Microsatellite population genetics of the emerald ash borer (Agrilus planipennis Fairmaire): Comparisons between Asian and North American populations

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ORIGINAL PAPER

Microsatellite population genetics of the emerald ash borer (*Agrilus planipennis* Fairmaire): comparisons between Asian and North American populations

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Abstract The emerald ash borer (EAB) (*Agrilus planipennis* Fairmaire) (Coleoptera; Buprestidae), is an invasive wood-boring beetle native to northeast Asia. This species was first detected in Michigan USA in 2002, and is a significant threat to native and ornamental ash tree species (*Fraxinus* spp.) throughout North America. We characterized seven polymorphic microsatellite markers for EAB and used these to investigate EAB population structure in the early invasive populations within North America and in comparison with Asia. We found 2–9 alleles per microsatellite locus, no evidence of linkage disequilibrium, and no association with known coding sequences, suggesting that these markers are suitable for population genetic analysis. Microsatellite population genetic structure was examined in 48 EAB populations sampled between 2003 and 2008 from five regions, three in the introduced range, Michigan (US) and Ontario and Quebec (Canada) and two Asian regions, China and South Korea, where EAB is native. We found significant genetic variation geographically but not temporally in EAB populations. Bayesian clustering analyses of individual microsatellite genotypes showed strong clustering among multiple North American populations and populations in both China and South Korea. Finally, allelic richness and expected heterozygosity were higher in the native range of EAB, but there was no difference in observed heterozygosity, suggesting a significant loss of alleles upon introduction but no significant change in the distribution of alleles within and among individuals.

Keywords Microsatellite · Invasive species · Genetic diversity · Genetic bottleneck

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Introduction

The emerald ash borer (EAB), *Agrilus planipennis* (Fairmaire), is an invasive, phloem-feeding beetle

species attacking ash trees (*Fraxinus* spp.) in the eastern and central United States (US) and southern Ontario and Quebec, Canada. Populations of EAB were first discovered in southeast Michigan in 2002 causing extensive ash tree mortality throughout the area (Haack et al. 2002, Wei et al. 2007, Wang et al. 2010). Dendrochronological analyses indicate that EAB was introduced to southeast Michigan at least 10 years earlier (Siegert et al. 2008). To date, EAB is known to attack at least half of the 16 species of *Fraxinus* native to North America. EAB is a formidable threat to *Fraxinus* spp. due to its ability to attack and kill healthy, vigorously growing ash trees ranging in size from sapling to mature trees (Poland and McCullough 2006). Ash trees are widespread and abundant in forested and riparian areas of North America, and ~8 billion ash trees are estimated at risk on the US timberlands alone (Nowak et al. 2003). In addition to being important wildlife species, ash cultivars are widely planted in urban areas and the hardwood is used in numerous contexts (MacFarlane and Meyer 2005; Poland and McCullough 2006).

The emerald ash borer is native to northeastern regions of Asia including China, South Korea, Japan, Mongolia, Russia and Taiwan (Haack et al. 2002). While studying ash and EAB in northern and eastern China, Liu et al. (2003) found that EAB population densities were highest in areas where ash species native to North America were planted. Prior to the 1960's, EAB was considered only a periodic pest of *Fraxinus* spp. native to Asia, and Zhang et al. (1995), which suggests the introduction of exotic ash species from North America to China resulted in more frequent and damaging EAB outbreaks than were previously known. During a subsequent study by Liu et al. (2007) in Jilin province, similar results were found with higher EAB densities attacking North American ash species (e.g. *Fraxinus pennsylvanica*) than Asian ash species (e.g. *Fraxinus chinensis*), supporting the notion that innate resistance in Asian ash species has coevolved with EAB. Similar findings were reported from a common garden trial in Michigan, with out-plantings of North American ash species dying sooner that those from Asia (Rebek et al. 2008). Endemic populations of EAB documented in Taiwan (Bray et al. 2011) and in the Russian Far East (Baranchikov et al. 2008) also appear to have a less profound effect on native ash species and a patchy distribution in these regions. Invasive populations of EAB have also been encountered in the environs of Moscow, Russia (Baranchikov et al. 2008).

Adult EAB lay eggs in bark crevices and between bark layers almost exclusively on ash species (Bauer et al. 2004; Cappaert et al. 2005). After egg hatch, neonates feed in the phloem and cambial regions of the tree, forming galleries in the sapwood. Mature larvae overwinter in pupation chambers chewed out of either the outer sapwood or outer bark; they pupate and emerge as adults during the following spring or summer from D-shaped exit holes characteristic of *Agrilus* spp. Larvae, requiring 2 years to complete development, overwinter in larval galleries, continue to feed the next year, and emerge the following spring (Cappaert et al. 2005). The proportion of the population of EAB that undergo a 2-year generation time depends on the age of the infestation and the geographic location (Cappaert et al. 2005). Adults are strong fliers and capable of dispersal flights, as demonstrated in flight mill studies where 20 % of gravid females were estimated to fly more than 10 km in 24 h (Taylor et al. 2010). The probability of EAB colonization was significantly affected by the abundance of ash trees and decreased with distance from the epicenter (Mercader et al. 2009; Siegert et al. 2010). Regardless of natural spread, long-distance spread of EAB individuals throughout North American is caused by human-mediated movement of EAB in ash firewood, logs, nursery stock, and other ash materials (Cappaert et al. 2005; Poland 2007).

In North American ash trees, EAB populations reach a mean density of 89 adults/m^2 ash phloem (range 17–170 adults/m^2), causing tree mortality through girdling (McCullough and Siegert 2007). As of 2007 in Michigan alone, EAB had caused the death of at least 20 million ash trees (Poland 2007). Less is known about changes in effective population size, population genetic structure, or selection for novel phenotypes since the introduction of EAB to North America. Furthermore, there is limited information on the region or population of origin of invasive North American individuals (Bray et al. 2011). A greater knowledge of these genetic and demographic parameters in EAB would greatly inform strategies used in the management and control of this species.

Molecular markers are useful tools for studying the patterns of variation that arise as the result of species invasions (Roderick 1996; Sakai et al. 2001). They can help to determine the source of introduced populations, their relative sizes, dispersal throughout the non-

native range and temporal changes in patterns of genetic variation since introduction of species such as EAB (Roderick and Navajas 2003). These factors may ultimately shed light on the mechanisms that have led to a successful introduction (Allendorf and Lundquist 2003; Estoup and Guillemaud 2010).

Species invasions can consist of a small number of colonizing individuals who are the sole contributors to the gene pool of the newly founded population (Sakai et al. 2001; Allendorf and Lundquist 2003). This colonization pattern can result in a small effective population size and high levels of genetic drift in the invasive populations relative to source populations (Nei et al. 1975; Grosberg and Cunningham 2001; Novack and Mack 2005; Dlugosch and Parker 2008). Alternatively, if multiple introductions occur and/or a larger number of individuals are introduced, genetic diversity, effective population size and the adaptive potential of the introduced population may be larger (Novack and Mack 1993; Lee 1999; Novack 2007). Furthermore, if the introduced species has some advantage in its new environment then a rapid increase in population size due to the success of the population and its corresponding genetic diversity can accumulate rapidly, leaving a signature of increased genetic diversity in the introduced range relative to the native range (Wares et al. 2005). In addition, Dlugosch and Parker (2008) and others point out that a genetic bottleneck during introduction can cause balanced epistatic genetic variance to be directly exposed to selection as additive genetic variance, leading to more rapid evolution in the new environment (Carson 1990).

Nuclear microsatellite makers are tandem repeats of nucleotide sequences distributed throughout the genome. These genetic markers offer an advantage over other classes of molecular markers because high mutation rates lead to high levels of allelic variability within populations (Selkoe and Toonen 2006). This is especially useful in studying species invasions where microsatellite polymorphisms can help to distinguish the magnitude, location and frequency of colonization events, as well as changes in levels of diversity and adaptive potential in introduced relative to native populations (Davies et al. 1999; Wares et al. 2005).

Population genetic analyses of insect species invasive to North America show that differences among species and introduction scenarios can result in different evolutionary outcomes. Carter et al. (2010) examined populations of the invasive Asian longhorned beetle (*Anoplophora glabripennis* Motschulsky) and showed that invasive North American individuals had lower levels of genetic diversity than Chinese populations, and that North American populations could result from multiple introduction events. Research on the invasive Argentine ant (*Linepithema humile* Mayr) in its introduced range in North America, showed that a strong genetic bottleneck upon introduction facilitated the success of the ant population in North America by reducing aggression among colonies (Tsutsui and Suarez 2000; Tsutsui et al. 2003). Furthermore, using microsatellite and mtDNA, they identified a specific geographic region (Rosario Argentina) within the native range as the source of North American populations (Tsutsui et al. 2001). Finally, a microsatellite population genetic analysis revealed that invasive populations of the walnut husk fly (*Rhagoletis completa*) had high levels of genetic variation in some introduced populations, suggesting that this species may not have undergone a severe genetic bottleneck and that multiple introduction events and/or a large number of founders were involved in the initial introduction (Chen et al. 2006).

Bray et al. (2011) conducted a population genetic analysis of EAB populations using AFLP and mitochondrial DNA markers to identify potential source populations for the introduction of EAB in North America and changes in genetic diversity since its introduction from Asia. Individuals were sampled from much of the native range of EAB and a portion of the introduced North American range, including Michigan and one population in Ontario. They found a close relationship between EAB populations in Michigan and those in the Chinese provinces of Hebei and Tianjin City, suggesting that North American populations may have originated in this region of China. Bray et al. also reported higher levels of genetic diversity in native Asian populations than in North American populations, but lower levels of population genetic variability in native than introduced populations, suggesting that a population bottleneck occurred upon introduction but also that populations may be more isolated from each other in the introduced than in the native range. However, only a portion of the native and introduced range was sampled and an increase in sampling of EAB may help improve our understanding of the invasion, subsequent range expansion, and diversification of EAB in North America. Furthermore,

expanding the analysis of invasion genetics to a larger toolkit of genetic loci will bring us closer to a genome-wide understanding of the patterns of genetic variation in native and introduced EAB populations.

In this study, we describe the development and characterization of seven polymorphic microsatellite markers from EAB. We then use these microsatellite markers to analyze population genetic variation of EAB throughout its native and introduced range, including new detailed sampling from Ontario and Quebec, two Canadian provinces with EAB infestations.

Methods

Sample collection

A map of EAB sampling locations and regional demarcation can be found in Fig. 1. Canadian EAB samples were collected as part of annual surveys by the Canadian Forest Service, and from outbreak sites where trees were removed (Table 1); these samples were obtained between August 14, 2003 and December 17, 2008. Samples of EAB from Michigan were collected as adults by the USDA FS NRS researchers from 2005 to 2007 or as larvae or pupae during a collection trip to Michigan in 2008. Specimens from China were collected as larvae from ash trees by Chinese and USDA FS NRS researchers from 2003 to 2007 and reared to adults in the laboratory (Liu et al. 2003, 2007); specimens from South Korea were collected as adults and pupae by USDA APHIS researchers from Korean ash (*Fraxinus chinensis var. rhynchophylla*). Larvae and pupae were usually stored in ethanol; adult samples were also stored in ethanol if collected directly, but were often collected from EAB monitoring traps initially where they dried out. Many samples were poorly preserved (particularly early Canadian samples from sticky traps), and thus it was difficult to recover DNA from these tissues, resulting in the under-sampling of some populations and regions relative to others. Nevertheless, the characterization of microsatellite markers, as well as the general description of population genetic structure of EAB, will enable future genetic analyses related to

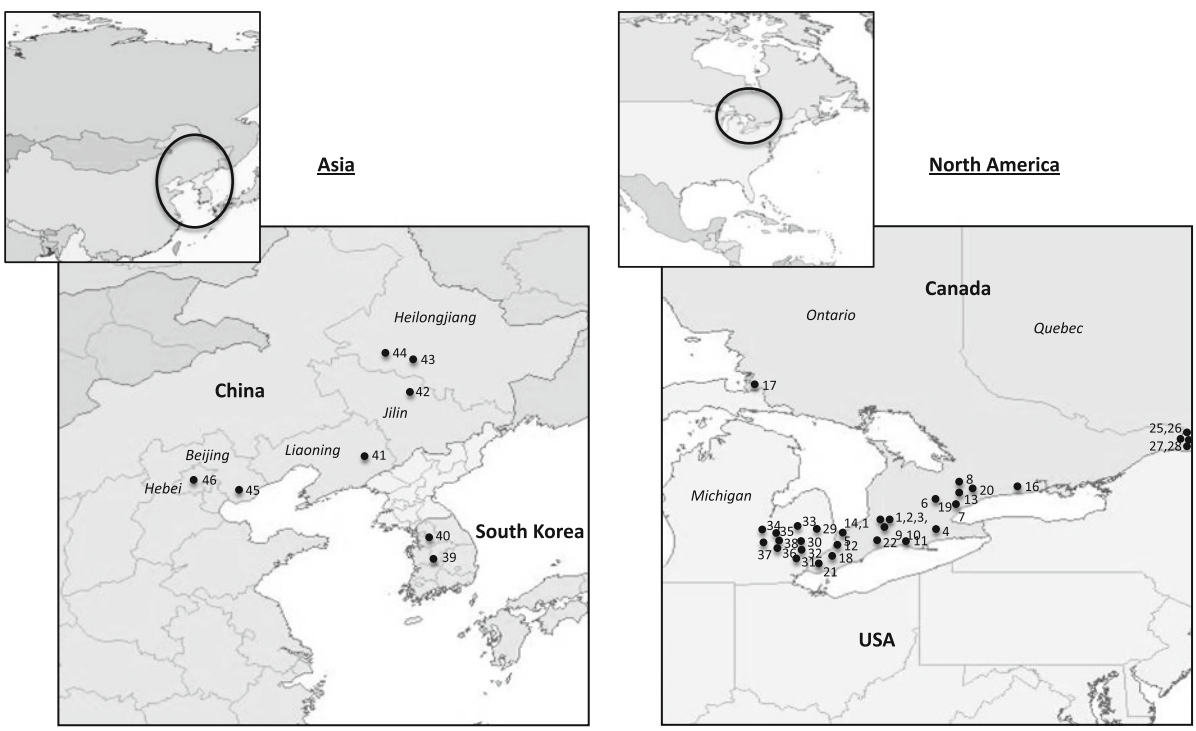

Fig. 1 A map of sampled Asian and North American emerald ash borer populations. The population numbers refer to the populations in Table 1. *Labels in bold font* represent country labels. Chinese and Canadian provinces as well as US states are labeled in *italics*

Table 1 Information for sampled EAB populations, including population abbreviations (Population), associated region of sampling (Region), year sampled (Year) and sample size (N)

	Population	Population description	Region	*N*	Years
1	07.CH	London Carriage Hill Park	Ontario	23	2007
2	07.CLN	London Carroll St.	Ontario	15	2007
3	07.DD	London Doon Drive	Ontario	6	2007
4	07.NB	Norfolk County	Ontario	5	2007
5	07.TOA	Toronto A	Ontario	3	2007
6	08.AR	Mississagua	Ontario	7	2008
7	08.DR	Brampton Dixie Road N	Ontario	18	2008
8	08.EY	Vaughan Emery Road	Ontario	22	2008
9	08.LHS	London Hospital A	Ontario	20	2008
10	08.LHT	London Hospital B	Ontario	7	2008
11	08.NC	Norfolk County	Ontario	17	2008
12	08.PLL	Port Lambton	Ontario	20	2008
13	08.RDR	Vaughan	Ontario	5	2008
14	08.SAL	Lambton County A	Ontario	19	2008
15	08.SBL	Lambton County B	Ontario	21	2008
16	08.SCC	Lambton County St Clair Conservation Authority	Ontario	23	2008
17	08.SMA	Sault St. Marie	Ontario	24	2008
18	08.WCK	Chatham-Kent County	Ontario	20	2008
19	08.WLL	Lambton County	Ontario	27	2008
20	07.TO	Toronto B	Ontario	3	2007
21	05.ME	MacGregor Essex County	Ontario	10	2005
22	06.DEC	Elgin County	Ontario	1	2006
23	07.CLM	London	Ontario	3	2007
24	07.LHS	London Hospital C	Ontario	18	2007
25	08.QC	Carignan	Quebec	8	2008
26	08.QCA	Carignan 1190 Rue Demers, lle Demeis	Quebec	29	2008
27	08.QCB	Carignan 144 Rue des Sapins, lle Demeis	Quebec	30	2008
28	08.QCC	Carignan 2142 Rue de Roses, lle Goyer	Quebec	29	2008
29	08.BMR	Bald Mountain Recreation Area, Lake Orion	Michigan	30	2008
30	06.IL	Island State Recreation Area, Brighton	Michigan	1	2006
31	05.CFP	County Farm Park, Ann Arbor	Michigan	2	2005
32	06.OG	Oak Grove State Game Area, Oak Grove	Michigan	3	2006
33	06.SL	Sullivan Lake, Tyrone	Michigan	4	2006
34	07.WAP	Washington Park, Lansing	Michigan	4	2007
35	06.SP	Stoddard Park, East Lansing	Michigan	2	2006
36	06.PR	Pinckney State Recreation Area, Pinckney	Michigan	3	2006
37	05.HM	Hudson Mills Metro Park, Dexter	Michigan	3	2005
38	07.LA	Livingston County Airport, Howell	Michigan	3	2007
39	08.KDJ	Daejeon	South Korea	3	2008
40	08.KYS	Yangsuri	South Korea	3	2008
41	03.C-BAL	Benxi-A, Liaoning	China	2	2003
42	07.CJJ	Jiutai, Jilin	China	3	2007
43	07.CHH	Harbin, Heilongjiang	China	4	2007
44	07.CLH	Laoshan, Heilongjiang	China	4	2007
45	06.CTH	Tangshan, Hebei	China	4	2006
46	07.CCB	Chaoyang, Beijing City	China	1	2006

the invasion of this species. In total we sampled 516 individuals from 48 sites, covering 5 regions: Ontario, Quebec, Michigan, China and South Korea.

Marker cloning

Initial tissue samples were sent as whole larvae, collected in Ontario between 2003 and 2006, to Genetic Identification Services (GIS, Chatsworth, CA, US) for cloning and isolation of microsatellite markers in May 2007. Digested DNA from the EAB was size selected for 300–700 base pair fragments and introduced into *E. coli* strain DH5α (ElectroMax™, Invitrogen) by electroporation. Colonies were then grown on agar plates for blue/white selection of positive colonies and enriched with CA, GA, AAC, and ATG microsatellite motifs. Positive colonies were sequenced using DYEnamic ™ ET Terminator Cycle Sequencing kit (Amersham Biosciences P/N US81050) and electrophoresis was performed on an Applied BioSystems Model 377 DNA sequencer. Primers were tested under standard PCR conditions and PCR cocktails contained 5.15 µl H_2O, 1 µl BioTaq Buffer, 1 µl Sucrose/Cresol Red, 0.4 µl MgCl, 0.8 µl DNTP's, 0.3 µl forward and reverse primers, 0.05 µl BioTaq and 1 µl template DNA. PCR reactions were denatured at 94 °C for 3 min, followed by 35 cycles at 94 °C for 40 s, 55–57 °C for 40 s 72 °C for 30 s, and a final extension step of 72 °C for 4 min. Products were run on a standard 1 % agarose gel and scored for presence or absence of bands and polymorphism.

Laboratory extraction

For the PCR trial and population genetic amplification, DNA was extracted in the lab with a STE Buffer DNA modified extraction protocol (Sambrook et al. 1989). This protocol was acquired from Dr. Carol Ritland (Genetic Data Center, University of British Columbia) and can be sent upon request to the corresponding author.

PCR conditions

Microsatellite sequences, primers, and test amplifications were obtained from GIS and primers for population genetic analysis were selected on the basis of polymorphism from test amplifications and repeat types. These primers include C5, C8, D120, D126, D130, D140 and AB6 (Table 2). For all but two primers, PCR reaction reached a maximum volume of 10.4 µl (see Table 3).

PCR products were run on 5.25 % polyacrylamide gels (SequaGel XR, National Diagnostics, Distributor Diamed), 18 or 25 cm in length and 0.25 or 0.4 mm in thickness, for 2–3.5 h on the LI-COR 4200 Auto-Sequencer (LI-COR Inc.). No difference was observed between gels of different thicknesses. Four standard ladders indicating 50–350 bp were loaded per gel. Gels were run between 1 and 3 times each, on both the 700 and 800 channels. Assignment of a primer to a particular channel was arbitrary. Gels were scored using SAGA Generation 2 (LI-COR Inc.). Scoring was done using both the intensity of the bands and the peaks given by the program.

Blast search for marker homologues

For each locus that was successfully cloned by GIS, and that we amplified in EAB for population genetic analyses, a BLASTn search was conducted for significantly similar nucleotide sequences. BLAST searches were conducted on 460–500 base pairs of trimmed sequence, containing the microsatellite, for primers C5, C8, D120, D126, D130, D140. One locus, AB6 was cloned from a separate source (Bray et al. 2008) and the cloned DNA sequence for this locus was unavailable, thus a BLAST search was not conducted. Each microsatellite sequence was also translated, and the resulting protein sequence was BLASTx searched for significant matches to protein-coding genes.

Population genetic data collection

Microsatellite characteristics and within population genetic variation

To test for a stepwise mutation model of microsatellite loci, allele size, frequency distributions for all seven loci were plotted in R (Development Core Team 2005, www.R-project.org). Distributions were assessed visually and with the Shapiro–Wilk normality test (Shapiro and Wilk 1965). Pair-wise analysis of linkage among loci within and over all populations was conducted in Genepop (Raymond and Rousset 1995a, b; Rousset 2008). Significance was assessed for each pair

Table 2 Measures of allelic richness for each population and locus

Population	C5	C8	D120	D126	D130	D140	AB6	Overall
07.CH	1.64	1.00	1.51	1.51	1.64	1.00	1.04	1.33
07.CLN	1.54	1.47	1.51	1.57	1.63	1.35	1.51	1.51
07.DD	1.54	1.00	1.54	1.41	1.53	1.00	1.36	1.34
07.NB	1.67	1.53	1.55	1.47	1.62	1.00	1.55	1.48
07.TOA	1.33	1.60	1.60	1.53	1.53	1.00	1.60	1.46
08.AR	1.66	1.43	1.52	1.70	1.52	1.00	1.60	1.49
08.DR	1.66	1.50	1.49	1.68	1.59	1.00	1.04	1.42
08.EY	1.51	1.43	1.43	1.51	1.50	1.00	1.44	1.40
08.LHS	1.50	1.13	1.24	1.47	1.57	1.00	1.46	1.34
08.LHT	1.44	1.10	1.32	1.58	1.52	1.00	1.32	1.33
08.NC	1.59	1.39	1.51	1.61	1.06	1.25	1.68	1.44
08.PLL	1.63	1.45	1.51	1.68	1.56	1.25	1.64	1.53
08.RDR	1.44	1.47	1.52	1.66	1.46	1.16	1.71	1.49
08.SAL	1.68	1.47	1.51	1.66	1.55	1.40	1.52	1.54
08.SBL	1.68	1.50	1.44	1.63	1.53	1.38	1.56	1.53
08.SCC	1.62	1.45	1.51	1.55	1.41	1.15	1.52	1.46
08.SMA	1.67	1.34	1.51	1.63	1.35	1.00	1.20	1.39
08.WCK	1.55	1.49	1.50	1.64	1.59	1.12	1.53	1.49
08.WLL	1.64	1.49	1.36	1.56	1.62	1.03	1.58	1.47
07.TO	1.67	1.50	1.53	1.47	1.44	1.17	1.55	1.47
05.ME	1.62	1.19	1.44	1.27	1.10	1.10	1.47	1.31
06.DEC	1.54	1.50	2.00	1.50	1.33	1.00	1.73	1.51
07.CLM	1.53	1.60	1.43	1.71	1.75	1.43	1.68	1.59
07.LHS	1.67	1.00	1.00	1.00	1.00	1.00	1.00	1.10
08.QC	1.54	1.53	1.52	1.48	1.19	1.10	1.79	1.45
08.QCA	1.63	1.47	1.51	1.54	1.13	1.13	1.60	1.43
08.QCB	1.62	1.50	1.51	1.55	1.00	1.00	1.61	1.40
08.QCC	1.46	1.45	1.51	1.55	1.35	1.43	1.68	1.49
08.BMR	1.69	1.48	1.49	1.58	1.51	1.13	1.61	1.50
06.IL	1.60	1.50	2.00	1.73	1.33	1.00	1.60	1.54
05.CFP	1.60	1.53	1.67	1.73	1.80	1.00	1.53	1.55
06.OG	1.60	1.00	1.53	1.53	1.73	1.00	1.73	1.45
06.SL	1.61	1.54	1.54	1.75	1.68	1.43	1.54	1.58
07.WAP	1.75	1.25	1.54	1.68	1.79	1.00	1.43	1.49
06.SP	1.53	1.60	1.60	1.57	1.60	1.00	1.33	1.46
06.PR	1.73	1.60	1.53	1.60	1.73	1.00	1.53	1.53
05.HM	1.73	1.33	1.60	1.80	1.60	1.00	1.73	1.54
07.LA	1.73	1.60	1.53	1.60	1.60	1.33	1.53	1.56
08.KDJ	1.60	1.60	1.60	1.93	1.60	1.80	1.53	1.67
08.KYS	1.62	1.59	1.55	1.68	1.55	1.68	1.76	1.63
03.C-BAL	1.71	1.54	1.60	1.68	1.83	1.00	1.73	1.59
07.CJJ	1.83	1.33	2.00	1.83	1.33	1.33	1.61	1.61
07.CHH	1.86	1.00	1.53	1.53	1.60	1.33	1.33	1.46

Table 2 continued

Population	C5	C8	D120	D126	D130	D140	AB6	Overall
07.CLH	1.00	1.00	1.43	1.25	1.54	1.00	1.71	1.28
06.CTH	1.25	1.25	1.00	1.68	1.43	1.25	1.57	1.35
07.CCB	1.73	1.00	1.54	1.64	1.68	1.25	1.71	1.51
Overall	1.49	1.15	1.17	1.40	1.55	1.00	1.46	1.32

Overall average allelic richnesses for each population and for each locus are shown in the last column and row of the table respectively

of loci using Fisher's method (Raymond and Rousset 1995a, b).

To assess the level of polymorphism within and among populations, allele size variation and allele frequency per population was analyzed using Genodive (Meirmans and Van Tienderen 2004). Allelic richness was calculated for all populations using Fstat (Goudet 1995). We calculated observed (H_o) and expected (H_e) heterozygosity for each locus over all loci and populations using Arlequin (Excoffier et al. 2005). To detect deviation from Hardy–Weinberg Equilibrium within populations, we calculated F_{is} in Genodive by the method of Weir and Cockerham (1984), and significance was tested with 10,000 permutations of the data.

Bayesian clustering analyses and assignment of genotypes

We used the model based clustering analysis STRUCTURE (Pritchard et al. 2000) to assess the most probable cluster membership for each individual from all populations from both the native and introduced range. Initially we ran one run for each k (population) from k = 1 to k = 9 using 1,000,000 iterations of the data and a burn-in period of 100,000. After initial sampling, we ran three independent runs, consisting of 2,000,000 iterations with a burn-in period of 200,000, for each k from k = 1 to k = 7. We ran the analysis assuming admixture among populations and no correlation among allele frequencies. We then analyzed populations in North America independently of Asian regions, in an attempt to detect a greater level of local clustering throughout the most heavily sampled region. Initially we ran one run from k = 1 to k = 7 using 1,000,000 iterations of the data and a burn-in period of 100,000. After initial sampling, we ran five runs for each value of K from k = 1 to k = 6, and each run consisted of 1,000,000 iterations with a burn-in period of 100,000. To select the appropriate model for each analysis (number of clusters), we took the mean likelihood value from three runs for each value of k (number of clusters) from k = 1 to k = 7 and calculated delta (Ln(P|D)) for each k as in Evanno et al. (2005).

Analysis of among population variation

To examine patterns of genetic variation among the 48 populations, pairwise F_{ST} estimates and Analyses of Molecular Variance (AMOVA) analysis were conducted using Genodive (Meirmans and Van Tienderen 2004). Pairwise F_{ST} estimates were calculated using the method of Weir and Cockerham (1984). Significance was assessed after a sequential Bonferroni correction for multiple tests.

Geographic sampling location were identified in Google Earth either using GPS waypoints or a description of the sampling location. We used transformed GPS waypoints in the program Gen-AlEx to calculate a geographic distance matrix among all sampling locations (Peakall and Smouse 2006). We analyzed the relationship between genetic distance and geographic distance among populations using Mantel tests of isolation by distance in Genodive (Meirmans and Van Tienderen 2004). Geographic distances were transformed with the natural logarithm to improve normality.

To determine which population grouping explains the largest proportion of the genetic variation, AMOVA were conducted using the method of Excoffier (1992). Analysis of among population, within population and among individual genetic variation was conducted on all populations sampled regardless of

Table 3 Measures of observed heterozygosity (H_o) for each population and locus

Population	C5	C8	D120	D126	D130	D140	A6	Overall
07.CH	0.92	0.00	1.00	0.96	0.92	0.00	0.04	0.55
07.CLN	0.56	0.50	1.00	0.67	0.65	0.43	0.47	0.61
07.DD	0.71	0.00	1.00	0.50	0.86	0.00	0.43	0.50
07.NB	1.00	0.83	1.00	0.60	0.50	0.00	0.33	0.61
07.TOA	0.33	0.33	1.00	0.67	0.00	0.00	0.33	0.38
08.AR	1.00	0.56	1.00	0.90	0.44	0.00	0.44	0.62
08.DR	0.50	0.73	0.71	0.68	0.54	0.00	0.04	0.46
08.EY	0.50	0.54	0.60	0.55	0.48	0.00	0.50	0.45
08.LHS	0.52	0.14	0.28	0.42	0.52	0.00	0.33	0.32
08.LHT	0.41	0.10	0.39	0.33	0.64	0.00	0.38	0.32
08.NC	0.67	0.50	1.00	0.71	0.06	0.17	0.72	0.55
08.PLL	0.64	0.50	1.00	0.63	0.52	0.21	0.70	0.60
08.RDR	0.60	0.22	0.92	0.82	0.67	0.17	0.33	0.53
08.SAL	0.55	0.45	0.62	0.65	0.41	0.43	0.55	0.52
08.SBL	0.75	0.52	0.46	0.50	0.42	0.23	0.57	0.49
08.SCC	0.64	0.42	0.39	0.54	0.36	0.16	0.50	0.43
08.SMA	0.60	0.29	1.00	0.50	0.45	0.00	0.14	0.43
08.WCK	0.62	0.52	0.65	0.85	0.61	0.13	0.60	0.57
08.WLL	0.67	0.40	0.40	0.59	0.57	0.03	0.67	0.48
07.TO	0.86	0.50	0.40	0.20	0.29	0.17	0.00	0.34
05.ME	0.80	0.20	0.60	0.30	0.10	0.10	0.30	0.34
06.DEC	0.75	0.50	1.00	0.50	0.33	0.00	1.00	0.58
07.CLM	0.67	1.00	0.50	0.00	1.00	0.00	1.00	0.60
07.LHS	0.43	0.15	0.18	0.42	0.58	0.00	0.46	0.32
08.QC	0.64	0.38	1.00	0.50	0.20	0.10	0.78	0.51
08.QCA	0.70	0.53	1.00	0.48	0.13	0.13	0.73	0.53
08.QCB	0.83	0.70	1.00	0.00	0.47	0.00	0.70	0.53
08.QCC	0.52	0.47	1.00	0.67	0.37	0.47	0.70	0.60
08.BMR	0.70	0.63	0.40	0.40	0.53	0.14	0.80	0.51
06.IL	0.67	0.50	1.00	0.33	0.33	0.00	0.33	0.45
05.CFP	0.33	0.00	1.00	1.00	1.00	0.00	0.67	0.57
06.OG	0.33	0.00	0.67	0.00	0.67	0.00	1.00	0.38
06.SL	0.50	0.75	0.75	0.25	1.00	0.50	0.25	0.57
07.WAP	0.50	0.25	0.75	0.75	1.00	0.00	0.50	0.54
06.SP	0.67	0.33	0.33	0.50	0.67	0.00	0.33	0.40
06.PR	1.00	1.00	0.67	0.67	1.00	0.00	0.67	0.71
05.HM	1.00	0.33	1.00	0.00	1.00	0.00	0.67	0.57
07.LA	0.67	1.00	0.67	0.33	1.00	0.33	0.00	0.57
08.KDJ	0.67	0.67	1.00	0.67	0.33	0.67	0.00	0.57
08.KYS	0.50	0.67	1.00	0.50	1.00	0.67	1.00	0.76
03.C-BAL	0.50	0.25	1.00	0.75	1.00	0.00	0.33	0.55
07.CJJ	1.00	0.67	0.67	0.67	0.33	0.00	0.33	0.52
07.CHH	0.00	0.00	0.50	0.25	0.75	0.00	1.00	0.36

Table 3 continued

Population	C5	C8	D120	D126	D130	D140	A6	Overall
07.CLH	0.25	0.25	0.00	0.25	0.50	0.25	1.00	0.36
06.CTH	0.67	0.00	0.25	0.50	0.75	0.25	0.50	0.42
07.CCB	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14
Overall	0.65	0.42	0.72	0.51	0.56	0.13	0.50	0.50

Overall observed heterozygosities for each population and for each locus are shown in the last column and row of the table respectively

sample size. Five population groupings were used to attempt to explain the both the spatial and temporal distribution of genetic variation in our sample.

1. North American (Michigan, Ontario, and Quebec) and Asian (South Korea and China) regions
2. North American regions (Michigan, Ontario, and Quebec)
3. Asian regions (South Korea, and China)
4. Grouped among years for populations in Ontario, Quebec and Michigan
5. Grouped among years for populations in Ontario

Exploration of amplification patterns and polymorphism by locus and by region

We explored differences in mean observed (H_o) and expected (H_e) heterozygosity and allelic richness by locus to determine if these measures were correlated. We plotted allelic richness, H_o and H_e data by locus in R to visually discern the impact of locus type on polymorphism. We conducted ANOVA, and a one tailed *t* test to determine whether a significant proportion of the variation in estimates of observed and expected heterozygosity and allelic richness could be explained by locus. We used a one tailed *t* test to examine the differences among all loci in values of allelic richness and heterozygosity. We adjusted alpha values for multiple tests using a sequential Bonferroni correction (Rice 1988).

We also explored relationships between allelic richness and observed (H_o) and expected (H_e) heterozygosity and the region. We predict that genetic diversity in North American populations would be lower than the Asian populations due to founder effects in the introduced range (Edmands 2001; Wares et al. 2005; Dlugosch and Parker 2008). First, to evaluate more generally whether genetic variation is lower in the introduced range, we pooled regions into a North American and an Asian group and conducted ANOVA to determine whether a significant proportion of the variation in allelic richness and measures of heterozygosity can be explained between larger geographic regions. We then ran an ANOVA to determine if a significant proportion of the variation in richness and heterozygosity can be explained among specific geographic regions (e.g. Ontario, Quebec, Michigan, Korea, China). Mean allelic richness and heterozygosity were then assessed for differences among regions using a one tailed *t* test. We adjusted alpha values for multiple tests using a sequential Bonferroni correction.

Results

Microsatellite identification

We received 46 loci from Genetic Identification Services known to harbor microsatellite motifs. These loci were cloned, sequenced and initially screened on agarose to identify possible polymorphisms. After preliminary screening we chose 6 of these loci plus one additional locus from a related study of EAB (Bray 2009). We optimized PCR conditions and assessed population level diversity for these microsatellite markers, and the remainder of this analysis will focus on these seven markers (six from the EAB library and one from Michigan State University) (Online Resource 1; Online Resource 2). Further investigation of the sequences from this microsatellite library could uncover a greater number of polymorphic microsatellite loci. The sequences of the 46 positive loci and their corresponding primers are available on GenBank (accession numbers JN604382.1—JN604431.1).

Microsatellite motifs cloned by GIS were di- and tri-nucleotide motifs. We genotyped one di-nucleotide locus (ct) and six tri-nucleotide loci (including cat, gat, caa, taa) (Online Resource 1). BLASTn and BLASTx comparisons of cloned EAB sequence produced no notable matches to known coding sequence.

Population genetic analysis

Sampling

We sampled 48 populations between 2003 and 2008 from five regions: China, South Korea, Michigan, Ontario, and Quebec (Fig. 1; Table 1). Overall 512 individuals were sampled at seven microsatellite loci and each population consisted of 1–30 individuals. Samples for which three or more genotypes were missing were excluded from the analysis and there is reason to suspect that these samples may have been inadequately preserved leading to low quality DNA extractions and unsuccessful amplification of microsatellite markers. These individuals were excluded from the total count of 512 individuals genotyped fully for all 7 markers. Although DNA extraction failure was likely a cause for this issue, we cannot rule out the presence of null alleles at these loci.

Characterization of within-population genetic variation

Microsatellite loci contained from 2 to 9 alleles. Only one locus D120 had two alleles, while the remainder of loci had between 4 and 9 alleles. Out of 42 alleles sampled, 25 alleles were found in North American populations and 39 alleles were found in Asian populations. Asian and North American populations shared 23 alleles, only three alleles were shared solely among North American populations, while 17 were restricted in Asian populations, 3 alleles were shared only between South Korea and North America and 3 different alleles were shared between only China and North America. Overall, in 6 out of 7 loci (D140, AB6, D130, D126, C8 and C5) Asian populations exhibited private alleles relative to North American populations. In some cases (allele 260 at locus D126) private alleles are shared between South Korean and Chinese populations, while in other cases (allele 55 at locus AB6) alleles are isolated to either China or South Korea.

To determine whether microsatellite loci conformed to a stepwise mutation model we pooled allele size for all populations for each locus and plotted allele size frequency distributions (Online Resource 4). If the size frequency distributions appeared to be normally distributed or to statistically conform to normality then these loci likely conform to a stepwise mutation model. However, if size frequency distributions are not normally distributed then these markers are not stepwise and instead conform to some unknown mutation model. A Shapiro–Wilk normality test showed that for all loci the null hypothesis of normal allele size frequency distributions can be rejected and that all loci show a deviation from normality (range of W values: 0.3177, 0.7844, $P = 2.2 \times 10^{-16}$). In all cases loci had bimodal or tri-modal distributions. In some cases loci (C5, C8, AB6, D130 and D140) have two or three frequency modes and several additional low frequency alleles distributed between the modes and in other cases (D120 and D126) low frequency alleles are absent.

We examined linkage disequilibrium among microsatellite loci by testing for pairwise linkage among markers. None of the loci in this sample appeared to be in significant linkage disequilibrium when populations were pooled or treated independently ($P = 0.27$–0.98).

In both North American and Asian populations allelic richness ranged from 1 to 2 (Table 2). Mean allelic richness in North American regions was 1.47 (Ontario), 1.50 (Michigan) and 1.44 (Quebec). Similarly the two Asian regions had an average rarefied allelic richness of 1.50 (South Korea) and 1.40 (China).

Observed heterozygosity ranged from 0 to 1 in all populations (Table 3). Mean observed heterozygosities in North American regions were 0.58 (Ontario), 0.62 (Michigan), and 0.58 (Quebec). Observed heterozygosities in Asian regions were on average lower than those in North America and ranged from 0.49 (South Korea) to 0.44 (China). Expected heterozygosity ranged from 0.03 (population in Ontario) to 1.00 (populations in Michigan, and South Korea; Table 4). Mean expected heterozygosities in North American regions were 0.48 (Ontario), 0.57 (Michigan), and 0.48 (Quebec). Average expected heterozygosity in Asian regions was 0.57 (China) and 0.64 (South Korea).

To determine whether populations conformed to the assumption of random mating, we tested populations

Table 4 Measures of expected heterozygosity (H_e) for each population and locus

Population	C5	C8	D120	D126	D130	D140	AB6	Overall
07.CH	0.64	0.00	0.50	0.50	0.63	0.00	0.04	0.33
07.CLN	0.54	0.47	0.50	0.57	0.63	0.34	0.51	0.51
07.DD	0.52	0.00	0.50	0.40	0.50	0.00	0.36	0.33
07.NB	0.63	0.50	0.50	0.45	0.63	0.00	0.57	0.47
07.TOA	0.33	0.67	0.50	0.50	0.67	0.00	0.67	0.48
08.AR	0.64	0.42	0.50	0.69	0.53	0.00	0.61	0.48
08.DR	0.66	0.49	0.48	0.68	0.59	0.00	0.04	0.42
08.EY	0.51	0.43	0.42	0.51	0.50	0.00	0.44	0.40
08.LHS	0.50	0.13	0.24	0.48	0.57	0.00	0.47	0.34
08.LHT	0.44	0.10	0.32	0.60	0.51	0.00	0.31	0.33
08.NC	0.59	0.38	0.50	0.61	0.06	0.25	0.68	0.44
08.PLL	0.63	0.45	0.50	0.68	0.56	0.25	0.64	0.53
08.RDR	0.43	0.49	0.50	0.65	0.46	0.16	0.75	0.49
08.SAL	0.68	0.47	0.51	0.66	0.55	0.40	0.52	0.54
08.SBL	0.68	0.50	0.44	0.63	0.53	0.39	0.56	0.53
08.SCC	0.62	0.45	0.51	0.55	0.41	0.15	0.52	0.46
08.SMA	0.67	0.34	0.50	0.63	0.35	0.00	0.20	0.39
08.WCK	0.55	0.49	0.50	0.63	0.59	0.12	0.53	0.49
08.WLL	0.64	0.49	0.36	0.56	0.62	0.03	0.57	0.47
07.TO	0.66	0.50	0.55	0.50	0.45	0.17	0.60	0.49
05.ME	0.61	0.19	0.43	0.27	0.10	0.10	0.48	0.31
06.DEC	0.50	0.50	–	0.50	0.33	0.00	0.70	0.42
07.CLM	0.50	0.50	0.42	0.83	0.71	0.50	0.63	0.58
07.LHS	0.49	0.15	0.17	0.40	0.55	0.00	0.46	0.32
08.QC	0.53	0.54	0.50	0.48	0.19	0.10	0.79	0.45
08.QCA	0.63	0.47	0.50	0.54	0.13	0.13	0.60	0.43
08.QCB	0.61	0.50	0.50	0.55	0.00	0.00	0.61	0.40
08.QCC	0.46	0.45	0.50	0.55	0.35	0.43	0.68	0.49
08.BMR	0.69	0.48	0.49	0.58	0.51	0.13	0.60	0.50
06.IL	0.58	0.50	–	0.83	0.33	0.00	0.67	0.49
05.CFP	0.67	0.67	0.50	0.67	0.75	0.00	0.50	0.54
06.OG	0.67	0.00	0.50	0.67	0.75	0.00	0.67	0.46
06.SL	0.63	0.50	0.50	0.83	0.63	0.42	0.58	0.58
07.WAP	0.79	0.25	0.50	0.67	0.75	0.00	0.42	0.48
06.SP	0.50	0.67	0.67	0.58	0.58	0.00	0.33	0.48
06.PR	0.67	0.50	0.50	0.58	0.67	0.00	0.50	0.49
05.HM	0.67	0.33	0.50	1.00	0.50	0.00	0.75	0.54
07.LA	0.75	0.50	0.50	0.67	0.50	0.33	0.67	0.56
08.KDJ	0.58	0.58	0.50	1.00	0.67	0.83	0.67	0.69
08.KYS	0.63	0.58	0.50	0.70	0.50	0.68	0.73	0.62
03.C-BAL	0.75	0.58	0.50	0.67	0.75	0.00	0.83	0.58
07.CJJ	0.83	0.00	0.50	0.50	0.58	0.33	0.33	0.44
07.CHH	0.00	0.00	0.42	0.25	0.50	0.00	0.67	0.26

Table 4 continued

Population	C5	C8	D120	D126	D130	D140	AB6	Overall
07.CLH	0.25	0.25	0.00	0.75	0.42	0.25	0.50	0.35
06.CTH	0.75	0.00	0.58	0.67	0.67	0.25	0.75	0.52
07.CCB	0.50	–	–	0.00	–	–	–	0.25
Overall	0.58	0.39	0.47	0.59	0.51	0.15	0.55	0.46

Overall estimates of H_e for each population and locus are found in the last column and row of this table respectively

for conformation to Hardy–Weinberg Equilibrium (HWE) using the F_{IS} test statistic. Estimates of F_{IS} ranged from −1.00 to 0.67 (Table 5). Few populations and loci showed a significant deviation from Hardy–Weinberg Equilibrium (HWE). Populations and loci that showed significant deviation from HWE were from Ontario, Quebec, and Michigan and China (07.CH, 07.CLN, 08.NC, 008.PLL, 08.QCA, 08.QCB, 08.QCC and 08.SMA; see Table 1 for population names). Locus D120 from populations in China, Ontario, Michigan and Quebec, showed a significant excess of heterozygous genotypes relative to equilibrium conditions ($F_{IS} = -1.00$). Locus D120 was also the only locus to show an overall deviation from HWE among all populations ($F_{IS} = -0.57$). Population 07.CH from Ontario showed a significant heterozygote excess at locus D126 and also a heterozygote excess among all markers ($F_{IS} = -0.91$ and -0.69). Two additional populations 08.QCA and 08.QCB showed a significant global deviation from HWE ($F_{IS} = -0.25$ and -0.34 respectively; Table 5).

Exploration of amplification patterns and polymorphism

We investigated the association between levels of diversity measured as observed (H_o) and expected (H_e) heterozygosity and allelic richness by both locus and region. A significant difference among loci in levels of microsatellite diversity would suggest that a particular locus or repeat types may be more suitable for certain analyses (such as paternity analyses) than others. Conversely, similarities of differences in levels diversity among regions can, for example, shed light on possible mechanisms of introduction of EAB in the introduced range.

ANOVA analyses of allelic richness and H_o and H_e among loci showed that a significant proportion of the variation could be explained among loci ($P = 6.4e^{-15}$, $P = 6.4e^{-15}$, $P = 2.2e^{-16}$ respectively; Fig. 2a, c, e). Locus D140 appeared to have lower values of H_o, H_e and allelic richness than any other locus, suggesting that this locus may not be suitable for analyses that require a high level of allelic or genotypic diversity (Fig. 2).

We expected diversity to be higher in the native (Asian) range than the introduced (North American) range of EAB, particularly if there were few introductions and a significant loss of diversity due to a genetic bottleneck upon introduction. If we find no significant difference in diversity among native and introduced regions or higher diversity in the introduced range then this may point to either multiple introductions, relaxed predation and selection in the introduced range and a rapid increase in genetic diversity relative to the native range, and/or sufficient time for accumulation of neutral diversity within introduced EAB populations (Lee 2002; Wares et al. 2005). There was no significant difference in either observed heterozygosity or allelic richness among the five sampled regions in an ANOVA among regions ($P = 0.15$, $P = 0.10$; Fig. 2b, d). Surprisingly, a significant proportion of the variation in expected heterozygosity was distributed among regions ($P = 0.003$; Fig. 2f). In this case H_e was higher in both Asian regions than in any North American regions. One-way *t* tests showed that variation was highest in South Korea for all three measures of diversity, however, it was only significantly larger for allelic richness and expected heterozygosity, suggesting that although genetic diversity may be higher in South Korea (and China H_e), the distribution of diversity (actual number of heterozygotes vs. homozygotes) does not differ among regions. One notable discrepancy between the results of allelic richness and expected heterozygosity is the difference in the measures of diversity in the Chinese populations. There is a greater expected heterozygosity in China relative to populations in North America (specifically

Table 5 Deviation from Hardy–Weinberg Equilibrium within populations, measured as F_{is}

Population	C5	C8	D120	D126	D130	D140	AB6	Overall
07.CH	−0.44	–	**−1.00**	**−0.91**	−0.55	–	0.00	**−0.69**
07.CLN	−0.08	−0.08	**−1.00**	−0.22	0.00	−0.27	0.10	−0.21
07.DD	−0.25	–	−1.00	−0.25	−0.67	–	−0.25	−0.50
07.NB	−0.54	−0.60	−1.00	−0.33	−0.20	–	0.39	−0.35
07.TOA	0.00	0.50	−1.00	−0.33	1.00	–	0.50	0.20
08.AR	−0.59	−0.20	−1.00	−0.47	0.22	–	−0.33	−0.41
08.DR	0.23	−0.56	−0.56	−0.09	−0.03	–	0.00	−0.16
08.EY	0.00	−0.18	−0.31	−0.07	0.11	–	−0.09	−0.08
08.LHS	0.02	−0.03	−0.06	−0.01	−0.09	–	0.25	0.03
08.LHT	−0.04	0.00	−0.20	0.38	−0.50	–	−0.20	−0.06
08.NC	−0.20	−0.28	**−1.00**	−0.16	0.00	0.32	−0.03	−0.25
08.PLL	−0.11	−0.07	**−1.00**	0.05	0.01	0.24	−0.33	−0.19
08.RDR	−0.33	1.00	−1.00	−0.19	−0.60	−0.14	0.36	−0.08
08.SAL	0.24	−0.03	−0.24	0.03	0.21	−0.13	0.01	0.03
08.SBL	−0.05	0.05	−0.12	0.24	0.25	0.41	−0.04	0.09
08.SCC	0.08	0.06	0.23	0.01	0.02	−0.07	−0.03	0.06
08.SMA	0.19	0.20	**−1.00**	0.31	−0.28	–	0.26	−0.06
08.WCK	−0.10	−0.10	−0.19	−0.35	−0.05	−0.06	−0.09	−0.15
08.WLL	−0.06	0.13	−0.10	−0.06	0.10	0.00	−0.19	−0.03
07.TO	−0.14	1.00	0.00	0.50	1.00	–	1.00	0.63
05.ME	−0.32	−0.06	−0.39	−0.13	0.00	0.00	0.37	−0.11
06.DEC	–	–	–	–	–	–	–	–
07.CLM	−0.33	−1.00	−0.33	1.00	−0.50	1.00	−1.00	−0.08
07.LHS	0.19	−0.03	−0.10	−0.08	0.02	–	0.19	0.06
08.QC	−0.11	0.30	−1.00	0.02	0.00	–	0.05	−0.12
08.QCA	−0.14	−0.16	**−1.00**	0.11	−0.06	−0.06	−0.21	**−0.25**
08.QCB	−0.36	−0.40	**−1.00**	0.16	–	–	−0.16	**−0.34**
08.QCC	−0.13	−0.05	**−1.00**	−0.25	−0.07	−0.06	−0.02	−0.23
08.BMR	−0.01	−0.33	0.18	0.31	−0.06	−0.06	−0.33	−0.04
06.IL	–	–	–	–	–	–	–	–
05.CFP	0.00	–	−1.00	−0.33	−0.33	–	0.00	−0.33
06.OG	0.50	–	−0.33	1.00	0.11	–	−0.50	0.18
06.SL	0.20	−0.50	−0.50	0.70	−0.60	−0.20	0.57	0.02
07.WAP	0.37	0.00	−0.50	−0.13	−0.33	–	−0.20	−0.11
06.SP	0.00	0.00	0.00	0.00	−0.33	–	0.00	−0.08
06.PR	−0.50	−1.00	−0.33	−0.14	−0.50	–	−0.33	−0.46
05.HM	−0.50	0.00	−1.00	1.00	−1.00	–	0.11	−0.07
07.LA	0.11	−1.00	−0.33	0.50	−1.00	0.00	1.00	−0.02
08.KDJ	−0.14	−0.14	−1.00	0.33	0.50	0.20	1.00	0.17
08.KYS	0.21	−0.14	−1.00	0.29	−1.00	0.02	−0.36	−0.23
03.C-BAL	–	0.00	−1.00	0.50	−0.33	–	0.50	0.07
07.CJJ	−0.09	–	−0.33	−0.33	−0.14	0.00	0.00	−0.16
07.CHH	–	–	−0.20	0.00	−0.50	–	−0.50	−0.36

Table 5 continued

Population	C5	C8	D120	D126	D130	D140	AB6	Overall
07.CLH	0.00	0.00	–	0.67	−0.20	0.00	−1.00	−0.03
06.CTH	0.11	–	0.57	0.25	−0.13	0.00	0.33	0.21
07.CCB	–	–	–	–	–	–	–	–
Overall	–	−0.11	**−0.57**	0.01	−0.08	0.05	−0.05	**−0.14**

Information on overall deviation from equilibrium for each population and locus can be found in the last column and row of this table. Categories with dashed lines (e.g. 07.CH at locus C8) are either monomorphic for a particular allele, are not sampled for this locus or like in the case of population 06.Dec, are only sampled for one individual

Bold values are significant at $P < 0.05$

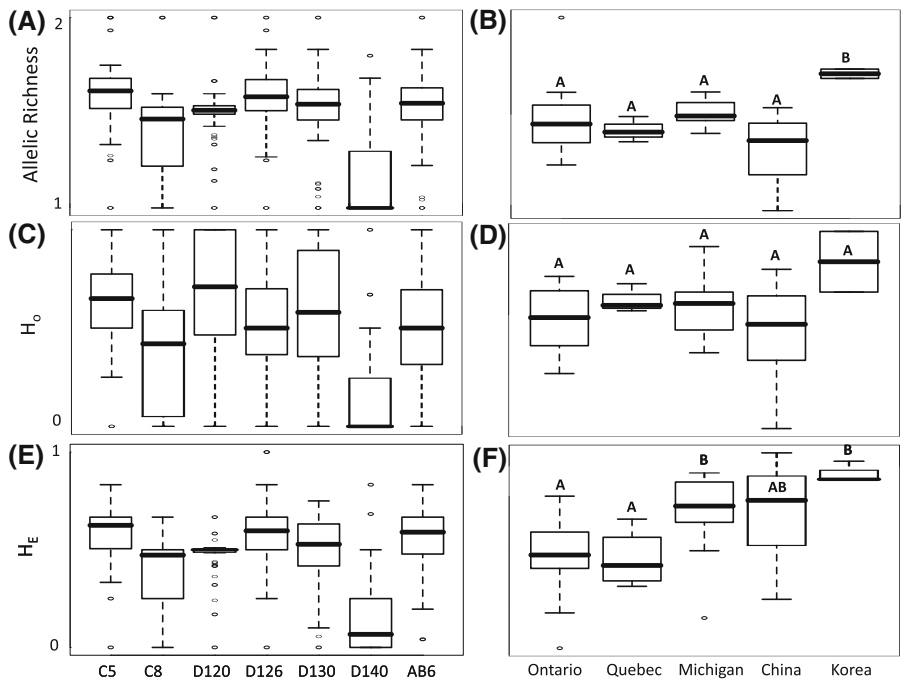

Fig. 2 Boxplots describing the relationship among regions and among loci for allelic richness, observed (HO) and expected heterozygosity (HE) for the emerald ash borer. Estimates of allelic richness and heterozygosity plotted by marker type (**a**, **c** and **e**) and by region (**b**, **d** and **f**). The *dark horizontal line* represents the median estimate of richness or heterozygosity. The *bottom and top of the box* show the 1st and 3rd quartiles. *Dotted lines* represent 2 standard deviations from the mean value and *open circles* denote outlying data points. The *letters above the boxplots* signify significance in one-way pairwise *t* tests of differences in diversity between regions. *Boxes with the same letters* were not significantly different from one another and *a change in letter notation* indicates significant regional differences in diversity

Quebec where $P = 0.003$); in fact the Chinese populations have the second highest level of expected heterozygosity in this sample. However, allelic richness from Ontario, Quebec and Michigan was slightly larger than that of the Chinese populations. This result may suggest that although we controlled for rarefaction in our measure of allelic richness, low sample sizes in all Chinese populations could have caused a downward bias in measures of average allelic richness among the populations in China, a bias that was not found when calculating expected heterozygosity. When regions were pooled into a North American and an Asian sample there was no significant difference in observed heterozygosity or allelic richness among regions ($P = 0.52$ and $P = 0.43$ respectively). In this case, mean heterozygosity and allelic richness in North American were slightly higher or equal to that of Asian populations, respectively. Alternatively,

there was a marginally significant difference in expected heterozygosity among continents ($P = 0.02$) and in this case expected heterozygosity was smaller in North America than in Asia.

Bayesian clustering analysis

We assessed the most probable genotype groupings using the Bayesian clustering analysis STRUCTURE. If there is a strong association among populations or regions then the majority of individuals in a particular location will show a high probability (70–100 %) of clustering with individuals from another population or region. These analyses may show associations among individuals in the native and introduced range of EAB, and in addition, they can identify the patterns and levels of population structure associated with a species invasion (Harter et al. 2004). Our analysis of population clustering was directed toward identifying associations between populations in North America and Asia, as well as identification of population structure within the sampled North American range of this species.

Results of the Bayesian clustering analyses using STRUCTURE on all North American and Asian regions suggest that there are two highly probable population clusters (Fig. 3). Although the maximum likelihood value was found at K = 6 populations (Ln(P|D) = −6,068.89, variance = 1,013.91), the delta K method of Evanno et al. (2005) suggests that K = 2 clusters is a better fit because it causes the greatest likelihood improvement from a lower K value in the analysis and has smaller variance than K = 6 (Ln(P|D) = −6,164.89, variance = 504.68). The majority of individuals from South Korea, China and Quebec have a higher probability (>70 %) of falling into the blue population cluster, while at least half the individuals from Michigan and Ontario have a higher probability of falling into the yellow population cluster. One population from Chaoyang, Bejing, China (07.CCB) had a moderate (~67 %) probability of falling into the yellow population cluster suggesting that individuals in this population may be more closely linked to individuals in the yellow cluster (Michigan and Ontario). When populations in North America were analyzed without populations in the Asian regions, individuals were assigned to 3 population clusters (Ln(P|D) = 5,651.1, variance = 571.9). Few individuals had greater than 70 % probability of being assigned to any one of the three clusters. The first cluster (blue) consisted mostly of individuals from the London Ontario Hospital (07.LHS, 07LHT and 08.LHS), individuals with a high probability of clustering with the red cluster were from populations in Quebec, and the remainder of individuals in this sample had low assignment probability (~50 %) to a third (yellow) population cluster, suggesting that there may be a lack of isolation among some North American populations.

Genetic variation among populations

Population pairwise estimates of F_{ST} ranged from 0 to 0.53 (Online Resource). After sequential Bonferroni correction none of the F_{ST} values were significantly different from zero. As there were no significant F_{ST} values we pooled populations into groups and conducted AMOVA analyses to further investigate frequency-based associations between North American and Asian regions and to examine temporal structure among populations within North America. If regional groupings explain a significant proportion of the genetic variation, then weak gene flow, strong genetic drift and/or selection may be acting to differentiate geographically distinct regions. Significant variation among temporal groupings of EAB in North America would suggest that genetic variation may have changed through time and that changes since early introduction (2003) may reflect adaptation or diversification in the introduced range. AMOVA showed that allelic variation among regions (F_{CT}) within North America (Michigan, Ontario, Quebec) and among regions in both North America and Asia (groupings 1 and 2 respectively) explained a significant proportion of the total genetic variation (Table 6). When the three North American regions only were considered, 3 % of the genetic variation was explained among regions ($F_{CT} = 0.03$, $P = 0.004$). When all 5 regions, both Asian and North American, were considered in an AMOVA, among region variation explained 4 % of the total genetic variation ($F_{CT} = 0.04$, $P = 0.017$). When Asian populations were considered independently of North American populations (Grouping 3) among group variation (China and South Korea) explained 7 % of the total genetic variation ($F_{CT} = 0.073$, $P = 0.00$), suggesting a relatively high degree of regional differentiation in Asian population in EAB. Results of AMOVA analyses in North America and in North America and Asia

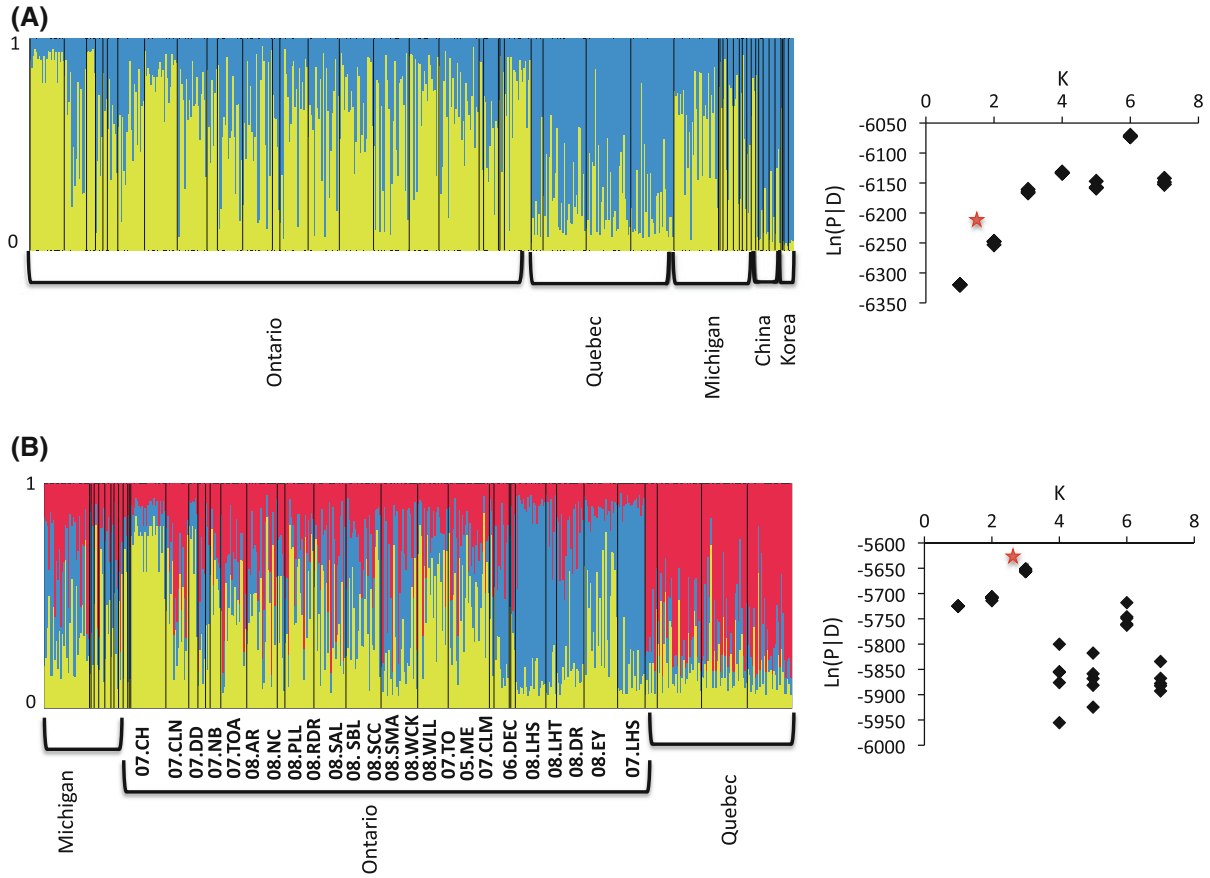

Fig. 3 Results of the heuristic Bayesian clustering analysis of emerald ash borer populations in STRUCTURE. The *colors* represent different clusters; **a** among the five regions and **b** among populations in North America. A plot of the likelihood scores for each K value is shown next to the graph of population clusters. The *star* represents the largest improvement of likelihood values from **a** K = 1 to K = 2 for and **b** from K = 2 to K = 3

combined suggest that a greater proportion of the total genetic variation can be explained among populations (F_{ST}) within groups than among the regional groupings (F_{CT}) themselves ($F_{ST} = 0.13$ $P = 0.00$). Similarly, when Asian populations were treated separately, among population variation (F_{ST}) explained a higher proportion of the total genetic variation than that explained by regional groupings ($F_{ST} = 0.15$). Overall, this result suggests a significant degree of allelic differentiation among populations within regions, relative to variation among regions. Finally, we found no evidence of temporal structure in our sample.

We tested for isolation by geographic distance to investigate patterns of allele sharing among populations. A significant signal of isolation by distance shows that populations that are geographically closest to each other have more similar allele and genotype frequencies, implying that dispersal, selection and/or genetic drift may be functioning to unite geographically proximal populations. Our mantel test revealed a significant signal of isolation by distance when all North American and Asian populations were included in the analysis ($r = 0.23$, $P = 0.007$). When we examined isolation by distance in only North American populations there was a non-significant but positive relationship between genetic distance and geographic distance ($r = 0.06$, $P = 0.19$). Finally, when we examined isolation by distance in Asian populations there was a significant negative relationship between geographic distance and genetic distance, suggesting that geographically distant populations were sometimes more genetically similar ($r = -0.47$, $P = 0.009$).

Table 6 AMOVA analysis of regional population grouping of emerald ash borer

Source of variation	Nested in	%var	Fixation index	*P* value
Group 1 among regions North America and Asia				
Within individual	–	0.94	0.06	–
Among individual	Population	−0.11	−0.13	1.00
Among population	Group1	0.12	0.13	0.00
Among group1	–	0.04	0.04	0.10
Group 2 among regions North America				
Within individual	–	0.96	0.04	–
Among individual	Population	−0.11	−0.13	1.00
Among population	Group2	0.12	0.13	0.00
Among group2	–	0.03	0.03	0.01
Group 3 among regions Asia				
Within individual	–	0.834	0.17	–
Among individual	Population	0.047	−0.06	0.90
Among population	Group3	0.141	0.15	0.00
Among group3		0.073	0.07	0.00
Group 4 among years North America				
Within Individual	–	0.97	0.03	–
Among Individual	Population	−0.11	−0.13	1.00
Among population	Group4	0.13	0.13	0.00
Among group4	–	0.02	0.02	0.61
Group 5 among years in Ontario				
Within individual	–	0.94	0.06	–
Among individual	Population	−0.09	−0.11	1.00
Among population	Group5	0.15	0.15	0.00
Among group5	–	0.00	0.00	0.55

Discussion

We cloned, sequenced, and characterized seven polymorphic microsatellite markers from the Emerald Ash Borer. We used these markers to genotype EAB individuals from North American (Canada and US) and Asian populations. Ultimately between 1 and 30 individuals were genotyped from 46 populations throughout the northeastern US, Canada, China and South Korea.

All populations for which greater than 15 individuals were sampled were from North America and the majority of these populations were sampled in 2007 and 2008 from Ontario. Three populations in Quebec and one in Michigan also had sample sizes greater than 15. Low sample sizes for all Asian and some North American populations could cause calculations of site-level genetic diversity to be over- (observed heterozygosity) or under- (expected heterozygosity, and allelic richness) represented in small populations relative to populations with larger sample sizes (Balloux and Lugon-Moulin 2002; Hellberg 2009). For this reason, we suggest caution in over interpreting our measures of within- and among-population variation, especially where few individuals were sampled. Bayesian clustering analysis is an exception (Pritchard et al. 2000). Since this analysis assigns individuals to populations, minimizing linkage among individual genotypes it is more reliable (

Excoffier and Heckel 2006). We therefore based our findings more heavily on the results of Bayesian clustering analyses than on the classical frequency based analyses such as F_{ST}.

The results of our population genetic analysis suggest that individuals in North America have lower levels of allelic diversity than those in Asia but that there is less of a discrepancy in relative frequency of observed heterozygosity. Our study added to the growing body of literature on EAB by collecting genetic data for multiple introduced Canadian

populations in the Provinces of Quebec and Ontario. These new data shows that populations from Quebec are unique relative to populations from Michigan and Ontario. Finally, we find high levels of genetic subdivision in native and introduced ranges of EAB when populations are pooled into regions. This finding suggests that broad scale isolation may be linked to the development of differential outcomes of genetic drift and selection in each region.

Utility of EAB microsatellite markers

The microsatellite markers described in this study and used in our population genetic analysis were polymorphic, with 2–9 alleles per locus. There were fewer alleles found in North American than in Asian populations suggesting that analyses for paternity and multiple mating should be done by selecting loci (such as D140 or AB6) with larger number of alleles in North American populations. One additional option would be to screen unanalyzed EAB microsatellite loci on GenBank (Accession Numbers JN604382.1—JN604431.1) in order to detect additional loci with high levels of polymorphism in North American populations.

Lack of association with known coding sequences suggests that EAB microsatellite markers are less likely to be subject to selection, thus may indeed be selectively neutral markers. Although microsatellites are often assumed to be neutral markers, researchers have found cloned sequences that include microsatellites associated with coding sequences and particular phenotypes (Li et al. 2002; Ranum and Day 2002). Therefore, investigation of association with known coding sequences before microsatellites neutrality is assumed is warranted. Furthermore, the lack of association with coding regions does not exclude the possible physical linkage of these loci to coding sequence (Selkoe and Toonen 2006).

Allele size frequency distributions significantly deviated from normality suggesting an overall lack of conformation to the stepwise mutation model. In EAB, the shape of allele-size frequency histograms were usually bimodal or unimodal and non-normally distributed. Analyses that reconstruct phylogenetic history of organisms usually assume stepwise mutation model, therefore the use of methods that employ a stepwise mutation model to microsatellites should be conducted with caution in EAB. Greater sampling of individuals and loci among populations could improve the normality of the samples by increasing the probability of sampling lower frequency alleles. For example, more intensive sampling of the native range of EAB species may reveal missing alleles that are not represented in our sample. The low sample size in Asian populations, and the high levels of diversity found there, suggests that much of the diversity accumulated over an evolutionary time-scale may not be present in our sample, which consists primarily of North American individuals.

Overall EAB microsatellite markers consisted of 2–9 alleles for each locus and population. This level is slightly lower than a recent microsatellite studies of introduced species where between 2 and 12 alleles per locus were sampled (Carter et al. 2009). The slightly lower level of diversity among our markers could have resulted from greater sampling of populations from North America, which may have incurred a genetic bottleneck and thus retained less genetic diversity than that present in the native range.

Patterns of amplification suggest higher diversity in the native range

When we explored patterns of genetic diversity among regions using ANOVA, geographic region explained a significant proportion of the variation in expected heterozygosity. However South Korean populations had significantly larger allelic richness and expected heterozygosity relative to some (H_o) or all (allelic richness) of the populations in North America. Furthermore, although observed heterozygosity was not significantly different among regions, South Korean populations had a larger number of heterozygotes than populations in North America. One interesting result of this analysis is that populations in China had lower levels of allelic richness and observed heterozygosity than all regions within North America, but higher levels of expected heterozygosity than populations in both Ontario and Quebec but not Michigan. These results suggests that if the main source of introduction was from Chinese populations, then North American populations did not suffer a large reduction in genetic diversity upon introduction (especially in Michigan). However, if some or all of the North American introductions were from South Korea then populations in North America underwent a much larger reduction in genetic diversity upon introduction especially in Quebec and Ontario. These

results are similar to Bray et al. (2011) who found higher levels of diversity in Asian populations relative to North American populations using AFLP markers. However, the differences in diversity among Asian regions in Bray et al. (2011) were less similar to our analysis: populations in South Korea had higher allelic diversity but lower expected heterozygosity relative to other regions within Asia (China and Japan), whereas in our analysis Korea always had the highest level of diversity.

Despite the small Asian sample size, the above result highlights the importance of understanding the source of individuals. If at least some North American individuals are of South Korean origin then higher levels of genetic diversity (H_e and Allelic richness) in South Korea and some populations in China suggests that North American populations have gone through a genetic bottleneck and suffered some amount of genetic drift since introduction. However, if North American populations were introduced primarily from China then levels of genetic diversity did not change drastically upon introduction. In this case the region of introduction dramatically influences the interpretation of changes in diversity since introduction.

Numerous studies have shown that the loss of rare alleles among markers such as microsatellites can be greater than the loss of observed population heterozygosity during a bottleneck (Spencer et al. 2000). A study by Wares et al. (2005) quantified the change in allelic diversity and heterozygosity among many studies of invasion genetics and found that there were slightly larger reductions in levels of allelic diversity than observed heterozygosity upon introduction. We found differences among measures of genetic diversity (H_e, H_o, Allelic Richness) in patterns of change in diversity from the native range to the introduced range in EAB. In this case, reductions in allelic richness and expected heterozygosity upon introduction were larger than observed heterozygosity. These results suggest that either the reduction of diversity was not sufficient to change patterns of observed heterozygotes but was sufficient to decrease measures of genetic diversity, or that the nature of our molecular markers led to proportionally larger losses of rare alleles relative to heterozygotes (Wares et al. 2005). Finally, while our investigation of changes in patterns of variation highlights some important observations and concepts in invasion genetics, we suggest caution in interpreting our result, as our sample size in Asia was small.

Clustering analysis reveals unique clustering of Quebec samples

Results of Bayesian clustering analyses in STRUCTURE suggest that the majority of individuals from populations in Quebec closely clustered with a majority of individuals in China and South Korea. Alternatively, $\sim$60 % of individuals from Michigan and Ontario grouped into a second population cluster which included fewer individuals from China, South Korea and also Quebec. This result suggests that individuals in Quebec are indeed unique relative to other North American regions. There are two possible explanations that could be used to interpret this result. First, populations from Quebec could be the product of an additional, more recent introduction of EAB into North America from an Asian population. One line of evidence in support of this scenario is that the low levels of diversity (H_O, H_E, and richness) in Quebec relative to Michigan and Ontario imply that this population may have been through a more recent genetic bottleneck (and thus the product of a more recent introduction) than the other North American regions. A second explanation is that because populations in Quebec are geographically distant from populations in Ontario and Michigan, a single or very few dispersal events from populations in Michigan and Ontario to Quebec resulted in EAB's introduction into Quebec. If this single introduction event led to a genetic bottleneck followed by high levels of genetic drift (with very little additional migration from the Michigan and Ontario), then populations in Quebec could show their unique clustering pattern as a result of stochastic processes related to genetic drift and gene flow, rather than because of a recent introduction from Asian populations (Spencer et al. 2000).

When North American populations were considered independently of Asian populations there were three highly probable population clusters made up of individuals from Quebec (red) in one cluster, individuals from the London Ontario Hospital (blue) from both 2007 and 2008, as well as individuals from Brampton, Ontario and Vaughn, Ontario, and finally, a third cluster (yellow) which consisted of individuals from the remaining populations in Ontario and Michigan (Fig. 3). One interesting outcome of the STRUCTURE analysis of North American populations is that Quebec falls into its own population cluster, a result that could be explained by the large geographic distance (and

associated genetic drift; ~500 km) from the main sample of North American individuals in Michigan and Ontario. However, another population almost equidistant from the main sample in Michigan and Ontario is Sault Ste Marie, Ontario (08.SMA), about 400 km west. This population (08.SMA) showed no strong isolation from populations in the main sampling region of Michigan and Ontario, despite being a similar distance as Quebec from the main source. This result suggests that the uniqueness of the Quebec populations is not only due to remoteness, but instead may be the result of another factor such as an independent migration event from a native source population, natural selection, extreme isolation, or differences in levels of genetic drift among regions.

Results of assignment analysis in Bray et al. (2011) using AFLP loci and two of the microsatellite markers described in this study, and with individuals sampled in 3 US states including Michigan, Illinois and Indiana, and one site in the Canadian Province of Ontario, finds that a large number of individuals could be reliably assigned to Hebei Province in China. While Bray et al. (2011) sampled the introduced range throughout Michigan, our investigation focused on populations throughout Ontario and Quebec, with more minor sampling of populations from Michigan. If there were multiple introductions of EAB into North America, and the location of these introduction events correspond to regional genetic differences among populations, then the results of assignment could point to different populations of origin within the native range. Differences in assignment results could also be the product of differences in marker type (AFLP vs. Microsatellites) but further tests will be required to identify the true origin of EAB individuals throughout the Canadian provinces and US states. Ultimately, results of both studies point to northeastern Chinese populations as a potential source for at least some of the North American populations, suggesting that research on the ecology and natural enemies of EAB should be focused in that region (Estoup and Guillemaud 2010).

High among population variation in the native and introduced range of EAB

We found that a significant proportion of the genetic variation could be partitioned among regions in EAB, suggesting that the levels of differentiation amongst North American and Asian regions and even among North American regions alone is sufficient to explain a significant proportion of the total genetic variation. An AMOVA among only the Asian regions explained an even larger proportion of the total genetic variation, suggesting that Asian regions (China and Korea) are genetically differentiated and that identification of a region of origin should not be hampered by lack of structure in the native range as it was for the Asian longhorned beetle in Carter et al. (2010).

Conclusion

Population genetic methods can be extremely useful in elucidating invasion pathways, especially when direct evidence of the invasion route is unavailable (Estoup and Guillemaud 2010). The results of this study provide a usable suite of microsatellite markers for investigation of the invasion genetics of the emerald ash borer. Results of the population genetic analyses using these markers suggest first that North American populations may have gone through a genetic bottleneck upon introduction but that the severity of this bottleneck depends on the source of introduced individuals. Secondly we have shown that individuals from different regions of North America (Quebec vs. Ontario) may be associated with different regions within the native range of EAB. Our ultimate goal was to present a microsatellite toolkit, which can be used to further track invasion pathways of EAB and to monitor contemporary changes in genetic variation throughout its newfound range. Specifically, with greater sampling of individuals these markers will be useful for inferences of invasion pathways using methods such as approximate Bayesian computation (Guillemaud et al. 2010) that allow hypotheses of invasion scenarios to be tested explicitly, and where unlinked microsatellite loci are an asset.

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