Genetic Diversity of Halyomorpha halys (Hemiptera, Pentatomidae) in Korea and Comparison with COI Sequence Datasets from East Asia, Europe, and North America

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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

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**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

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**Resumo**

El chinche marmolino 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 la 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 haplótipo H22 fue predominante en Corea, H1 fue predominante en China, Grecia, Hungría, Italia, Canadá y los EEUU, y H3 fue predominante 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 marmolino café, diversidad genética, haplótipo, citocromo oxidasa mitocondrial I, Corea
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. 2012a, b; Haye et al. 2014). Halyomorpha halys has been recorded outside its original geographic range in Greece (Milonas & Partisinevelos 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
of the COI gene was amplified using primers LCO1490 (5'-GGTCAA-
CAATATAAAGATATG-3') and HCO2198 (5'-TAAACTCAGGGTGAC-
CAAAACTA-3') (Folmer et al. 1994) with AccuPower 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 thermocycler (Gene Technologies, Ltd., Essex,
United Kingdom) with the following cycle: 95 °C for 5 m (initial dena-
turation); 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 se-
quenced in both directions using an automated sequencer (ABI PrismH
3730 XL DNA Analyzer). Chromatograms were examined for miscal-
s and ambiguities and assembled into contigs using SeqMan™Pro (ver-
sion 7.1.0, 2006, DNASTAR Inc., Madison, Wisconsin, USA). Sequences
were individually checked to avoid the use of pseudogenes from pro-
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 col-
llected 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 previ-
ously published COI sequences (Gariepy et al. 2014b, 2015; Cesari et
al. 2015) also were analyzed. COI sequences without collection infor-
mation 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 previ-
ous 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.

| Native range | Haplotypes | Total |
|--------------|------------|-------|
| East Asia    | Korea      | 15    |
|              | Japan      | 75    |
|              | China      | 1     |
|              | France     | 1     |
|              | Greece     | 1     |
|              | Germany    | 1     |
|              | Hungary    | 1     |
|              | Italy      | 1     |
|              | Switzerland| 1     |
|              | Total      | 89    |
| Non-native range | Haplotypes | Total |
| Europe       | France     | 18    |
|              | Germany    | 83    |
|              | Italy      | 32    |
|              | Switzerland| 225   |
|              | Total      | 225   |
| North America | Haplotypes | Total |
| USA          | Canada     | 49    |
|              | USA        | 25    |
|              | Total      | 74    |
| Total        |            | 452   |
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 most 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).

*Halymorpha 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. halys*.

### Table 2. Genetic diversity measures for *Halymorpha 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                   | 1                    | 1                             | 0.145 ± 0.090                   | 0.148 ± 0.217                           | 0.00024 ± 0.00015                |
| USA         | 27                  | 2                   | 3                    | 2                             | 0.078 ± 0.051                   | 0.078 ± 0.152                           | 0.00013 ± 0.00008                |
| Canada      | 51                  | 2                   | 3                    | 3                             | 0.043 ± 0.024                   | 0.071 ± 0.144                           | 0.00012 ± 0.00007                |
| France      | 139                 | 3                   | 3                    | 3                             | 0.724 ± 0.036                   | 2.341 ± 1.297                           | 0.00381 ± 0.00017                |
| Greece      | 57                  | 8                   | 7                    | 7                             | 0.024 ± 0.023                   | 0.024 ± 0.082                           | 0.00004 ± 0.00004                |
| Hungary     | 84                  | 1                   | 2                    | 2                             | 0.337 ± 0.082                   | 0.428 ± 0.395                           | 0.00070 ± 0.00023                |
| Italy       | 40                  | 3                   | 3                    | 3                             | 0.261 ± 0.035                   | 0.549 ± 0.454                           | 0.00089 ± 0.00012                |
| Switzerland | 225                 | 5                   | 4                    | 1                             | 0.724 ± 0.036                   | 2.341 ± 1.297                           | 0.00381 ± 0.00017                |
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 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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