LONGLEAF PINE HYBRIDIZATION: IS THERE A GROWING PROBLEM?

James P. Barnett, Rabiu Olatinwo, D. Paul Jackson, and Stacy Blomquist

Abstract—Longleaf pine (*Pinus palustris*) seedlings grown from seeds collected from two seed orchards in 2014 showed evidence of hybridization with loblolly pine—as much as 80 percent had some stem elongation in the container nursery. This stem elongation, however, was not to the extent that has typically characterized bareroot seedlings of Sonderegger pine (*P.* x *sondereggeri*), the recognized hybrid of longleaf and loblolly (*P. taeda*) pine. Seedlings from these collections were quantified and outplanted. These outplanted seedlings were tracked to determine if they were Sonderegger pine and analyzed by DNA techniques to confirm their taxonomic status. The results indicate that hybridization has not occurred. Seedling development following outplanting and DNA analyses indicate these seedlings are not markedly different from those of typical longleaf pine. The reason for the unusual stem elongation in the nursery has not been determined.

INTRODUCTION

In the summer of 2015, the manager of a container nursery in southern Georgia reported that up to 80 percent of longleaf pine (*Pinus palustris*) seedlings from two seed orchards being grown under contract were showing significant stem elongation. This is the traditional and accepted method for determining if longleaf seedlings have been hybridized with loblolly pine (*P. taeda*). Such hybrids, named Sonderegger pines (*P.* x *sondereggeri*) (Chapman 1922), are typically culled at the nursery due to the poor form and quality they show when outplanted (Wakeley 1954). Based on this reputation, many landowners decline to plant Sonderegger seedlings. However, the elongation observed in the container nursery had not developed to the extent (12 to 15 cm) noted in bareroot nurseries where other seedlings have previously been classified as Sonderegger pines (fig. 1).

The two orchards are major sources of seeds for the longleaf pine reforestation program in the Southern Region of the Forest Service, U.S. Department of Agriculture. The Craig Seed Orchard, located in south Mississippi, is owned by the Mississippi Forestry Commission but provides longleaf seeds to the Southern Region. The Stuart Seed Orchard, located at Stuart Nursery on the Kisatchie National Forest in central Louisiana, provides seed for West Gulf reforestation in Louisiana and Texas. When stem elongation was observed on longleaf seedlings from these orchards, the immediate questions were: if these seedlings are Sonderegger pines, is the genetic base for longleaf pine being compromised, and should these seedlings be outplanted? Also, is this a 1-year phenomenon or an indication of possible longer term effects of climate change?

Because of the serious implications, scientists from the Forest Service, Southern Research Station's (SRS) Research Work Units (RWUs) 4552, 4159, and 4158 at Pineville, LA, joined a Longleaf Pine Hybridization Team[1] to (1) conduct DNA analyses of the seed sources in question to determine whether hybridization occurred; (2) monitor the morphological development of the seedlings before and after outplanting; (3) determine if weather conditions at time of pollination may have increased the opportunity for cross-pollination; and (4) provide scientific data for managers to make decisions on the use of the seedlings. Through a multi-pronged approach, we hoped to learn if hybridization between longleaf and loblolly pines in the two orchards had occurred and if DNA analysis of seeds can be used to determine the status of hybridization in stored seeds. To do this, SRS scientists in Pineville coordinated the development of DNA markers for the determination of

[1] Longleaf Pine Hybridization Team members were James Barnett, Emeritus Scientist, SRS RWU-4159; Brian Strom, Research Entomologist, and Rabiu Olatinwo, Research Plant Pathologist, SRS RWU-4552; D. Paul Jackson, Associate Professor, Louisiana Tech University, School of Agricultural Sciences and Forestry; Stacy Blomquist, Public Affairs Officer, Kisatchie National Forest; Alex Mangini, Entomologist, Forest Health Protection; Susana Sung, Research Plant Physiologist, SRS RWU-4158; and Jessica McKenney, Research Associate, Louisiana State University AgCenter.

Author information: James P. Barnett, Retired Chief Silviculturist and Emeritus Scientist, U.S. Department of Agriculture, Forest Service, Southern Research Station, Pineville, LA 71360; Rabiu Olatinwo, Research Plant Pathologist, U.S. Department of Agriculture, Forest Service, Southern Research Station, Pineville, LA 71360; D. Paul Jackson, Associate Professor, Louisiana Tech University, School of Agricultural Sciences and Forestry, Ruston, LA 71272; and Stacy Blomquist, Public Affairs Specialist, U.S. Department of Agriculture, Forest Service, Kisatchie National Forest, Pineville, LA 73260.

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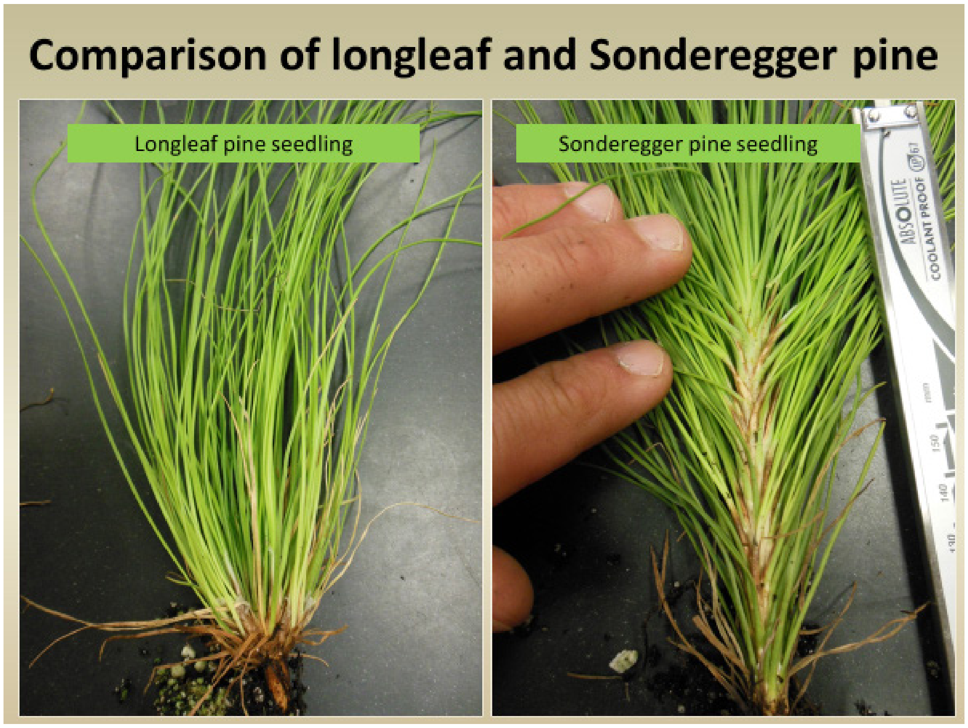


Figure 1—Standard visual differences between container longleaf and Sonderegger pine seedlings.

hybridization with geneticists from the SRS Southern Institute of Forest Genetics (RWU-4160) at Saucier, MS, and the Southern Region in Atlanta, GA. Collaboration with Louisiana Tech University's School of Agricultural Sciences and Forestry facilitated the study of seedling morphological development. Staff at the Pineville offices of Southern Region, Forest Health Protection and the Kisatchie National Forest provided expertise, resources, and a planting site at the Stuart Seed Orchard.

METHODS

Seedlot Acquisition

Since the status of about 12,000 container longleaf pine seedlings was in question, an immediate objective was to determine if hybridization of seed from cones collected in 2014 from the Craig and Stuart seed orchards had occurred (fig. 2). Several hundred container seedlings from these seedlots were selected randomly, taken from the nursery, and shipped to Louisiana Tech University in late July 2015. Another collection of cones from the Craig Seed Orchard was made in the fall of 2015. Mike Lee of the Mississippi Forestry Commission coordinated the cone collection, and the Forest Service's cone and seed processing facility at Brooklyn, MS, processed the seeds. Seedlings were also grown from stored seeds from the Craig Seed Orchard's 2013 and 2014 crops and from the Stuart Seed Orchard's 2014 crops for another evaluation of seedlings from the seedlots. Seeds from these sources were grown at the International Forest Company nursery at Evans, LA. These seedlings were subjected to the same detailed measurements as those from the earlier evaluations.

In addition to growing seedlings again from both 2014 collections from the Craig and Stuart seed orchards, seeds from Craig Seed Orchard's 2013 and 2015 collections were sown to be evaluated. This was done to determine if the possible hybridization was just a 1-year occurrence or a developing trend. Because no published information on the growth and development of container-grown Sonderegger seedlings is available, plants from Stuart Seed Orchard that met the traditional criteria for hybridization and would have been historically culled were instead outplanted to provide a comparison for evaluating the development of those grown from the 2014 sources in question.

Seedling Morphology in the Nursery

Sonderegger seedlings in the past were noted in beds of bareroot longleaf pines by their prominent stem elongation of 12 to 15 cm —clearly, they stood up above the surrounding longleaf plants that remained in the grass stage. This is the only current means to quantify Sonderegger pine seedlings in the nursery. Through the summer and fall, monthly measurements of needle length and stem elongation were made for individual seedlings to document their growth and development. Subsets of these seedlings were outplanted in the fall of 2015 so that growth patterns could be determined and related to results of DNA analyses.

Figure 2—The location of the two seed sources in question are near the same latitude. The Louisiana site is the Stuart Seed Orchard of the Kisatchie National Forest, and the Mississippi site is the Craig Seed Orchard of the Mississippi Forestry Commission.

Craig Seed Orchard 2014 seedlot—In August 2015, four trays with 198 container longleaf pine seedlings each from the Craig Seed Orchard were taken to Louisiana Tech University for the evaluation of seedling morphology. Seedling morphological measurements included root collar diameter, height (stem between cotyledons and the tip of the terminal shoot bud), longest needle length, and fascicle and primary needle presence. The seedlings were measured on August 10, September 21, and November 9, 2015. Stem elongation was recorded to the nearest half centimeter.

Stuart Seed Orchard 2014 seedlot—In October 2015, two trays with 198 container longleaf pine seedlings each from the Stuart Seed Orchard were taken to Louisiana Tech University for the evaluation of seedling morphology. The same measurements described above were performed on October 5 and November 20, 2015.

Craig Seed Orchard 2013 seedlot—In October 2015, one tray with 198 container longleaf pine seedlings from the Craig Seed Orchard was brought to Louisiana Tech University for the evaluation of seedling morphology. The same measurements were performed on these seedlings on October 5 and November 20, 2015.

Seedling Development After Outplanting

The first outplanting of seedlings in the fall of 2015 was lost due to feral animals that pulled most of the seedlings from the ground. Seedlings from the same sources were grown at a Louisiana container nursery in following years from stored seed and the planting site moved to the Stuart Seed Orchard where they were more easily protected and monitored.

Seedlings were randomly outplanted to monitor their individual development. Further randomization was achieved by sorting seedlings by their stem elongation measurements (least to most) and then numbering them sequentially. A random sequence of numbers was generated from 1 to the maximum number (343 for the Craig Seed Orchard 2014 seedlot, 185 for the Stuart Seed Orchard 2014 seedlot). Seedlings were arranged in trays by their sequence number, brought to the field, and outplanted in that sequence (Jackson and others 2020). To provide a comparison with known Sonderegger seedlings, seedlings culled because of hybridization from a Stuart Seed Orchard 2016 seedlot were outplanted to compare their performance with the study seedlings from the Craig Seed Orchard 2014 seedlot. Heights of these seedlings were measured after the first growing season.

DNA Evaluation

Samples of needle tissue were taken from seedlings of known size and needle development for DNA analysis. Barbara Crane, Regional Geneticist of the Southern Region, also authorized analysis of 48 tissue samples from this crop to be done by the National Forest Genetics Laboratory (NFGEL) at Placerville, CA. This lab has the expertise and tools needed to separate southern pine species using chloroplast technology.

Early discussions with geneticists from SRS RWU-4160 and the Southern Region about their assistance in conducting the needed DNA analyses of the samples indicated limitations due to both the status of marker development for the species and the timely availability of unit resources to conduct the analyses. With the recent hire of Rabiu Olatinwo as a Research Plant Pathologist at SRS RWU-4552 in Pineville, an effort began to develop DNA techniques for separating longleaf from known loblolly and Sonderegger pines on site. Through his efforts, procedures for establishing appropriate DNA markers for longleaf and loblolly pine were developed. This study provided an evaluation of his approach with the somewhat different techniques developed by SRS RWU-4160 and Southern Region personnel.

To determine if the seedlings with some limited stem elongation were indeed hybridized, seedlings were analyzed and had their DNA compared by using two chloroplast DNA markers with a set of specific primers for each (one for longleaf pine and one for loblolly pine) (Olatinwo and others 2020). Olatinwo and others (2020) extracted DNA from each seed using the Qiagen DNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. The DNA from known longleaf pine and loblolly pine were included in the polymerase chain reaction (PCR) amplification to serve as positive controls in evaluating the seed samples. Amplification of DNA was performed in 10 μl PCR reaction in an Eppendorf Mastercycler® Pro PCR machine (Eppendorf AG, Hamburg, Germany). Gel electrophoresis was performed to examine amplified products by loading 5 μl PCR products on 1-percent agarose gels. The agarose was stained with ethidium bromide after 20 minutes of electrophoresis, and the resulting bands were visualized under ultraviolet (UV) illumination to confirm positive or negative amplifications. A band indicates positive amplification, while no band indicates negative. A positive with the longleaf marker identifies a sample as a longleaf pine, while a negative with longleaf marker indicates the sample is not a longleaf pine. Similarly, a positive with the loblolly marker identifies a sample as a loblolly or a Sonderegger pine, while a negative with the loblolly marker indicates the sample is not a loblolly or a Sonderegger pine. Therefore, if a sample is longleaf-marker positive, it is not a Sonderegger hybrid.

Weather Patterns During Pollination

Weather patterns at or near the orchards, especially temperatures, during the 2013 pollination period for longleaf and loblolly pine were evaluated. Data comparison to weather measurements from this and earlier years were made to try to understand if hybridization of these seed sources may have occurred. Boyer (1973, 1978) established that heat accumulation can be used to accurately predict peak pollen shed. Degree-day heat sums can be derived from local temperature data. The procedure can provide early warning of unusually early or late flowering. Degree-day heat sums for longleaf and loblolly pine accumulate from base temperatures of 50 and 55 °F, respectively. Peak pollen shed is predicted when 1,208 degree days for longleaf and 636 degree days for loblolly accumulate. This procedure was used to determine peak pollen shed times in the spring of 2013 that led to the development of the two sets of cones in 2014. Because actual data documenting pollination times for longleaf and loblolly pine are not available, Boyer's heat-sum models were used to predict pollination periods for these species and the overlap using historical weather data from nearby weather stations. These analyses provide some understanding of climatic changes that may have resulted in changes of pollination time overlap between longleaf and loblolly. Importantly, evaluations were made to evaluate whether the year 2013 (the pollination year for the questionable seed from the two orchards) was likely to have pollen/receptivity overlap between the two species.

RESULTS

Seedling Morphology in the Nursery

At the time of the last measurement, 79 percent of a sample of 343 seedlings from the 2014 Craig Seed Orchard seedlot had initiated some level of stem elongation with a range from 1.5 cm to 13.0 cm (table 1). A stem measurement of 1.0 cm was characterized as being normal for longleaf pine due to the hardening of the area for normal bud set. At the time of the last measurement for the 2014 Stuart Seed Orchard

Table 1—Seedlings grown in 2015 from 2014 Craig and Stuart Seed Orchard seedlots with stem elongation in the nursery

	Craig Seed Orchard seedlot	Stuart Seed Orchard seedlot
Elongation (*cm*)	Number (*percent*)	Number (*percent*)
≤1.0	71 (21)	22 (12)
1.1–2.5	105 (31)	73 (39)
2.6–5.0	143 (42)	74 (40)
5.1–7.5	21 (6)	16 (9)
≥7.6	3 (<1)	0 (0)
Total	343	185

Seedlings were measured in November 2015 before outplanting.

seedlot, 88 percent of a sample of 185 seedlings had initiated some level of stem elongation with a range from 1.1 cm to 7.0 cm (table 1). Finally, at the time of the last measurement of the 2013 Craig Seed Orchard seedlot, only two seedlings (1.9 percent) of a sample of 102 seedlings had initiated stem elongation exceeding 1.0 cm, and they measured only 2.5 cm and 3.0 cm. These results are typical for a normal longleaf pine seed source.

Seedling Development After Outplanting

Outplanted seedlings from the Craig Seed Orchard 2014 crop performed like longleaf in the field trial—the only seedling with appreciable elongation was a known hybrid in the seedlot. The Sonderegger seedlings had stem elongation when planted and continued to grow after planting with most growing to about 24 cm after 1 year (table 2).

DNA Evaluation

The results of gel electrophoresis of PCR products from the longleaf-marker primers used in evaluating DNA samples from 64 pine seedlings (29 from the Stuart Seed Orchard 2014 seedlot, 17 from the Craig Seed Orchard 2014 seedlot, and 18 from Craig Seed Orchard 2013) showed no indication of hybridization (fig. 3). Natural hybridization between longleaf and loblolly was not detected in the sample of seedlings evaluated from the three seedlots included in this study; however, the genetic markers and the corresponding specific primers developed for detection of a "pure" longleaf from a Sonderegger hybrid or loblolly will facilitate routine evaluation of suspect pine seedings in the future. The methodology will also allow evaluation of hybridization in the seeds themselves.

Weather Patterns During Pollination

The application of the heat-sum data to the Craig and Stuart seed orchards provide the following information. In years 2002, 2005, 2006, 2009, 2012, and 2013, longleaf pine reached peak flowering before loblolly. Conversely, in years 2001, 2003, 2004, 2007, 2008, 2010, 2011, 2014, 2015, and 2016, loblolly pine reached peak flowering before or at the same time as longleaf pine.

The graphic data are reflected in these flowering times that are provided in figure 4. These data confirm Boyer and Woods' (1973) conclusion that pollination times for longleaf and loblolly frequently overlap, and which species reaches peak pollen shed first varies from year to year. Our data do not provide any clarity on whether pollination time had any influence on the 2014 seed crop seedling developmental issue.

DISCUSSION

The report in 2015 that a large portion of seedlings from two longleaf pine seed sources collected in 2014 may have been hybridized naturally with loblolly pine raised concern among forest geneticists and silviculturists. The results of our DNA analyses and the field outplanting studies indicate that hybridization was not responsible for the stem elongation phenomenon. DNA analyses conducted locally and by both the SRS RWU-4160 scientists and the National Forest Genetics Laboratory specialists indicate that hybridization did not occur. The outplanted seedlings have remained in the grass stage and are performing as typical longleaf pine seedlings. The seedlings grown from the 2014 Craig and Stuart seed orchard seedlots do not meet the Sonderegger characterization. Some limited stem elongation occurred

Table 2—Development of seedlings after one growing season from the Craig Seed Orchard 2014 seedlots compared to culled Sonderegger seedlings from the Stuart Seed Orchard 2016 seed source

Craig Seed Orchard 2014 seedlot			Stuart Seed Orchard 2016 Sonderegger seedlings[a]		
Stem elongation (*cm*)	Number of seedlings	*Percent*	Height (*cm*)	Number of seedlings	*Percent*
≤2.0	162	72	≤10	1	<1
2.1–3.0	37	16	11–20	29	22
3.1–4.0	21	9	21–30	78	60
4.1–5.0	4	2	31–40	22	17
5.1–6.0	0	0	41–50	1	<1
≥6.1	1[b]	<1	≥51	0	0
Total	225	100	Total	131	100

[a] The Sonderegger seedlings had stem elongation when planted and continued to grow after planting.

[b] The only Craig Seed Orchard seedling with appreciable elongation was a known hybrid in the seedlot.

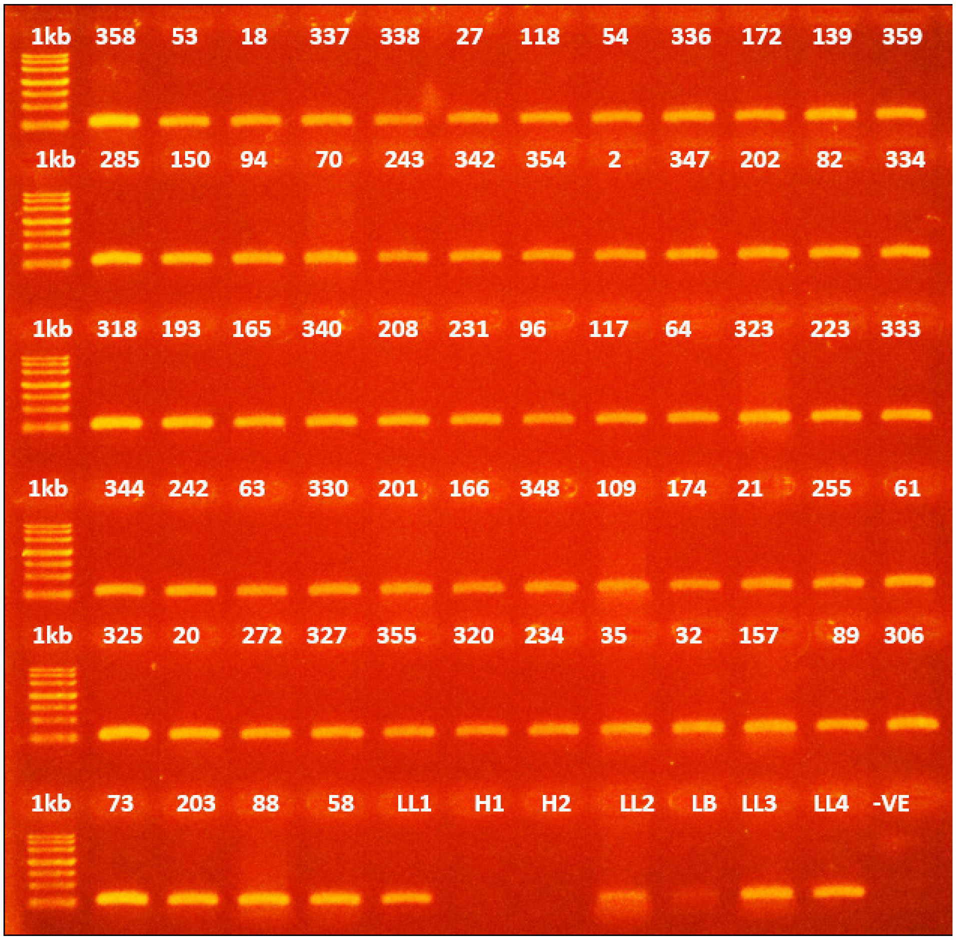


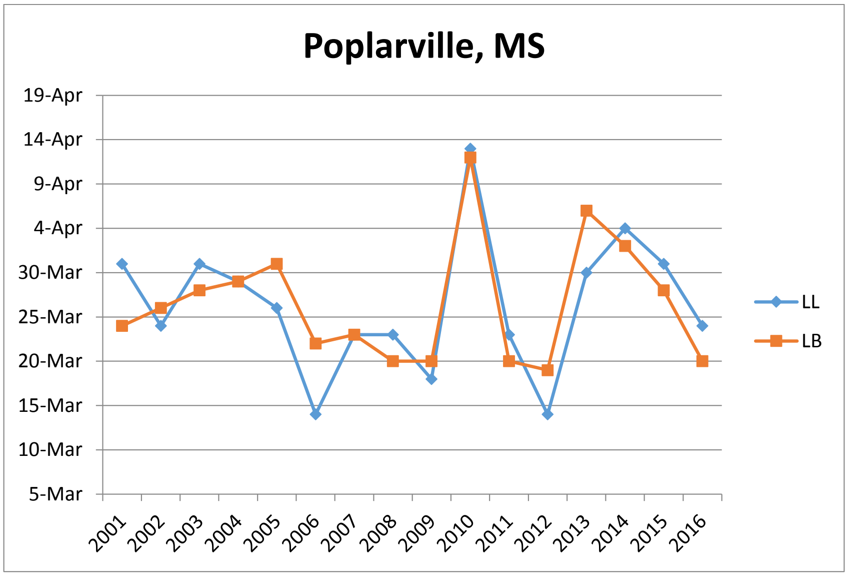
Figure 3—DNA samples LL1–LL4 are known to be longleaf positive, H1 and H2 are from a known Sonderegger hybrid, LB is from a loblolly seedling, and -VE was included as a control.

in the container nursery in 2015, but what caused this phenomenon? One premise is that this development may have occurred as the result of some nursery application. The nursery manager indicated, however, that this development was limited to these two seedlots among several seed sources being grown. Another thought is that it resulted from a shift in pollination times between longleaf and loblolly pine that might have favored hybridization.

Unfortunately, we have no answer to the question of what caused this stem elongation phenomenon in the nursery. Most likely it was triggered by some nursery-related application, but we have no direct evidence. There could be other possible mechanisms. For example, could increasing temperatures resulting from climate change be affecting the pollination sequences of the two species? Little information has been published about the growth and development of Sonderegger pine seedlings, particularly for container-grown plants.

Given that natural hybridization between the two species is known to occur, but the Sonderegger pine hybrid is considered to have lower quality than either parent, should these seedlings be planted?

A series of evaluations has begun to determine if these seedlings will continue to elongate and develop more traditional Sonderegger characteristics or remain in the grass stage typical of longleaf pine. It became obvious that it would take time to outplant and evaluate the seedlings and to develop the genetic markers needed for the DNA analyses. Because of this uncertainty, the land managers involved decided not to plant the seedlings, and all remaining seedlings not used for this study—nearly 12,000—were destroyed. We believe our outplanted studies, including plantings of known Sonderegger seedlings, will provide needed information on the growth and development of Sonderegger seedlings and the quality of these trees in established stands.



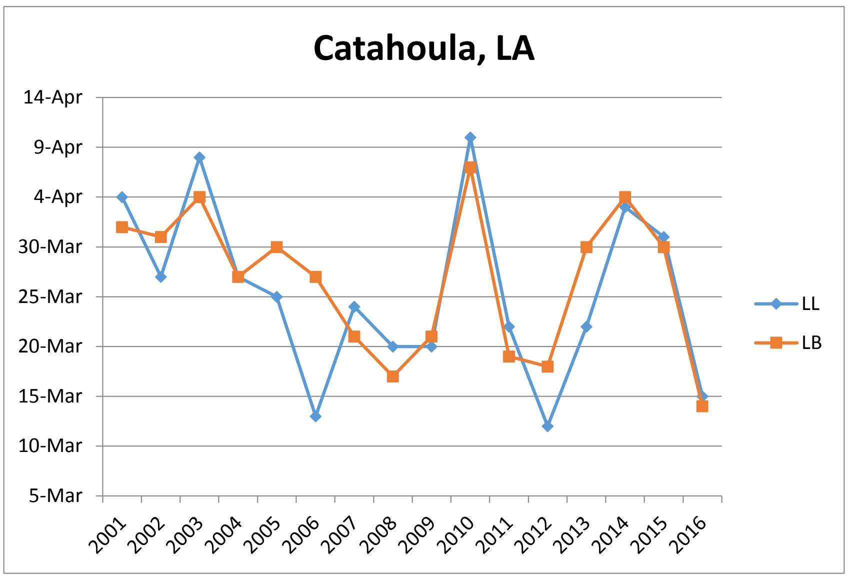


Figure 4—A comparison of projected peak pollination dates (based on historical weather data from nearby weather stations in Poplarville, MS, and Catahoula, LA) for longleaf and loblolly pines in the Craig and Stuart Seed Orchards under evaluation. Overlap in pollination times occurs frequently and seems to have little consistent effect on the quantity of Sonderegger seedlings produced.

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