

Tracing the origin of US brown marmorated stink bugs, *Halyomorpha halys*

Jiawu Xu · Dina M. Fonseca · George C. Hamilton ·
Kim A. Hoelmer · Anne L. Nielsen

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Abstract Identifying the origin of a biological invasion has important applications to the effective control of the invaders. This is more critical for invasive agricultural pests that cause severe economic losses. The brown marmorated stink bug, *Halyomorpha halys*, originally from East Asia, has become a principal agricultural pest in the US since its first detection in Pennsylvania in 1996. This species is responsible for crop failures on many mid-Atlantic farms and current control efforts rely on heavy insecticide applications

because no other options are available. To examine the genetic diversity and identify the source region of the US introductions, we sequenced portions of the mitochondrial cytochrome *c* oxidase subunit II gene, 12S ribosomal RNA gene and control region in populations from the US, China, South Korea and Japan. We detected high genetic divergence among native populations and traced the origin of US *H. halys* to the Beijing area in China. We observed much lower genetic diversity in exotic compared to native populations—two mitochondrial haplotypes in 55 US specimens versus 43 haplotypes in 77 native specimens. A single introduction of small propagule size matches the invasion history in the US. For the effective control of the US population, we suggest that surveys on egg parasitoids and insecticide resistance in natives should focus on the Beijing area in China.

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J. Xu · D. M. Fonseca (✉) · G. C. Hamilton ·
A. L. Nielsen
Department of Entomology, Rutgers University, 93
Lipman Dr., New Brunswick, NJ 08901, USA
e-mail: dinafons@rci.rutgers.edu

A. L. Nielsen
e-mail: nielsen@AESOP.Rutgers.edu

J. Xu · D. M. Fonseca
Center for Vector Biology, Rutgers University, 180 Jones
Ave., New Brunswick, NJ 08901, USA

K. A. Hoelmer
USDA ARS Beneficial Insects Introduction Research
Unit, 501 South Chapel St., Newark, DE 19713, USA

A. L. Nielsen
Rutgers Agricultural Research and Extension Center, 121
Northville Rd., Bridgeton, NJ 089302, USA

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Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), also called the yellow–brown stink bug and referred to as *H. picus* or *H. mista* in earlier Asian literature, is native to East Asia (China, Korea and Japan), and was reported in the

United States for the first time in 1996 from Allentown, Pennsylvania (Hoebeke and Carter 2003). Since then, this species has also been detected in Switzerland and Canada (Quebec and Ontario), and intercepted in New Zealand (Wermelinger et al. 2008; Harris 2010; Fogain and Graff 2011). In its native range, *H. halys* is an occasional agricultural pest of tree fruits and soybeans (Hoffman 1931; Kobayashi et al. 1972; Funayama 2004). In the US, it is now a widespread agricultural pest occurring in all states east of the Mississippi River as well as several west coast states (Leskey et al. 2012). *H. halys* has become the dominant pentatomid species in many mid-Atlantic areas (Nielsen and Hamilton 2009; Nielsen et al. 2011), causing \$37 million loss to apples in 2010 (Seetin 2011) as well as unreported losses to a variety of ornamentals, vegetables and field crops (Leskey and Hamilton 2010; Kuhar et al. 2012). Moreover, great potential for similar economic losses exists in southern, western and mid-western states (Holtz and Kamminga 2010).

Pest management programs, especially for invasive agricultural species, are complex programs developed over many years to minimize insecticide use and costs. The severe agricultural damage by *H. halys* has led many farmers to rely heavily on insecticides with up to fourfold increase in applications (Leskey et al. 2012), increasing the risk of insecticide resistance and concerns regarding food safety and environmental pollution. Alternative management strategies such as biological control are in urgent need but lacking. Traditionally, natural enemies of a pest, either endemic or purposefully introduced, have been used to control invasive populations (Kidd and Jarvis 2007). Several natural enemies of *H. halys*, primarily hymenopteran egg parasitoids, have been reported in Japan (Kawada and Kitamura 1992; Arakawa and Namura 2002) and China (Yang et al. 2009). The predominant egg parasitoid of *H. halys* in northern China is *Trissolcus halyomorphae* (Hymenoptera: Scelionidae), with an average parasitism rate of 50 % (Yang et al. 2009). In the US, endemic enemies have minimal impact on *H. halys*, thus surveys for egg parasitoids are ongoing in native East Asia. To obtain useful knowledge on insecticide resistance and efficient natural enemies of an invasive pest, a prerequisite is to identify its source region(s), where surveys can be started subsequently.

Molecular genetic markers, both mitochondrial and nuclear ones, are powerful tools to examine diversity

and define the source region(s) of an invasive pest, and thus to understand the mechanisms behind invasions (Ficetola et al. 2008). For these purposes, extensive and representative sampling in both native and exotic ranges as well as employing informative genetic markers is the key to success. In the case of invasions originated from divergent and structured native populations, the exact source region(s) and multiple introductions can often be identified (e.g., Kolbe et al. 2004); otherwise, the source region may be defined to a large native area and multiple introductions may be indiscernible.

The present study aims to (1) examine the genetic structure and assess the genetic diversity of *H. halys* in native East Asia and in the US, (2) identify the source region(s) of the US introductions, and (3) estimate the number of introductions and the likely size of founding populations. We also discuss possible mechanisms underlying the rapid infestation of *H. halys* in the US. The results of this study have important applications to the efficient control of this invasive agricultural pest.

Materials and methods

Specimens of *H. halys*

Halyomorpha halys specimens were collected in East Asia and across the US in 2004–2008 (Table 1; Fig. 1). Adults and large nymphs were preferred over small nymphs whenever possible to decrease the possibility of sampling siblings. Native *H. halys* was sampled from seven locations in China—Beijing (BJCN, Haidian District in Beijing), Xi'an (XACN, Shaanxi Province), Xuzhou (XZCN, Jiangsu Province), Nanjing (NJCN, Jiangsu Province), Hefei (HFCN, Anhui Province), Fuzhou (FZCN, Fujian Province) and Kunming (KMCN, Yunnan Province), three nearby sites combined into one sample in South Korea—Seoul/Suwon area (SWKR, Gyeonggi Province), and one location in Japan—Tsukuba City (TKJP, Honshu), with sample size of 5–21. These samples were collected from urban ornamentals, botanical gardens, agricultural crops and urban structures during the fall when *H. halys* aggregated prior to overwintering. In locations Xi'an, Fuzhou and Kunming, which are at the edges of its distribution in China (Zhu et al. 2012), all nymphs were included

because it was difficult to find *H. halys* even on the well-known host plants such as *Paulownia tomentosa*.

The US *H. halys*, all adults, were obtained from Massachusetts (MA: town of Bridgewater, Plymouth County), New York (NY: New York City, New York; Staten Island, Richmond), New Jersey (NJ: Mt. Royal, Gloucester; Basking Ridge, Somerset; Freehold, Monmouth; Hillsborough, Somerset; Somerset, Somerset), Pennsylvania (PA: Edgeworth, Allegheny; Monaca, Beaver; Allentown, Lehigh), Maryland (MD: Bethesda, Montgomery; Silver Spring, Montgomery; Rockville, Montgomery; Hagerstown, Washington; Williamsport, Washington), Delaware (DE: Dover, Kent; Wilmington, New Castle), West Virginia (WV: Shepherdstown, Jefferson), Virginia (VA: Lynchburg, Lynchburg; Roanoke, Roanoke), Mississippi (MS: Stoneville, Cleveland) and California (CA: San Marino, Los Angeles), with 1–6 specimens from each county and 2–12 from each state (Fig. 1). Most of these specimens were donated by the general public, through a web based reporting system (www.njaes.rutgers.edu/stinkbug) that we developed for documenting the distribution of *H. halys*. This website derives its usefulness from the fact that in the US, adult *H. halys* overwinters inside residences. The general public is an excellent source of information regarding the spread

and infestation levels of *H. halys*, reporting thousands of sightings annually and mailing us many of the specimens used in this study. Specimens were either stored at -20°C or in 95 % ethanol, and deposited in the Rutgers University Insect Museum. Genomic DNA was extracted from thoracic tissue with DNeasy tissue kit (Qiagen, Valencia, CA) following the manufacturer's procedures. For testing the possible infection with *Wolbachia*, DNA was also extracted from the ovaries of a few specimens (see below).

Genetic data

We sequenced portions of three mitochondrial DNA genes, the cytochrome *c* oxidase subunit II (COII), and a fragment starting on the 12S ribosomal RNA and spanning part of the control region (12S/CR). To further narrow down the source region(s), sequences of cytochrome *c* oxidase subunit I (COI) gene were also collected from several select specimens. Nuclear markers were not included because highly variable markers such as microsatellites are still lacking for the species. Considering the infection of cytoplasmic parasites such as *Wolbachia* in other Pentatomidae species (Kikuchi et al. 2008), which leads to a decreased mtDNA variability due to selective sweeps

Table 1 Sample information and mtDNA diversity of *H. halys*

Location	Years	Abbreviation	<i>N</i>	<i>Hn</i>	<i>Hd</i>	$\theta\pi$
Beijing, China	2006	BJCN	6	5	0.93	0.0015
Xi'an, China	2006, 2007	XACN	5	3	0.4	0.0026
Xuzhou, China	2006	XZCN	6	6	1	0.0038
Nanjing, China	2006	NJCN	13	9	0.91	0.0021
Hefei, China	2006	HFCN	7	4	0.81	0.0011
Fuzhou, China	2006	FZCN	6	2	0.33	0.0012
Kunming, China	2007	KMCN	5	3	0.7	0.0011
Suwon, South Korea	2005	SWKR	8	7	0.96	0.0023
Tsukuba, Japan	2007	TKJP	21	10	0.87	0.0027
Massachusetts, US	2007	MAUS	6	2	0.33	0.0003
New York, US	2007	NYUS	3	1	0	0
New Jersey, US	2007, 2008	NJUS	12	2	0.17	0.0002
Pennsylvania, US	2004, 2006, 2007	PAUS	8	1	0	0
Maryland, US	2006, 2007, 2008	MDUS	9	2	0.22	0.0002
Delaware, US	2007, 2008	DEUS	3	2	0.67	0.0006
West Virginia, US	2007	WVUS	3	1	0	0
Virginia, US	2006, 2007	VAUS	6	2	0.53	0.0004
Mississippi, US	2007	MSUS	2	1	0	0
California, US	2007	CAUS	3	1	0	0

N sample size, *Hn* number of haplotypes, *Hd* haplotype diversity, $\theta\pi$ nucleotide diversity

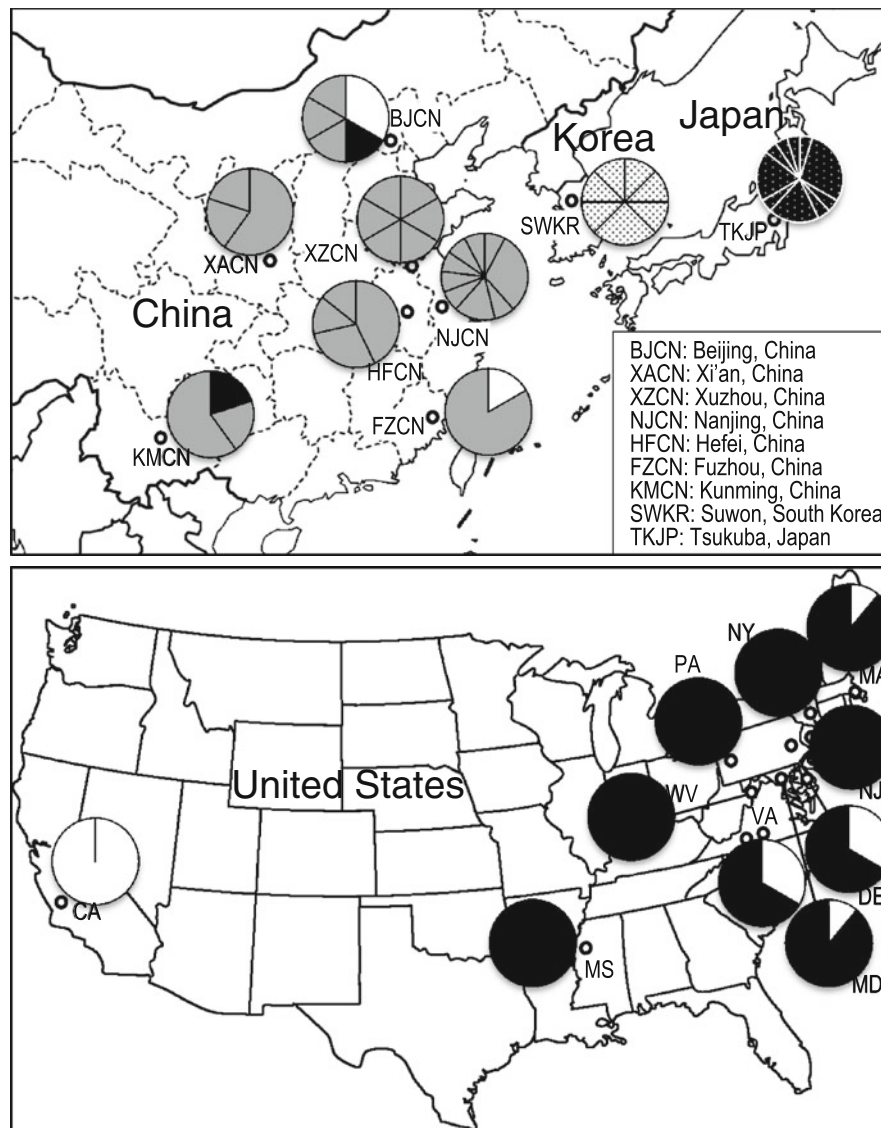


Fig. 1 Maps of East Asia and the US showing the sampling locations and mtDNA haplotype distribution of *H. halys*. Native specimens were collected from China (CN), South Korea (KR) and Japan (JP). The US specimens were sampled from Massachusetts (MA), New York (NY), New Jersey (NJ), Pennsylvania (PA), Maryland (MD), Delaware (DE), West

Virginia (WV), Virginia (VA), Mississippi (MS) and California (CA). Two haplotypes identified in the US are shown in *white* (H1) and *black* (H2). Haplotypes unique to China, South Korea and Japan are shown in *grey*, *stippled white* and *stippled black*, respectively. No shared haplotypes were detected among the three native countries

(Engelstädter and Telschow 2009), we also tested for the possible presence of *Wolbachia* in the species, particularly in the ovaries.

Mitochondrial genes

Partial COII (559 bp) and 12S/CR (592 bp) genes were amplified with primers HhalysCO2F2 (5'-TAACCC-

AAGATGCAAATTCT-3') and HhalysCO2R2 (5'-CCATATATAATTCCTGGACGA-3'), and HalCRf (5'-TTCCTAATCCTACTATTTAAGC-3') and HalCRr (5'-GGTAACTTTATAAGAGGTCG-3'), respectively. These primers were designed based on *H. halys* sequences in GenBank (AY679135 and FJ685650). Complete COI gene (1,542 bp) was amplified with primers HhalCOIF (5'-CGCCTAAAAATTCAGC

CAC-3'; newly designed) and COIR (Xu and Fonseca 2011). PCR was composed of 1 × PCR buffer, 2.5 mM of MgCl₂, 200 μM of each dNTP, 0.2 mg/ml of BSA, 0.2 μM of each primer, and 1 unit of *AmpliTaq*[®] (Applied Biosystems, Foster City, CA). A two-step PCR program, denaturation for 3 min at 96 °C followed by 10 cycles of 30 s at 94 °C, 30 s at 48 °C and 45 s at 72 °C, then 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 45 s at 72 °C, and a final extension of 10 min at 72 °C, was used to amplify COII. 12S/CR and COI were amplified with a program of denaturation for 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 45/90 s at 72 °C (12S/CR 45 s and COI 90 s), and a final extension of 10 min at 72 °C. PCR products were treated with ExoSAP-IT[®] (USB, Cleveland, OH) and cycle sequenced for analysis on an ABI 3100 automated sequencer (Applied Biosystems). Sequences were cleaned and checked with Sequencher 5.0 (Gene Codes, Ann Harbor, MI), and aligned with MUSCLE (Edgar 2004).

Wolbachia detection

The possible infection with *Wolbachia* was tested with universal primers for *Wolbachia wsp* 81F/*wsp* 691R (Zhou et al. 1998) by standard PCR, and with primers FtsZ-F/FtsZ-R by long PCR (Jeyaprakash and Hoy 2000). Standard PCR conditions were the same as above but with 35 cycles of 60 s at 94 °C, 60 s at 55 °C and 60 s at 72 °C. Long PCR amplification followed the program of Jeyaprakash and Hoy (2000). We used DNA of the mosquito *Culex pipiens* as a positive control because it is infected with *Wolbachia*. We tested specimens from native and exotic ranges, and included both thoracic tissues and ovaries of three female individuals.

Data analysis

Phylogeny and network of haplotypes

We examined the relationships among mtDNA haplotypes by creating a Bayesian phylogenetic tree with MrBayes 3.1 (Ronquist and Huelsenbeck 2003). Gene partition was used for COII and 12S/CR, and codon position (cp) partition (cp1+2 and cp3) was further used for COII. The best-fit models of sequence evolution were determined with MrModeltest (Nylander 2004). Analysis was run for 10 million

generations using 4 chains with a sampling frequency of 1/1000. The results were visualized and checked using Tracer 1.4 (Rambaut and Drummond, 2003), and a burn-in of 2,500 was discarded. Parsimony network of the haplotypes was constructed with TCS (Clement et al. 2000), using all sequences from native samples. We excluded exotic specimens to avoid sampling bias, because there were only two haplotypes identified across all the US specimens, which were observed and included in native populations (see “Results”). Nested clade analysis (NCA) for populations from China was further conducted to infer possible evolutionary processes that played significant roles in forming the present genetic structure. Associations between haplotypes and geography were tested with GeoDis (Posada et al. 2000).

Population analysis

Population genetic diversity, as indexed by the number of haplotypes (*Hn*), haplotype diversity (*Hd*) and nucleotide diversity ($\theta\pi$), was assessed with DnaSP 5.0 (Librado and Rozas 2009). Genetic differentiation among populations was estimated by the fixation index Φ_{ST} as implemented in Arlequin 3.5 (Excoffier and Lischer 2010). The most appropriate model of sequence evolution for this analysis was estimated with Modeltest (Posada and Crandall 1998), and HKY + I was the selected model. Because this model is not implemented in Arlequin, the more inclusive Tamura-Nei (TrN, Tamura and Nei 1993) model was used for all relevant analyses. We examined the statistical significance of the estimates with 10,000 permutations. Significance levels for multiple tests were adjusted with sequential Bonferroni corrections (Rice 1989). To show the relationships among populations, a principal coordinate analysis (PCA) was created with GenAlEx 6.4 (Peakall and Smouse 2006), based on pairwise genetic distances among populations. Population genetic structure was assessed with an analysis of molecular variance (AMOVA) in Arlequin. The significance of the measures was examined with 1,000 permutations.

We examined the historical demographic expansion in natives with Fu's *F_s* neutrality test (Fu 1997) and mismatch distribution based on COII gene, as implemented in DnaSP. Fu's *F_s* value is sensitive to demographic expansion, which usually leads to large negative values. Mismatch distribution was used to

distinguish between smooth unimodal distribution and multimodal distribution (Rogers and Harpending 1992), which respectively indicate historical expansion and population equilibrium. We estimated population expansion time with $\tau = 2\mu t$, where τ is the crest of mismatch distribution, μ is nucleotide substitution rate and t is the time in generations. We assumed a mutation rate of 6.2×10^{-8} per site per generation—an experimental estimation for mutation of *Drosophila* mtDNA (Haag-Liautard et al. 2008)—for the COII gene of *H. halys*, because no estimation for *H. halys* is available. To transfer the time in generations to years, we adopted a generation time of two generations a year for native *H. halys*.

Results

We identified 18 haplotypes (H1–18) defined by 17 polymorphic sites in a 534 bp COII fragment, and 20 haplotypes (H1–20) defined by 20 polymorphic sites in a 550–552 bp 12S/CR fragment (83–84 bp of 12S and 467–468 bp of CR), from a total of 132 *H. halys* specimens—48 of China, 8 of South Korea, 21 of Japan and 55 of the US (Table S1). In the US, a single COII haplotype (H1) and two 12S/CR haplotypes (H1 and H2) were detected from 55 specimens. Sequences of haplotypes were deposited in GenBank (accession # KF112000–KF112037). Mutations in COII resulted in four amino acid changes, two in Japanese specimens ($_{28}\text{Ala} \rightarrow \text{Val}$ and $_{32}\text{Thr} \rightarrow \text{Ser}$) and two in Chinese specimens ($_{63}\text{Met} \rightarrow \text{Thr}$ and $_{172}\text{Ileu} \rightarrow \text{Met}$). Four indels (insertion and deletion) and a 20 bp highly mutant region (eight mutation sites in four specimens of XACN and one specimen of TKJP) were observed in 12S/CR. The mean nucleotide difference and genetic distance (uncorrected) among haplotypes were 3.3 and 0.0062 for COII, and 4 and 0.0065 for 12S/CR. When the sequences of COII and 12S/CR were combined, we identified 43 haplotypes (H1–43) in the 132 specimens (Table 2).

In the native range, we detected 26, 7 and 10 haplotypes in China, South Korea and Japan, respectively, with 2–10 haplotypes from each location. There were no shared haplotypes among the three countries (Table 2; Fig. 1). In the 26 haplotypes from China, five were present in more than one location, i.e., H1 in BJCN and FZCN, H2 in BJCN and KMCN, H4 in BJCN, XZCN and NJCN, H12 in XZCN and NJCN,

and H13 in XZCN and FZCN. Populations XACN and HFCN contained only private haplotypes and seem to be isolated from others. In the 55 *H. halys* specimens across 10 US states, we only found two haplotypes—H1 and H2; H2 was much more common ($N = 46$) than H1 ($N = 9$). These two haplotypes coexisted in the coastal states MA, NJ, MD, DE and VA; H2 also occurred singly in the inland states PA, WV and MS as well as in NY, a coastal state, and H1 occurred singly in the west coast in CA (Fig. 1). Interestingly, in native regions, H1 and H2 only coexisted in BJCN, but also occurred separately in FZCN and KMCN. This distribution pattern pinpoints BJCN as the source origin, though we cannot completely exclude FZCN and KMCN as possible origins if multiple introductions have occurred. The COI sequences (1,542 bp), however, further excluded FZCN as the origin, because the single individual in FZCN with COII+12S/CR haplotype H1 had a different COI haplotype (H3) from those in the US, and those from BJCN and KMCN (Table S2). Two specimens of BJCN with COII+12S/CR haplotype H1 had different COI haplotypes (H1 and H2). In total, we recovered three COI haplotypes (GenBank accession # KF112038–KF112040) from nine specimens (five from China and four from the US), differing from each other at 1–2 mutation sites. KMCN and FZCN are at the edge of the species' distribution, and it was difficult to find *H. halys* there; KMCN is therefore unlikely to be an alternative source region for the US introductions. Haplotypes H1 and H2 in FZCN and KMCN were more likely a result of long distance dispersal from BJCN (possibly the precursor of intra-continental dispersal), rather than indigenous ones (see “Phylogenetic and network analyses”).

Phylogenetic and network analyses

Phylogenetic analysis revealed structuring among haplotypes, but most of the posterior probabilities supporting the topology were lower than 0.95 (not well supported; Fig. 2). Despite the low support, we observed divergence among China, South Korea and Japan, as indicated by the fact that most of their haplotypes were clustered into private lineages (Maximum Likelihood phylogeny resulted in the same topology with low support, not shown). Two lineages were also observed in China—one consisting of haplotypes (H3–19) in north China and the other

Table 2 Geographical distribution of mtDNA haplotypes of *H. halys*

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	H34	H35	H36	H37	H38	H39	H40	H41	H42	H43	Sum						
BJCN	2	1	1	1	1																																										6			
XACN						3	1	1																																								5		
XZCN			1					1	1	1	1	1																																				6		
NJCN			1								4		1	2	1	1	1	1	1																													13		
HFCN																					3	2	1	1																							7			
FZCN	1										5																																				6			
KMCN		1																							1	3																					5			
SWKR																											1	1	1	2	1	1	1															8		
TKJP																																																21		
MAUS	1	5																																													6			
NYUS																																																3		
NJUS	1	11																																														12		
PAUS																																																8		
MDUS	1	8																																														9		
DEUS	1	2																																														3		
WVUS																																																3		
VAUS	2	4																																														6		
MSUS																																																	2	
CAUS	3																																															3		
Sum	12	48	1	3	1	3	1	1	1	1	1	5	6	1	2	1	1	1	1	1	3	2	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	132			
COII	H1	H1	H2	H3	H4	H3	H3	H3	H3	H5	H1	H4	H6	H3	H7	H3	H4	H2	H3	H8	H3	H4	H1	H5	H1	H1	H9	H10	H3	H11	H1	H12	H1	H13	H14	H14	H15	H11	H13	H11	H16	H17	H18							
12S/CR	H1	H2	H1	H1	H1	H3	H4	H5	H6	H2	H7	H8	H1	H2	H8	H8	H6	H9	H9	H1	H10	H10	H10	H10	H11	H12	H13	H14	H13	H13	H15	H13	H13	H16	H4	H17	H18	H19	H20	H4	H19	H19	H19							

consisting of haplotypes in both north (H1–2, 7, 10–11 and 13–14) and south China (H20–26). FZCN in south China had two haplotypes both shared with populations in north China. A parsimony network (95 % confidence level, 14 steps) indicated that haplotypes in China, South Korea and Japan each formed a network, and the three networks were linked by one mutation (Fig. 3A). Chinese haplotypes formed a star-like network, with those from BJCN, XZCN and NJCN in the center (Fig. 3), implying north China (BJCN, XZCN and NJCN) as the center of distribution (ancestral clades) and population expansion in its demographic history. NCA for populations in China inferred restricted gene flow but with some long distance dispersal for clade 2-1, which includes all populations except HFCN (Table 3; Fig. 3B). Long distance dispersal, likely facilitated by human movement, could explain why the marginal populations FZCN and KMCN shared haplotypes with the central ones BJCN and XZCN.

Population analysis

Genetic diversity in the native populations, particularly the haplotype diversity (*Hd*), was generally high (*Hd* = 0.81–1) except in populations at the edge of the species’ distribution, i.e., XACN, FZCN and KMCN, which showed much lower diversity (*Hd* = 0.33–0.7; Table 1). In contrast, genetic diversity in the US populations was extremely low, even zero in half of the populations because only a single haplotype was

detected. This indicates that population size of the introductions was quite small, and a population bottleneck has occurred due to a founder effect upon introduction.

Strong genetic divergence was observed across all populations ($\Phi_{ST} = 0.616, P < 0.01$) or across native populations ($\Phi_{ST} = 0.518, P < 0.01$, Table 4). Pair-wise Φ_{ST} values among populations further supported their significant differentiation; populations XACN, HFCN, FZCN, SWKR, TKJP and US were isolated from each other, and from the other Chinese populations (Table 5). PCA based on population genetic distance clearly showed the existence and divergence of five distinct groups in native range (Fig. 4). We therefore separated the natives into five groups, (1) BJCN, XZCN, NJCN, HFCN and KMCN, (2) XACN, (3) FZCN, (4) SWKR and (5) TKJP, to further explore their differentiation (Table 4). The Φ_{CT} value among the five groups was 0.331 and significant, further supporting the differentiation in native populations. *H. halys* in the three native countries each formed different groups, with at least three groups observed in China—XACN, FZCN and the others.

Demographic expansion was detected in native populations with the neutrality test and mismatch distribution based on COII gene. Fu’s *F*s values for all native, Chinese, Korean and Japanese populations were negative, and significant for all natives and Korean population (Table 6). When we combined the COII and 12S/CR sequences for this analysis, the Fu’s *F*s ranged from –2.71 to –33.02 and were significant

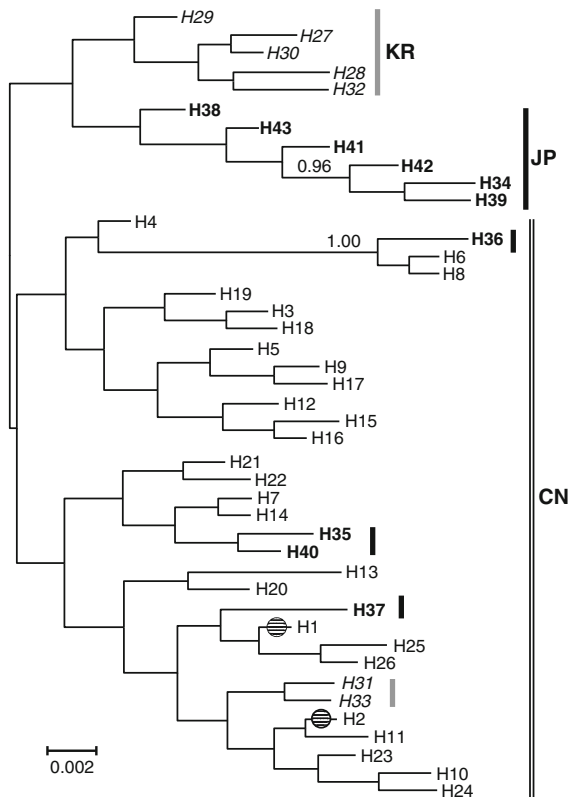


Fig. 2 Bayesian phylogenetic tree of mtDNA haplotypes of *H. halys*. Bayesian posterior probabilities >0.95 are shown above branches. Haplotypes of China, South Korea and Japan are respectively labeled with normal, *italic* and **bold fonts**, and grouped separately with *double*, *grey* and *black lines*. The two haplotypes found in the US are indicated with *lined circles*

(not shown). Mismatch distributions for all natives, Chinese, Korean and Japanese populations were unimodal and fitted well with the expected distribution under expansion model (Fig. 5). These results indicate demographic expansion in the natives. The expansions in all natives and the Japanese population were estimated to start around 11 thousand years ago (ka), and about 6 and 15 ka for the Chinese populations and the Korean population, within the interglacial time of the Holocene (0–12 ka) following the last glacial maximum (LGM, ~20 ka).

Wolbachia detection

We found no evidence for the presence of *Wolbachia* in *H. halys*, either in thoracic or ovary tissue, by both standard PCR and long PCR.

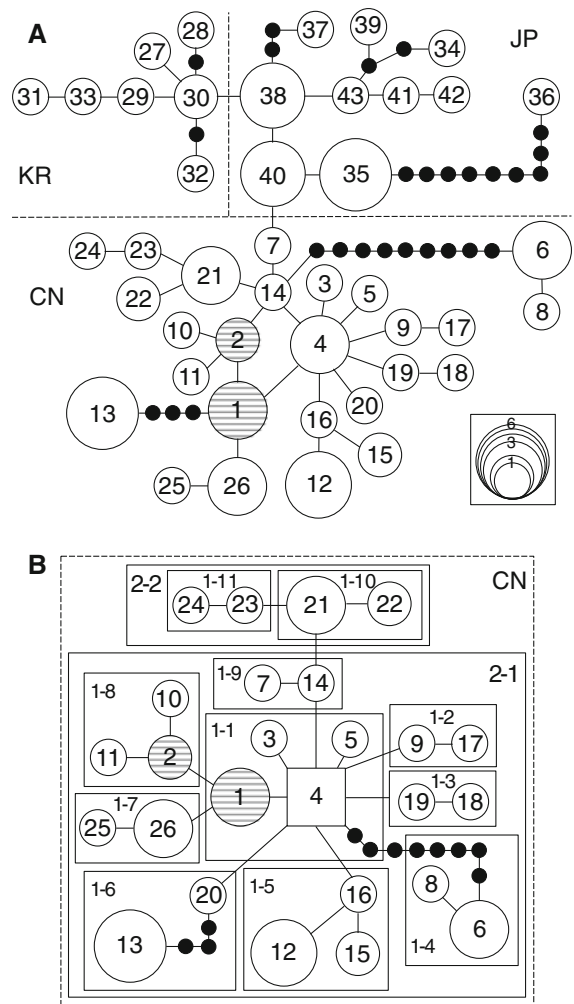


Fig. 3 **A** Parsimony network of mtDNA haplotypes of *H. halys*, and **B** haplotype network with nested clade design for *H. halys* in China. Each haplotype is indicated with a number in a circle. Numbers correspond to the haplotype numbers in Table 2. Haplotype circle size is proportional to its frequency in native populations (see scale on the right low corner of A). The haplotype in a square refers to the ancestral haplotype. Black dots represent undetected haplotypes. The two haplotypes found in the US are shown with *lined circles*

Discussion

Among native populations we detected significant genetic structure and divergence as well as high genetic diversity within populations except those at the edge of the species’ distribution. In contrast, genetic diversity in the US populations was much lower, with only two haplotypes identified across the continental US as of 2008—approximately 10 years

Table 3 Nested contingency result based on 10,000 permutations

Clade	χ^2	Probability	Inference chain	Inferred population process
2-1	121.63	< 0.001	1-2-3-5-6-7-Yes	Restricted gene flow but with some long distance dispersal

Only the clade with significant geographic association is shown ($P < 0.05$)

post introduction. Based on private haplotypes and phylogeographic analysis of native populations, we traced the origin of the US *H. halys* to the Beijing area in China, which has important applications to the effective control of this invasive pest.

Genetic structure of *H. halys* in East Asia

The mtDNA analysis of *H. halys* revealed a clear genetic structure in native East Asia. We detected few haplotypes shared among populations and significant genetic divergence among populations of China, South Korea and Japan. Furthermore, we observed genetic isolation in populations of China such as XACN, FZCN, KMCN and HFCN. These results indicate limited gene flow or migration among populations, which is also supported by the NCA inference of ‘restricted gene flow’. Based on phylogeny and

network analysis of haplotypes, we inferred north China (BJCN, XZCN and NJCN) as the distributional center in China, which agrees with that predicted from ecological niche modeling (Zhu et al. 2012). The haplotypes shared between marginal populations (FZCN and KMCN) and populations at the center (BJCN, XZCN and NJCN) likely resulted from human mediated dispersals from central to marginal areas, which is consistent with the NCA inference of ‘with some long distance dispersal’. The high genetic diversity in the central populations but low in marginal ones further corroborates this dispersal pattern. Both neutrality test and mismatch distribution indicated demographic expansion in the native populations, which was estimated to have started around 6000–15,000 years ago—a time within the interglacial period after the LGM. This explains why the haplotype phylogeny has low statistical support.

Introduction of *H. halys* to the US

Halyomorpha halys has invaded the US and expanded to 39 states in the past 15 years, causing unprecedented damages to agriculture (Leskey and Hamilton 2010; Seetin 2011; Kuhar et al. 2012). The first and critical question on its invasion is where did this invasive stink bug come from, followed by questions such as how many introductions have occurred and what was the likely size of the founding populations? Two mtDNA haplotypes (H1 and H2) were identified in the US populations, both coexisting in eastern

Table 4 Summary of hierarchical AMOVA of *H. halys*

Source of variation	df	Sum of squares	% of variation	Fixation index
(a)				
Among populations	9	125.5	61.57	
Within populations	122	88.48	38.43	$\Phi_{ST} = 0.616^{**}$
(b)				
Among populations	8	93.31	51.78	
Within populations	68	80.92	48.22	$\Phi_{ST} = 0.518^{**}$
(c)				
Among groups	4	71.89	33.06	$\Phi_{CT} = 0.331^*$
Among populations within groups	4	21.41	22.05	$\Phi_{SC} = 0.329^{**}$
Within populations	68	80.92	44.89	$\Phi_{ST} = 0.551^{**}$

AMOVA partitioned among (a) all populations, (b) native populations, and (c) five groups of native populations (1 BJCN + XZCN + NJCN + HFCN + KMCN, 2 XACN, 3 FZCN, 4 SWKR, 5 TKJP)

* $0.01 < P < 0.05$, ** $P < 0.01$ after 1,023 permutations

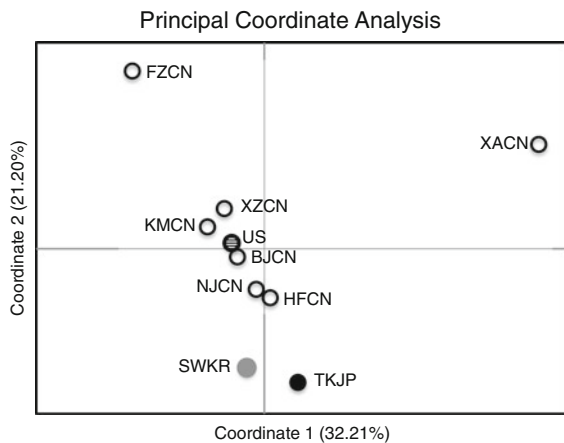


Fig. 4 Principal coordinate analysis (PCA) on *H. halys* populations based on population pairwise genetic distances. Populations of China, South Korea, Japan and the US are indicated with empty, grey, black and lined circles, respectively

coastal states and each also occurring singly in eastern inland states (H2) and west coast CA (H1). The unique mtDNA haplotypes in each native population, and the significant genetic differentiation among native populations and native countries, allow us to pinpoint the Beijing area in China (BJCN, the only native location where the two US haplotypes were found to coexist) as the likely source region for the US *H. halys*. We further excluded Kunming (KMCN) and Fuzhou (FZCN) as alternative origins by combining COI sequence analysis and phylogeographic analysis of the Chinese populations. This result disagrees with the proposed origins in northern Japan or western Korea based on climate matching models (Zhu et al. 2012). The US mid-Atlantic region, particularly eastern Pennsylvania and New Jersey where the exotic *H.*

halys was first reported, is the purported epicenter of current distribution in the US (Hoebeke and Carter 2003). We found two haplotypes coexisting in this area, which implies that a single introduction with the two haplotypes may summarize the US invasion history. Specimens in California and eastern inland states appear more likely the result of long-distance dispersal events from the east coast population facilitated by human transportation (e.g., Jones and Lambdin 2009), rather than new invasions from the native range. The single haplotype observed in these locations may result from genetic drift or selection during and after introduction. Since we detected only one haplotype in eight specimens from Pennsylvania, although it is the believed epicenter, examination of more samples from here will be instructive.

Compared with the high genetic diversity in native populations such as BJCN (five haplotypes in six individuals, $Hd = 0.93$, $\theta\pi = 0.0015$), we observed extremely low diversity in US populations (two haplotypes in 55 individuals, $Hd = 0.28$, $\theta\pi = 0.0005$). As we discussed above, Beijing (BJCN) is the likely source region of the US introduction. In BJCN, five haplotypes were identified from six individuals, and frequencies of H1 and H2 were 1/3 and 1/6, respectively. This implies that the current US population may result from a single successful introduction with a small population size (as small as two females; theoretically, in any 18 females from the source region, haplotypes H1 and H2 will be observed, because the frequency of H1 coexisting with H2 is 1/18), rather than multiple introductions or a large population. More haplotypes would be expected if the latter scenarios had

Table 5 Pairwise Φ_{ST} values among populations of *H. halys*

	BJCN	XACN	XZCN	NJCN	HFCN	FZCN	KMCN	SWKR	TKJP
XACN	0.675								
XZCN	-0.076	0.53							
NJCN	0.168	0.666	0.117						
HFCN	0.531	0.742	0.339	0.527					
FZCN	0.662	0.804	0.401	0.667	0.803				
KMCN	0.338	0.743	0.194	0.494	0.691	0.72			
SWKR	0.38	0.688	0.297	0.436	0.617	0.696	0.545		
TKJP	0.341	0.618	0.309	0.404	0.493	0.657	0.502	0.333	
US	0.648	0.922	0.535	0.764	0.811	0.904	0.754	0.826	0.697

Significant Φ_{ST} values after sequential Bonferroni corrections are shown in bold ($P = 0.05$)

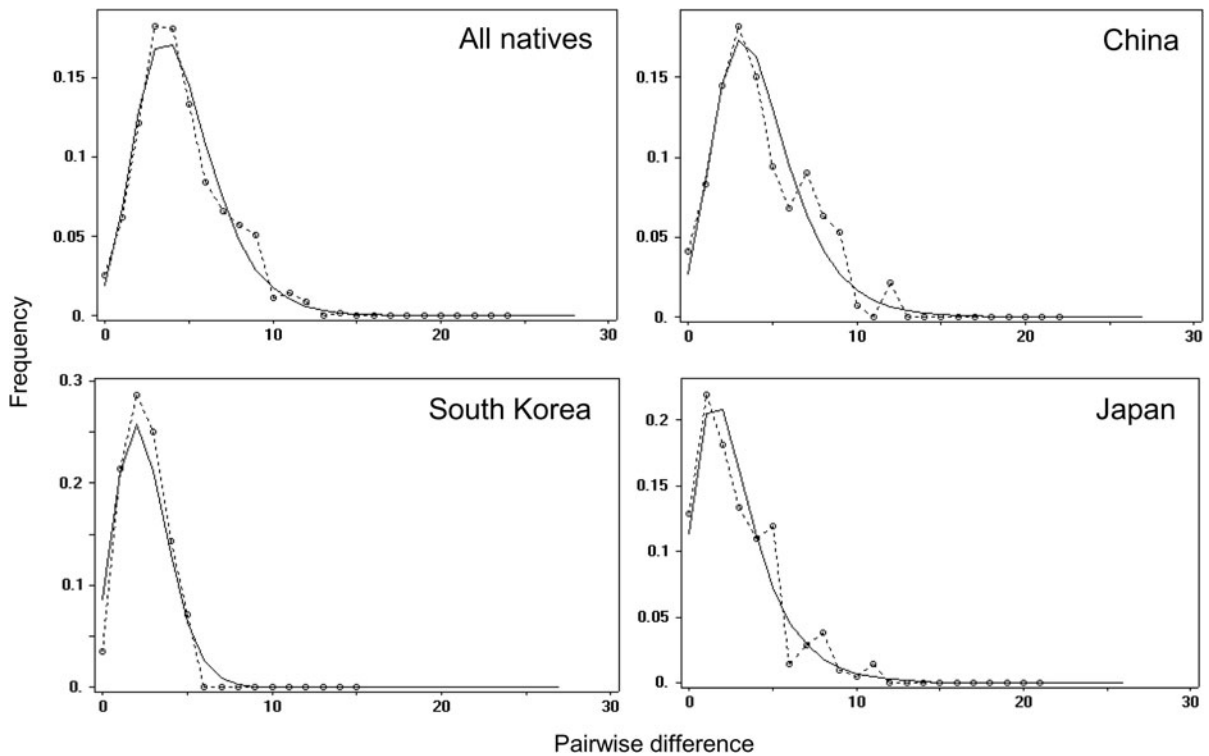


Fig. 5 Mismatch distribution of native populations of *H. halys*. The observed pairwise differences and expected values under growth-decline model are shown with *dashed* and *solid* lines, respectively

Table 6 Genetic diversity, Fu's neutrality test (F_s) and mismatch distribution parameter τ for native *H. halys* based on COII sequences

	N	H_n	H_d	$\theta\pi$	Fu's F_s	τ	Expansion time (ka)
All natives	77	18	0.891	0.0045	-7.234*	1.476	11.145
China	48	8	0.81	0.0037	-0.538	0.81	6.116
South Korea	8	6	0.929	0.0037	-2.676*	1.964	14.830
Japan	21	7	0.762	0.0028	-2.064	1.476	11.145

Number of specimens (N), haplotype numbers (H_n) and diversity (H_d), and nucleotide diversity ($\theta\pi$) are indicated. Expansion time shows as 1,000 years ago (ka)

* Significant at $P = 0.05$

happened, unless there existed strong selection on the mitochondrial genome in the exotic range. Nuclear markers such as amplified fragment length polymorphism (AFLP) and microsatellites would provide further insight into the nuclear genetic diversity and selection on the mitochondrial genome.

Identifying the source region has important applications to the control of *H. halys*. Egg parasitoids of *H. halys*, especially those in the *Trissolcus* genus, are the primary candidates for biological control and are expected to have the greatest impact on mitigating

population growth. Our results strongly suggest that surveys for egg parasitoids should focus on north China, specifically the Beijing area. *Trissolcus halyomorphae*, which has the highest reported parasitism rate for *H. halys*, was first identified in Beijing, China from natural populations (Yang et al. 2009); it has similar developmental thresholds as *H. halys* and thus should be a well adapted biological control agent for the US population. As an agricultural pest, *H. halys* has caused severe damage to crops, especially tree fruits, with mid-Atlantic growers experiencing

20–90 % economic losses. Reacting to an emergency situation, up to 16 insecticide applications are made each season against *H. halys*. Although insecticide resistance has not been reported in the US, we should be mindful that it might have been introduced with the founding population, or it may arise quickly given that the population has experienced such a strong genetic reduction. Our results suggest that surveys for possible insecticide resistance in source regions, particularly in the Beijing area, are necessary.

Successful invasion with low genetic diversity

A few invasive insects have extremely low mtDNA genetic diversity across a wide exotic range, including Colorado potato beetle *Leptinotarsa decemlineata* (Grapputo et al. 2005), Argentine ant *Linepithema humile* (Corin et al. 2007), the red imported fire ant *Solenopsis invicta* (Caldera et al. 2008), Russian wheat aphid *Diuraphis noxia* (Shufran et al. 2007) and the kudzu bug *Megacopta cribraria* (Jenkins and Eaton 2011). *L. decemlineata* was introduced to Europe in the 1920s, where it has become an important pest of solanaceous crops. Across all European populations, single mtDNA haplotype is matched with low nuclear genetic diversity as revealed by AFLP analysis (Grapputo et al. 2005). Haplodiploid ant species are expected to share maternal mitochondrial haplotypes because of a single queen in one colony; exotic populations of both *L. humile* and *S. invicta* appear to have spread from a single introduction (Corin et al. 2007; Caldera et al. 2008). Similarly *D. noxia* with a single haplotype in the US is consistent with a single introduction from South Africa (Shufran et al. 2007). Yet the single maternal haplotype of exotic *M. cribraria* may be explained by the single *Wolbachia* haplotype found in the US and Japanese populations (Jenkins and Eaton 2011).

Many invasive species have experienced a decrease of genetic diversity in their exotic habitats, but low genetic variation does not necessarily endanger fitness, in contrast, it may highlight rare alleles that increase fitness or invasiveness (Dlugosch and Parker 2008). In the blueberry maggot *Rhagoletis mendax*, for example, low genetic diversity is the direct result of selection on individuals that matched the fruiting schedule of commercial blueberries (Teixeira and Polavarapu 2003). In Argentine ant *L. humile*, inter-colony competition decreases with relatedness and

reduction of genetic variation (Tsutsui et al. 2000). Colorado potato beetle *L. decemlineata* thrived by finding the same food species in southern Europe as in its native range in the southwestern US, where it had specialized in feeding on commercial potatoes (Grapputo et al. 2005).

Lack of genetic diversity in the US *H. halys* cannot be explained by a *Wolbachia* sweep, since we found no evidence for infestation with this intracellular parasite. As a distributional center in the US, eastern Pennsylvania and New Jersey share similar climatic conditions with the native distributional center (Zhu et al. 2012). In the mid-Atlantic area, egg parasitism rates of *H. halys* are lower than those of native pentatomid species (e.g., Koppel et al. 2009), as well as those of *H. halys* in native China (Yang et al. 2009). Local climatic conditions and lack of effective natural enemies possibly have played a major role in the explosive growth of *H. halys* in the US mid-Atlantic region. In addition, the association of an agricultural pest like *H. halys* with human-made constructions, which remain warm during winter seasons, may be an important adaptation that enabled it to successfully establish and thrive in the mid-Atlantic region. Like *H. halys*, the invasive kudzu bug *M. cribraria* also exploits the agriculture/urban structures for overwintering, and its population densities have increased rapidly in the invaded areas. We hypothesize that proximity between homes and crops in the small farms in Pennsylvania and New Jersey is a critical ecological factor driving the expansion of *H. halys* in the US. Breaking this link, by targeting populations indoors or when they enter or exit homes, may be an effective way to control this pest.

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