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Occurrence of Shortleaf × Loblolly Pine Hybrids in Shortleaf Pine Orchards: Implications for Ecosystem Restoration

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Shortleaf pine (*Pinus echinata* Mill.) is an important conifer in much of the southeastern United States. However, the species and its associated ecosystems are in decline, and recent evidence about hybridization with loblolly pine (*Pinus taeda* L.) raises concerns that the species may be at risk of further losses due to introgression. Although shortleaf pine is not widely planted for timber production, the US Department of Agriculture (USDA) Forest Service, state forestry agencies, some conservation organizations, and private landowners use artificial regeneration to regenerate shortleaf pine for various purposes including restoration of shortleaf pine-dominated ecosystems. Given the threat of introgression with loblolly pine, we tested federal and state shortleaf pine seed orchard trees (i.e., grafted clones) and wind-pollinated seedlings (from various nurseries sourced from several of the tested orchards) for hybrid character, using a previously developed microsatellite DNA test. We found that 8 to 10% of the USDA Forest Service orchard clones and 0 to 10% of state agency clones genetically resembled F1 hybrids or first-generation backcrosses to shortleaf pine (SLBC1). Frequencies of hybrid classifications in seedlings were generally similar to those of their seed orchard parent trees (0–10%), although seedlings from the Oklahoma Forestry Services nursery contained an unusually high proportion of apparent F1 hybrids (4%), possibly due to the proximity of a loblolly pine seed orchard and loblolly pine plantations to the shortleaf pine seed orchard of origin. Based on these results, we recommend that shortleaf pine seed orchard managers consider steps to mitigate the genetic impact of trees classified as either F1 or SLBC1 hybrids to maintain the genetic integrity and desired phenotypic traits (i.e., fire, drought, and ice tolerance) of their shortleaf pine seeds, ensuring survival and adaptation of the species and its ecosystem to future climate variation.

Keywords: shortleaf pine, loblolly pine, introgression, microsatellites, artificial regeneration

Shortleaf pine (*Pinus echinata* Mill.) is an important forest tree and timber-producing species found throughout the southeastern United States. Its natural range is the largest of the southern yellow pines (subgenus *Pinus*, section *Trifoliae*, subsection *Australes*), extending further north and west than that of the other major species—loblolly pine (*Pinus taeda* L.), longleaf pine (*Pinus palustris* Mill.), and slash pine (*Pinus elliottii* Engelm.). Shortleaf pine occurs naturally on mesic to xeric sites and is a dominant forest species in western Arkansas and eastern Oklahoma. Prior work showed that shortleaf pine hybridizes naturally with loblolly pine, a species that often occupies the same sites as shortleaf pine but tends to predominate on wetter sites (Zobel 1953, Hicks et al. 1972).

Landowners and managers grow both shortleaf and loblolly pines for commercial timber production, but loblolly pine is much more widely planted with more than 1 billion seedlings planted in some years (Wear and Greis 2012). Whereas private companies, through their university-led tree improvement cooperatives, supply large quantities of improved loblolly pine seed, the US Department of Agriculture Forest Service (USDAFS) and state forestry agencies

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This article uses metric units; the applicable conversion factors are: hectares (ha): 1 ha = 2.471 ac; meters (m): 1 m = 3.28 ft.

Table 1. Materials for this study originated from USDAFS and state seed orchards.

Orchard (abbreviation)	Nearest municipality/ forest	Latitude and longitude	Material deployment zone
Mount Ida Seed Orchard (MISO)*	Mount Ida, AR	34°33′ N, 93°33′ W	Ouachita and Ozark NFs; districts in North Alabama NFs
Stuart Seed Orchard (SSO)*	Bentley, LA/Kisatchie National Forest	31°30′ N, 92°28′ W	NFs in Texas and Kistachie NFs in Louisiana
Clinton/Norris Dam Seed Orchard (CNDSO)	Norris, TN	36°13′ N, 84°4′ W	The state had to completely eliminate their orchard due to a pitch canker epidemic; previously, materials were available for purchase
John P. Rhody Nursery (JPRN)	Gilbertsville, KY	37°1′ N, 88°17′ W	Seed has not been collected for years
Magnolia Springs Seed Orchard (MSSO)	Kirbyville, TX	30°42′ N, 94°3′ W	East Texas and southeastern Oklahoma
Bluff City Seed Orchard (BCSO)	Bluff City, Arkansas	33°40′ N, 93°9′ W	Mostly Arkansas, but the state has previously sold material to buyers in Louisiana, New York, and Georgia
Baldwin Seed Orchard (BSO)	Baldwin State Forest,GA	33°4′ N, 83°15′ W	Mostly Georgia, but the state has recently sold a large amount of seed to the South Carolina Forestry Commission
Oklahoma Forest Tree Improvement Center (OFTIC)	Idabel, OK	33°41′ N, 94°45′ W	Eastern Oklahoma and western Arkansas

NF, National Forest.
*Seeds from the USDAFS.

provide most of the shortleaf pine seed for artificial regeneration. The USDAFS currently owns 95% of all known shortleaf pine orchard resources and actively manages approximately 500 acres across four geographically separated seed orchard complexes, each of which has seed sources originating from those states and the states that border them. The orchards are composed of both first- and second-generation material (although only Mount Ida Seed Orchard in Arkansas has second-generation material), representing seed sources from 12 southern states and containing upward of 50 clones per breeding zone. The USDAFS sells excess shortleaf pine seed to many state agencies and is able to provide scion material to various partners to build additional seed orchards. At present, the USDAFS, several state agencies, and many partners are involved in developing the Shortleaf Pine Initiative[1] to further support shortleaf pine ecosystem restoration.

Using microsatellite DNA markers, Stewart et al. (2012) showed that hybrid character in natural populations of both loblolly pine and shortleaf pine significantly increased between the 1950s and the 2000s. Hybridization and introgression can threaten natural species, even to the point of extinction (Rhymer and Simberloff 1996, Wolf et al. 2001). Examples of this include species of trout (Dowling and Childs 1992), mallard ducks (Rhymer et al. 1994), and mountain mahogany (Rieseberg and Gerber 1995). Given the time period in which the increase in hybridization has occurred, the causes for the elevated hybrid character are most likely anthropogenic. Tauer et al. (2012) speculated that the contributing causes include fire exclusion and succession to hardwoods, habitat fragmentation, planting of loblolly pine on shortleaf pine sites, and hybridized seed from seed. Stewart et al. (2015) showed that fire exclusion will increase hybridization over time, since regular fire selects against hybrid seedlings and saplings.

We worry that during the period when the shortleaf pine superior tree selection programs were ongoing (1970s and 1980s), natural hybrids were occasionally mistaken for pure shortleaf pine and incorporated into the seed orchards. Since much of the seed produced by the USDAFS and state agencies is intended for operational reforestation with ecosystem restoration and other ecological applications, it is important that the sources of the seed be largely free of hybrids. Maintaining the genetic integrity of shortleaf pine is critical to the restoration of resilient, biodiverse forests. In addition, compared to loblolly pine, shortleaf pine is more fire, drought, cold, and ice tolerant (Guldin 1986), providing better traits for long-term survival in varying environments. Because resilience to disturbance and potential climate change is preferred for most restoration activities, it is desirable that foresters plant, to the extent possible, genetically pure shortleaf pine seedlings. In addition, some of the restoration activities occur north or west of the range of loblolly pine where shortleaf pine is the naturally occurring and desired foundational forest tree species. To more fully understand the potential for hybridization and introgression within seed orchard and nursery stocks of shortleaf pine, we investigated the degree of hybridization in the parental trees (i.e., grafted clones) making up the seed orchards and in their nursery seedling crops. A thorough understanding of the situation in each seed orchard is important to have before recommending courses of action for seed orchard and nursery managers.

Materials and Methods

Source Material

Shortleaf pine material for this study came from USDAFS and state forestry agency seed orchards (Table 1). Seed orchard employees mailed us needles either on ice or packed with silica desiccant. From each seed orchard, we tested all clones (287 in total) present, by sampling one ramet. For those clones that were classified as F1 or first-generation backcrosses into shortleaf pine (SLBC1), orchard managers collected additional ramet samples for us to test to ensure clonal identity of the initial ramet sample.

International Forest Company provided 40 seedlings originating from USDAFS and state agency orchards (20 seedlings labeled IF1193 from BCSO; 20 seedlings labeled IF1191, Ozark seed source collected in 2005, from MISO), Oklahoma Forestry Services provided 100 seedlings originating from the OFTIC orchard, the Missouri Department of Conservation provided 40 seedlings originating from the MISO Orchard, the Kentucky Division of Forestry provided 40 seedlings originating from the JPRN orchard, and the South Carolina Forestry Commission provided 40 seedlings originating from the BSO orchard (Georgia Forestry Commission.) All samples arrived as bareroot seedlings, and needle tissue samples were frozen and stored at −20°C.

Morphological Measurements

Shortleaf pine seedlings often exhibit a basal crook, which is a short segment of stem just above the rootcollar that lies flat on the ground. Crook characteristics for each seedling received were measured on arrival in the laboratory. Lower angle refers to the angle from the ground for the lower turn of the basal crook, using the

Table 2. Frequencies of hybridization for each of the USDAFS and state seed orchards sampled were very similar.

Sample	By clones		By ramets		State seed orchards					
	MISO	SSO	MISO	SSO	CNDSO	MSSO	JPRN	BCSO	BSO	OFTIC
	[no. (%)]									
F1	2 (1)	1 (3)	283 (2)	6 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
SLBC1	9 (7)	2 (7)	994 (8)	12 (5)	1 (3)	0 (0)	1 (4)	0 (0)	2 (10)	0 (0)
SLBC2	10 (7)	2 (7)	1,225 (10)	18 (8)	2 (5)	0 (0)	2 (7)	2 (18)	0 (0)	0 (0)
SLBC3	17 (12)	4 (14)	1,401 (11)	36 (15)	5 (13)	2 (67)	2 (7)	3 (27)	4 (20)	2 (10)
SL	100 (72)	20 (69)	8,974 (70)	162 (69)	31 (79)	1 (33)	22 (81)	6 (55)	14 (70)	18 (90)
Total	138	29	12,877	234	39	3	27	11	20	20

For USDAFS orchards, both clone and ramet counts are present. For state seed orchards, only clone count is present. Each clone is represented by a varying quantity of ramets in each seed orchard.

taproot as the guide for straight down. Upper angle refers to the upper turn of the basal crook, and the angle is also relative to the ground. Crook length was measured to the nearest 1 cm. Whereas we could not measure individuals without basal crooks, we classified seedlings as having strong crooks when they had a difference between the upper and lower angles of 60° or more. The remainder ($>0°$ to $\leq 60°$) were classified as having weak crooks.

DNA Extraction

Needle tissue was disrupted in one of two ways. For samples sent on ice, 150 mg of tissue was ground in a mortar and pestle with liquid nitrogen. For samples sent with desiccant, 50 mg of leaf tissue was dried for at least 7 days in silica gel desiccant beads (Dri Splendor; Miracle Coatings, Anaheim, CA) and ground in a ball mill with chromium-steel beads. DNA was extracted with the DNeasy plant mini kit (Qiagen, Valencia, CA) with 150 μl of final effluent.

Microsatellite Markers

Twenty-five microsatellite primer pairs were used in this study: PtSIFG_0493, PtSIFG_1018, PtSIFG_0424, PtSIFG_0437, PtSIFG_1207, PtSIFG_1295, PtSIFG_1190, PtSIFG_0587, PtSIFG_0265, PtSIFG_0440, PtSIFG_1008, PtSIFG_1166, RPTest8, PtRIP_0388, PtRIP_0619, PtRIP_0211, and PtRIP_0079 from Echt et al. (2011); PtTX4205 and PtTX3052 from Auckland (2002); PtRIP_0629 from Nelson et al. (2006); RPTest9 from Williams et al. (2001); PtTX3034, PtTX2123, and PtTX3013 from Elsik et al. (2000); and SsrPt_ctg4487b from Chagné et al. (2004). Polymerase chain reaction and gel electrophoresis conditions are provided in Stewart et al. (2010). Allele names were standardized using reference samples and a scoring system similar to that described in Deemer and Nelson (2010).

Analysis

Marker data were analyzed using Structure 2.3.4 (Hubisz et al. 2009). The marker data from Stewart et al. (2012) were added to the data set to include known loblolly pine examples for reference purposes. Settings were 20,000 cycles of burn-in and 50,000 Monte Carlo Markov chain cycles after burn-in with the inferred alpha ancestry model and initialized POPINFO (which was set using the Stewart et al. [2012] sample). We used two populations ($K = 2$) to represent the two parental species, loblolly pine and shortleaf pine. Since $Q_{loblolly} = 1 - Q_{shortleaf}$, only one Q value, $Q_{loblolly}$ was used for analysis. For discussion purposes, trees were classified by their Q value into species and various hybrid classifications as follows: shortleaf pine for $Q_{loblolly} = 0$ to 0.046, as F1 hybrid backcrossed to shortleaf pine 3 times (SLBC3) for $Q_{loblolly} = 0.047$ to 0.094, as backcrossed to shortleaf pine 2 times (SLBC2) for $Q_{loblolly} = 0.095$ to 0.118, as backcrossed to shortleaf pine once (SLBC1) for $Q_{loblolly} = 0.119$ to 0.375, or as F1 hybrid for $Q_{loblolly} = 0.376$ to 0.625. We detected no individuals with $Q_{loblolly} > 0.625$.

Results and Discussion

Seed Orchards

For the USDAFS seed orchards, we classified 8% of shortleaf pine clones from Mount Ida and 10% of shortleaf pine clones from Kisatchie as either F1 or SLBC1 hybrids. We classified the remaining trees as SLBC2 (backcrossed two times to shortleaf pine) or SLBC3 (backcrossed three times to shortleaf) or as nonhybrid shortleaf pine trees (Table 2; Figure 1). There were 1,295 (9.9%) F1-classified and SLBC1-classified ramets in the USDAFS orchards, most of which were in the MISO because of the much greater number of total ramets there. Additional tests of three more ramets of each clone showed consistency with the original ramet that we tested, indicating that the initial samples were representative of the clones. The USDAFS should consider steps such as rogueing and sorting seed out from especially introgressed orchard trees to reduce future introgression in shortleaf pine seedlings. Overall, none of the state seed orchard clones tested were F1-classified hybrids, and only 4 of the 120 were SLBC1-classifed hybrids (Table 2, Figure 2). Given the small number of clones with significant hybrid components, removing ramets classified as SLBC1 from seed orchards would be modestly beneficial and seemingly worth the effort, given the low cost.

Although the SLBC2-classified (~7/8 shortleaf pine) and SLBC3-classified (~15/16 shortleaf pine) trees may include some loblolly pine character, most of their genomic material appears to be shortleaf pine. If orchard managers can mitigate crosses between these less introgressed seed orchard trees and the more introgressed F1- and SLBC1-classified trees, then their impact on seedling genetics can be minimized; i.e., pollination of SLBC2 clone by shortleaf pine results in SLBC3. Stewart et al. (2015) showed that many pine seedlings and saplings categorized as SL, SLBC3, and SLBC2 survived recurring prescribed fires and persisted, whereas F1 and SLBC1 seedlings generally did not persist. At the very least, inclusion of SLBC2-classified or greater shortleaf pine character seedlings at sites with artificial regeneration should help to establish good shortleaf pine populations with genetic integrity for future natural selection and regeneration.

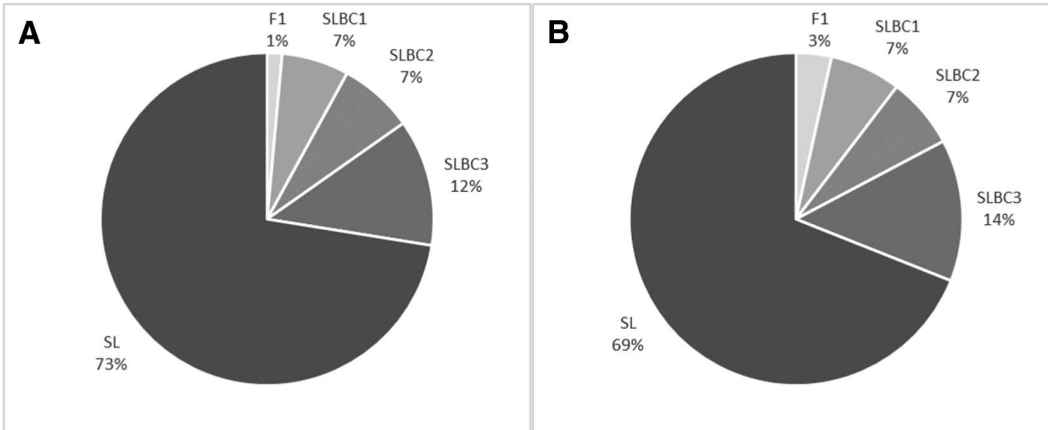

Figure 1. Similar hybrid frequencies were found in seed orchard trees from US Forest Service seed orchards Mount Ida Seed Orchard (A) and Kisatchie Seed Orchard (B).

Quality shortleaf pine seedlings are important for federal, state, and private shortleaf pine ecosystem restoration efforts. The species, once described as dominating the forests of the southeastern quarter of the United States (Mohr and Roth 1897), continues to decline in dominance and land coverage (Oswalt 2012). Because shortleaf pine is at risk of further decline because of introgression (Stewart et al. 2012), avoiding the planting of hybrid seedlings should be paramount to all interested parties such as federal and state land management agencies and private landowners who intend to restore native ecosystems.

As the USDAFS and state forestry agencies rogue the hybrid clones in their shortleaf pine seed orchards, genetic diversity of the remaining clones and resulting wind-pollinated families should be evaluated. Seed zones that are underrepresented or absent from the seed orchards should be incorporated into orchards. The USDAFS and some state agencies are already in the process of exchanging scion material to replenish respective orchards. In addition, the USDAFS will be measuring a portion of their 155 shortleaf progeny tests to provide data and materials for selecting desirable trees for second-generation orchard establishment.

Special considerations should be made for the role of shortleaf pine in southeastern US forest management. Although loblolly pine is the predominant species used in plantation silviculture (over 11 million ha) (Wear and Greis 2012), shortleaf pine is a historically important component of southeastern US forests (Mohr and Roth 1897, Tauer et al. 2012). Given its tolerance to drought, fire, ice, and fusiform rust (Guldin 1986), it will be an important component in a resilient future of southeastern US forests and woodlands. Maintaining diversity in shortleaf pine seed sources, both within and between regions, is important for restoration of ecosystems and will help to mitigate the potential effects of climate, whether local or warmer climate-adapted sources are used. New planting zone maps for shortleaf pine and other species have been developed to help land managers make decisions about what species to plant where[2] so that they will survive and reproduce in the next several hundred years.

Seedlings

The hybrid character of shortleaf pine seedlings varied by source-seed orchard and was generally in alignment with the source-seed orchards themselves, when data for both the seed orchards and the seedlings were available (Table 3). Seedlings from OFTIC represent a notable exception to this trend, as there were more hybrid seedlings than hybrid seed orchard trees (4% F1- and 1% SLBC1-classified hybrids compared with 0% F1- or SLBC1-classified hybrids in the seed orchards).

The Oklahoma Forestry Services seed orchard is adjacent to the agency's loblolly pine seed orchard and a private loblolly pine plantation (Justin Jones, Oklahoma Forestry Services Forest Trees Improvement Center, May 15, 2013), and the seed orchard is within the natural range of loblolly pine, resulting in a significant proportion (4% in our study) of Oklahoma-sourced shortleaf pine seedlings being sired by loblolly pine. Pollen contamination is a potential problem for seed orchards, although aside from moving the seed orchard or removing nearby loblolly pine pollen sources through harvesting trees, little can be done to confront the problem (Wang et al. 1991, Adams et al. 1997). Stewart et al. (2013) reported that the pollen from nearby loblolly pine plantations in particular affects the rate of hybridization in naturally regenerating shortleaf pine stands. In contrast to the Oklahoma and South Carolina seed orchards, the seed orchards that provided seed for International Forest Company (MISO and BCSO) and the states of Missouri (MISO) and Kentucky (JPRN) are beyond the natural range of loblolly pine which apparently reduced the likelihood of contamination from neighboring loblolly pine trees.

Although shortleaf pine seedlings do not necessarily have basal crooks when they are grown in nursery conditions (Wakeley 1954), probably because dense seedbed plantings limit the ability of crook formation, F1- and SLBC1-classified hybrids had fewer and weaker basal crooks than less hybridized seedlings (Table 4). In naturally regenerating shortleaf pine stands, seedlings and saplings exhibit the basal crook trait that facilitates better resprouting after surface fires (Mattoon 1915). Loblolly pine seedlings do not have this characteristic, and hybrids of shortleaf pine and loblolly pine tend to have intermediate or weak basal crooks (Lilly et al. 2012, Will et al. 2013). The trait probably plays an important role in excluding hybrids from sites with regular fire return intervals (Stewart et al. 2015). Because seedlings produced in nursery settings do not reliably produce this trait, managers may need to limit the use of fire on sites with artificial shortleaf pine regeneration or plant seedlings deeper to protect the dormant buds, unless they observe that the seedlings form crooks after planting. According to our data, nursery managers also cannot rely on culling seedlings without crooks from

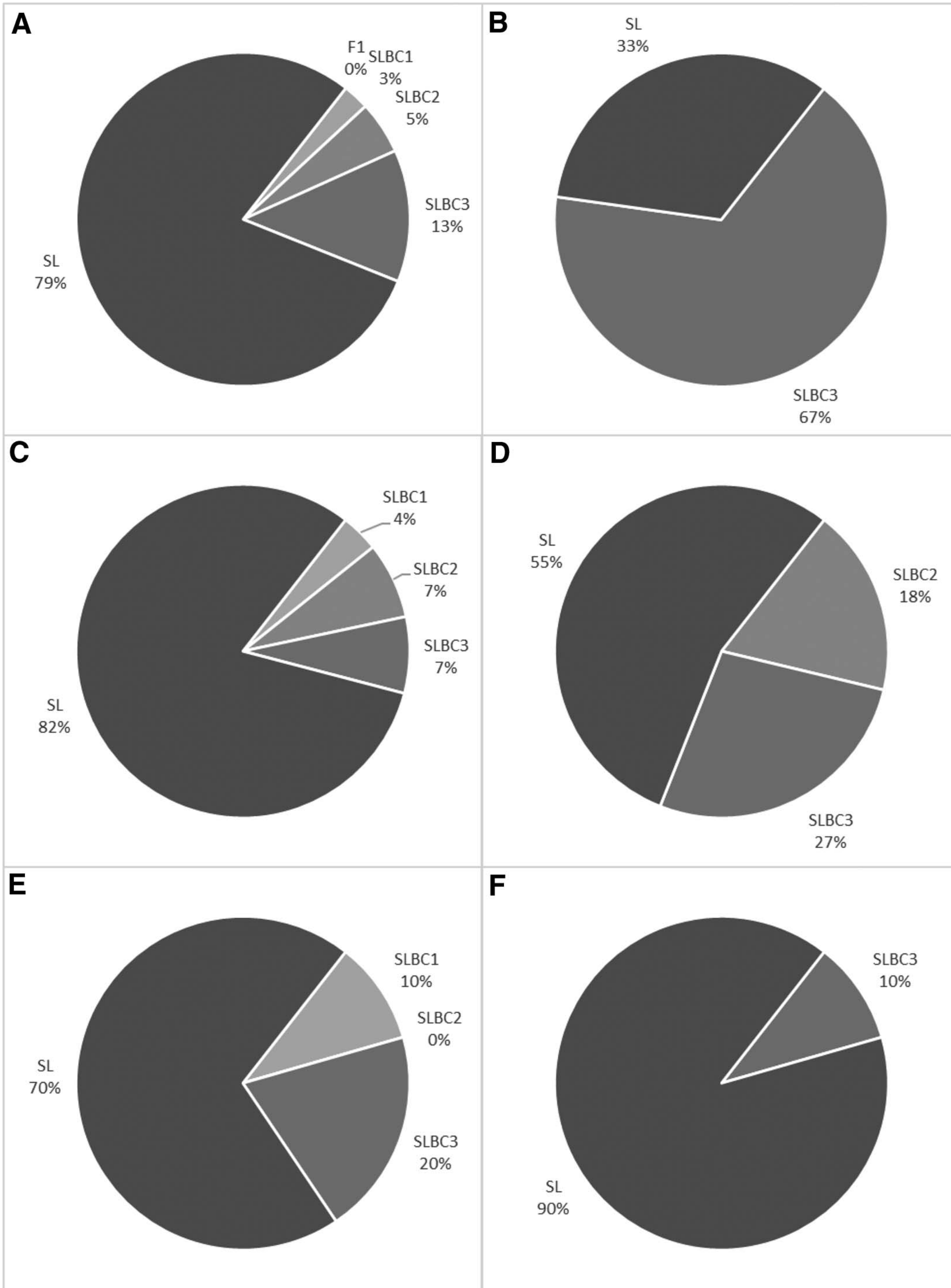

Figure 2. State seed orchards had little hybridization in their shortleaf pine parent populations. Few differences among the populations from CNDSO (A), MSSO (B), JPRN (C), BCSO (D), BSO (E), and OFTIC (F) were noted.

their inventory to reduce hybrids since they would remove far too many nonhybrid shortleaf pine seedlings.

Conclusion

Whereas most of the shortleaf pine seedlings produced by nurseries using federal and state seed orchard-produced seed are nonhybridized shortleaf pine, even low levels of hybrid character are a potential problem for current and future efforts to rebuild resilient pine forests in the southeastern United States. By identifying the scope of the problem and mitigating crosses between highly introgressed individuals, we can increase the overall shortleaf pine character of artificial regeneration projects throughout the region. We recommend phasing out clones with hybrid character from seed orchards and mitigating pollination from existing hybrid seed orchard trees. In addition, agencies will need to consider how they would prefer replacing those clones in the future.

Overall, pollen contamination with loblolly pine did not appear to be a major problem. However, the frequency of hybrids was greater for the orchards in Oklahoma and South Carolina,

Table 3. **Percent hybrid classification found in each set of seedlings varied some by source seed orchard, most representing the rough proportions from their seed orchard sources.**

Sample	OKSeed	IF1191	IF1193	MOSeed	KYSeed	SCSeed
	. . . .	. . . . . .	. . . (%) . . .	. . . . . .	. . . . . .	. . . . . .
F1	4	0	0	0	0	3
SLBC1	1	10	5	8	0	3
SLBC2	4	15	5	11	3	6
SLBC3	11	10	5	11	8	10
SL	80	65	85	69	89	77
Total	95	20	20	36	37	31

Oklahoma seedlings, however, had more hybrid character than the seed orchard trees from which they originated. IF1191 and IF1193 are two groups of seedlings from Arkansas and USDAFS seed orchards grown by International Forest Company.

Table 4. **F1 hybrid seedlings and shortleaf pine backcross 1 hybrid seedlings lack strong basal crooks, but the lack of a basal crook is not a good indication that a nursery-grown seedling is a hybrid.**

Characteristic	F1	SLBC1	SLBC2	SLBC3	SL
	. . . . . .	. . . . . .	. . . (%) . . .	. . . . . .	. . . . . .
No crook	80	25	33	30	34
Weak crook	20	75	53	52	54
Strong crook	0	0	13	17	13

which were surrounded by loblolly pine plantations. Maintaining shortleaf pine seed orchards in areas without nearby loblolly pine is critical to minimize pollination of shortleaf pine by loblolly pine. Although no guidelines or research exists for shortleaf pine and loblolly pine pollination distances in seed orchards, studies of similar situations in loblolly pine alone (Friedman and Adams 1985), Scotch pine (Wang et al. 1991), and Douglas-fir (Adams et al. 1997) have been completed. In those cases, it was found that pollination occurred within 122 m (Friedman and Adams 1985). Therefore, it seems reasonable for the USFS to consider cutting down loblolly pines from around the periphery of their shortleaf pine seed orchards. In addition, a continued fire regime of frequent prescribed burns will further help to eliminate hybrids that have been planted in shortleaf pine reforestation efforts.

Endnotes

1. For more information, see http://shortleafpine.net/ and http://www.sref.info.
2. For more information, see http://planthardiness.ars.usda.gov/.

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