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AFLP, mtDNA, AND MICROSATELLITE ANALYSIS OF EMERALD ASH BORER POPULATION STRUCTURE FROM ASIA AND NORTH AMERICA

By

Alicia Marie Bray

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ABSTRACT

AFLP, mtDNA, AND MICROSATELLIT ANALYSIS OF EMERALD ASH BORER POPULATION STRUCTURE FROM ASIA AND NORTH AMERICA

By

Alicia Marie Bray

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is a devastating invasive pest of North American ash trees (*Fraxinus* spp.) that was first discovered outside of its native range of Northeastern Asia in 2002 (Haack et al. 2002). With the unintended assistance from human movement of infested ash material, EAB spread swiftly from its initial discovery in the Detroit area of Michigan and Windsor, Ontario to currently include 13 states in the United States and two provinces in Canada. Understanding the population biology of an invasive species such as EAB could provide valuable information on geographic origin and location of possible effective biological control agents, estimate host range potential, and provide evidence of the main mode of spread. This study had three main goals: 1) obtain samples from throughout the native and introduced ranges of EAB, 2) characterize the genetic population structure of EAB in its native and introduced range using mitochondrial partial gene sequencing and DNA fingerprinting using amplified fragment length polymorphisms, and 3) develop and characterize microsatellite loci to further assess population dynamics and invasion history.

To accomplish the first goal, a network of collaborators, including myself, was developed to obtain samples throughout the native of China, South Korea, and Japan and introduced ranges in the United States, Canada, and western

Russia. This effort yielded a collection consisting of 1799 specimens from across 7 states in the United States, 114 in Ontario, Canada, 12 in Moscow, Russia, 274 specimens in China, 17 in South Korea, and 3 in Japan.

To accomplish the second goal, individual insects were characterized with partial mtDNA cytochrome oxidase subunit I sequence (481 bp) and four AFLP primer pair combinations yielding 273 loci. COI sequences detected one common haplotype found in China, South Korea and all samples in N. America, as well three unique haplotypes in China, and four haplotypes from South Korea that all differed from the common sequence by 2-4 nucleotides. In addition, a single EAB from Japan differed from the common sequence by 22 nucleotide changes (3.7%). The majority of the AFLP genetic variability was within populations and not among populations. Very weak genetic structure was detected. Average pairwise Φpt across all populations in N. America revealed the lowest population differentiation between Dagong and Tangshan, China (Φpt = 0.0877 and 0.0848, respectively). Over 67%individual beetles from N. America were assigned either to Dagong or Tangshan, China by assignment tests.

The final goal to develop and characterize microsatellite loci evaluated 41 primer pair combinations that successfully amplified in EAB, however, none of these loci yielded variation to assess within and between populations in Asia. Microsatellite loci variation of EAB is presented on a subset of samples in this study on two loci developed by a Dr. Jenny Cory (Simon Fraser University, BC, Canada). These data did not provide enough information to distinguish between single or multiple EAB introductions into N. America.

Dedicated with love to my husband and parents for their unwavering support and guidance through all the obstacles that life gives, and to my daughter for her amazing smile that brightens each and every day.

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TABLE OF CONTENTS

LIST OF TABLES..... x

LIST OF FIGURES..... xii

CHAPTER 1
INTRODUCTION..... 1
Invasive species..... 1
Ecological impact of invasive species..... 2
Control strategies of invasive species..... 4
Detecting evolutionary change associated with invasions..... 6
Variation within a population..... 6
Molecular markers to assess genetic variation..... 7
Genetic structure of populations in the native ranges. 10
Geographic structure of introduced species..... 12
Genetic makeup of introduced populations..... 14
Population change after introduction..... 14
Geographic origin of invasive species 15
Number of introductions of invasive species..... 18
Hybridization of introduced populations with native species..... 19
Emerald Ash Borer..... 20
Control Methods of EAB in North America..... 22
Detection of EAB..... 23
Chemical control of EAB..... 23
Biological control..... 24
Objectives..... 26

CHAPTER 2
GEOGRAPHIC DISTRIBUTION OF EMERALD ASH BORER..... 27
Introduction.....27
Host range.....31
EAB life history..... 33
Methods for EAB collection in introduced and native range... 34
EAB collection with collaborators..... 34
Collection of North American EAB samples.....40
Collection of Asian EAB samples..... 41
Results of emerald ash borer collection.....49
Discussion..... 56

CHAPTER 3
AFLP AND mtDNA ANALYSIS OF EAB POPULATIONS IN ASIA AND NORTH AMERICA..... 57

Introduction..... 57
Materials and methods..... 63
Sample collection..... 63
DNA isolation..... 68
Mitochondrial DNA gene sequence..... 68
Amplified fragment length polymorphism (AFLP)..... 70
Results..... 74
mtDNA variability..... 74
AFLP variability..... 80
Discussion..... 93

CHAPTER 4
MICROSATELLITE ANALYSIS OF EMERALD ASH BORER..... 100
Introduction..... 100
Materials and methods..... 101
Sample collection and DNA isolation..... 101
Microsatellite marker development..... 103
Data analysis..... 104
Results and discussion..... 105

APPENDICES 113
Appendix A: COI sequence haplotypes..... 114
Appendix B: Deposition of voucher specimens..... 126

LITERATURE CITED..... 129

LIST OF TABLES

Table 2.1.a. Field location survey records for trees examined for the presence of Emerald Ash Borer in South Korea.....42

Table 2.1.b. Field location survey records for trees examined for the presence of Emerald Ash Borer in China.....44

Table 2.1.c. Field location survey records for trees examined for the presence of Emerald Ash Borer in Japan.....47

Table 2.2. Emerald ash borer specimens obtained/collected in North America from 2003-2008.....50

Table 2.3. Emerald ash borer specimens obtained/collected in Asia from 2003-2008.....54

Table 3.1. Emerald ash borer specimens collected/obtained from 2003-2008...64

Table 3.2. Mitochondrial DNA variability of emerald ash borer in Asia and North America.....75

Table 3.3. AFLP and mtDNA COI results from AMOVA analysis of Asian and North American collection locations of emerald ash borer.....79

Table 3.4. Pairwise F_{ST} between populations of mtDNA COI haplotypes from emerald ash borer based on Kimura 2-parameter distance.....81

Table 3.5. Descriptive statistics for Asian and North American EAB collection locations with (N) number of individuals using 273 total AFLP loci.....82

Table 3.6. Pairwise Φ_{pt} between Asian populations with AFLP data set of 273 loci from emerald ash borer.....84

Table 3.7. Pairwise Φ_{pt} between North American populations with AFLP data set of 273 loci from emerald ash borer.....85

Table 3.8. Pairwise Φ_{pt} between Asian and North American populations with AFLP data set of 273 loci from emerald ash borer.....86

Table 3.9. Structure analysis results.....92

Table 3.10. Assignment test of individuals of the introduced range of emerald ash borer in North America against collection locations from the native range in Asia.....94

Table 4.1. Locality information for EAB individuals included in the mocrosatellite analysis.....102

Table 4.2. Characteristics of 2 microsatellite loci isolated from emerald ash borer of 162 individuals from Asian and North American populations.....106

Table 4.3. Genetic diversity of two microsatellite loci in emerald ash borer populations from Asia and North America.....107

Table 4.4. Genetic differentiation (pairwise F_{st}) of emerald ash borer populations in China and North America.....108

LIST OF FIGURES

Figure 2.1. Emerald ash borer introduced distribution range in North America reported as of July 2009.....29

Figure 2.2. Emerald ash borer distribution in China, South Korea, and Japan based on literature recordings and field surveys conducted from 2003-2006.....30

Figure 2.3. *Fraxinus* spp. host tree distribution of emerald ash borer in China...32

Figure 2.4. Collection locations of emerald ash borer in the native range of Asia 35

Figure 2.5.a. Collection locations for emerald ash borer in North America.....37

Figure 2.5.b. Expanded view of collection locations for emerald ash borer in North America..... 38

Figure 3.1. Strict consensus of six parsimonious trees for 9 emerald ash borer haplotypes 77

Figure 3.2. Haplotype network for 326 emerald ash borer individuals collected in Asia and North America based on partial sequences (481 bp) of the mitochondrial cytochrome oxidase I.....78

Figure 3.3. Neighbor-joining analysis of EAB AFLP data set based on pairwise Φ_{pt} values.....88

Figure 3.4. Neighbor-joining analysis of EAB AFLP data set based on pairwise Φ_{pt} values for the native range in Asia and introduced range in North America.....89

Figure 3.5. Structure analysis results representing the most probable number of populations of EAB, K = 4 through K = 7 in Asia.....90

Figure 4.1. Neighbor-joining analysis of EAB microsatellite loci (two) based on pairwise FST values for Asian and North American populations.....110

Figure 4.2. Test for isolation by distance in the North American EAB populations based on the analysis of microsatellite markers (2 loci).....111

CHAPTER I:

INTRODUCTION

Invasive Species

Introduced and established exotic species are an increasing challenge throughout the world, given enhanced globalization and trade. Invasive species can have serious implications on the global environment (Elton 1958; Simberloff 2005; Perrings et al. 2005). There are countless examples of human action and movement causing unprecedented movement of organisms to novel habitats having adverse impacts in the new environment, ranging from examples such as the near extinction of the American chestnut due to the invasive plant disease chestnut blight imported accidentally from Asia to North America (Elton 1958) to the extinction or endangered status of most birds on the island of Guam due to the accidental introduction of the brown tree snake from Australia/New Guinea (Burdick 2005). Many introductions of species into North America are intentional, and a few are even beneficial, such as the crops and livestock that currently yield 98% of the US food supply and generate $800 billion per year (Pimental et al. 2000). However, many non-native species, either accidentally or intentionally introduced, have become invasive costing, approx. $137 billion per year in losses, damage, and management. An invasive species is characterized as an established, widespread exotic species and in sufficient numbers to threaten native species (Colautti and MacIsaac 2004). While the financial responsibility to detect and eradicate an introduced species rests solely on the country in which they are newly discovered (Simberloff 2005), many only become noticed when

they become invasive in the new geographic range. The potential for species introduction increases with the volume of trade to or from a particular region (Semmens et al. 2004; Levine and D'Antonio 2003), increasing the probability of introducing a potentially invasive organism.

Ecological impact of invasive species

Once an introduced species becomes invasive, a variety of ecological consequences may occur (Kenis et al. 2009). Successful invaders tend to out-compete native species for space and resources; possibly due to fewer direct competitors, predators and prey defenses causing displacement, or introgression or extinction (Mooney and Cleland 2001; Sakai et al. 2001). For example, introduced purple loosestrife, *Lythrum salicaria* Linnaeus, grows rapidly in wetlands throughout its introduced range, creating monospecific stands in which native wetland species are unable to compete for space or food, and thus become rare or eliminated (Strefeler et al. 1996). This has long-lasting effects not only for the plant life, but also for the animal life that use the native species for shelter or food. If animals are unable to adapt or disperse to new habitat their population will decline. Hybridization between introduced species and wild native relatives is another consequence of introductions and can lead to the transfer of invasive traits (Strefeler et al. 1996). Mallard ducks (*Anus platyrhynchos* Linnaeus) hybridize with a variety of other species in their introduced range (Hawaii, Australia, and New Zealand), including the Hawaiian duck (*Anus wyvilluana* Sclater) or the New Zealand gray duck (*Anas superciliosa superciliosa* Gmelin) (Rhymer and Simberloff 1996). This hybridization has led to the

reduction in populations of the New Zealand gray and Hawaiian duck populations.

Argentine ants (*Linepithema humile* Mayr), now widely distributed in North and South America, Africa, and Europe, displace native ants species presumably because they are better at food acquisition (Tsutsui et al. 2001). The traits that have led to the success of the ants are thought to have been caused by a genetic bottleneck in the introduced ranges leading to a reduction of intraspecific aggression and formation of supercolonies (Tsutsui et al. 2000; Tsutsui and Case 2001). Due to the increased land needed to maintain supercolonies, Argentine ants have changed the landscape where they are found. The red imported fire ant, *Solenopsis invicta* Buren, accidentally imported into the U.S. from South America in the early 1900's, also changes the landscape in introduced regions causing a variety of ecological impacts including displacement of native species, killing poultry and reptiles, and medical complications due to painful bites (Ross et al. 2007; Shoemaker et al. 2006). Significant ecological changes have also been observed due to the intentional introduction of the gypsy moth, *Lymantria dispar* Linnaeus, from France to North America in 1869 for silk production. Repeated defoliation causing tree decline and death has occurred across hardwood forests in eastern N. America (Reineke et al. 1999). This has reduced native animal species, including mammals, birds and insects, dependent on tree resources for survival (Elkinton and Liebhold 1990).

Ecological damage can occur during range expansion and invasion of species to new continents in cropping systems. For example, the Colorado

potato beetle (*Leptinotarsa decemlineata* Say) (CPB), native to Mexico and feeding on buffalo bur, expanded its range north into the U.S. in the mid 1800's where it feeds on various solanaceous plants including the potato (Grapputo et al. 2005). CPB was also accidentally introduced into Europe in the 1920's. It quickly spread through the continent. The spread of this pest has caused severe loss in crop yield both in North America and Europe, exacerbated by extensive insecticide resistance. The western corn rootworm (*Diabrotica virgifera virgifera* LeConte) (WCR) has a similar range expansion in N. America and invasion in Europe to the pattern observed in CPB. It is thought to have originated in Nebraska, Colorado, and Kansas expanding its range north feeding on cultivated corn (Kim and Sappington 2005). It was accidentally introduced into Yugoslavia in the 1990's and has since been found in France, Italy, Switzerland, Belgium, the United Kingdom and the Netherlands. Outbreaks of the western corn rootworm have caused severe crop loss in both North America and Europe (Kim and Sappington 2005; Miller et al. 2005). Increased land use for host crops and the speed of spread and development of insecticide resistance in both CPB and WCR cause severe damage to crop systems.

Control strategies of invasive species

Given the potential for major environmental consequences, considerable effort is placed on eradication or control of introduced species that become invasive. Successful eradication projects have common characteristics and are generally more cost effective than the mechanical, chemical or biological control methods later (Simberloff 2005). The little fire ant, *Wasmannia auropunctata*

Roger, had displaced and reduced native ant populations on Marchena Island, Galápagos since being introduced in the 1900's. A comprehensive and widespread eradication project was initiated that included a toxic commercial pesticide AMDRO specific to the fire ant to reduce and eliminate it from all regions it was found on the island. Within two years, the fire ant was almost eliminated and native ant species began to recolonize the area (Causton et al. 2005). Eradication of three of four invasive fruit fly species (*Bactrocera* spp.), devastating mango and breadfruit production, was successful in the Republic of Nauru. This program was implemented by using protein bait application, male annihilation technique, managing fallen fruit, and developing more stringent quarantine regulations to reduce the risk of re-introduction (Allwood et al. 2002). These two programs (*W. auropunctata* and *Bactrocera* spp.) may have been successful for a variety of reasons. Both programs were on islands, which would limit the spread and geographic range expansion potential of the invading species, and in each case the eradication effort was developed and implemented fairly quickly after the problem was discovered. In each case there was also a widespread and comprehensive eradication plan that included a variety of collaborators. All of these qualities increased the likelihood of successful eradication. However, even in the absence of one of these factors, successful eradication may be possible if designed well and enough labor and financial support are in place (Simberloff 2005).

Detecting evolutionary change associated with invasions

Variation within a population

Diversity is a key component to every environment and population. Because habitat is not continuous over a species' range, many populations are composed of metapopulations, where many subpopulations fluctuate in isolation from each other (Hanski 1999). Subpopulations randomly become extinct and recolonize through time as populations drift and migrate (McCullough 1996). Genetic variation within a species population is thought to be the foundation for its continued survival in an ever-changing climate (Frankham 1996).

Invasive species have special characteristics in their population dynamics compared to populations in the native regions since they typically originate from a relatively small population when introduced (Sakai et al. 2001; Kenis et al. 2009). Many ecological hypotheses have been suggested to explain why introduced species become invasive and proliferate, such as, absence of natural enemies, absence from competitors, and/or open ecological niches in the new environment (Orians 1986).

There could also be a genetic basis to explain why certain species become invasive. Most introduced species have experienced a population bottleneck at the time of introduction; i.e. the number of individuals in the introduced population is relatively small compared to the native population, thus the genetic composition of the founder population is greatly reduced (Allendorf and Luikart 2007; Sakai et al. 2001). Therefore, the probability of reduced genetic variability, measured by the amount of heterozygosity and loss of rare

alleles, is increased as the founding population size and number of founding populations decrease. This reduction of variation could lead to founder effects with the genetic composition in the introduced population changed from the native range. Reduced genetic variation could also increase the rate of change in allele frequency leading to genetic drift (Allendorf and Luikart 2007). On the other hand, there could be an increase in genetic variation (increased heterozygosity and numbers of alleles) in an introduced population if multiple introductions from different source populations or if the single founding population was from multiple source populations (Baker 1992; Kolbe et al. 2004; Allendorf and Luikart 2007). This could occur if a mixture of populations occurred in the native region, such as combining goods and products at a port before shipment. Determining the genetic structure and composition of an invasive species is important since an increase or decrease in variation could influence management practices. For example, introduction of a natural enemy from the single source population would have a higher probability of controlling the invasive species with little genetic variation than a population with high genetic variation (Conord et al. 2006).

Molecular markers to assess genetic variation

Molecular markers, such as mtDNA gene sequences, nuclear gene sequences, amplified fragment length polymorphic DNA (AFLP), and microsatellites, are powerful tools to assess genetic variation in populations and identify sources of biological invasions (Roderick 2003). Fonseca et al. (2001) used multiple molecular markers to study the invasion of *Aedes japonicus*

japonicus Theobald into the USA. The authors used random amplified polymorphic DNA (RAPD) and mtDNA sequencing of NADH dehydrogenase subunit 4 to compare the genetic diversity of insects in the introduced range and the native range in Asia, and to identify the source population responsible for the introduction into the USA. Grapputo et al. (2005) utilized a similar approach using both mtDNA sequences and variation at AFLP loci to pinpoint the source population of Colorado potato beetle into Europe from N. America. These studies are examples of how to use multiple molecular markers to identify the source of the invading population and assess genetic variation between native and introduced populations.

Mitochondrial DNA has been studied extensively in animal systems, including insects (see Harrison 1989; Caterino et al. 2000 for reviews). Absence of recombination within mtDNA and its maternal mode of inheritance (exceptions: Wallis 2000; Andolfatto et al. 2003) make mtDNA useful in detecting population-bottleneck events caused by an introduction incident. Numerous primers are available for amplifying insect mtDNA genes, making the amplification and sequencing of mtDNA a straightforward process using PCR (Simon et al. 1994). Mitochondrial DNA alleles found in a population can be sequenced and analyzed via phylogenetic analysis.

AFLP genotyping (Vos et al. 1995), or genetic fingerprinting, has come into use for studying population structure and differentiation, estimating population genetic parameters (Reineke et al. 1998; Cardoso et al. 2000), and studying closely related species (Albertson et al. 1999). The AFLP technique

yields information from many loci distributed throughout the nuclear genome (Mueller and Wolfenberger 1999). AFLP data can be analyzed both within a phylogenetic framework and from a population genetics perspective. AFLP data can also be used to carry out assignment tests (Campbell et al. 2003).

Microsatellite loci, also known as short tandem repeats (STRs), are characterized by the presence of multiple copies of a di-, tri-, or tetranucleotide DNA sequences (e.g., $(CA)_n$, or $(ATGC)_n$). Microsatellite loci are distributed throughout the genome (Bruford and Wayne 1993), and analysis of microsatellite variation is used to study a variety of problems (Balloux & Lugon-Moulin 2002). Microsatellites are widely used to assess the genetic structure of populations (e.g., Paupy et al. 2004) and study the geographic origin and subsequent spread of introduced species (e.g., Müller 2001). Microsatellite markers have some distinct advantages over other types of molecular markers, such as DNA sequences of mitochondrial or nuclear genes, and various genotyping or DNA fingerprinting techniques (e.g., RAPDs and AFLPs). Microsatellites are codominant markers (both alleles in heterozygous diploid individuals can be scored) and alleles at microsatellite loci can be scored accurately and fairly quickly via a polymerase chain reaction-based strategy.

One important use of molecular data to assess an invasive species is to determine the geographic origin of the putative source population(s) thought to be responsible for an introduced population. Population assignment tests are an informative method that attempts to assign an individual to the most likely population from which it originated. These tests are used to answer a variety of

questions including: identifying dispersal patters in a metapopulation (Berry et al. 2004), confirming population structure of native and/or invasive populations (Elderkin et al. 2004; Paupy et al. 2004), assessing population structure to design eradication strategies (Abdelkrim et al. 2005), estimating the origin of invasive species (Bonizzoni et al. 2001; Genton et al. 2005; Tsutsui et al. 2001), and to assess if individuals are from an established population or recently migrated to an area (Kim et al. 2006). These tests are typically performed using microsatellite data, however, AFLP's are considered a useful alternative for species where microsatellite markers are unavailable or logistically difficult to obtain (Campbell et al. 2003).

Genetic structure of populations in the native ranges

Bernardi et al. (1993) used both nuclear and mtDNA genes as well as restriction fragment length polymorphism (RFLP) loci to understand the population structure of the native teleost fish, *Fundulus heteroclitus* Linnaeus, in North America ranging from Canada to Florida. Phylogenetic relationships of the populations were compared using maximum parsimony analysis for all markers. Each marker yielded similar results by detecting genetic separation between northern and southern populations of teleost fish with a mixture of populations in the region of overlap. The authors concluded that the different markers were useful to obtain independent support for the separate populations. Chong et al. (2000) found geographic structure among five populations of Malaysian river catfish, *Mystus nemurus* Cuvier & Valenciennes, by analyzing samples with AFLP and RAPD techniques. Fifteen to thirty individuals from each population

(one population on the island of Borneo and the remaining four populations from the west side of Peninsular Malaysia) were characterized using both techniques to determine the similarity of individuals within a population, genetic distance between populations, and to carry out phylogenetic analyses based on calculated similarity indices and genetic distance. The authors found that both AFLP and RAPD analysis provided similar geographic structure, with the Borneo Island population clustering alone. However, only AFLP analysis was able to detect subpopulation structure of the populations from Peninsular Malaysia into three identifiable subgroups. The authors concluded AFLP analysis was more efficient in detecting structure in populations that are closely related than RAPD.

Population structure of gypsy moth collected in the native range from four cities in western Japan was estimated with microsatellite markers (Koshio et al. 2002). The authors identified three microsatellite markers that yielded high polymorphism with unique alleles detected in three distinct populations. However, there was variation within a population as well as between populations and the authors concluded that more loci would be needed to resolve populations into separate groups. Population structure also was evaluated for *Aedes aegypti* Linnaeus in Cambodia using multiple molecular methods, AFLP, microsatellite, and isozyme markers (Paupy et al. 2004), in part to determine if all markers yielded similar population structure measured by genetic differentiation (F_{st}). The authors determined all molecular markers were able to detect two distinct populations in Cambodia when compared by an estimation of genetic differentiation, F_{st}, however, the strength of differentiation was up to five times

higher in AFLP data compared to microsatellite and isoenzyme markers. In this study, as seen before, it was important to evaluate different genetic markers to ensure the estimate of population structure was valid and consistent patterns of structure were detected.

Geographic structure of introduced species

Molecular markers are also useful to study biological introductions. Genetic analysis can provide insight on a variety of questions pertaining to invasive species, including 1) determine the geographic structure in native and introduced ranges, 2) genetic make-up of the introduced population, 3) genetic change after introduction, 4) geographic origin, 5) number of introductions, and 6) assess hybridization of introduced populations with native species. Determining the answers to these questions will provide valuable information on the biology and ecology of the invasive species as well as possibly impacting the development of eradication or management strategies (Allendorf and Lundquist 2003; Caldera et al. 2008)

Grapputo et al. (2005), with mtDNA COI and AFLP with two primer pair combinations, evaluated the genetic diversity of the CPB (*L. decemlineata*) throughout its native range in North America and introduced range in Europe. The mtDNA variability was high through N. America with 20 unique haplotypes, however, there was a single fixed haplotype found in Europe providing evidence of a bottleneck event in the introduced population. AFLP analysis revealed higher genetic diversity in N. America than in the introduced population in Europe with the average level of polymorphism measured 67.47 and 47.98%,

respectively and average expected heterozygosity of 0.354 and 0.175, respectively. The authors also found that specimens from North America and Europe separate into two groups according to their continent of collection based on Nei's genetic distance (D). They concluded the reduced mtDNA and AFLP variability in the introduced range in Europe compared to the native environment from N. America could have been caused by one introduction or several small introductions from the same source population.

D. virgifera virgifera, provides a unique situation in that it is historically native to Nebraska, Colorado and Kansas but increased its range and became invasive to the east coast within 60 years (Kim and Sappington 2005). It was also accidentally introduced from N. America into Europe where it was initially detected in 1992 (Miller et al. 2005). Kim and Sappington (2005) employed seven microsatellite loci to characterize WCR population structure from the native and range expansion populations in the United States using a total of 595 individuals sampled from ten populations across the current range. Genetic differentiation was not detected among populations sampled with the microsatellite loci used in the study with very little genetic differentiation detected across the range (global $F_{st} = 0.006$). There was also very little differentiation among populations with the majority of pairwise F_{st} values not detecting significant variation. The authors attributed the lack of differentiation to continued migration throughout the current range to maintain gene flow or there was insufficient time for population divergence to be detected at the seven loci used in the study.

Genetic makeup of introduced populations

Assessing the genetic structure of an introduced population may provide important baseline information on the genetic makeup of the invading population (Sakai et al. 2001; Allendorf and Lundquist 2003). For example, genetic relatedness was evaluated for the invasive bark beetle, *Tomicus piniperda* Linnaeus, of pine (*Pinus* spp.) to determine if introduced populations were genetically similar (Carter et al. 1996). Isozyme loci and RAPD were used to assess variation at eight localities in five states (Illinois, Indiana, Michigan, Ohio and New York). RAPD analysis revealed the Illinois population separated from the other four locations with a mean genetic distance of 0.895 while the other four locations having a mean genetic distance of 0.595 among populations when Illinois was excluded. Isozyme data analyzed with maximum parsimony analysis supported the RAPD analysis with the Illinois population isolated from the rest of the introduced populations. Given both RAPD and isozyme markers discovered a separate population in Illinois differed from the remaining introduced population, it is likely there was a separate introduction event in Illinois. This information could be useful in developing management strategies since the same control method may not be successful in the separate introduced lineages (Allendorf and Lundquist 2003).

Population change after introduction

After introduction into a novel environment, the introduced population may undergo rapid genetic change in response to the local environment (Mooney and Cleland 2001). *Drosophila subobscura* Collin is an excellent example of an

introduced population evolving in a new environment (Huey et al. 2000). *D. subobscura* was introduced to North and South America during the 1970's from its native range in the Old World. Although populations throughout the native range exhibited an increase in body size with latitude, populations in the introduced range did not exhibit this trend at the initial time of introduction (Huey et al. 2000). The authors collected specimens from varying latitudes in both the introduced range in N. America and in the native range in Europe two decades after introduction and reared each population in a common garden for up to six generations to ensure differences between populations were due to genetic differences rather than unknown environmental causes. Twenty years after the initial introduction into North America, *D. subobscura* populations had evolved to exhibit a latitudinal cline; wing length increases with latitude similar to the Old World populations. This study signifies the importance to re-examine the variation in an introduced population through time to understand how the populations may be responding to local environmental conditions.

Geographic origin of invasive species

Molecular markers provide researchers with powerful tools to identify sources of biological invasions (Roderick 2003). Microsatellite markers, mtDNA and satellite telemetry were used to determine the source populations of Canada Geese, *Branta Canadensis* Linnaeus, in new colonies in Greenland (Scribner et al. 2003). Assignment tests based on likelihood analysis were used to show the Greenland population arose from East Ungava, and refuted the hypothesis that the population arose from the North Atlantic population as inferred from flight

patterns. Cognato et al. (2005) used mtDNA cytochrome oxidase subunit I gene (COI) partial sequence to determine the geographic origin of a destructive insect, red turpentine beetle (*Dendroctonus valens* LaConte) introduced into China from North America. Parsimony and statistical parsimony analyses of the haplotypes determined populations in China were more closely related to populations from the Pacific Northwest than to populations from Mexico and Michigan. The population diversity was also unusually high in China suggesting that there was a large founding population or multiple introductions.

Navia et al. (2005) evaluated the geographic origin and ancestral host association of the invasive coconut mite, *Aceria guerreronis* Keifer, with mtDNA 16S and nuclear ribosomal internal transcribed spacer sequences. They compared individuals from populations throughout the current range, 12 countries from North and South America, 8 countries in Africa as well as Sri Lanka and India for genetic variability and haplotype diversity. The authors determined the genetic variability from populations in North and South America was high, but only a single mtDNA 16S haplotype in Africa, Sri Lanka, and India. The level of genetic variation measured by % nucleotide diversity observed in the ITS region was also higher in S. America than in Africa and southern Asia (1.90, 0.70, and 0.49, respectively). This lower genetic variation led the authors to hypothesize that these countries contain the introduced populations while North and South America are most likely the native range. This result also indicated a recent host switch of the mite onto coconut since the plant is native to the Indo-Pacific region and not from North or South America (Navia et al. 2005).

The Argentine ant provides another example of the utility of molecular markers to estimate the geographic origin of an invasive species. The Argentine ant has become invasive throughout most of the world currently inhabiting 15 countries on six continents. Determining the source of introduced populations would provide valuable information on where to focus research for development of biological control. Tsutsui et al. (2001) used mtDNA cytochrome *b* and seven microsatellite loci to compare samples from the native range in Brazil and Argentina to samples from introduced areas including California, Hawaii, Maui, Louisiana, Bermuda, Australia, South Africa, Chile and Italy. Population assignment tests were used to estimate the log-likelihood that each individual genotype was found in potential source populations and employed to make inferences about the geographic localities of source populations. Similar mtDNA haplotypes were observed in all of the introduced populations, all native populations in Argentina, and a single population in Brazil, while distinct haplotypes were observed in other Brazilian individuals that separated from the other haplotypes using a maximum parsimony analysis. Microsatellite analysis provided a finer resolution than mtDNA between populations in the native and introduced ranges. In the native range, pairwise genetic distances increased as geographic distance increased forming a cluster of individuals from Brazil and three separate clusters defining populations from Argentina (northern Rio Parana, southern Rio Parana, and Rio Uruguay). The only native site that grouped with introduced populations based on pairwise genetic distances was a population from Rosario, Argentina. Assignment tests based on microsatellite

loci also determined the majority of the samples throughout the introduced range were assigned to samples collected in Rosario, Argentina. The authors concluded this location was the most likely source of the majority of introduced populations throughout the world. This multi-locus approach provided strong evidence of the geographic origin of introduced populations and should be incorporated in future studies of invasive species whenever possible.

Number of introductions of invasive species

Miller et al. (2005) compared WCR populations from the native range in N. America to introduced populations in Europe to determine the source of the introduced populations in Europe. They used data from eight microsatellite loci to compare genetic variation from populations on both continents and tested hypotheses for the pattern of introduction based on computer simulations with approximate Bayesian computation analysis (ABC). ABC analysis is able to test different introduction scenarios by developing a linear regression model of computer-generated parameter values using simulated summary statistics and then replacing the observed summary statistics into the equation (Beaumont et al. 2002). The authors determined there was strong evidence for at least three separate introductions of WCR into Europe (Paris, France, northwestern Italy, and central and southeastern Europe) from N. America. The analysis of the number of introductions such as this has strong implications for management strategies since eradication could only be successful if the pathways of introduction are eliminated to stop future invasions of WCR.

Hybridization of introduced populations with native species

Population genetic variation has also been studied for a variety of invasive species to determine effects of introductions on the genetic makeup of related native species. For example, Strefeler et al. (1996) studied hybridization between native and introduced *L. salicaria*. They proposed a low level of genetic introgression, as revealed by isozyme data. However, Houghton-Thompson (2001) found no evidence for hybridization in purple loosestrife when she evaluated the species by AFLP.

For the invasive tamarisk plant (*Tamarix chinensis* Laureiro and *Tamarix ramosissima* Ledebour), Gaskin and Schaal (2002) used an intron of a nuclear gene, phosphoenolpyruvate carboxylase (*Pep*C) to determine population dynamics. The variable intron region revealed novel hybrid plants in the introduced range in the United States, but few hybrids were found in the native range in Eurasia. They continued to describe how this information can be helpful in developing control strategies; in this case, the hybrid species with novel allele combinations may have changed the biology of the plant enough to render current biological control agents ineffective.

As noted above, molecular markers provide important information for understanding the dynamics of invasive species. Incorporation of genetic analysis to assess the population biology of invasive species may be critical to develop practical management strategies for control (Sakai et al. 2001). Some of these methods will be used in this dissertation to characterize the population dynamics of an invasive species introduced into North America.

Emerald ash borer

The United States and Canada are currently battling a devastating invasive pest, the emerald ash borer (EAB), *Agrilus planipennis* Fairmaire, which is native to Eastern Asia. The pest was detected in North America in 2002 (Haack et al. 2002), and was initially found in southeast Michigan, northern Ohio, and in southwestern (Windsor) Ontario (Scarr et al. 2002). Based on dendrochronological reconstruction, Siegert et al. (2007) postulated that EAB was introduced in the early to mid 1990's into Wayne County, MI. Since the initial collection in 2002 in the Detroit area of Michigan and Windsor, Ontario, EAB has been discovered over a wide area including 15 states and two Canadian provinces: Illinois, Iowa, Indiana, Kentucky, Maryland, Michigan, Minnesota, Missouri, New York, Ohio, Pennsylvania, Virginia, West Virginia, Wisconsin, Ontario and Quebec, Canada (Cooperative Emerald Ash Borer Project 2009).

EAB is native in a vast region of Asia, including northern China, Japan, Korea, Mongolia, the Russian Far East, and Taiwan (Yu 1992; Chinese Academy of Sciences 1986). Several species were synonymized to what is currently accepted to be EAB, *A. planipennis*, including *A. marcopoli* Obenberger in China, *A. molco-poli*, *A. feretrius* Obenberger in Taiwan, and *A. marcopoli* subsp. *ulmi* Kurosawa in Japan (Jendek 1994). This has implications on possible host range of EAB since the host range in China includes various *Fraxinus* spp. (Oleaceae) (Yu 1992; Chinese Academy of Sciences 1986), while a broader host range is found in Korea, Mongolia and Japan that includes some species of walnut

(Juglandaceae) (*Juglans* spp.), elm (Ulmaceae) (*Ulmus* spp.), and wingnut (Juglandaceae) (*Pterocarya* spp.), in addition to *Fraxinus* spp. (Akiyama and Ohmomo 1997). Given the various host species in Asia, Anulewicz et al. (2008) conducted a study to determine the extent of the host range in the introduced region of N. America to better predict the potential extent of the damage that may be caused. They determined EAB in Michigan successfully oviposited and developed on a variety of N. America ash species, however, did not oviposit and/or develop in other tree species tested, including *Syringa reticulata* (Oleaceae) Japanese tree lilac, *Juglans nigra* (Juglandaceae) black walnut, *Ulmus americana* (Ulmaceae) American elm, *Carya ovata* (Juglandaceae) shagbark hickory, and *Celtis occidentalis* (Ulmaceae) hackberry.

Ash trees (*Fraxinus* spp.) are very common in the eastern hardwood forests and urban landscapes of N. America. Ash was one of the primary tree types used to replace American elms lost during the Dutch Elm disease epidemic in the latter half of the 20th century (MacFarlane and Meyer 2005). It has been estimated that Michigan has 693 million ash trees throughout its forests and urban environments (estimated value > $18.92 billion) that are susceptible to EAB (USDA-APHIS 2003). Ash trees in the N. America, including Michigan, have a variety of economic values including nursery sales, timber, baseball bats, hockey sticks, and recreational use in state forests. Estimated loss in the Michigan nursery industry was over $2 million in revenue the first year EAB was detected (USDA-APHIS 2003). EAB has been responsible for killing millions of ash trees in N. America (Cooperative emerald ash borer project 2009).

EAB larvae destroy ash trees (*Fraxinus* spp.) by feeding on phloem. Adults feeding on ash foliage cause little defoliation. EAB develop in one or two years under ash bark until emerging as adults beginning in May (Russell et al. 2003). Damage caused by EAB occurs when beetle density increases within a tree and nutrient flow in the phloem is staunched. High densities of larvae create feeding galleries that cover the majority of the cambium area girdling the tree causing nutrient flow to cease, resulting in death of the tree (Russell et al. 2003). EAB are able to develop in all ash trees greater than 2.5 cm, however, the majority of EAB in a given area emerge from large trees (greater than 26 cm diameter at breast height). Therefore, reducing the number of large ash trees may be crucial in managing EAB in forested areas (McCullough and Siegert 2007).

Control Methods of EAB in North America

EAB eradication attempts have not been successful. This consisted of cutting and disposing of infested trees, and removing all ash trees in 0.8 km zone around outlier populations in the U.S. to slow down the natural spread of the pest. Therefore, alternative methods of control are being evaluated, including trapping (Poland et al. 2006; Crook et al. 2007; Poland and McCullough 2007), chemical control (Cappaert et al. 2007; McCullough et al. 2007b; Rebek and Smitley 2007; Tanis et al. 2007), and biological control (microbial and natural enemies) (Liu et al. 2003; Bauer et al. 2004; Bauer et al. 2005; Marshall et al. 2005; Yang et al. 2005; Zhang et al. 2005; Yang et al. 2006; Liu et al. 2007; Liu and Bauer 2008; Wang et al. 2008).

Detection of EAB

Several detection methods for EAB are being developed, including stress induced girdling of trees, purple panel traps (used by USDA APHIS), and purple panel traps baited with a leaf blend lure (Poland et al. 2006) and/or Manuka oil (similar to ash bark volatiles) (Poland and McCullough 2007; Crook et al. 2007). Purple traps placed in areas with high sun exposure and baited with Manuka oil and leaf blend lures are very effective in capturing EAB (McCullough et al. 2007a). Traps placed along the edge of a tree line or in open fields, as well as above ground (13 m) are most effective (Francese et al. 2008).

Cerceris fumipennis (Hymenoptera: Crabronidae), a known predator of buprestid species, has been shown to effectively detect EAB in low and high-density populations (Marshall et al. 2005). Females search for prey to provision the nest; able to orientate to a new area and actively search for prey within 24 hours of transport. Since it attacks a variety of buprestid beetles and is not typically found in great numbers, this predator will be most effective monitoring new areas of infestation and not effective at controlling EAB populations.

Chemical Control of EAB

Chemical control is being evaluated for control of EAB suppression and protection of host trees. Imidacloprid applied by soil drench (Rebek and Smitley 2007; Cappaert et al. 2007) and trunk injections in the fall and spring (Tanis et al. 2007) were tested for effectiveness for tree protection. Imidacloprid is only mildly effective in reducing EAB larval activity, resulting in only partial control and tree mortality was still likely. Trunk injection with emamectin benzoate shows this

product is more effective with 100% adult mortality on foliage and over 99% control of larvae within infested trees (McCullough et al. 2007b). This could provide effective control of EAB for high-value urban ash trees. However, these methods are expensive, labor intensive, and environmentally risky for the protection of forests.

Biological Control

Invasive insect species may be successfully controlled with biological control: the introduction and establishment of pathogens, parasites, or predators from the pest's home of origin. Although the presumed source of the infestation is China (due to international trade), beetles may have originated from other Asian countries. Even though the specific origin was unknown when EAB was initially detected, a search for biological control agents across the native range was initiated (Liu et al. 2003). Several parasitoids were identified that attack EAB. *Spathius agrili* (Hymenoptera: Braconidae), a larval ectoparasitoid found in China, develop up to 2 generations per year on EAB (Liu et al. 2003; Yang et al. 2005; Wang et al. 2008). Two generations of an egg parasitoid, *Oobius agrilli* (Hymenoptera: Eulophidae) were found in high density attacking EAB in northeastern China (Liu et al. 2003; Zhang et al. 2005; Liu et al. 2007). Another biological control agent was located in the same location in northeastern China; the larval endoparasitoid, *Tetrastichus planipennisi* (Hymenoptera: Eulophidae), attacked EAB in four separate generations per year (Yang et al. 2006; Liu et al. 2007). *O. agrili* and *T. planipennisi* reduced populations by over 70% in 2005 (Liu et al. 2007). *Balcha indica* (Hymenoptera: Eupelmidae), an invasive

parasitoid from Southeast Asia, has also been recorded to attack EAB (Gibson 2005).

There have also been biological control agents found native to North America that have attacked EAB. For example, Liu and Bauer (2008) evaluated an entomopathogentic fungus, *Beauveria bassiana* strain GHA, sold as BotaniGard ES, by foliar and trunk sprays on EAB colonized ash trees. Over 40% reduction in EAB population was achieved in treated trees with 47% reduction of larval density. These authors concluded that *B. bassiana* might be an effective method to slow the spread of EAB and possibly contain an infestation in an outlier site.

Despite the potential for chemical control of EAB with emamectin benzoate, and control with biological control agents identified from Asia and N. America, the spread of EAB continues by natural dispersal and human transport. Given the fact that natural enemies found from the same source population of EAB will have a higher probability of success in control strategies, one goal of the work described in this dissertation was to test EAB populations throughout its native range in Asia to determine the specific origin of N. American EAB. This knowledge will be useful in focusing effort on continued establishment of biological control agents from the same region of origin to increase the likelihood of success to control EAB in the introduced range.

Objectives

The overall objectives of this study were 1) to determine the geographic origin of North American populations of EAB, 2) to determine if North American EAB populations were the result of a single or multiple introductions, and 3) to determine the spread of EAB from the source of its introduction in North America. In an attempt to answer these questions, I used genetic methods to determine the geographic origin and monitor the spread throughout the introduced range.

CHAPTER II

GEOGRAPHIC DISTRIBUTION OF EMERALD ASH BORER

Introduction

Emerald Ash Borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), was first detected in Southeast Michigan near Detroit in May and June 2002 (Haack et al. 2002). Collected adults were initially evaluated by Gary Parsons (Department of Entomology, Michigan State University) and identified as an *Agrilus* sp. unlike species known to the Great Lakes region. The beetles or images were evaluated by five other US beetle experts to identify these specimens to the species level. All five experts agreed on the identification of the genus, suggesting it was most likely of Asian origin; however, species identification remained unclear. Digital images, followed by specimens, were then evaluated by an Asian *Agrilus* spp. expert in Slovakia, Eduard Jendek, (State Forest Products Research Institute, Slovakia) for identification. He positively identified the specimens as *A. planipennis* in July 2002 (Haack et al. 2002). Following the positive species identification, adult EAB were collected in Windsor, Ontario and subsequently confirmed to be *A. planipennis* by Richard Westcott (Oregon Department of Agriculture). Since the initial collection in 2002 in the Detroit area of Michigan and Windsor, Ontario, EAB has been discovered in other areas of North America. In 2003 it had been discovered in Northern Ohio; in 2004 Maryland, and Indiana; in 2006 throughout most of Michigan's Lower Peninsula and in areas of the Upper Peninsula Michigan. By the end of 2007, EAB range extended into Illinois, Pennsylvania, and West Virginia. Within

the next year (2008) populations were discovered in Missouri, Virginia, Wisconsin, as well as Quebec, Canada with the current range in 2009 to include locations in Iowa, Minnesota, New York, and Kentucky (Cooperative Emerald Ash Borer Project 2009; Figure 2.1).

Information on EAB was limited before its discovery in N. America (Liu et al. 2003; Wei et al. 2004). EAB is native to eastern Asia, including northeastern China, Inner Mongolia, Japan, Korea, Mongolia, the Russian Far East, and Taiwan (Figure 2.2) (Chinese Academy of Science 1986, Wei et al. 2004, Yu 1992). Several populations of EAB have been recognized as separate species in the literature. In China, EAB is commonly referred to as *A. marcopoli* Obenberger, in Korea and Japan *A. marcopoli ulmi* Kurosawa, and *A. feretrius* Obenberger in Taiwan (Haack et al. 2002). These species were synonymized by Jendek (1994) as *A. planipennis*. Although details were not provided for the rationale for synonomyzing the species, it was stated the decision was based on the overlap of morphological characteristics of type specimens for each species. Adult EAB are normally 8.5-13.5mm in length in native China (Yu 1992) and 11.5-15mm in native Japan (Akiyama and Ohmomo 2000). The adults are generally metallic green in color with purple on the dorsal surface of the abdomen. Color morphs differing from the standard metallic green include metallic bronze, gold, copper, or ruby color in the native range (Yu 1992; Akiyama and Ohmomo 2000), however, morphs differing from green are rare in the introduced range (personal observation).

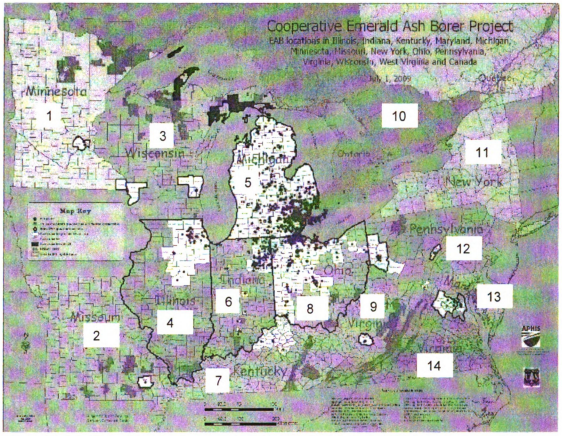

Figure 2.1. Emerald Ash borer introduced distribution range in North America reported as of July 2009. Dots are locations where EAB has been detected. Numbers correspond to states or province: 1-Minnesota, 2- Missouri, 3-Wisconson, 4-Illinois, 5-Michigan, 6-Indiana, 7-Kentucky, 8-Ohio, 9-West Virginia, 10-Ontario, 11-New York, 12-Pennsylvania, 13-Maryland, 14-Virginia. (Figure taken from Cooperative Emerald Ash Borer Project 2009).

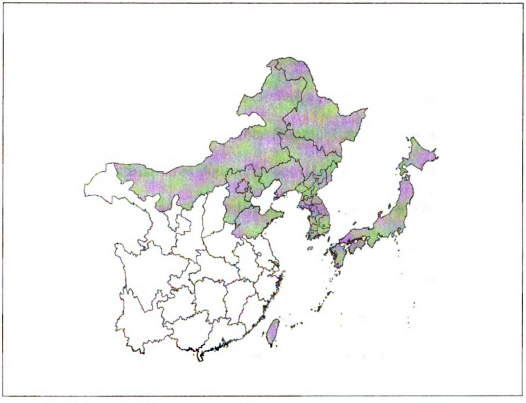

Figure 2.2. Emerald ash borer distribution in China, South Korea, and Japan based on literature recordings and field surveys conducted from 2003-2006 in shaded areas

Host range

In Asia, EAB has been reported primarily on *Fraxinus* spp. (Oleaceae) hosts with some variation depending on location. In China EAB has been reported on *F. chinensis* subsp. *chinensis* Robx., *F. chinensis* subsp. *rhychophylla* Hance, and *F. mandshurica* Ruprecht. but causes less damage to these native species than to N. American spp. (Liu et al. 2003; Wei et al. 2004). The distribution of native ash in China includes central and eastern China extending from the southern to northern border (Wei et al. 2004; Figure 2.3). EAB, however, aggressively attacks ash trees imported into China from North America for use primarily as ornamental trees. *F. velutina* Torr (Velvet ash) was first imported into China in the 1950's, while *F. americana* Linnaeus (white ash), *F. pennsylvanica* var. *lanceolata* Marsh (green ash), and *F. pennsylvanica* Marsh (red ash) were imported in the 1960's. These imported species of ash must be managed in China as EAB may kill entire plantings (Wei et al. 2004). The host range of EAB in Korea includes *F. mandshurica*, *F. rhynchophylla, F. chiisanensis* Nakai, and *F. sieboldiana* Blume (Haack et al. 2002). The host range in Japan includes *F. japonica* Maximowicz (Japanese ash), *F. spaethiana* Lingelsh (Späth's ash), *F. apertisquamifera* Hara, *F. longicuspis* Siebold and Zucc., *F. platypoda* Oliver and *F. lanuginosa* Koidzumi. In addition, EAB records exist for collections from walnut and elm, including *Juglans mandshurica* var. *sieboldiana* Maximowicz and var. *sachalinensis* Kitamura (Juglandaceae) (Manchurian walnut), *Pterocarya rhoifolia* Siebold and Zucc. (Juglandaceae) (Japanese Wingnut), and *Ulmus davidiana* var. *japonica* Nakai and *U. propinqua*

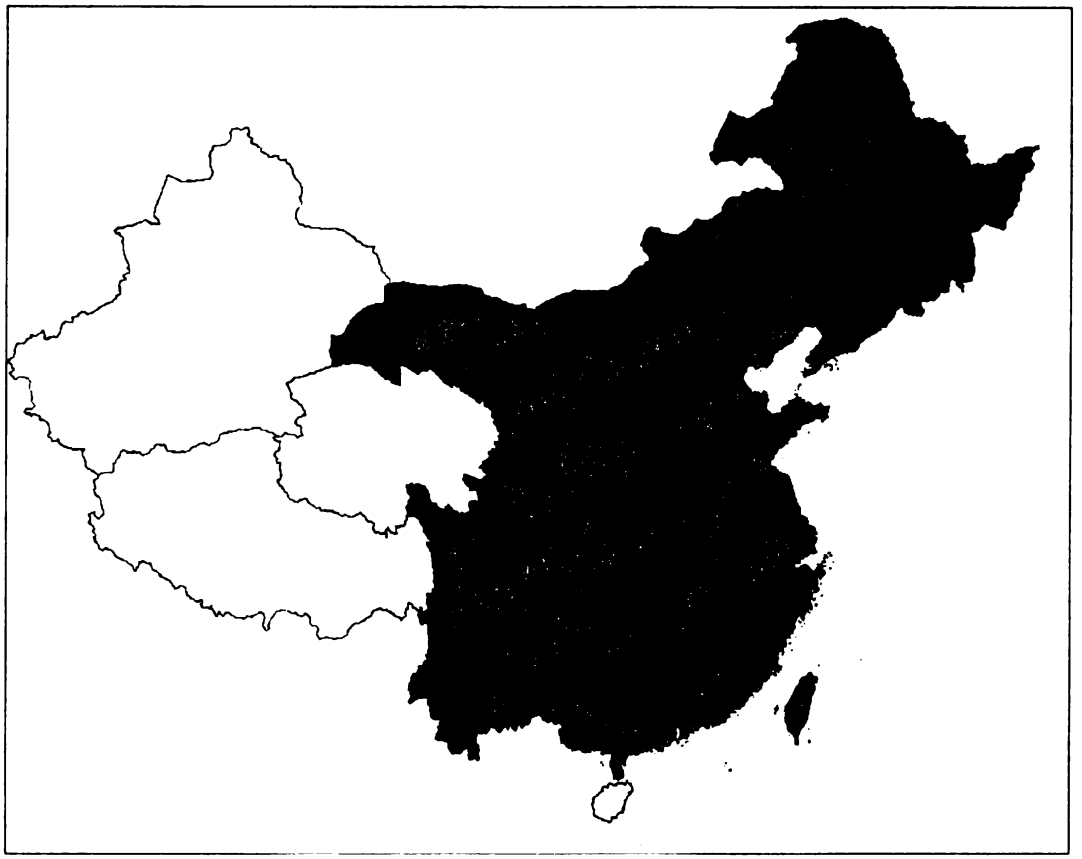

Figure 2.3. *Fraxinus* spp. host tree distribution of emerald ash borer in China (shaded areas). Distribution based on Wei et al. 2004

Koidz (Ulmaceae) (Japanese elm) (Akiyama and Ohmomo 2000; Haack et al. 2002).

In the introduced range of N. America, EAB has developed successfully on all ash species encountered to date (Anulewicz et al. 2008): *F. Americana, F. pennsylvanica, F. nigra* Marshall (black ash), *F. quadrangulata* Michaux (blue ash), and *F. profunda* Bush (pumpkin ash). EAB populations in N. America have not been found to develop on nonash species, including walnut or elm species (Anulewicz et al. 2008).

EAB Life History

Biological information on EAB is scarce (Haack et al. 2002); however the following information is based on literature from the Chinese Academy of Science (1986) and Yu (1992). Adult EAB typically emerge and are active from mid May to August. Males and females live approx. two and three weeks, respectively after emergence from the host tree. Adults feed on ash foliage (*Fraxinus* spp.) during the daytime preferably when it is warm and sunny. Mating occurs after 7-14 d of feeding with copulation lasting 20-90 min. Females oviposit eggs individually in bark crevices 7-9 d after the first mating with a lifetime production of 68-90 eggs. Eggs are found in early June to late July maturing for 7-21 d. Larvae hatch from eggs and bore through bark to the cambium layer where they feed in a serpentine pattern creating an S-shaped gallery while developing through four instars. One generation per year or one generation every two years occurs in both the native and introduced ranges. When univoltine, EAB overwinter as mature larvae, however, when development occurs over two years

EAB overwinter as early instar larvae the first winter and mature larvae the second winter. EAB pupate in spring lasting 14-21 d (at 24°C) and emerge as adults after another 7-14 d (Petrice and Haack 2006).

Methods for EAB collection in introduced and native range

EAB were sampled for this study from native populations in China, Japan, and South Korea (Figure 2.4) and introduced populations in Illinois, Indiana, Michigan, Ohio, Pennsylvania, West Virginia and Ontario (Figure 2.5). Samples were also collected from an introduced location in Moscow, Russia.

EAB collection with collaborators

Due to the large geographic distribution of EAB, a network of collaborators was established to assist in insect collection. Leah Bauer PhD (USDA Forest Service), Houping Liu PhD, and Therese Poland PhD (USDA Forest Service) throughout China and Michigan, Roger Fuester (USDA-ARS) in China, Paul Schaefer (USDA-ARS) in Japan, Bob Haack PhD (USDA-FS) with Oleg Kulinich PhD in Russia, and David Williams in South Korea coordinated collection of Asian samples (Figure 2.4). Additional collaborators, Deb McCullough PhD and David Smitley PhD, of the Department of Entomology at MSU and several employees of the Michigan Department of Agriculture collected individual beetles from populations throughout Michigan. Lucy Hunt (Ohio Department of Agriculture), Mark Cinnamon (Illinois Department of Agriculture), Benjamin West (Indiana Department of Natural Resources), and Dick Bean (Maryland Department of Agriculture) assisted in beetle collection in Ohio, Illinois, Indiana, and Maryland respectively. Ches Caister, Plant Protection officer Southwest

Figure 2.4. Collection locations for Emerald Ash Borer in the native range of Asia (2002-2007). Province or provincial level cities with collection locations are as followed: Tianjin City (1-Dagong, 2- Hangu), Liaonging (3 and 4- Shenyang, 5- Benxi), Jilin (6-Laoniujuan, 7-Changchun City, 8- Jiutai, 9- Jilin City), Hebei (10- Tangshan), Beijing (11-Chaoyang), Heilongjiang (12 – Harbin), Miyagi (13- Shirioshi City), Chungchong-nam do (14- Daejeon), Gyeonggi-do (15- Suwon), Gyeongsang-bukto (16- Sangju), Gangwon-do (17- Inge, 18- Samcheok)

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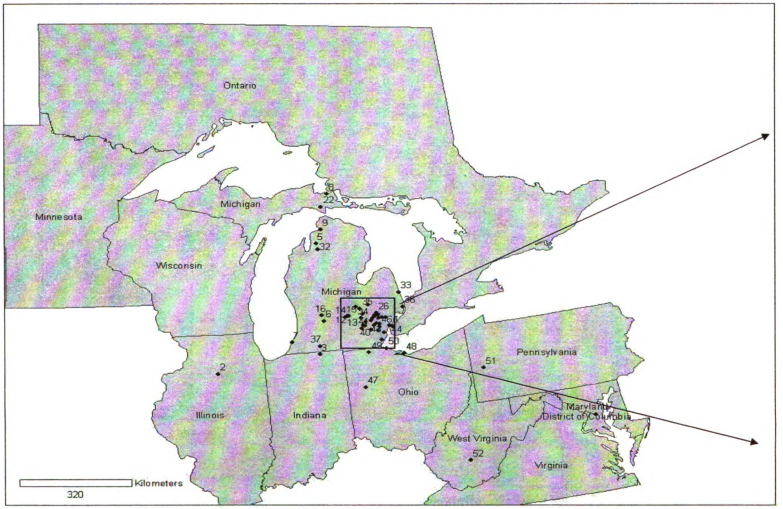


Figure 2.5.a. Collection locations for emerald ash borer in North America (2002-2007). Boxed area is expanded in figure 2.5.b.

Figure 2.5.b. Expanded view of collection locations of emerald ash borer in southeast Michigan and Winsor, Ontario (2002-2007).

region, collected specimens in Windsor, Ontario. R.M. Turcotte (USDA-FS) collected specimens in West Virginia. Nathan Siegert PhD (Dept. of Entomology, MSU) and Sven-Erik Spichiger (Pennsylvania Department of Agriculture) collected specimens in Pennsylvania. North America collection locations are shown in figure 2.5.

All collaborators were requested to follow the following field collection technique. Visual inspections of *Fraxinus spp.* were conducted at breast height for evidence of EAB activity, such as, the characteristic D-shaped exit hole or external tree evidence of larval activity of bark cracks or woodpecker holes. Adult beetles were collected in the field from mid May to late July with a sweep net; samples were killed and stored in 90-100% ethanol or killed by freezing and stored in ethanol and shipped to the lab in East Lansing, MI. To collect larvae, bark was removed from trees with a drawknife to survey for larval serpentine galleries. This was done at the field site on standing trees, or trees were cut into logs (60 cm sections of cut tree) in the field and returned to East Lansing, MI for bark removal with knives and chisels. When a gallery was located, the larvae or pupae were removed with forceps and placed in 90% ethanol or on larval diet (developed by Juli Gould, USDA-APHIS; Liu et al. 2007) for further development. All dead insects were sent to East Lansing, MI for genetic analysis. Live insects were shipped to the USDA-FS lab in East Lansing, MI for development on media or in logs for natural enemy evaluation (project conducted by Dr. Bauer (USDA-FS) and Dr. Liu (MSU). Live specimens that did not contain natural enemies

were preserved in 90% ethanol for genetic analysis.

Collection of *North American EAB samples*

In addition to samples collected by collaborators, samples were collected directly. EAB were collected in the summer of 2005 and 2008 in Lansing, MI from *F. pennsylvanica*. Four trees were felled in 2005 and three felled in 2008, cut into two feet logs and transported to the laboratory. Bark was removed from three logs from each tree. Larvae were removed and killed in liquid nitrogen, or frozen at -20°C or -80°C and stored in 95% ethanol at -20°C. Six additional logs from each tree (24 total) in 2005, held at 4°C to simulate winter conditions, were used for EAB adult development. After 2-4 months in chill, the infested logs were placed into heavy cardboard tubes capped with plastic ends at 24°C to stimulate pupation and adult development. Emerged adults were collected on a daily basis, killed and stored at -80°C.

Collection of EAB was also conducted in Shipshewana, Indiana in January 2005. *Fraxinus spp.* were located on a woodlot adjacent to farm land and visually observed for EAB activity. Adult emergence holes (D-shaped) were observed on 10 trees; therefore, bark was peeled at breast height to locate and expose larval galleries. Larvae and pre-pupae were removed and placed individually into wells of 24-well tissue plates and allowed to develop into adults. Sampling was also conducted in Oregon, OH and N. Baltimore, OH in January 2005. Urban street trees were felled and visually observed for D-shaped adult exit holes. Bark was peeled in the field and larvae and pre-pupae were removed and placed into individual wells for further development. Six 60 cm logs were

also maintained at 5°C until used to rear adults as described above. In June 2005, a *Fraxinus spp.* tree farm in Windsor, Canada was inspected for adult EAB. All trees were approx. 8-12 feet tall. Adult EAB were collected with an aerial net from the leaf canopy and killed in 70% ethanol for transport to East Lansing, MI in accordance with the Canadian Food Inspection Agency requirements.

Collection of Asian EAB samples

One of the most challenging aspects of this research project was obtaining samples from the native range of EAB in Asia. Because it was very difficult for the foreign collaborators to take time to collect themselves, a sampling trip was conducted in the native range of EAB in South Korea, China and Japan in the summer of 2006. The focus for site selection was on locations EAB was documented in the literature, collected in the past, and from personal communication with collaborators. Collection occurred mostly in natural forests and urban trees in South Korea, plantation and urban trees in China, and natural forests in Japan (Table 2.1). All sites were visually inspected for D-shaped exit holes and crown dieback. If symptoms were found, bark was removed at breast height to locate larval galleries.

Several locations in South Korea were identified for survey with the primary host tree sampled being *F. rhynchophylla*. Visual survey for host trees were conducted at Mt. Juri near Jinju. One tree with significant dieback at the Gyeongsang University Experimental Station was felled and sectioned into one meter logs for transport to Gyeongsang National University for bark removal.

Table 2.1.a. Field location survey records for trees examined for the presence of Emerald Ash Borer in South Korea.

Province, City,	Collector, Location (if known)	Host species	Forest type	Tree condition	Description of site
Gangwon-do					
Hoengseong	Tai-Wan Kim c/o Dave Williams	Unknown	Unknown	Unknown	Unknown
Inge	Dave Williams Maebongjae	*F. rhynchophylla*	Forest	Girdled	Unknown
Samcheok	Dave Williams	Unknown	Pulp mill	N/A	Resting on tree trunks
Chungchong-nam do					
Daejeon	Dave Williams	*F. rhynchophylla*	Urban, street trees	Unknown	Ash trees along roads near buildings
Gyeonggi-do					
Suwon	Alicia Bray	*F. rhynchophylla*	Urban	Unknown	*Fraxinus spp.* seed tree farm
Gyeongsang-namdo					
Near Jinju	Alicia Bray East Mt. Juri	*F. rhynchophylla*	Natural forest	Very good-fair	Mixed hardwood forest

Table 2.1.a. Continued

Province, City	Collector, Location (if known)	Ash species	Forest type	Tree condition	Description of site
Gyeongsang-namdo					
Near Jinju	Alicia Bray Gyeongsang National University Experimental Station – Mt. Juri	*F. rhynchophylla*	Natural forest	Very good-fair	Mixed hardwood forest
Near Jinju	Alicia Bray Jurisan National Park Near Jungsanni Ranger Station	*F. rhynchophylla*	Natural forest	Very good-fair	Mixed hardwood forest
Gyeongsang-bukto					
Near Sangju	Alicia Bray Gabjang, Mt. Sangju	*F. rhynchophylla*	Natural forest	Very good-fair	Mixed hardwood forest
Near Sangju	Alicia Bray Woraksan National Park Mt. Wolak	*F. rhynchophylla*	Natural forest/ plantation	Very good-fair	Mixed hardwood forest; near *Pinus spp.* seed farm
Jeikkabuk-do					
Near Muju	Alicia Bray Mt. Muju	*F. rhynchophylla*	Natural forest	Very good-fair	Mixed hardwood forest

Table 2.1.b. Field location survey records for trees examined for the presence of Emerald Ash Borer in China.

Province, City	Collector, Location (if known)	Ash species	Forest type	Tree condition	Description of site
Heilongjiang					
Harbin	Houping Liu	Unknown	Unknown	Unknown	Unknown
Tianjin City					
Dagong	Houping Liu	*F. velutina*	Plantation	Fair	Imported trees from N. America, 10-12yrs old trees
Hangu	Houping Liu	Unknown	Unknown	Unknown	Unknown
Jilin					
Jilin City-a	Houping Liu	Unknown	Unknown	Unknown	Unknown
Jilin City-b	A. Bray/ R. Fuester Song Hua Lake Park	*F. mandshurica* *F. rhynchophylla*	Natural forest	Good	Mixed forest with maple (*Acer* spp), oak (*Quercus* spp.), and pine (*Pinus* spp.)
Jilin City-c	A. Bray/R. Fuester Jiang Nan Forestry Center	*F. mandshurica*	Plantation	Good-poor	Plantation adjoining mixed forest
Jiutai	Bauer et al.	Unknown	Unknown	Unknown	Unknown

Table 2.1.b. Continued

Province, City	Collector, Location (if known)	Ash species	Forest type	Tree condition	Description of site
Changchun City-a	Bauer et al. Jingyuetan Forest Park Laoniujuan	*F. pennsylvanica*	City Park	Good	Mixed with oak (*Quercus* spp.) and pine (*Pinus* spp.)
Changchun City-b	A. Bray/R. Fuester Jingyuetan Forest Park	*F. chinensis* *F. mandshurica*	City Park	Good-poor	Mixed with pine (*Pinus* spp.)
Changchun City-c	A. Bray/R. Fuester Nanhu Park	*F. mandshurica*	City Park	Good-fair	Mixed with pine (*Pinus* spp.), poplar (*Populus* spp.), and willow (*Salix* spp.)
Changchun City-d	A. Bray/R. Fuester Tu Men Ling	*F. mandshurica*	Roadside	Good	Outside city planted planted along road
Hebei					
Tangshan City-a	Bauer et al.	Unknown	Unknown	Unknown	Unknown
Tangshan City-b	R. Fuester Zhen Zhuang-Zi villiage	*Fraxinus spp.*	Unknown	Unknown	Unknown
Liaonging					
Benxi City-a	Bauer et al.	Unknown	Unknown	Unknown	Unknown

Table 2.1.b. Continued

Province, City	Collector, Location (if known)	Ash species	Forest type	Tree condition	Description of site
Benxi City-b	R. Fuester Dong Ying Fang	*F. rhyncophylla*	Urban	Good-fair	Planted along roadside
Shenyang-a	Bauer et al. Yushutun	Unknown	Unknown	Unknown	Unknown
Shenyang-b	R. Fuester Wu Ai Street	*F. velutina*	Urban	Good-poor	Monoculture of ash along street
Shenyang-c	R. Fuester Cai Ta Street	*F. velutina*	Urban	Good-poor	Monoculture of ash along street
Beijing					
Beijing	Bauer et al. Chaoyang	Unknown	Uknown	Unknown	Unknown

Table 2.1.c. Field location survey records for trees examined for the presence of Emerald Ash Borer in Japan.

Prefecture, City	Collector, Location (if known)	Ash species	Forest type	Tree condition	Description of site
Aomori					
Aomori	A. Bray	Various spp.	Natural forest	Very good-good	Mixed forest
Iwaki-san	A. Bray Mt. Iwaki	Various spp.	Natural forest	Very good-good	Mixed forest
Akita					
Odate	A. Bray	Various spp.	Natural forest	Very good-good	Mixed forest
Iwate					
Morioka	A. Bray	Various spp.	Natural forest	Very good-good	Broadleaf deciduous forest
Miyako City	A. Bray	Various spp.	Natural forest	Very good-good	Mixed forest
Miyagi					
Shiroishi City-a	Minemitsu Kaneko c/o P. Schaefer-2004	Unknown	Natural forest	Very good-good	Mixed forest
Shiroishi City-b	A. Bray/ Minemitsu Kaneko-2006 Forest of Azalea	*F. lanuginosa*	Natural forest	Very good-good	Mixed forest

Table 2.1.c. Continued.

Prefecture, City	Collector, Location (if known)	Ash species	Forest type	Tree condition	Description of site
Sendai	A. Bray/ Mt. Zao	Various spp.	Natural forest	Very good-good	Mixed forest
Yamanashi					
Otsuki	A. Bray	Various spp.	Natural forest	Very good-good	Mixed forest
Tokyo					
Tanashi	A. Bray University of Tokyo Experimental Station	*Fraxinus spp.*	Plantation	Very good	Small planting of various spp.
Chiba					
Chiba	A. Bray University of Tokyo Experimental station	Various spp.	Natural forest	Very good-good	Mixed forest

Several more sites were visually surveyed for host trees and collection including Mt Muju, Mt. Sangju, Mt. Wolak, and Suwon (Table 2.1.a).

In China, nine collection locations were surveyed and sampled from four provinces: Liaoning, Jilin, Hebei, Heilongjiang, and two provincial-level cities: Tianjin City and Beijing (Table 2.1.b). Collection locations were chosen, in part, in accordance to sites previously successfully sampled within five years (Liu et al. 2003) and personal communication with local foresters. Surveying concentrated on native host species including *F. mandshurica, F. rhynchophylla, F. chinensis,* and introduced host species *F. velutina* and *F. pennsylvanica* (Liu et al. 2003).

Finally, five geographic locations were surveyed and sampled in Japan concentrating on native host trees known from the literature *F. japonica*, *F. spaethiana*, *F. apertisquamifera*, *F. longicuspis, F. platypoda, F. lanuginosa*, *J. mandshurica* var. *sieboldiana* and var. *sachalinensis*, *P. rhoifolia*, and *U. davidiana* var. *japonica* (Akiyama and Ohmomo 1997; Table 2.1.c). Visual surveys in Chichibu and Morioka were restricted to trees along roadsides for safety precautions due to the presence of native poisonous snakes and bears.

Results of emerald ash borer collection

For this study, an extensive EAB collection was assembled. Because EAB was first detected in Michigan (Haack et al. 2002), widespread surveys were conducted for the presence of EAB in this state. For this project, I obtained samples throughout the current range of EAB distribution to include EAB from 43 sites in 21 counties, including two in the upper peninsula for a total of 1652 EAB individuals (Table 2.2). Samples were also obtained with collaborators or

Table 2.2. Emerald ash borer specimens obtained/collected in North America from 2003-2008

Country	State/ Province (or equiv.)	County	Locality	# of sites in locality sampled	Collector	#Adults	#Larvae	#Pupae	Map#
Canada	Ontario	Essex	Windsor	1	Caister/Bray	107	7	0	1
U.S.A	Illinois	LaSalle	Peru	1	Cinnamon	0	10	0	2
	Indiana	LaGrange	Shipshewana	1	Bray	0	14	0	3
	Maryland	Prince George's		2	Bean	1	12	0	4
	Ohio	Auglize		1	ODA	0	12	0	47
		Erie/Lorain		1	ODA	0	11	0	48
		Fulton	Swanton	1	Bray/ODA	0	27	0	49
		Lucus	Oregon	1	Bray/ODA	5	0	0	50
	Pennsylvania	Butler	Cranberry	2	Siegert/Spichger	1	49	0	51
	West Virginia	Fayette		1	Turcotte	0	5	0	52
	Michigan	Antrim		1	MDA	0	1	0	5
		Barry		1	MDA	0	3	0	6
		Berrien		1	MDA	0	3	0	7
		Chippewa	Brimley	1	Siegert	0	10	0	8
		Emmet	Petoskey	1	Siegert	115	0	0	9
		Genesee	Flint	3	Simon	36	0	0	10
		Ingham	East Lansing	3	Bauer	79	0	0	11-13

Table 2.2. Continued

Country	State/ Province (or equiv.)	County	Locality	# of sites in locality sampled	Collector	#Adults	#Larvae	#Pupae	Map#
			Lansing	4	Bauer/Bray	163	42	0	14
			Okemos	2	Bauer	77	0	0	15
		Iosco		1	MDA	0	7	0	-
		Kent	Bowne Twp	1	MDA	3	0	0	16
		Livingston	Brighton	1	Bauer	59	0	0	17
			Howell	2	Bauer	87	0	0	18-19
			Oak Grove	1	Bauer	20	0	0	20
			Pinckney	1	Bauer	20	0	0	21
		Mackinac	Moran	1	McCullough	0	21	0	22
		Macomb	Shelby Twp	1	Bauer	3	0	0	-
			Waterford	1	Bauer	21	0	0	23
		Monroe	Monroe	1	Kovach	5	0	0	24
		Oakland	Bloomfield	1	Newhouse/Smitley	0	24	0	25
			Clarkston	1	Harrow	3	0	0	26
			Milford	1	Bauer	67	34	3	27
			Northville	1	Bauer	44	0	0	28
			Orchard Lake	1	Bauer	4	0	0	29
			White Lake	1	Bauer	4	0	0	30
			Troy	1	Newhouse/Smitley	0	19	0	31
				1	Newhouse/Smitley	0	22	0	-
		Roscommon		1	MDA	0	1	0	32
		Sanilac		1	MDA	0	4	0	33
		Shiawassee	Corunna	1	Leshefski	0	9	0	34
			Durand	1	Leshefski	25	8	0	35

Table 2.2. Continued

Country	State/ Province (or equiv.)	County	Locality	# of sites in locality sampled	Collector	#Adults	#Larvae	#Pupae	Map#
		St. Clair		1	MDA	22	0	0	36
		St. Joseph		1	MDA	0	6	0	37
		Washtenaw	Ann Arbor	4	Bauer	130	0	0	38-40
			Chelsea	1	Newhouse/Smitley	1	0	0	41
			Dexter	1	Bauer	67	0	0	-
			Willis	1	Bauer	18	0	0	-
		Wayne	Belleville	3	Bauer	210	0	0	42
			Brownstown	1	Bauer	36	0	0	43
			Detroit	1	Bauer	25	10	9	44
			Livonia	1	Bauer	43	0	0	45
			Plymouth	1	Bauer	21	0	0	-
			Westland	1	Newhouse/Smitley	0	8	0	46

collected directly from Illinois (one site; 10 individuals), Indiana (1; 14), Maryland (2; 13), Ohio (4; 55), Pennsylvania (2; 50), West Virginia (1; 5), and Windsor, Canada (1; 114) (Table 2.2). Thus, the collection presented here represents most of the current known range of EAB in N. America. Samples were not obtained for this project from more recently detected (2008-2009) sites in Kentucky, Minnesota, Missouri, New York, Virginia, Wisconsin, and Quebec, Canada (Cooperative emerald ash borer project 2009), due to time limitations and resource availability.

In the summer of 2006, field surveys for EAB in South Korea were carried out at 11 sites in six provinces spanning the range of peninsula (Table 2.1.a). Surveys included a range of locations to include urban street trees, plantation forests and natural forests to include a range of possible habitats for EAB. EAB adults were collected from six of the 11 sites in four provinces (Table 2.3). Host tree associations could not be verified for these specimens since only adults were obtained; however, all were collected in close vicinity of *F. rhynchophylla*. Trees, mainly *F. rhynchophylla*, that exhibited signs of EAB infestation (crown dieback, bark splitting, and D-shaped exit holes) were peeled of bark to locate larval galleries. Several trees peeled were heavily infested with larvae; however, identification of the collected samples indicated that they were in the family *Cerambycidae* and not *A. planipennis*. Even though EAB larvae were not detected at the time of collection, the presence of D-shaped exit holes on the trees leads to the hypothesis that EAB (or a different Buprestid spp.) did infest the tree in the past.

Table 2.3. Emerald ash borer specimens obtained/collected in Asia from 2003-2008

Country	Province (or equiv.)	Locality	Collector	#Adults	#Larvae
China	Heilongjiang	Harbin	Liu/Bauer/Zhao	4	3
	Tianjin City	Dagong	Liu/Bray/Fuester	22	94
		Hangu	Liu/Bauer/Zhao	0	11
	Jilin	Jilin City	Liu/Bauer/Zhao	0	4
		SongHua Lake Park	Bray/Fuester	0	13
		Jiang Nan Forestry Center	Bray/Fuester	0	8
		Laoniujuan	Liu/Bauer/Zhao	0	20
		Jiutai	Liu/Bauer/Zhao	0	20
	Hebei	Tangshan	Liu/Bauer/Zhao	0	20
		Zhuang-Zi	Fuester	0	21
	Liaonging	Benxi	Liu/Bauer/Zhao	0	6
		DongYingFang	Fuester	2	0
		Shenyang	Fuester	1	5
		Shenyang	Liu/Bauer/Zhao	0	10
	Beijing	Chaoyang	Liu/Bauer/Zhao	0	10
South Korea	Gangwon-do	Hoengseong	Williams	1	0
		Samcheok	Williams	1	0
		Inge	Williams	5	0
	Chungchong-nam do	Daejeon	Williams	9	0
	Gyeongsang-bukto	Sangju	Bray	1	0
	Gyeonggi-do	Suwon	Bray	1	0
Japan	Miyagi	Shiroishi City	Schaefer/Bray	1	0
Russia		Moscow	Kulinich	12	0

In China, field surveys for EAB in all *Fraxinus* spp. present were conducted by collaborators or directly at 19 sites in four provinces and two provincial-level cities (Table 2.1.b). Larvae were successfully collected from 15 of the sites, representing all provinces (Table 2.3). Evidence of past EAB activity, crown dieback in edge trees, exit holes, and larval galleries, was found at the remaining 4 sites although samples were not collected. Historical records of EAB host range include Shandong province (CAS 1986, Yu 1992), however surveys within the province have not been successful (Liu et al. 2003).

Surveys for EAB in Japan included all historical host species including, ash, walnut and elm species. Extensive surveys for EAB were conducted at 11 sites in seven prefectures (Table 2.1.c). One location, Forest of Azalea near Shiroishi City, was found to have an active population. One adult was collected by collaborators Mr. Kaneko and Dr. Schaefer at this location in 2004 as well as two larvae from *F. lanuginose* by this project in 2006. The larvae collected were relatively small, possibly 1st or 2nd instars, and difficult to identify to species. There is another metallic woodboring beetle known in the same area to attack *F. lanuginosa*, *Agrilus koyoi* Ohmomo, which is difficult to distinguish from EAB in early instars. Very little detail is known about the biology of *A. koyoi* since it was recently described in 2002 by Ohmomo (Jendek 2007). It is much smaller than *A. planipennis* being 5.1-6.2 mm adult length compared to 8.5-15mm, however, it is similar in color being metallic green to bronze, have the same host tree, and located in the same forest near Shiroishi City. Two adults of *A. koyoi*, were

collected in 2006 at this location confirming an active population of both species in the area.

Discussion

Current efforts for emerald ash borer eradication and control have not been successful in slowing the spread of this destructive pest. High quality basic knowledge on the biology of an introduced pest and the speed at which action is taken will facilitate efforts to slow the spread of any invasive pest (Simberloff 2005).

Given the large native region of EAB including eastern Russia, Korea, China, Japan and Taiwan, there could be important biological differences between these populations that could impact the management strategy of EAB in North America. The collection efforts of this study have yielded an extensive collection of EAB from the native and introduced ranges. Although surveys throughout much of the native range included regions in China, South Korea, and Japan, samples were rare in South Korea and Japan, reducing the probability the origin of the introduced population was from these two countries. This hypothesis is further evaluated by genetic methods in the following chapters.

CHAPTER III

AFLP and mtDNA COI analysis of EAB Populations in Asia and North America

Introduction

Michigan has 693 million ash trees (*Fraxinus* spp.) throughout its forests and urban environments with an estimated value > $18.92 billion (USDA-APHIS 2003). Ash has a variety of economic values including nursery sales, timber, sports equipment, tool handles as well as recreational use in state and federal forests. For example, ash trees were one of the primary replacement trees in urban landscaping to replace the American elms lost during the Dutch Elm Disease epidemic in the latter half of the 20th century (MacFarlane and Meyer 2005).

The United States and Canada are currently battling a destructive invasive pest of ash, the emerald ash borer (EAB), *Agrilus planipennis* Fairmaire, which is native to Eastern Asia. Larvae of EAB destroy northeastern species of North American ash trees by feeding below the bark on phloem tissue (Anulewicz et al. 2008). EAB develop within one to two years inside the tree until emerging as an adult beginning in May creating a characteristic D-shaped exit hole in the bark. High densities of larvae create feeding galleries that girdle the tree causing nutrient flow to cease, causing death of the tree within as little as two years (Russell et al. 2003).

EAB was detected in North America in 2002 (Haack et al. 2002), and was initially found in counties of southeast Michigan and southeastern Ontario (Scarr

et al. 2002) and one northern county of Ohio in 2003. It is suspected that EAB was introduced in the mid-1990's and potentially may have been introduced in the early-1990's (Siegert et al. 2007). The known current range of EAB in the United States includes 15 US states as well as several counties in Ontario and Quebec, Canada (Chapter 2 Figure 2.1).

Invasive species such as EAB are an increasing problem as global trade and travel increase (Simberloff 2005). A species can become invasive when it is introduced into a new environment free from natural enemies and competitors, becoming released from population constraints (Orians 1986, Sakai et al. 2001). Even though the incidences of species introduction continue to rise, the obstacles they must overcome to become established and invasive remain the same. Invaders must locate a suitable habitat, resources and potential mates to continue the population apart from the native range (Elton 1958). Introduced populations may also have to overcome decreased genetic variation due to small founding populations that could lead to inbreeding depression (Allendorf and Lundquist 2003, Allendorf and Luikart 2007). However, decreased genetic variation has been found to be beneficial, such as in Argentine ants with the genetic stock of the introduced population having a competitive advantage over native ant populations (Tsutsui et al. 2000; Tsutsui and Case 2001). In some cases, there could also be an increase in genetic variation if the introduced population is an admixture from multiple introductions or a single introduction of multiple source populations that are genetically distinct from each other (Allendorf and Luikart 2007). This has been observed in a variety of invasive

organisms including the invasive weed, *Verbascum thapsus* (Dlugosch and Parker 2008) and the lizard, *Anolis segrei* (Kolbe et al. 2004). In both examples, the authors detected increased genetic variation in the introduced range compared to the native range presumably due to multiple introductions. Knowledge of genetic variation has important implications for development of an effective management strategy for invasive species (Allendorf and Lundquist 2003, Sakai et al. 2001). Understanding the population biology of an invasive species could provide information on suitable habitat, potential location(s) of effective biological control agents, or help predict the effectiveness of a control agent (Kambhampati and Rai 1991; Lee 2002; Allendorf and Lundquist 2003; Schefffer and Grissell 2003,).

Molecular markers have been used to elucidate the origin and number of introductions (Grapputo et al 2005), invasion history (Elderkin et al. 2004), genetic variation of an introduced species (Roderick 1996; Sakai et al. 2001), and to develop and evaluate control strategies (Szalanski and Owens 2003; Conord et al. 2006; Kim et al. 2006). For example, mitochondrial DNA gene sequencing has been used to detect genetic constraints caused by an introduction event (Graputto et al. 2005). Mitochondrial DNA is haploid and maternally inherited (exceptions: Wallis 2000; Andolfatto et al. 2003); therefore it may reveal haplotype confirmation of geographic origin of invasive species as well as events of strong genetic drift (Avise 1994; Villablanca et al. 1998). However, it may not be able to detect genetic variation on a recent time scale (Grapputo et al 2005; Shapiro et al. 2008). Cognato et al. (2005) successfully

used the mtDNA cytochrome oxidase subunit I to determine the geographic origin of the red turpentine beetle, *Dendroctonus valens* LeConte, that was introduced into China. Genetic variation was assessed from individuals from 32 populations across the native range in North America and in the introduced range in China. Haplotypes found in China were most closely related to haplotypes found in the Pacific North-West of the United States. High genetic variation was detected in the introduced range measured by haplotype diversity (0.94), indicating the founding population may have been large or there have been multiple introductions.

Genetic variation may also be assessed using nuclear markers exhibiting higher variation than mtDNA due to biparental inheritance (Villablanca et al. 1998). One such marker is DNA fingerprinting by amplified fragment length polymorphisms (AFLP); it is able provide a high number of polymorphic loci throughout the whole genome of an individual without prior genetic knowledge of the organism (Vos et al. 1995). This allows for rapid detection of polymorphism in a population that is not possible with gene or DNA fragment sequencing (Mueller and Wolfenbarger 1999). This technique has successfully provided information about population variation including, the genetic variability of insect populations across large geographic areas (Conord et al 2006; Clark et al. 2007), identification of strains within the same species (Dalirsefat and Mirhoseini 2007), genetic structure of an invasive species in an introduced range (Elderkin et al. 2004), and identification of geographic origin of invasive species (Reineke et al 1999; Benavides et al. 2005).

Although the examples above describe studies using one molecular marker to elucidate population structure and variation, the addition of markers with different inheritance patterns can provide a more complete understanding of the population variation. For example, Salvato et al. (2002) used AFLP and mtDNA markers to assess variation and population structure in two sibling species of the winter pine processionary moth (*Thaumetopoea pityocampa* and *Th. wilkinsoni*). Strong genetic differentiation between the two species as well as geographic structuring of *Th. pityocampa* was detected with both markers. AFLP and mtDNA markers were also successfully used to assess phylogeographical patterns of *Adelges cooleyi* in Western N. America (Ahern et al. 2009). Paupy et al. (2004) compared AFLP's, microsatellite and isoenzyme markers in *Aedes aegypti* from Phnom Pehn, Cambodia and determined the effectiveness of these markers for inference of population structure. All markers revealed the same population structure; however, AFLP analysis revealed up to five times greater genetic differentiation than the other two. AFLP and mtDNA markers have been used to estimate the geographic origin and population structure of an invasive species. Grapputo et al. (2005) used both to assess the variation in Colorado potato beetles in the native range of N. America and introduced range in Europe. The European populations only had a portion of the AFLP native variability and only a single mtDNA haplotype. Hence, they concluded that the introduced populations underwent a bottleneck. These examples provide evidence on the importance of assessing genetic variation and

structure with different marker systems to obtain a clear understanding and corroboration of hypotheses.

To provide further evidence of population origin of invasive species, population assignment tests may be used with molecular data to assign an individual to the most likely population it originated (first described by Paetkau et al. 1995). This method has been used to answer a variety of questions including: identifying dispersal patterns in a metapopulation (Berry et al. 2004), confirming population structure of native and/or invasive populations (Elderkin et al. 2004; Paupy et al. 2004), assessing population structure to design eradication strategies (Abdelkrim et al. 2005), estimating the origin of invasive species (Bonizzoni et al. 2001; Genton et al. 2005; Tsutsui et al. 2001), or to assess if individuals are from an established population or recently migrated to an area (Kim et al. 2006). These tests are often performed using microsatellite data, however, AFLPs are useful alternative for species where microsatellite markers are unavailable or logistically difficult to obtain (Campbell et al. 2003).

The purpose of this study was to characterize the genetic population structure of EAB in its native range in Asia and introduced range in N. America. A primary goal was to identify as specifically as possible the geographic location of the source EAB population(s) that gave rise to North America's EAB infestation. Although the presumed source of the infestation is China (based on specific trade records), beetles may have originated from other Asian countries within its native range. Population structure and variability of EAB was assessed using AFLP and mtDNA COI markers. Understanding the genetic structuring in

the native and introduced range could be helpful to determine movement patterns to focus management efforts (Sakai et al. 2001) and estimating the success of introduced biological control agents or developing chemical controls (Bourguet et al. 2000).

Materials and methods

Sample collection

Larvae, pupae and adult EAB were collected from introduced populations in Illinois, Indiana, Maryland, Michigan, Ohio, Pennsylvania, West Virginia and Windsor, Canada and from native populations in China, Japan, and South Korea (Table 3.1). A network of collaborators from several government agencies and academic institutions was established to assist in insect collection. Field collected EAB were killed in 70% EtOH and shipped to Michigan State University. Upon arrival, samples were transferred to 90% ethanol and stored in a -20°C freezer until needed. Some samples from Michigan were reared to adult and killed in liquid nitrogen. To do this, ash bolts, 60 cm sections of trunk or branch, were obtained from many locations in Michigan for insect rearing in the USDA Forest Service laboratory in East Lansing, MI. Bolts were cut in the field, transferred to the laboratory and stored at 4°C until rearing of larvae into adults. Rearing adult EAB was accomplished by placing individual bolts in a circular cardboard container closed at both ends with a plastic cap, allowing light to enter, and maintained at room temperature (approx. 20-24°C). Emerged adult EAB were killed with liquid nitrogen.

Table 3.1. Emerald Ash Borer Specimens Collected/Obtained From 2003-2008. Number of samples used for mtDNA and AFLP analysis included.

Country	State/Province (or equiv)	County	Locality	Total # Ind (N) collected	mtDNA Anal. (N)	AFLP Anal. (N)
China	Beijing		Chaoyang	10		2
China	Hebei		Tangshan	20	11	16
China	Hebei		Tangshan – Zhenzhu Zhenstulangai	21	4	6
China	Heilongjiang		Harbin	7	4	5
China	Jilin		Changchun City - Jing Yue Tan Park	17	4	5
China	Jilin		Changchun City - Laoniujuan	20		1
China	Jilin		Jilin City - Jiang Nan Forestry Center	8		
China	Jilin		Jiutai	20	20	20
China	Liaonging		Benxi	6	2	6
China	Liaonging		Shenyang	10	4	4
China	Liaonging		Shenyang - Yushutun	10	9	9
China	Tianjin City		Dagong	116	23	24
China	Tianjin City		Hangu	11	1	1
Japan			Shiroishi City	3	1	2
South Korea	Chungchong-nam do		Daejeon	9	5	4
South Korea	Gyeonggi-do		Suwon	1		1
South Korea	Gyeongbak		Sanju	1		1
South Korea	Gangwon-do		Inje	5	5	2
South Korea	Gangwon-do		Samcheok	1	1	0
Russia			Moscow	12	2	
Canada	Ontario	Essex	Windsor	114	25	26

Table 3.1. (cont.)

Country	State/Province (or equiv)	County	Locality	Total # Ind (N)	mtDNA Anal. (N)	AFLP Anal. (N)
U.S.A	Illinois	LaSalle	Peru	28	2	14
U.S.A	Indiana	LaGrange	Shipshewana	34	16	16
U.S.A	Maryland	Prince George's		12		
U.S.A	Michigan	Antrim		1		
U.S.A	Michigan	Barry		3		
U.S.A	Michigan	Berrien		3		
U.S.A	Michigan	Chippewa	Brimley	10		
U.S.A	Michigan	Emmet	Petoskey	115	11	10
U.S.A	Michigan	Genesee	Flint, U of M campus	36		
U.S.A	Michigan	Ingham	East Lansing - Harrison Road (HR)	32		
U.S.A	Michigan	Ingham	East Lansing - Stoddard Park (SP)	37		
U.S.A	Michigan	Ingham	East Lansing - Trowbridge Rd (TR)	10		
U.S.A	Michigan	Ingham	Lansing – Alicia Bray's backyard	42	30	29
U.S.A	Michigan	Ingham	Lansing - Crego park	32		
U.S.A	Michigan	Ingham	Lansing – Washington Park	30		
U.S.A	Michigan	Ingham	Lansing - Willoughby Park (WP)	101		
U.S.A	Michigan	Ingham	Okemos	42	4	
U.S.A	Michigan	Ingham	Okemos - Ferguson Park (FP)	35		
U.S.A	Michigan	Iosco		7		
U.S.A	Michigan	Kent	Bowne Twp section 36	3		
U.S.A	Michigan	Livingston	Brighton - Island Lake (IL)	59	3	
U.S.A	Michigan	Livingston	Howell - Brighton State Rec area (BR)	20		
U.S.A	Michigan	Livingston	Howell - Livingston Airport (LA)	67		

Table 3.1. (cont.)

Country	State/Province (or equiv)	County	Locality	Total # Ind (N)	mtDNA Anal. (N)	AFLP Anal. (N)
U.S.A	Michigan	Livingston	Oak Grove - State Game Area (OG)	20		
U.S.A	Michigan	Livingston	Pinckney - Pinckney State Rec area (PR & SL)	20		
U.S.A	Michigan	Mackinac	Moran	21	11	21
U.S.A	Michigan	Macomb	Shelby Twp - Stony Creek park	3	1	
U.S.A	Michigan	Macomb	Waterford - Rotary park	21	4	
U.S.A	Michigan	Monroe	Monroe	5		
U.S.A	Michigan	Oakland	Bloomfield - Bay Point golf course	24	1	
U.S.A	Michigan	Oakland	Clarkston	3		
U.S.A	Michigan	Oakland	Milford- Kensington Park	104	6	
U.S.A	Michigan	Oakland	Northville - Maybury Park	44	1	
U.S.A	Michigan	Oakland	Orchard Lake	4	2	
U.S.A	Michigan	Oakland	White Lake - Indian Springs park	4	3	
U.S.A	Michigan	Oakland	Troy	19	2	
U.S.A	Michigan	Oakland		22	1	
U.S.A	Michigan	Roscommon		1		
U.S.A	Michigan	Sanilac		4		
U.S.A	Michigan	Shiawassee	Corunna	9	2	
U.S.A	Michigan	Shiawassee	Durand	33	2	
U.S.A	Michigan	St. Clair		22		
U.S.A	Michigan	St. Joseph		6		
U.S.A	Michigan	Washtenaw	Ann Arbor - County Farm Park (CFP)	43	2	
U.S.A	Michigan	Washtenaw	Ann Arbor - Delhi park	12	3	

Table 3.1. (cont.)

Country	State/Province (or equiv)	County	Locality	Total # Ind (N)	mtDNA Anal. (N)	AFLP Anal. (N)
U.S.A	Michigan	Washtenaw	Ann Arbor - Hellnen Rd tree nursery	12		
U.S.A	Michigan	Washtenaw	Ann Arbor - Matthaei park	63	8	
U.S.A	Michigan	Washtenaw	Chelsea	1		
U.S.A	Michigan	Washtenaw	Dexter - Hudson Mills park (HM)	67		
U.S.A	Michigan	Washtenaw	Willis	18	2	
U.S.A	Michigan	Wayne	Belleville - Lower Huron Metro Park	163	3	
U.S.A	Michigan	Wayne	Belleville - Oakwoods	21	3	
U.S.A	Michigan	Wayne	Belleville - Willow park	26	3	
U.S.A	Michigan	Wayne	Brownstown - Lake Erie park	36	3	
U.S.A	Michigan	Wayne	Detroit	44	4	
U.S.A	Michigan	Wayne	Livonia - Bicentenial park	43	22	21
U.S.A	Michigan	Wayne	Plymouth - Fox Hills country club (FH)	21	13	13
U.S.A	Michigan	Wayne	Westland	8	1	
U.S.A	Ohio	Auglize	South Bound	12	2	4
U.S.A	Ohio	Erie/Lorain		11	2	
U.S.A	Ohio	Fulton	Swanton - Jones Optometry	27	18	6
U.S.A	Ohio	Lucus	Oregon	5	2	2
U.S.A	Pennsylvania	Butler	Cranberry	50	7	17
U.S.A.	West Virginia	Fayette		5	5	5
TOTAL				2260	326	293

The aim was to collect at least ten individual beetles from each location. However, this was not possible for all locations (e.g., South Korea, Japan, and West Virginia, Table 3.1).

DNA isolation

Total genomic DNA was extracted from larval tissue (with the GI tract removed to avoid potential contamination from gut contents) or from adult femur muscle tissue using a QIAamp DNA Mini Kit (QIAGEN cat. no. 51304) as described by the manufacturer with the following modifications. Larval tissue, two abdominal segments, was combined with 20 µL protease K and 180 µL QIAGEN ATL buffer in a 1.5 mL microcentrifuge tube and homogenized with a pestle (1mL pipet tip closed by flame). If an adult hind-leg was used, the femur was removed from the thorax and cut in half perpendicularly with a sterile razor blade to expose tissue and then combined with the protease K and QIAGEN ATL buffer. Tissue homogenates were incubated overnight at 56°C on a rocking platform. DNA was eluted from the spin columns by applying 80 µL QIAGEN AE elution buffer to the spin column, incubation at room temperature for 5 min, and centrifugation at 8000 rpm for 1 min. Elution step was repeated for a total volume of 160 µL. Samples were stored at -20°C. Quantification of DNA was carried out on a subset of samples using a Nanodop system (Thermo Scientific).

Mitochondrial DNA gene sequence

The partial sequence of cytochrome oxidase I (COI) was amplified for all EAB individuals in the sample (Table 3.1). Primers C1-J-1751 (5'-GGA TCA CCT GAT ATA GCA TTC CC- '3) and C1-N-2191 (5'-CCC GGT AAA ATT AAA

ATA TAA ACT TC-'3), desicribed by Simon et al. 1994, were used to amplify a partial COI nucleotide sequence of approx. 450 bp. PCR reactions (25µL) contained 9.3 µL ddH_20, 2 µL 10X Taq polymerase buffer (Invitrogen), 1.6 µL 50 mM $MgCl_2$ (Invitrogen), 0.4 µL Taq DNA polymerase (Invitrogen 5 units/µL), 2 µL 10mM each deoxynucleotide triphosphates (dATP, dTTP, dCTP, and dGTP), 1.5 µL of 10 µmol oligonucleotide forward and reverse primer, and 2.7 µL sample DNA. Reactions were carried out on a PE9700 Thermal Cycler with the following protocol: 5 min at 95°C, 35 cycles of 120 sec at 94°C, 90 sec at 52°C, and 120 sec at 72°C, followed by a final extension for 7 min at 72°C. PCR products were purified using QIAGEN MinElute PCR Purification Kit (cat no. 28004) to remove unincorporated primers and nucleotides. Purified product (5 µL) was combined with 4 µL ddH_20 and 3 µL of 10 µmol primer and sent to the MSU Genomics Technology Support Facility for forward and reverse sequencing. Alignment of obtained sequences was performed using SeqEd Ver 1.0.3 (Applied Biosystems).

Phylogenetic analysis of mitochondrial COI haplotypes was carried out by parsimony analysis of sequence data using PAUP*4 (Swofford 2000). A heuristic search was performed using tree-bisection-reconnection branch swapping, equal weighting of characters, and random addition of replicates.

Haplotype relationships were also analyzed using statistical parsimony with the computer program TCS program 1.21 (Clement et al. 2000). This analysis allows the possibility the ancestral population is observed in the sample set and potentially the most frequently observed. Haplotype relationships from

TCS were displayed as a network. Haplotype diversity was also determined using the TCS program.

Analysis of molecular variance (AMOVA) using ARLEQUIN 3.0 (Excoffier et al. 2005) was used to determine if population structure between the collection locations in Asia and N. America was observed using the mtDNA haplotypes. Within- and between- population variation was estimated considering the total EAB samples as 11 populations (seven populations in China, two in South Korea, one in Japan, one in Russia and 230 individuals pooled together in one population in N. America). Φ_{st} in pairwise population comparisons were considered to be significantly different at the $p = 0.05$ level.

Amplified Fragment Length Polymorphism (AFLP)

AFLP plant mapping protocol (Applied Biosystems Part #402083) was used to generate fragments for fingerprinting of individual EAB. Restriction fragments were generated by digesting genomic DNA with EcoRI and MseI restriction endonucleases and ligating the fragments to specific EcoRI and MseI adaptors. This created a large number of fragments used for subsequent selective amplification. An enzyme master mix was prepared by combining 0.1 µL 10X T4 ligase buffer, 0.1 µL 0.5 M NaCl, 0.05 µL 1 mg/mL bovine serum albumin (BSA), 1 unit MseI, 5 units EcoRI, 1 unit T4 DNA ligase, and brought to a total volume of 1 µL with ddH_2O. The restriction/ligation master mix was prepared by combining 1 µL 10X T4 ligase buffer, 1 µL 0.5 M NaCl, 0.5 µL 1 mg/mL BSA, 1 µL MseI adaptor, 1 µL EcoRI adaptor, and 1 µL enzyme master mix. Reactions were carried out by combining 5.5 µL genomic DNA with 5.5 µL

master mix and incubating for 2.5 hr at 37°C. The product of this digestion was diluted with 89 µL of $TE_{0.1}$ buffer (20 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Reduction of the number of fragments was accomplished by PCR amplification with pre-selective primers; a MseI complementary primer (MseI adaptor with the recognition site and 3'C) and an EcoRI complementary primer (EcoRI adaptor with recognition site and 3'A). Solutions were prepared by adding 4 µL dilute restriction/ligation product to 1 µL pre-selective primer pairs and 15 µL core mix (Applied Biosystems). Pre-amplification solutions were placed in a Applied Biosystems GeneAmp PCR System 9700 thermal cycler with the following program: one cycle of 72°C for 2 min; 20 cycles of 94°C for 1 sec, 56°C for 30 sec, and 72°C for 2 min; with a final extension step of 60°C for 30 min . Pre-amplification products were diluted with 89 µL of $TE_{0.1}$ buffer for final selective amplification. Four primer pair combinations used for selective amplification were run on each EAB sample (*Eco*RI-AGG/*Mse*I-CTT, *Eco*RI-ACT/*Mse*I-CAG, *Eco*RI-ACT/*Mse*I-CTT, *Eco*RI-AGG/*Mse*I-CAG. Selective amplification solutions were prepared by combining 3.0 µL diluted pre-selective amplification product, 1.0 µL 5µM MseI primer, 1.0 µL 1µM EcoRI primer (fluorescent labeled), and 15.0 µL AFLP core mix and run in the above thermal cycler with the following program: one cycle of 94°C for 2 min; 10 cycles of 94°C for 20 sec, 66°C for 30 sec, 72°C for 2 min decreasing by one degree at the second step each cycle; 20 cycles of 94°C for 20 sec, 56°C for 30 sec, and 72°C for 2 min; with a final extension step of 60°C for 30 min. Selective amplification PCR products were submitted to the MSU Genomics Technology Support Facility for electrophoretic

separation of DNA fragments on an Applied Biosystems ABI PRISM ® 3100 Genetic Analyzer, and analyzed using Applied Biosystems GeneScan Analysis Software.

Profiles were observed with GeneScan 3.1 software (ABI Applied Biosystems) for scoring of fragments between 50-500 bp in length. Scoring fragments smaller than 50 bp increases the likelihood of homoplasy (Ahern et al. 2009) and therefore these were eliminated from the analysis. Fragments greater than 500 bp were not observed in any profile. Fragments (peaks) were coded manually as present (1) or absent (0) for each individual locus and compiled in a Microsoft Excel spreadsheet for subsequent analysis. Loci were scored as present if the peak height was greater than 100 (Ahern et al. 2009).

Descriptive statistics, including the percent polymorphic loci and average heterozygosity for each population, as well as pairwise genetic differentiation (F_{st}) were obtained using AFLP-SURV (Vekemans et al. 2002). A Bayesian method of analysis with non-uniform prior distribution of allele frequencies and assumed Hardy-Weinberg equilibrium was used to determine allele frequencies. The proportion of polymorphic loci and expected heterozygosities were calculated for each population (Lynch and Milligan 1994). Higher values of polymorphism and expected heterozygosity are expected in Asian populations than N. American populations if the introduced population was the result of a single introduction or several small introductions from the same geographic source. Overall genetic differentiation, F_{st}, was calculated with 500 permutations to determine differentiation among populations at the 1% level (Lynch and Milligan 1994). A

high value of overall F_{st} (higher than 0.15 (Wright 1951; Connor and Hartl 2004) is considered to indicate strong differentiation among populations. Pairwise genetic differentiation (F_{st}) between each population was calculated to determine which populations were significantly differentiated from each other. Low pairwise F_{st} values (close to zero) were interpreted to mean that two populations are either not differentiated from each other or the loci used were unable to detect differentiation.

Significance of pairwise Φ_{pt} values and population structure were analyzed by AMOVA (999 permutations) using GenAlEx 6.1 (Peakall and Smouse 2006) with presence/absence data in a Microsoft Excel file. The analysis was performed for Asian and N. American collection locations with each collection location defined as a population to determine proportion and significance of variation within and between populations. Mantel tests were also performed to determine if a pattern of isolation-by-distance model of population structure is observed in the native and introduced range.

The number of distinct genetic groups (K) throughout the native range given no *a priori* information was estimated using STRUCTURE 2.2 (Pritchard et al. 2000). This program uses Bayesian algorithms to determine the proportion of genetic variation in each sample from the estimated number of populations. The admixture ancestry model was used for a different number of parent populations (K = 1-8) and each run was conducted with a burn-in value of 50000 and 450000 iterations for data collection. The value of K with the lowest posterior probability is considered the probable number of parent populations (K) for a given data set.

Genetic similarity among EAB populations were displayed via neighbor-joining (NJ) analysis using pairwise Φpt distances with MEGA 4 (Tamura et al. 2007). Results from neighbor-joining analysis and structure analysis were compared to determine if similar structuring was observed.

Population assignment tests were performed to determine the geographic origin of N. American EAB samples from Asian populations using the AFLPOP version 1.1 program (Duchesne and Bernatchez 2002). The program does not permit missing data; therefore only individuals with all loci scored for all primer pair combinations were entered for analysis.

Results

mtDNA variability

Partial COI gene sequencing of 326 EAB from 15 collection locations in Asia and 37 locations in N. America (Table 3.1) resulted in nine different haplotypes (Table 3.2; Appendix 1). Seven haplotypes differed from the common haplotype (N=316) by a single bp change, one haplotype by two bp, with the final haplotype differing by 21 bp changes (Shirioshi City, Japan). Twenty-nine nucleotide changes occurred at 25 nucleotide sites along the 481bp length. Third codon position changes accounted for the majority of differences (96%) with a single change at the first codon position. All changes were synonymous with respect to amino acid translation.

Parsimony analysis of the nine haplotyes based on 1 parsimony informative character, 24 variable uninformative characters, and 456 constant

Table 3.2. Mitochondrial DNA variability of emerald ash borer in Asia and North America. Number of individuals (N) for each location and haplotype

		Haplotypes									
Populations	N	H1	H2	H3	H4	H5	H6	H7	H8	H9	Haplotype diversity (*h*)
USA and Canada	230	230	-	-	-	-	-	-	-	-	0
Moscow, Russia	2	2	-	-	-	-	-	-	-	-	0
Dagong and Hangu, China	24	24	-	-	-	-	-	-	-	-	0
Tangshan, China	15	15	-	-	-	-	-	-	-	-	0
Chanchun City, China	4	4	-	-	-	-	-	-	-	-	0
Jiutai, China	20	20	-	-	-	-	-	-	-	-	0
Harbin, China	4	4	-	-	-	-	-	-	-	-	0
Shenyang, China	13	11	-	-	2	-	-	-	-	-	0.282±0.142
Benxi, China	2	-	1	1	-	-	-	-	-	-	1.000±0.500
Daejeon, South Korea	5	2	-	-	1	1	-	-	1	-	0.900±0.161
Inje and Samcheok, South Korea	6	4	-	-	-	-	1	1	-	-	0.700±0.218
Shirioshi City, Japan	1	-	-	-	-	-	-	-	-	1	0

characters yielded 6 most parsimonious trees which had < 50% bootstrap values. The strict consensus of these six trees was unresolved (Figure 3.1).

Statistical parsimony network analysis yielded two groups (Figure 3.2). All samples in N. America and the majority in China had the same haplotype, H1 (Table 3.2, Figure 3.2). Three other haplotypes were observed in China; two haplotypes in Benxi, H2 and H3, and the third haplotype in Shenyang, H4. The common haplotype, H1, was observed in South Korea with five other haplotypes, H4-H8. A single sample from Shirioshi City, Japan differed from the common haplotype by 3.7% and separated on its own into the second group (Figure 3.2).

Genetic structuring of populations was assessed by AMOVA. Collection locations in Asia were pooled into a single population if they were within 50 km of each other (Tangshan and Zhenzhu zhenstulangai; Changchun City and Jiutai; Shenyang and Yushutan; Samcheok and Inge). Significant population structuring was detected between the six populations in China with 32% of the variation explained between populations (F_{st} = 0.3207, P < 0.001). When the analysis was expanded to the nine total populations in Asia (China, S. Korea, and Japan), 77% of the variation was explained between populations (F_{st} = 0.7716, P < 0.001) (Table 3.3). Structuring the populations into 3 groups according to country revealed significant structuring with 90% of the variation explained between countries (F_{ct} = 0.9045, P < 0.005). When the introduced populations from N. America and Moscow, Russia were added to the into AMOVA analysis for a total of five groups by country detected 74% of the variation due to country grouping (F_{ct} = 0.7424, P < 0.001) and 13% of the

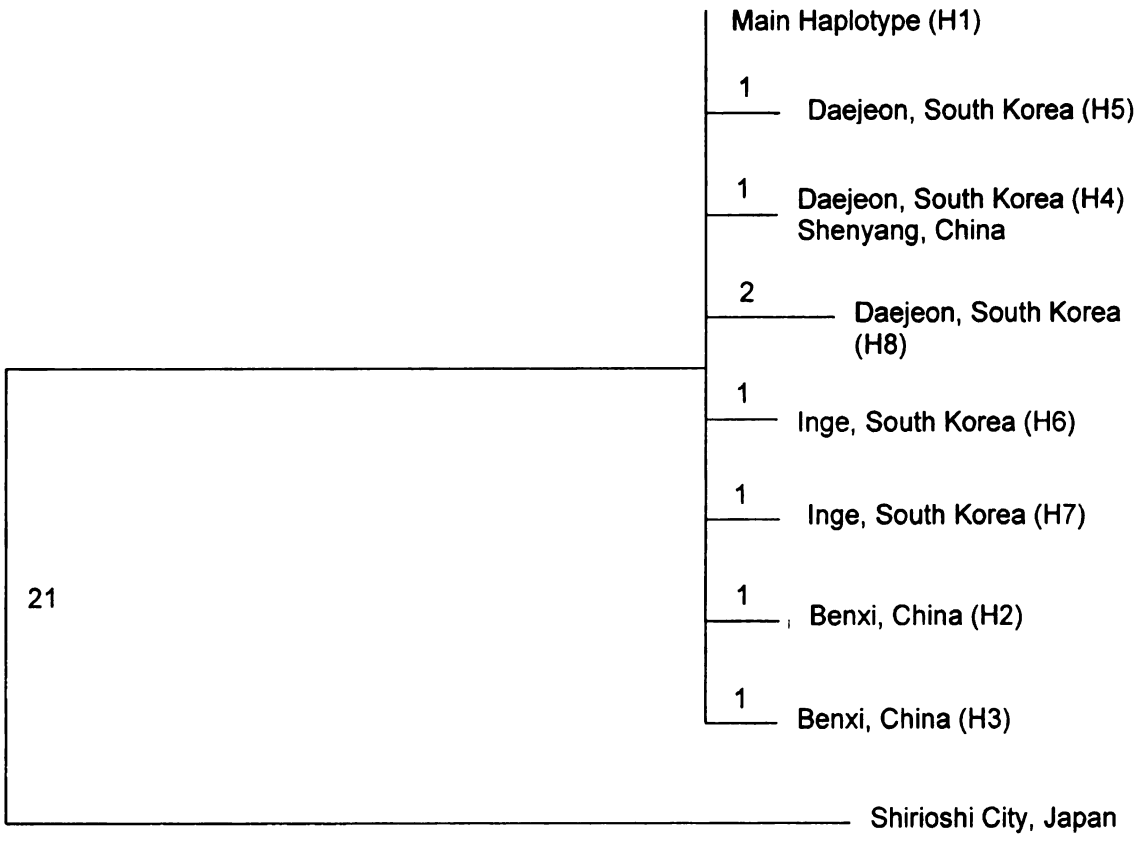


Figure 3.1. Strict consensus of six parsimonious trees for 9 emerald ash borer haplotypes. The main haplotype is a single individual representing 316 samples that share this haplotype collected in China, South Korea, North America, and Moscow, Russia.

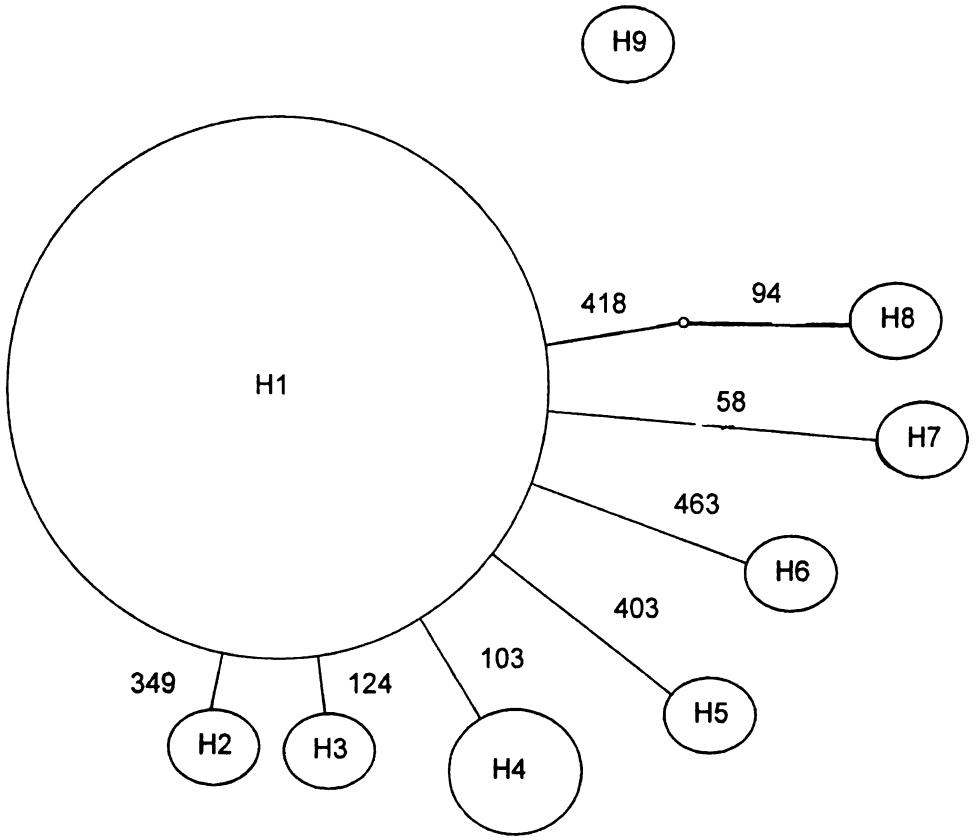


Figure 3.2. Haplotype network for 326 Emerald Ash Borer individuals collected in Asia and North America based on partial sequences (481 bp) of the mitochondrial cytochrome oxidase I gene. Numbers on the lines between haplotypes represent single nucleotide mutations

Table 3.3. AFLP and mtDNA COI results from AMOVA from Asian and North American collection locations of EAB.

Marker	Range	Source of variation	df	Sum of squares	Variance components	Percentage of variation
mtDNA	Native	Among locations	8	23.824	0.301	77.16
		Within locations	85	7.584	0.089	22.84
		Total	93	31.408	0.391	
		Fixation index (F_{ST})	0.772 (P<0.001)			
	Introduced	Among locations	10	0.000	0.000	0
		Within locations	173	0.000	0.000	
		Total	183			
		Fixation index (F_{ST})	0.000 (P=0.000)			
AFLP	Native	Among locations	9	697.202	2.484	5
		Within locations	99	5153.798	52.059	95
		Total	108	5851.000	54.543	
		Fixation index (Φpt)	0.046 (P=0.002)			
	Introduced	Among locations	10	882.787	3.209	8
		Within locations	173	6135.784	35.467	92
		Total	183	7018.571	38.676	
		Fixation index (Φpt)	0.083 (P=0.001)			

variation between populations within groups. The subdivision was due to two populations in China (Benxi and Shenyang), and both populations in S. Korea and Japan. Pairwise F_{st} analysis did not detect significant differentiation between the majority of populations (Table 3.4).

AFLP variability

AFLP analysis was conducted with four primer pair combinations yielding 273 polymorphic loci scored for 109 samples from 10 locations in Asia and 184 samples in 11 locations in N. America (Table 3.1). Two loci were unique to the Japan samples and one locus was unique to the South Korea samples. Of the eight collection locations in China, polymorphism level (% polymorphic loci) was detected highest in Shenyang (64.8%) and lowest in Benxi (35.9%) while estimated heterozygosity ranged from 0.136 in Benxi to 0.265 in Harbin (Table 3.5). In South Korea, polymorphism level and estimated heterozygosity was 47.6% and 0.169 respectively. Two individuals sampled in Japan recorded 100% and 0.376 respectively. In the introduced range in N. America, polymorphism was lowest in West Virginia (33.7%) to 53.5% in Petoskey, Michigan. Expected heterozygosity ranged from 0.095 to 0.194 in West Virginia and Canada, respectively (Table 3.5). Difference in estimated heterozygosity was marginally significant between Asian and N. American locations (Mann-Whitney U test P= 0.043), however, when the value from Japan was removed they are not significantly different (Mann-Whitney U test P= 0.080). Although the mean level of polymorphism was slightly higher in Asia (55.92% with Japan; 51.02% without Japan) than North America (46.34%), it was not significantly different (Mann-

Table 3.4. Pairwise F_{st} between populations of mtDNA COI haplotypes from emerald ash borer based on Kimura 2-parameter distance. Populations 1-6 are from China, 7 and 8 from South Korea, 9 from Japan, and 10 from Russia. Number of individualsare listed in parentheses

Populations	1	2	3	4	5	6	7	8	9	10	11
1. Dagong and Hangu (24)	-										
2. Hebei (15)	0.000	-									
3. Benxi (2)	0.850*	0.774*	-								
4. Shenyang (13)	0.156	0.099	0.493	-							
5. Jilin (24)	0.000	0.000	0.850*	0.156	-						
6. Heilongjiang (4)	0.000	0.000	0.381*	-0.621	0.000	-					
7. Daejeon (5)	0.353*	0.237*	0.041	0.056	0.353*	-0.055	-				
8. Inje and Samcheok (6)	0.263	0.164	0.225	0.073	0.263*	-0.083	0.016*	-			
9. Shirioshi City (1)	1.000	1.000	0.910	0.987*	1.000	1.000	0.928	0.970	-		
10. Moscow (2)	0.000	0.000	0.000	-0.246	0.000	0.000	-0.293	-0.307	-1.000	-	
11. North America (230)	0.000	0.000	0.983*	0.622*	0.000	0.000	0.850*	0.790*	1.000*	0.000	-

* Significant F_{st} value at the 5% level

Table 3.5. Descriptive statistics for Asian and North American EAB collection locations with (N) number of individuals using 273 total AFLP loci.

Population	(N)	% polymorphic loci (p)	Expected heterozygosity (H) ± SE
Asia			
China			
Dagong	25	64.1	0.19±0.01
Tangshan	22	61.9	0.18±0.01
Beijing	2	36.6	0.19±0.01
Benxi	6	35.9	0.14±0.01
Shenyang	13	64.8	0.17±0.01
Jilin	6	45.4	0.17±0.01
Jiutai	20	40.3	0.15±0.01
Harbin	5	62.6	0.26±0.01
South Korea	8	47.6	0.17±0.01
Japan	2	100	0.38±0.01
North America			
Canada	26	61.2	0.19±0.01
Michigan			
Plymouth	13	40.3	0.14±0.01
Livonia	21	48.7	0.15±0.01
Lansing	29	45.1	0.15±0.01
Petoskey	10	53.5	0.15±0.01
Moran	21	49.1	0.15±0.01
Ohio	12	52.0	0.19±0.01
Indiana	16	39.6	0.14±0.01
Illinois	14	41.4	0.16±0.01
Pennsylvania	17	45.1	0.16±0.01
West Virginia	5	33.7	0.09±0.01

Whitney U test P= 0.251 with Japan; Mann-Whitney U test P= 0.412 without Japan). Overall Φpt was 0.094 (P=0.001), signifying slight differentiation between collection locations.

AMOVA detected genetic differentiation between the populations from the native and introduced ranges with 5% of the total variation attributed to variation between country (P = 0.001) and 7% attributed to variation within country (P = 0.001). When the analysis was conducted without regional information to determine the level of differentiation between the 10 Asian populations and 11 N. American populations, 9% of the differentiation was attributed to among population variation (P = 0.001). The amount of variation was higher among N. America populations than among Asian populations when considered separately (Φst = 0.083, P = 0.001 and Φst = 0.046, P = 0.002, respectively) (Table 3.3). Pairwise Φpt among Asian populations did not detect any differentiation between several populations (Φpt = 0.000) with the highest differentiation between Japan and Jilin, China (Φpt = 0.461) (Table 3.6). Pairwise Φpt among N. American populations did not detect any differentiation between Cranberry, Pennsylvania and Peru, Illinois (Φpt = 0.000) with the highest differentiation between Fayette Co., West Virginia and Petoskey, Michigan (Φpt = 0.236) (Table 3.7). Pairwise Φpt between Asian and N. American populations ranged from no differentiation detected between several population comparisons (Φpt = 0.000) with the highest differentiation between Fayette Co., West Virginia and Beijing, China (Φpt = 0.624) (Table 3.8).

Table 3.6. Pairwise Φ_{pt} between Asian populations with AFLP data set of 273 Loci from emerald ash borer. Populations 1-8 are from China, 9 from South Korea, 10 from Japan. Number of individuals are listed in parentheses

Populations	1	2	3	4	5	6	7	8	9	10
1. Beijing (2)	-									
2. Benxi (6)	0.000	-								
3. Dagong and Hangu (25)	0.000	0.000	-							
4. Heilongjiang (5)	0.138	0.159	0.090*	-						
5. Chanchun City, Jilin (6)	0.216	0.042	0.057	0.206*	-					
6. Juitai (20)	0.052	0.010	0.024	0.179*	0.145*	-				
7. Shenyang (13)	0.000	0.000	0.000	0.096	0.075	0.006	-			
8. Tangshan (22)	0.000	0.000	0.021*	0.096	0.029	0.089*	0.000	-		
9. South Korea (8)	0.020	0.012	0.005	0.148*	0.121*	0.009	0.000	0.058	-	
10. Shirioshi City (2)	0.204	0.256	0.105	0.248*	0.461*	0.136	0.078	0.270*	0.146	-

* Significant Φ_{pt} value at the 5% level

Table 3.7. Pairwise Φ_{pt} between North American populations of emerald ash borer with AFLP data set of 273 loci. Number of individuals are listed in parentheses

Populations	1	2	3	4	5	6	7	8	9	10	11
1. Canada (26)	-										
2. Illinois (14)	0.026	-									
3. Indiana (16)	0.118*	0.148*	-								
4. Lansing, MI (29)	0.019	0.056*	0.055*	-							
5. Livonia, MI (21)	0.093*	0.132*	0.086*	0.064*	-						
6. Moran, MI (21)	0.031*	0.040*	0.073*	0.016	0.033*	-					
7. Ohio (12)	0.052*	0.057*	0.097*	0.043	0.066*	0.030	-				
8. Pennsylvania (17)	0.029	0.000	0.105*	0.047*	0.110*	0.022	0.031	-			
9. Petoskey, MI (10)	0.042	0.053*	0.233*	0.102*	0.170*	0.103*	0.095*	0.047*	-		
10. Plymouth, MI (13)	0.099*	0.209*	0.257*	0.105*	0.222*	0.150*	0.204*	0.207*	0.265*	-	
11. West Virginia (5)	0.124*	0.205*	0.086*	0.060	0.099	0.127*	0.160*	0.184*	0.281*	0.236*	-

* Significant Φ_{pt} value at the 5% level

Table 3.8. Pairwise Φ_{pt} between Asian and North American populations with AFLP data set of 273 Loci from emerald ash borer. Populations 1-8 are from China, 9 from South Korea, 10 from Japan. (Population 1-Beijing, 2-Benxi, 3-Dagong and Hangu, 4-Heilongjiang, 5- Chanchun City, Jilin, 6-Jiutai, 7-Shenyang, 8-Tangshan). Number of individuals are listed in parentheses

Populations	1 (2)	2 (6)	3 (25)	4 (5)	5 (6)	6 (20)	7 (13)	8 (22)	9 (8)	10 (2)
Canada (26)	0.000	0.003	0.016	0.136*	0.076	0.060*	0.014	0.039*	0.016	0.155
Illinois (14)	0.000	0.000	0.030	0.169*	0.058*	0.105*	0.040	0.007	0.085*	0.308*
Indiana (16)	0.348*	0.206*	0.155*	0.346*	0.279*	0.248*	0.209*	0.141*	0.282*	0.580*
Lansing, MI (29)	0.047	0.033	0.062*	0.178*	0.113*	0.108*	0.073*	0.063*	0.102*	0.285*
Livonia, MI (21)	0.181	0.147*	0.126*	0.236*	0.183*	0.207*	0.160*	0.127*	0.206*	0.406*
Moran, MI (21)	0.067	0.060*	0.072*	0.194*	0.096*	0.157*	0.089*	0.053*	0.127*	0.356*
Ohio (12)	0.075	0.090*	0.074*	0.135*	0.118*	0.173*	0.102*	0.060*	0.158*	0.372*
Pennsylvania (17)	0.003	0.000	0.039*	0.173*	0.056*	0.126*	0.066*	0.014	0.102*	0.354*
Petoskey, MI (10)	0.130	0.070	0.035	0.212*	0.165*	0.111*	0.059	0.059*	0.084*	0.347*
Plymouth, MI (13)	0.264*	0.253*	0.190*	0.323*	0.311*	0.229*	0.151*	0.189*	0.179*	0.433*
West Virginia (5)	0.624*	0.298*	0.166*	0.373*	0.466*	0.224*	0.186*	0.181*	0.250*	0.586*

* Significant Φ_{pt} value at the 5% level

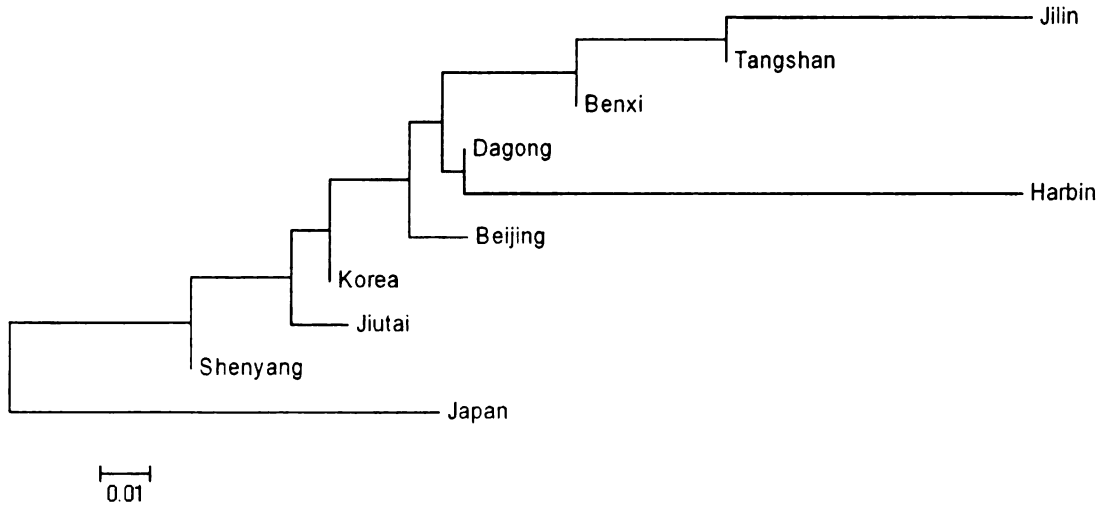
Neighbor-joining analysis based on pairwise Φpt of Asian populations did not reveal clear differentiation between populations based on geographic separation (Figure 3.3a). Analysis of N. American populations also did not divulge clear differentiation between populations based on geographic separation expected if introduced populations were isolated by distance (Figure 3.3b). Analysis of all populations revealed genetic similarity associated with geographic proximity (Figure 3.4).

Structure analysis of Asian samples determined the most probable hypothesis for the 10 collection locations is the genetic data developed from six populations (Table 3.9, Figure 3.5). Structure was not realized because there were no unique AFLP patterns to specific populations. Samples from Dagong, China exhibit the most variation among the collection locations (Figure 3.5). Clear separation of geographic regions was not possible given the level of admixture from each of the collection locations (Figure 3.5).

A Mantel test conducted on Asian populations revealed a marginally significant correlation between genetic and geographic distances ($r = 0.772$, $P = 0.026$). However, this relationship was not significant when the Japan population was removed from the analysis ($r = 0.234$, $P = 0.190$). N. American populations also showed a marginally significant correlation between genetic and geographic distances ($r = 0.471$, $P = 0.029$).

Population assignment tests were performed to assess the most likely hypothesis for the geographic origin of N. American individuals using the 10 Asian collection locations as potential sources. Individuals from N. American

A.



B.

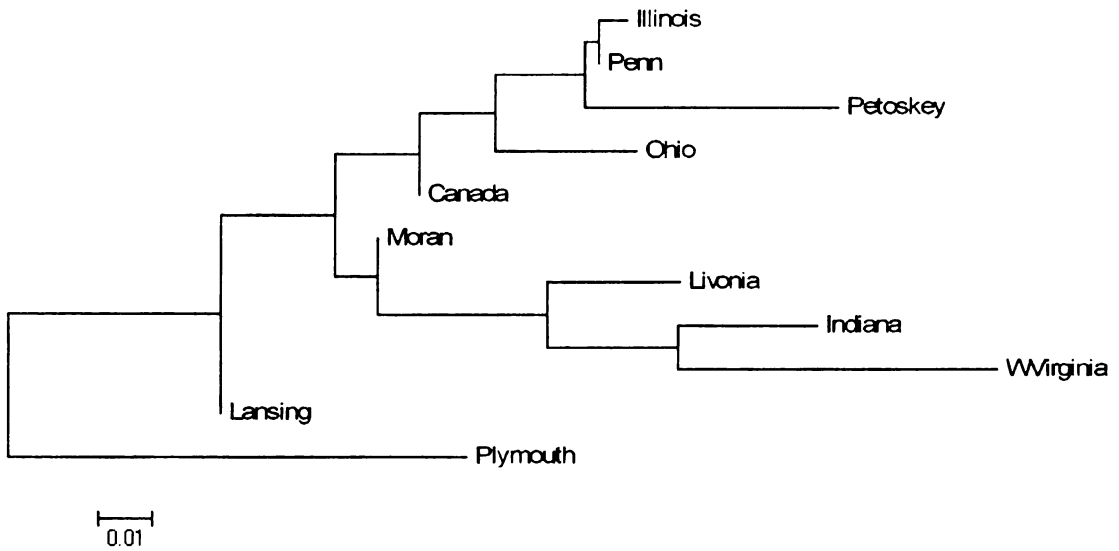


Figure 3.3. Neighbor-joining analysis of EAB AFLP data set based on pairwise Φpt values for A) native range in Asia and B) introduced range in North America.

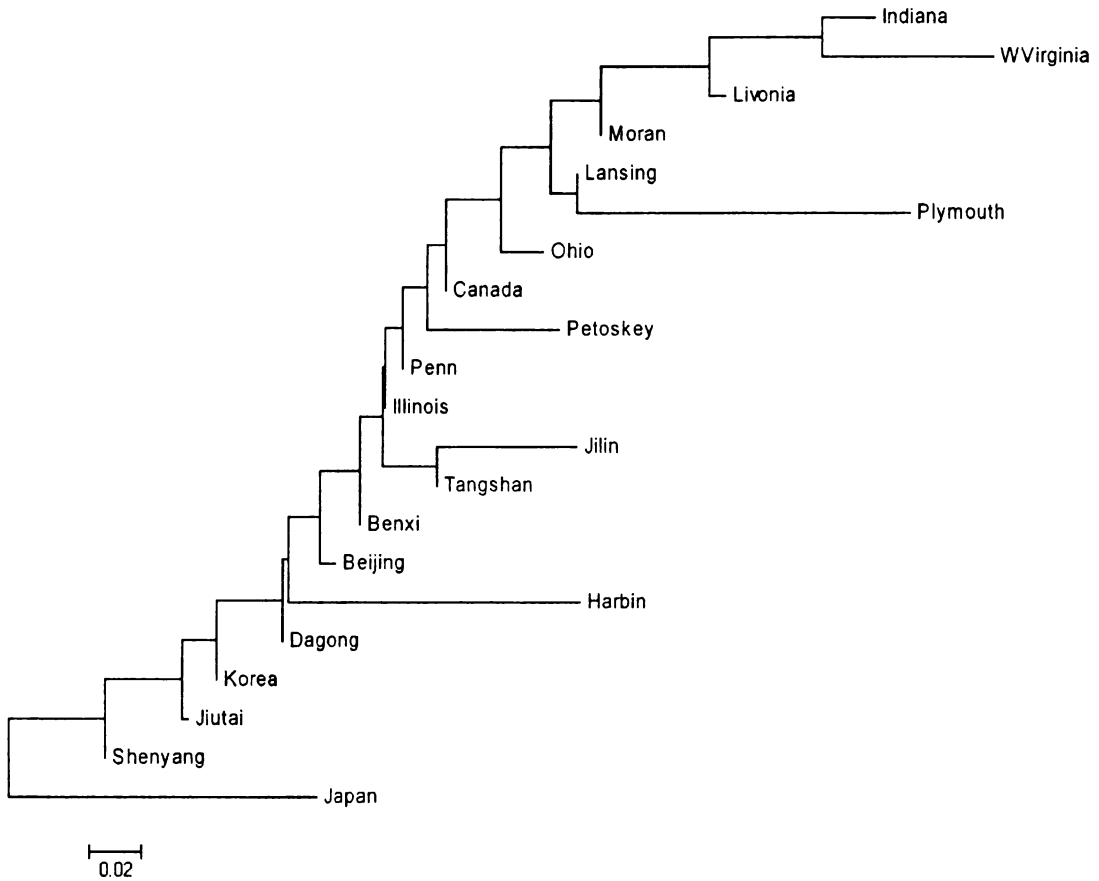
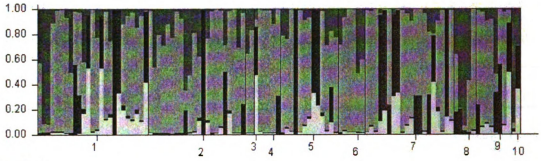


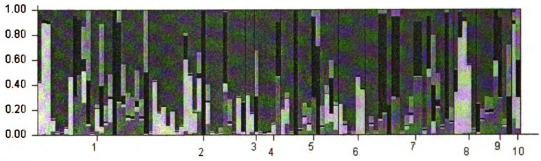
Figure 3.4. Neighbor-joining analysis of EAB AFLP data set based on pairwise Φpt values for the native range in Asia and introduced range in North America.

Figure 3.5. Structure analysis results representing the most probable number of populations of EAB, K= 4 through K=7 in Asia. Shades are assigned automatically by the program with a separate color for each inferred population with the proportion of the samples variation on the *y*-axis. Collection locations, denoted on the *x*-axis, 1-8 were obtained in China (Population 1-Dagong, 2-Tangshan, 3-Beijing, 4-Benxi, 5-Shenyang, 6-Jilin, 7-Juitai, 8-Harbin), location 9 in South Korea and location 10 in Japan.

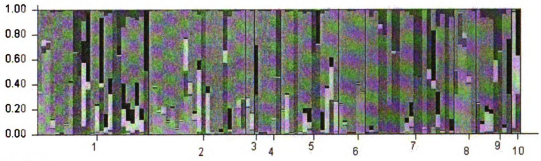
A. K=4



B. K=5



C. K=6



D. K=7

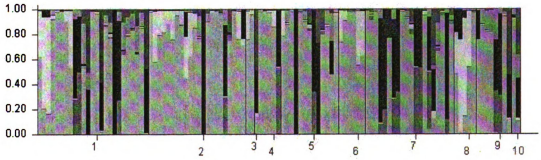


Table 3.9. Structure analysis results. AFLP samples collected from Asia were used to test the hypotheses samples originated from varying number of populations (K) by Bayesian analysis. Data presented in log likelihood with the lowest score representing the highest posterior probability number of populations.

Population number (K)	Log likelihood
2	-48741.5
3	-47080.0
4	-46457.3
5	-55289.2
6	-45888.1
7	-45894.6
8	-46872.6

populations were assigned to Tangshan, China with the most frequency ranging from 12.5% in Petoskey, Mi to 80% assignment in W. Virginia with a mean percentage of assignment of 45.51% (Table 3.10). Individuals were also assigned to Dagong, China with a frequency as high as 53.3% from Windsor, Canada with a mean assignment of 22.26% (Table 3.10).

Discussion

There was very little COI variation detected in most locations in China as a single haplotype was found in the majority of locations and only three haplotypes detected in two locations that differed from the main haplotype observed (Table 3.2). The majority of unique haplotypes were found in locations in South Korea with these locations showing higher haplotype diversity compared to China with a final haplotype detected in Japan. Since there were few samples from locations where unique haplotypes were observed, ranging from one individual in Japan to 13 in Shenyang, China, it is possible the haplotype diversity in these locations are an underestimate of the true diversity of the location.

A reduction in mitochondrial variation is common in introduced insect population studies (Kambhampati and Rai 1991; Villeblanca et al. 1998; Tsutsui et al. 2000; Downie 2002; Scheffer and Grissell 2003; Corin et al. 2007). For example, the Colorado potato beetle (*Leptinotarsa decemlineata* Say), had 20 haplotypes detected throughout the native range in N. America compared to a single haplotype found in the introduced range in Europe (Grapùtto et al. 2005). Another example of reduced mitochondrial variation due to a founder event was found in a moth pest of cacao, *Conopomorpha cramerella* Snellen, in the Malay

Table 3.10. Assignment test of individuals of the introduced range of emerald ash borer in North America against collection locations from the native range in Asia

Collection location:	Individual assignment:									
	Dagong	Tangshan	Beijing	Benxi	Shenyang	Jilin	Jiutai	Heilongjiang	South Korea	Japan
Plymouth, MI	0	4	0	1	0	0	2	0	0	0
Livonia, MI	5	10	0	0	0	2	1	0	0	0
Lansing, MI	5	11	0	2	0	0	1	0	0	0
Petoskey, MI	3	1	0	0	0	0	4	0	0	0
Moran, MI	3	6	0	2	1	2	2	0	0	0
Ohio	3	2	0	1	0	2	2	0	0	0
Indiana	5	7	0	3	0	1	0	0	0	0
Illinois	0	7	0	2	1	1	0	0	0	0
Pennsylvania	0	6	1	4	0	1	1	0	0	0
West Virginia	1	4	0	0	0	0	0	0	0	0
Windsor, Canada	8	4	0	1	0	0	2	0	0	0

Archipelago. Shapiro et al. (2008) found only six COI haplotypes across widespread islands concluding the reduction of variation compared to similar studies (Juan et al. 1998; Mun et al. 2003) was very low and the result of a bottleneck event. This pattern of reduction of COI variation is consistent with the data found in this study for introduced EAB. All samples throughout the introduced range of USA and Canada shared the single main haplotype found in Asia. Since the main COI haplotype is found throughout EAB's native range, this could be explained by either a unique introduction event with an unknown number of individuals into N. America or multiple introductions of the sample haplotype (Sakai et al. 2001; Graputto et al. 2005).

Given the only COI haplotype found in N. America is found throughout most of the native range in Asia, added genetic information was needed to characterize the population structure of EAB. AFLP analysis has been a powerful tool to estimate population genetic relationships of insects (Meng et al. 1996; Reineke et al. 1999; Ravel et al. 2001; Elderkin et al. 2004; Conord et al. 2006; Dalirsefat and Mirhoseini 2007; Clark et al. 2007). Reduced genetic variation was weakly confirmed by AFLP analysis, however the reduction was only marginally significant by expected heterozygosity and not statistically significant by % polymorphic loci, demonstrating the introduced populations have retained genetic variation with nuclear loci (Sakai et al. 2001). Genetic variation retained by nuclear loci but reduced in mtDNA could have been caused by the unique introduction of a large founding population or multiple introductions (Allendorf and Lundquist 2003; Grapputo et al. 2005; Ahern et al. 2009). Similar

results have been found in other invasive pests with reduced genetic variation in the introduced compared to native ranges. For example, the Argentine ant had significant reduction of genetic variation was detected in introduced populations in N. America, Australia, Chile, Italy, and South Africa compared to the native range in Brazil and Argentina (Tsutsui and Case 2001; Tsutsui et al. 2001). The Eurasian spiny waterflea also demonstrated evidence of reduced genetic variation in the introduced range in N. America compared to the historical range in Europe (Colautti et al. 2005). However, reduction of genetic variation is not always observed in all invasive pests. For example, zebra and quagga mussel populations introduced into N. America showed similar genetic variability to populations in the native range of Eurasia and did not show evidence of a bottleneck event during introduction (Stepien et al. 2002).

Weak population structure was detected using AFLP in both native and introduced populations of EAB. This structure was not consistent with an isolation-by-distance hypothesis characteristic to natural spread after initial introduction and more indicative of human-mediated transport (Sakai et al. 2001). In addition, greater genetic differentiation was detected in the introduced populations ($\Phi st = 0.083$ vs. $\Phi st = 0.046$) suggesting multiple introductions into N. America (Sakai et al. 2001). Average pairwise Φpt was lower in the native range (0.089) compared to the introduced range (0.105) meaning the Asian populations are more similar to each other than the N. American populations are to each other (Mann-Whitney U test P= 0.023). This could have several interpretations including 1) the introduced population(s) was the result of multiple

introductions (Sakai et al. 2001; Allendorf and Lundquist 2003; Kolbe et al. 2004), 2) the sampling in the native range does not represent the full variation and differentiation between locations (Kim and Sappington 2005), 3) there is more gene flow between populations in the native range than in the introduced range, 4) population comparisons in the introduced range are caused by incomplete sorting of nuclear variation (Hartl and Clark 1997; Ahern et al. 2009), or 5) introduced populations have developed local adaptations causing populations to diverge (highly unlikely given EAB was introduced into N. America within the last 20 years). Incorporation of additional samples throughout the native and introduced ranges and/or additional genetic markers are needed to differentiate between these hypotheses.

Determining the geographic origin of an invasive insect can be challenging (Roderick and Navajas 2003, Cognato et al. 2005, Shapiro et al. 2008). Both mtDNA and AFLP analysis suggest the most likely origin of EAB is from a region of China, more specifically near Dagong and Tangshan. Even with small sample sizes, the genetic diversity detected in a couple populations in China and S. Korea was not observed in the introduced range while the main haplotype was fixed in higher sample sizes in Dagong and Tangshan. Although pairwise Φpt between locations in Asia and N. America indicated significant differentiation between several locations in the introduced range to Dagong and Tangshan (Table 3.8), the average pairwise Φpt across all populations in N. America revealed the lowest population differentiation between these two populations (Φpt = 0.0877 and 0.0848, respectively) with all other average pairwise Φpt

values ranging from 0.1044 against Shenyang, China to 0.3818 against Japan.

Assignment tests of individual beetles from N. America were assigned over 67% of individuals were assigned to either of these two collection locations, while there were no individuals assigned to the location in Shiroishi City, Japan or any location from South Korea. More extensive sampling to include more individuals from known source populations, incorporation of samples from locations in the native range not included in this study (for example Far eastern Russia), as well as including more samples across the current introduced range of EAB may help strengthen the assignment strength of individuals to the most likely source population.

In conclusion, this is the first study to assess genetic variation of EAB populations in its native and introduced range. Molecular analysis of an invasive species is essential to understand the geographic origin, population structure, and develop management strategies. Given eradication is most successful before a species has become established and widespread (Ruesink et al. 1995; Allendorf and Lundquist 2003; Perrings et al. 2005; Simberloff 2005), it is unlikely EAB can be eradicated due to its widespread distribution in N. America. Therefore, increased understanding of population structure, estimating direction and mode of dispersal, and determining the origin can be important (Grapputo et al. 2005). The use of mtDNA and AFLP markers was useful in assessing the population structure in Asia and N. America, as well as estimating the geographic origin of the introduced population. This study demonstrated weak population structure in both Asia and N. America; however this structure was not

characteristic of population separated by geographic distance. In addition, the majority of the AFLP genetic variability is within individual populations and not among populations, possibly suggestive of minor gene flow (Clark et al. 2007), or human-mediated transport. Stronger restrictions on long-distance transport of ash material should be incorporated into any management strategy. The reduction in mtDNA and AFLP variability and estimated geographic origin of Dagong and Tangshan, China provides evidence that the introduced population should be susceptible to the biological control agents from that region (Sakai et al. 2001; Allendorf and Lundquist 2003).

CHAPTER IV

MICROSATELLITE ANALYSIS OF EMERALD ASH BORER

Introduction

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire, is a serious exotic pest of North American ash trees (*Fraxinus* spp.) that was first discovered outside of its native range of Northeastern Asia in 2002 (Haack et al. 2002). Since its initial discovery in Michigan and Ontario, it has spread to Illinois, Indiana, Kentucky, Maryland, Minnesota, Missouri, New York, Ohio, Pennsylvania, Virginia, West Virginia, Wisconsin, and Quebec (Cooperative Emerald Ash Borer Project 2009). Greater than seven billion ash trees are in the United States with > 1.13 billion located in Michigan, Ohio and Indiana (Liu et al. 2003). The economic value of these trees is considerable, with an estimated value of $18.9 billion in Michigan alone (USDA-AHPIS 2003). Since the most likely introduction into N. America was in the early to mid 1990's based on dendrochronology (Siegert et al. 2007) the spread of EAB has almost certainly been expedited by unintentional human movement of infested ash material (USDA APHIS 2006). Information on EAB was limited before the introduction into N. America (Liu et al. 2003; Wei et al. 2004) because it was not considered a pest in the native range of Northeastern China, Far eastern Russia, Japan, Korea, Mongolia, and Taiwan (Chinese Academy of Science 1986; Yu 1992). EAB host range in Asia includes several *Fraxinus* spp. (Chinese Academy of Science 1986; Yu 1992) as well as *Juglans mandshurica* var. *sieboldiana*, *J. mandshurica* var. *sachalinensis, Pterocarya rhoifolia* and *Ulmus davidiana* var.

japonica in Japan (Akiyama and Ohmomo 2000; Sugiura 1999). EAB is not considered a pest species in its home range, however; it has caused serious damage to imported N. American *Fraxinus* spp. plantations in China causing many to be removed (Liu et al. 2003; Wei et al. 2004).

Molecular markers, in general, are useful in understanding the introduction and expansion of invasive species (Roderick 1996; Sakai et al. 2001). More specifically, microsatellite markers have been useful in studying invasive species, notably in determining their geographic origins (Tsutsui et al. 2001; Fonseca et al. 2004; Scribner et al. 2003; Kim et al. 2006; Caldera et al. 2008); identifying bottlenecks and gene flow (Tarr et al. 1998; Colautti et al. 2005); estimating the size and number of introductions (Bonizzoni et al. 2001; Walker et al. 2003; Miller et al. 2005); and evaluating genetic population structure and dispersal (Vargo 2003a,b; Kim and Sappington 2005; Herborg et al 2007). Despite EAB's ecological and economic impact in N. America, relatively little is known about its invasion history and geographic origin. In this chapter, I describe research to isolate, identify, and characterize microsatellite markers to better understand the demographics of EAB in its native Asian range and its introduced range in N. America.

Materials and methods

Sample collection and DNA isolation

EAB were collected from native populations in China, South Korea and Japan and from introduced populations in N. America (Table 4.1) with the assistance of a network of collaborators from several government agencies and

Table 4.1. Locality Information for EAB Individuals Included in the Microsatellite Analysis

Country	State/Province	County	Locality	Pop # (*)	# ind (N)
China	Hebei		Tangshan	1	19
China	Jilin		Chanchun	2	6
China	Jilin		Jiutai	2	15
China	Liaoning		Benxi	3	5
China	Liaoning		Shenyang	3	8
China	Tianjin		Dagong	4	8
China	Beijing		Beijing	4	2
Russia			Moscow		2
South Korea	Chungchong-nam do		Daejeon		1
South Korea	Gyeonggi-do		Suwon		1
USA	Illinois		Peru	5	13
USA	Indiana	LaGrange	Shipshewana		6
USA	Maryland	Prince George's			3
USA	Michigan	Chippewa	Brimley		4
USA	Michigan	Emmet	Petoskey		3
USA	Michigan	Ingham	East Lansing	6	2
USA	Michigan	Ingham	Lansing	6	12
USA	Michigan	Livingston	Brighton	7	1
USA	Michigan	Livingston	Howell	7	1
USA	Michigan	Livingston	Pinckney	7	3
USA	Michigan	Oakland	Bloomfield	7	1
USA	Michigan	Washtenaw	Ann Arbor	7	6
USA	Michigan	Washtenaw	Dexter	7	2
USA	Michigan	Wayne	Belleville	7	1
USA	Michigan	Wayne	Westland	7	1
USA	Michigan	Mackinac	Moran	8	10
USA	Ohio	Fulton and Wood			5
USA	Pennsylvania	Butler	Cranberry	9	15
Canada	Ontario	Essex	Windsor	10	6
Total					162

Notes-

* Population number in Fstat Analysis; some localities pooled

academic institutions. Samples were collected in the field and then stored in 90-100% ethanol and sent to the lab for analysis. Total genomic DNA was extracted from larval tissue (with the GI tract removed to avoid potential contamination from gut contents) or from adult femur muscle tissue using a DNA mini-kit (Qiagen cat. No 51304) as described by the manufacturer with minor modifications. Populations with few individuals and primers which did not amplify DNA in all samples were excluded from statistical analysis in this report due to low statistical power. However, these data are available for future studies.

Microsatellite marker development

Ninety-six plasmid clones were developed in collaboration with Dr. Travis Glenn at the University of Georgia's Savannah River Ecology Laboratory (Glenn and Schable 2005). In brief, extracted DNA from four larvae (Lansing, MI) was used for enrichment of di-, tri-, and tetra nucleotide microsatellite motifs and development of clones of DNA region. Sequences of clones were forwarded to me for primer design and optimization. Primers were designed using the program PRIMER (vers. 3.0; Rozen & Skaletsky 2000), with ideal primers being 18-26 base pairs long with 45-50% GC content. Test amplifications at each locus were performed on a PE9700 Thermal Cycler (Applied Biosystems) using unlabeled primers to amplify alleles from eight individuals from two collection locations in Michigan (Lansing and Belleville). In each case, varying $MgCl_2$ concentrations and annealing temperatures were tested to determine optimal PCR conditions. PCR products were visualized on 1.5% agarose gels. Primers designed to amplify alleles at 41 microsatellite loci yielded successful PCR

amplification in the test samples. Twenty-five of these were selected as candidates to detect variation in EAB populations; the remaining 16 loci were excluded because the primer pair either yielded more than two PCR products or the amplified product was not in the expected size range (the primer pair amplified a different region of the genome). The forward primer for each of the 25 candidate loci was 5' fluorescence-labeled and PCR was performed at optimum conditions developed for each specific primer pair using DNA templates from a test group of 16 individuals collected throughout the native range in China to assess polymorphism among these loci. PCR products from these reactions were analyzed using an ABI 3130 capillary DNA analyzer at the Research Technology Support Facility (RTSF) at Michigan State University,

At the time this dissertation research, another researcher Dr. Jenny Cory (Simon Fraser University, British Columbia, Canada) produced her own set of candidate microsatellite loci for EAB. A collaboration was established with Dr. Cory to develop and test each others candidate loci to assess for variation and increase the likelihood of success in locating polymorphic loci for EAB. This study screened four of these loci for variation within the test population of Asian samples and found that primer pairs at two loci (C-C5 and C-C8) were polymorphic in test samples and used for further characterization of EAB genetic variation in 162 individuals from Asia and N. America.

Data analysis

Microsatellite allele data were analyzed first using the program MSA (Microsatellite Analyzer, vers. 4.05; Dieringer and Schlotterer 2003), which

allowed determination of basic population statistics, including observed number of alleles and average observed and expected heterozygosity. FSTAT (Goudet 2001) was used to estimate EAB population differentiation between four regions in China and six regions in N. America (Table 4.1). Population differentiation was measured using the parameter, F_{ST}, which measures the reduction of heterozygotes from the entire dataset due to differentiation among subpopulations; population differentiation increases as F_{ST} increases from zero.

Results and Discussion

Forty-one microsatellite loci were successfully amplified in EAB, however, none were polymorphic with respect to a test group of 16 Asian individuals. None of the primer pairs at the 25 candidate loci yielded polymorphism in our test sample that would allow the assessment of variation within and between populations in Asia. Loci yielded a limited number of alleles (1-3) or did not amplify in all Asian test samples. Therefore, variation in EAB was based on data from the two loci, C-C5 and C-C8 (developed at Dr. Cory's laboratory), and a study sample of 162 individuals drawn from 24 localities in Asia and N. America (Table 4.1). The observed and effective number of alleles was eight and seven alleles and 6.1678 and 4.6231 over all populations, respectively (C-C5 and C-C8; Table 4.2). The expected and observed heterozygosity, number of alleles and allelic richness per locus per population are given in Table 4.3.

F_{ST} values informed structure among N. American and Chinese populations. Most interesting, the population from Jilin Province (Chanchun and Jiutai) appears most dissimilar to each of the six N. American populations (Table

Table 4.2. Characteristics of 2 microsatellite loci isolated from emerald ash borer of 162 individuals from Asian and North American populations. Loci were developed by Dr. Jenny Cory (Simon Fraser University, British Columbia, Canada)

Locus	Primer sequence (5'-3')	Repeat	Allele size range (bp)	Sample size	H_O/H_E	N_O	N_E
C-C5	F: TCGATGCAACAAAGACCTC	$(ACC)_9$	130-154	162	0.7705/0.7084	8	6.1678
	R: GTGCTTTTAGGTTTTGCTGTG						
C-C8	F: CGTCGATTAACTTTTAGTTCAG	$(ACC)_6(AAT)_2$	208-235	162	0.5382/0.5674	7	4.6231
	R: CCAGAAGATGCTGTAGTTGAAG						

H_o = observed heterozygosity; H_E = expected heterozygosity; N_o = number of alleles; N_E = number of effective alleles

Table 4.3. Genetic diversity of two microsatellite loci in emerald ash borer populations from Asia and North America (Loci developed by Dr. Jenny Cory, Simon Fraser University, BC, Canada)

		China						
		Tianjin	Hebei	Beijing	Liaoning		Jilin	
		Dagong	Tangshan	Beijing	Benxi	Shenyang	Jiutai	Chanchun
C-C5	H_O	0.5	0.578	1	0.6	0.857	0.6	0.5
	H_E	0.725	0.633	0.666	0.711	0.791	0.501	0.56
	N_O	5	4	2	3	5	5	4
	A	1.725	1.633	1.666	1.711	1.791	1.501	1.56
C-C8	H_O	0.285	0.47	1	0.4	0.333	0.2	0.333
	H_E	0.527	0.513	1	0.355	0.484	0.336	0.439
	N_O	2	2	2	2	2	2	3
	A	1.527	1.513	2	1.355	1.484	1.336	1.439

		North America						
		Ann Arbor, MI	Livingston Co., MI	Lansing, MI	Moran, MI	Illinois	Pennsylvania	Windsor, Canada
C-C5	H_O	0.833	0.75	0.5	0.444	0.615	0.533	1
	H_E	0.787	0.607	0.423	0.542	0.63	0.514	0.733
	N_A	4	3	4	4	4	3	3
	A	1.787	1.607	1.423	1.542	1.63	1.514	1.733
C-C8	H_O	0.166	0	0.583	0.625	0.636	0.769	0.166
	H_E	0.409	0.355	0.489	0.491	0.601	0.667	0.53
	N_A	2	2	2	3	3	4	2
	A	1.409	1.355	1.489	1.491	1.601	1.667	1.53

H_o = observed heterozygosity; H_E = expectted heterozygosity; N_o = number of alleles;
A = Allelic richness

Table 4.4. Genetic differentiation (pairwise F_{st}) of emerald ash borer populations in China and North America*

	Hebei	Jilin	Liaonging	Tianjin/ Beijing	Illinois	MI - Ingham	MI - SE	MI - Moran	Pennsylvania	Windsor, Canada
1. Hebei (N = 19)	0	0.0902	-0.007	-0.0458	0.1247	0.003	0.0118	0.1273	0.2121	0.0189
2. Jilin (N = 21)		0	0.071	0.1033	0.2337	0.1489	0.2232	0.3661	0.3182	0.1231
3. Liaonging (N = 12)			0	-0.0276	0.0672	0.0818	0.0322	0.1036	0.1369	-0.023
4. Tianjin/Beijing (N = 10)				0	0.1006	-0.0061	-0.0227	0.0853	0.1879	-0.0101
5. Illinois (N = 13)					0	0.2355	0.076	0.2038	-0.018	-0.0576
6. MI - Ingham (N = 14)					463	0	0.0569	0.2195	0.3302	0.1448
7. MI - SE** (N = 16)					500	103	0	0.0494	0.1497	0.0015
8. MI - Moran (N = 10)					806	399	468	0	0.2522	0.1544
9. Pennsylvania (N = 15)					828	537	434	901	0	0.0198
10. Windsor, Canada (N = 6)					569	146	71	496	433	0

N = sample size

* Distances between North American populations (in km) shown below the diagonal

** SE = Individuals were collected from southeastern Michigan including Livingston, Oakland, Washtenaw, and Wayne counties. This area is suspected to be near the initial introduction into Michigan (Siegert et al. 2007)

4.4). The Illinois population appears most similar to Liaonging Province. However it is difficult to identify a single population that is most similar to the other N. American populations. These microsatellite data do not provide enough information to distinguish between a single EAB introduction or multiple EAB introductions into N. America.

Neighbor-joining analysis based on pairwise F_{ST} of Asian and North American populations did not reveal clear differentiation between populations based on geographic separation (Figure 4.1). The topology of the genetic tree is most likely due to several negative pairwise F_{ST} treated as zero's by the program MEGA and not likely due to biological similarity. Negative values are most likely due to greater within population variation than among population variation (Conner and Hartl 2004). Greater number of loci and individuals are needed to reduce this error.

To assess the main mode of spread in N. America, pairwise F_{ST} values were evaluated as a function of geographic distance between any two given N. American populations. If the spread of EAB was due to natural movement, genetic differentiation is expected to increase as geographic distance increases (Sakai et al. 2001), however, little relationship between genetic and geographic distance would provide evidence humans mediated transport is likely. If there is any isolation by distance in this set of populations, it is very slight. There is a slight relationship among F_{st} and distance, but this relationship is not significant (Figure 4.2). This non-significant relationship suggests that humans have aided

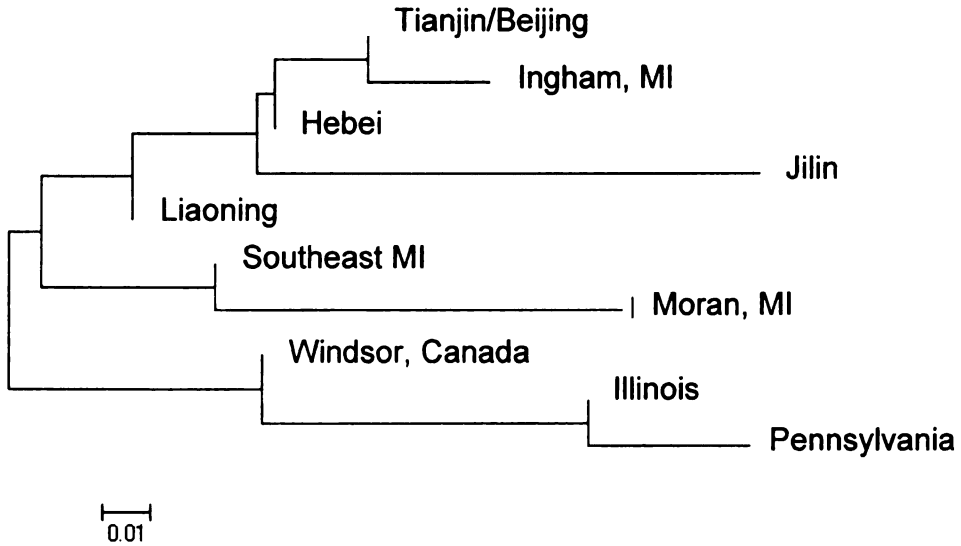


Figure 4.1. Neighbor-joining analysis of EAB microsatellite loci (two) based on pairwise F_{ST} values for North American EAB Populations.

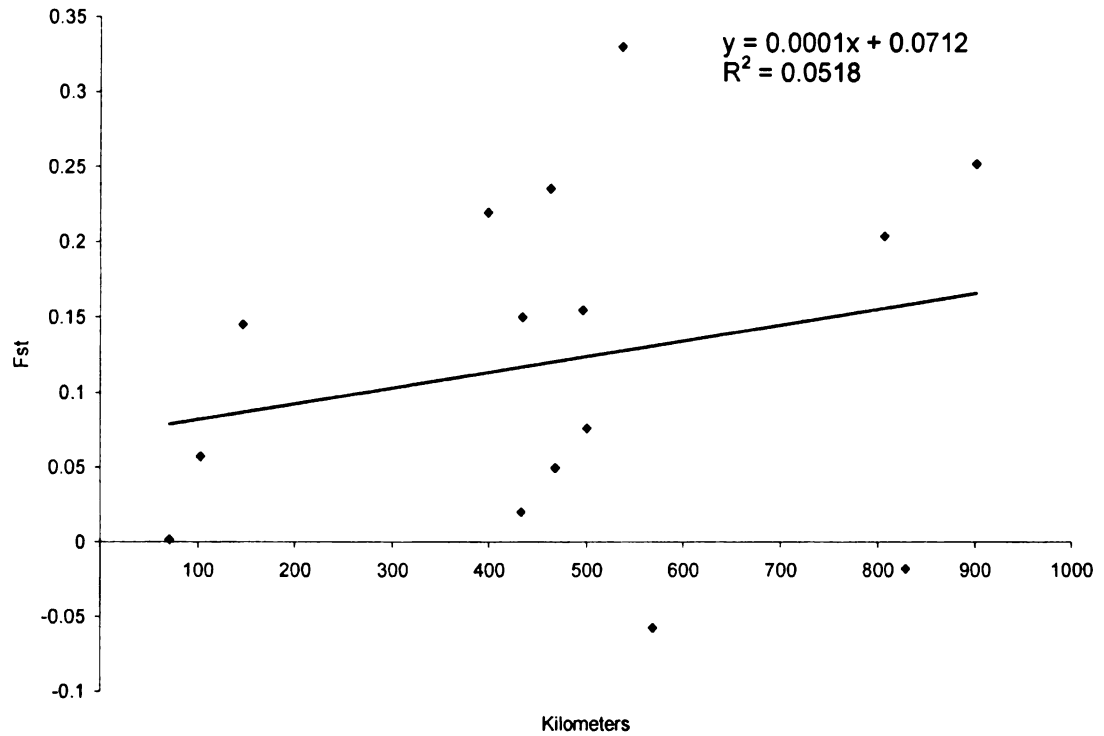


Figure 4.2. Test for Isolation by Distance in the North American EAB Populations based on the analysis of microsatellite markers (2 loci).

the movement of EAB in N. America, however, it cannot be ruled out that natural dispersal is a factor in the movement of EAB across some of these distances.

Analysis of genetic variation at polymorphic microsatellite loci holds promise for answering questions about both the structure of EAB populations at various spatial scales and about the basic biology of EAB. Even this dataset, based on an analysis of 162 individuals at two genetic loci, has yielded some interesting and thought-provoking results. Development of more microsatellite loci and increased samples sizes will be needed to give a better understanding of the invasion dynamics of EAB. EAB microsatellite markers also have applicability with respect to answering some basic questions about the biology of EAB, e.g., how many individuals are responsible for the eggs found on a single tree? The outcome of further analysis thus has direct impacts on control strategies since that could help concentrate efforts on the leading edge of migration for control rather than on many long-range dispersal events caused by humans.

APPENDIX

APPENDIX A

COI sequence haplotypes

Table A-1. Haplotype data for variation in the mtDNA cytochrome oxidase 1 gene of emerald ash borer. Lines denote conserved nucleotides that are identical to those in haplotype H1. Variable nucleotide positions are lettered. n = the frequency of each haplotype.

		Nucleotide position																										
Haplotype	n	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
H1	316	T	G	G	A	T	C	A	C	C	T	G	A	T	A	T	A	G	C	A	T	T	C	C	C	C	C
H2	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53
H1	G	A	A	T	A	A	A	T	A	A	T	A	T	A	A	G	A	T	T	C	T	G	G	C	T	A	C
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	G	-

Table A-1: continued

	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78
H1	T	C	C	C	T	C	C	C	T	C	T	T	T	A	A	C	A	T	T	A	C	T	T	T	T
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103
H1	A	A	T	A	A	G	A	A	G	A	A	T	A	G	T	C	G	A	A	A	G	A	G	G	A
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	C

Table A-1: continued

	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124
H1	G	C	A	G	G	G	A	C	T	G	G	T	T	G	G	A	C	A	G	T	A
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	A	-	-	-	-	-	-	-	-	A	-	-	-	-	-	C

	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145
H1	T	A	T	C	C	A	C	C	A	T	T	A	G	C	G	G	C	T	A	A	T
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	C	-	-	-	-	-	-	-	-	-	-	-	A	-	-	C	-	-	-

Table A-1: continued

	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166
H1	A	T	T	G	C	C	C	A	T	A	G	A	G	G	G	G	G	C	T	C	T
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-

	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187
H1	G	T	T	G	A	C	T	T	A	G	C	A	A	T	T	T	T	T	A	G	A
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A-1: continued

	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208
H1	C	T	T	C	A	T	T	T	A	G	C	T	G	G	A	A	T	C	T	C	C
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229
H1	T	C	A	A	T	T	C	T	A	G	G	G	G	C	A	A	T	T	A	A	T
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A-1: continued

	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250
H1	T	T	T	A	T	T	A	C	A	A	C	A	G	T	A	A	T	T	A	A	T
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271
H1	A	T	G	C	G	A	G	C	A	A	T	A	G	G	A	A	T	A	A	C	C
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A-1: continued

	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292
H1	T	T	A	G	A	C	C	A	A	A	C	A	C	C	T	C	T	A	C	T	A
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313
H1	G	T	C	T	G	A	T	C	T	A	T	T	G	C	T	A	T	T	A	C	A
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-

Table A-1: continued

	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334
H1	G	C	C	C	T	T	C	T	T	C	T	T	C	T	T	T	T	A	T	C	C
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	C	-	-	-	-	-

	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355
H1	T	T	A	C	C	T	G	T	T	C	T	T	G	C	G	G	G	A	G	C	A
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A-1: continued

	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376
H1	A	T	T	A	C	C	A	T	A	C	T	T	T	T	A	A	C	A	G	A	T
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	C	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397
H1	C	G	A	A	A	C	T	T	A	A	A	T	A	C	A	T	C	A	T	T	C
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A-1: continued

	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418
H1	T	T	T	G	A	C	C	C	G	G	C	T	G	G	A	G	G	A	G	G	A
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G
H9	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-

	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439
H1	G	A	C	C	C	T	A	T	T	C	T	T	T	A	T	C	A	A	C	A	T
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A-1: continued

	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460
H1	C	T	C	T	T	T	T	G	A	T	T	T	T	T	T	G	G	T	C	A	C
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481
H1	C	C	C	G	A	A	G	T	T	T	A	T	A	T	T	T	T	A	A	T	T
H2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

APPENDIX B

Deposition of voucher specimens

Appendix B

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 2009-05

Title of thesis or dissertation (or other research projects):

AFLP, mtDNA, and Microsatellite Analysis of Emerald Ash Borer Population Structure from Asia and North America

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name(s) (typed)
Alicia Marie Bray

Date 18/Aug/2009

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America. Bull. Entomol. Soc. Amer. 24: 141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.

Copies: Include as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

Appendix B

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Agrilus planepennis	5616 Annapolis Dr. Lansing, MI		10						MSU
Agrilus planepennis	Willoughby Park, Lansing, MI					1			MSU
Agrilus planepennis	Willoughby Park, Lansing, MI						1		MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Alicia Marie Bray

Date 18/Aug/2009

Voucher No 2009-05

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator 18 AUG 2009

Date

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LITERATURE CITED

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