# Saving Green Ash[1]

**J. Romero-Severson[2] and Jennifer L. Koch[3]**

### Abstract

The emerald ash borer (EAB, *Agrilus planipennis*) continues to kill ash trees in North America at an alarmingly fast pace. Although EAB is a threat to all species of ash (*Fraxinus*) in the United States, green ash (*F. pennsylvanica*) is among the most susceptible. Among the most commonly planted landscape trees in the United States, green ash is also an important species in riparian forests, rural agricultural systems and urban woodlands. Within 4 to 6 years from the time of first detection, the damage caused by EAB larval feeding can kill 100 percent of the green ash trees in a stand. Green ash is genetically incompatible with EAB-resistant Asian ash species, ruling out a simple backcrossing program to transfer resistance from species. However, a small number of green ash trees (~0.05 percent) have survived long term EAB attack. Careful testing of grafted clones of these “lingering ash” selections provides compelling evidence of a defensive response against EAB. The defenses lingering ash trees possess enable longer survival, but do not prevent premature death. Individual lingering ash trees employ different types of defense responses. Combining these defenses through breeding is expected to produce progeny that combat EAB more effectively than the original parent trees and presumably allow for long-term survival. However, if we do not act now to prevent the death of lingering ash, we will risk losing this invaluable genetic variation forever. An interdisciplinary strategy that combines long-term monitoring to identify lingering ash, wise application of genomic tools, and an EAB resistance breeding program will rescue an irreplaceable genetic resource and provide an accelerated route to the restoration of this important species.

## Introduction

Emerald ash borer (EAB, *Agrilus planipennis*) poses an acute threat to the *Fraxinus* species in North America (Herms and McCullough 2014). Green ash (F. *pennsylvanica*) and white ash (*F. americana*), attractive and fast-growing, were widely planted in urban forests and suburban landscapes to replace the American elm, *Ulmus americana* (Poland and McCullough 2006). Native *Fraxinus* species are widely used for shelterbelts in northern climates and for riparian buffer zones. Although EAB attacks all the major species of ash in the United States and Canada, green ash appears to be more susceptible (Anulewicz et al. 2007). The EAB was first detected in the region around Detroit, Michigan and Windsor, Ontario in 2002 and has since spread to 27 states in the United States and three Canadian provinces (http://www.emeraldashborer.info/). EAB kills 99 to 100 percent of green ash trees in forest stands within 4 to 7 years of first detection and kills urban green ash plantings as fast or faster (Knight et al. 2013, Kooster et al. 2014). The near synchronous loss of green ash across broad areas is having a cascade of negative impacts, including direct financial losses to industry, billions of dollars in tree removal cost to local governments, and the rapid loss of naturally occurring riparian forests comprised mainly of green ash (Gandhi and Herms 2010a, 2010b; Hausman et al. 2010, Knight et al. 2013, Kovacs et al. 2010). Without effective and timely intervention, EAB invasion threatens the survival of one of the most widely distributed hardwood in the riparian forests of eastern North America.

Accidentally imported exotic pests and diseases have adversely affected a host of native forest trees over the last several hundred years. In the case of the EAB, doing nothing will mean that green ash will likely become extinct over a large part of a vast native range. We propose an approach that implements a breeding plan based on intensive phenotyping, rapid deployment of improved trees, and wise use of genomics tools. This approach will help conserve the rapidly disappearing green ash gene pool, provide

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[2] University of Notre Dame, Notre Dame, IN 46556.
[3] Northern Research Station, USDA Forest Service, Delaware, OH 43015.
Corresponding author: jromeros@nd.edu.

source materials for early testing, and provide the basis for breeding for resistance. An approach like this can be successful, as evidenced by the white pine blister rust breeding program in the northwestern United States (Liu et al. 2016; Sniezko et al. 2014, 2016), and many others programs worldwide (FAO 2015).

## A Consideration of Strategies

### Containment and Control Are Not Sufficient

The response to EAB initially focused on eradication, prevention of further dispersal, and diminishing EAB pressure through insecticides and biocontrol (Poland and McCullough 2006). As containment efforts around the point of introduction in Michigan were defeated, management strategy shifted from extirpation to slowing ash mortality through the reduction of EAB populations using selective removals of ash trees, insecticide treatments, and other containment strategies (McCullough et al. 2015, Mercader et al. 2016, Mercader et al. 2011). Containment strategies may spread out financial impacts, but do not change the outcome or restore ash to native ecosystems.

Currently four Asian parasitoids are being released in the United States in an attempt to control EAB population levels. Although studies show successful establishment of these parasitoids in some areas, this approach alone will not save green ash. The release range of one parasitoid is limited by its lack of cold tolerance (Duan et al. 2010). Another parasitoid is limited to attacking larvae only in young ash trees due to its inability to oviposit on thick barked trees (Abell et al. 2012). Recent studies report parasitism rates in the United States similar to those reported on North American ash species growing in Asia (Duan et al. 2015, Liu et al. 2007). Despite the high level of parasitism of EAB in Asia, North American ash species in Asia still experience mortality due to EAB infestation. Biocontrol alone will not provide a solution in the absence of host resistance (Duan et al. 2015, Duan et al. 2012, Herms and McCullough 2014). However, increasing population level of EAB resistance in green ash, in conjunction with biocontrol of EAB populations, could allow the establishment of a new equilibrium that ensures the survival of North American ash. Implementation of a resistance breeding program can achieve this by rescueing the ash gene pool before it is lost.

### Technology in Absense of Breeding Program will Not Save Ash

Many people assume that cutting edge genetic technology will be the key to saving green ash. While high throughput genotyping has spurred the development of insect-resistant high-value row crops, this success is due to heavy reliance on long-term traditional breeding programs, made more efficient with high throughput genotyping, high throughput phenotyping, and better statistical tools. In the absence of a breeding program that will produce well-characterized phenotypes, appropriate germplasm, and replicated testing, the latest technologies will not save the ash of North America.

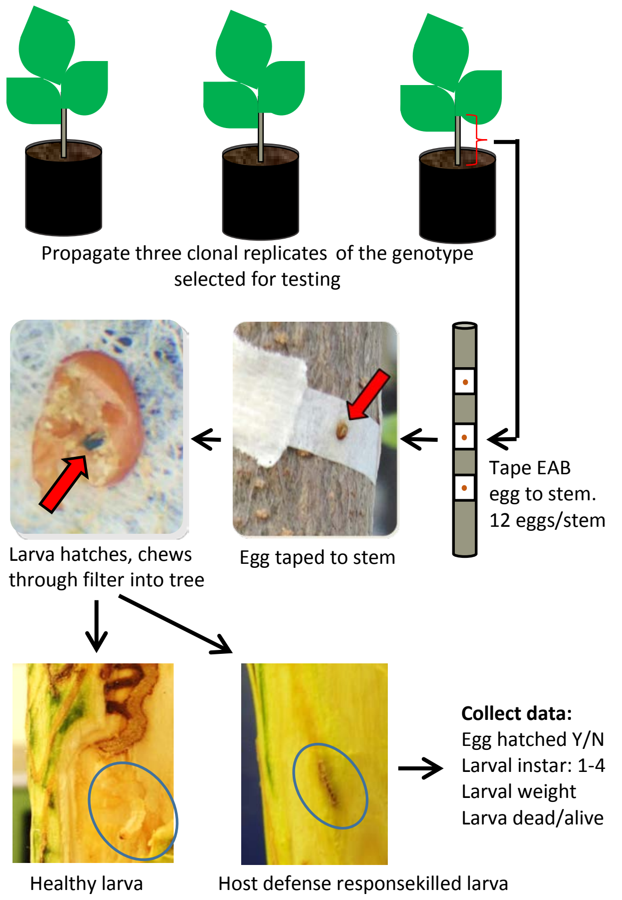
### Evidence That Natural Resistance Exists

Certain species such as Manchurian ash (*F. mandshurica*), tolerate EAB without sustaining major damage. This immediately suggests employing the classic plant hybrid breeding strategy of crossing with a resistant species. Unfortunately this is not feasible because green ash and Manchurian ash are genetically incompatible. However, there is another approach that shows promise.

A small number of green ash trees (<1 perecent) survive EAB attack many years longer than nearby trees of the same species. These ‘lingering ash’ show evidence of less severe EAB infestation, often accompanied by evidence of vigorous wound healing, and maintain a healthy crown for years after local conspecifics have died (Knight et al. 2013). Knight and her colleagues (Klooster et al. 2014) identified trees in or near a subset of permanent plots established in southeastern Michigan and northeastern Ohio based on two criteria: 1) a healthy canopy 2 years after the mortality rate of the stand exceeded 95 percent, and 2) a minimum diameter at breast height (DBH, 1.37 m from the ground) of 10 cm, indicating they were over the minimum size preferred by EAB (>3 cm), when the infestation was at peak levels (Wei et al. 2007). Some of these plots continue to be monitored yearly (Knight et al. 2012). In another study, a helicopter fly-over was done in natural areas just outside the core infestation zone to identify ash trees that were still alive (Marshall et al. 2013). The criteria for selection of these trees were a healthy canopy and a minimum DBH of 24 cm, to exclude the possibility of trees that may have re-sprouted after the main bole was killed. The status of these trees was verified by examination from the ground.

EAB egg bioassays (fig. 1) were done on grafted replicates of four ‘lingering green ash’ genotypes along with one susceptible wild green ash tree (PE-36) and the susceptible green ash cultivar ‘Summit’. An EAB-resistant Manchurian ash was included as well. Dissections were performed to determine the outcome for each of the 12 eggs placed on each grafted plant, whether larvae lived or died and if they died how far they developed (larval instar L1, L2, L3, or L4). Larval outcome was modeled as a multinomial response using a general linearized mixed model. Differences among ash genotypes were significant (p = 0.0002). When larvae killed by host defense response were modeled as an outcome, one lingering ash clone (PE-L19) killed significantly more larvae (p <0.0001) than the susceptible control ‘Summit’. When larval weight was modeled as an outcome, the larvae in the clone PE-L22 had significantly lower larval weights (p = 0.0163) than the susceptible control. These initial results suggested that lingering ash employ different mechanisms to enable longer survival in the field (Koch et al. 2015). Although lingering green ash genotypes permitted more EAB larval development in additional egg bioassay experiments than the resistant Manchurian ash, some lingering ash clones consistently killed more early instar larvae (35 to 50 percent) than the susceptible green ash controls (0 to 10 percent). While lingering ash trees are clearly not as resistant as Manchurian ash, they do possess a partial resistance that permits longer survival. Given this preliminary evidence for multiple mechanisms, lingering ash (once EAB phenotypes have been confirmed), could form the basis of a breeding program for ‘stacking’ or pyramiding the multiple allelic variants that may be responsible for the multiple mechanisms of partial resistance. Phenotypic confirmation is essential, as past work has shown that ~20 percent of lingering ash identified in a forest setting show no evidence of partial resistance when clonal replicates are tested by egg bioassay. Other mechanisms of resistance have been shown to exist (e.g., EAB prefer to avoid some trees) so these clones are retained for later tests (Koch et al. 2015, Peterson et al. 2015). Preserving lingering ash by grafting and confirming the EAB phenotype rescues the genes and gene variants that confer these defenses before they are permanently lost. Conservation of this valuable resource is a major step towards the long term goal of producing trees that can withstand EAB long enough to maintain populations in a forest setting.

**Figure 1**—Egg bioassay protocol. The grafted genotype is preserved in untreated grafted clones.



There are other examples of infrequently occurring individuals of native tree species having genetic resistance to non-native invasive insects. Some American beech trees (*Fagus grandifolia*) are resistant to the nonnative beech scale insect (*Cryptococcus fagisuga*). The insect creates entry wounds permitting infection with *Neonectria* spp. This insect-disease complex results in beech bark disease. After Koch and colleagues demonstrated that scale resistance is heritable (Koch et al. 2010), scale-resistant clones were planted in regional seed orchards to enable beech restoration of state and national forests severely impacted by this disease. Other investigators have recently shown that some eastern and Carolina hemlocks (*Tsuga canadensis* and *T. caroliniana*) have resistance to the non-native hemlock wooly adelgid (*Adelges tsugae*) (Oten et al. 2014).

## A Strategy to Save Green Ash

We propose a breeding plan based on intensive phenotyping to identify the best lingering ash trees, establishing orchards planted with polycrossed progeny (resulting from many different combinations of the best lingering ash trees as parents), managing such orchards to encourage early flowering, and using genomic tools to serve the program, rather than drive it. This plan will save the lingering ash gene pool, establish a sound breeding program, insure genetic diversity, and lay the foundation for functional genomics studies. The plan we outline below could be fully implemented in the same amount of time (8 to 10 years) it would take for the development of a full set of genomics tools for green ash (sequenced and assembled genome, deep transcriptome and functional annotation). A ‘genomics only’ approach would not deliver breeding populations, would not provide immediately useful information in the absence of such populations, and would fail to rescue the existing lingering green ash gene pool. We propose an integrated strategy that included functional genomics as well as traditional breeding. We argue that a breeding program based on the phenotyping approach outlined here (visual identification of “candidate” lingering ash followed by confirmation with the egg bioassay) can proceed without any genomics other than the development of DNA “passports” that uniquely identify each individual. A genomics approach alone produces nothing that saves the lingering ash gene pool.

### Goals, Strategies, and Objectives

The proximate goal of our plan is to save and characterize the lingering ash gene pool. The ultimate goal is to save green ash. The strategy is to 1) save naturally occurring resistance alleles from extinction by finding lingering ash trees, grafting them and planting them in containers for testing and breeding; 2) combine the unique alleles of each lingering ash selection by using them to produce progeny in controlled cross pollinations with at least two other lingering ash parents; establish the sets of progeny from each of these lingering ash families in a “polycross” seed orchard where they can naturally cross pollinate with members of many different families, producing many different combinations of alleles, and 3) maintain high genetic diversity within these polycross populations and manage for early flowering. We have four objectives:

1. Using container-grown lingering ash parents, generate at least two different full sibling families per parent and establish these progeny in a planting as a polycross population.
2. Confirm our preliminary result supporting the hypothesis that cross-pollination between parents with different lingering phenotypes produces some progeny with EAB defensive responses more effective than either parent.
3. Generate scalable, transferrable DNA passports for all lingering ash germplasm in the project.
4. Develop regional programs to identify additional lingering ash parents and establish polycross orchards.

### A Testable Hypothesis

If there are multiple genes with allelic variants that act additively to inhibit the growth of or kill EAB, then individuals having partial resistance could have some, but not all, of the “optimum” variants that contribute to resistance (fig. 2). If this is true, then among a large number of full sib progeny of two individuals having presumably different mechanisms of partial resistance, there are likely to be some individuals who have higher levels of resistance than either parent and some individuals who have lower levels than either parent. These transgressive phenotypes, if verified in independent tests of clonal replicates, would support the hypothesis of multiple genes with allelic variants that act additively to inhibit the growth of or kill EAB. Intercrossing or polycrossing lingering ash would ‘stack the deck’ to produce some individuals capable of more effective defensive responses than their parents.

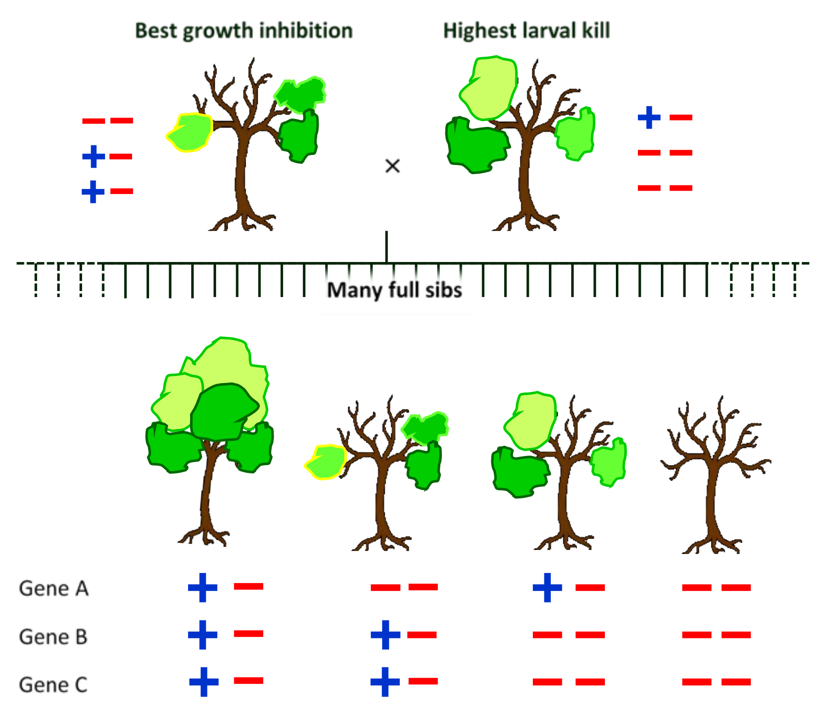


Figure 2—Transgressive phenotypes, as detected by replicated egg bioassays, indicate allelic variants at multiple genes. Highly simplified cartoon with progeny drawn as if they were mature trees.

## The Next Step

The next step in our plan is to remove individuals from the polycross population who have lower levels of resistance than their parents, and allow the remaining trees with the best defensive responses to intercross with each other.

Seedlings produced by the intercross and polycrosses can be outplanted for early testing in EAB-infested areas. As polycross orchards mature, seed could be distributed to cooperators. Many of these seeds will have little EAB resistance, but some will have much higher levels of resistance than the original parent populations. This approach does not involve marker-assisted selection (MAS) or genome-wide association (GWAS). MAS and GWAS are premature at this stage of a breeding project and may be ineffective in this system. Functional studies do have value, but only if identification of superior phenotypes has been done in replicated tests that include susceptible controls. Indirect selection using DNA markers works only in well-characterized systems, where the variation in the phenotype due to environmental influences is well understood (Bian et al. 2014, Muranty et al. 2014). Genomic selection, using a high density collection of markers distributed across the genome also works, but only in those systems where 1) dense genotyping tools exist for the species of interest, 2) the phenotype is thoroughly characterized and accurately measured, 3) extremely large pedigrees exist, 4) a long term breeding program exists, and 5) funding is available to back the effort over the long term (Isik 2014).

## Transferrable and Scalable Marker Systems

The cost-effective integration of a tree breeding program with wise use of DNA markers requires a marker system that has two features that most high-throughput genotyping approaches lack: scalability and transferability. Our proposed breeding program will have a DNA- passport (a genetic fingerprint) for every individual, to insure that the relationship of phenotypic data to genotypic data is not compromised. These specific sets of sequences need to be scalable so that they can be generated again and again, for any number of trees, from one to thousands. In species where interspecific hybridization occurs naturally, the markers need to be transferrable, i.e., likely to be informative across species within sections and even across all the species in a genus. This will enable leverage of the information gained in one species to other species within the genus and in the case of using markers from expressed genes, may provide relevant functional information. Previous work has shown that the informative EST-SSRs we developed from the green ash transcriptome are informative in other Meloides in the *Fraxinus* genus and most are informative in sections *Ornus* and *Fraxinus* as well (Noakes et al. 2014). For our plan, these markers have a dual purpose: to enable monitoring of genetic diversity in polycross orchards to limit narrowing of the genetic base and to serve as DNA passports.

A DNA-based genetic passport would consist of those sequences that capture the most polymorphism with the least number of sequences while at the same time uniquely identifying every individual in the project. A DNA passport must be easily verifiable. The verification technology must be scalable (i.e., a passport can be generated for only one tree at one time, or for many trees, as needed). A DNA passport must also be designed so that any instrument capable of DNA sequencing can generate it, i.e., platform-independent. The methods most frequently employed for sequencing or genotyping do not meet both of these requirements. Reduced representation sequencing (RRS) technologies, high-throughput exome or whole genome sequencing and SNP chips enable discovery of unique DNA-based fingerprints, but lack scalability. Genomic SSR and EST-SSR markers, amplified a few at a time then size fractionated using capillary electrophoresis, are highly scalable and in the case of EST-SSR markers, highly transferrable (Noakes et al. 2014). However, genotyping is slow and labor-intensive. Amplicon size estimation, while highly accurate within the same technology platform, can vary across platforms.

A targeted sequencing approach (capture by hybridization or bait-capture) has the potential to meet both requirements. Bait-capture using microsatellite-containing EST sequences is scalable, transferrable, and platform independent, in that each tree has a fingerprint consisting of a set of sequences, any or all of which may be generated from any number of trees using the sequencing technology of choice. Capture by hybridization (Sun et al. 2013, Zhou and Holliday 2012) in which *Fraxinus* EST-SSR sequences are the baits is a promising approach. The *Fraxinus* genus has two publically available transcriptomes: the *F. pennsylvanica* transcriptome (http://www.hardwoodgenomics.org/node/68249), and the *F. excelsior* transcriptome (http://www.ashgenome.org/transcriptomes). However, other approaches that are just as good or better, and cheaper, will almost certainly be available soon. A caveat is that the expense of generating a DNA passport in not limited to the cost of sequencing, which is almost trivial. The expense lies in the DNA library prep kits and the salary cost for entering data into a usable, secure, and easily retrievable form.

### Collaboration, Cooperation and Partnership

Green ash is the most wide-ranging hardwood tree in eastern North America. A serious effort to save green ash and the other North American *Fraxinus* species will require regional cooperation. Confirming that defensive responses of some of the progeny in a first generation cross are superior to either parent, without destroying these genotypes (which is technically possible, but requires space for many grafted clones), will help attract funding and collaborators.. The program we envision will be amenable to a participatory breeding approach with partners that may include private citizens, state and federal agencies, and nonprofit organizations.

## Conclusion

The ultimate goal of this plan is restoration of a green ash resource that can survive long enough to ensure self-sustaining populations with minimal loss of genetic diversity in the riparian forest ecosystems of eastern North America. The plan we have outlined here is dependent on securing funding to support a sustained effort that may take 3 or more years before the first results are analyzed and published. Involving the public gardens in this effort could greatly assist in garnering public support for a longer term project. Almost everyone who lives in the suburbs or a city in the upper Midwest has seen dying ash trees as a result of EAB. The partnership of The American Public Gardens Association with the U.S. Department of Agriculture Forest Service includes provisions for conservation education programs. “Saving green ash” could become a part of those programs.

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