*Lycopersicon esculentum* (tomato), *Capsicum* spp. (peppers), *Helianthus annuus* (sunflower)], field crops [*Glycine max* (soybean), *Phaseolus vulgaris* (common bean), *Zea mays* (maize)], and a number of forest and ornamental trees and shrubs (i.e. *Acer* spp., *Fraxinus excelsior*, *Paulownia tomentosa*, *Ailanthus altissima*, *Buddleia davidii*, *Cupressus* spp., *Hibiscus* spp., *Lonicera* spp., *Rosa rugosa*, *Salix* spp.) (Lee et al. 2013; Haye et al. 2014; Rice et al. 2014).

1.3.4 Damages

Halyomorpha halys uses the rostrum to pierce fruits, leaf surfaces or young stems (Fig. 1.6). As consequence of the suctions, fruits present small necrotic spots under the skin and/or pod malformations (Nielsen and Hamilton 2009).

The damages found after *H. halys* feeding can vary based on the attacked fruit, generally include abscission, discolorations, scars, deformities, depressions, black spots, and brown flesh in fruits (Fig. 1.7). Damaged fruits appear unpleasant to the sight and not marketable. In other cases, the fruits report internal damages that compromise their development. In both cases the outcome is a high economic loss.

In invasive areas and Japan, *H. halys* is a serious public nuisance and household pest due to the ability of adult specimens to aggregate in private and public/commercial buildings (Lee et al. 2013; Haye et al. 2014; Rice et al. 2014).

In the USA, *H. halys* is a serious agricultural pest becoming the dominant pentatomid species in ornamentals, vegetables and field crops (Nielsen et al. 2017; Leskey and Nielsen 2018). An outbreak on apple orchards during 2010 caused $37 million loss in the mid-Atlantic region (Leskey et al. 2012)

So far in Europe damages are reported only in Italy where they started to occur during 2015 in commercial fruit orchards in the Emilia-Romagna region, first area of introduction in Italy (Maistrello et al. 2014, 2017; Bortolotti et al. 2015; Bariselli et al. 2016). Since then, damages have been reported in Piedmont, Lombardy, Veneto and Friuli-Venezia Giulia regions (Pansa et al. 2013; Bariselli et al. 2016; Lupi et al. 2017; Bosco et al. 2018; Candian et al. 2018; Moraglio et al. 2018). Important losses involved organic orchards and, other than fruit deformities and damages, the

presence of the stinkbug excreta on fruits made them unmarketable (Fig. 1.8; Bariselli et al. 2016). Affected crops included several pear varieties, apples, kiwi, and persimmons, and further losses have been recorded among rice, soybean, corn, and sunflower extensive crops (Maistrello et al. 2014, 2017; Bariselli et al. 2016).

1.3.5 Current distribution

The native range of *H. halys* encompasses, Far East of Russia, China, Japan, and Korea (Hoebeke and Carter 2003; Lee et al. 2013). In China, this species is broadly distributed through the subtropical and temperate regions (Zhu et al. 2012). In Japan, it occurs on the islands of Shikoku, Honshu, and Kyushu, no record in Hokkaido (Watanabe et al. 1994). *H. halys* is present across South Korea (Bae et al. 2008); nevertheless, records from North Korea are missing (Haye et al. 2015). The first record of established population outside Asia, dates back to 1996 in Pennsylvania, although the first confirmed identification of established populations was made in 2001 in eastern Pennsylvania (Hoebeke and Carter 2003; Lee et al. 2013). Since then its range has increased and, at present, *H. halys* is reported 42 states of the U.S. from the east to the west coast, Alaska and Hawaii included (Kriticos et al. 2017). *H. halys* first record in Canada came from the Province of British Columbia in 1993 but established populations were officially confirmed in 2012 in the Greater Toronto and Hamilton Areas of Ontario (Gariepy et al. 2014). To date, *H. halys* is also present in Southern Alberta, Quebec, Saskatchewan, Ontario, Prince Edward Island, and Nova Scotia (Gariepy et al. 2014; Kriticos et al. 2017). The presence of this species in South America is reported only for Chile (Faúndez and Rider 2017).

The first established population of *H. halys* in Europe was reported in Zurich, Switzerland, in 2007 (Wermelinger et al. 2008) but likely the first introduction dates back to 2004 (Haye et al. 2014). During the same year, a second specimen was caught near Balzers, in southern Liechtenstein (Arnold 2009), probably derived from the expansion of the Swiss population. At present *H. halys* is present in 11 Swiss Cantons, with massive presence in the cities of Zurich, Basel, Bern and Lugano (Haye et al. 2014).

During 2011, several individuals were found in Central Athens, Greece, (Milonas and Partsinevelos 2014). A single adult was detected in Konstanz, Germany, in 2012, (Heckmann 2012), to date the species has a restricted distribution.

In France, *H. halys* was first detected in the Alsace region during 2012 (Callot and Brua 2013), established populations are present in Île-de-France (Garrouste et al. 2014), South France (Maurel et al. 2016) and in Corsica (Kriticos et al. 2017).

In 2013 adults and nymphs were found in Budapest, Hungary (Vétek et al. 2014), and *H. halys* it is

nowadays largely distributed across the central and northern part of the country (Vétek and Korányi 2017). During the same year, several overwintering adults were found in Sochi, Russia, likely introduced from Europe (Italy) with ornamental plants for landscaping the XXII Olympic Winter Games. After two years, in 2015 *H. halys* spread to Abkhazia, and Georgia with massive outbreaks recorded during October 2016. In 2015, the detection of *H. halys* established populations occur in Bucharest, Romania (Macavei et al. 2015), in Austria (Rabitsch and Friebe 2015), and in Belgrade, Serbia (Šeat 2015). In Spain it was recorded for the first time in the city of Girona, Catalonia, during 2016 (Dioli et al. 2016), and so far represent the most western occurrence in Europe. In the same year its presence is confirmed in Slovakia (Hemala and Kment 2017) and Bulgaria (Simov 2016). The next year, 2017, massive occurrence of *H. halys* populations were reported in Slovenia in localities around Nova Gorica (Rot et al. 2018), Croatia (Šapina and Jelaska 2018), and Turkey (Çerçi and Koçak 2017).

In the United Kingdom only occasional interception at the London airport were reported (Malumphy 2014), nevertheless the presence of established populations was confirmed during 2019 (Defra 2019).

In Italy, *H. halys* was detected for the first time on September 2012 in the province of Modena, Emilia- Romagna region (Maistrello et al. 2014). An extensive survey started in 2013 to detected established populations in northern Italy, confirmed the presence in Emilia Romagna, Lombardy and Piedmont regions (Pansa et al. 2013; Maistrello et al. 2014; Cesari et al. 2015). During 2014, it started to occur in the North Eastern part of the country with detections of large populations in Veneto and Friuli Venezia-Giulia regions (Maistrello and Dioli 2014; Benvenuto et al. 2015). In 2015 its presence is confirmed in Tuscany (Maistrello et al. 2017; Cesari et al. 2018) and Latium (Maistrello et al. 2017). In 2016 stable populations are present in Trentino-South Tyrol (Hunterhurner 2016; Maistrello et al. 2017) and Sardinia (Dioli et al. 2016). Occasional records were reported for Central regions of Marche, Abruzzo and Umbria and Southern regions of Campania, Apulia, Calabria and Sicily (Maistrello et al. 2016, 2018; Carapezza and Lo Verde 2017; Cianferoni et al. 2018).

1.4 Figures

Figure 1.1. *Halyomorpha halys* (Stål, 1855), adult specimen on a petri dish.

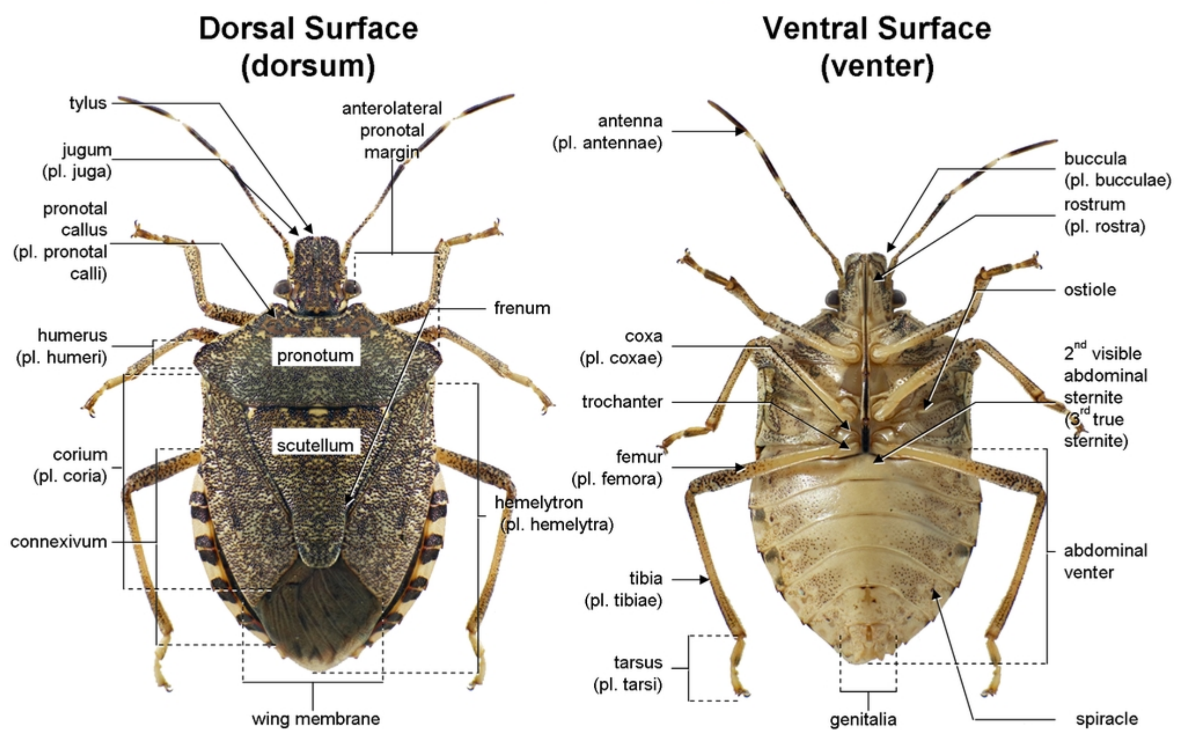

Figure 1.2. Dorsal and ventral view of an adult specimen of *Halyomorpha halys*. From Paiero et al. (2013).

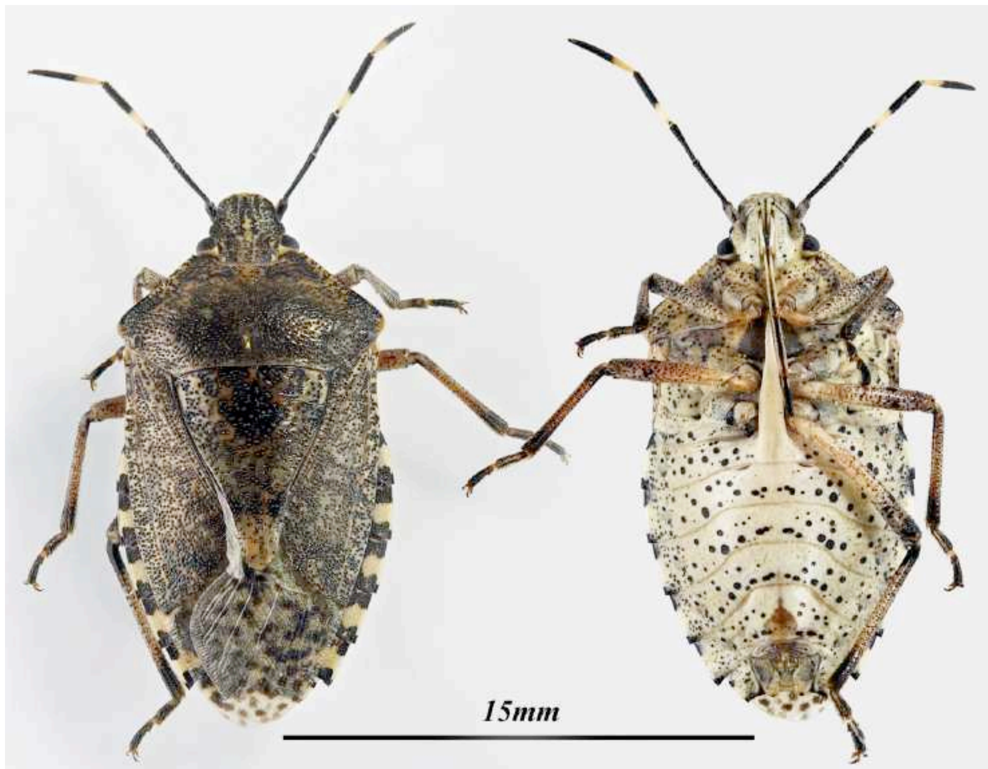

Figure 1.3. Dorsal and ventral view of an adult specimen of *Rhaphigaster nebulosa*. From: www.aramel.free.fr.com.

Figure. 1.4. *Halyomorpha halys* maturing eggs and first instar nymphs. Left: maturing eggs on underside of an apple leaf, about 32 hours before hatching. Right: Brown marmorated stinkbug first instar wingless nymphs clustered on egg mass, eight hours after hatching. From: http://bugguide.net. Copyright © 2010 ©Wil Hershberger.

Figure 1.5. *Halyomorpha halys* life instars and adult male and female. Composite of images, to scale, of second instar nymph through adult. From: http://bugguide.net. Copyright © 2010 ©Wil Hershberger.

Figure 1.6. *Halyomorpha halys* feeding on fruits and vegetable products. Left: an adult and a nymph feeding on an apple. Right: adults feeding on a tomato. Copyright Elena Costi.

Figure 1.7. *Halyomorpha halys* damages on fruit products. Left: external BMSB damage to nectarine. Centre: external injury to an apple. Right: internal evidence of surface feeding of an apple. Note sunken areas on the surface. From: www.ext.vt.edu.

Figu**re 1.8.** *Halyomorpha halys* damages on Italian pears. Left: adult specimen feeding on a pear. Centre: pear deformities. Right: pears covered by *H. halys* excreta. From: Bortolotti et al. (2015).

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CHAPTER 2

A genomic perspective on population structure and invasion dynamics of the brown marmorated stink bug *Halyomorpha halys* (Stål, 1855) (Hemiptera, Pentatomidae) in Italy

2.1 Abstract

The brown marmorated stink bug *Halyomorpha halys* (Stål, 1855) is an East Asian invasive pest which is spreading worldwide, most notably to North America and many European countries, with the first record in Italy dating to 2012. In addition of being a serious pest of agricultural crops, it is also present in urban areas, which hampers eradication efforts. The genetic diversity of Italian, Greek and Romanian populations has been investigated so far using mitochondrial DNA (mtDNA) markers (*cox1* and *cox2*), providing the first data on patterns of introduction and dispersal of this species, and showing that one haplotype is common and widespread in Italy and also in other countries. The present study aimed to deepen the knowledge of the genetics of *H. halys* individuals characterised by the most widespread mitochondrial haplotype (TH1) previously identified by Cesari et al. (2018), dissecting local population structure and patterns of introduction/dispersal. The study applied the RADseq method on 30 specimens from seven Italian regions and Greece selected from those previously analysed in Cesari et al. (2018). The individual-based analysis of genome-wide RADseq markers (1422 SNPs) led to the detection of two main clusters that clearly separate Emilia-Romagna individuals from those of North Italy and Tuscany. Nevertheless, a deeper analysis hinted a further separation in four genetic subclusters: two included individuals from different geographic regions, a third cluster included only individuals from Emilia-Romagna, while the fourth one included only specimens from Veneto region and was characterized by the highest within-cluster differentiation. These results proved that the individuals with the TH1 haplotype are not a homogeneous population, rather have originated from multiple invasion events. The results highlighted also the high mobility of the species, as evidenced by the presence of two geographically mixed clusters, likely enhanced by human activities. The lack of information from other countries made it impossible to trace back the exact sources of invasion. These genomic analyses proved to be more effective than single or few mitochondrial loci in disentangling the population dynamics of the recent invasion events, providing important knowledge to guide proper pest management strategies.

Keywords: RAD sequencing; Invasive species; Multiple invasions; Jump dispersal; *Halyomorpha halys*.

2.2 Introduction

The rate of spread of invasive alien species (IAS) has increased dramatically in the last centuries as a consequence of the rising of globalisation of trade and traffic and climate changes. The intentional or fortuitous introduction of a species beyond its native range is among the main drivers of impacts on local biodiversity, economy and health-care (Sax et al. 2005; Hulme 2009; Pyšek and Richardson 2010; Keller et al. 2011; Seebens et al. 2017). Disentangling the biological and social factors underpinning the success of a species to establish in a new environment and become invasive is crucial to increase knowledge on biological invasions and better guide stakeholders in their management decisions (Elton 1958; Sax et al. 2005; Hulme 2009; Keller et al. 2011; David et al. 2013; Chown et al. 2015).

The inherent features of a species intended as its biology, ecology, phenotypic plasticity, and genetic diversity of populations, contribute to its response and survival to a new environment (Elton 1958; Sakai et al. 2001). Nevertheless, the sole consideration of these aspects without an historical perspective on native and invasive populations origin and diffusion can lead to bias in the estimation of the invasive outcome (Goodwin et al. 1999). A species with a wide range of distribution in its native area is composed of populations adapted to different habitats that may differentiate them from other populations of the same species. These differences can enhance one population to outcompete another one during the invasion process along with the dynamics beyond its introduction event and the factors facilitating its spread and diffusion through native and invasive ranges (Allendorf and Lundquist 2003).

Population genetics applied to biological invasions is nowadays the most stated approach to rapidly identify an invasive species, define the genetic diversity of the introduced population(s) and trace back its invasion history by detecting its origin and patterns of introduction and diffusion across the globe (Estoup and Guillemaud 2010; Lawson Handley et al. 2011; Cristescu 2015). The integration of genetic data with information from other fields (i.e. observational, ecological, population modelling) is central to define the factors that have led the species of interest to become invasive and possibly forecast its future success. Genetic methods rely on the comparison of selected mitochondrial and/or nuclear markers or genome-wide single nucleotide polymorphisms (SNPs) of the introduced population(s) with those from inferred ranges of origin, either native or introduced that act as secondary source (Estoup and Guillemaud 2010; Lombaert et al. 2010; van Boheemen et al. 2017).

Halyomorpha halys is an invasive agricultural and household pest with a high rate of diffusion worldwide. The native range of this stink bug encompasses, Japan, China, Taiwan, North and South

Korea. First reported in Mid-Atlantic regions of the United States of America in the mid-1990s, is nowadays established in 43 US states and Canada, in 15 European countries, Turkey, Caucasus and recently started spreading in Chile, South America (Cianferoni et al. 2018; Maistrello et al. 2018). The close association with human activities allows this species to travel long distances as stowaway of private and commercial means of transport (Gariepy et al. 2015; Haye et al. 2015) thus, increasing the risk of new introductions in many countries.

In its native range, *H. halys* is an occasional or outbreak pest of several crops such as apple, pear, persimmon, and soybeans with restricted damages probably due to the presence of co-evolved organisms that limit the growth of populations (Lee et al. 2013). The only exception is represented by Japan where *H. halys* causes extensive damages to various fruits and soybean and has become a serious pest of apples other than being a common nuisance pest (Toyama et al. 2010). Despite the wide diffusion, the most significative agricultural and nuisance pest problems have been recorded in in the mid-Atlantic region of U.S.A and in the Emilia-Romagna region in Italy. Here this stink bug affects many orchard crops, vegetables, grapes, row crops, ornamentals, and nursery crops with outbreaks peaks that caused economic losses in agri-food industries (Costi et al. 2017; Maistrello et al. 2017; Leskey and Nielsen 2018).

Several studies started after the introduction and spreading of *H. halys*, mainly focused on tracing the origin of the populations and the pattern of their diffusion through the analysis of molecular markers of invasive and native populations. As a common strategy in the analysis of non-model species with a worldwide distribution, these studies focused on one or few loci of mitochondrial DNA (Sakai et al. 2001; Hebert et al. 2003; Le Roux and Wieczorek 2009; Lawson Handley et al. 2011; Kirk et al. 2013). Xu et al. (2014) analysed genetic diversity of specimens collected in U.S.A. and across the native range of China, Korea, and Japan based on portions of cytochrome oxidase II (*cox2*) and mtDNA control region (CR) genes. In two successive studies Gariepy et al. (2014, 2015) analysed portions of cytochrome oxidase I (*cox1*) and cytochrome b (*cyt b*) genes from specimens collected in Canada and Switzerland, and portions of cytochrome oxidase I (*cox1*) gene from specimens collected in Switzerland, France, Greece, Italy and Hungary. Lee et al. (2018) analysed a portion of the cytochrome oxidase I (*cox1*) gene in specimens from Korea and U.S.A. while Valentin et al. (2017) widened the analysis resequencing the specimens from Xu et al. (2014) and additional specimens from U.S.A., Japan, Korea and China for a portion of the cytochrome oxidase I (*cox1*) gene.

In Europe Cesari et al. (2015, 2018) conducted an extensive study on specimens collected across all invaded Italian regions, in Greece and Romania investigated through the analysis of portions of cytochrome oxidase I (*cox1*) and cytochrome oxidase II (*cox2*) genes. The latest study from 2018

(Cesari et al. 2018) integrated current and previous observational data in space and time with molecular information to identify the genetic diversity of *H. halys* in Italy and Europe, its invasion history, and the potential pathways of entry and diffusion. Their results revealed that one combined (*cox1* + *cox2*) haplotype, called TH1, was the most common in almost all analysed Italian regions, Romania and Greece. In Italy, they correlated the occurrence of this haplotype with the detection of newly established populations in a timeframe from 2012, year of the first reported detection of the species in Italy (Maistrello et al. 2014), to 2016. Their analysis showed that until 2013 the TH1 haplotype was limited to the Emilia-Romagna region and the presence of the species was limited to restricted areas in this region and in Piedmont and Lombardy regions. Conversely, from 2014 to 2016 this haplotype became more frequent and widespread along with the massive presence of *H. halys* throughout North Italy and Tuscany. The integration of these data with those from previous studies, traced the presence of the TH1 haplotype also in the U.S.A. and China. According to the pattern of diffusion of the TH1 haplotype across Italian territories, Cesari et al. (2018) hypothesised that the Emilia-Romagna region was the entering point of this haplotype in Italy and then, the established population started to expand by an active dispersion process throughout all Northern Italian regions and Tuscany. Furthermore, the presence of the TH1 haplotype in China and in the U.S.A. led the authors to assume two introduction scenarios for the introduction of *H. halys* in the Emilia-Romagna region. The first assumed a direct introduction from China while the second assumed an introduction from the U.S.A. as a secondary invasion by "bridgehead effect".

Although the analysis based on one or few maternally inherited loci, as mitochondrial cytochrome oxidase I, proved to be very informative in the study of the invasion history of *H. halys* it may underrepresent the existing genetic variation. This is particularly noticeable when the majority of investigated populations show very low or no sign of variation across a wide range of distribution. In absence of further information that could provide hints about the presence of structured populations, especially in geographically connected areas, the risk of underestimating the introduction events and population dynamics is high (Hebert et al. 2003; Dlugosch and Parker 2008; Estoup and Guillemaud 2010; Pyšek and Richardson 2010; Jinbo et al. 2011; Cristescu 2015).

However, genotyping-by-sequencing (GBS) techniques, like the Restriction site-associated DNA sequencing (RADseq) (Baird et al. 2008) that involve the use of thousands of randomly distributed markers across genomes, have the potential to increase the molecular resolution to outline the invasion history of a species and identify events associated with the invasion process (Estoup and Guillemaud 2010; Cristescu 2015).

RADseq is a common method to investigate the genomic diversity of non-model organisms (Baird et al. 2008; Kirk et al. 2013; Vergara et al. 2015; Goubert et al. 2016; Trucchi et al. 2016; Elfekih et

al. 2018; Lemopoulos et al. 2019). It makes use of restriction enzymes to generate reduced representation libraries (RRLs) consisting of sized selected fragments at the restriction sites. These libraries represent a subset of the genome that can be screened to identify thousands of single nucleotide polymorphisms (SNPs) allowing the comparison of allele diversity and frequency on individual or population-based analysis.

Therefore, this study aimed at dissecting the population structure and patterns of introduction and dispersal of *H. halys* individuals characterised by the most widespread mitochondrial haplotype TH1 by applying the RADseq method on a representative Italian and Greek sample from the previous study of Cesari et al. (2018).

2.3 Materials & Methods

2.3.1 Samples

In this study 30 *Halyomorpha halys* genomic DNA (gDNA) extracts among those utilised in Cesari et al. (2015, 2018) were selected for RAD sequencing analysis. The selection was restricted to specimens characterised by the TH1 mitochondrial haplotype as reported in the same studies (Cesari et al. 2015, 2018) and presenting a good quality DNA for genomic analysis. The DNA concentration was measured using the Qubit 2.0 fluorometer (Life Technologies), and the quality was checked using the NanoDrop® ND-1000 spectrophotometer (Life Technologies) and visually inspected after gel electrophoresis.

The individuals from which the gDNA was extracted were collected between 2013 and 2017 in localities from 15 Italian provinces of 7 regions (*Emilia-Romagna*: Bologna, Modena, Piacenza, Ravenna, Reggio Emilia; *Tuscany*: Livorno; *Piedmont*: Cuneo, Turin; *Lombardy*: Brescia, Milan, Varese; *Trentino-South Tyrol*: Bolzano; *Friuli Venezia-Giulia*: Pordenone, Udine; *Veneto*: Belluno) covering all the northern regions where the species is present, and in one Greek locality (Athens) (Fig. 2.1, Tab. 2.1).

2.3.2 RAD sequencing

RAD libraries were generated following the RAD sequencing protocol from Baird et al. (2008). Around 300 ng of genomic DNA per extract were selected and added to a final volume of 40 µl to obtain 30 samples at the same DNA concentration. The samples were digested with the restriction enzyme SbfI-HF® (CCTGCAGG) (NEB), then ligated to a unique barcoded P1 adapter prior to pooling them in 4 separated libraries (2 libraries with 7 individuals each, 2 with 8 individuals each). The four libraries were sheared by sonication on a Bioruptor® Plus (Diagenode) using 6 cycles (30 s

ON, 3 s OFF; 2°C, high power) to obtain the target size range fraction of 300–600 bp. Sonicated products were purified by capture on AMPure XP (Beckman Coulter) magnetic beads (beads solution: DNA = 0.8:1). This technique was used in all purifications required in the following steps and concentrated to 30 μL each with QIAquick PCR purification kit (Qiagen), libraries were size selected by gel electrophoresis and manual excision and purified again with the Wizard Gel and PCR cleaning kit (Promega). Blunt-end repairing, and poly-A tailing were performed using the Quick Blunting™ kit and Klenow exo- (NEB). After a second unique barcoded P2 adapter ligation, the libraries were enriched by a 21-cycle PCR (98°C 30 s; [98°C 10 s | 65°C 30 s | 72°C 30 s] 21 cycles; 72°C 5 min). To reduce amplification bias, PCR four aliquots of 25 μL per library were separately amplified and then pooled again. Libraries were quantified by a fluorimetric-based method (Qubit 2.0, Invitrogen), and molarity was checked on an Agilent Bioanalyzer chip (Invitrogen). The final pooled libraries were sequenced using 150-bp paired-end reads on an Illumina NextSeq 500 lane at the Quick Biology Inc. centre (Pasadena, CA).

2.3.3 Bioinformatic analyses

Raw Illumina sequence data were processed using the scripts included in the *Stacks* 1.46 package (Catchen et al. 2013) on the Beagle server at the CiBio Centre, University of Porto, Portugal. Raw reads were quality filtered and demultiplexed according to individual barcodes using the script *process_radtags* with default settings. PCR clones (17.6% of raw reads) were removed using the *clone_filter* script in the *Stacks* pipeline. The genome of *H. halys* (assembly Hhal_2.0, RefSeq assembly accession: GCF_000696795.2) downloaded from the assembly section of GenBank was used as reference for mapping cleaned reads using the Burrows-Wheeler Alignment Tool as implemented in BWA-MEM (Li 2013). Assembly of RAD loci and genotype calling were obtained by running the *ref_map* wrapper script in *Stacks* using default settings. The program *Stacks* requires a priori information about the population to which individuals belong meant as a group of individuals that gather together, i.e. based on geographic origin, same laboratory strain, physical features. To perform an individual-based analysis instead, each individual need to be processed as a single population (Catchen et al. 2013; Rochette and Catchen 2017). Thus, the individuals in this analysis were processed as single populations (sensu *Stacks)*, with the *-popmap* program flag. Referenced mapped outputs were post processed by the *populations* script with a threshold allowance for missing data across individuals at a locus of 50%, and for each RAD-tag only one SNP was retained using the program flag -*write_random_snp* (if there were two or more SNPs in the sequence, *Stacks* would randomly choose one to analyse).

2.3.4 Genetic structure: Bayesian clustering and multivariate analysis

Population structure was first estimated using the program STRUCTURE v. 2.3.4 (Pritchard et al. 2000; Falush et al. 2003, 2007) assuming individual admixture and correlated allele frequencies within individuals. Simulations were run with Markov Chain Monte Carlo (MCMC) of 1000000 iterations after a burn-in of 100000. Number of clusters (K) was allowed to vary from 1 to 16, and 10 independent simulations were run for each K value to check for consistency in the results. The rate of change in the log probability of data between successive K values (ΔK), as described by Evanno et al. (2005) was estimated with STRUCTURE HARVESTER (Earl and VonHoldt 2012) to determine the most likely number of clusters. To determine the population assignment probability of each individual across all simulations for the identified K value, data were run on the CLUMPAK server (http://clumpak.tau.ac.il/index.html; Kopelman et al. 2015). Spatial interpolation of ancestry coefficients was plotted on a geographic map adapting Q-matrices to the scripts from the POPSutilities.R suite of functions (Jay et al. 2012) and visualised in R (R Core Team 2017). Puechmaille (2016) affirm that STRUCTURE could led to erroneous results in cluster assignments as result of an individual-based rather than population-based analysis. To double-check STRUCTURE's results and obtain solid inferences, the fineRADstructure package v 0.3.2 r109 (Malinsky et al. 2018) was used. This program was developed to process RAD data and utilises the *Stacks* output *haplotypes.tsv* file to quantify the ancestry sources in each individual. In this study fineRADstructure was allowed to retain a maximum of 15% of missing loci to be included in the PCA from the *haplotypes.tsv* file. After the filtering for missing loci, a total of 22 individuals was retained for the analysis (Tab. 2.1). The resulting co-ancestry matrix was then visualized in Rstudio. Signals of genetic discontinuities in continuous populations and estimation of spatially varying individual admixture proportions were computed with the R packages tess3r (Caye et al. 2018) and LEA (François 2016), computing the *snmf* function (Frichot and François 2015) for K from 1 to 16, and 200 independent simulations for each K.

The statistical analyses were carried out using the R package *adegenet* v2.1.0 (Jombart 2008). The Principal Component Analysis (PCA) was computed on allele frequencies with the function *dudi.pca*, missing data were replaced by the mean of allele frequencies (Rochette and Catchen 2017). In addition, the genetic diversity was displayed on a PCA plot with the function *colorplot*. All plots were generated using the *ggplot2* package (Hadley 2016). To incorporate spatial relationships among the individuals and check for a correlation between geographic distances and genetic distances the spatial principal component analysis (sPCA) was computed. sPCA was generated using the function *spca* specifying as connection network the Delauney triangulation graph (type 1) to define spatial connectivity. The resulting sPCA scores were plotted onto a

personalised raster map of Europe downloaded from the NOAA web site (http://maps.ngdc.noaa.gov/viewers/wcs-client/). Since isolation by distance (Wright 1921) and range expansion (Peter and Slatkin 2013) can be computed only among geographically connected populations that share a uniform space, the individuals from Greece were removed in these two analyses. Isolation by Distance (IBD) were tested across the Italian individuals with the *mantel.randtest* function computing the Mantel test between the matrix of Nei's genetic distances (Nei and Roychoudhury 1974) and the matrix of Euclidean geographic distances, with 9999 permutations and using Greek individuals as outgroup. Based on STRUCTURE results, IBD signals were also checked separately on two subsets including respectively all the individuals from Emilia Romagna and all the individuals from Piedmont, Lombardy, Trentino South-Tyrol, Veneto, Friuli-Venezia-Giulia and Tuscany. To test for the occurrence of a recent range expansion among analysed individuals from Italy, the R package *rangeExpansion* (Peter and Slatkin 2013, 2015) was used and the test performed on three datasets as in IBD analysis.

2.4 Results

2.4.1 RAD sequencing data

After initial filtering steps, the data related to two individuals (PIE15 from Piedmont and LOM19 from Lombardy; Tab. 2.1) were removed due to low sequencing coverage (mean coverage 7.3X and 5X), the average number of paired reads retained per individuals was 1,042,714 (SD 558,267) with a mean percentage of mapping of 95%. Across the data related to 28 remaining individuals, the total number of single SNP loci retained via the *Stacks* pipeline was 1,422 with a mean coverage per allele per individual of ca. 19X.

2.4.2 Population genomics structure

The results from STRUCTURE, STRUCTURE HARVESTER and CLUMPAK identified two clusters (K=2) at the uppermost level (Figs. 2.2, 2.3, 2.4). The first cluster (blue) is composed by all the individuals from the Emilia-Romagna region, with the exception of one individual (ER8) from Modena province and one individual from Ravenna (ER10) province that are included in the second cluster (purple) along with the individuals from the other Italian regions and Greece.

The LEA and tess3r analyses also indicated two (K=2) as the most supported number of clusters according to the lowest value of cross-entropy (equivalent to ΔK). The two clusters scored by LEA were in agreement with the results from STRUCTURE while, in tess3r the two clusters differed for one individual (ER8) included in the first cluster with all the others from Emilia-Romagna (Figs.

2.5, 2.6, 2.7).
Nevertheless, in both LEA and tess3r analyses, progressive values of cross-entropy (Fig. 2.5) reach a plateau at 4 (K=4), suggesting further subdivisions of the two previous clusters in four partitions.
In particular, LEA identified one cluster with only Emilia-Romagna individuals (the same cluster scored in K=2), a second cluster with all the individuals from Veneto, a third cluster with the individuals from Piedmont and Greece and a fourth cluster with the individuals from Lombardy, Trentino-South Tyrol, Friuli-Venezia-Giulia, Tuscany and the two individuals ER8 and ER10 from Emilia-Romagna (Fig. 2.8).
Whit regard to tess3r, the program identified almost the same clusters of LEA. The first cluster was the same scored for K=2 with only individuals from Emilia-Romagna, the other three clusters were identical of those found by LEA except for one individual from Piedmont (PIE16) not assigned to any cluster (Fig. 2.9).
The coancestry matrix from fineRADstructure was computed on 22 individuals (Tab. 2.1; Fig. 2.10); the reduced dataset excluded from the analysis 5 individuals from Emilia-Romagna (ER3, ER5, ER6, ER7, ER8) and one individual from Friuli Venezia-Giulia (FVG22) that presented percentages of missing loci >15%. fineRADstructure also scored the presence of two main clusters that separate the Emilia-Romagna individuals from all the others Italian individuals, Greek individuals and the individual ER10 from Emilia-Romagna, and a further subdivision in four groups. In accordance with the previous analyses, of the four clusters, two of them were well-defined; the first was formed by Veneto individuals and the other one by Emilia-Romagna individuals, both characterised by high levels of intrapopulation coancestry. The third cluster included the individuals from Trentino South-Tyrol, Friuli Venezia-Giulia, the individual from Tuscany and one individual from Lombardy (LOM18) while the fourth cluster gather individuals with lower levels of assignment and includes one individual from Emilia-Romagna (ER10) and one individual from Lombardy (LOM19) along with individuals from Greece and Piedmont.
In the PCA (Fig. 2.11), the first two eigenvectors held the largest percentage of variance among the individuals, principal component 1 = 8.0% and principal component 2 = 6.9% revealing the presence of four genetic clusters. One cluster, consistent in all previous analyses (STRUCTURE, LEA, tess3r and fineRADstructure), included only Emilia-Romagna individuals with the exception of the ER8 and ER10, a second cluster included only individuals from Veneto region and was characterized by the highest within-cluster differentiation, a third cluster gathered individuals from Piedmont, Greece and the ER8 individual, and the fourth one was formed by ER10 individual and individuals from all the others Italian regions. This grouping was almost in agreement with the structure-like results from LEA and tess3r analyses.

The sPCA of Italian and Greek individuals (Figs. 2.12, 2.13) showed a significant correlation between genetic and geographic distances (p-value = 0.003), which revealed a global structure mainly explained by the first global principal component λ1 (Fig. 2.12). The main separation occurred between the individuals from Emilia-Romagna and all the rest of considered Italian regions (Fig. 2.13).

The results from Isolation by Distance (IBD) analysis indicated no patterns of IBD across all Italian areas, neither in the subsets generated according to STRUCTURE results for K=2. In all cases, the Mantel regression plot displayed discontinuities in high-density clouds of points, this pattern is more consistent with the presence of patches (Fig. 2.14).

The results from range expansion analysis computed on all individuals Indicated signals of range expansion only in Emilia-Romagna (p-value = 0.04) (Fig. 2.15) pointing the origin of expansion to an area nearby Modena province (coordinates: 44.49957, 10.78365). The analysis on the two subsets, generated according to STRUCTURE results for K=2, again indicated signals of range expansion only in the subset with Emilia-Romagna individuals (p-value = 0.03) but no signal in the subset with all the other individuals (p-value > 4) (Fig. 2.15). These signals support the presence of two main groups, one limited to Emilia Romagna and another well differentiated from this.

2.5 Discussion

The global spreading of *H. halys* is giving rise to concerns due to the critical impacts that its presence might generate in newly invaded areas. The intimate connection with trade and traffic and the biodiversity homogenisation related to climate change have the potential to enhance the diffusion of this species beyond the limit of its current range (Fraser et al. 2017; Kriticos et al. 2017). Like many invasive species (Ciosi et al. 2008; Lombaert et al. 2010; Schmack et al. 2019), *H. halys* populations in new areas are characterised by a reduced genetic diversity compared to those from native areas (Cesari et al. 2018; Lee et al. 2018). This evidence is consistent with an expected decrease of diversity in populations that went through a founder effect since the first introduction events usually involve a small number of individuals that represent only a fraction of the original genetic variability present in native populations (Estoup et al. 2016). The patterns emerging from previously published data focused on mitochondrial loci of this species in introduced countries depict a history of successful establishment and natural range expansion of these relatively homogeneous populations. Even though, evidences of multiple introductions of *H. halys* from native and introduced areas are widely reported along with cases of jump dispersal due to passive transport (Gariepy et al. 2014, 2015; Xu et al. 2014; Cesari et al. 2015, 2018; Zhu et al.

2016; Valentin et al. 2017; Lee et al. 2018).

Interpreting the invasion history of these homogeneous populations at one or few loci can be challenging, especially in case of wide ranges of distribution. The absence of distinct genetic and geographic boundaries separating the populations can lead to wrong conclusions regarding the invasion processes involved in their success.

The use of reduced representation libraries (RRLs), generated by RADseq to deepen the degree of the genetic variability extent in target individuals or populations, can overcome the limitation observed in previous methods.

The focus of this study was the reanalysis of a representative subsample of Italian individuals characterised by a single combined haplotype TH1 as emerged from the previous study of Cesari et al. (2018) to detect the presence of underscored genetic variability and population structuring.

Present results showed that the introduction and spreading dynamics of *H. halys* across Italian territories are even more complex than previously hypothesised. In contrast with previous results that identified these individual as a single homogeneous group, data obtained with RADseq clearly pointed out to the presence of two distinct genetic groups that separate individuals from the Emilia-Romagna region from those originating from North Italy, Tuscany and Greece (Figs. 2.4, 2.6, 2.7, 2.10, 2.13). Despite some differences in the clusters assignment for one individual (ER8) highlighted in STRUCTURE and structure-like clustering results (Figs. 2.4, 2.6, 2.7), the assignment values based on the genetic similarities for all the other individuals clearly indicates that the two groups derive from genetically different populations. This separation is coherent with the historical data and with the temporal occurrence of the TH1 haplotype within Italy according to Cesari et al. (2018). In fact, until 2014 this haplotype was only present in the Emilia-Romagna region while, after that year started to appear with a low frequency in other regions, especially Friuli Venezia-Giulia (geographically distant from Emilia-Romagna) and Lombardy. The occurrence in the other areas bordering the Emilia-Romagna region started later, during 2015 and 2016 (Cesari et al. 2018). The uncertainties related to the assignment of the ER8 individual might be related to the presence of a higher percentage of missing loci as evidenced during the filtering step in fineRADstructure that excluded this individual from the analysis (see Results 2.4.2).

Noteworthy, analyses with fineRADstructure (Fig. 2.10), PCA (Fig. 2.11), and progressive clustering with LEA and tess3r (Figs. 2.8, 2.9) provided deeper information, suggesting the presence of at least four substructures in the analysed dataset even if they did not converge to the same solution. These programs are more sensitive to detect the differences that might derivate from recent migration events from sources genetically related and for which a clear genetic separation did not occur (Durand et al. 2009; Caye et al. 2018). A similar pattern emerged from the study of

Vergara et al. (2015) on the stone marten (*Martes foina*, Erxleben, 1777) genetic structure in the Iberic peninsula. In their case, STRUCTURE, structure-like programs and PCA identified two main clusters but the presence of weak further partitioning led the authors to identify a contemporary genetic substructuring of one cluster due the presence of three rivers that acted as barriers to gene flow. In present study, the further partition does not involve the Emilia-Romagna cluster, reinforcing the hypothesis of a single introduction event from one distinct source that characterised these individuals. On the contrary, the explanations for the other three subclusters are more difficult to depict in absence of similar data to compare and lack of obvious barriers to gene flow. Nevertheless, the high levels of shared coancestry that separate individuals from the Veneto region from the rest of the clusters might indicate an undetected introduction event of few individuals from a different unknown source. A weak suggestion for the cluster that gather the individuals from the Piedmont region and Greece come from the mitochondrial analysis from Cesari et al. (2018). Their study evidenced a pattern of genetic diversity in the Piedmont region that differentiate its populations from the neighbouring regions, this might indicate a different origin for these individuals. A common origin from a more strictly related population might also explain the similarities between individuals from Greece and the Piedmont region even though it is impossible to determine the source and direction of the introduction without larger samples from these areas and genomic information from other countries. For the same reasons, it is impossible to determine the introduction events and the actual invasion pathways underlying the fourth geographically admixed cluster that encompasses the individuals from Lombardy, Trentino-South Tyrol, Friuli-Venezia-Giulia, Tuscany regions. The only exception is represented by the ER10 individual from Emilia-Romagna that clearly represent a case of jumping dispersal from an area of Northern Italy to Emilia-Romagna, probably due to passive transport.

An interesting result comes from the sPCA analysis (Fig. 2.13) that tested the correlation between geographic and genetic diversity. The program evidenced a geographic correlation that separate the Emilia-Romagna region from the rest of the considered regions. However, the shape of such pattern can be better explained considering invasion dynamics, ecological factors and previous observational data of *H. halys* in Italy. Recently, Maistrello et al. (2018) demonstrate that the most abundant populations were concentrated along the main road or railway lines and cities. In addition, in the Emilia-Romagna region the distribution of the population showed a strict connection also with the presence of open field crops near the main cities. Based on an extensive survey, Maistrello et al. (2018) further demonstrated that in these areas occurs the main concentration of the Emilia-Romagna population.

The absence of strong isolation by distance (Fig. 2.14) signals in all analysed datasets may be

correlated to a combination of low natural expansions and high levels of human-mediated diffusion as recently demonstrated in the study of Goubert et al. (2016) on the population genetics of the invasive species *Aedes albopictus* Skuse, 1894. These factors exclude the involvement of geographic distances as strong barriers to gene flow and for the emergence of a clear isolation by distance pattern.

The results from range expansion analysis (Fig. 2.15) corroborate the hypothesis that the analysed *H. halys* individuals originate from at least two separate introduction events and followed different pathways of expansion and dispersal.

Summarising the invasion dynamics of *H. halys* according to present data, the TH1 haplotype first appeared in the Emilia-Romagna region. This is also the first Italian region where this species has been found in 2012 (Maistrello et al. 2014). The first established individuals with this haplotype originated a population that went through a fast growth and a rapid diffusion from the origin point in an area between Modena and Reggio Emilia provinces to nearby areas in strong association with the presence of main road/railway lines and agricultural orchards as mentioned above. In contrast with the original hypothesis, present results do not provide any evidence of an expansion of this haplotype outside the regional borders of Emilia Romagna. The genetic similarities among the individuals from Northern Italy, Tuscany and Greece hint a shared origin. Unfortunately, the RADseq data to compare from other countries where the TH1 mitochondrial haplotype is dominant and native areas are still missing. Hence, it is impossible to infer the exact origin of these two clusters neither the validity of a further separation.

Even with a small dataset, this study represents the first attempt to apply genomic approaches to deepen investigate the real diversity and structure underlying the most common and widespread mitochondrial haplotype, bringing out to our knowledge unrecorded introduction events and movements of *H. halys* populations.

Future efforts should be devoted to this type of data in order to access detailed information on the invasion process and adaptive potential of invasive insects thus, applied biosurveillance efforts will benefits from detailed insights that genomic data bring to our understanding of biological invasions.

Applying RAD sequencing to the study of economical relevant invasive alien species such as *H. halys* can exceed the information obtained only by one or few genetic markers (i.e. mitochondrial, nuclear) improving the pest management programs and reducing the economic costs. However, this requires an increase use of this method to obtain comparable data either from native and/or introduced areas. In addition, the quantity and quality of samples required to obtain strong supported information are limiting factors that doesn’t match the practical issues of dealing with this kind of species. Generally, the finding and collection of invasive species, especially in the first

phases of their diffusion do not take into account the need of an adequate preservation for molecular analysis thus, most of the time, the specimens are too degraded or the genomic quality does not fit the standard required for this type of analysis. Considered this, the solution should be a better integration between different types of studies.

2.6 Table & Figures

Table 2.1. List of the analysed individuals with geographic information and year of the sampling. § individuals excluded from the analysis for low coverage; * individuals retained for the fineRADstructure dataset.

Sample name	Country	Region	Province	Geographic coordinates	Year
ER1*	Italy	Emilia-Romagna	Bologna	44.4949, 11.3426	2014
ER2*			Modena	44.6346, 10.8718	2013
ER3			Modena	44.5955, 10.8591	2013
ER4*			Modena	44.5957, 10.8593	2013
ER5			Modena	44.6471, 10.9252	2013
ER6			Modena	44.5621, 11.0342	2013
ER7			Modena	44.5443, 10.7847	2014
ER8			Modena	44.4781, 11.008	2014
ER9*			Piacenza	45.0524, 9.6987	2016
ER10*			Ravenna	44.4134, 11.9781	2017
ER11*			Ravenna	44.4157, 12.1966	2017
ER12*			Reggio Emilia	44.7288, 10.6294	2017
TUS13*		Tuscany	Livorno	43.5519, 10.308	2015
PIE14*		Piedmont	Cuneo	44.5970, 7.6114	2014
PIE15§			Torino	45.2015, 7.7773	2014
PIE16*			Torino	45.0703, 7.6869	2014
LOM17*		Lombardy	Milano	45.4612, 9.1878	2014
LOM18*			Brescia	45.5416, 10.2118	2014
LOM19§			Varese	45.8206, 8.8250	2015
TST20*		Trentino South-Tyrol	Bolzano	46.4952, 11.3541	2016
FVG21*		Friuli Venezia-Giulia	Udine	46.0649, 13.2307	2014
FVG22			Udine	46.2075, 13.1169	2015
FVG23*			Udine	46.1266, 13.0329	2014

FVG24*			Udine	46.0167, 12.9792	2014
FVG25*			Pordenone	46.0705, 12.5868	2015
VEN26*		Veneto	Belluno	46.1424, 12.2167	2015
VEN27*			Belluno	46.1425, 12.2167	2015
VEN28*			Belluno	46.1426, 12.2167	2015
GRE29*	Greece	Attica	Athens	37.9838, 23.7275	2015
GRE30*			Athens	37.9838, 23.7276	2015

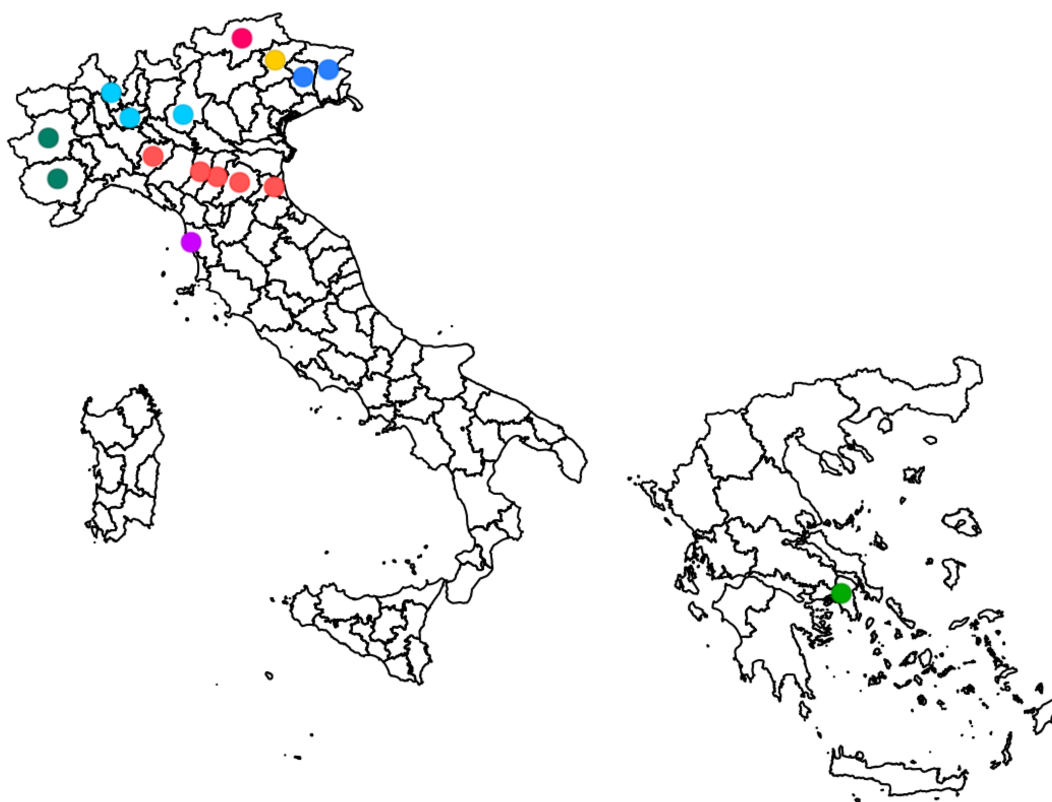

Figure 2.1. Italian and Greek sampling provinces. Italian regions of each province: Emilia-Romagna, *salmon;* Tuscany, *purple*; Piedmont, *dark green*; Lombardy, *light blue*; Trentino-South Tyrol, *pink*; Friuli Venezia-Giulia, *blue* Veneto*, yellow*; Greece, *green*.

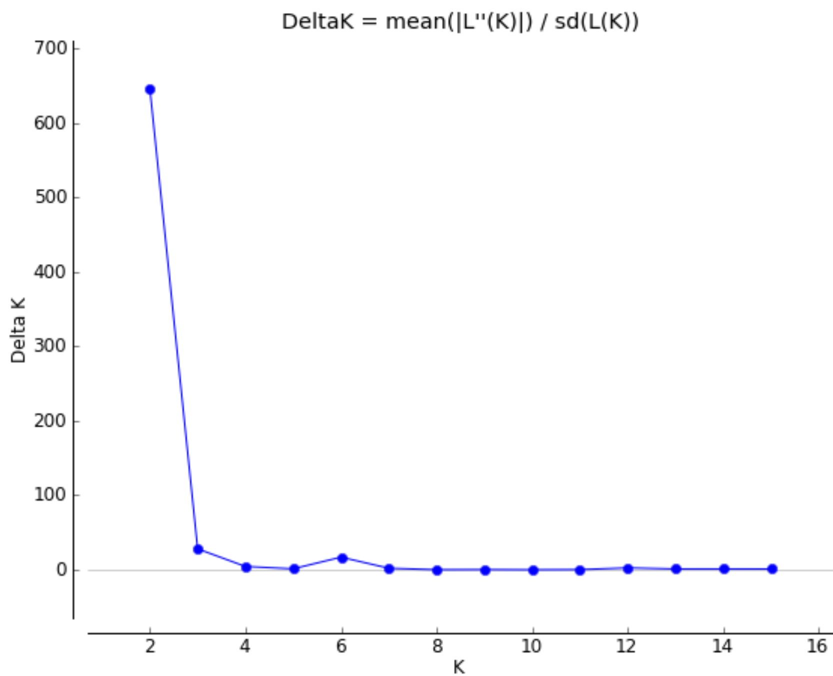

Figure 2.2. Structure Harvester analysis. Graph of Delta K (ΔK) indicating 2 as the number of clusters with the highest probability.

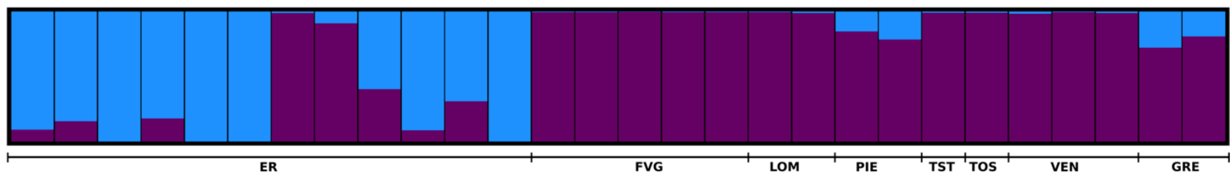

Figure 2.3. Clumpak analysis. Barplot of assignment for K=2. Blue: Cluster 1, Purple: Cluster 2. Bars group individuals (bars) according to their region or country of origin. ER = Emilia-Romagna, FVG = Friuli Venezia-Giulia, LOM = Lombardy, PIE = Piedmont, TST = Trentino South-Tyrol, TOS = Tuscany, VEN = Veneto, GRE = Greece.

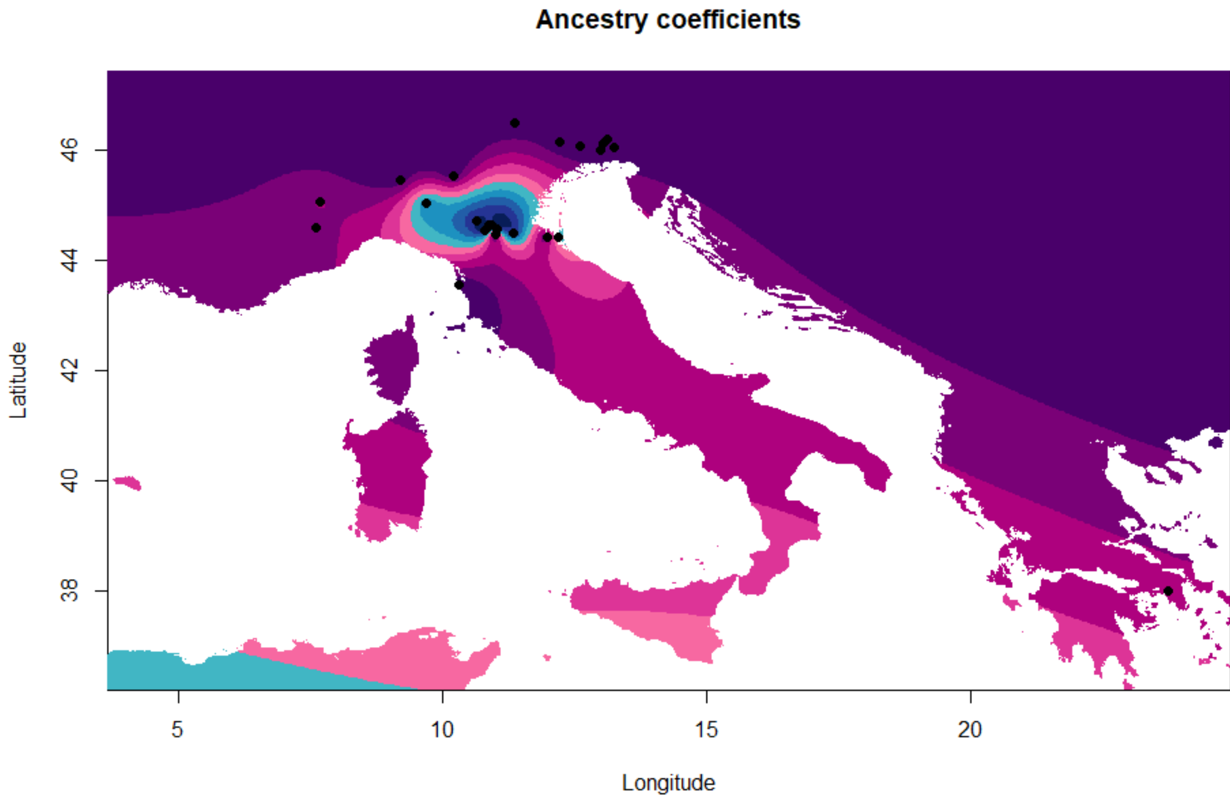

Figure 2.4. STRUCTURE analysis. Map of ancestry coefficients distribution for K=2. Different colour shades indicate values from 0.5 (lighter) to 1 (darker). Black dots indicate sampling sites.

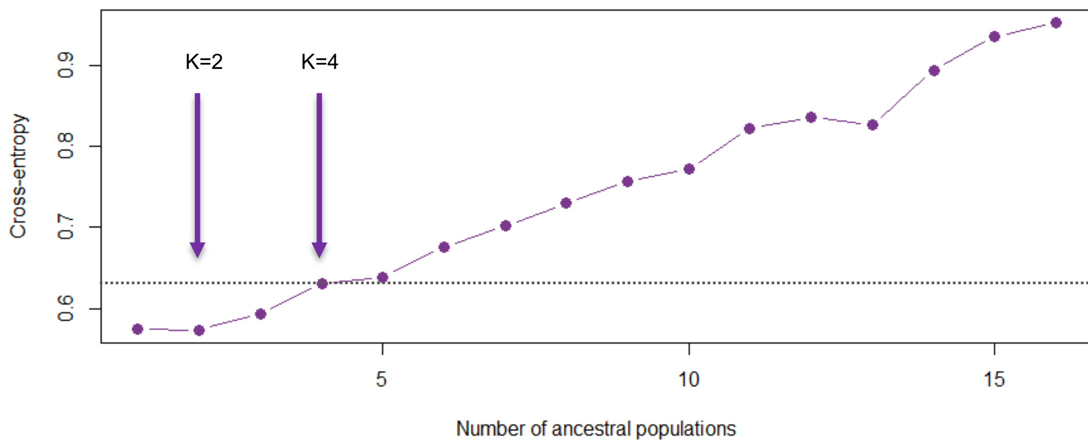

Figure 2.5. LEA and tess3r cross-entropy combined graph. The lowest value indicates the most supported partitioning.

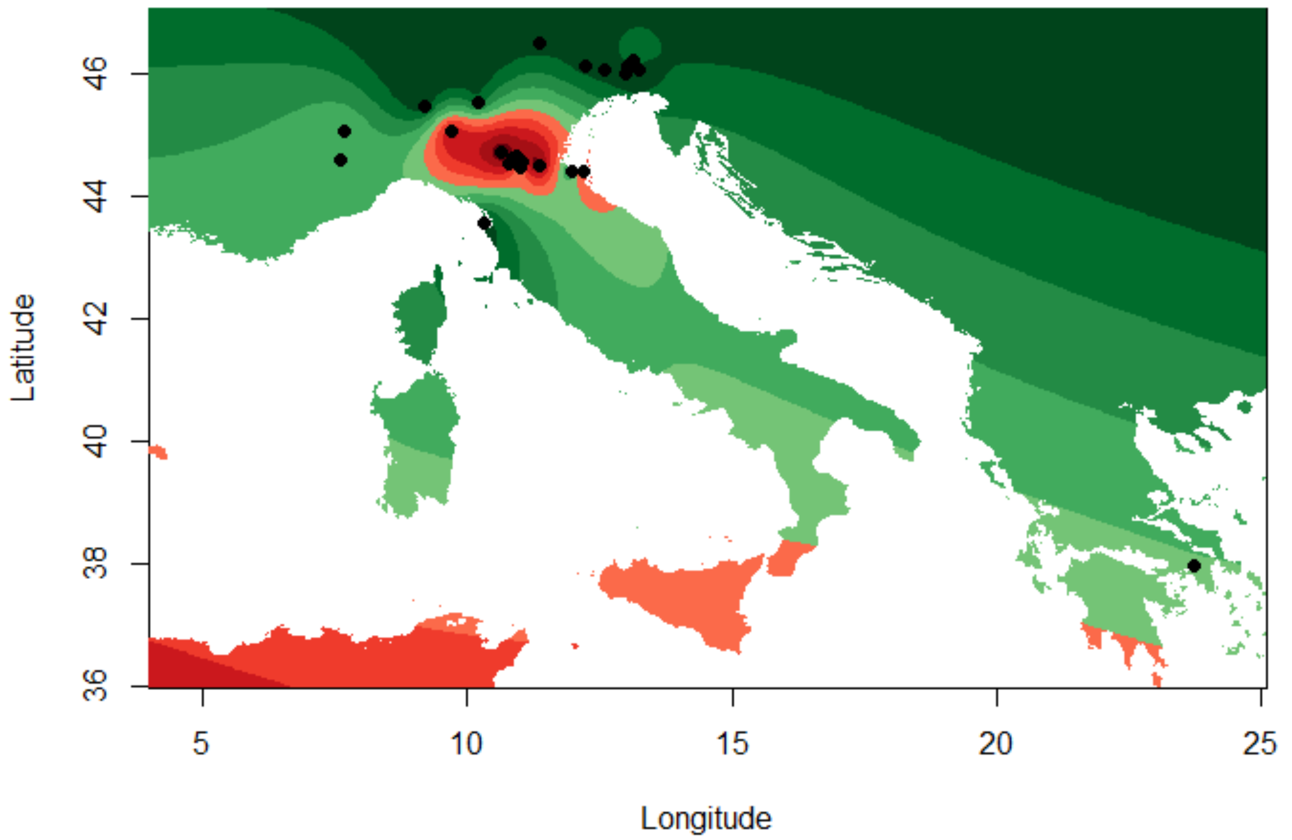

Figure 2.6. LEA analysis. Map of ancestry coefficients distribution for K=2. Different colour shades indicate values from 0.5 (lighter) to 1 (darker). Black dots indicate sampling sites.

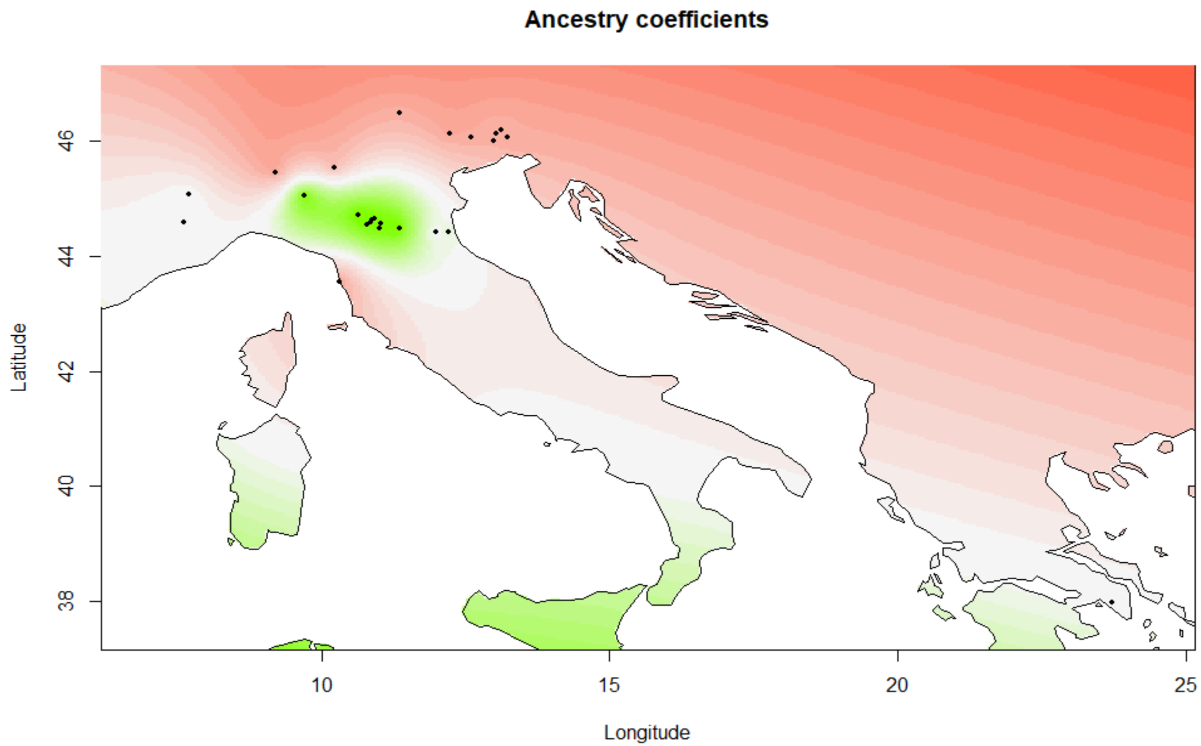

Figure 2.7. tess3r analysis. Map of ancestry coefficients distribution for K=2. Different colour shades indicate values from 0.5 (lighter) to 1 (darker). Black dots indicate sampling sites.

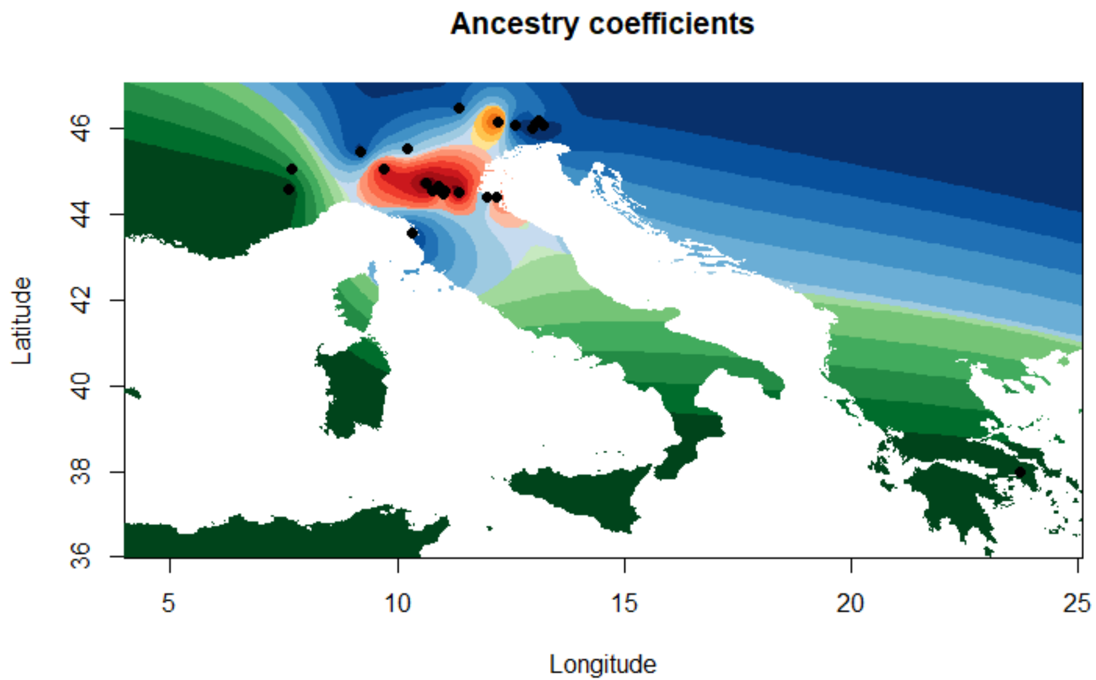

Figure 2.8. LEA analysis. Map of ancestry coefficients distribution for K=4. Different colour shades indicate values from 0.5 (lighter) to 1 (darker). Black dots indicate sampling sites.

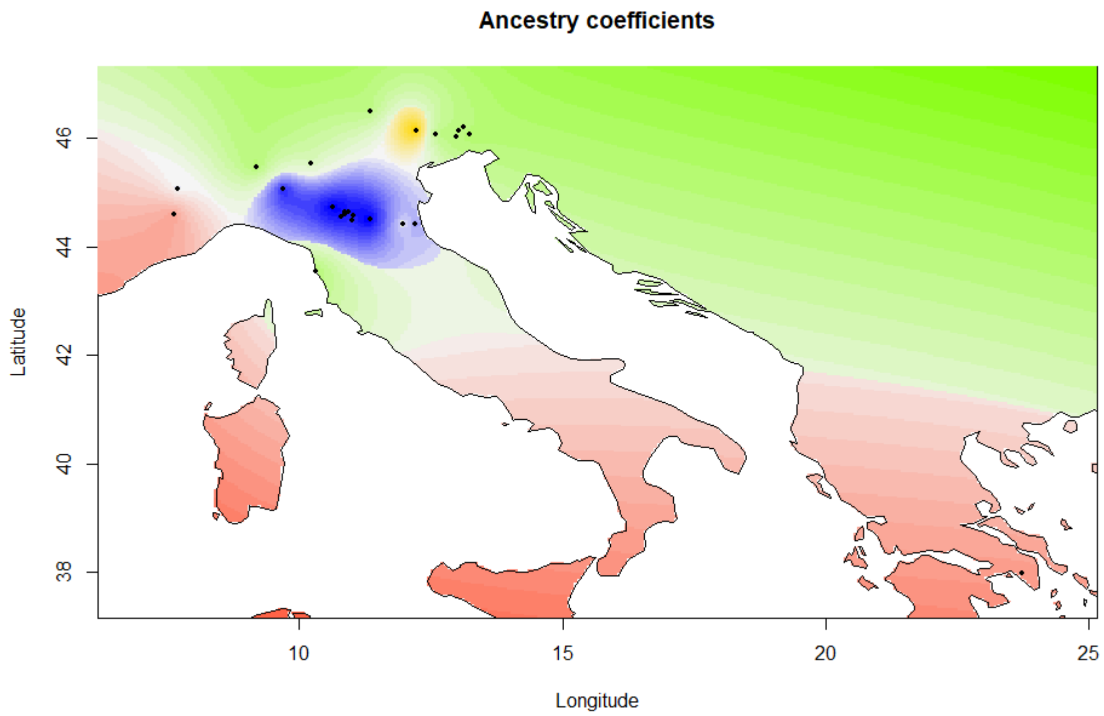

Figure 2.9. tess3r analysis. Map of ancestry coefficients distribution for K=4. Different colour shades indicate values from 0.5 (lighter) to 1 (darker). Black dots indicate sampling sites.

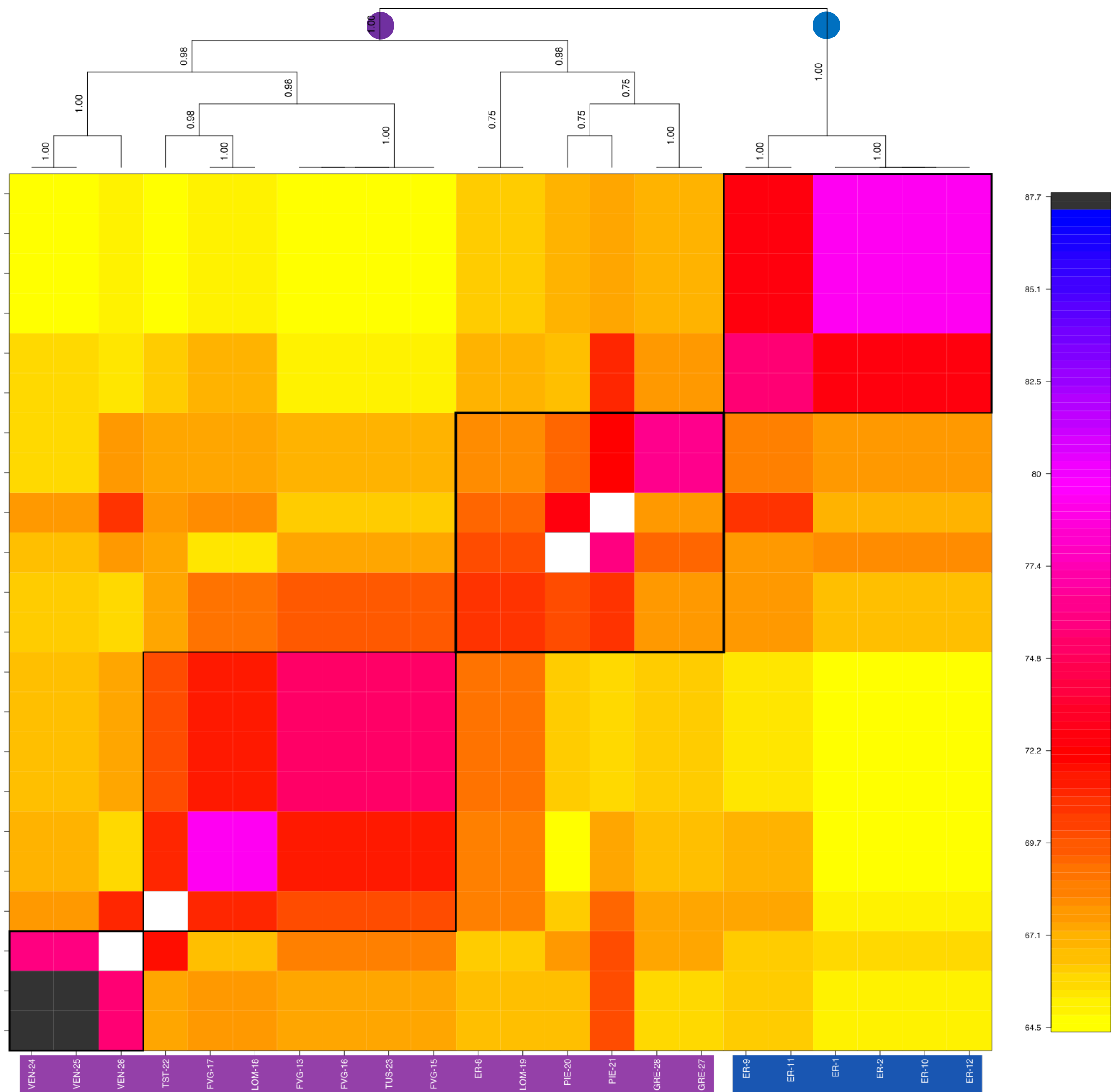

Figure 2.10. fineRADstructure analysis. Graph of coancestry matrix. On the right the bar with coancestry scores (max score on top, min score on bottom). On the top the cladogram, numbers indicate likelihood of each branch. On the bottom the analysed individuals separated by different colours to identify the two main clusters, *blue* = Cluster 1, *purple* = Cluster 2. The four clusters are evidenced by black squares in the matrix. Acronyms of individuals as in Table 2.1.

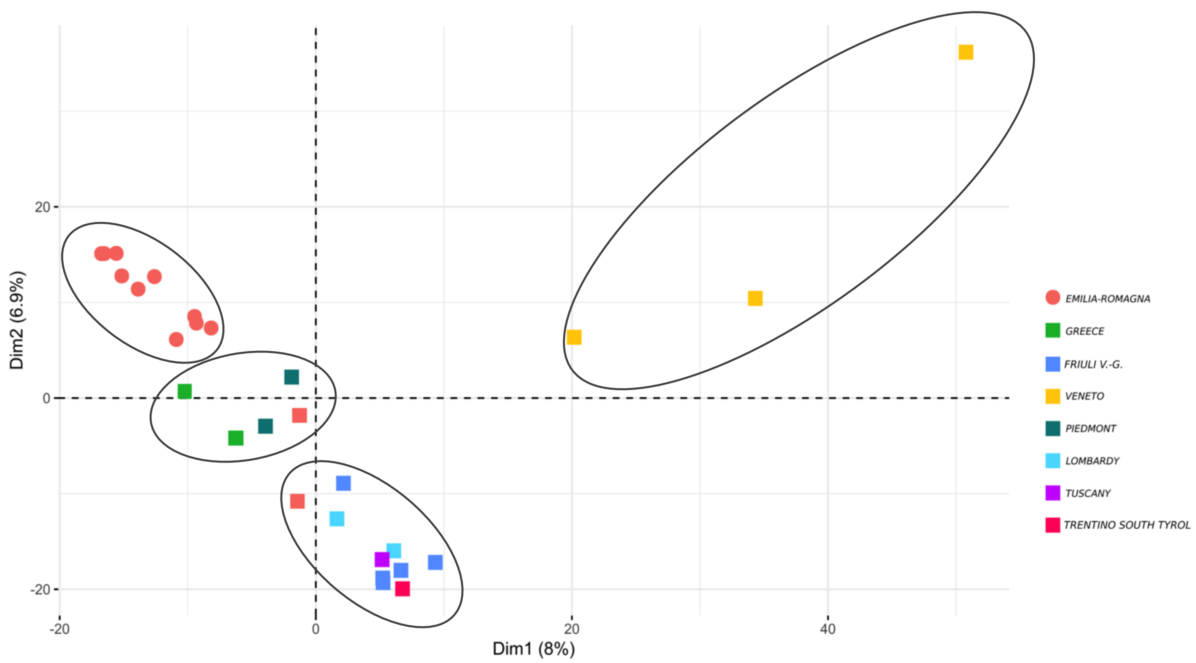

Figure 2.11. PCA analysis. Plot of the first two axes (PC1 and PC2), analysed individuals are color-coded based on the sampling region. Different shapes (circles and squares) separate the two main clusters according to Bayesian analyses; ellipses indicate the clusters retrieved in the PCA.

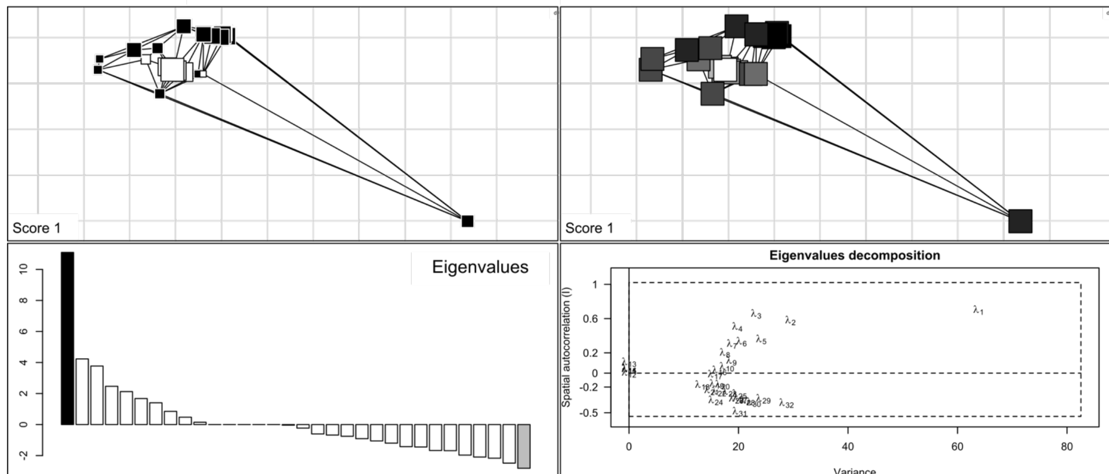

Figure 2.12. sPCA analysis. Summary analysis of sPCA results. Top left, connection network of absolute values spatially distributed, white squares indicate positive values, black squares indicate negative values, the dimension of squares indicate high (large squares) or low (small squares) degrees of absolute values. Top right, same representation of the previous connection network using variant grey levels instead of square dimension. Bottom left, plot of sPCA eigenvalues, positive values define the global structure, negative values define the local structure. Bottom right, plot of sPCA scores of eiegenvalues defined by variance (x axis) and Moran's Index (I) (y axis).

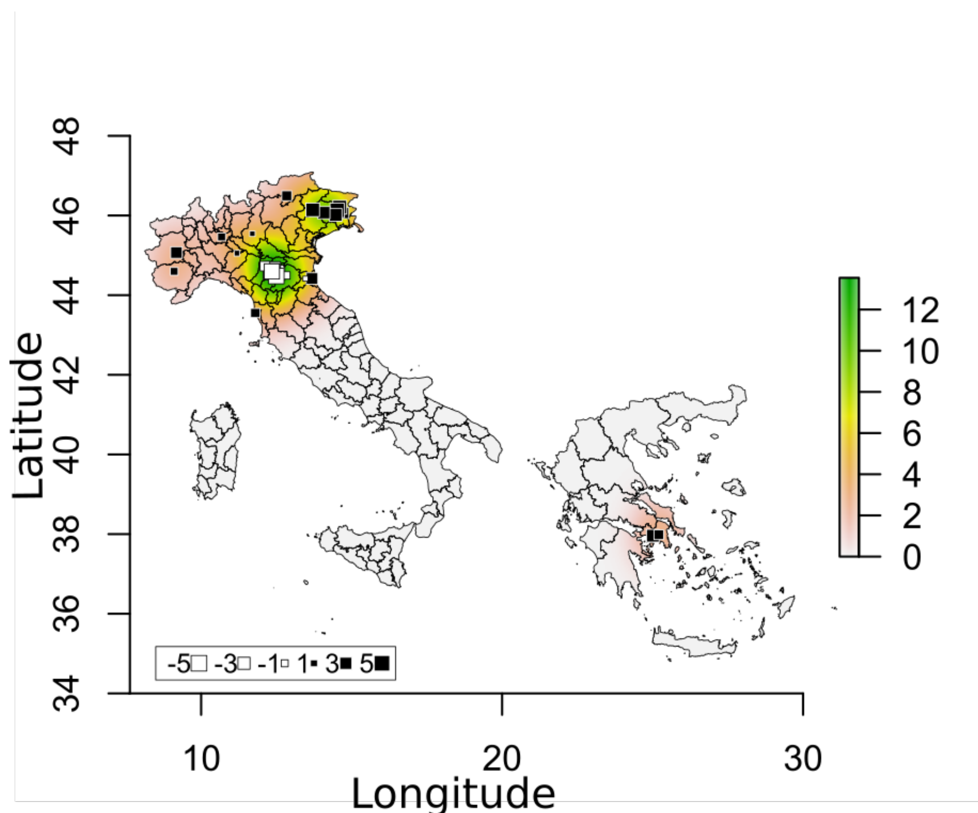

Figure 2.13. sPCA analysis. Geographic display of first PC values. The bar in the graph indicates positive values (white squares) and negative values (black squares). White and black squares indicate two separate clusters. The colour shades indicate the number of individuals, the bar on the right shows the number of individuals per colour.

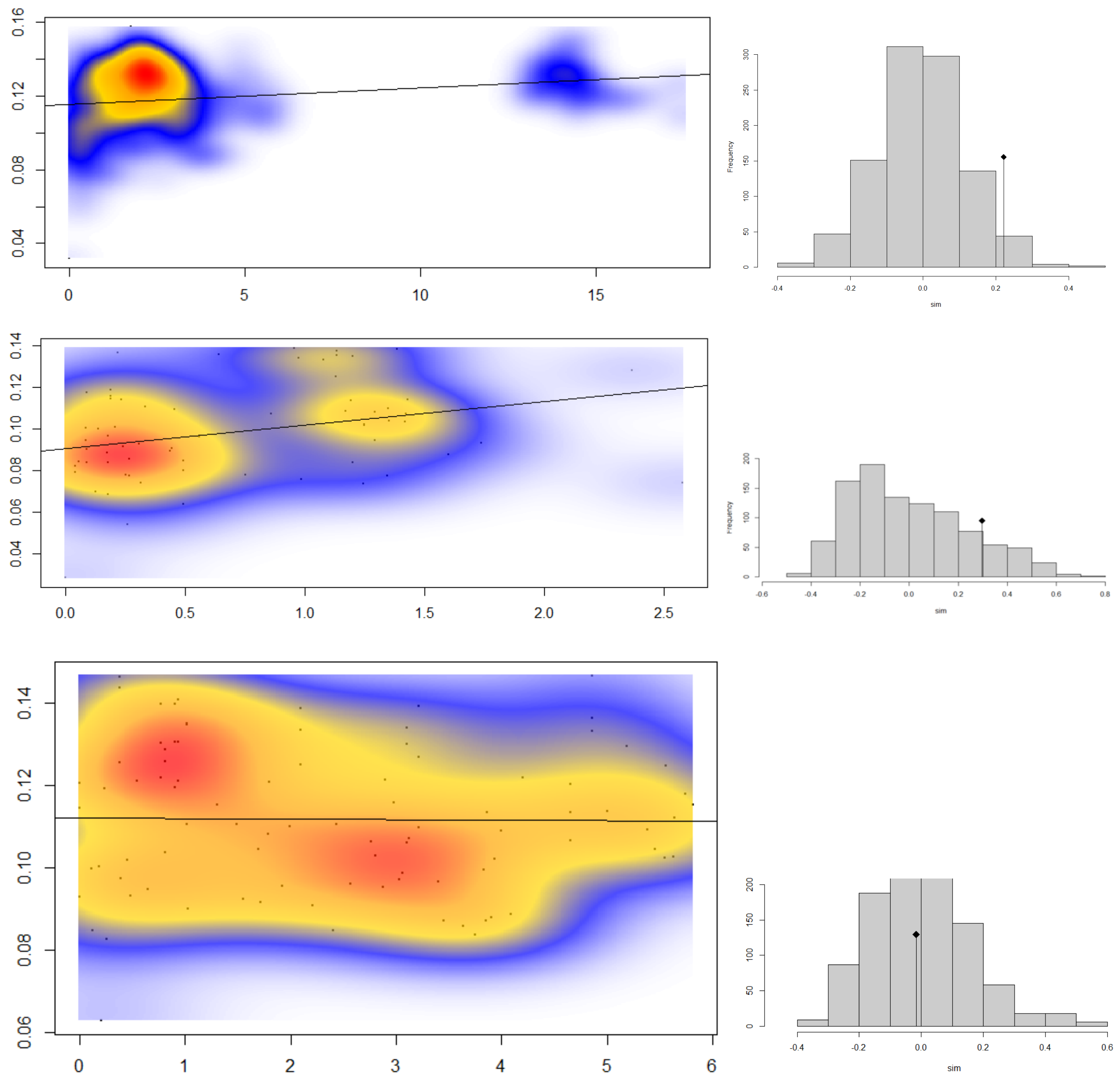

Figure 2.14. IBD analysis. On the left, scatterplots of Mantel correlation between matrix of geographic distances (x axis) and genetic distances (y axes), regression line in black. Colours indicate relative density of the points, warmer (orange-red) colours = high densities, colder (blue) colours = low densities. On the right, histograms of permutated values in absence of spatial structure, y axis = frequency, x axis = simulated Mantel test, the black dot is the original value of the correlation between the distance matrices, values inside the reference distribution are not significative.

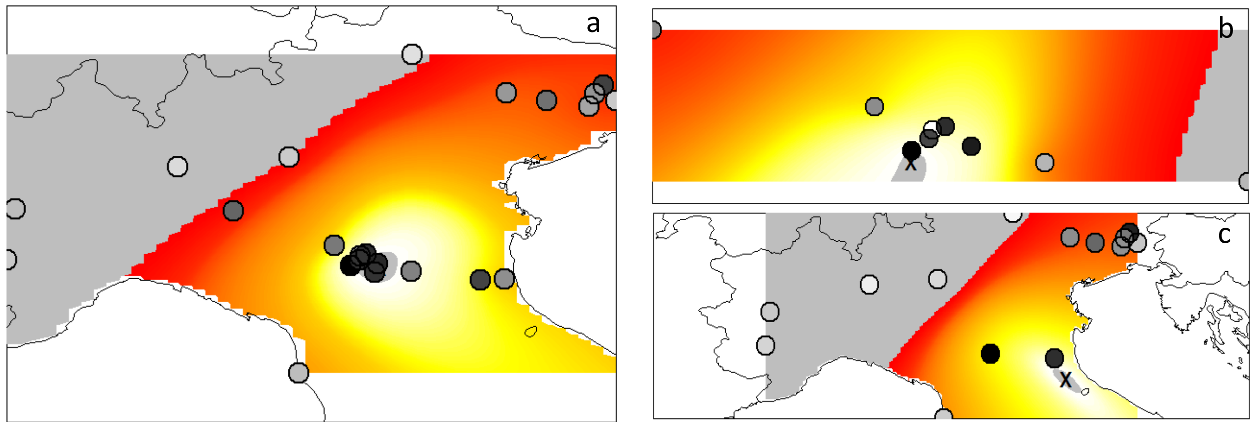

Figure 2.15. RangExpansion analysis. Map of range expansion for analysed datasets. a. Dataset complete, b. cluster 1, c. cluster 2. Brighter yellow indicates the likely origin of expansion; orange, red and grey indicate least likely origin.

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CHAPTER 3

Molecular gut content analysis on urban arthropods for the identification of *Halyomorpha halys* (Stål, 1855) (Hemiptera, Pentatomidae) predators

3.1 Abstract

The globalisation of trade and traffic, along with climate change, are favouring the spread of Invasive Alien Species (IAS) across the globe. The establishment of IAS in non-native areas is often responsible for severe impacts on biodiversity, human health, and economy.

In recent years, the brown marmorated stink bug *Halyomorpha halys* (Stål, 1855) has become a worldwide concern. Apart from its native range in East Asia, it is nowadays present in North and South America, and in Europe, from Spain to Caucasus. Urban environments are a suitable habitat for this highly polyphagous species due to the availability of a broad variety of plants and the presence of man-made structures where it can aggregate for overwintering. Thus, the massive occurrence of *H. halys* in such environment has demanded new rapid solutions for its management avoiding harmful effects on human health and non-target organisms. Like many newly introduced insects, assessing the relationship with local generalist arthropod predators is challenging mainly because of their small dimensions and concealed or nocturnal feeding habits. However, modern molecular techniques can help for the identification of native predators to limit the pest population without the direct observation of their interactions.

This study was aimed at identifying generalist arthropod predators of *H. halys* in urban environments using a species-specific Real-time PCR protocol. The Real-time PCR was used to detect *H. halys* DNA in the gut contents of different native arthropod predators collected in urban parks of the city of Reggio-Emilia (Emilia Romagna, Italy). The protocol called BMITS1 from Valentin et al. (2016) was used to amplify a 96 bp section of the *H. halys* ITS1 rDNA with a specific TaqMan minor groove binder (MGB) probe. The analysis scored 23 positive results making possible the identification of 10 predator species among insects and arachnids. Present data prove that the species-specific Real-time PCR assay can detects degraded target DNA, making it a valuable tool for the implementation of pest management strategies. Possible disadvantages of the protocol and troubleshoot were discussed along with the applicative aspects of the findings at the urban control of this invasive species.

Keywords: Real-time PCR, Molecular gut content, Invasive species, Arthropod predators, *Halyomorpha halys*.

3.2 Introduction

The introduction and establishment of Invasive Alien Species (IAS) in urban areas is often enhanced by the presence of a complex network of roads, railways, and airports that facilitate their rapid diffusion (Maistrello et al. 2018). Cities, by their own, represent a suitable habitat in terms of abundance of resources, presence of shelter, better climatic conditions and release from competition since the local communities suffer from a certain degree of habitat disturbance (Kuhman et al. 2010; Gaertner et al. 2017). In this kind of environment, IAS can proliferate and alter either the biotic or abiotic components causing severe impacts on ecosystem services (i.e. food/vegetable pests, pathogens, and vectors of pathogens/diseases), eventually replacing co-specific local species becoming predominant. Furthermore, urban centres can act as pest incubators for the spreading of invasive species to peri-urban, agricultural and natural areas (McLean et al. 2017).

Pest management in urban areas is challenging since it must take care of the secondary impacts on the environment and human and animal health (Myers et al. 1998; Cook et al. 2007; DiTomaso et al. 2017). Moreover, cities are a set of public and private lands, thus the agencies responsible for management programs need to take account of the possible resistance by the public to this type of intervention. As emerged from an extensive survey conducted in South California (USA), residents were more disposed to accept the use of biological control instead of chemical pesticides or biorational insecticides (Jetter and Paine 2004). Therefore, long-term and sustainable solutions based on biological control by local antagonists should be explored (Symondson et al. 2002).

Arthropod communities include a large number of generalist predators that may already be present in a habitat that is experiencing a pest invasion. Generally, opportunistic feeding species tend to rapidly shift from native to invasive species, when the abundance of the latter increase, without suffering any adverse effect when the population of alien species decrease (Bompard et al. 2013; Jaworski et al. 2013).

The accurate investigation of arthropod prey-predator interactions, based exclusively on field studies or laboratory analysis, is difficult to achieve and may lead to an underestimation of the complex network of involved variables. Arthropods frequently have small dimensions, concealed and/or nocturnal habits or the predation events occur in places difficult to access (i.e. canopy, dens). Besides, the morphological identification of the gut content is impossible in case of fluid feeder predators like spiders or hemipterans. To overcome the limitations of direct observation, molecular techniques can be applied to study the diet of generalist predators through the analysis of their gut content (Symondson 2002; Lawson Handley et al. 2011).

Molecular gut content analysis relies on the identification of the detected prey DNA at a taxonomic

specific level, the information obtained can be used to trace back the whole trophic community or a specific target species (i.e. IAS) by using different methods.
Next generation sequencing methods can uncover species composition or a single prey species from a variety of predators through metabarcoding analysis, recovering millions of copies of a target sequence from many genomes (Mollot et al. 2014; Harms-Tuohy et al. 2016; Sullins et al. 2018; Casey et al. 2019; Siegenthaler et al. 2019). Another common and accurate method is the detection and amplification of a gene or a portion of it from the undigested DNA of a target species in the gut of its predator by Polymerase Chain Reaction (PCR) (Symondson 2002; Greenstone et al. 2010; Clare 2014). The obtained amplicons have been used to study the preys range of a generalist predator (Blankenship and Yayanos 2005; Pons 2006; Eitzinger et al. 2013) or to detect effective generalist predators for the management of IAS (Chen et al. 2000; Hoogendoorn and Heimpel 2001; Furlong 2015).
A recent method, the Real-time PCR, streamlines the conventional PCR cutting out the sequencing and sequence analysis steps by harnessing the fluorescence emitted from specific dyes during the amplification process. The dyes are combined to species-specific primers or probes designed to start replication only in case of complete match with the target DNA, and the results are then immediately displayed by the Real-time PCR machine. The advantages of this method made it one of the most used for the analysis of arthropod trophic interactions (Lundgren et al. 2009; Troedsson et al. 2009; Valentin et al. 2016) and for the identification of generalist predators of pest species (Yu et al. 2004; Zhang et al. 2007; Gomez-Polo et al. 2015; Dhami et al. 2016; Unruh et al. 2016; Valentin et al. 2016).
The rapid identification of predators is crucial in case of IAS because once settled, the DNA amplification protocol can be employed rapidly by many laboratories and the obtained results are helpful to design effective pest management programs.
The brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål, 1855) (Hemiptera, Pentatomidae), is an IAS native to East Asia. Its diffusion ranges is now very wide: North America (U.S.A., Canada) (review in Leskey and Nielsen (2018)), South America (Chile; Faúndez and Rider 2017), Europe (Switzerland, Germany, France, Italy, Greece, Hungary, Austria, Serbia, Romania, Spain, Slovakia, Bulgaria, Slovenia, Croatia, Bosnia and Herzegovina, United Kingdom, Georgia, Abkhazia and South Russia) (Cianferoni et al. 2018; 2019) In recent years, *H. halys* has become a concerning agricultural and urban pest in all the infested countries, causing significant environmental and socioeconomic impacts.
The factors driving its invasion success are tightly related to its biological and behavioural traits, and the landscape features of invasive areas. *H. halys* is a phytophagous and highly polyphagous

species with a broad range of hosts (over 200 wild, ornamental, and agricultural plants) (Lee et al. 2013). In nature, it tends to overwinter in sheltered, dry cavities of trees while in anthropic areas it tends to overwinter in human-made structures (residential and commercial buildings) and it has high dispersal rates (autonomous flight and human-assisted) (Wiman et al. 2015). *H. halys* has been intercepted multiple times in private cars, trucks, freight shipments (Zhu et al. 2012; Cianferoni et al. 2018; Maistrello et al. 2018), and beside this, adults are strong flyers with a flight capacity up to 5 km within 24 h or more (record 117 km) while nymphs can walk up to 20 m within 24 h (Wiman et al. 2015).

Studies conducted in Italy (Cesari et al. 2018; Maistrello et al. 2018) and U.S.A. (Wallner et al. 2014; Valentin et al. 2017; Leskey and Nielsen 2018) proved that the first establishments were mostly in urban areas likely facilitated by the presence of transport infrastructure.

In Italy, the massive presence of aggregating individuals on urban vegetation (i.e. gardens, public parks), and inside private and public buildings of many cities, represents a threat due to its potential dispersal to the surrounding areas (Maistrello et al. 2016, 2017, 2018; Costi et al. 2017). The potential expansion of this pest to agricultural areas, where the risk of considerable economic losses is high, is of major concern. During 2015, an *H. halys* outbreak caused near 80% of production losses in pear orchard of Emilia Romagna (Italy; Maistrello et al. 2016), although damages were reported also in nectarine and apple orchards, and rice crops in North Western Italy (Pansa et al. 2013; Lupi et al. 2017; Bosco et al. 2018; Candian et al. 2018).

Investigating the response of native predators to the *H. halys* invasions in urban areas may provide crucial information about the health of the invasive populations and outline the impact that native species may have on containing its further expansion. So far, the identification of native predator and parasitoids species, that can affect the population dynamics of *H. halys* in Italy, has been investigated through observational surveys of sentinel egg mass in agroecosystems (Haye et al. 2015; Moraglio et al. 2018; Costi et al. 2019) and laboratory trials (Castracani et al. 2017).

The protocol called BMITS1 developed by Valentin et al. (2016) is based on a species-specific Real-time PCR assay for the detection of degraded *H. halys* DNA. This protocol utilises primers specific to *H. halys* and a TaqMan fluorescent probe to amplify a 96-bp segment of the Internal Transcribed Spacer 1 (ITS1) of ribosomal DNA.

Hence, the aims of this study were to evaluate the predatory potential of these arthropods to *H. halys* using the species-specific Real-time PCR, BMITS1, protocol to detect *H. halys* DNA in the gut content of different native arthropod predators collected in urban parks of the city of Reggio-Emilia (Italy).

3.3 Materials & Methods

3.3.1 Samples collection

The 50 specimens of generalist arthropod predators analysed in this study were provided by collaborators from the Laboratory of Applied Entomology of the "Centro Interdipartimentale per il Miglioramento e la Valorizzazione delle Risorse Biologiche Agro-alimentari" (BIOGEST-SITEIA) of the University of Modena and Reggio Emilia in Reggio Emilia (UNIMORE), Italy. The samplings were conducted in two urban parks, Rodano park (44°40'39.4"N, 10°39'51.5"E) and Mauriziano park (44°41'01.5"N, 10°40'27.9"E), in the city of Reggio Emilia, Italy (Tab. 3.1). The sampling areas were selected based on the presence of *H. halys* and different host plant species for it, and their proximity to buildings that can act as overwintering shelter and agricultural fields. The survey started in 2017 and ended in 2018, samplings were performed every 15 days, from May to September concomitantly to the presence of *H. halys* on shrubs/trees using tree beating and sweep net (in grassy areas).

For each area, all collected potential predators (Figs. 3.1, 3.2) were separated and individually placed in sterile vials, labelled with information reporting date, name of the species and presence/absence of BMSB in the sampling area. From collected material, 50 individuals among insects and arachnids (25 for each park) were selected for molecular analyses. The animals were immediately killed by freezing at -20°C and rinsed with 100% ethanol before sending them to the Laboratory of Evolutionary Zoology (Department of Life Science, UNIMORE, Modena, Italy).

A katydid of the species *Eupholidoptera chabrieri* (Charpentier, 1825) from collected insect specimens were kept alive and in starvation for two days at the laboratory BIOGEST-SITEIA (Department of Life Science, UNIMORE, Reggio Emilia, Italy), then subjected to a laboratory no-choice feeding trials with different life stages of BMSB and 30 min after a feed, killed by freezing at -20°C to obtain positive controls for the Real-time PCR assays. All the arthropods collected for this study were identified as nonendangered and nonprotected species.

3.3.2 Gut dissection, DNA extraction and Real-time PCR assays

To avoid the contamination of the samples with *H. halys* DNA possibly present in the lab, all dedicated equipment for gut dissection and DNA extraction was sterilized prior to each experiment. Moreover, each specimen was accurately put in a sterile 1.5 ml tube and washed by rotating the tube for 1 min with a solution containing 0.001 Triton X-100, then put in a new sterile 1.5 ml tube and washed for one min with ultra-distilled water to remove all impurities and reduce the risk of contamination.

The gut was isolated from all collected insects by placing each specimen on a sterile Petri dish and dissecting the head and the last segment of the abdomen (telson) utilising a sterile cutter and flame-sterilised tweezers to separate the gut from the rest of the body, then each gut was placed into a 1.5 ml tube and kept on ice for subsequent DNA extraction. Spiders and opilionids utilise extra-oral digestion for prey consumption and digestion of liquefied material. Their gut takes place in many different parts of the body (midgut diverticula extend throughout the prosoma and legs) making impossible the dissection of the whole gut. Therefore, they were utilised as a whole if they could easily fit into a 1.5 ml tube otherwise legs and prosoma were removed by dissection with a flame-sterilized cutter to fit the body within the 1.5 ml tube.

For this study, the selected positive controls were the genomic DNA (gDNA) extracted from the gut content of the katydid individual *E. chabrieri* fed under laboratory conditions and the gDNA extracted from the head of an *H. halys* specimen reared in lab. The selected negative controls were gDNA extracted from legs and heads of two of widely diffused pentatomid species in Italy, the species *Nezara viridula* L. and the palearctic species *Rhaphigaster nebulosa* Poda, 1761. All pentatomid specimens used as negative controls were collected in Modena (Italy) and stored in 100% ethanol at -20°C prior to molecular analysis.

The total gDNA was extracted right after animal sterilization and, for insects, after gut isolation with a Dneasy Blood & Tissue kit (Qiagen Sciences, Germantown, MD, USA) following the protocol "Total DNA from Animal Tissue" (Dneasy Blood & Tissue Handbook, July 2006). The gDNA was re-suspended with 200 µl of Qiagen buffer ATE and then the amount of detectable DNA was measured with a NanoDrop® ND-1000 spectrophotometer (Life Technologies). The same protocol was used for total DNA extraction of positives and negatives controls.

To detect traces of *H. halys* among the DNA extracted from potential predators, a high-sensitivity Real-time PCR assay (BMITS1 protocol) was used. The assay was specific to the BMSB conserved region of the rDNA internal transcribed spacer 1 (ITS1) as described in Valentin et al. (2016). Real-time PCR reactions were performed in replicates of two for each sample in 20 µl reactions using 500 nM of each primer BMITS1F 5'-CGAGGCCGCCGATGA-3'; BMITS1R 5'-CCCACGAGCCGAGTGATC-3'), 250 nM of the TaqMan™ fluorescent probe (BMITS1TM 5'-CAGGCAATGAAGCACA-3') with a dye label (VIC) on the 5' end and a minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3' end, 1× TaqMan™ Fast Advanced Master Mix with Uracil-N glycosylase (UNG) and 2 µl of undiluted gDNA. The optimized Real-time PCR reaction protocol was conducted at an initial step of 50°C for 2 min to activate the UNG, a denaturing step of 95°C for 20 s, followed by 40 cycles of denaturing for 10 s at 95°C, annealing

for 20 s at 67°C, extension for 30 s at 72°C and a final extension at 72°C for 7 min. All reactions were run on a BioRad CFX96 Real-time PCR system (Bio-Rad Laboratories, Inc.) and results were analysed using CFX Maestro™ Software version 1.1 (Bio-Rad). The Software compares the final relative fluorescence units (RFUs) for each well with positive and unknown content of target sequence to the RFU levels of wells with negative controls and identifies the positives when their RFU values are greater than the average RFU values of the negative controls plus the default cut off value.

Present study was conducted in collaboration with the Laboratory of Applied Entomology, of the BIOGEST-SITEIA, Reggio Emilia, Italy, as part of the survey on presence of potential native generalist predators for *H. halys* predators in Italy.

3.4 Results

Despite the DNA degradation due to digestion processes, the gut content analysis of all generalist predators tested with the BMITS1 amplification protocol resulted in 23 positive specimens for the presence of *H. halys* DNA (Tabs. 3.1, 3.2); 9 of them were from Mauriziano park while, 14 of them were from Rodano park. In all assays, the amplification curves (Fig. 3.3) of *H. halys* positive control always started before the positive control with digested *H. halys* DNA as expected, while negative controls always started later than positive controls therefore confirming the species-specificity of the primers and probe to the target sequence.

Furthermore, the presence of positives and negatives hits in specimens of the same species and from the same sampling date excluded the possibility of external contamination with *H. halys* DNA either during the sampling collection and the molecular preparation protocols in laboratory. The absence of contamination was also confirmed by the absence of any signal in the no template controls (NTCs) in all reactions.

Overall, positive results were found across almost all assayed arthropods leading to the identification of ten generalist predator species feeding on *H. halys*, five among insects and five among arachnids. More specifically, considering insect results, traces of BMSB were found in five out of 16 specimens of *Forficula auricularia* L.; in seven out of nine specimens of *Arachnocephalus vestitus* Costa, 1855; in the single specimen of *Yersinella raymondi* (Yersin, 1860) and of *Nagusta goedelii* (Kolenati, 1857) and in both specimens of *Harmonia axyridis* (Pallas, 1773). The results from arachnids identified BMSB traces in one out of three specimens of *Mitopus morio* (Fabricius, 1779) and in the two specimens of *Opilio canestrinii* (Thorell, 1876); on one out of 10 specimens of *Anyphaena* sp., in both specimens of *Philodromus* sp. and in the single

specimen of *Calositticus* sp.; whereas no traces were found in the three arachnid specimens belonging to *Pistius truncatus* (Pallas, 1772), *Dictyna* sp. and *Tetragnatha* sp.

3.5 Discussion

Since its first detection in Italy, *H. halys* has become a serious threat to local agroecosystems, causing severe economic losses in many parts of its introduced range. Tracking down the patterns of its introductions, a strict correlation with human activities and urban areas emerged. Indeed, many Italian cities are dealing with the massive presence of this invasive species. The benefits offered by the high vegetable diversity occurring in urban areas and the presence of repaired structures to overwinter, enhance the survival of introduced populations. The constant increase of *H. halys* populations and their dramatic mobility are raising serious concerns about the dispersal of new propagules to surrounding areas. Native generalist predators are the focus of the investigation because of the impact that they might have on population dynamics. Therefore, a rapid analysis for a sustainable long-term management strategy is needed. The use of molecular gut content analysis for the determination of native predators offers the ideal solution to obtain rapid results to apply for biological control.

In this study the gut content of generalist native arthropods collected in two urban parks of Reggio Emilia (Italy) was analysed using a species-specific Real-time PCR protocol.

The results showed that the gut content analysis through Real-time PCR is a fast, and relatively inexpensive method, to obtain reliable information about the trophic dynamics of an IAS like *H. halys*.

For this type of analysis, one of the main drawbacks of field collection is that the information about the elapsed time between the prey ingestion and the predator capture is missing. This has a crucial relevance, since the detectability of prey DNA starts to decrease after a long permanence in the digestive tract as a consequence of the degradation operated by digestive enzymes (Symondson 2002; Dhami et al. 2016). Nevertheless, this method was able to detect traces of the BMSB target DNA in 23 samples over 50, and to identify 10 predator species among insects and arachnids. Storing the samples in refrigerated conditions right after their collection may have reduced the degradation process by slowing the enzymatic activities and prevented the DNA alteration by physical agents (i.e. temperatures shifts, UV lights) (Juen and Traugott 2006; Oehm et al. 2011; Macías-Hernández et al. 2018). It is important to underline that this technique only detect the presence of the DNA of a target species, thus there are different ways to interpret negative results. For example, the analysed specimens from which the gDNA has been extracted belonged to a

species that i) is not a predator of the target one; ii) is a predator of the target one but the analysed specimens did not eat the target species; iii) is a predator of a specific life stage of the target species (i.e. eggs, nymphs or adults) that develops earlier/later than the sampling date; iv) is a predator of the target species but was in a fasting state; v) is a predator of the target species but the DNA of the target species was to degraded for its detection. Based on this, the interpretation of results should be supported by the comparison of the results within the same analysis or with any other information from previous studies and observations. These precautions should be extended also to the interpretation of positive results considering that the analysed specimens could belongs to a species that i) can be considered an actual predator only if more than one specimen is tested as positive in order to exclude false positive; ii) is a predator of a specific life stage of the target (i.e. eggs, nymphs or adults); iii) is an occasional predator that generally feeds on different species.

In this study, the discrimination of the predator species was careful assigned based on the overall assessment of the results and on the information about *H. halys* predators from other studies.

Looking at the positive results, all the five analysed insect species can be easily identified as predators since each tested positive to target DNA in all (*Yersinella raymondi*, *Harmonia axyridis*, *Nagusta goedelii*) or most (*Forficula auricularia*, *Arachnocephalus vestitus*) of the specimens. These results are in agreement with previous findings by Morrison et al. (2016) and Pote and Nielsen (2017). In the first study, they were able to determine the predators by analysing the feeding damages on sentinel egg masses of *H. halys* in USA mid-Atlantic orchards and vegetable crops, and through laboratory trials with over 25 taxa of arthropod predators collected in the field. As a result, they found that the most efficient insect predators on egg masses were katydids (Orthoptera: Tettigoniidae) like *Y. raymondi*, ground beetles (Coleoptera: Carabidae), crickets (Orthoptera: Gryllidae) very similar to *A. vestitus* and earwigs (Dermaptera: Forficulidae), the same *F. auricularia* retrieved in present study. The second study evaluated the predatory potential of 10 taxa of generalist predators collected in New Jersey (USA) agroecosystems by exposing them to egg masses, 1st and 2nd instars of *H. halys*. According to their results, the main predators on eggs were grasshoppers (Orthoptera: Acrididae), katydids (Orthoptera: Tettigoniidae), the ladybug *Coccinella septempunctata* (L.) (Orthoptera: Coccinellidae) with the same biology of *H. axyridis,* and the spined soldier bug *Podisus maculiventris* (Say, 1832) (Hemiptera: Pentatomidae). While the predators of nymphs were mostly hemipterans including *P. maculiventris*, damsel bugs *Nabis* spp. (Hemiptera: Nabidae) and assassin bugs (Hemiptera: Reduviidae) like *N. goedelii*. Contrary to above-mentioned studies, it is impossible to discriminate through the molecular gut content analysis on which *H. halys* life stage the detected predators fed. During the season when the specimens were collected, all *H. halys* life stages were present in the areas, according to Dott. Bulgarini (pers.

comm.) and to the biology of this species in Italy as reported in Costi et al. (2017). Further investigations are required to verify on which life stages (i.e. 3rd, 4th, 5th instars, adult) these species are able to prey.

With regards to arachnids, positive results were found in at least one specimen (*M. morio*) or all (*O. canestrinii*) of the two analysed opilionid species, whereas in spiders, positive results were found in *Anyphaena* sp., *Philodromus* sp. and *Calositticus* sp.; while each specimen of the three species *Dictyna* sp., *Tetragnatha* sp. and *P. truncatus* were negative to the presence of target DNA. Opilionids are generalist predators that fed on a large variety of insect preys included nymphs of hemipteran (Phillipson 1960; Acosta and Machado 2007), therefore it is possible to consider them predators of *H. halys.* Spiders instead are more difficult to identify. In previous studies, Morrison et al. (2016, 2017) found evidence of predation on *H. halys* eggs by jumping spiders (Araneae: Salticidae) of the same family of *Calositticus* sp. (the positive in this study), but they also found that several families of web-building spiders such as Theridiidae, Pholcidae and Agelenidae were able to predate on adults. Thus, the unequivocal presence of *H. halys* DNA in *Calositticus* sp., *Anyphaena* sp. and *Philodromus* sp. combined with the small dimensions of these spiders suggests that they might be occasional predators of the first instars of this species. On the contrary, the information about the biology of the spiders that did not yielded positive results and the absence of previous investigations are not sufficient to conclude whether they are or not possible predators for *H. halys*.

To summarise, all the identified predators belong to widespread and abundant species that are quite common in disturbed habitat like urban environments, are characterised by a very generalist feeding habit and most of them are already predators of other heteropteran species. These features clearly fit the requirement needed for a species to be used as biological control agent against an invasive species (Carlsson et al. 2009; Jaworski et al. 2013; Furlong 2015).

The molecular gut content analysis performed through species-specific Real-time PCR protocols is a prompt and reliable tool to detect the DNA of IAS among a selected group of potential predators. Without solid information about the biology and ecology of both invasive species and native predator, the results should be interpreted carefully. However, this type of analysis is advised to dramatically reduce the time needed to design a fast and effective pest management strategy.

Based on the results, it is possible to explore the possibility to act the containment of *H. halys* populations in Italian urban centres and limit its further expansion avoiding the impacts that this species is causing. The discovery of possible native control agents in small sample areas like urban parks is a signal that the urban biodiversity is an important resource and therefore must be protected.

3.6 Tables & Figures

Table 3.1. List of the 50 generalist arthropod predators analysed with data on sampling sites, systematic assignment, identified species and date of sampling of each, + positive or – negative Real-time PCR results for *H. halys* DNA detection.

Locality	Taxa	Species	Sampling date	DNA detection
Mauriziano Park	Hemiptera	*Nagusta goedelii*	16/08/2018	+
	Dermaptera	*Forficula auricularia*	13/09/2018	-
	Dermaptera	*Forficula auricularia*	10/05/2018	+
	Dermaptera	*Forficula auricularia*	07/06/2018	+
	Dermaptera	*Forficula auricularia*	13/09/2017	+
	Dermaptera	*Forficula auricularia*	19/07/2018	+
	Dermaptera	*Forficula auricularia*	13/09/2018	+
	Dermaptera	*Forficula auricularia*	24/05/2018	-
	Dermaptera	*Forficula auricularia*	13/09/2018	-
	Dermaptera	*Forficula auricularia*	13/09/2018	-
	Dermaptera	*Forficula auricularia*	13/09/2018	-
	Dermaptera	*Forficula auricularia*	07/06/2018	-
	Dermaptera	*Forficula auricularia*	07/06/2018	-
	Dermaptera	*Forficula auricularia*	07/06/2018	-
	Dermaptera	*Forficula auricularia*	07/06/2018	-
	Orthoptera	*Arachnocephalus vestitus*	13/09/2018	-
	Orthoptera	*Arachnocephalus vestitus*	27/09/2018	-
	Opiliones	*Opilio canestrinii*	19/07/2018	+
	Opiliones	*Opilio canestrinii*	13/09/2018	+
	Araneae	*Anyphaena* sp.	30/08/2018	-
	Araneae	*Anyphaena* sp.	27/09/2018	-
	Araneae	*Anyphaena* sp.	16/08/2018	-
	Araneae	*Philodromus* sp.	24/05/2018	+
	Araneae	*Dictyna* sp.	24/05/2018	-
	Araneae	*Tetragnatha* sp.	13/09/2018	-
Rodano Park	Coleoptera	*Harmonia axyridis*	13/09/2018	+
	Coleoptera	*Harmonia axyridis*	27/09/2018	+
	Dermaptera	*Forficula auricularia*	27/09/2018	-
	Dermaptera	*Forficula auricularia*	13/09/2018	-
	Orthoptera	*Arachnocephalus vestitus*	30/08/2018	+
	Orthoptera	*Arachnocephalus vestitus*	13/09/2018	+
	Orthoptera	*Arachnocephalus vestitus*	27/09/2018	+
	Orthoptera	*Arachnocephalus vestitus*	13/09/2018	+

Orthoptera	*Arachnocephalus vestitus*	13/09/2018	+
Orthoptera	*Arachnocephalus vestitus*	13/09/2018	+
Orthoptera	*Arachnocephalus vestitus*	13/09/2018	+
Orthoptera	*Yersinella raymondi*	13/09/2018	+
Araneae	*Anyphaena* sp.	02/08/2018	-
Araneae	*Anyphaena* sp.	30/08/2018	+
Araneae	*Anyphaena* sp.	30/08/2018	-
Araneae	*Anyphaena* sp.	30/08/2018	-
Araneae	*Anyphaena* sp.	13/09/2018	-
Araneae	*Anyphaena* sp.	13/09/2018	-
Araneae	*Anyphaena* sp.	27/09/2018	-
Araneae	*Philodromus* sp.	07/06/2018	+
Araneae	*Calositticus* sp.	27/09/2018	+
Araneae	*Pistius truncatus*	13/09/2018	-
Opiliones	*Mitopus morio*	13/09/2018	-
Opiliones	*Mitopus morio*	13/09/2018	-
Opiliones	*Mitopus morio*	13/09/2018	+

Table 3.2. List of analysed species, number of analysed individuals for each species and number of positives to *H. halys* DNA.

Order	Family	Common name	Species	Number of analysed specimens	Number positives to *H. halys*
Dermaptera	Forficulidae	Earwig	*Forficula auricularia*	16	5
Orthoptera	Mogoplistidae	Hairy scale-cricket	*Arachnocephalus vestitus*	9	7
Orthoptera	Tettigonidae	Katydid	*Yersinella raymondi*	1	1
Coleoptera	Coccinellidae	Asian ladybeetle	*Harmonia axyridis*	2	2
Hemiptera	Reduvidae	Assassin bug	*Nagusta goedelii*	1	1
Opiliones	Phalangiidae	Harvestman	*Mitopus morio*	3	1
Opiliones	Phalangiidae	Harvestman	*Opilio canestrinii*	2	2
Araneae	Anyphaenidae	Anyphaenid sac spiders	*Anyphaena* sp.	10	1
Araneae	Phylodromidae	Crab spider	*Philodromus* sp.	2	2
Araneae	Dyctinidae	Mesh web weavers	*Dictyna* sp.	1	0
Araneae	Tetragnathidae	Stretch spider	*Tetragnatha* sp.	1	0
Araneae	Salticidae	Jumping spider	*Calositticus* sp.	1	1
Araneae	Thomisidae	Crab spider	*Pistius truncatus*	1	0

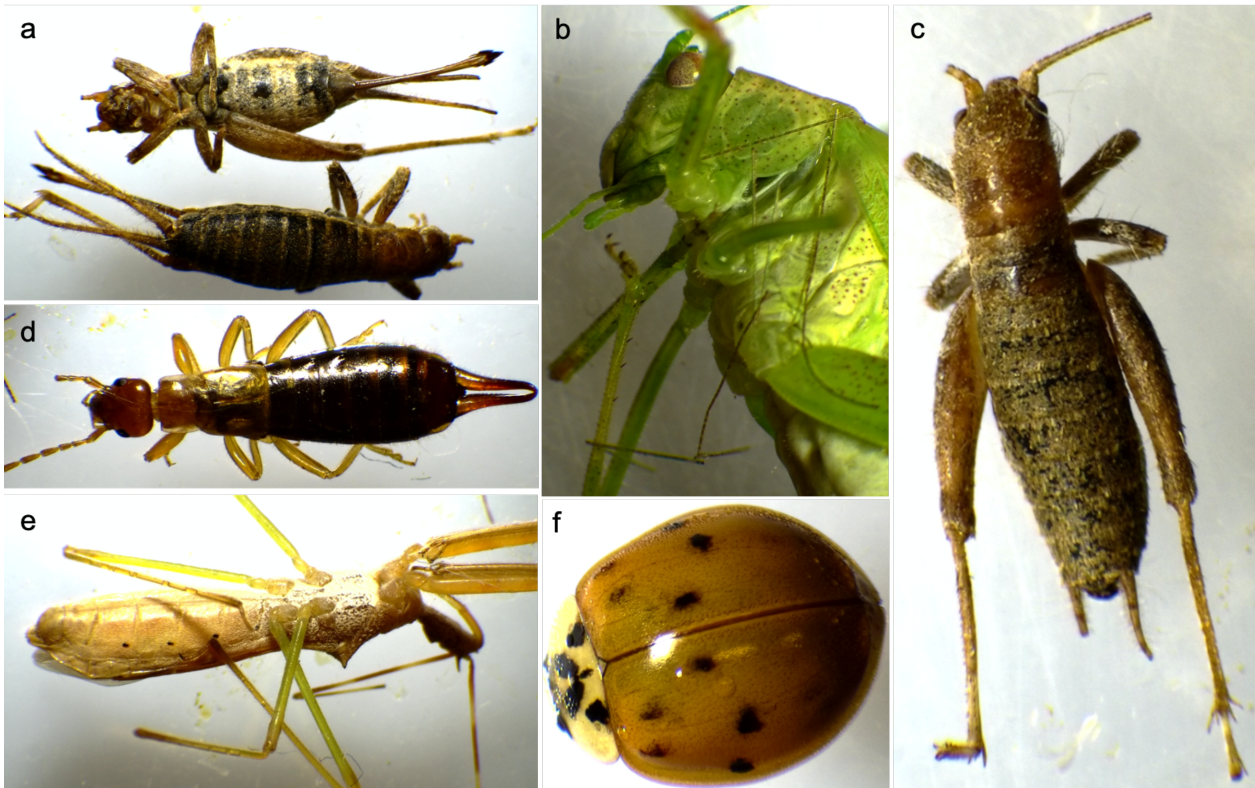

Figure 3.1. Some of the collected insect predator species: a, c. *Arachnocephalus vestitus* Costa, 1855; b. *Phaneroptera falcata* Poda, 1761; d. *Forficula auricularia* L.; e. *Nagusta goedelii* Kolenati, 1857; f. *Harmonia axyridis* Pallas, 1773.

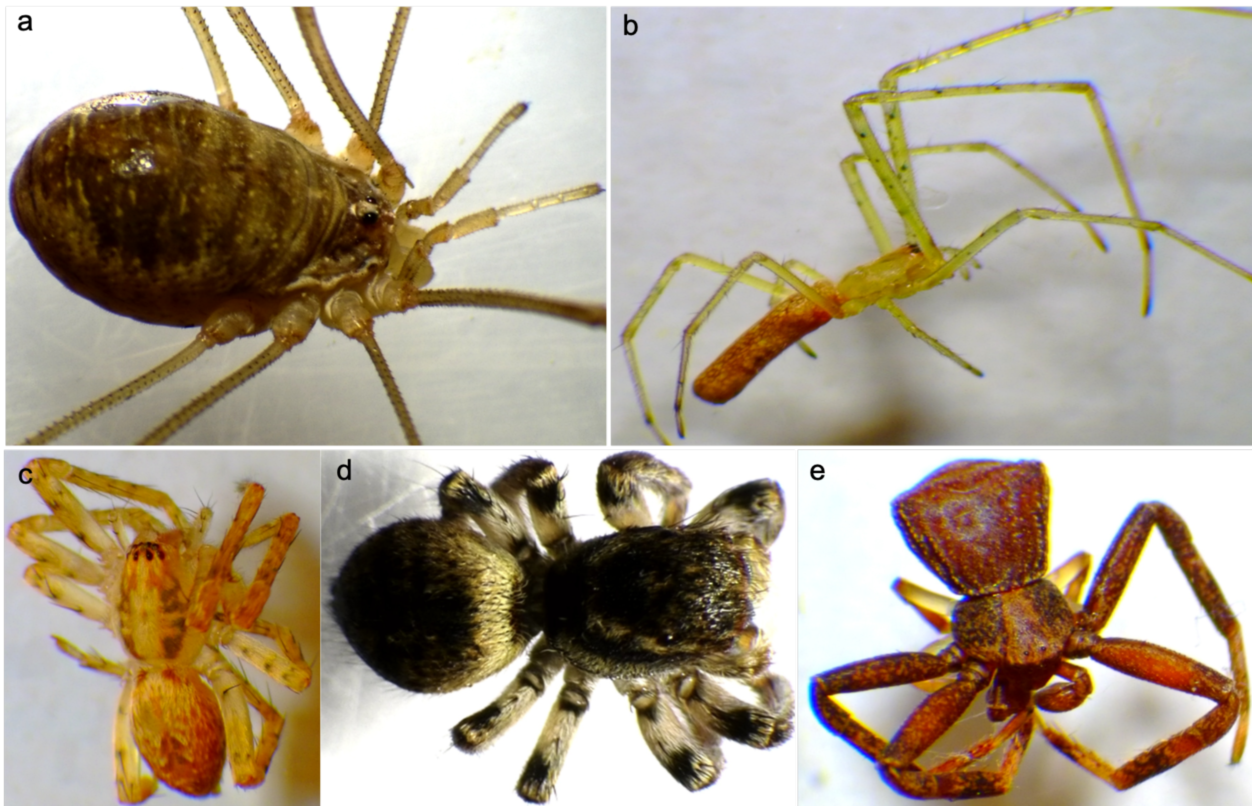

Figure 3.2. Some of the collected arachnid predator species: a. *Opilio canestrinii* Thorell, 1876; b. *Tetragnatha* sp.; c. *Anyphaena* sp.; d. *Calositticus* sp.; e. *Pistius truncatus* Pallas, 1772.

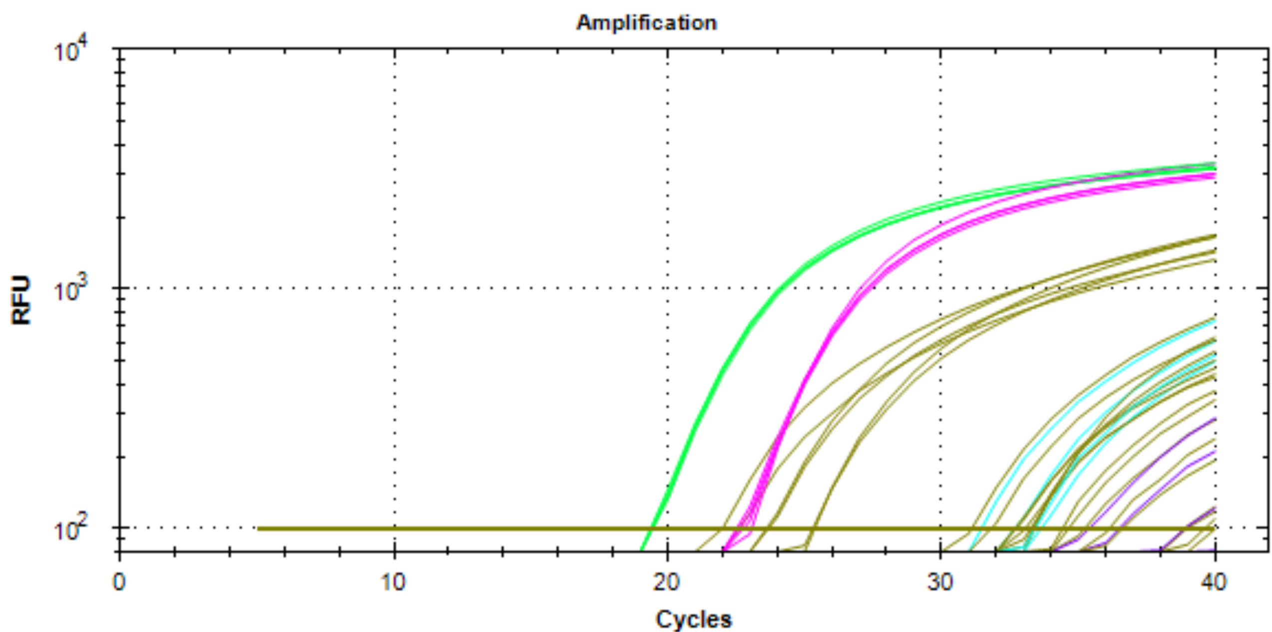

Figure 3.3. Amplification curve of one Real-time PCR assay. Green, *H. halys* gDNA positive control; pink, *E. chabrieri* gDNA positive control; olive green, gut content gDNA of arthropods; light blue, *R. nebulosa* gDNA negative control; purple, *N. viridula* gDNA negative control. The NTCs signal is absent. RFU = Relative fluorescent units; Cycles = Number of Real-time PCR cycles.

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CHAPTER 4

Real-time PCR to detect the DNA of *Halyomorpha halys* (Stål, 1855) (Hemiptera, Pentatomidae) from the guano of generalist Italian chiropterans

4.1 Abstract

Halyomorpha halys (Stål, 1855) is an invasive alien species native to eastern Asia. Its establishment outside the native area ranges from North America (Canada and U.S.A.), South America (Chile) and Europe, where it is spreading rapidly across all countries reaching Turkey and the Caucasus (Georgia, Abkhazia and South Russia). Due to its phytophagous and highly polyphagous habit, it is causing economic impacts in the United States and Italian agroecosystems, and it became a serious threat to local biodiversity. Moreover, *H. halys* is a household pest all over its invasive range due to its aggregatory behaviour during overwintering. As an extremely mobile species, *H. halys* progeny are hard to detect allowing them to proliferate in new environments, enhanced by the absence of closely evolved natural enemies (i.e. pathogens, parasitoids, predators) to limit populations growth and spread.

DNA-based methods can overcome the need for *a priori* information about the presence of a species and its trophic relationships by detecting its genetic signature in a large variety of biological and environmental samples. This study tested the predatory potential of 13 Italian chiropteran species by detecting *H. halys* DNA in guano samples using a species-specific Real-time PCR assay. Analysed samples were collected in roosts and bat-boxes from historical dwellings, and natural and agricultural areas of three Italian regions: Piedmont, Aosta Valley and Tuscany. The protocol developed by Valentin et al. (2016) and based on a TaqMan minor groove binder (MGB) species-specific probe was used for identifying and amplifying a 96 bp fragment of *H. halys* ITS1 rDNA. Four positive hits from agricultural sites in Piedmont and two positive hits in Tuscany parks were found, leading to the determination of at least seven chiropteran species feeding on *H. halys*. In Tuscany, it has also been possible to early detect this pest species in an area where its presence was never reported before. Present results prove that species-specific Real-time PCR analysis can detect degraded DNA, making it a valuable tool to address different biological questions like the early detection of pests and their potential native predators. Possible disadvantages of the protocol and troubleshoot were discussed along with the applicative aspects of the results for the *H. halys* management.

Keywords: Real-time PCR, Guano, Chiroptera, Native predators, Early detection, *Halyomorpha halys*.

4.2 Introduction

The brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål, 1855) (Hemiptera, Pentatomidae), is an invasive alien species native to Far East of Russia, Japan, China, Taiwan, North and South Korea. The first report of its presence outside the original area of distribution has been recorded in Pennsylvania, United States of America, in the mid-1990s. Nowadays, *H. halys* is present in 43 North American states and seven Canada provinces (Cianferoni et al. 2018; Leskey and Nielsen 2018) and, from 2017 is present in Chile, South America (Faúndez and Rider 2017).

In the European continent *H. halys* was detected for the first time in Switzerland in 2004 (Haye et al. 2015) and, from 2011 to the present, resulted established in Germany (Heckmann 2012), France and Corsica (Callot and Brua 2013, Kriticos et al. 2017), Italy (Maistrello et al. 2012), Greece (Milonas and Partsinevelos 2014), Hungary (Vétek et al. 2014), Russia (Gapon 2016), Austria (Rabitsch and Friebe 2015), Serbia (Šeat 2015), Romania (Macavei et al. 2015), and Georgia (Gapon 2016), Spain (Dioli et al. 2016), Slovakia (Hemala and Kment 2017), Bulgaria (Simov 2016), Abkhazia (Gapon 2016), Slovenia (EPPO 2018), Turkey (Çerçi and Koçak 2017), Croatia (Šapina and Jelaska 2018), Bosnia and Herzegovina (Zovko et al. 2019), United Kingdom (Defra 2019).

Focusing on the Italian distribution, *H. halys* is widespread all over northern Italian regions, mountains included, with an increasing expansion in central Italy (Tuscany, Marche, Latium and Abruzzo), albeit occasional records were reported for southern Italy and the main islands (Dioli et al. 2016; Cianferoni et al. 2018; Maistrello et al. 2018).

The life-history of *H. halys* and its strict association with human-modified ecosystems make it a successful invasive species, able to tolerate a broad range of environmental stresses and habitat variables. The BMSB is a phytophagous and highly polyphagous pest with a host range that encompasses over 200 species of plant, many of which have a great agricultural and commercial importance (Lee et al. 2013; Haye et al. 2014; Rice et al. 2014; Wallner et al. 2014; Costi et al. 2017; Maistrello et al. 2017; Nielsen et al. 2017; Leskey and Nielsen 2018; Mauvisseau et al. 2019). Italy and USA are the two countries were the most severe damages to agroecosystems were recorded.

In both cases, the affected areas experienced a dramatic increase of *H. halys* populations before agricultural and economic impacts started. In the United States, the economic loses started since 2006 to tree fruit, berry crops, grapes, vegetables, field crops and ornamentals, with damage estimated at $37 million in 2010 alone (Leskey et al. 2012). In Italy, damages were reported since 2014 in Emilia-Romagna and economic loses started the next year affecting Northern Italian apple,

nectarine, pear, kaki, rice, hazelnut and corn industries (Bariselli et al. 2016; Lupi et al. 2017; Maistrello et al. 2017; Bosco et al. 2018; Candian et al. 2018). The severity of the attacks is enhanced by the species behaviour, BMSB easily flies from the edge of the orchard to the surrounding vegetation concentrating in certain areas due to the aggregation pheromone and, in case of adverse condition, tend to shelter in man-made structures. As a result, the usage of insecticides has disrupted organic farm and Integrated Pest Management programs, also leading to a drastic decline of the local biodiversity and the potential predators (Leskey and Nielsen 2018).

The increase of *H. halys* diffusion and the concrete impact that cause have risen the necessity to enact more effective strategies for managing and containing the established populations, thus adopting solutions to prevent further expansions without affecting local biodiversity. One of these strategies includes the detection of native predators to be exploited as pest control agents.

Bats are placental mammals of the order Chiroptera, representing the only active flyer among the mammal group, with over 1.300 species worldwide (Simmons and Cirranello 2019), distributed all over biogeographic realms, except for Antarctica. Habitat fragmentation coupled with landscape modification and anthropogenic disturbance is changing the nesting behaviour of many bat species, leading many of them to prefer urban (i.e. buildings, cellars, barns, bridges) or anthropogenic habitats (i.e. mines and quarries) as roosting sites (Kurta and Teramino 1992; Avila-Flores and Fenton 2005; Jung and Kalko 2011; Hale et al. 2012; Russo and Ancillotto 2015; Threlfall et al. 2016). Although the loss of habitat is one of the main cause of bat populations decline, it is worth to remark the endangered status of over 70% of Italian species (Rondinini et al. 2013).

Over 70% of bat species are insectivorous, as all European and Italian species. To sustain the energy required for flight, bats need to assume per day a quantity of insects equivalent to 30%-50% of their body weight (Elliott 2000). Around 35 bat species are listed in Italy and most of them are opportunistic feeders, meaning they feed on the most abundant preys (Beck 1995; Lanza 2012) and, according to several studies, on pest species (Kalka et al. 2008; Williams-Guillén et al. 2008; Böhm et al. 2011; Boyles et al. 2011; Ricucci and Lanza 2014).

In the field of biological invasion, efficient and accurate identification of a prey-predator relationship is generally challenging, as it relies on a large number of variables, among others the knowledge of the pest presence in a certain habitat and the biological habits of both agents (Elton 1958; Sakai et al. 2001; Symondson et al. 2002; Colautti et al. 2004; Sax et al. 2005; Pyšek and Richardson 2010; Keller et al. 2011; David et al. 2013). The prompt detection of these aspects is crucial for enacting effective pest management programs.

Traditional strategies based on observational surveys require managing efforts that are not feasible in terms of time and cost. However, advances in molecular methods have made possible to detect

highly degraded DNA from environmental samples (i.e. soil, water, air, faeces, biological remains). These samples, better known as environmental DNA (eDNA), are advantageous since they can detect a rare species (i.e. early introductions) without any *a priori* information about its presence or its ecological relationships (Symondson 2002; Clare et al. 2009; Dejean et al. 2012; Taberlet et al. 2012). Many studies have driven their attention to disentangling the prey identity in faecal samples exploiting molecular methods from a large variety of animals (Symondson 2002; Pompanon et al. 2012; Clare 2014). Generally, these type of studies have two different achievements, detect all the prey present in a sample by the use of next-generation sequencing reconstructing the whole trophic interaction (Pompanon et al. 2012; Vesterinen et al. 2013; Guillerault et al. 2017; Esnaola et al. 2018) or trace back the genetic signature of a specific target species either through polymerase chain reaction (PCR) and sequencing (Clare et al. 2014; Peters et al. 2015; Dell'Agnello et al. 2019) or Real-time PCR (O'Meara et al. 2014; Dhami et al. 2016; Valentin et al. 2016; Maslo et al. 2017; Tsuji et al. 2018).

In relation to *H. halys* control, Valentin et al. (2016) designed a novel species-specific Real-time PCR assay for the detection of *H. halys* DNA in the guano of insectivorous bats. They developed primers specific to this species able to amplify a 96-bp section of the ITS1 region of ribosomal DNA. The method, called BMITS1, includes a TaqMan fluorescent probe to increase the assay specificity, since a complete match between target region and probe are required for the reaction to success.

The previous study from Maslo et al. (2017) investigated the predatory potential of the big brown bat (*Eptesicus fuscus* Beauvois, 1796) and its use as an agent of pest surveillance in US agroecosystems by analysing guano content through Real-time PCR. Their results showed consistent seasonal predation of BMSB and the ability to detect the presence of *H. halys* in the guano, 3-4 weeks earlier than traditional pest monitoring tools.

Therefore, the objectives of this study were to i) evaluate the predatory potential these chiropterans as pest control agents, ii) evaluate the early detection potential of the assay for presence of new propagule of *H. halys* detecting traces of BMSB DNA using the species-specific BMITS1 Real-time PCR assay on guano samples from 15 Italian chiropteran species.

4.3 Materials & Methods

4.3.1 Samples collection

The study was conducted in collaboration with colleagues from the Laboratory of Applied Entomology of the "Centro Interdipartimentale per il Miglioramento e la Valorizzazione delle

Risorse Biologiche Agro-alimentari" (BIOGEST-SITEIA) of the University of Modena and Reggio Emilia (UNIMORE), Reggio Emilia, Italy.

A total of 27 chiropteran guano samples were collected in 16 different Italian sites (Tab. 4.1). Fifteen chiropteran guano samples were collected in the Italian provinces of Asti, Cuneo, Turin, Verbania and Vercelli (Piedmont region) and one sample in Aosta (Aosta Valley region) by collaborators from the Theriological Station of Piedmont (Carmagnola, TO). In these regions, the sampling sites were all historical buildings and churches hosting well-known reproductive colonies of the species *Rhinolophus ferrumequinum* (Schreber, 1774) and five conservation-relevant species of the genus *Myotis*: *Myotis blythii* (Tomes, 1857), *Myotis capaccinii* (Bonaparte, 1837), *Myotis daubentonii* (Kuhl, 1817), *Myotis emarginatus* (Geoffroy, 1806); *Myotis myotis* (Borkhausen, 1797). Four additional samples from Cuneo province in Piedmont were collected by Dr. Mara Calvini of the Italian Farming Union *Coldiretti,* from bat-boxes placed near conventional and organic fruit orchards of apples, peaches and pears. Since bat-boxes can be occupied by different genera and species at the same time, the identification of the occupying individuals was conducted by visual determination of the species and/or by the identification of the species-specific echolocation ultrasound signals through bat-detector. In this way, it has been possible to attribute collected guano pellets to individuals of the species *Pipistrellus kuhlii* (Kuhl, 1817), *Eptesicus serotinus*, Schreber, 1774, *Myotis blythii* (Tomes, 1857), *Myotis myotis* (Borkhausen, 1797), *Myotis nattereri* (Kuhl, 1817) and *Plecotus* sp. Seven other samples were collected in Tuscany region, from localities in the province of Pistoia: three of them were from natural caves in the Biogenetic Natural Reserve of "Pian degli Ontani" hosting reproductive colonies of the migratory species *Nyctalus leisleri* (Kuhl, 1817) and four of them were from disused quarries in meadows areas hosting resident colonies of *Miniopterus schreibersii* (Kuhl, 1817) and *Rhinolophus hipposideros* (Bechstein, 1800) (Tab. 4.1). These samples were collected by Dr. Gianna Dondini and Dr. Simone Vergari of the Nature and Archaeological Museum of Pistoia Apennine (Cutigliano, PT).

All samples were collected before sunrise and/or during night when the animals were absent, in order to avoid any disturbance of the colony; guano pellets were selected from all guano piles beneath bat roosts at each site or from all guano piles in each bat-box, carefully placed in sterile plastic jars, labelled with information reporting date of collection, locality, roost typology and species of bats present at that site, and stored before sending them at the Laboratory of Evolutionary Zoology of the University of Modena and Reggio Emilia where they were stored at -20°C for molecular analyses.

In order to have a positive control for molecular analyses, consisting of a sample with degraded *H. halys* DNA, guano pellets from one bat of the species *P. kuhlii*, exclusively fed with the BMSB

were collected. The individual was a young male in recovery at the Theriological Station of Piedmont, after two days of starvation it was fed for two consecutive evenings with a *H. halys* homogenate daily prepared with reared individuals, then guano pellets were collected the morning after feeding, immediately stored at -20°C and sent in a refrigerated box to the Laboratory of Evolutionary Zoology.

4.3.2 DNA extractions and Real-time PCR assays

For each sample, whole genomic DNA (gDNA) was extracted from 200 mg of guano pellet with a QiaAmp fast DNA stool mini kit (Qiagen Sciences, Germantown, MD, USA) following the human DNA analysis protocol (Quik-Start Protocol, December 2017). The gDNA was resuspended with 200μl of Qiagen buffer ATE and then the amount of detectable DNA was measured with a NanoDrop® ND-1000 spectrophotometer (Life Technologies). To avoid the contamination of the guano samples with *H. halys* DNA potentially present in the lab, all dedicated equipment for DNA extraction was sterilized prior to each experiment.

To detect traces of *H. halys* DNA among the DNA extracted from the guano, a high-sensitivity Real-time PCR assay was employed. The assay was specific to the *H. halys* conserved region of the rDNA internal transcribed spacer 1 (ITS1) as described in Valentin et al. (2016). Real-time PCR reactions were performed in replicates of two for each sample in 20 μl reactions using 500 nM of each primer BMITS1F 5'-CGAGGCCGCCGATGA-3'; BMITS1R 5'-CCCACGAGCCGAGTGATC-3'), 250 nM of the TaqMan™ fluorescent probe (BMITS1TM 5'-CAGGCAATGAAGCACA-3') with a dye label (VIC) on the 5' end and a minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3' end, 1× TaqMan™ Fast Advanced Master Mix with Uracil-N glycosylase (UNG), and 2 μL of undiluted gDNA. All Real-time PCR assays were performed including: positive controls as standards to distinguish between true positive and/or false negative; negatives controls to test the specificity of the probe to the target DNA sequence and to check for the presence of false positive; No Template Controls (NTC) to exclude any contamination during the reaction preparation. The selected positive controls were the genomic DNA from guano of the bat fed with the stink bug and the genomic DNA extracted from *H. halys* specimens reared in lab. The selected negative controls were gDNA extracted from two of widely diffused pentatomid species that share habitat and feeding preferences with *H. halys* in Italy, the holarctic species *Nezara viridula* L. and the palearctic species *Rhaphigaster nebulosa* Poda, 1761. All pentatomid specimens used as negative controls were collected in Modena (Italy) and stored in ethanol 100% at -20°C prior to molecular analysis. The gDNA from *H. halys*, *N. viridula* and *R.*

nebulosa was extracted from the legs and heads of each animal with a DNeasy Blood & Tissue kit (Qiagen Sciences, Germantown, MD, USA) following the protocol "Total DNA from Animal Tissue" (DNeasy Blood & Tissue Handbook, July 2006). The gDNA was re-suspended with 200µl of Qiagen buffer ATE and then the amount of detectable DNA was measured with a NanoDrop® ND-1000 spectrophotometer (Life Technologies).

The optimized Real-time PCR reaction protocol was conducted at an initial step of 50°C for 2 min to activate the UNG, a denaturing step of 95°C for 20 s, followed by 40 cycles of denaturing for 10 s at 95°C, annealing for 20 s at 67°C, extension for 30 s at 72°C and a final extension at 72°C for 7 min. Reactions were run on a BioRad CFX96 Real-time PCR system (Bio-Rad Laboratories, Inc.) and results were analysed using CFX Maestro™ Software version 1.1 (Bio-Rad). The software compares the final relative fluorescence units (RFUs) for each well with positive and unknown content of target sequence to the RFU levels of wells with negative controls and identifies the positives when their RFU values are greater than the average RFU values of the negative controls plus the default cut off value.

4.4 Results

The species-specific BMITS1 Real-time PCR assay on total genomic DNA extracts from the guano of Italian chiropterans, targeted *H. halys* DNA in six samples out of 27 (Tab. 4.2).

Four genomic extracts tested positive to target DNA belonged to four samples collected in bat-boxes located in agricultural sites in Piedmont and generated by six different bat species as reported in Tab. 4.1. The two further genomic extracts tested positive to *H. halys* DNA were samples from two sites of the Natural Biogenetic Reserve of "Pian degli Ontani" (Tuscany) and belonged to individuals of the species *Nyctalus leisleri.*

Despite six positive results, six samples presented traces of DNA not attributable to *H. halys* hence, classified by the software as negatives to the presence of target DNA, while, 15 samples were characterised by too degraded DNA to yield any amplification at all. This could be expected in DNA samples that were subjected to digestive processes, and physical and biological degradations once they are released on the ground.

Overall, the amplification performances were consistent with the expectations since the amplification curves of the positive control with the whole *H. halys* DNA always started before the positive control with the digested *H. halys* DNA in all assays, while negative controls always started later than positive controls (Fig. 4.1), confirming the species-specificity of the primers and probe to the target sequence. Furthermore, No Template Controls (NTCs) and negative controls were consistently negative for BMSB, indicating the absence of cross-contamination during

amplification workflow.

4.5 Discussion

The rate of diffusion of *H. halys* across Italian territories is extremely high and, it appears to be enhanced either by biological or human-mediated processes. This species can shelter in hidden spots, surviving a broad range of environmental stresses; therefore, the presence of new individuals goes undetected or underestimated, even less the ecological interaction with the native biota (Cesari et al. 2018; Maistrello et al. 2018). The timescale for the identification of prey-predator relationships based on observational data and the morphological analysis of prey fragments in chiropteran guano are not compatible with the timeframe required for a prompt pest management (Clare et al. 2009).

The application of targeted molecular approaches reduces time and efforts required for a pest survey, increasing the identification specificity of the invasive species and its native generalist predators (Clare et al. 2009, O'Meara et al. 2013, Dhami et al. 2016, Valentin et al 2016, Maslo et al. 2017).

The main objectives of this study were the identification of native bats feeding on *H.* halys and the early detection of *H. halys* in new areas, using a non-invasive Real-time PCR protocol on environmental DNA, in this context informative results were yielded.

The detection of BMSB DNA in guano samples indicates that native insectivorous bats underwent to prey switching in at least two Italian regions. This phenomenon is likely related to the largest abundance of the invasive alien species (IAS) in analysed areas. It is well known that successful IAS usually outweigh native species abundance, especially in environments that suffer from habitat disturbance, like agroecosystems and urban areas, where the ecological dynamics are more unstable (Kolar and Lodge 2001; Sax et al. 2005; Carlsson et al. 2009; Estoup and Guillemaud 2010; Pyšek and Richardson 2010). Moreover, changing prey target in short time it is not unusual for generalist predators such as bats, provided that the new prey fits in their feeding capabilities (Symondson et al. 2002; Carlsson et al. 2009; Pyšek and Richardson 2010; Bompard et al. 2013).

As expected, all the three analysed samples from Cuneo orchards (Piedmont region) yielded positive results confirming the agricultural pest characteristic of this species. The massive presence of *H. halys* in agroecosystems of Cuneo province is well documented along with the damage that causes (Maistrello et al. 2014, Lupi et al. 2017, Bosco et al. 2018, Candian et al. 2018). It is worth to note that the guano samples were collected from bat boxes adjacent the orchards and inhabited by six different bat species (*Pipistrellus kuhlii*, *Eptesicus serotinus*, *Myotis blythii*, *Myotis myotis*, *Myotis nattereri*, *Plecotus* sp.). Even though it is impossible to discriminate the actuals predator

species without more focused analysis, results of DNA detection are strong enough to consider them all potential active predators of BMSB. This result is consistent with previous studies from North American agroecosystems where the local big brown bats *Eptesicus fuscus* was found to feed on *H. halys* (Valentin et al. 2016; Maslo et al. 2017).

On the contrary, the presence of target DNA in two samples from Tuscany was unexpected. The guano was collected in two roosts known to be inhabited by colonies of the migratory Lesser noctule (*Nyctalus leisleri*). By the time this experiment was conducted, no information about *H. halys* presence in the Natural Biogenetic Reserve of "Pian degli Ontani" was reported. This might not be indicative of the establishment of the BMSB in this area, rather denotes an underestimation of the species expansion towards natural habitats. The absence of significative results (Tab. 4.2) from samples collected in the surrounding sites does not allow us to define the extension of the settlement. At the same time, it is impossible to provide a correlation between the extent of predation by *N. leisleri* on limiting the invasive population and the potential of diffusion of *H. halys*.

There are different implications regarding the amplification resulted negative after Real-time PCR. Considering the origin of the guano samples, five belonged to individuals of the Geoffroy's bat (*Myotis emarginatus*) from Piedmont, while one belonged to individuals of the Schreibers's bent-winged bat (*Miniopterus schreibersii*) from Tuscany. The most conservative hypothesis is that the abundance of *H. halys* population in those areas is not substantial to become part of the diet of these bats. Alternatively, the feeding habit of these species does not include *H. halys* or did not go through prey switching, even admitting a consistent presence of individuals in their hunting range.

As mentioned above, the absence of amplification in 15 samples is likely related to the very low quality of available DNA to be detected by the probe.

Even though heteropteran exoskeleton is supposed to survive the chewing and digestive processes, many other factors can affect amplification success when dealing with faecal samples. Many studies based on DNA analysis from animal excreta investigated the processes that can alter DNA quality and quantity, compromising its traceability during the analysis (Symondson 2002; Deagle et al. 2006; Clare et al. 2009; Oehm et al. 2011; O'Meara et al. 2014). Among these factors would be necessary to consider the elapsed time between the feed and the sampling, and between the sampling and the storage of the sample at the optimal conditions. During these steps, the already degraded DNA can go toward further degradation processes operated by biological agents (i.e. bacteria and fungi) and physical processes (i.e. UV lights, temperature oscillations, humidity). In the case of non-invasive sampling as in this study, addressing the first question is almost impossible without direct observation of the feeding process and immediate collection of the droplets.

Nevertheless, it should be given more attention to the sampling step, avoiding keeping the sample in the suboptimal condition in order to reduce the ongoing degradation process.
Once taken into account the different problems of this method on guano samples, it is possible to improve each part of the protocol increasing the quality of the results. Using a species-specific Real-time PCR assay to identify target DNA in predator samples proved to be fast and reliable in addressing either the early detection of the invasive species or its native predators.
An increasing number of studies support the application of this method in invasion biology (Yu et al. 2004; Walsh et al. 2005; Clare et al. 2009; Dhami et al. 2016; Valentin et al. 2016) to obtain quick results compared to traditional methods (Symondson 2002; Venzon et al. 2002; Sheppard et al. 2004; Foottit et al. 2008; Clare et al. 2009; Furlong 2015; Petráková et al. 2016; Rondoni et al. 2017). The fast identification of the new individual spots and the native control agents are crucial factors for a prompt and effective pest control management. Based on these results, extending the use of bat-boxes can address multiple questions in all the considered environments. Italian bats can be exploited to limit the population growth of the invasive *H. halys* and thereby as a mean of population conservation for those species that are threatened by habitat disturbance.

4.6 Tables & Figures

Table. 4.1. Table reporting geographic information, typology of the bat roosts, date of sampling and species of chiropteran present at each site for all analysed samples.

Region	**Province**	**Assigned acronym**	**Locality**	**Type of roost**	**Sampling date**	**Species of chiropteran**
Aosta Valley	AO	GAO1	Aosta chatedral	building	27/06/2016	*R. ferrumequinum*
Piedmont	AT	GAT1	Passerano Marmorito	building	23/06/2016	*M. emarginatus*
	AT	GAT2	Serravalle castle	building	23/06/2016	*M. emarginatus*
	TO	GTO1	Reggia Venaria	building	26/06/2016	*M. emarginatus*
	TO	GTO2	Reggia Vanaria	building	26/06/2016	*M. emarginatus*
	TO	GTO3	Agliè castle	building	19/06/2016	*M. myotis, M. blythii*
	TO	GTO4	Verrua Savoia	building	20/06/2016	*M. blythii, M. myotis*
	TO	GTO5	S.M. Maddalena church,Casalborgone	building	23/06/2016	*M. emarginatus*
	TO	GTO6	Reggia Venaria	building	26/06/2016	*M. myotis, M. blythii*
	TO	GTO7	Reggia Venaria	building	26/06/2016	*M. myotis, M. blythii*
	CN	GCN1	Mulino di Revello	building	15/06/2016	*M. emarginatus*
	CN	GCN2	Mulino di Revello	building	15/06/2016	*M. emarginatus*
	CN	GCN3	Staffarda abbey	building	15/06/2016	*M. myotis, M. blythii*
	CN	GCN4	Racconigi	building	22/07/2016	*M. emarginatus*
	CN	GCN5	Tarantasca	bat-box	13/10/2016	*P. kuhlii, E. serotinus, M. blythii, M. myotis, M. nattereri, Plecotus* sp.
	CN	GCN6	Paniale-Caraglio	bat-box	12/10/2016	*P. kuhlii, E. serotinus, M. blythii, M. myotis, M. nattereri, Plecotus* sp.
	CN	GCN7	Palazzasso-Caraglio	bat-box	12/10/2016	*P. kuhlii, E. serotinus, M. blythii, M. myotis, M. nattereri, Plecotus* sp.
	CN	GCN8	Palazzasso-Caraglio	bat-box	12/10/2016	*P. kuhlii, E. serotinus, M. blythii, M. myotis, M. nattereri, Plecotus* sp.
	VB	GVB1	Isola Bella - Stresa	building	18/06/2016	*M. capaccinii, M. myotis, M. blythii, M. daubentonii*
	VC	GVC1	Castello Rovasenda	building	18/06/2016	*M. emarginatus*
Tuscany	PT	GPT1	Cutigliano-Pian degli Ontani	natural cave	29/09/2016	*N. leisleri*

PT	GPT2	Cutigliano-Pian degli Ontani	natural cave	29/09/2016	*N. leisleri*
PT	GPT3	Pian degli Ontani	natural cave	29/09/2016	*N. leisleri*
PT	GPT4	Pieve a Nievole	disused quarry	07/10/2016	*M. schreibersii*
PT	GPT5	Pieve a Nievole	disused quarry	07/10/2016	*M. schreibersii*
PT	GPT6	Pieve a Nievole	disused quarry	07/10/2016	*M. schreibersii, R. hipposideros*
PT	GPT7	Monsumanno Terme	disused quarry	07/10/2016	*R. hipposideros*

Table 4.2. List of the 27 analysed samples reporting geographic information, type of roost of each bat colony, identified bat species at each roost, assigned acronym for molecular analyses, + positive Real-time PCR results, - negative Real-time PCR results, N.D. not detected Real-time PCR results.

Region	Acronym	Type of roost	Species of chiropteran	*H. halys* DNA detection
Aosta Valley	GAO1	building	*R. ferrumequinum*	N.D.
Piedmont	GAT1	building	*M. emarginatus*	N.D.
	GAT2	building	*M. emarginatus*	-
	GTO1	building	*M. emarginatus*	-
	GTO2	building	*M. emarginatus*	-
	GTO3	building	*M. myotis, M. blythii*	N.D.
	GTO4	building	*M. blythii, M. myotis*	N.D.
	GTO5	building	*M. emarginatus*	N.D.
	GTO6	building	*M. myotis, M. blythii*	N.D.
	GTO7	building	*M. myotis, M. blythii*	N.D.
	GCN1	building	*M. emarginatus*	-
	GCN2	building	*M. emarginatus*	-
	GCN3	building	*M. myotis, M. blythii*	N.D.
	GCN4	building	*M. emarginatus*	N.D.
	GCN5	bat-box	*P. kuhlii, E. serotinus, M. blythii, M. myotis, M. nattereri, Plecotus* sp.	+
	GCN6	bat-box	*P. kuhlii, E. serotinus, M. blythii, M. myotis, M. nattereri, Plecotus* sp.	+
	GCN7	bat-box	*P. kuhlii, E. serotinus, M. blythii, M. myotis, M. nattereri, Plecotus* sp.	+
	GCN8	bat-box	*P. kuhlii, E. serotinus, M. blythii, M. myotis, M. nattereri, Plecotus* sp.	+
	GVB1	building	*M. capaccinii, M. myotis, M. blythii, M. daubentonii*	N.D.
	GVC1	building	*M. emarginatus*	N.D.
Tuscany	GPT1	natural cave	*N. leisleri*	+
	GPT2	natural cave	*N. leisleri*	+
	GPT3	natural cave	*N. leisleri*	N.D.
	GPT4	disused quarry	*M. schreibersii*	N.D.
	GPT5	disused quarry	*M. schreibersii*	-
	GPT6	disused quarry	*M. schreibersii, R. hipposideros*	N.D.
	GPT7	disused quarry	*R. hipposideros*	N.D.

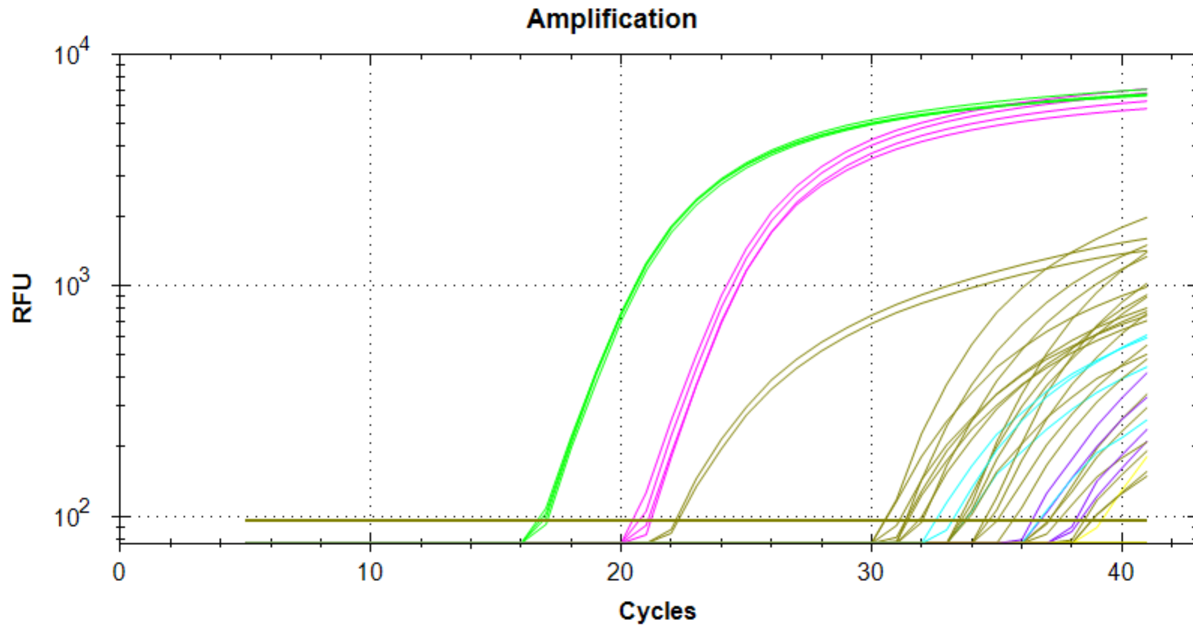

Figure 4.1. Amplification curve of one Real-time PCR assay. Green, *H. halys* gDNA positive control; pink, *P. kuhlii* gDNA positive control; olive green, guano gDNA; light blue, *R. nebulosa* gDNA negative control; purple, *N. viridula* gDNA negative control, yellow, NTCs. RFU = Relative fluorescent units; Cycles = Number of Real-time PCR cycles.

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CHAPTER 5

Conclusions

5.1 Conclusions

The overall purpose of present dissertation was the study of the invasive alien species *Halyomorpha halys* (Stål, 1855) (Hemiptera, Pentatomidae) in Italy focusing on its population structure and invasion dynamics and to its possible native predators, using two distinct molecular approaches: the Restriction site associated DNA sequencing (RADseq) and the Real-time PCR. *Halyomorpha halys* is becoming a serious agricultural and urban pest allover its invaded territories with concerning spread rates either on short or long distances. Understanding the dynamics through which the populations of *H. halys* enter and settle themselves into new areas and how the native species can limit its population growth may provide valuable insights applicable to the management of future invasive species. Therefore, these molecular methodologies were selected as implementing tools for the pest management strategies of this species and thus, setting the stage for the development of adaptable protocols for different case studies.

Assessing the population structure and invasion dynamics of *H. halys* with the RADseq method was one of the main goal of this dissertation. Genomic data from reduced representation libraries (RRLs) are far longer more informative than those obtained from the analysis of one or few genetic loci since they can analyse a higher number of Single Nucleotide Polymorphisms (SNPs) across the genome. As in the case of the present study, such extensive analysis was able to reject the hypothesis advanced by Cesari et al. (2018). The original statement ascribed the origin of the most widespread mitochondrial TH1 haplotype in Italy to a single introduction event occurred in the Emilia-Romagna region (IT) followed by the spread of individuals through neighbouring regions. On the opposite, the genomic data presented in this thesis highlighted two or more sources of invasion in Italy and high mobility of the individuals likely enhanced by human activities. Having the correct information is crucial to better direct pest management options such as the adoption of more strict controls on the movements of goods and commodities from invaded areas.

The identification of *H. halys* predators in Italy was other main goal of this dissertation. Two different types of analysis were performed using a single method, the species-specific Real-time PCR assay developed by Valentin et al. (2016). The method was applied to perform a molecular gut content analysis on native generalist arthropod predators collected in urban areas to check for the presence of *H. halys* DNA traces. The analysis enabled the identification of different species among insects and arachnids feeding on this invasive species thus providing new important information about the trophic relationships of *H. halys* with the native arthropod fauna few years after its settlement in Italy. The correct identification of different predator native species is valuable to implement programs of rearing and releasing of biological pest control agents, avoiding the use of

pesticides in urban environments. Similarly, the Real-time PCR assay was performed on eDNA represented by guano from Italian chiropteran species to prove their predator potential, but also to test whether the method was valid for the early detection of *H. halys* in areas with no records about its presence. Even in this case, the analysis led to the detection of *H. halys* DNA traces in different samples from different Italian regions, corroborating the evidence that bats are active predators of this species and they can be exploited as biological pest control agents. The results led also to the early detection of this species in a natural area, namely the Biogenetic Natural Reserve of "Pian degli Ontani", Tuscany, Italy. Therefore, the method can be easily applied to monitor the diffusion of this species and direct management efforts to prevent the establishment of *H. halys*.

In conclusion, molecular approaches proved to be valid in addressing various problematics in invasive species studies. Considering overall results, important knowledge was achieved for the study of the biological invasion of *H. halys*, with remarkable implications for a fast and more effective management.

In light of this accomplishment, the adoption of molecular strategies should become a standard procedure to address either theoretical or practicable knowledge on the study of biological invasions of invasive alien species.

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