

Genetic Integrity of Longleaf and Shortleaf Pine Seed Orchards and Seed Banks

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Abstract

Longleaf pine (*Pinus palustris* Mill.) and shortleaf pine (*Pinus echinata* Mill.) are priority species targeted for increased restoration on the national forests in the Southern Region of the U.S. Department of Agriculture, Forest Service. The genetic integrity of both species is important to ensure adaptation, survival, and resilience of future forests. Longleaf x loblolly pine hybrids (*Pinus* × *sondergeri* H.H. *Chapm. ex Sudw. [palustris* × *taeda*]) and shortleaf x loblolly pine hybrids are known to occur in the general forests, but at a rate of less than 5 percent. Climate change can trigger extreme fluctuations in temperatures, which could influence flower receptivity and result in greater potential for increased inter-species hybridization. This hybridization may compromise the genetic purity of a species and present challenges to successful restoration. It is important to know the genetic identity of the seedlings we are deploying in operational plantings, and the seed being sold to State partners. The Southern Region National Forest System Genetics program chose to DNA fingerprint longleaf and shortleaf pine parents (clones) in the regional seed orchards to assess genetic purity. Final results showed no hybrid fingerprint for the 250 longleaf clones tested and a hybrid fingerprint for 17 of the 619 shortleaf clones tested. The regional seed bank inventory for longleaf and shortleaf pines was also DNA fingerprinted. The seed tested had been collected across multiple years and seed zones. Final results showed a hybrid fingerprint for less than 3 percent of the seed. This paper was presented at the Joint Annual Meeting of the Southern Forest Nursery Association and the Northeast Forest and Conservation Nursery Association (Pensacola, FL, July 17–19, 2018).

Introduction

The U.S. Department of Agriculture, Forest Service National Forest System (NFS) in the Southern Region (R8) provides oversight for the management of approximately 800,000 ac (323,750 ha) of longleaf pine (*Pinus palustris* Mill.) and 1,440,000 ac (582,750 ha) of shortleaf pine (*Pinus echinata* Mill.), across 13 southern national forests. Approximately 97 percent of the longleaf pine ecosystem and 53 percent of the shortleaf pine ecosystem have been lost over the past century (Wear and Greis 2013). A range map for longleaf and shortleaf pine reflects the current geographic distributions of each (figure 1). There is a priority emphasis on accelerated restoration of these species and associated ecosystems on R8's national forests. Multiple agencies, organizations, and partners are also engaged in restoration of these species, such as the Longleaf Alliance (<https://longleafalliance.org/>), America's Longleaf (<http://www.americaslongleaf.org/>), the Shortleaf Pine Initiative

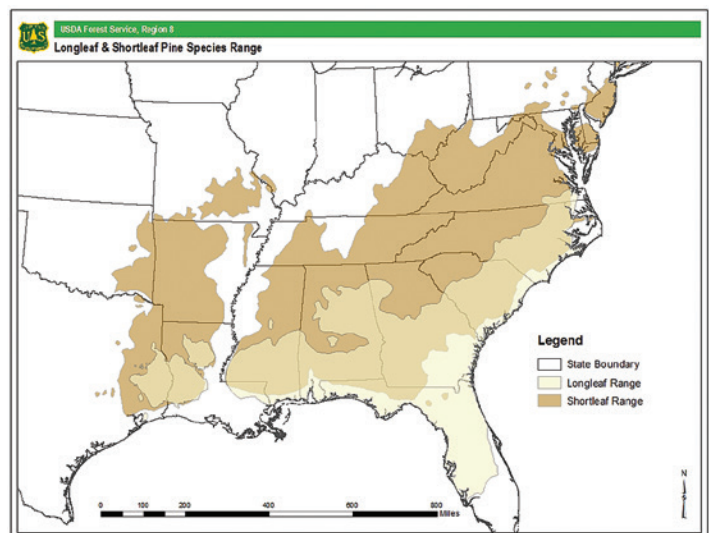


Figure 1. Current geographic range map of longleaf and shortleaf pine. (Created by Chelsea Leitz, USDA Forest Service, 2019)

(<http://www.shortleafpine.net/>), The Nature Conservancy (<https://www.nature.org/>), and others.

R8 NFS's reforestation trends reflect approximately 50 percent artificial regeneration and 50 percent natural regeneration. Artificial regeneration activities are expected to increase at an accelerated pace to support increasing restoration targets, therefore more seed will be needed. Genetic integrity (purity) of a species is important to ensure adaptation, survival, and resiliency of forests. If the genetic purity of a species has been compromised, this may present challenges to successful restoration and resiliency (Ledig and Kitzmiller 1992). Both current and future planted forests in R8 are often managed on a 100-year rotation cycle, so adaptation, survival, and resiliency are critical.

Within the past several years, a southern nursery experienced an increase in unusual pine seedling morphologies. Concerns and questions arose about the genetic purity and identity of the seed, and hybridization was suspected. Research to assess suspected increased hybridization in the general forests, seed orchards, and seedling crops was already ongoing (Tauer et al. 2012, Stewart et al. 2016). Longleaf x loblolly pine hybrids (*Pinus ×sonderegeri* H.H. Chapm. ex Sudw. [*palustris* × *taeda*]) and shortleaf x loblolly pine hybrids are known to occur in the general forests, but at a low rate of less than 5 percent (Chapman 1922, Tauer et al. 2012). Climate change can trigger extreme fluctuations in temperatures, which could influence flower receptivity and result in the potential for increased inter-species hybridization. The suspect seed did not come from R8 seed orchards; however, are the concerns over seed purity lead the R8 NFS Genetics program to initiate a project to validate the genetic purity of our germplasm (i.e., orchard trees and seed bank inventory). These genetic resources represent multiple seed sources and seed collections spanning 25 years. The objective of the project was to DNA fingerprint longleaf and shortleaf pine parents in all the seed orchards and the seed bank inventory to assess genetic purity and identify any hybrids that may exist.

Questions and Concerns About Hybrid Seedlings

Several questions and concerns have arisen regarding establishment of hybrid seedlings on the landscape. These questions include the following:

- Will hybrid seedlings adapt or be maladapted? Will they survive and reproduce? Is there hybrid vigor?
- What are the growth rates? What is the wood quality? What is the longevity/life span?
- Will the seed physiology change, e.g., germination, viability, stratification requirements?
- Will the hybrid seed provide adequate sustenance for the wildlife that depends on this food source?
- Will red cockaded woodpeckers build cavities in a hybrid tree?
- Will hybridization increase as climate change and extreme fluctuations in temperatures occur? What are the effects on phenology and flower receptivity? How will this affect orchard management?
- Are we looking at future forests that contain more natural hybrids? Will this support or deter forest resiliency? Will hybrids be as resilient as their progenitors to catastrophic weather events in the South, e.g., hurricanes, tornadoes, ice/snow?
- Will silvicultural methods need to be modified if hybrids increase on the landscape? Both longleaf and shortleaf pines are fire-dependent species. Currently, prescribed burning is the most economic and efficient silvicultural tool used to manage these forests. Loblolly pine is not fire tolerant, so then will the hybrids survive fire?
- Longleaf and shortleaf pines occupy very different geographic sites and soil types, from extreme coastal to Piedmont to mountain geographic regions, respectively. Will hybrids adapt, migrate, or die on various sites?

The seed harvested from the orchards and stored in the seed bank is used in restoration on the national forests. Excess seed from this seed bank is occasionally sold to State agencies. R8 NFS Genetics program manages the highest percentage of known longleaf and shortleaf genetic resources (seed orchards, seed production areas, progeny tests) that exist in the South (table 1). Scion material is shared with external partners who are establishing seed orchards. It behooves our program to know the genetic purity of the orchard trees and seed, so that the identity of the seedlings being planted on Federal and non-Federal forested lands is also known.

Table 1. Summary of longleaf and shortleaf pine genetic resources (seed orchards, seed production areas, progeny tests) acreage in the South.

Agency	# Longleaf pine seed orchards / seed production acres	# Shortleaf pine seed orchards / seed production acres	Number of progeny tests
Forest Service	540 / 272	527 / 0	35 longleaf 155 shortleaf
State	225 / 0	70 / 0	unknown
Industry	47 / 125	0 / 0	unknown
Private	unknown	Unknown	unknown

Methodology

Field Collections

R8 NFS longleaf pine seed orchards are located near Benton, LA, Wiggins, MS (figure 2), and Mt. Pleasant, SC. Shortleaf pine seed orchards are located near Mount Ida, AR (figure 3), Benton, LA, Wiggins, MS, and Murphy, NC. Seed orchard and field personnel collected needle samples from two ramets of each clone

(family) in each orchard. Needle samples were taken from first-year needles in the top third of the crown. Samples were collected from a total of 250 longleaf pine clones and 619 shortleaf pine clones. Longleaf pine sources represented Alabama, Florida, Louisiana, Mississippi, South Carolina, and Texas. Shortleaf pine sources represented Alabama, Arkansas, Georgia, Kentucky, Louisiana, Mississippi, Missouri, North Carolina, South Carolina, Tennessee, and Virginia. Needle tissue was shipped overnight to the National Forest System Genetics Lab (NFGEL) at the Institute of Forest Genetics, Placerville, CA.

In addition to needle samples, longleaf and shortleaf pine seed samples from the R8 NFS Ashe seed bank (housed near Wiggins, MS, figure 4) were shipped to NFGEL for DNA testing. Seed sources tested represented Alabama, Arkansas, Florida, Georgia, Kentucky, Louisiana, Mississippi, Missouri, North Carolina, South Carolina, Tennessee, Texas, and Virginia. Seed collection years spanned 1987 to 2017, and approximately 200 seed from each source and year were shipped (table 2).



Figure 2. Longleaf pine seedling seed orchard in Mississippi (Photo by Barbara Crane, 2012)



Figure 3. Second-generation shortleaf pine seed orchard in Arkansas. (Photo by Barbara Crane, 2016)



Figure 4. Ashe seed bank inventory drums in Mississippi (Photo by Barbara Crane, 2015)

Laboratory Work

Simple sequence repeat (SSR) DNA markers were used to fingerprint the orchard needle samples and seed bank samples. The Southern Research Station Southern Institute of Forest Genetics (SIFG), Saucier, MS developed the longleaf pine markers (Echt and Josserand, 2018). The shortleaf pine markers were developed in collaboration with Oklahoma State University (Stewart et al. 2012). Three markers were developed from GenBank chloroplast DNA sequences that together identify species-specific profiles (haplotypes) among longleaf, shortleaf, and loblolly pines. In addition to distinguishing among species, these markers allow easy and fast assays to identify longleaf x loblolly pine and shortleaf x loblolly pine hybrids with a high degree of confidence, because chloroplast DNA is only inherited through pollen in pines. Loblolly pine chloroplast DNA, as the pollen parent, was the differential indicator marker for detecting hybrids in the samples.

SIFG invested substantial time and work in the initial development of the markers that could be used in the DNA fingerprinting and hybrid identification. Over 2 years, prior to the NFGEL work, many samples had to be initially screened to find relevant markers that would differentiate the species. Needle and seed samples for more than 2,000 longleaf pines, more than 1,000 shortleaf pines, and nearly 300 loblolly pines were screened (Echt et al. 2013). We have seen only one shared haplotype at 1 percent frequency in longleaf pine and 0.1 percent in shortleaf pine. There were no shared haplotypes with loblolly pine. These results indicate that these chloroplast markers are useful to estimate proportions of pollen contamination in seed lots and identify orchard trees that are likely to be hybrid. To estimate the full extent of species specificity, additional sampling and testing is being considered for each species.

NFGEL staff extracted DNA from the seed orchard needle samples. For the seed samples, the seed was first germinated, then the DNA was extracted from both the megagametophytes and the embryos. Qiagen DNA kits (<https://www.qiagen.com/us/>) were used for extracting the DNA. Applied Biosystems ABI machines were used to run the DNA samples with the markers. An example of an SSR marker profile to identify the different DNA fingerprint for loblolly pine, longleaf pine, and longleaf x loblolly suspected hybrid can be seen in figure 5.

Table 2. Summary results of DNA fingerprinting on longleaf and shortleaf pine families and seed to assess genetic purity and identify hybrids.

Species	Number of seed orchard families tested	Sources tested (orchard families and seed)	Seed years tested*
Longleaf	250 (2 ramets each)	AL, FL, LA, MS, SC, TX	1981 – 2017
Shortleaf	619 (2 ramets each)	AL, AR, GA, KY, LA, MS, MO, NC, SC, TN, TX, VA	1981 – 2017
Results: how many families had a hybrid fingerprint?	0 longleaf in all sources 0 shortleaf in all sources except LA 17 families shortleaf LA source	Less than 3% of the seed in each species	

*Not every year of seed tested due to lack of seed.

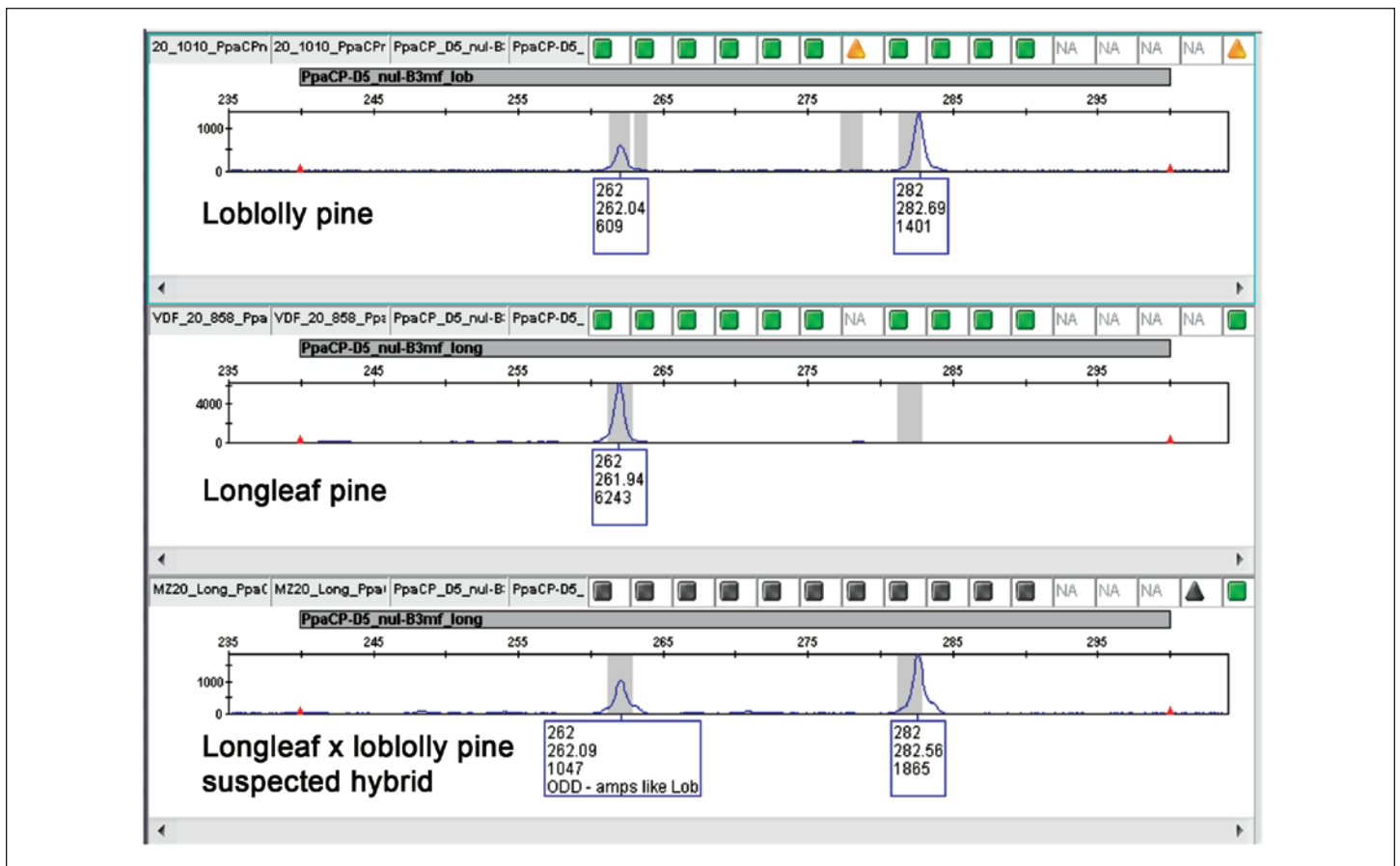


Figure 5. SSR marker profile to identify DNA fingerprints for loblolly, longleaf, and the longleaf x loblolly hybrid. (By Sedley Josseland, 2017)

Results

The objective of the project, to DNA fingerprint orchard trees and seed bank inventory for genetic purity and identify any hybrids, was accomplished. R8 NFS Genetics program, in cooperation with NFGEL and SIFG, used three DNA markers to fingerprint all longleaf and shortleaf pine clones in the seed orchards (two ramets from each clone).

Longleaf Pine Germplasm

All 250 longleaf clones, representing six seed sources, in the Louisiana and Mississippi orchards showed no hybrid DNA fingerprint (table 2). Twelve additional trees in the Louisiana longleaf seed orchard, with the unusual branchy “wolf tree” phenotype (suspected of being hybrids, figure 6), were also tested and showed no hybrid DNA fingerprint.

For the seed tested, less than 3 percent showed a hybrid DNA fingerprint. It is reasonable to surmise that there was minor pollen contamination in the longleaf orchard from the loblolly pine orchard. The phenology window for late-ripening longleaf

flowers and early ripening loblolly pollen most likely coincided, thereby creating a hybridization event. Cones are collected by breeding zone or source rather than by mother tree, so the seed is bulked. This collection method maximizes genetic diversity in the seed. Phenology data for the late-flowering longleaf clones and loblolly clones with early pollen



Figure 6. Trees with branchy “wolf” phenotypes at the longleaf pine seed orchard in Louisiana. (Photo by Barbara Crane, 2014)

maturation will be reviewed. The R8 NFS seed bank is not concerned with this very small percentage of hybrid seed, because nursery practices include culling unusual longleaf seedling phenotypes (e.g., elongated stem potentially indicative of a hybrid) at the grading table.

Shortleaf Pine Germplasm

All shortleaf clones, representing 12 sources, in the Arkansas, Mississippi, and North Carolina orchards, showed no hybrid DNA fingerprint, for both first- and second-generation orchards, for a total of 602 pure orchard clones (table 2). The shortleaf pine results are updates to the findings by Stewart et al. (2016).

Approximately 17 shortleaf clones located in the Louisiana orchard showed a hybrid DNA fingerprint. Those clones have since been eliminated from the orchard. It is reasonable to suggest that during the 1960s superior tree identification campaign, some chosen candidates were hybrids but were mistaken for shortleaf pine. Additionally, during first-generation tree breeding activities in the 1980s, it is possible that loblolly pollen was mistakenly used on shortleaf mother trees, resulting in hybrids in progeny tests. If those individuals from the progeny tests were then selected to be grafted in the second-generation orchard, that may explain why some hybrids showed up in the second generation shortleaf orchard as well.

For the seed tested, less than 3 percent showed a hybrid DNA fingerprint. It is reasonable to surmise that there was minor pollen contamination in the shortleaf orchard from the loblolly pine orchard. The phenology window for late ripening shortleaf flowers and early ripening loblolly pollen most likely coincided, hence a hybridization event. As with the longleaf germplasm, phenology data for the shortleaf clones with late flowering and loblolly cones with early pollen maturation will be reviewed. R8 NFS seed bank is not concerned with this very small percentage of hybrid seed, since nursery practices include culling unusual shortleaf seedling phenotypes (e.g., elongated stem potentially indicative of a hybrid) at the grading table.

Discussion

Longleaf and shortleaf pine ecosystems have been identified as top priorities for restoration in the

Southern Region, per each National Forest System Forest Plan. Accelerated restoration will require increased seed supplies, with seed of known quality, source, and genetic integrity. Quality seed is an important factor in the production of quality seedlings and field survivability (Barnett et al. 2002). Knowledge about the source and genetic identity of the seedlings will support successful restoration, when planting on the appropriate sites for both Federal and non-Federal forested lands. The genetic integrity of a species will favor survival, adaptation, and resiliency of the future forests. National and regional policy states that locally adapted, genetically appropriate seed sources are best to use for now. But as climate change impacts increase, the seed sources may be combined and seed movement guidelines will change to accommodate updated deployment strategies (Crane et al. 2011, Erickson et al. 2012).

The longleaf and shortleaf pine first-generation orchards were established in the 1960s, and the second-generation shortleaf orchards were established in the 1980s. Seedling seed orchards and progeny tests for both species were established throughout the 1980s and 1990s. Seed has been harvested from all orchards since the 1970s. A continuous seed supply, for multiple species, has been banked for use in reforestation and restoration on the southern national forests.

Quality seed is needed to support accelerated artificial regeneration efforts for both longleaf and shortleaf pine. Seed is often scarce and in high demand. The cone cycle frequency of both species further complicates seed availability. Longleaf pine has a bumper cone crop approximately every 5 years (figure 7), and shortleaf pine bumper cone crops occur every 5 to 7 years. Bumper cone crop years yield seed that is high quality, high vigor, and has excellent germination (Barnett et al. 2002). Cone harvest methods differ for each species. Longleaf pine cones are collected using a tree shaker (cones fall to the ground), whereas shortleaf pine cones require the use of bucket trucks or lifts to cut the cones from branch tips. Cones then have to be transported to a cone extractory, where seed is removed. Seed extraction from the cones requires experience and skill with the cone dry kilns, seed gravity tables, and X-ray machines. Longleaf pine cones must be processed and seed extracted within 2 weeks of collection; otherwise the seed will begin



Figure 7. Longleaf pine bumper cone crop in Louisiana. (Photo by Barbara Crane, 2014)

to degrade because of its thin seed coat (Barnett et al. 2002). By comparison, shortleaf pine cone processing follows a more routine protocol, with a more flexible timeframe in which to process cones and still extract quality seed.

Most pine seed must be dried down to the proper moisture content of less than 10 percent to ensure storage longevity (Barnett et al. 2002). If properly handled and processed, longleaf pine seed can be stored for 10 to 15 years and shortleaf pine seed can be stored for 15 to 20 years. The R8 NFS Genetics program ships samples of the newly harvested seed to the National Seed Lab (Dry Branch, GA; <https://www.fs.usda.gov/ns/>) to be tested for initial germination and viability. At 5-year intervals after that, the seed bank re-tests seed lots for germination and viability. This testing protocol allows us to track seed quality and degradation over time. After the seed has reached its maximum shelf life, it is disposed of according to agency regulations. Most seed bank, seed orchard, and nursery personnel are aware that the infrequency of cone crops, improper handling or processing of cones or seed, and limited storage shelf life of seed can contribute to seed scarcity and compromised seed quality.

As plans for accelerated restoration move forward, it is important to be cognizant of the genetic resources (i.e., seed orchards, seed production areas, and progeny tests) available that can provide a sustainable supply of seed (Crane and Barbour, 2009, Crane et al. 2015). This knowledge will help assess the capacity, identify needs, and ensure availability

of multiple seed sources to plant in various seed zones. A survey template to assess southern genetic resources was developed by R8 NFS Genetics program, for both longleaf and shortleaf pine (figure 8). Surveys were circulated over the past decade to a number of participants, including Federal and State agencies, universities, nongovernmental organizations, private industry and private nurseries, and tree improvement and nursery cooperatives. The survey results were summarized (table 1), and the information will help us address questions such as the following:

- Does the South have enough seed to support accelerated restoration efforts?
- Who has ownership of the genetic resources? Private, public, Federal, State?
- What is the quantity and condition of these resources? What is the age of the resource? Are the resources being managed? Have they been mothballed? Or have they been abandoned?
- Are all seed zones covered? Are any seed zones missing?
- Eastern seed zones are being updated; how will this affect seed supply and deployment e.g., Eastern Seed Zone Forum (<http://eszf.sref.info/>)?
- Are there challenges to seed processing and kiln capacity? Are there bottlenecks? Are there adequate facilities? Storage shortfalls?
- Are skills being retained? Is there succession training to develop new personnel and provide continuity of experience and skills?
- What about climate change? Are there enough seed sources to address changing climates and subsequent changing seed zones? How will this affect deployment and what will be the guidance for deployment?

In the long term, options may be considered for additional DNA marker development and more intensive DNA testing of orchard trees and future seed crops. These options, however, are expensive and time-consuming. Neither the longleaf nor shortleaf pine genomes have been mapped. Genome mapping, especially for outbred organisms that have high genetic diversity, like longleaf and shortleaf pines, will take several years and more than \$1 million. Questions remain about the number of genes in each genome and what those genes control.

Statement	
Seed Orchard Resources	ACRES
First Generation	
Second Generation	
Advanced Generation	
Seed Production Areas	
Current Orchard Management	YES NO
Original orchards retained, not managed, no seed collected	
Orchards retained, not managed, some seed collected	
Orchards retained, limited management, seed collected	
Orchards retained, actively managed, seed collected	
Orchards retained, actively managed, seed collected, additional genetics work underway or planned	
Orchards removed	
New orchards recently established on _____ acres	
Never had shortleaf orchards	
Seed Inventory	POUNDS/ 1st or 2nd GEN
Approximate annual seed collection (averaged for last five years)	
Approximate pounds of seed in storage	
Seed Age	
Program Intentions Next Five Years	YES NO
Maintain status quo	
Increase management intensity and seed collection activity	
Discontinue shortleaf efforts, remove orchards	
Mothball orchards for the time being	
Kiln Facility (Write in YES or NO and LOCATION)	
Geographic sources for shortleaf in your program:	

Geographic area where seed/seedlings are adapted for out-planting:	

Additional Comments:	

Figure 8. Longleaf pine genetic resources survey template. (By Barbara Crane, 2010)

Conclusions

There is uncertainty with climate change, and what the impacts will be on future forests. Will there be more natural hybrids? No one knows for sure. There are several management strategies in play that can deter hybrid seedling establishment on the forested landscape:

- R8 NFS Genetics program will continue to provide guidance on deploying the most appropriate genetic material, using pure seed, adhering to appropriate seed zones and seed movement guidelines (both current and updated), and monitoring future seed crops for any signs of hybridization. The program, in cooperation with partners, will work on increasing the genetic resources (seed orchards, seed production areas, seed banks) and support general forest collections to augment seed orchard collections.

- R8 NFS Silviculture programs will continue to use prescribed burning as a management tool, and monitor any evidence of hybrid seedlings during seedling survival checks.
- Nursery personnel will continue to cull any unusual seedling phenotypes at the grading tables.

One additional paramount management strategy is succession planning and training of new people in forest genetics. Genetics programs are critical in supporting successful reforestation and restoration by providing genetics expertise and genetically appropriate quality seed. Unfortunately, many challenges exist in maintaining the agency's forest genetics skills and expertise and its tree improvement and nursery programs. These challenges include: declining resources (funding, personnel); loss of skilled seed orchard, seed bank, and nursery personnel; aging seed orchards and/or lack of seed orchards representing all seed zones and seed sources; loss of forest genetics, tree physiology, and seed biology expertise; lack of, or failing, infrastructures; and loss of nurseries (Wheeler et al. 2015).

Summary

As practitioners struggle with how to restore and manage populations that are threatened with climate change, applied-academic partnerships can achieve both restoration and research goals. Through translational collaboration, we can increase the impact of our work by combining our resources to get projects done while also studying their efficacy. Engagement of professionals interested in forest genetics is an important component in this effort because it increases opportunities to collect more extensive and longer term data using different cohorts over time. However, the greatest benefit may be to stimulate interest in a diverse cadre of students to encourage them to continue on to professional careers in our disciplines and become a component of an informed citizenry.

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