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RAPID COMMUNICATION

Occurrence and genetic diversity of new populations of *Halyomorpha halys* in Europe

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Abstract Originating in East Asia, the brown marmorated stink bug, *Halyomorpha halys*, has established in North America and Europe where it has become an urban and agricultural pest. Although initial populations in Europe were only reported in Switzerland, *H. halys* has expanded its range to include areas in France, Greece, Hungary, and Italy. Analysis of a portion of the mitochondrial COI gene was used to evaluate the genetic diversity of these populations and to investigate the movement and spread of different COI haplotypes of this pest in Europe. A total of 9 haplotypes are now confirmed in Europe. Four of these haplotypes have not been previously reported elsewhere, and one of these haplotypes has not been previously reported in Europe but is known from Korea. The *H. halys* population in Greece was the most diverse, with 7 of the 9 haplotypes occurring in the area of collection. Haplotype H1, which is known from North America, Italy, and China, was also present in samples collected in France, Greece, Hungary, and Switzerland. In fact, the H1 haplotype was the dominant haplotype detected in Budapest, Hungary and also occurred frequently in the samples from Athens, Greece. Patterns of haplotype diversity indicate the movement of successful invasive populations to generate secondary invasions within Europe ('bridgehead effect'), as well as the occurrence of multiple invasions from Asia. Scenarios regarding the movement and spread of particular haplotypes in Europe are discussed.

Keywords *Halyomorpha halys* · Brown marmorated stink bug · Invasive species · Genetic diversity · Haplotype · DNA barcoding · Cytochrome oxidase I · Pentatomidae

Key message

- *Halyomorpha halys* is a serious agricultural pest worldwide, and has been established in Switzerland for over 10 years. Recent spread to other countries in Europe is cause for concern due to the increased threat to agricultural crops.
- Analysis of the genetic diversity of recently established populations in France, Greece, Hungary, and Southern Switzerland may shed light on the movement and spread of this pest and aid in the identification of pathways of entry.

Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål, 1855) (Hemiptera: Pentatomidae) is widely distributed in East Asia, and has become an invasive pest in North America and Europe (Hoebeke and Carter 2003;

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Wermelinger et al. 2008; Gariepy et al. 2014a; Haye et al. 2014a). *Halyomorpha halys* is known to feed on over 120 host plants, including a number of economically important field, fruit, and vegetable crops, as well as ornamental plants (Leskey et al. 2012a, b; Haye et al. 2014a). *Halyomorpha halys* is a serious pest in the USA where it causes economic loss in tree fruit, including apples and peaches, as well as field crops such as sweet corn and soybean (Leskey et al. 2012b; Rice et al. 2014). With a rapidly expanding range in introduced areas of both Europe and Canada (Haye et al. 2015), and increasing interceptions in Australia and New Zealand (Walker 2009; Duthie 2012; MacLellan 2013; Ward 2013), *H. halys* is poised to become a serious global pest.

Halyomorpha halys has been established in Switzerland for at least 10 years, with the first record in Zurich in 2004 (Haye et al. 2014a) and confirmed establishment of breeding populations in the same region in 2007 (Wermelinger et al. 2008). By the end of 2013, *H. halys* was established in 11 Cantons in Switzerland (Haye et al. 2014b). Until recently, the Swiss population was the only known established population of *H. halys* in Europe. Although one individual *H. halys* has been reported in Germany (Heckmann 2012) and one in Liechtenstein (Arnold 2009), breeding populations were not previously detected outside of Switzerland. However, this changed in 2011 when *H. halys* was reported in residential homes in Athens, Greece (Milonas and Partsinevelos 2014). This was followed by the detection of breeding populations in the Alsace region of France in 2012 (Callot and Brua 2013) as well as in Paris in 2013 (Garrouste et al. 2014). Populations were reported in Northern Italy in 2012, and by 2013 *H. halys* was considered widely established in this region (Pansa et al. 2013; Maistrello et al. 2014; Cesari et al. 2015). In the fall of 2013, *H. halys* was detected for the first time in Budapest, Hungary (Vétek et al. 2014). In these recently invaded areas in Europe, *H. halys* is currently considered an urban and household pest due to its affinity to enter man-made structures for overwintering. However, economic damage has been reported in pepper crops in the Canton of Aargau in Switzerland (Sauer 2012) and in commercial fruit orchards in Italy (Maistrello et al. 2014). Following establishment in North America in 2001, *H. halys* was generally reported as a nuisance pest (Hoebeke and Carter 2003); however, by 2010 high levels of economic damage were observed in the northeastern USA, where it is now considered a serious economic pest of a wide variety of crops (Leskey et al. 2012a, b). A similar scenario may occur in Europe, where serious economic damage is reported several years following confirmed establishment.

The recent and fairly rapid expansion of the geographic range of *H. halys* in Europe since 2011 may be the result of outward spread of known populations in Switzerland. As the Swiss population has been established for over 10 years, we would expect gradual invasion into bordering countries. However, the incidence of established, breeding populations of *H. halys* in countries that do not directly border Switzerland (e.g., Greece, Hungary) suggests the possibility of additional introductions from other locations. In addition, the occurrence of this pest in several new locations over a fairly short period of time raises the possibility that multiple introductions may be responsible for some of these recent reports. Introduced species often display reduced genetic variability during colonization of a new environment (Mayr 1954), which may or may not impact their successful establishment (Sax and Brown 2000). Although a genetic bottleneck effect was detected in invasive populations of *H. halys* in North America and Europe (Gariepy et al. 2014b; Xu et al. 2014), this effect clearly has not prevented the successful colonization of *H. halys* in these areas. Some founding populations are particularly successful and serve as a source of secondary invasions. This “bridgehead effect” can often lead to a burst of global invasions following an initial invasion event (Lombaert et al. 2010; Lawson Handley et al. 2011). The recent spread of successfully established *H. halys* in Europe warrants further investigation to determine the origin and spread of these populations.

DNA sequence data for the mitochondrial genes has been used to trace the origins and spread of a number of introduced insect pests (Grapputo et al. 2005; Corin et al. 2007; Barr 2009; deLeón et al. 2011; Auger-Rozenberg et al. 2012), including *H. halys* (Gariepy et al. 2014b; Xu et al. 2014; Cesari et al. 2015). Previous genetic studies on *H. halys* from Asia and the USA have used the Cytochrome Oxidase II (COII) gene, coupled with the Cytochrome Oxidase I (COI) gene to narrow down the source region of the invasive US population (Xu et al. 2014). Additional studies have relied primarily on the COI gene to evaluate genetic diversity of *H. halys* in Europe (Gariepy et al. 2014b; Cesari et al. 2015), Canada, and Asia (Gariepy et al. 2014b). To expand upon previous knowledge of the genetic diversity of *H. halys* in Europe, and to investigate the possibility of additional introductions of *H. halys* in newly invaded areas, we analyzed a portion of the mitochondrial COI gene from samples collected in regions where *H. halys* was most recently reported [Alsace region (France), Athens (Greece), Budapest (Hungary), and the Canton Ticino (southern Switzerland)]. Haplotype analysis of specimens from these regions may shed some light on the continued introduction and spread of *H. halys* in Europe, and may help identify pathways of entry and movement within Europe.

Materials and methods

Collection of specimens

A total of 308 *H. halys* specimens were collected from areas where new reports of this pest have recently surfaced. In the Alsace region of France, 139 adults were collected from *Carpinus betulus* trees in a small park area in the city of Schiltigheim (N48°36′30 E7°44′57; Département Bas-Rhin), just north of Strasbourg, on 15 September 2013. In Greece, 57 *H. halys* specimens were collected from residential areas in the north suburb of Athens, Kifisia (N38°04′52 E23°48′47) during the first half of October 2014 as they were trying to enter public and private buildings for overwintering. A total of 84 *H. halys* nymphs and adults were collected from the fruits of *Acer negundo* L. and *Amorpha fruticosa* L. within the area of the Research and Experimental Farm of Soroksár, Budapest (N47°23′51 E19°08′50) on 25 September 2014. In southern Switzerland, 28 individuals were collected on various trees and shrubs on 12 September 2013 in residential areas in the city of Lugano (N46°00′16 E8°57′27; Canton Ticino). Following collection, all *H. halys* specimens were preserved in 95 % ethanol for molecular analysis.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from a single leg of each *H. halys* specimen using a Chelex extraction method (Walsh et al. 1991). Briefly, a single leg from each individual insect was placed in a 200-uL well of a 96-well microplate in 100 uL of 5 % solution of Chelex 100 Molecular Grade Resin (Bio-Rad Laboratories) and 2 uL of 20 mg/mL Proteinase K. A negative extraction control containing the Chelex and Proteinase K solutions, but no insect tissue, was included in each microplate. Each microplate was sealed and incubated overnight at 55 °C, followed by 10 min at 99 °C. Samples were centrifuged at 13,000 rpm for 5 min to pellet the Chelex solution, and 50 uL of the supernatant (containing DNA) was transferred to wells in a new plate, taking care not to transfer the Chelex residue along with the sample. Microplates containing the extracted DNA were stored at −20 °C until further analysis. PCRs were performed in a 25 uL volume containing 0.125 uL of Taq Platinum, 2.5 uL of 10× PCR buffer, 1.25 uL of 50 mM $MgCl_2$, 0.125 uL of 10 uM dNTPs (Invitrogen), 0.25 uL of 10 uM forward and reverse primer (respectively), 19.5 uL ddH20, and 1 uL of template DNA. A 658-bp sequence of the mitochondrial gene Cytochrome C oxidase subunit 1 (COI) was amplified by PCR using primers LCO1490 and HCO2198 (Folmer et al. 1994).

PCR products were subject to electrophoresis on a 1.5 % agarose gel (UltraPure Agarose, Invitrogen) containing 10,000× stock GelRed (Biotium) diluted at 1:10,000 in the agarose gel solution. Resulting PCR products were visualized on a BioDoc-it imaging system (UVP) and purified using ExoSAP-IT (USB Corporation) following the manufacturer's instructions. The PCR products were bidirectionally sequenced (using the above primers) on an ABI 3730 DNA Analyzer at the Robarts Research Institute (London Regional Genomics Centre, Ontario, Canada). Forward and reverse sequences were assembled, aligned using Clustal W algorithm (Thompson et al. 1994) and edited using Codon Code Aligner program, version 4.0.4.

A separate extraction, amplification, and sequencing of an additional leg from specimens which produced unique, singly occurring haplotypes as well as haplotypes that were previously unreported, was repeated to ensure that sequence variation was not an artifact generated by errors in the PCR or contamination.

Distribution and diversity of haplotypes of *Halyomorpha halys*

Mitochondrial COI sequences from *H. halys* specimens were grouped based on the geographic area of collection: France, Greece, Hungary, and southern Switzerland (Canton Ticino). In addition, sequence data from previous collections in the Swiss Cantons of Basel, Bern, and Zurich (Gariepy et al. 2014b) were combined with sequence data from the present collections in Canton Ticino (southern Switzerland) in order to provide more complete, up-to-date diversity measures for the country. Measures of genetic diversity were calculated for each of these geographic regions using DnaSP v5.10.01 (Librado and Rozas 2009) and ARLEQUIN v3.1 (Excoffier et al. 2005), including: number of haplotypes, number of haplotypes unique to a given geographical area, haplotype diversity, and nucleotide diversity.

Using the software package TCS v1.21 (Clement et al. 2000), statistical parsimony cladogram networks of *H. halys* COI haplotypes were generated for the present collections. In order to place the present collections in a global framework, the same approach was used to generate a global haplotype network based on data from previous collections in Asia, Europe, and North America (Gariepy et al. 2014b; Xu et al. 2014; Cesari et al. 2015; publicly available genetic data on Genbank).

Results

All 308 specimens collected from France, Greece, Hungary, and southern Switzerland yielded a 658-bp DNA sequence for the 5' end of the COI gene. A total of 8 haplotypes were found, and a list of their occurrence (Table 1) and a comparison with known global records is provided (Table 2). Collectively, two haplotypes were most frequently observed in our samples and comprised 34 % (H1) and 54 % (H3) of the samples collected in Europe. The remaining 6 haplotypes (H8, H22, H30, H31, H32, H33) were observed in 0.3–7 % of the samples collected. The occurrence and frequency of each haplotype in each country is shown in Table 1 and Fig. 1 [combined data from present study and previous studies (Gariepy et al. 2014b; Cesari et al. 2015)].

Specimens collected in France were not only predominately haplotype H3 (97.8 %), but also demonstrated the occurrence of haplotypes H1 (0.7 %) and H8 (1.5 %) in the region. Samples from Greece were the most diverse, displaying 7 of the 8 haplotypes—four of these haplotypes (H30, H31, H32, H33; Genbank accession: KR070748–KR070751) were only found in Greece and have not been found elsewhere in Europe or Asia thus far (Tables 1, 2; Fig. 1). The two most common haplotypes in Greece were H33 (40 %) and H1 (32 %), followed by H32 (14 %), H3 (7 %), and H22 (3.5 %); H30 and H31 occurred singly in these collections. The Hungarian samples were comprised of two haplotypes (H1 and H3), but the samples were predominantly (~99 %) haplotype H1. Specimens collected in southern Switzerland in the Canton of Ticino ($n = 28$) consisted of 3 haplotypes—H1 (7 %), H3 (89 %), and H8 (4 %) (Table 1), whereas combined data for all of Switzerland (including data from Gariepy et al. 2014b and Cesari et al. 2015) consisted of 4 haplotypes—H1 (0.9 %), H3 (85 %), H8 (13.7 %), and H9 (0.4 %) (Table 3).

Genetic diversity measures for *H. halys* are shown in Table 4, including haplotype diversity (the probability that two randomly chosen haplotypes are different) and nucleotide diversity (the average number of nucleotide differences per site between two randomly chosen DNA sequences) (Nei and Li 1979). Within our European samples, haplotype and nucleotide diversity was 0.521 ± 0.022 (SD) and 0.00137 ± 0.00106 (SD), respectively. These standard diversity measures were highest among the specimens collected in Greece compared to the other European countries investigated. Samples collected in Switzerland demonstrated that this is the second most diverse population among our European samples. Genetic diversity measures for Hungary and France were considerably lower than those obtained from Greece and Switzerland (see Table 4).

Haplotype networks for samples collected in the present study are shown in Fig. 2, along with their placement within a global haplotype network (Fig. 3).

Discussion

Many introduced species experience genetic bottlenecks upon colonization of new areas. Although this may decrease the fitness of established individuals, some species have become extremely successful colonizers despite strong founder effects (Sax and Brown 2000). In some cases, multiple introductions can enhance genetic diversity, thereby allowing the population to overcome founder effects associated with the initial introduction (Miller et al. 2005; Ciosi et al. 2008; Lawson Handley et al. 2011). Reduced genetic variability in introduced populations of *H. halys* has been observed, with the existence of a single COI haplotype and two COII haplotypes in specimens collected from the USA and Canada (Gariepy et al. 2014b; Xu et al. 2014), and four COI haplotypes in Europe (Gariepy et al. 2014b; Cesari et al. 2015). In contrast, in its native Asian range at least 21 COI haplotypes and 18 COII haplotypes have been documented for *H. halys* (Gariepy et al. 2014b; Xu et al. 2014). However, although genetic diversity of *H. halys* in Asia has been investigated, the studies are preliminary and it is likely that the haplotype diversity has been underestimated. For example, a relatively large number of samples ($n = 110$) has been assessed from the Hebei/Beijing region in China (Gariepy et al. 2014b); however, the geographic range of *H. halys* is far more extensive and further coverage is necessary to document the occurrence of all haplotypes across the native range. In contrast, Xu et al. (2014) analyzed *H. halys* specimens from a broad geographic range in China, Korea, and Japan; however, under-sampling is a concern, as sample size from each population was low (ranging from 5–21 specimens, with a mean sample size of 8 specimens). Goodall-Copestake et al. (2012) have shown that under-sampling can provide inaccurate estimates of genetic diversity measures, and a larger number of individuals

Table 1 Cytochrome Oxidase I (COI) haplotypes detected and corresponding area(s) of collection in Europe

	H1	H3	H8	H22	H30	H31	H32	H33	Total
Schiltigheim, France	1	136	2	0	0	0	0	0	139
Athens, Greece	18	4	0	2	1	1	8	23	57
Budapest, Hungary	83	1	0	0	0	0	0	0	84
Lugano, Switzerland	2	25	1	0	0	0	0	0	28
Total	104	166	3	2	1	1	8	23	308

Table 2 List of haplotypes occurring in Europe based on present study and previous studies (Gariepy et al. 2014b; Cesari et al. 2015) as well as the occurrence and status of the same haplotypes in Asia and North America

Haplotype	Non-native range of *H. halys*		Native range of *H. halys*	Genbank accession number
	Europe	North America	Asia	
H1	France Greece Hungary Italy Switzerland	USA Canada	China	KF273380
H3	France Greece Hungary Italy Switzerland	Canada: detected in a shipment from China; not established	China	KF273382
H8	France Italy Switzerland	Unknown	Unknown	KF273387
H9	Switzerland	Unknown	Unknown	KF273388
H22	Greece	Canada: detected in a shipment from Korea; not established	Korea	KF273401
H30	Greece	Unknown	Unknown	KR070749
H31	Greece	Unknown	Unknown	KR070748
H32	Greece	Unknown	Unknown	KR070750
H33	Greece	Unknown	Unknown	KR070751

If a given haplotype has not been detected in a country, then its status is listed as “unknown”

Note that additional haplotypes (H2, H4–H7, H10–H23) are known from Asia but have not been found elsewhere

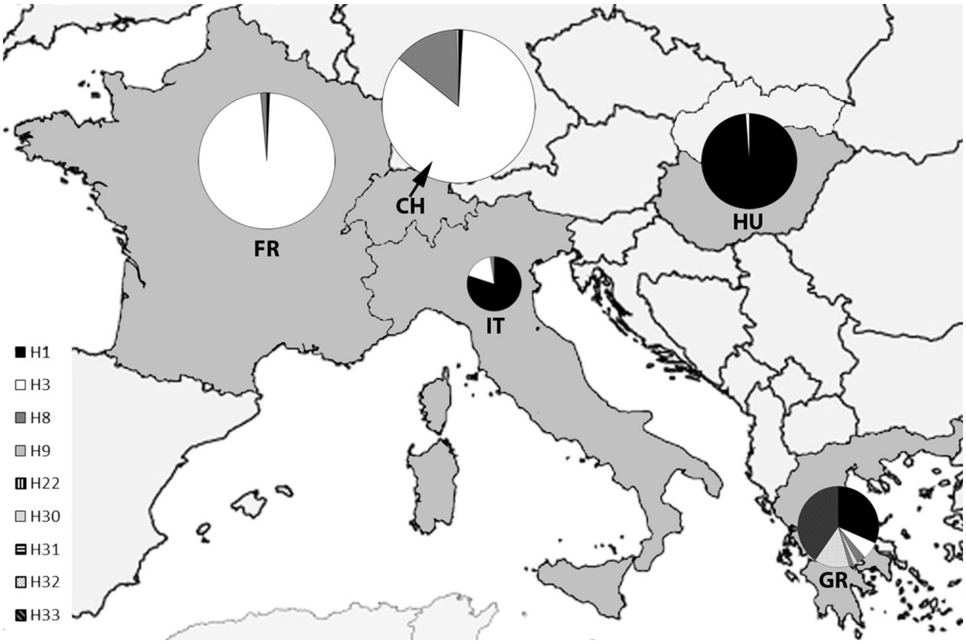

Fig. 1 Map of Europe showing the countries where BMSB is established (shaded in *gray*). The haplotype frequency of *H. halys* is shown with pie charts sized proportionally to the sample size from each country [France, FR ($n = 139$); Greece, GR ($n = 57$); Hungary, HU ($n = 84$), Italy, IT ($n = 40$); and Switzerland, CH ($n = 225$)]. Based on data from the present study, Gariepy et al. (2014b) and Cesari et al. (2015)

($n \geq 25$ as general rule) should be sampled from a population to document the presence of lower-frequency mitchondrial haplotypes.

The limited information on the genetic diversity of *H. halys* from its native Asian range makes it difficult to compare these measures quantitatively on a global scale.

Table 3 Cytochrome oxidase I (COI) haplotypes detected in Switzerland

Collection location	H1	H3	H8	H9	Total
Switzerland: North (Cantons Basel, Bern, and Zurich)[a]	0	164	30	1	195
Switzerland: South (Canton Ticino)[b]	2	27	1	0	30
Combined total[a,b]	2	191	31	1	225

[a] Based on samples ($n = 195$) from Gariepy et al. (2014b)

[b] Based on samples from the present study ($n = 28$) and samples from Cesari et al. (2015) ($n = 2$)

Table 4 Genetic diversity measures of *Halyomorpha halys* collected in Europe

Population	*N*	Polymorphic sites	Haplotypes	Unique haplotypes[a]	Haplotype diversity (±SD)	Mean # pairwise nucleotide differences (±SD)	Nucleotide diversity (±SD)
France Schiltigheim	139	3	3	0	0.043 ± 0.023	0.071 ± 0.144	0.00011 ± 0.00024
Greece Athens	57	8	7	4	0.724 ± 0.036	2.34 ± 1.30	0.00356 ± 0.0022
Hungary Budapest	84	1	2	0	0.024 ± 0.023	0.024 ± 0.082	0.00004 ± 0.00014
Switzerland[b]	223	4	4	1	0.263 ± 0.0348	0.508 ± 0.432	0.00077 ± 0.00072
Lugano	28	3	3	0	0.204 ± 0.098	0.280 ± 0.310	0.00043 ± 0.00052
Europe[b]	503	10	9	5	0.521 ± 0.022	0.902 ± 0.629	0.00137 ± 0.00106

[a] Haplotypes unique to a particular geographic area

[b] Includes previous data from Swiss populations of *H. halys* (Gariepy et al. 2014b)

As such, using a portion of the mitochondrial COI gene, we investigated the genetic diversity of invasive populations of *H. halys* within Europe. We recorded the occurrence of new haplotypes and identified haplotypes that match with existing data from previous haplotype studies where available, with the understanding that many haplotypes in the native Asian range have yet to be described. Previous haplotype analyses of established European populations of *H. halys* in Switzerland and Italy reported the occurrence of a total of 4 COI haplotypes [H3, H8, H9: Gariepy et al. (2014b); H1, H3, H8: Cesari et al. (2015)]. In contrast, a total of 8 COI haplotypes (H1, H3, H8, H22, H30, H31, H32, H33) were detected in the present study from samples collected in areas of France, Greece, Hungary, and southern Switzerland. When data from both the present study and previous European studies (Gariepy et al. 2014b; Cesari et al. 2015) are considered, a total of 9 COI haplotypes are now known from Europe. By expanding the haplotype analysis to include samples collected in the newly reported areas of establishment, 5 additional haplotypes have been detected in Europe. It is important to note that our sample sizes differed between regions (ranging from 28 to 139 specimens per population) based on the availability of specimens at the time of collection. Additional sampling efforts over a longer period of time in recently invaded areas may uncover haplotypes that were not detected in the present study. Although our population samples exceed the $n > 25$ "rule of thumb" (Goodale-Copestake et al. 2012) a larger sample size would permit greater resolution of the haplotype diversity. This is of particular importance to ensure the detection of rare haplotypes which are major contributors to haplotype diversity despite the fact that they are only present in a small fraction of the population (Slater et al. 2015).

The geographic distribution of haplotypes from the present study and previous European studies (Gariepy et al. 2014b; Cesari et al. 2015) shows that haplotype H1 and H3 occur in all countries investigated (France, Greece, Hungary, Italy, and Switzerland; see Fig. 1 and Table 2). However, H1 is more prominent in Greece, Hungary, and Italy, whereas H3 is more prominent in France and Switzerland. Interestingly, samples from Greece were more diverse than samples from the other countries. These samples ($n = 57$) represented 7 of the 9 haplotypes, with haplotypes H1 and H33 occurring most frequently. All five of the newly detected haplotypes in Europe were found in Greece, and multiple individuals were detected for three of the five haplotypes (H22, H32, H33). One of these haplotypes (H22) is previously known from samples originating in Korea (Gariepy et al. 2014b), whereas the remaining four haplotypes (H30–H33) have not been previously described in the literature, nor are they published in publicly

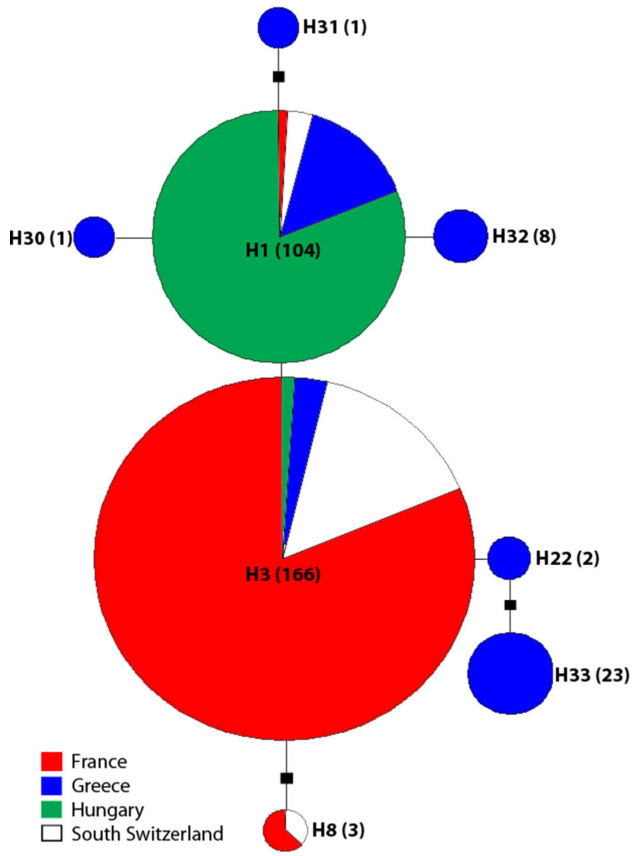

Fig. 2 Statistical parsimony cladogram network representing the relationships among haplotypes for a 658-bp fragment of the cytochrome oxidase I (COI) gene from *Halyomorpha halys* collected in regions where this pest has recently been found. *Each circle* is labeled by haplotype number, and the frequency of each haplotype is provided (*in brackets*). The size of each circle is proportional to the frequency of each haplotype. *Differing colors* in the pie diagrams indicate the country in which the samples were collected, as indicated in the figure legend. *Small*, *black squares* represent intermediate haplotypes not detected in the samples. (Color figure online)

available sequence databases (e.g., Genbank, BOLD). Based on their placement in the global haplotype network (Fig. 3), H30, H31, and H32 are more closely connected with samples from China, whereas H33 clusters more closely with specimens from Korea and Japan. Additional studies and characterization of *H. halys* haplotypes in the native Asian range are necessary to confirm this and to resolve the Asian origin of all currently unidentified haplotypes that occur in the invaded range of this pest.

Switzerland was the second most diverse population, with 4 of the 9 haplotypes represented. For the first time, the occurrence of haplotype H1 was recorded among Swiss (2 of 28 specimens collected in Canton Ticino) and French (1 of 139 specimens from the Alsace region) populations, albeit at a low frequency. This haplotype is known to occur in China, and represents the primary haplotype in invaded areas in North America (Gariepy et al. 2014b; Xu et al. 2014). Recently, Cesari et al. (2015) reported haplotype H1 as the dominant haplotype in northern Italy. Haplotype H1 is also the dominant haplotype found in Budapest, Hungary, representing 99 % of the samples collected, and was also the second most common haplotype in Athens, Greece, representing approximately 32 % of the samples collected in this region. Until now, H1 has not previously been recorded in Switzerland (the presumed first point of establishment in Europe) despite the fact that extensive collections have been made across its reported range in Basel, Bern, and Zurich (Gariepy et al. 2014b). This emphasizes the importance of tracking the movement and spread of new populations, as continued and repeated introduction of additional populations from the native Asian range and/or from other established populations in the introduced range (e.g., North America or other European countries) is possible.

Given the patterns of haplotype diversity in Europe (see Fig. 1) and the fact the first known population of *H. halys* was discovered in Switzerland, we speculate that populations of haplotype H3 and H8 in Switzerland were initially introduced from Asia, and then spread outwards to neighboring countries (France and Italy) by passive and active movement. As haplotype H1 was not previously recorded from Switzerland and is present at low frequency in the Swiss population, this haplotype likely established elsewhere in Europe (Italy, Greece, and/or Hungary) from North America and/or China, and then continued to spread to new locations within Europe, including Switzerland and France. There is at least one documented interception of *H. halys* (all Haplotype H1) in Europe in a shipment arriving in Germany from the United States (Pennsylvania) (Freers 2012; Gariepy et al. 2014b). Although there is no documented proof of established populations of *H. halys* in Germany, this interception shows the potential for secondary introductions. The widespread occurrence of Haplotypes H1 and H3 in Europe may be consistent with the "bridgehead effect," in which widespread secondary invasions arise from particularly successful invasive populations (Lombaert et al. 2010; Lawson Handley et al. 2011).

The genetic diversity measures for *H. halys* in Greece were more similar to the diversity measures from the native range of *H. halys* than to the diversity measures from other invaded areas in Europe and North America (Gariepy et al. 2014b; Xu et al. 2014) However, it is important to note that these Asian collections were preliminary and further sampling would be needed to obtain a more accurate interpretation of the diversity measure for Asia. Nonetheless, the high level of diversity in *H. halys* in Greece may suggest multiple and/or large introductions of *H. halys* into Greece from Asia (and/or other established populations).

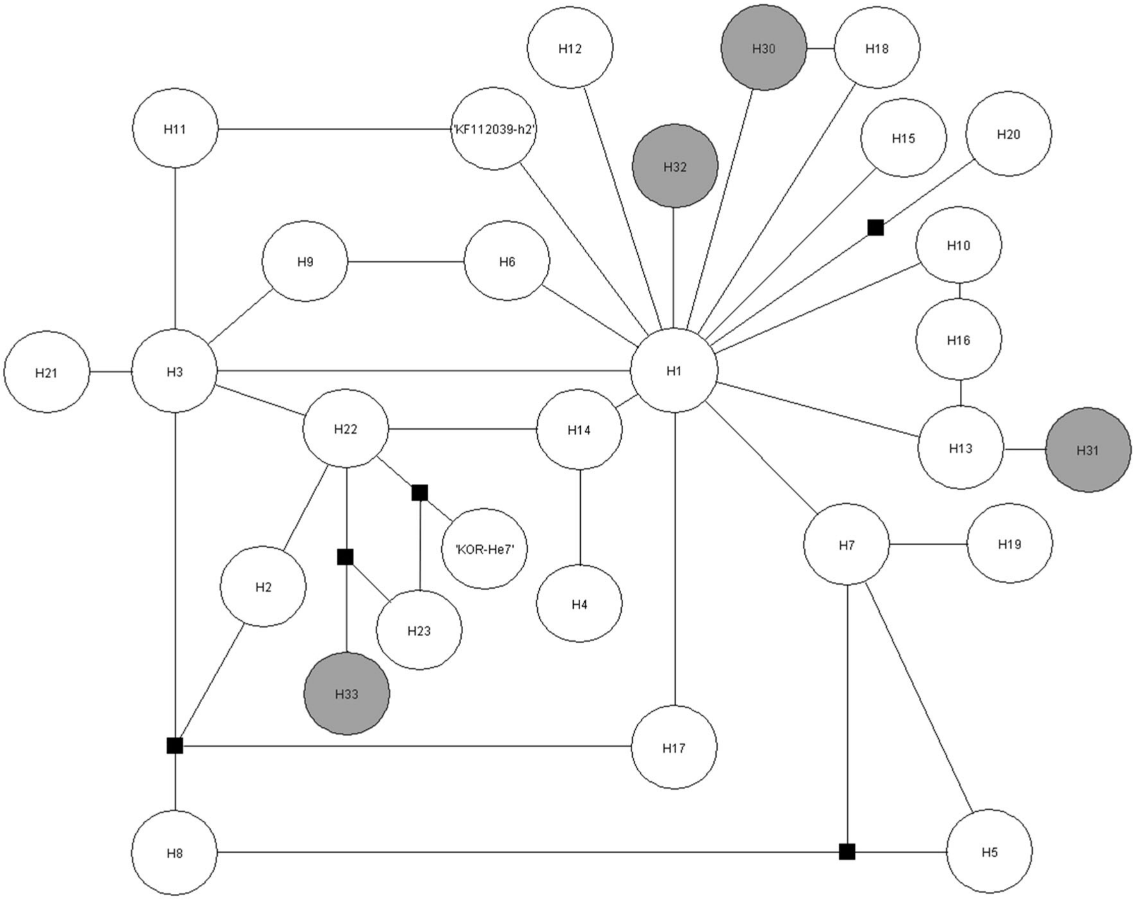

Fig. 3 Connectivity of haplotypes from *Halyomorpha halys* specimens collected from Europe and Asia based on sequence data from the present study and publicly available sequences on Genbank for a 658-bp fragment of the COI gene. Diagram represents only haplotype connectivity and does not provide a quantitative representation of these haplotypes. Previously unreported haplotypes are shaded in *gray*. *Small*, *black squares* represent intermediate haplotypes not detected in the samples. Note that KOR-He7 and KF112039-h2 correspond to genbank accession numbers KC510116 and KF112039; all other haplotypes and their associated accession numbers are as reported by Gariepy et al. (2014b)

We can speculate that the H1 and H3 populations in Greece were derived from populations introduced from China, other established populations within Europe, and/or North America (H1). The occurrence of haplotype H22 points towards introduction and establishment of a population from Korea. However, the point source of H30, H31, H32, and H33 remains unknown. This was also the case in previous studies on haplotype diversity in Switzerland, where the area of origin remains unknown for two haplotypes (H8 and H9) (Gariepy et al. 2014b). The area of origin of these haplotypes will likely be clarified once additional specimens from Asia are obtained and analyzed. This may be particularly important for haplotypes which occur frequently in the invaded area, but for which no match to the haplotypes in the area of origin have been found (e.g., H8 in Switzerland and H33 in Greece).

There is often very little historical and observational data available on the movement of invasive populations, and therefore genetic data can provide insight into the source, routes, and mechanisms of spread throughout invaded areas (Lawson Handley et al. 2011). It is widely recognized that the shelter-seeking behavior of *H. halys* often leads to aggregations of large numbers of adults in vehicles, shipping containers, and luggage (Duthie 2012), and international transport hubs are recognized as stepping stones for insect pest invasions (Floerl et al. 2009; Bacon et al. 2012). For example, in Greece, the port city Piraeus is located within the Athens urban area and is one of the world's busiest passenger ports, the Mediterranean's third largest container port and is well connected with major Asian ports (www.seatrade-global.com). As such, the likelihood of cargo with *H. halys* adults arriving in Piraeus is high. With free intra-European trade, a well-established Trans European Transportation Network (www.nordregio.se), and gaps in border controls, 'hitchhiking' invasive species (such as *H. halys*) that enter Europe at a major transport hub could pose significant invasion risks to the rest of Europe (Bacon et al. 2012). As *H. halys* has become a high-profile pest, historical records, and observational data are becoming more readily available, and these can be used to put the molecular genetic data in context with the movement and spread of this pest. To date, this study represents the most comprehensive analysis of *H. halys* populations in Europe and puts the European populations in perspective with the global distribution of known *H. halys* haplotypes. Now that baseline information on the diversity of *H. halys* has been defined for newly established regions, additional surveys moving outwards of the initial point of these detections will be important to refine estimates of haplotype diversity in Europe (based on greater sample sizes), determine how widespread this pest is in each country, and categorize the presence of *H. halys* as a potential threat to agricultural productivity or as a nuisance pest in each of these regions.

Author contributions

Research was designed by TDG and TH. TH, GV, and PM carried out research in Europe. Molecular analysis was done by TDG and AB. TDG wrote the manuscript and all authors read and approved the manuscript.

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