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Authors: Lee, Wonhoon, Guidetti, Roberto, Cesari, Michele, Gariepy, T.D., Park, Yong-Lak , et al.

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Genetic diversity of *Halyomorpha halys* (Hemiptera, Pentatomidae) in Korea and comparison with *COI* sequence datasets from East Asia, Europe, and North America

Wonhoon Lee[1], Roberto Guidetti[2], Michele Cesari[2], T.D. Gariepy[3], Yong-Lak Park[4], and Chang-Gyu Park[5,]*

Abstract

The brown marmorated stinkbug, *Halyomorpha halys,* is an invasive insect pest in North America and Europe that attacks crop species and causes substantial economic damage. To evaluate the genetic diversities and distributions of different *H. halys* populations in East Asia, North America, and Europe, *COI* sequences obtained from 79 new specimens from Korea and 10 from the USA were compared with 725 existing *COI* sequences. In total, 45 haplotypes were detected in populations from 10 countries. Sixteen haplotypes from Korea (H34–H49) and 2 from the USA (H50 and H51) were novel. Korean populations exhibited the 2nd highest diversity among the 10 countries, with only Greece exhibiting higher diversity. Haplotype H22 was prominent in Korea, H1 was prominent in China, Greece, Hungary, Italy, Canada, and USA, and H3 was prominent in France and Switzerland. Of the 18 haplotypes found in Korea, 1 was shared with China (H2) and 1 with Greece (H22). Haplotype diversity patterns showed that Korean populations were genetically distinct from populations in China, Europe, and North America. This suggested that populations in Europe and North America arose through multiple invasions from China and that (with the exception of Greece), Korean populations did not spread to other countries. This study represents a comprehensive analysis of *H. halys* populations in Korea and places these populations in a global context that includes other native populations in East Asia and invasive populations in Europe and North America.

Key Words: Brown marmorated stink bug, genetic diversity, haplotype, mitochondrial *cytochrome oxidase I*

Resumo

El chinche marmorino café *Halyomorpha halys* es un insecto plaga invasor de América del Norte y Europa que ataca a las especies de cultivos y causa importantes daños económicos. Para evaluar la diversidad genética y las distribución de diferentes poblaciones de *H. halys* en Asia Oriental, América del Norte y Europa, se compararon las secuencias de *COI* obtenidas de 79 nuevos especímenes de Corea y 10 de EE. UU. con 725 secuencias de *COI* existentes. En total, se detectaron 45 haplotipos en poblaciones de 10 países. Seis haplotipos de Corea (H34-H49) y dos de los EE.UU. (H50 y H51) fueron nuevos. Las poblaciones coreanas exhibieron la segunda mayor diversidad entre los 10 países, con sólo Grecia exhibiendo una mayor diversidad. El haplotipo H22 fue prominente en Corea, H1 fue prominente en China, Grecia, Hungría, Italia, Canadá y los EEUU, y H3 fue prominente en Francia y Suiza. De los 18 haplotipos encontrados en Corea, uno fue compartido con China (H2) y uno con Grecia (H22). Los patrones de diversidad de haplotipos mostraron que las poblaciones coreanas son genéticamente distintas de las poblaciones de China, Europa y Norteamérica. Esto sugiere que las poblaciones en Europa y América del Norte surgieron a través de múltiples invasiones de China y que (con la excepción de Grecia), las poblaciones coreanas no se esparcieron a otros países. Este estudio representa un análisis exhaustivo de las poblaciones de *H. halys* en Corea y coloca a estas poblaciones en un contexto global que incluye otras poblaciones nativas de Asia Oriental y poblaciones invasoras en Europa y América del Norte.

Palabras Clave: chinche marmorino café, diversidad genética, haplotipo, citocromo oxidasa mitocondrial I, Corea

The brown marmorated stinkbug, *Halyomorpha halys* (Stål, 1855) (Hemiptera: Pentatomidae), is native to East Asia and is widely distributed in Korea, China, Taiwan, and Japan. *Halyomorpha halys* is an invasive insect in North America (Hoebeke & Carter 2003) and Europe (Wermelinger et al. 2008; Haye et al. 2014a). Approximately 120 host plant species for *H. halys* were noted to date, including several ornamental

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[1] Department of Plant Medicine and Institute of Agriculture & Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea; E-mail: wonhoon@gnu.ac.kr (W. L.)

[2] Department of Life Sciences, University of Modena and Reggio Emilia, via G. Campi 213/d, 41125, Modena, Italy; E-mail: roberto.guidetti@unimore.it (R. G.); michele.cesari@unimore.it (M. C.)

[3] Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, London, Ontario N5V 4T3, Canada; E-mail: tara.gariepy@agr.gc.ca (T. D. G.)

[4] Entomology Program, Division of Plant and Soil Sciences, West Virginia University, Morgantown, West Virginia 26506, USA; E-mail: Yong-Lak.Park@mail.wvu.edu (Y. L. P.)

[5] Crop Protection Division, Department Agro-Food Safety and Crop Protection, National Instititue of Agricultural Sciences, RDA, Jeollabuk-do 55365, Republic of Korea; E-mail: changgpark@korea.kr (C. G. P.)

*Corresponding author; Email: changgpark@korea.kr

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plants and fruit and vegetable crops (Leskey et al. 2012a, b; Haye et al. 2014a). *Halyomorpha halys* is not a severe agricultural pest in its native East Asia, but causes substantial economic losses in fruits and field crops in North America and Europe, where it is considered a serious pest (Leskey et al. 2012b; Rice et al. 2014). *Halyomorpha halys* has been recorded outside its original geographic range in Greece (Milonas & Partsinevelos 2014), Switzerland (Haye et al. 2014b), France (Callot & Brua 2013), Italy (Pansa et al. 2013; Maistrello et al. 2014; Cesari et al. 2015), Hungary (Vétek et al. 2014), Romania (Macavei et al. 2015), USA (Xu et al. 2014), Canada (Haye et al. 2015), and India (Nikam & More 2016), and interception of this species was reported in Australia and New Zealand (Walker 2009; Duthie 2012; MacLellan 2013; Ward 2013). *Halyomorpha halys* is one of the most serious pest insects in the world.

A genetic bottleneck effect was reported in invasive populations of *H. halys* in North America and Europe (Gariepy et al. 2014a, b; Xu et al. 2014). Invasive species sometimes exhibit low genetic variability during colonization of a new environment (Mayr 1954; Sax & Brown 2000). However, the bottleneck effect in *H. halys* in North America and Europe did not prevent successful establishment in those areas. The bridgehead effect, where several global invasion incidents occur subsequent to an initial invasion event (Lombaert et al. 2010; Handley et al. 2011), suggests that widespread invasions can stem from a single particularly successful invasive population that acts as the source of colonists for remote new territories (Lombaert et al. 2010). Understanding the genetic diversity of worldwide *H. halys* populations will aid understanding of invasion and dispersion events in Europe and North America.

Mitochondrial gene sequences were recently used to examine the origins and spread of invasive *H. halys* in North America and Europe (Gariepy et al. 2014b, 2015; Xu et al. 2014; Cesari et al. 2015). Xu et al. (2014) analyzed genetic variation in *H. halys* collected in Asia and USA using 3 mitochondrial genes: *cytochrome c oxidase subunit II* (*COII*), *12S ribosomal RNA*, and *cytochrome c oxidase subunit I* (*COI*). Gariepy et al. (2014b, 2015) and Cesari et al. (2015) analyzed *COI* sequences to evaluate the genetic diversity of *H. halys* among European countries. These studies suggested that *H. halys* samples collected in Europe and North America were introduced from East Asian countries, primarily China (Gariepy et al. 2014b, 2015; Xu et al. 2014). However, relatively few specimens from autochthonous regions of Korea and Japan were included in these analyses. For example, only 1 Korean sample and 1 Japanese sample were included in 1 study (Gariepy et al. 2014b), and these were intercepted at Canadian ports and no information was provided regarding their local origins in Korea and Japan. Xu et al. (2014) included 8 Korean and 20 Japanese specimens, but samples were collected only from a single region in each country (Suwon in Korea and Tsukuba in Japan). Additional sampling of East Asian *H. halys*, particularly from Korea and Japan, is needed to trace the origins of *H. halys* establishment in Europe and North America. In this study, a new analysis of 79 specimens collected from Korea and 10 specimens collected from the USA was conducted. Sequences were compared to previously published data to produce an expanded analysis of *H. halys* genetic diversity. This analysis sheds light on the pathways of continued introduction and spread of *H. halys* in Europe and North America.

Materials and Methods

COLLECTION OF SPECIMENS

In total, 79 *H. halys* specimens were collected from autochthonous areas of Korea in 7 provinces (Chungcheongnam-do, Chungcheongbuk-do, Gyeonggi-do, Gyeongsangnam-do, Gyeongsangbuk-do, Gangwon-do, and Jeollabuk-do) during 2011 to 2013. Ten specimens of *H. halys* were collected from 3 states in the USA (West Virginia, New Jersey, and Maryland). Specimen details such as collection site (Fig. 1) and date are provided in Supplementary Table S1. All samples were hand-picked or captured in the field and either placed in 100% ethanol immediately upon collection or kept alive until processing. To avoid genetic contamination, each specimen was washed in running distilled water at least 5 times, then preserved in 100% ethanol at –80 °C until needed for DNA extraction and genetic analysis. Voucher specimens are stored in 100% ethanol at –80 °C in the collection of the Institute of Insect Sciences at the National Academy of Agricultural Science, Korea.

DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

Genomic DNA was extracted from each specimen (usually from 1 hind leg) using a DNeasy Blood & Tissue Kit (Qiagen, Inc., Dusseldorf, Germany) as described by the manufacturers. A 615 bp section

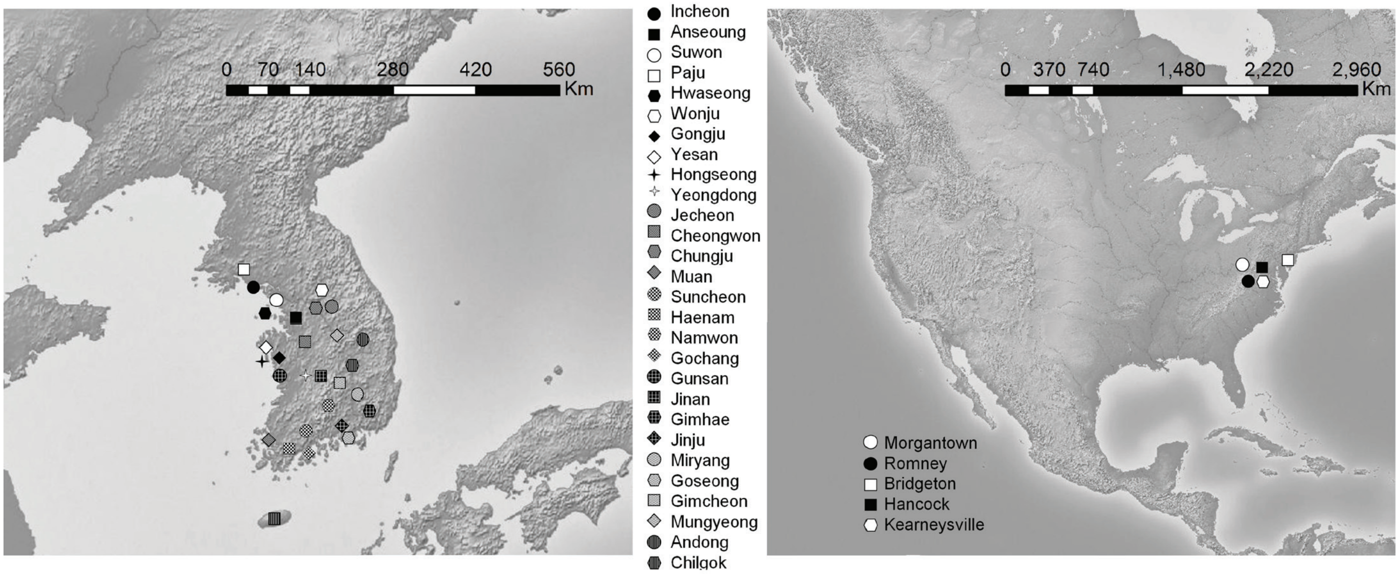

Fig. 1. *Halyomorpha halys* collection sites in Korea and the USA.

of the *COI* gene was amplified using primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994) with AccuPowerH PCR PreMix (Bioneer Corp., Daejeon, Korea) in 20 µl reaction mixtures containing 0.4 µM of each primer and 0.05 µg of genomic DNA template. PCR was performed using a GS1 thermo-cycler (Gene Technologies, Ltd., Essex, United Kingdom) with the following cycle: 95 °C for 5 m (initial denaturation); 34 cycles at 95 °C for 30 s, 45 to 50 °C for 30 s, 72 °C for 30 s; and 72 °C for 5 m (final extension). PCR products were analyzed using 1.5% agarose gel electrophoresis and purified using a QIAquick PCR purification kit (Qiagen, Inc.). Purified PCR products were directly sequenced in both directions using an automated sequencer (ABI PrismH 3730 XL DNA Analyzer). Chromatograms were examined for miscalls and ambiguities and assembled into contigs using SeqMan™Pro (version 7.1.0, 2006, DNAstar Inc., Madison, Wisconsin, USA). Sequences were individually checked to avoid the use of pseudogenes from protein coding frame-shifts (Zhang & Hewitt 1996). Consensus files were aligned using Clustal X 1.83 (Thompson et al. 1997). The 89 new *COI* sequences generated from the 89 *H. halys* individuals were deposited in GenBank (Supplementary Table S1).

MOLECULAR DIVERSITY OF HALYOMORPHA HALYS HAPLOTYPES

The *COI* sequences generated from the 89 *H. halys* specimens collected in this study were grouped according to their geographic area of collection in Korea and the USA. To produce comprehensive, up-to-date, diversity measures for a wide range of locations, 725 previously published *COI* sequences (Gariepy et al. 2014b, 2015; Cesari et al. 2015) also were analyzed. *COI* sequences without collection information were excluded from analysis. Datasets comprised sequences from 10 countries: Canada, USA, Japan, China, Korea, Hungary, France, Switzerland, Italy, and Greece. Measures of genetic diversity (number of haplotypes, number of haplotypes unique to a given geographical area, haplotype diversity, and nucleotide diversity) were calculated for each country in a geographical area using DnaSP v5.10.01 (Librado & Rozas 2009) and ARLEQUIN v3.1. The software package TCS v1.21 (Clement et al. 2000) was used to construct statistical parsimony cladogram networks of *H. halys COI* haplotypes for the new specimens from Korea and the USA. To place the newly collected sequences in a broader context, the same approach was used to generate a global haplotype network that also included data from previous collections in East Asia, Europe, and North America (Gariepy et al. 2014b, 2015; Cesari et al. 2015).

Results

A 615 bp region of the *COI* gene was amplified from 89 *H. halys* specimens from Korea (79 samples) and the USA (10 samples). Sixteen haplotypes from Korea (H34–H49) and 2 from the USA (H50–H51) were novel. Haplotype denotations followed Gariepy et al. (2014b, 2015), and Cesari et al. (2015). Comparisons between haplotypes identified from the 89 samples and previously reported haplotypes are shown in Table 1. Of the 45 different haplotypes observed in this and previous studies, the 2 most frequently occurring haplotypes constituted 43.37 % (H3) and 35.01% (H1) of the 814 *COI* sequences. Haplotype H1 was detected in North America (Canada and USA), Europe (France, Greece, Hungary, Italy, and Switzerland), and Asia (China). Haplotype H3 was observed only in Europe (France, Greece, Hungary, Italy, and Switzerland), and Asia (China). The remaining 43 haplotypes (H2–H23 and H30–H51) were relatively rare, constituting 0.28 to 4.27% of the 814 *COI* sequences.

Table 1. Details of newly collected and previously identified specimens from East Asia, Europe, and North America (Gariepy et al. 2014b, 2015; Cesari et al. 2015) and their *COI* haplotypes. Novel haplotypes identified in this study are shown in bold text.

			Haplotype																																										Total
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	30	31	32	33	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**	
Native range	East Asia	Korea		15																				41						2	1	1	1	1	5	2	1	1	3	1	1	1	1	1	1			80
		China	75	1	14	1	1	1	3			1	1	1	3	1	1	1	1	1	1	1	1																									110
		Japan																							1																							1
Non-native range	Europe	France	1		136					2																																						139
		Greece	18		4																			2		1	1	8	23																			57
		Hungary	83		1																																											84
		Italy	32		7					1																																						40
		Switzerland	2		191					31	1																																					225
	North America	Canada	49					1								1																																51
		USA	25																																											1	1	27
Total			285	16	353	1	1	2	3	34	1	1	1	1	3	2	1	1	1	1	1	1	1	43	1	1	1	8	23	2	1	1	1	1	5	2	1	1	3	1	1	1	1	1	1	1	1	814

The occurrence and frequency of each haplotype in each of 10 countries is shown in Table 1 (data combined with results from previous studies [Gariepy et al. 2014b, 2015; Cesari et al. 2015]). The highest numbers of different haplotypes were observed in Korea and China, which exhibited 18 (40%) and 19 (42.2%) of the 45 haplotypes, respectively. The 2 most common haplotypes in Korea were H22 (51.2 %) and H2 (18.7%), followed by the novel haplotypes H39 (6.2 %), H43 (3.7%), H34 (2.5%), and H40 (2.5%). Haplotypes H35–H38, H41–H42, and H44–H49 each occurred once. A private haplotype (H23) was seen in the single Japanese specimen examined. Three haplotypes were observed in each of the USA and Canada (H1, H50, and H51, and H1, H6, and H14, respectively). The H1 haplotype, which was seen in both the USA and Canada, was predominant in the USA (92.5%). In Europe, Greece exhibited the highest diversity, with 7 haplotypes (H1, H3, H22, and H30–H33). Four haplotypes were found in Switzerland (H1, H3, H8, and H9), 3 haplotypes were found in France and Italy (H1, H3, and H8), and only 2 haplotypes were seen in Hungary (H1 and H3).

Genetic diversity measures for *H. halys*, including haplotype diversity and nucleotide diversity (Nei & Li 1979), are shown in Table 2. Haplotype and nucleotide diversities were 0.703 ± 0.049 (SD) and 0.00173 ± 0.00019 (SD), respectively, in the Korean samples isolated in this study. Genetic diversity in the Korean population was higher than in the Chinese population and was 2nd only to Greece among the 10 countries examined. Genetic diversity in Canada, Hungary, and France was considerably lower than in other countries (Table 2).

A haplotype network for the 841 *COI* sequences is shown in Fig. 2, alongside their placement within a global haplotype network.

Discussion

Low genetic variability in the *COI* gene was observed in the introduced populations of *H. halys* in European and North American countries examined to date (Gariepy et al. 2014b, 2015; Xu et al. 2014; Cesari et al. 2015). Higher diversity was seen in populations of *H. halys* in its native East Asian range, where 19 *COI* haplotypes were previously documented (Gariepy et al. 2014b). However, to date, studies of *H. halys* genetic diversity in East Asia focused on samples from China, hampering attempts to determine the origins of global *H. halys* diversity. To provide a broader perspective, this study examined *H. halys* specimens collected in the USA and several native areas in Korea. Genetic diversity was assessed using *COI* sequences from the collected specimens and from previously characterized populations from East Asia (Japan, Korea, and China), North America (USA and Canada), and Europe (France, Greece, Italy, Hungary, and Switzerland). The specimens collected in Korea and the USA for this study harbored novel haplotypes as well as previously identified haplotypes (Gariepy et al. 2014b, 2015; Cesari et al. 2015).

Twenty haplotypes (H1–H7 and H10–H22) from East Asia, 9 haplotypes (H1, H3, H8, H9, H22, and H30–H33) from Europe, and 3 haplotypes (H1, H6, and H14) from North America were identified previously (Gariepy et al. 2014b, 2015; Cesari et al. 2015). Twenty-one haplotypes were identified from samples collected in Korea (H2, H22, and H34–H49) and the USA (H1, H50, and H51) in this study. The increased sampling in these countries facilitated the detection of 16 (H34–H49) and 2 (H50 and H51) novel haplotypes in Korea and USA, respectively.

Forty-five haplotypes were identified in total from this and previous studies (Gariepy et al. 2014b, 2015; Cesari et al. 2015). Examination of geographic distribution demonstrated that 2 haplotypes, H1 and H3, occurred more frequently and were more widespread than other haplotypes. Both the H1 and H3 haplotypes were found in China and Europe (France, Greece, Hungary, Italy, and Switzerland), and haplotype H1 was also detected in the USA and Canada. Haplotype H1 was more prominent in China, Greece, Hungary, Italy, Canada, and the USA, whereas haplotype H3 was more prominent in France and Switzerland.

As would be expected for samples collected in native areas, populations from Korea and China contained higher numbers of haplotypes than populations from the other countries. Eighteen haplotypes were identified from 80 *COI* sequences in samples from Korea. Haplotypes H2 and H22, which occurred most frequently, were observed previously. Haplotype H22 was previously found in samples from Greece (Gariepy et al. 2014b, 2015), and haplotype H2 was reported in China (Gariepy et al. 2014b). The remaining 16 haplotypes (H34–H49) were novel, and were not previously described in the literature or published in publicly available sequence databases. Six of the 16 novel haplotypes were identified in multiple specimens (H2, H22, H34, H39, H40, and H43) and the remaining 10 haplotypes were found in single individuals. Nineteen haplotypes were identified from 110 *COI* sequences in samples from China, with haplotype H1 occurring most frequently. With the exception of haplotype H1, most Chinese haplotypes were found in 1 to 2 specimens. Of the 36 haplotypes identified in East Asia, only 1 haplotype (H2) was found in both the Korean population (15 of 80 specimens) and the Chinese population (1 of 110 specimens).

Halyomorpha halys genetic diversity measures differed between Korea and the 2 other Asian countries examined in this study (China and Japan), although it should be noted that only one *COI* sequence from Japan was included. In addition, although haplotype H22 was shared between Korea and Greece, diversity measures in the native range of *H. halys* in Korea differed substantially from those in invaded areas in Europe and North America (Gariepy et al. 2014b; Xu et al. 2014). However, further sampling from native areas (e.g., China, Taiwan, Korea, and Japan) would provide a more accurate picture of *H.*

Table 2. Genetic diversity measures for *Halyomorpha halys* populations from East Asia, Europe, and North America assessed using a 615 bp *COI* sequence. Specimens were newly collected or were identified previously (Gariepy et al. 2014b, 2015; Cesari et al. 2015).

Country	Number of specimens	Polymorphic site(s)	Number of haplotypes	Number of unique haplotype(s)	Mean haplotype diversity (± SD)	Mean pairwise nucleotide differences (± SD)	Mean nucleotide diversity (± SD)
Korea	80	17	18	16	0.703 ± 0.049	1.063 ± 0.711	0.00173 ± 0.00019
China	110	16	19	14	0.521 ± 0.056	0.745 ± 0.555	0.00121 ± 0.00018
Japan	1			1			
USA	27	2	3	2	0.145 ± 0.090	0.148 ± 0.217	0.00024 ± 0.00015
Canada	51	2	3		0.078 ± 0.051	0.078 ± 0.152	0.00013 ± 0.00008
France	139	3	3		0.043 ± 0.024	0.072 ± 0.144	0.00012 ± 0.00007
Greece	57	8	7		0.724 ± 0.036	2.341 ± 1.297	0.00381 ± 0.00017
Hungary	84	1	2		0.024 ± 0.023	0.024 ± 0.082	0.00004 ± 0.00004
Italy	40	3	3		0.337 ± 0.082	0.428 ± 0.395	0.00070 ± 0.00023
Switzerland	225	5	4	1	0.261 ± 0.035	0.549 ± 0.454	0.00089 ± 0.00012

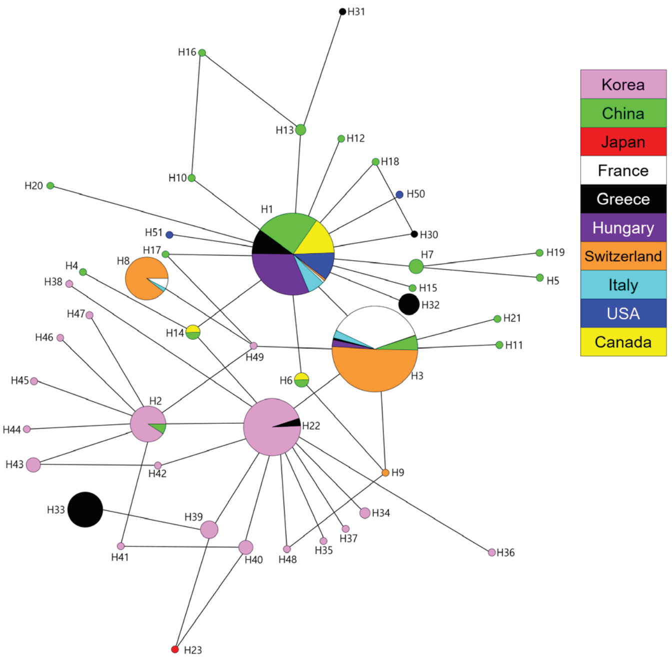

Fig. 2. Statistical parsimony cladogram network representing relationships among the 45 haplotypes for a 615 bp fragment of the *COI* gene of *Halyomorpha halys*. Each circle is labeled with haplotype number, and the size of each circle is proportional to the frequency of each haplotype [H3 (n = 353); H1 (n = 285); H22 (n = 43); H8 (n = 34); H33 (n = 23); H2 (n = 16); H32 (n = 8); H7, H9–H13, and H43 (n = 3); H6, H14, H34, H39, and H40 (n = 2); H4–H5, H12, H15–H21, H23, H30–H31, H35–H38, H41, H42, and H44–H51 (n = 1)]. Differing colors indicate countries in which samples were collected.

halys diversity in East Asia and provide a context for dispersion to other parts of the world. For example, Gariepy et al. (2015) suggested that co-occurrence of haplotype H22 pointed towards introduction and establishment of a population in Greece from Korea.

The global haplotype network (Fig. 2) was divided into 2 large clusters: 1 comprised of Korean and Japanese specimens, and the other comprised of Chinese, European, and North American specimens. Only Greek samples were present in both clusters. This pattern suggests that the *H. halys* populations in Europe and North America derive from multiple invasion events from China. It is also possible to hypothesize that, with the exception of Greece, *H. halys* populations originating from Korea are not widespread in other countries, although a specimen from Korea with haplotype H22 was intercepted in Canada (Gariepy et al. 2014b).

This study represents a comprehensive analysis of *H. halys* populations in Korea and places these populations in a global context that includes other native populations in East Asia and invasive populations in Europe and North America. This study contributes to our understanding of the global distribution of known *H. halys* haplotypes. The novel haplotypes identified in this study contributed to revised estimates of *H. halys* diversity, indicating that additional sampling moving outwards from points of detection is needed to further refine estimates of haplotype diversity in invasive populations in Europe and North America.

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