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ORIGINAL PAPER

Genetic analyses of the Asian longhorned beetle (Coleoptera, Cerambycidae, *Anoplophora glabripennis*), in North America, Europe and Asia

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Abstract The Asian longhorned beetle, (Coleoptera, Cerambycidae, *Anoplophora glabripennis* (Motschulsky)), is endemic to China and Korea and an important invasive insect in North America and Europe. We analyzed mitochondrial DNA sequence data of invasive populations of *A. glabripennis* in North America and Europe, and microsatellite allele frequency data of beetles from North America. We show that populations in New York City and Long Island NY; New Jersey, Chicago, IL, and Toronto, Canada have limited genetic diversity compared to populations in China. In addition, the data suggest that separate introduction events were responsible for many of the populations in North America and for European populations in Austria, France, Germany and Italy. Populations on Long Island, NY are suspected to have been initiated by the transport of cut wood from New York City. *A. glabripennis* beetles found in Jersey City, NJ appear to be derived from an expansion of the New York City, NY population, whereas beetles found in Linden, NJ are an expansion from the Carteret, NJ population. Limited genetic diversity did not stop this invasive insect from establishing damaging populations in North America. Founders of introduced *A. glabripennis* populations in North America and Europe are likely derived from populations in China that are themselves invasive, rendering difficult the identification of source populations. Invasiveness in an insect's natural range could be an important predictor of potential pest status of introduced populations.

Keywords *Anoplophora glabripennis* · Asian longhorned beetle · Invasive species · Mitochondrial DNA · Microsatellite loci

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Introduction

Alien species are a major contributor to the loss of biodiversity on the North American continent (Crooks and Soulé 1999; Wilcove et al. 1998; Enserink 1999; Krushelnycky and Gillespie 2008). In Europe increased awareness of the impact of invasive species on the loss of biodiversity lead to the formation of the European Strategy on Invasive Alien Species (Hulme et al. 2009). These invaders often have important economic effects (Pimentel et al. 2000; Sakai et al. 2001). In the United States, Pimentel et al. (2001) estimated a cost of $97 billion for 79 major biological

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invasions. Invasions are often considered to consist of three stages—arrival, establishment and spread with success at each stage governed by different biological characteristics (Liebhold and Tobin 2008).

Most incidents of invasion go undetected for a few to many generations, and the source population and history of an invasion must often be inferred indirectly. Molecular markers are useful for characterizing patterns of genetic variation in invasive species, potentially revealing signatures of an expanding population and providing evidence about the numbers of founders and their sources (Ficetola et al. 2008; Eales et al. 2008). Reduced genetic variation is predicted in newly founded populations due to population bottlenecks, in which only a limited subset of genotypes establish a new population (Nei et al. 1975). However, populations of alien invaders with low genetic diversity could include genotypes that thrive in novel environments and become locally adapted (Ahern et al. 2009; Tsutsui et al. 2000). It has been tentatively suggested that most invasive populations harbor enough additive genetic variation to respond to selection, which may be an important factor in population growth and spread (Wares et al. 2005; Carrol 2007). In some situations an increase in additive genetic variance, after a population bottleneck, allows for a rapid response to selection (Wade et al. 1996; Cheverud et al. 1999; Naciri-Graven and Goudet 2008). Although there are some consistent patterns of genetic diversity across studies of important invasive species (Dlugosch and Parker 2007), many populations have unique genetic signatures. For example, an analysis of mitochondrial DNA haplotypes of alien red turpentine beetle populations that were introduced into China showed unexpectedly high levels of genetic diversity, with unique haplotypes in most populations (Cognato et al. 2005).

Records show 585 species of non-native insects are feeding on trees and shrubs in the United States and Canada (Langor et al. 2009). Among these are damaging bark- and wood-boring beetle species that have already established in North American forests or exist as populations in urban areas and threaten to spread (Haack 2006; Poland and McCullough 2006). In Europe, 19% of the exotic phytophagous insects on woody plants are Coleoptera with the majority living on deciduous trees (Mattson et al. 2007).

Here we report genetic data for invasive populations of the polyphagous wood-boring Asian longhorned beetle (Coleoptera, Cerambycidae, *Anoplophora glabripennis* (Motschulsky)), which was first discovered in North America in 1996 (Cavey et al. 1998; Haack 2003, 2006; Haack et al. 1997). Although until recent times the Asian longhorned beetle had a restricted geographical distribution in its native range, it is now recorded throughout China (except the central province of Qinghai) and in Korea (Lingafelter and Hoebeke 2002; Hu et al. 2009). Figure 1 shows the distribution of *A. glabripennis* in its native range and the sites of invasive populations. Because of an increase in trade with Asia that involves untreated solid wood packing materials (Bartell and Nair 2003; Westphal et al. 2008), opportunities for transport of bark and wood-boring beetles have grown, and *A. glabripennis* has been detected at warehouses in 14 states in the United

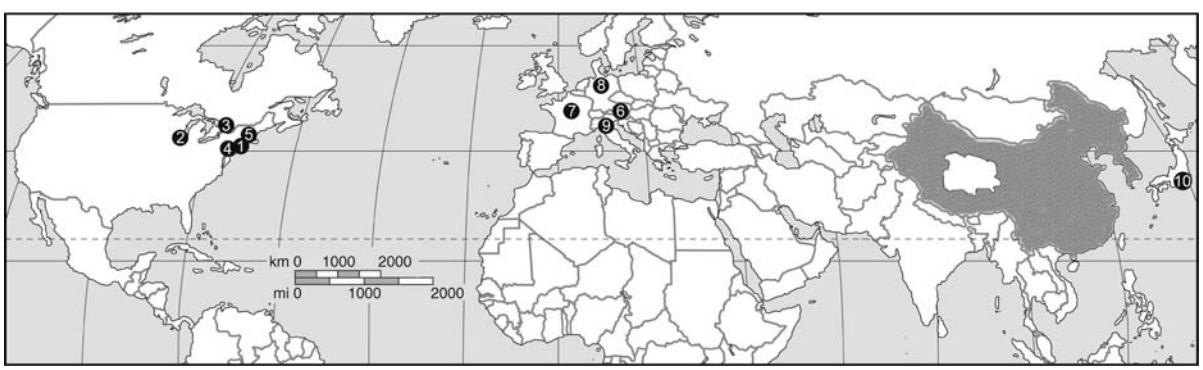

Fig. 1 Geographic distribution of *A. glabripennis* in China and Korea (*shaded area*) and sites of invasive populations (*circles*). *1* New York City and Long Island, NY and Jersey City, NJ; *2* Chicago, IL; *3* Toronto, Canada; *4* Carteret and Linden, NJ and Prall's and Staten Islands, NY; *5* Worcester, MA; *6* Austria; *7* France; *8* Germany; *9* Italy; *10* Japan

States (USDA-APHIS 2008) and has also been introduced into Europe [Austria (Tomiczek et al. 2002); France, Germany (Hérard et al. 2006); Italy (Maspero et al. 2007)], and Japan (Takahashi and Ito 2005).

The original North American discovery of *A. glabripennis* in Brooklyn, NY was quickly followed by findings at other sites in New York (Queens and Long Island; Fig. 2). Subsequent surveys revealed *A. glabripennis* in Chicago (1998), additional sites in Queens, Manhattan and Islip, Long Island (1999, 2000); Jersey City, NJ (2002); Toronto, Canada (2003); Carteret and Linden, NJ (2004, 2006); additional sites in Linden, NJ (2006); on Prall's and Staten Islands, NY (2007) and in Worcester, MA (2008) (Poland et al. 1998; Sawyer 2007; Hu et al. 2009). In 2005 a few *A. glabripennis* were found inside a warehouse on pallets shipped to Sacramento from China and beetles may have escaped the warehouse (Wasserman 2005).

Anoplophora glabripennis is a generalist, attacking deciduous tree species primarily in the genera *Acer, Aesculus, Betula, Fraxinus, Platanus, Populus, Salix,* and *Ulmus* (Sawyer 2003; Pan 2005). Female *A. glabripennis* lay eggs singly under the bark on the trunk, branches and exposed roots of host trees, and larvae feed first on the cambium layer and then bore into the wood for continued feeding and pupation (Lingafelter and Hoebeke 2002; Keena 2005). Adults emerge to feed on leaves, petioles and twigs before reproducing (Keena 2002; Smith et al. 2002).

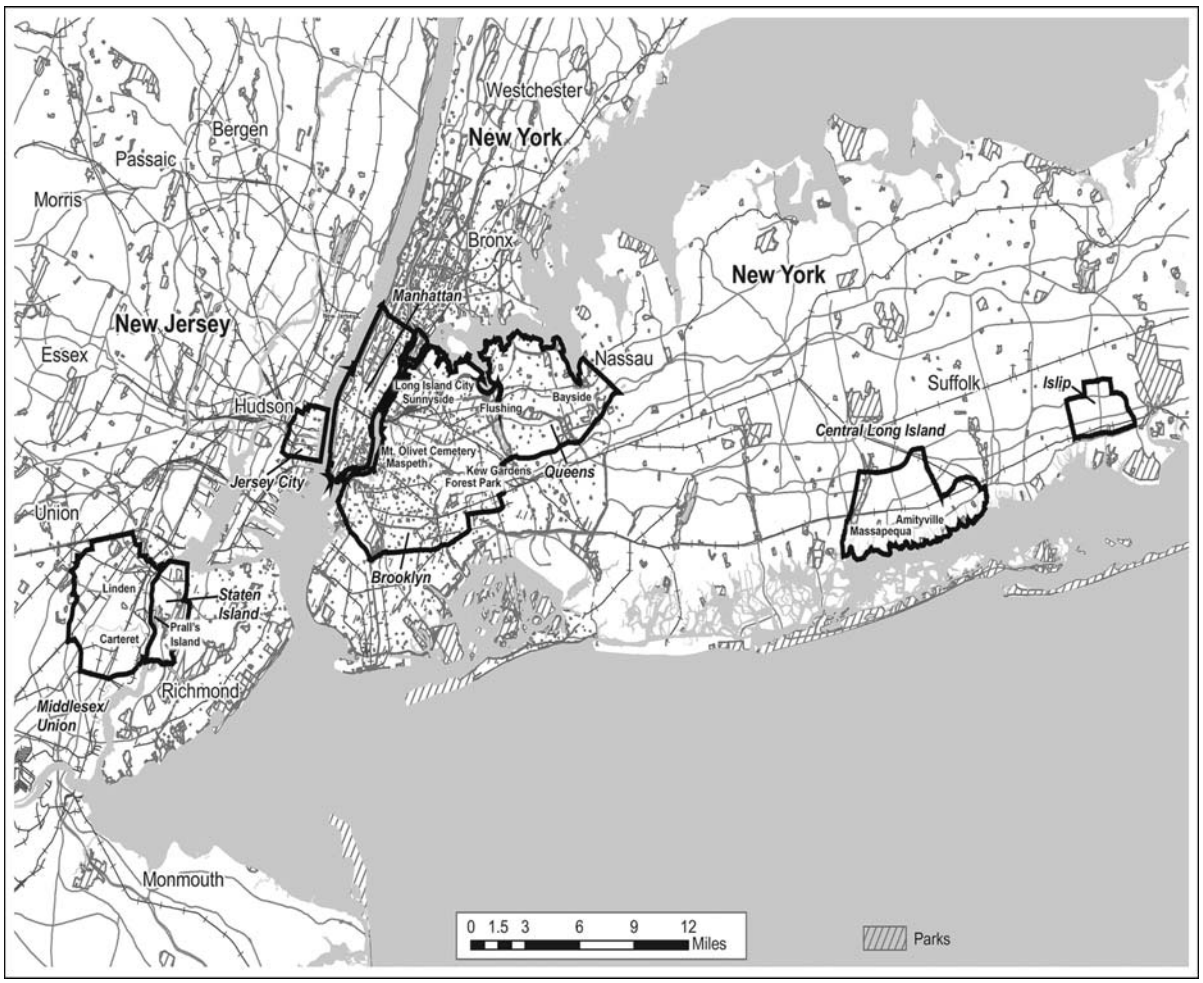

Fig. 2 Map of New York City, Long Island and New Jersey showing sites where *A. glabripennis* have been discovered. *Dark lines* indicate regulated areas that limit the movement of cut wood

Here we use two molecular markers (mitochondrial DNA and microsatellite loci) to determine amounts and patterns of variation in invasive populations of *A. glabripennis*. We use our data to infer whether *A. glabripennis* was introduced independently into many of the sites where it has been found in North America and Europe or whether its' distribution as an invasive can be explained by one or a few introductions followed by movement within each continent.

Materials and methods

Specimens of *A. glabripennis* were collected alive between 1996 and 2007, placed in 95% alcohol and stored at 4°C. A small number of specimens were frozen alive before being placed in alcohol and stored at 4°C. DNA was extracted from 1 week to years later. Total DNA was extracted using DNeasy tissue kits® (QIAGEN). DNA was extracted from adult beetles and larvae, either by removing a leg plus muscle tissue from adults or a segment of the immature abdomen. A subset of samples has been deposited as vouchers with the Cornell University Insect Collection lot #1262.

Mitochondrial DNA sequencing

As a maternally inherited, rapidly evolving DNA marker that lacks recombination, mitochondrial DNA has been extensively used to examine population structure (Avise 2000). Although not without limitations (e.g., see Ballard and Whitlock 2004), mitochondrial DNA data has provided important insights into the history of invasive insect populations (Grapputo et al. 2005; Scheffer and Grissell 2003; Puillandre et al. 2008; Cai et al. 2008). We analyzed DNA samples from invasive populations of *A. glabripennis* in North America using mitochondrial DNA sequence data to evaluate genetic diversity and relatedness within and among these introduced populations. We also examined mitochondrial DNA sequences of a few *A. glabripennis* from populations in Europe. In the absence of a definitive phylogeny for the genus *Anoplophora*, we chose *Anoplophora chinensis*, a commonly collected and likely close relative of *A. glabripennis* as the outgroup species. Patterns of variation in introduced populations are compared to patterns seen in potential source populations in China and Korea. DNA samples from 283 *A. glabripennis* (211 from the United States, 57 from Canada and 15 from Europe; Table 1) were amplified and sequenced for mitochondrial DNA, using six species-specific primers at optimized annealing temperatures (Carter et al. 2009b). Primer overlap was such that each nucleotide site had 2 to 6 times coverage. We sequenced 1,317 base pairs of cytochrome c oxidase 1, 65 base pairs of t-RNA-Leucine and 224 base pairs of cytochrome c oxidase 2 mitochondrial DNA genes. Each PCR was run in a volume of 14 µl, and contained 6.7 µl of water, 1.5 µl of 10× PCR buffer [20 mM Tris–HCl (pH 8.4), 500 mM KCl (Invitrogen; Carlsbad, CA)], 3 µl of 2.5 mM dNTP, 0.75 µl of 50 mM $MgCl_2$, 0.06 µl of *Taq* DNA polymerase (Invitrogen), 0.6 µl of primers at 10 mM and 20 ng of DNA. PCR products were cleaned with PCR mini-elute kits® (QIAGEN). Five µl sequencing reactions contained 1.88 µl of water, 0.25 µl of 5 M Betaine (Sigma), 1 µl of Ready Reaction and 5× buffer (Applied Biosystems), 0.12 µl of 10 mM primer and 1 µl of PCR products. Sequencing reactions were cleaned with Sephadex (Sigma) and directly sequenced on an ABI 3730 sequencer. Sequences were assembled into contiguous arrays, edited using Seqman™, and aligned with Megalign (Lasergene® 7.0).

Sequences for the invasive populations were entered into TCS v. 1.2.1 (Clement et al. 2000) where a gene geneology was estimated using the method of statistical parsimony (probability > 0.95) to produce a haplotype network. For Bayesian analysis, sequences from China and Korea (Carter et al. 2009b) were reduced to a data set of unique haplotypes and combined with a set of unique haplotypes from sequences from the invasive populations. No information is lost by doing so. Bayesian analysis was performed using Mr. Bayes v. 3.1.2 (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003). Using Mr. Modeltest2 v. 2.3 (Nylander 2004) a GTR + I+G substitution model (general time reversible model with gamma-distributed rate variation across sites and a proportion of invariable sites) was the best fit model to the data. The program was run for 1,000,000 generations and sampled every 100 generations. For

Table 1 Genbank accession numbers, number of samples and number of haplotypes for mitochondrial DNA sequence data by collecting site for *A. glabripennis* analyzed

Location[a]	n[b]	h_d[c]	Accession numbers
Bayside, NYC (BS)	6	1	EU914413-14, EU914556-59
Brooklyn, NYC (BR)	19	2	EU914502, EU914508, EU914523-24, EU914543-49, EU914552 EU914591-97
Flushing, NYC (FL)	2	1	EU914406, EU914427
Forest Park, NYC (FP)	9	1	EU914447-55
Kew Garden Hills, NYC (KG)	5	1	EU914442-46
Long Island City, NYC (LIC)	5	2	EU914423-24, EU914438-39, EU914555
Manhattan, NYC (MN)	18	1	EU914485-87, EU914505-06, EU914525, EU914567-71 EU914574-75, EU914606-4610
Maspeth, NYC (MS)	12	2	EU914407-10, EU914412, EU914425, EU914428-31, EU914435-36
Mt. Olivet Cemetery, NYC (OL)	11	1	EU914482-83, EU914534-41, EU914553
Prall's Island, NY (PI)	6	1	EU914526-31
Staten Island, NY (SI)	2	1	EU914532-33
Sunnyside, NYC (SS)	5	1	EU914426, EU914437, EU914440, EU914560-61
Amityville, LI, NY (AM)	13	5	EU914479-81, EU914581-90
Islip, (LI) NY (IS)	2	1	EU914572-73
Massapequa, LI, NY (MP)	16	2	EU914488-95, EU914598-605
Carteret, NJ (CT)	22	3	EU914463-78, EU914562-66, EU914611-13
Jersey City, NJ (JC)	14	1	EU914401-914405, EU914456-62, EU914542, EU914554
Linden, NJ (LN)	30	3	EU914496-501, EU914503-4, EU914507, EU914509-22 EU914550-51, EU914576-79, EU914614
Sacramento, CA (SC)	2	2	EU914484, EU914580
Ravenswood, IL (CH)	12	1	EU914411, EU914415-22, EU914433-34, EU914463
Toronto, Ontario, CN (TO)	57	1	EU914615-671
Brannau, Austria	4	2	EU91674-77
Gien, France	5	1	EU914678-82
Neukirchen, Germany	4	2	EU914683-6
Milano, Italy	2	2	EU914672-3

[a] *NYC* New York City, *NY* New York, *LI* Long Island, *NJ* New Jersey, *ILL* Illinois, *CN* Canada

[b] Number of samples

[c] Haplotype diversity

other priors we used the default settings with a burnin of the first 25% of the total number of generations. At 1,000,000 generations log-likelihood values had stabilized and converged from the two runs, the split frequencies approached zero and the potential scale reduction factor values were close to zero. Program runs at 3,000,000 generations gave the same results. The consensus tree was opened and annotated in TreeView v. 1.6.6 (Page 1996). A neighbor-joining analysis (1,000 psuedo-replicates) was calculated in PAUP* v. 4.0 (Swofford 2003) to produce a consensus tree using the same model as was used for the Bayesian analysis. Neighbor-joining bootstrap support values were placed on the Bayesian tree.

Microsatellites DNA markers

Microsatellites are highly polymorphic, presumably neutral genetic markers that are useful for tracing patterns of establishment and spread of organisms

and for inferring recent population history (Roderick and Navajas 2003; Schlotterer 2004). Microsatellite allele frequencies of invasive populations in North America were analyzed to determine relatedness among populations. A total of 340 *A. glabripennis* (NY, NJ, Chicago and California sites) was genotyped for 15 microsatellite loci (Carter et al. 2009a). Primers for one additonal locus (alb15; Genbank accession no. DR108921) are: GGCCTATTTTGAT GCGAGTG (forward) and GGCACTACCTGCTAC ACAGC (reverse), fluorescently labeled with PET (Applied Biosystems). We chose to focus on North American invasive populations because only there did we have extensive samples from different sites in close proximity. Briefly, PCR reaction volumes were 10 µl with 20 ng of DNA in 2.0, 1.7 µl of water, 1 µl of 10× PCR buffer, 2.0 µl of 5 M betaine, 0.2 µl of 10 mM dNTP mix, 0.1 µl of *Taq* polymerase, 1 µl each of the reverse specific primer and the tag sequence primers at 3.2 pMol/µl, and 1 µl of the forward specific primer at 0.8 pMol. Cycling was carried out with the optimal annealing temperature. Cycling included an initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturing at 95°C, annealing for 1 min, and extension at 72°C, each for 1 min, and a final elongation step of 4 min at 72°C. All PCR products were genotyped in formamide on a 3730×l DNA Analyzer and compared to a Liz 500 (−250) size standard. Eight standard DNA templates were run on each 96 well plate to check product migration rate. Microsatellite data were collected and binned with Genemapper® v. 4.0. Diversity information was calculated in PowerMarker v. 3.25 (Liu and Muse 2005) and included the frequency of the most common allele and expected and observed heterozygosities. Mean allelic richness was calculated in FSTAT v. 1.2 (Goudet 1995). Using Genepop v. 1.2 (Raymond and Rousset 1995) we calculated deviations from Hardy–Weinberg equilibrium [Fisher's Method; $P \leq 0.05$ with sequential Bonferonni correction (Rice 1989)] for each locus and deviations from genotypic disequilibrium ($P \leq 0.05$) for each locus pair across all populations. Pairwise F_{ST} values for all pairs of populations, Nei's average number of pairwise differences within and between populations (Nei and Li 1979), and the significance of the derived genetic distances were tested with 16,000 permutations in Arlequin v. 3.0 (Excoffier et al. 2005; $P \leq 0.001$). A neighbor-joining (NJ) tree was constructed (Rogers 1972) by bootstrapping (500 replicates) over all marker loci, and the trees were summarized to produce a consensus tree in the program Mega v. 3.0 (Kumar et al. 2004). We implemented the program Structure v. 2.2 (Pritchard et al. 2000; Falush et al. 2003) to calculate the probability of each individuals' assignment to each of K hypothetical populations or clusters, where K is initially undefined. This program uses a Bayesian approach to simultaneously estimate population (cluster) allele frequencies and cluster membership (Pritchard et al. 2000). Using the model with admixture, individuals can be assigned to multiple clusters with the sum of the membership coefficients equal to one. For $K = 1$–10, we ran ten simulations at each K value. For each run we used a burnin period of 50,000 and collected data for 950,000 iterations. Following the method of Evanno et al. (2005), we calculated ΔK based on the second order rate of change in the log probability of data with respect to the number of population clusters from the Structure analysis. From the value of $\Delta K = 2$ population substructure was indicated. We chose the run with the greatest likelihood at $K = 2$ to assign individuals into two subpopulations and we analyzed those groups separately in Structure to determine the correct value of K. We then used the Structure admixture analysis again to assign the proportion of each beetle's genotype originating from each hypothetical cluster.

To look for reductions in genetic diversity during founding events of these invasive populations, two-group, one-sided tests for differences in allelic richness, observed heterozygosity (H_o) and within sample gene diversity (H_s) were calculated in FSTAT between *A. glabripennis* from invasive populations in New York City, NJ and on Long Island and *A. glabripennis* from multiple populations in China and one population in Korea (Carter et al. 2009b). For each test statistic the program calculates the average for each group (over samples and loci), the difference between the averages of the two groups, and the significance of the difference using a one-sided permutation test (Goudet 1995).

To make inferences about sources for beetles from invasive populations, we compared multilocus genotypes of *A. glabripennis* from North America to

Table 2 Mitochondrial DNA sequence diversity and number of individuals of each haplotype of *A. glabripen*nis by sample site in North America

Haplotype	Nucleotide site	Number of samples per site[a]																						
	_111111	NY	NY	NY	NY	NY	NY	NY	NY	NY	NY	NY	NY	NY	LI	LI	LI	NJ	NJ	NJ	CA	CN	ILL
	_11222334556788803445 5	BS	BR	FL	FP	KG	LIC	MN	MS	OL	PI	SI	SS	AM	IS	MP	CT	JC	LN	SC	TO	CH		
	_40389979589652693148 13																							
	_26225826627348718432 51																							
1	CCCACAGTCTCGCATTAATTCT		17	2	8	5		18	11	11			6	8	2	5		14	1				
2	..T...A...T..........	6					4		1					2		11				1		12		
3	.T........TAT.....CCT.																				57			
4	..TTAGACT.TA..CCTG.CTC										6	2					20		24					
5	..TTAGACT.TA....TG.CTC		2																2					
6	..........T..........													1										
7	T..T.GA.TATA.G........																		1					
8	..TTAGACT.T..........						1																	
9	..TTAGACT.TA..CCTG.CT.															1								
10	...TAGACT.TA..CCT..CT.															1								
11	..TTA.ACT.TA.......CT.													1										
12	..TTAGACT.TA.......CT.													1										

[a] *NY* New York state, *LI* Long Island, *NJ* New Jersey, *CA* California, *CN* Canada, *ILL* Illinois, *BS* Bayside, *BR* Brooklyn, *FL* Flushing, *FP* Forest Park, *KG* Kew Gardens Hills, *LIC* Long Island City, *MN* Manhattan, *MS* Maspeth, *OL* Olivet Cemetery, *PI* Prall's Island, *SI* Staten Island, *SS* Sunnyside, *AM* Amityville, *IS* Islip, *MP* Massapequa, *CT* Carteret, *JC* Jersey City, *LN* Linden, *SC* Sacramento, *TO* Toronto, *CH* Chicago

multilocus genotypes of *A. glabripennis* from provinces in China and Korea using GeneClass v. 2.0 (Piry et al. 2004). Only beetles with all 32 alleles (no missing data) were used to make comparisons, and genotypes were assigned based on the Bayesian criteria of the score with the greatest likelihood [Rannala and Mountain (1997) threshold 0.05; score cutoff set to 90% probability].

Results

Mitochondrial DNA Sequence data

In North American populations the number of mitochondrial DNA haplotypes per locality ranged from 1 to 5 (Table 1). Of the 1,606 bp of mitochondrial DNA that were sequenced, only 22 sites (1.4%) were variable (Table 2). Twelve unique haplotypes were found in a sample of 258 *A. glabripennis* (Table 2).

Of four common haplotypes (marked by an * in Fig. 3), one haplotype is found at many sites in and around New York City (Brooklyn, Flushing, Forest Park, Kew Garden Hills, Manhattan, Maspeth, Olivet Cemetery and Sunnyside), at the three sites on Long Island, in Jersey City, NJ and in one beetle from Linden, NJ. A second haplotype is found in New York City, NY (Bayside and Long Island City) Long Island, NY (Amityville and Massapequa), is the only haplotype present in Chicago and is found in one beetle from California. In Linden and Carteret, NJ and on Prall's and Staten Islands, NY, the majority (90%) of beetles harbor a third major haplotype. A few beetles from Linden and Carteret, NJ and Brooklyn, NY have haplotypes that are separated by one or two mutations (see Fig. 3). Finally all beetles from Toronto carried a single haplotype distinct from that found in any other *A. glabripennis* in North America, but identical to a haplotype found in Germany. In Europe, seven haplotypes were found in a total of 15 samples (Table 3; Fig. 3), and each was found in a single country but 3 of the 4 countries had two haplotypes.

A Bayesian analysis of mitochondrial DNA sequence haplotypes from populations of *A. glabripennis* in Asia produced a tree in which populations from most provinces in China and the one site sampled in Korea are each characterized by multiple haplotypes, with these haplotypes scattered throughout the tree. This pattern presumably reflects significant genetic admixture among Asian populations due to human-driven translocations. We included haplotypes from North America, Europe and Asia in a single tree (Fig. 4; clade probability values (>0.5) above the branches and bootstrap support values (>50%) below the branches) to look for possible source populations for North American introductions. In fact, we do find a number of haplotypes that are identical between China and North America or

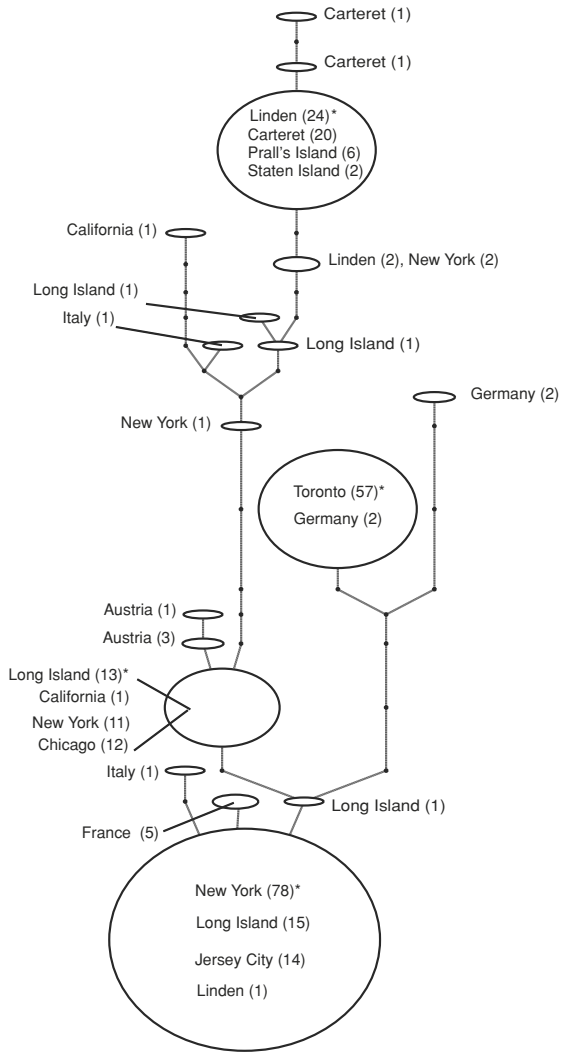

Fig. 3 Parsimony network summarizing the estimated relationships between the sequenced haplotypes of invasive populations of *A. glabripennis*. *Labels* indicate collection sites followed by sample size in parentheses. "New York" refers to sites in New York City (Bayside, Brooklyn, Flushing, Forest Park, Kew Garden Hills, Manhattan, Maspeth, Olivet Cemetery, and Sunnyside). Long Island sites are Amityville, Islip and Massapequa

Fig. 4 Bayesian consensus tree of 19 mitochondrial DNA unique haplotypes from invasive populations of *A. glabripennis* and 37 unique haplotypes from populations of *A. glabripennis* in China and Korea. *A. chinensis* is the outgroup species. Numbers *above* the branches indicate clade probability values (>0.50) while numbers *below* the branches indicate neighbor-joining bootstrap support (>50%). The *scale bar* indicates the branch lengths (expected number of sites) from the Bayesian analysis ▶

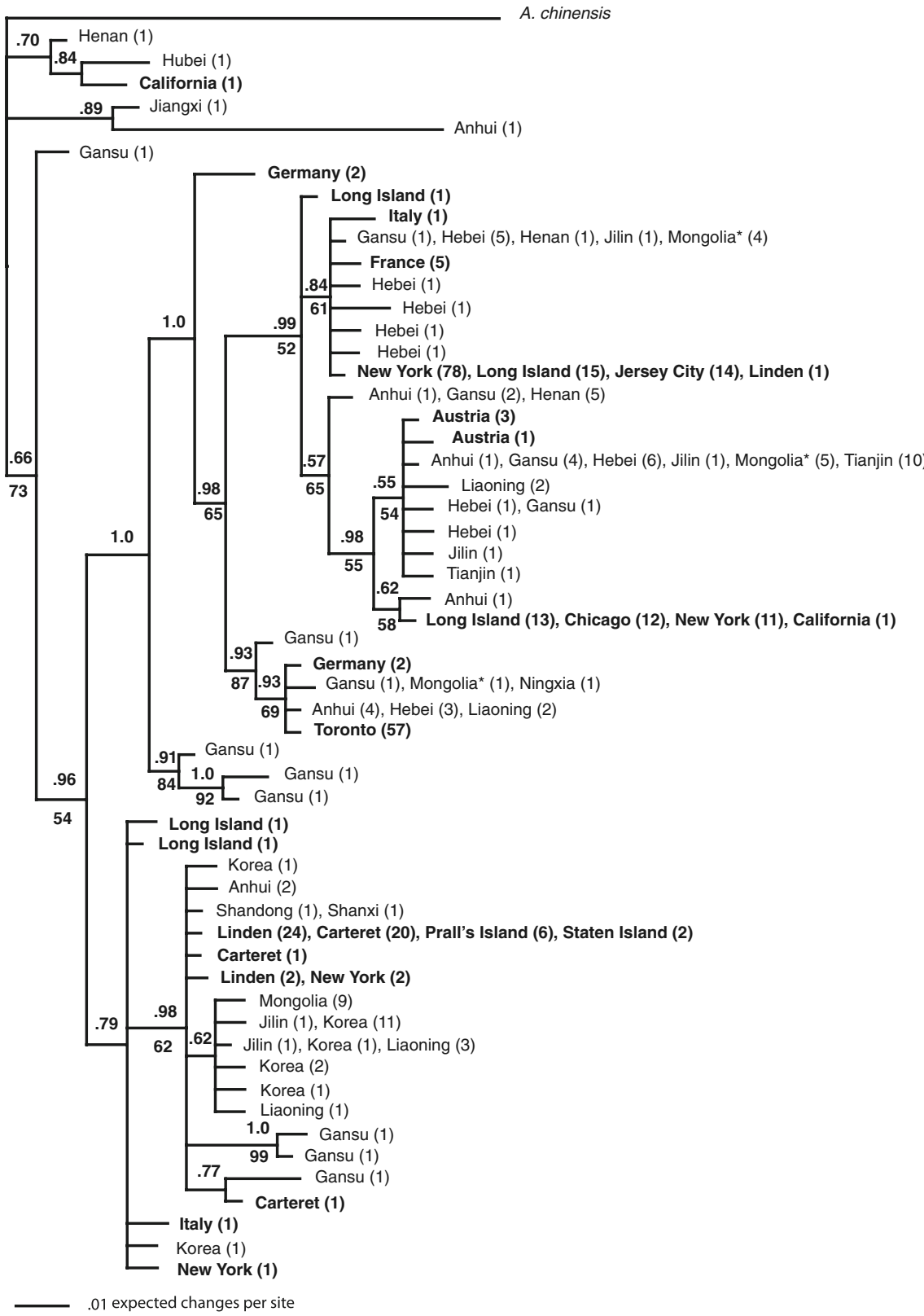
A. chinensis
.70
Henan (1)
.84
Hubei (1)
California (1)
.89
Jiangxi (1)
Anhui (1)
Gansu (1)
Germany (2)
Long Island (1)
Italy (1)
Gansu (1), Hebei (5), Henan (1), Jilin (1), Mongolia* (4)
France (5)
.84
Hebei (1)
61
Hebei (1)
1.0
.99
Hebei (1)
52
Hebei (1)
New York (78), Long Island (15), Jersey City (14), Linden (1)
Anhui (1), Gansu (2), Henan (5)
Austria (3)
Austria (1)
.66
Anhui (1), Gansu (4), Hebei (6), Jilin (1), Mongolia* (5), Tianjin (10)
73
.57
65
.55
Liaoning (2)
.98
65
54
Hebei (1), Gansu (1)
1.0
Hebei (1)
.98
Jilin (1)
55
Tianjin (1)
.62
Anhui (1)
58
Long Island (13), Chicago (12), New York (11), California (1)
Gansu (1)
.93
Germany (2)
87
.93
Gansu (1), Mongolia* (1), Ningxia (1)
69
Anhui (4), Hebei (3), Liaoning (2)
Toronto (57)
.91
Gansu (1)
84
1.0
Gansu (1)
.96
92
Gansu (1)
54
Long Island (1)
Long Island (1)
Korea (1)
Anhui (2)
Shandong (1), Shanxi (1)
Linden (24), Carteret (20), Prall's Island (6), Staten Island (2)
Carteret (1)
Linden (2), New York (2)
.98
Mongolia (9)
.79
Jilin (1), Korea (11)
62
.62
Jilin (1), Korea (1), Liaoning (3)
Korea (2)
Korea (1)
Liaoning (1)
1.0
Gansu (1)
99
Gansu (1)
.77
Gansu (1)
Carteret (1)
Italy (1)
Korea (1)
New York (1)
.01 expected changes per site

Table 3 Mitochondrial DNA sequence diversity and number of individuals of each haplotype of *A. glabripennis* by sample site in Europe

Haplotype	Nucleotide site	Number of samples			
		Locations			
	_11111	Italy	France	Austria	Germany
	_1223356702445				
	_33197996559481				
	_42082673469325				
1	CCGAGTCGCTATTC		5		
2	.TA.A.T...T...			3	
3	..AG....T.....	1			
4	TTA.A.T...T...			1	
5	..A...TAT..CCT				2
6	..AGACTAT.....	1			
7	..A...TA.C..CT				2

Europe. A haplotype common to five Chinese provinces (Gansu, Hebei, Henan, Jilin and Mongolia) is identical to the haplotype defining the majority of beetles from New York City, NY and Jersey City, NJ. A haplotype found in one specimen in each of Shandong and Shanxi provinces is identical to the haplotype defining the majority of beetles from Carteret and Linden, NJ and Prall's and Staten Islands, NY. One haplotype from Austria is identical to a haplotype common in 6 Chinese provinces (Anhui, Gansu, Hebei, Jilin, Mongolia, and Tianjin) and one haplotype from Germany and the haplotype found in Toronto are identical to a haplotype found in three provinces (Anhui, Hebei and Liaoning). Most North American haplotypes are similar (one or two mutational steps removed) to haplotypes found in one or more provinces of China, or Korea.

Microsatellite data

Tests of microsatellite data for deviation from Hardy–Weinberg equilibrium across all populations (NY, NJ, Long Island, California and Illinois) indicated significant heterozygote deficiency for 60% (of 80) of the comparisons. Pair-wise comparisons across 16 loci and five populations (NY, NJ, Long Island, Illinois and California) showed significant genotypic disequilibrium in 45% of 116 comparisons ($P < 0.004$). All major allele frequencies were ≥ 0.66 (Table 4). These results are not unexpected in recently founded

Table 4 Combined diversity information by collecting site for *A. glabripennis* genotyped at 16 microsatellite loci, including the sample size, mean allele number, major allele frequency, expected and observed heterozygosities and allelic richness

Population	No[a]	N_A^b	M_{AF}^c	H_e^d	H_o^e	A_R^f
Bayside, NYC	8	2.44	0.69	0.39	0.19	1.81 (7)
Brooklyn, NYC	26	2.38	0.80	0.27	0.15	1.50 (22)
Flushing, NYC	6	1.81	0.82	0.25	0.15	1.39 (3)
Forest Park, NYC	14	1.81	0.82	0.22	0.16	1.44 (14)
Kew Gardens Hills, NYC	6	1.75	0.82	0.24	0.21	1.52 (6)
Long Island City, NYC	11	2.63	0.69	0.41	0.21	1.88 (9)
Manhattan, NYC	17	2.06	0.80	0.26	0.17	1.50 (14)
Maspeth, NYC	23	2.63	0.78	0.29	0.16	1.54 (18)
Olivet, NYC	16	2.88	0.77	0.31	0.14	1.52 (8)
Prall's Island, NY	7	1.44	0.88	0.16	0.06	1.28 (6)
Staten Island, NY	2	1.25	0.89	0.12	0.09	1.19 (2)
Sunnyside, NYC	17	2.75	0.71	0.38	0.19	1.58 (11)
Amityville, NY (Long Island)	17	2.44	0.71	0.36	0.15	1.72 (13)
Islip, NY (Long Island)	2	1.81	0.70	0.34	0.16	1.81 (2)
Massapequa, NY (Long Island)	22	2.25	0.78	0.30	0.17	1.58 (18)
Carteret, NJ	32	2.25	0.80	0.27	0.16	1.48 (22)
Jersey City, NJ	27	2.06	0.80	0.27	0.17	1.51 (23)
Linden, NJ	73	2.31	0.86	0.20	0.10	1.38 (71)
Sacramento, CA	2	2.00	0.66	0.38	0.38	2.00 (2)
Ravenswood, IL	12	2.69	0.68	0.40	0.23	1.80 (5)
Loudonville, OH	1	1.00	1.00	0.00	0.00	–[g]
Seattle, WA	1	1.33	0.83	0.33	0.17	–[g]

[a] Number of beetles genotyped

[b] Mean number of alleles

[c] Major allele frequency

[d] Expected heterozygosity

[e] Observed heterozygosity

[f] Allelic richness (sample size)

[g] Not calculated ($n = 1$)

and bottlenecked populations where mating may not be random and in which there have been few generations of recombination (Reich et al. 2001). Each population presumably has an excess of common alleles because of recent population bottlenecks (Falush et al. 2003).

A table of pairwise F_{ST} values and a NJ tree based on 16 microsatellite loci (Table 5; Fig. 5) reveals patterns of similarity consistent with those found in the mitochondrial DNA analysis. Jersey City, NJ is most similar (smallest F_{ST} values) to some of the sites in New York City (Flushing and Kew Garden Hills); the New Jersey sites Linden and Carteret, and the New York sites of Prall's Island and Staten Island, are most similar to each other, as are the New York City sites Bayside and Long Island City. Some sites in New York City, NY (Manhattan, Brooklyn and Forest Park) are also most similar to each other.

Based on allele frequencies at 16 microsatellite loci, we used the program Structure to assign individuals to clusters, estimating number of clusters and cluster allele frequencies at all loci. Our analysis gives a conservative estimate of $K = 4$ hypothetical clusters. Many of the same groupings emerge from the Structure analysis (Fig. 6) as are apparent in the mitochondrial DNA tree. Beetles sampled from the New York City sites (Queens, Manhattan and Brooklyn), NY and Jersey City, NJ and a subset of the individuals from the Long Island sites of Amityville and Massapequa are one cluster (shown in yellow). The ancestry of other beetles from Long Island is primarily from a different cluster (shown in blue). A third cluster includes beetles from the Bayside and Long Island City sites in New York City and a single individual from Maspeth, in New York City (shown in green). Beetles from the Carteret and Linden sites in NJ and from Prall's and Staten Islands sites in NY are assigned primarily to a fourth cluster (shown in red). The two individuals from California and all of the Chicago individuals appear to be of mixed origins with respect to microsatelllite allele frequencies.

Allelic richness (3.28), observed heterozygosity (0.29) and expected heterozygosity (0.47) were all significantly greater ($P \leq 0.05$) in populations of beetles from China than in invasive populations of beetles (2.21, 0.15, 0.27, respectively).

Using Geneclass, 94 of 276 *A. glabripennis* from populations in North America were assigned with $\geq$90% probability to source areas in different provinces in China. Of the ten beetles from the three sites on Long Island, one was assigned to Gansu, 4 to Hebei, one to Jilin, one to Inner Mongolia, and 3 to Shanxi provinces. Thirty beetles from sites in New York City were assigned to Shanxi province, 8 beetles to Hebei province, 5 beetles to Jilin province and one beetle to Tianjin province. Strikingly different assignments

Table 5 Pairwise F_{ST} when all loci are combined for *A. glabripennis* across sampling sites in North America

	CT	JC	LN	AM	LIC	BA	BR	FL	FP	IS	KG	MN	MS	MP	OL	PI	SS	SI	ILL
JC	**0.498**																		
LN	0.065	**0.505**																	
AM	**0.358**	**0.078**	**0.372**																
LIC	**0.394**	**0.220**	**0.458**	0.123															
BA	**0.447**	**0.379**	**0.518**	**0.274**	0.087														
BR	**0.439**	**0.092**	**0.451**	**0.092**	**0.191**	**0.381**													
FL	0.512	0.018	**0.524**	0.123	0.235	**0.373**	0.074												
FP	**0.510**	**0.192**	**0.526**	**0.183**	**0.186**	**0.377**	**0.110**	0.176											
IS	0.479	0.317	**0.509**	0.168	0.176	0.323	0.309	0.365	0.309										
KG	**0.472**	0.048	**0.496**	0.065	0.185	0.381	0.071	0.124	0.171	0.227									
MN	**0.396**	**0.147**	**0.401**	0.078	**0.182**	**0.355**	0.048	0.136	**0.145**	0.276	0.12								
MS	**0.458**	0.076	**0.477**	0.084	**0.182**	**0.369**	0.056	0.119	**0.219**	0.306	0.08	**0.087**							
MP	**0.469**	**0.312**	**0.491**	**0.189**	**0.297**	**0.390**	**0.396**	**0.384**	**0.468**	0.296	**0.339**	**0.394**	**0.361**						
OL	**0.454**	0.130	**0.459**	**0.121**	**0.297**	**0.395**	**0.102**	**0.120**	**0.231**	0.298	0.103	**0.081**	0.047	**0.397**					
PI	**0.220**	**0.408**	0.146	**0.263**	**0.391**	**0.482**	**0.368**	**0.497**	**0.496**	0.477	**0.409**	**0.319**	**0.397**	**0.45**	**0.398**				
SS	**0.420**	**0.091**	**0.451**	0.059	**0.161**	**0.365**	**0.079**	0.156	**0.145**	0.169	0.008	**0.101**	**0.085**	**0.329**	**0.145**	**0.362**			
SI	0.287	0.433	0.233	0.288	0.364	0.424	0.437	0.544	0.55	0.419	0.465	0.423	0.467	0.389	0.503	0.316	0.419		
ILL	**0.345**	**0.234**	**0.391**	**0.108**	**0.163**	0.211	**0.255**	**0.267**	0.356	0.259	**0.233**	**0.234**	**0.218**	**0.21**	**0.279**	**0.283**	**0.214**	0.29	
CA	0.388	0.388	0.425	0.249	0.245	0.228	0.406	0.407	0.461	0.148	0.344	0.353	0.378	0.377	0.391	0.342	0.327	0.259	0.18

Significantly different populations are indicated by bold type ($P < 0.001$)

AM Amityville, *BA* Bayside, *BR* Brooklyn, *CA* California, *CT* Carteret, *FL* Flushing, *FP* Forest Park, *ILL* Illinois, *IS* Islip, *JC* Jersey City, *KG* Kew Gardens Hills, *LIC* Long Island City, *LN* Linden, *MN* Manhattan, *MP* Massapequa, *MS* Maspeth, *OL* Olivet Cemetery, *PI* Prall's Island, *SI* Staten Island, *SS* Sunnyside

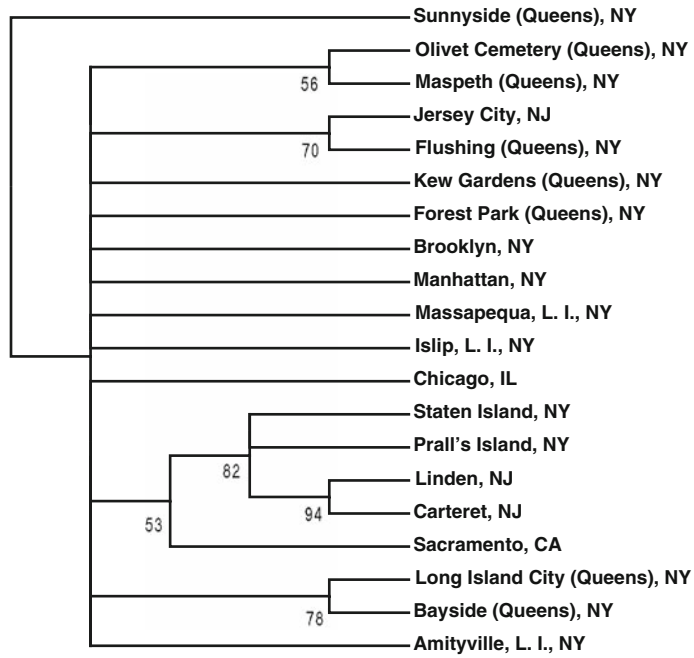

Fig. 5 Neighbor-joining consensus tree based on allele frequencies of 16 microsatellite loci from invasive populations of *A. glabripennis*. *Numbers* indicate bootstrap support. *LI* Long Island, *NJ* New Jersey, *NY* New York State. The single samples from Ohio and Washington are not included

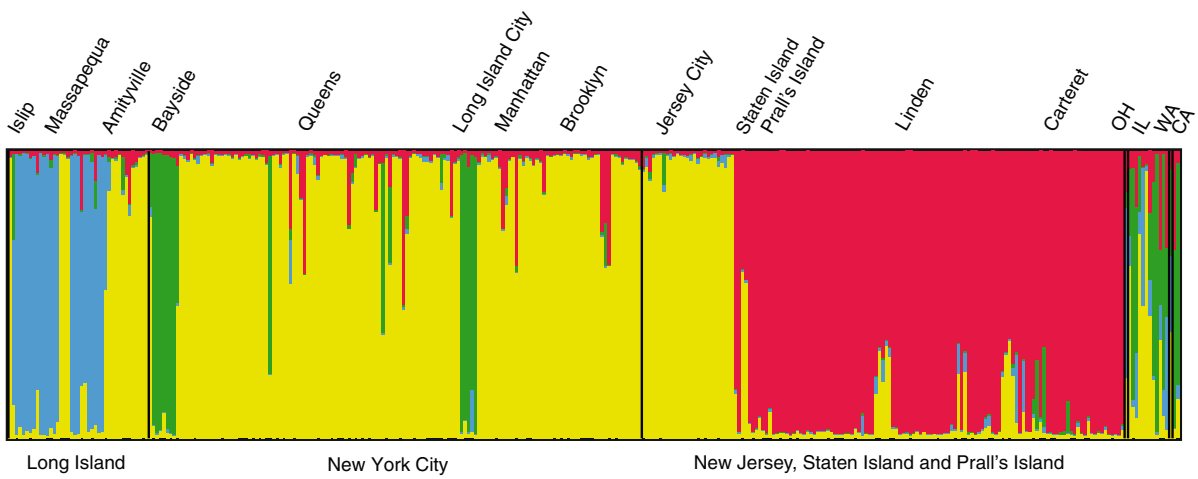

Fig. 6 Summary plot of estimated membership coefficients for individual *A. glabripennis* beetles in each of $K = 4$ hypothetical clusters from the Structure analysis. Each individual is represented by a *single vertical line* broken into K colored segments, with lengths proportional to membership in each of the K inferred clusters. *Labels* above the figure are the collection sites

were made for beetles from sites in Carteret and Linden. Thirty-six beetles (97%) from these sites were assigned to Hebei province with 1 beetle assigned to Jilin Province. Two beetles (67%) from Chicago were assigned to Hebei province and one beetle (33%) was assigned to Inner Mongolia province. The remaining beetles could not be assigned with ≥90% probability.

Discussion

Introduction history, expansion and spread

Given the life cycle of *A. glabripennis*, invasive populations likely have a small intrinsic rate of increase, with absolute numbers increasing slowly in

the first few years before detection (Crooks and Soulé 1999; Crooks 2005). *Anoplophora glabripennis* in North America and Europe was likely introduced on infested wood packing materials in the 1980s or early 1990s (Haack et al. 1997; Sellers 2004; Hérard et al. 2006) allowing opportunities for expansion and spread (Sawyer 2007; Sawyer and Panagakos 2009). Mitochondrial DNA sequence data and microsatellite allele frequency data generally provide concordant views of the genetic structure of United States Asian longhorned beetle populations.

Sequence data suggest that separate introductions of beetles from Asia are responsible for the appearance of *A. glabripennis* in New York City, NY (Manhattan, Brooklyn, and Queens; Fig. 2; New York) and Jersey City, NJ, and in Carteret and Linden in New Jersey and Staten Island and Prall's Island in New York. Moreover, sequence data also indicates multiple transport events of beetles from New York City to Long Island, given that two of the Long Island haplotypes were found at different sites in New York City (see Table 2). This is consistent with *A. glabripennis* detection history. Beetles were found in Amityville, Long Island shortly after the initial discovery in Brooklyn in 1996, and found in Islip, Long Island in 1999. In the greater New York City area only four haplotypes were found in 92 beetles sampled, suggesting that few beetle haplotypes were introduced and/or reproduced and spread. Based on detection dates, spread first occurred throughout Brooklyn and sites in Queens, and beetles subsequently moved to Manhattan and Jersey City. Of course, from the observed patterns, it is difficult to rule out the possibility of independent introductions of similar or identical haplotypes from Asia, rather than regional spread within North America. Thus, genetic data can only provide a minimum estimate of the number of times that beetles have arrived from abroad. Mitochondrial DNA sequence data also indicate a separate introduction event in Toronto. Although the mitochondrial DNA haplotype found in Chicago is also found at sites in New York, we believe that these beetles represent a separate introduction event into Chicago because of direct evidence from the site. In 1998 wood packing crates from China with beetle exit holes were found at a local business in Ravenswood, IL (USDA Forest Service 2004).

In the New York City area, beetles may well have been transported with infested trees to Amityville for disposal or sale as firewood (Haack et al. 1997). The most common haplotype found on Long Island is known from the Brooklyn, Flushing, Kew Garden Hills, Manhattan, Maspeth, Mount Olivet Cemetery and Sunnyside sites in New York City, any one of which might have been the source site. The second most common haplotype on Long Island was found also at the Bayside, Long Island City and Maspeth sites in New York City. Three additional singleton haplotypes, from beetles collected in 2005, were found on Long Island but not elsewhere. Because a quarantine zone of the infested New York areas was implemented in March, 1997 and was expanded as beetles continued to be detected at new sites in NY and NJ (USDA-APHIS 2007), these haplotypes likely arrived on Long Island prior to establishment of the quarantine zone.

Based on the small sample sizes available, each European population has a unique mitochondrial DNA haplotype or set of haplotypes. These haplotypes are not closely related; indeed most of the European haplotypes are more similar to haplotypes in North America or China (see Fig. 4). Thus, the pattern of genetic variation in Europe also provides evidence for multiple introduction events, as has been suggested (Hérard et al. 2006).

Mitochondrial DNA haplotype diversity was substantially less in invasive populations than in Asian populations. A previous study found 37 different mitochondrial DNA haplotypes in a sample of 131 beetles from Asia (Carter et al. 2009b), whereas here we document only 12 unique haplotypes in 258 beetles from North America. These 12 haplotypes appear to be a sample from across the spectrum of haplotypes found in China, a result consistent with expectations if only small numbers of beetles were the source of each of the presumably independent introductions.

Microsatellite data indicate only two population clusters in New York City, NY/Jersey City, NJ; one of which is also found at two sites on Long Island. This result is consistent with the scenario that few beetles founded the New York City population and that some were moved to Long Island. Few beetles likely also founded the Carteret, NJ population and subsequently spread to Linden, NJ and Staten and Prall's Islands, NY. One microsatellite population cluster is found only on Long Island. We can only

suggest that these beetles were transported to Long Island early in the 1990s, before intense sampling began in the New York City area. The substantial reductions in measures of microsatellite diversity are also consistent with expectations of small founding populations.

Source populations and general implications

The sources of the founders of *A. glabripennis* populations in North America and Europe are themselves likely invasive populations in China. There is substantial mitochondrial DNA variation found within populations in China and an absence of private mitochondrial DNA haplotypes within populations (Carter et al. 2009b). Thus, mitochondrial DNA alone is unlikely to pinpoint the location of source populations in China.

With microsatellite markers we found no private microsatellite alleles defining populations in China (Carter et al. 2009b). Because microsatellite genotypes are based on multiple highly polymorphic markers, we were able to demonstrate differences in probable source populations for beetles found in Manhattan, Queens and Brooklyn compared to beetles from Carteret and Linden. We were not able to pinpoint, however, probable source populations due to limitations of the data.

The physical environment, natural enemies, and host resources are important factors that can help explain the spread of alien invaders such as the Asian longhorned beetle (Shea and Chesson 2002). The physical environment in disturbed metropolitan areas is often favorable for population growth because of higher average temperatures, limited competition from other wood-boring beetles and absence of species-specific and general predators and parasitoids. These factors allow rapid population growth which can act as a buffer against random mortality (Lee 2002). Furthermore, the flora of eastern North America is similar to that in eastern China, with 85% of the plant genera in North America also occurring in China (Qian et al. 2003). In North America trees in urban areas are abundant, susceptible, healthy hosts with little antibiosis resistance (Nowak et al. 2001). Preferred host trees at risk for attack are abundant in New York City, NY and Jersey City, NJ (44–47% of trees; Nowak et al. 2001) and in the eastern deciduous forest region (54%; Bartell and Nair 2003). Novel selective pressures are absent in North America that might have stopped these invasions (Suarez and Tsutsui 2008).

Populations of alien *A. glabripennis* with low genetic diversity introduced into the United States have been able to expand to damaging levels. Population bottlenecks have not seemed to compromise the ability of Asian longhorned beetles to reproduce and spread. Biological characters, such as high survival of immature stages due to protection in the host trees (mortality rates between 0.1 and 0.3, Bartell and Nair 2003) and an ability to expand host range in a new environment ((Nowak et al. 2001; Sawyer 2003) may be primarily responsible.

A recent study used field data from nine US cites and national tree cover data to estimate the national urban impact of *A. glabripennis* and found 1.2 billion trees would die in those cities with a value of $669 billion (Nowak et al. 2001). Prevention of new introductions of *A. glabripennis* with methyl bromide fumigation or heat treatment of solid wood packaging materials (pallets, crates, dunnage, etc.), as required by International Phytosanitary Measure ISPM 15, and early aggressive action against outbreaks by setting up quarantine zones to stop the movement of contaminated wood and destroying infested trees, will likely limit establishment of Asian longhorned beetles in North America.

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