OVERCOMING OBSTACLES TO INTERSPECIES HYBRIDIZATION OF ASH

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Tree species that share a long co-evolutionary history with insects and pathogens are likely to have developed mechanisms of resistance that allow them to coexist. When insects and pathogens are introduced to different parts of the world, high levels of susceptibility can be observed, presumably in part due to the lack of co-evolutionary history between the insect (or pathogen) and host. In such cases, use of non-native tree species as a source of resistance for introgression into native susceptible tree species can be quite helpful. Examples of interspecific hybrids include hybrid hemlocks with resistance to hemlock wooly adelgid and hybrid chestnut trees resistant to chestnut blight (Hebard 2006, Montgomery et al. 2009). If first-generation hybrids show good resistance to the insect or pathogen, then a back-cross breeding program may be initiated to produce seedlings for restoration that preserve the introgressed resistance in a progeny set with most of the characteristics of the native parent species. This approach is being used by The American Chestnut Foundation and would be the model approach used to produce emerald ash borer (EAB)-resistant North American ash species.

The success of this strategy depends on the existence of genetically controlled resistance to EAB in Asian ash species and the ability to inter-breed these resistant species with susceptible North American ash species. Evidence of EAB resistance in Asian ash species has been reported (Liu et al. 2007), but controlled testing is limited to a single horticultural selection of *F. mandshurica* (Rebek et al. 2008). Little is known about interspecies hybridization in ash, with the exception of two cultivars ('Northern Gem' and 'Northern Treasure') that have been described as hybrids between Manchurian ash and black ash (*F. nigra*) (Davidson 1999). Northern Treasure has been reported as EAB-susceptible (Rebek et al. 2008). We have begun to establish a collection of Asian ash species (and other exotic species) at the U.S. Forest Service, Northern Research Station, in Delaware, OH, to identify species with EAB resistance for use as parents in a breeding program. We have performed controlled cross-pollinations with more than 30 species combinations of ash with little success. We have identified several challenges to controlled pollinations to produce hybrids, and our program is addressing them.

Obstacles to interspecific crosses in *Fraxinus* include between-species differences in reproductive biology and genetics, such as differing ploidy levels, differing pollination systems and phenology, and potential genetic incompatibility (Wright 1957, Wallander 2001). Although the majority of ash species are 2n=46, important exceptions influence hybrid breeding programs. The exceptions include white ash (*F. americana*) 2n=46, 92, or 138; pumpkin ash (*F. profunda*) 2n=138; velvet ash (*F. velutina*) 2n=46 or 92; and Chinese ash (*F. chinensis*) 2n=92 or 138. While ploidy level differences do not always result in complete incompatiblity, the resulting F_1 progeny are often sterile. Accordingly, we need to carefully match known ploidy levels when choosing parents for initial crosses. The ploidy levels of blue ash (*F. quadrangulata*) and Manchurian ash (*F. mandshurica*) are unknown, so cytogenetic work is being conducted to establish the ploidy number of these species. Preliminary results indicate both species are 2n=46. Genetically determined incompatibility is another natural barrier to hybridization. Genetic incompatibility may be structural, such as when there are different chromosome numbers or gross differences in genome organization (major deletions or transpositions that prevent proper chromosome pairing at mitosis and meiosis), or it may be biochemical, such as the expression of S-glycoproteins (Broothaerts and Nerum 2003, Wheeler et al. 2009).

Differences in pollination system (insect, wind-pollinated) represent significant barriers to natural cross hybridization but may be relatively easily overcome by controlled cross-pollination techniques. Storing pollen and manually pollinating flowers allows the crossing of species without regard to their natural pollination system. It is not known how genes controlling the pollination system might segregate in an F_1 or F_2 hybrid population, so our initial efforts have focused largely on species pairs with similar reproductive biology. Many ash species are dioecious or androdioecious, meaning that both male and female trees must be collected and established, increasing the minimum number of trees required for breeding. Differences in phenology are closely related to differences in reproductive biology and create real barriers to natural hybridization. By maintaining known female trees as potted ramets for long periods, we are able to control the timing of flowering. The female trees are kept in a cold storage area until fresh pollen of the desired species has been collected or until local trees have shed the bulk of their pollen. It has been suggested that the large size of the genus *Fraxinus* will make crosses between sub-genera impossible (E.Wallander, pers. comm.). However, there are few to no cross-compatibility data to determine exactly what the maximum allowable phylogenetic distance is between parents that results in successful hybridizations.

Perhaps the greatest restriction to hybridization identified to date is the limited number of Asian ash accessions available in the United States. The United States has only a limited amount of ash from China, Korea, Japan, and eastern Russia, and much of it is not yet reproductively mature. Some species are represented by only one or two accessions in all the U.S. gardens and arboreta, a limitation that in some cases became apparent only after our thorough review of accession records. Continued thorough review of accession records and DNA analysis will be necessary to ensure unique material is being accessioned. Seed collections from China were distributed widely throughout the United States. For example, the North American China Plant Exploration Consortium (NACPEC) is made up of many member gardens and arboreta that all share collected seed. A single Manchurian ash seed collection in 1997 (NACPEC97048) is documented as 25 specimens under 9 accession numbers in 7 different gardens. A more recent seed collection effort by NACPEC, headed by the Morton Arboretum (Chicago, IL), included a wide range of ash species in China; it should be a valuable resource once the seeds germinate and the trees become sexually mature.

Yet another challenge is presented once successful pollinations produce potentially hybrid seedlings. To rule out pollen contamination (including self-pollination), DNA-based methods to confirm the hybrid parentage of seeds produced from controlled crosses are being optimized. Despite the expectation that self-compatibility and pollen contamination would be rare, each cross may result in only a few seeds, so hybrid confirmation is essential. Our initial efforts to confirm several putative hybrids using SSR or AFLP markers uncovered some inconsistencies in the parent trees. Several trees seemed to cluster inappropriately based upon their marker genotype.

We expanded the number of reference samples included for each species of interest and began to carefully document the phenotypes of the exotic ash in our research collection. The ITS region of a subset of individuals was sequenced and these data confirmed that our AFLP-based groupings were consistent with the published phylogenies (Wallander 2008). Based upon both AFLP and ITS sequence results, however, several samples in question were incorrectly identified at the species level.

We were able to trace several incorrectly identified accessions back to the Chinese Academy of Sciences Botanical Garden in Beijing. In one case, the seed was directly distributed from the Chinese Academy to arboreta in the United States in the 1980s, and in another case seed was distributed to a botanical garden in another country in 1963, which then supplied materials to the United States. All accessions derived from this seed source that we have analyzed to date have been determined to be incorrectly identified as *F. chinensis.* In fact, they appear to be *F. pennsylvanica*. Furthermore, several seed lots imported from China by reputable U.S. seed companies have also been shown to be incorrectly identified as either *F. chinensis* or *F. bungeana*. To date, we have found eight specimens and seedlots that were incorrectly identified as either the Asian species *F. chinensis* or *F. bungeana*, all of which are consistent with *F. pennsylvanica*, based on AFLP genotyping. It appears that seed has been routinely collected from *F. pennsylvanica* growing in China and distributed as *F. chinensis* and *F. bungeana*. The seeds from these species are very similar, which has likely contributed to the mistaken identities.

This work accents the importance of confirming the identity of closely related species when initiating a hybrid breeding program or other scientific experiments. For example, comparisons of proteomics, metabolomics, and transcriptomics between EAB-resistant Asian ash species and EAB-susceptible North American ash species are of less value if the species of interest have not been identified correctly. Furthermore, these findings also demonstrate that the number of Asian ash accessions currently in the United States may be even more limited than records show.

It is our long-term goal to overcome the obstacles described here to establish an interspecific breeding program using exotic ash species as sources of EAB resistance that can be introgressed into native North American species. F_1 hybrids with EAB resistance may have immediate value to the nursery industry as street trees. F_1 progeny, as well as subsequent generations, will also be valuable genetic resources for determining the heritability and molecular mechanisms of resistance. Backcross generations created through careful selection of parents for both North American characteristics and EAB resistance may someday provide the resources needed to re-establish EAB-resistant North American ash species in our forests. Gene conservation activities involving North American ash species are a critical component of such a breeding program so that genetic diversity across the native range is preserved for use in advanced generations of breeding.

LITERATURE CITED

Broothaerts, W.; Van Nerum, I. 2003. **Apple self-incompatibility genotypes: an overview**. Acta horticulturae. 622: 379-387.

Davidson, C.G. 1999. **'Northern Treasure' and 'Northern Gem' hybrid ash.** HortScience. 34: 151-152.

Hebard, F. V. 2006. **The backcross breeding program of the American chestnut foundation**. Journal of the American Chestnut Foundation. 19: 55-77.

Liu, H.; Bauer, L.S.; Miller, D.L.; Zhao, T.; Gao, R.; Song, L.; Luan, Q.; Jin R.; Gao, C. 2007. **Seasonal abundance of *Agrilus planipennis* (Coleoptera: Buprestidae) and its natural enemies *Oobius agrili* (Hymenoptera: Encyrtidae) and *Tetrastichus planipennisi* (Hymenoptera: Eulophidae) in China.** Biological Control. 42: 61-71.

Montgomery, M. M.; Bentz, S.E.; Olsen, R. T. 2009. **Evaluation of hemlock (*Tsuga*) species and hybrids for resistance to *Adelges tsugae* (Hemiptera: Adelgidae) using artificial Infestation.** Journal of Economic Entomology. 102: 1247-1254.

Rebek, E.J.; Herms, D.A.; Smitley, D.R. 2008. **Interspecific variation in resistance to emerald ash borer (Coleptera: Buprestidae) among North American and Asian ash (*Fraxinus* spp.)** Plant-Insect Interactions. 37: 242-246.

Wallander, E. 2001. **Evolution of wind-pollination in *Fraxinus* (Oleaceae) – an ecophylogenetic approach.** Gothenburg, Sweden: University of Gothenburg. 129 p. Ph.D. dissertation.

Wallander, E. 2008. **Systematics of *Fraxinus* (Oleaceae) and evolution of dioecy.** Plant Systematics and Evolution. 273: 25-49.

Wheeler, N.; Nielsen, D.; Smith, C.; Tomkins, J.; Abbott, B.; Atkins, M.; Blackmon, B.; Staton, M.; Ficklin, S.; Hebard, F.; Sisco, P.; Carlson, J., Barakat, A.; Powell, W., Baier, K., Anagnostakis, S.; Kubisiak, T.; Burdin, C.; Sederoff, R. 2009. **Annotated EST-based SSR and SNP genetic maps for Chinese chestnut**. [online]. Available: http://www.fagaceae.org/sites/default/files/nov09conf/program.pdf. (Accessed December 15).

Wright, J. W. 1957. **New chromosome counts in *Acer* and *Fraxinus*.** Morris Arboretum Bulletin. 8: 33-34.