

## ABSTRACT

CASTILLO ZAMORA, ANA CRISTINA. Assessment of Genetic Variation of Growth and Water-Use Efficiency (WUE) in Mature Longleaf Pine (*Pinus palustris* Mill.). (Under the direction of Dr. Barry Goldfarb.)

The genetic and physiological quality of seeds and seedlings available for longleaf pine (*Pinus palustris* Mill.) restoration is a key concern, as nearly 62,000 hectares of longleaf pine were planted in 2014 alone. Longleaf pine forests are an endangered, biodiversity hotspot in the US Southeast. However, longleaf pine trees may live over 400 years and, thus, are likely to experience great climate variability, especially in the face of climate change. A key to long-term success of longleaf pine restoration will be planting the appropriate genetic material in longleaf pine plantations that is well adapted to environmental stress. One potential strategy to maximize the resilience of this ecosystem to climate change is to plant trees that exhibit good growth while minimizing water loss, i.e. high water-use efficiency (WUE). Our study assesses genetic variation of growth and WUE as well as growth -WUE relationships in longleaf pine, which is a first step towards determining the potential for breeding for and/or planting trees with high WUE in this species.

We measured  $\Delta^{13}\text{C}$  – a commonly used isotopic signature that can be used as a proxy for WUE – in 107 resin-extracted, longleaf pine cores representing nine genetic families. The trees were planted in 1960 by the USDA Forest Service at the Harrison Experimental Forest in Saucier, Mississippi and were 54 years old at the time of our experiment. Diameter at breast height (DBH) and height were measured at ages 7, 17, 30, and 40, with the exception of height at age 30. The tree cores were divided into segments corresponding to ages 7-17, 18-30 and 31-40, representing early, intermediate, and late growth of the trees.

Significant genetic variation in diameter at breast height (DBH) among families was observed in this longleaf pine population. There were strong phenotypic correlations between DBH at an early age and DBH at maturity, which would be useful for selection of superior trees for tree improvement programs. However, families were not significantly different for height. Height at age 7 was weakly correlated with height at age 40. Therefore, DBH appears to be a more reliable measure of tree growth than height for predicting performance from young trees.

We identified significant genetic variation of WUE in longleaf pine that merits further exploration for potentially identifying trees that can withstand drought stress. WUE declined as trees aged from 7 to 40 years old, which paralleled significant decreases in DBH and height growth rates. However, mean family growth rates were not correlated with  $\Delta^{13}\text{C}$ . We hypothesize that this is because water is typically not a limiting factor for tree growth in this region. Family variation in growth traits and WUE, but no apparent relationship between them, suggest it could be possible to select families with both superior growth and WUE.

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Assessment of Genetic Variation of Growth and Water-Use Efficiency (WUE) in Mature  
Loblolly Pine (*Pinus palustris* Mill.)

by  
Ana Cristina Castillo Zamora

A thesis submitted to the Graduate Faculty of  
North Carolina State University  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

Forestry

Raleigh, North Carolina

2016

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## **DEDICATION**

To my parents Teresa Zamora and Juan Manuel Castillo, who have worked so hard and selflessly their entire lives. Thank you for love and unyielding support.

## **BIOGRAPHY**

Ana Cristina Castillo was born in 1991 in a small, rural town in Michoacán, Mexico. She grew up in Watsonville, California where she attended Pájaro Valley High School and discovered her interest in studying the natural world. In 2009, Ana moved to New York City to attend Columbia University. A summer spent living in the El Yunque National Forest in Puerto Rico sparked her interest in studying forest ecology. She returned to El Yunque the following summer and worked as a field technician for the El Yunque Chronosequence Project. Ana completed her first research project as part of her senior thesis with Dr. Pamela Templer at Boston University. Her project focused on studying the effects of increased soil frost on nutrient uptake in by trees in Northeastern forests of the United States. Ana graduated in 2013 with a BA in Environmental Biology and a concentration in Sustainable Development. In 2014, Ana began her M.S. program at North Carolina State University in Raleigh, NC in the Forestry and Environmental Resources Department. Upon completing her Master's degree in Forestry, she plans to continue her career in research in applied forest science.

## ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Barry Goldfarb for sharing his wisdom with me and providing immense support and guidance while completing my program. I owe many thanks to my committee members Drs. Fikret Isik, Kurt Johnsen, and Stacy Nelson for helping ensure I completed my research successfully. I would also like to acknowledge the USDA Forest Service Southern Research Station for funding this project.

I am very grateful for Joshua McRae's assistance processing samples in the lab. I also owe special thanks to James Roberds and Karen Sarsony from the USDA Forest Service who were extremely helpful in coordinating the collection of tree cores and preparation of samples for analysis. Kevin Wise and Dr. Stephen Kelley provided helpful guidance and assistance in devising a protocol for chemical extractions of the tree cores. I also would like to thank the Tree Improvement Cooperative for allowing me to use their lab space and equipment.

Special thanks to my fellow office mates and graduate students for their friendship and academic support during my time at NC State. Finally, I owe immense thanks to my partner and biggest supporter in life, Gus Engman.

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## INTRODUCTION

Longleaf pine (*Pinus palustris* Mill.) once dominated the coastal plains of the Southeastern United States covering 25 million hectares, but its area has been reduced to less than 3% of its original extent (Gilliam and Platt, 2006). Longleaf pine's historical range spans the coastal plains of Virginia to East Texas (Jose et al., 2006). Longleaf pine ecosystems are adapted to a wide range of site conditions ranging from xeric sandhills to wet, poorly drained flatwoods, as well as montane areas of northern Alabama (Jose et al., 2006). The North American Coastal Plain (NACP) which includes longleaf pine ecosystems has recently been recognized as a global biodiversity hotspot (Critical Ecosystem Partnership Fund, 2015). Notably, 1,816 plant species are endemic to the NACP and over 30 endangered and threatened species live in these savannah-like ecosystems (Diop et al., 2009; Noss et al., 2015).

The decline of longleaf pine forests was largely due to anthropogenic pressures. Longleaf pine was heavily logged starting in the late 1800's through the 1930's and stands were cleared to make way for agriculture (Jose et al., 2006). Reforestation of these lands has been primarily with commercial pine plantations of short rotation species such as loblolly pine (*Pinus taeda* L.) and slash pine (*Pinus elliottii* Engelm.) (Van Lear et al., 2005). Longleaf pine savannahs are maintained by fire and, thus, fire suppression has been a main driver in the lack of natural regeneration of the species (Van Lear et al., 2005). Because of its high biodiversity and levels of anthropogenic pressures, the International Union for

Conservation of Nature (IUCN) has declared longleaf pine ecosystems as *endangered* on their Red List of Endangered Species (Farjon, 2013).

### *Longleaf pine restoration*

In 2007, the USDA Forest Service, Department of Defense, and U.S. Fish and Wildlife Service facilitated the formation of a Regional Working Group that developed the *Range-Wide Conservation Plan for Longleaf Pine* (Regional Working Group for America's Longleaf 2009). The main goal of the conservation plan is to increase longleaf pine forests to about 3.2 million hectares by 2024. The Steering Committee of the Regional Working Group launched the America's Longleaf Restoration Initiative (ALRI), which collectively implemented restoration work on about 769,000 hectares including approximately 62,000 hectares of newly established longleaf pine plantations in 2014 (Longleaf Partnership Council, 2014).

Restoration of longleaf pine ecosystems can be challenging, but much success has been achieved with improvements in seedling establishment and ecosystem management, including prescribed fire (Jose et al., 2006). Despite high levels of longleaf pine plantation establishment, there is limited knowledge on the genetic and physiological quality of the seedlings being planted across the U.S. Southeast. Compared to the other, more commercially valuable, species such as loblolly and slash pine, there has been less effort toward developing and deploying genetically improved planting stocks of longleaf pine.

### *Genetics of longleaf pine*

Longleaf pine likely originated from a single refugium in southern Texas or northeastern Mexico, and then migrated eastward across the Southeastern US after the

Pleistocene (Schmidting and Hipkins, 1998). A decreasing trend in heterozygosity in seed sources from west to east and low differentiation among longleaf pine populations has been observed. Early studies of genetic variation in longleaf pine found the average family-within-area effect was greater than the geographic effect for height, DBH, and plot volume (Wells and Snyder, 1976). Furthermore, trees from seed sources in central Georgia, southern Mississippi, and central Louisiana have been found to exhibit the fastest growth (Wells and Wakeley, 1970). Measures of genetic diversity appear unrelated to climate variables (Schmidting and Hipkins 1998). There are no ecotypic differences in longleaf pine based on site conditions and no significant differences in survival or growth between the eastern and western populations, as occurs in loblolly pine (Schmidting 1999).

The amount of genetic variation in longleaf pine is similar to other southern pines, making it a suitable candidate for genetic improvement (Schmidting and Hipkins, 1998; Schmidting, 1999). However, there are inherent difficulties in breeding longleaf pine due to its juvenile “grass stage” when the seedling’s apical bud is protected by its characteristic long needles and aboveground, vertical growth is suppressed. Typical tree improvement programs begin by selecting superior, open-pollinated trees and determining their age using an increment core. The progeny is tested using a common garden test and usually results in significant genetic gains in the first round of selection. Longleaf pine may spend up to 9 years in the grass stage making it difficult to determine the exact age of the trees and their growth potential using the traditional selection method (Haywood, 2007). Current efforts in longleaf pine tree improvement are primarily carried out with progeny tests of open-pollinated, vigorous, well-formed trees. Seeds are collected from these trees and plantings of

seedlings are established to determine their genetic potential. Two key traits of longleaf pine that determine its growth and survival have been identified – the duration of the grass stage and resistance to brown-spot (*Mycosphaerella dearnessii* Barr.) (Snyder et al., 1977). These traits have been found to exhibit high family heritabilities ranging from  $h^2 = 0.47$  to 0.68 for emergence from the grass stage (Layton and Goddard, 1982), and  $h^2 = 0.57$  for brown-spot resistance (Snyder and Derr, 1972).

One of the earliest genetic experiments on longleaf pine was established in 1960 at the USDA Forest Service Harrison Experimental Forest (HEF) in Mississippi to evaluate parent-progeny inheritance trends of growth, form, and pest resistance traits (Snyder and Namkoong, 1978). However, this progeny test has yet to be analyzed for genetic variation of physiological adaptive traits such as water-use efficiency. Most recently, longleaf pine provenance tests were established in Virginia in 2006 to test the performance of seed sources from a seed orchard in North Carolina and natural stands in North Carolina, South Carolina, Georgia, Florida, Alabama, and Mississippi (Johnsen et al., 2015). This study found that survival and mean height at age 5 was lowest at a cut-over site that did not receive additional water input. Growth decreased in a clinal pattern (north to south), but clinal variation was no longer apparent by age 5. Virginia seed sources performed the best at age 3 and exhibited far higher WUE than other more southern seed sources.

#### *Climate response of longleaf pine*

Dendrochronological methods have been used to study the historical effects of climate on longleaf pine growth, specifically its response to temperature, precipitation, and short-term drought. Generally longleaf pine has been observed to exhibit a positive growth

response to precipitation in the growing season (Henderson and Grissino-Mayer, 2009), but there are inconsistencies in the measured response to changes in temperature. The first tree ring study of longleaf pine found a strong positive correlation between radial growth and growing season rainfall, but no correlation between radial growth and temperature (Lodewick et al. 1930). Coile (1936) found growth to be positively correlated with early spring rainfall of the current year and negatively correlated to August temperature. Results from Meldahl et al. (1999) support past findings and show that total ring width in longleaf pine is primarily determined by current growing season rainfall, most markedly in March and September. Furthermore, a negative growth response to temperature and severity of drought was observed by Zahner (1989). Drought severity can be measured using the Palmer Drought Severity Index (PDSI) and Palmer Hydrological Drought Index (PHDI) which are drought indicators based on a supply-and-demand model of soil moisture using temperature and precipitation, reflecting long-term drought (U.S. Drought Portal). A comprehensive chronology of longleaf pine from 1921 to 1987 found that August rainfall, September temperature, and February Palmer Drought Severity Index (PDSI) were the best predictors of radial growth (Devall et al., 1991). Similarly, Henderson and Grissino-Mayer (2009) found a strong correlation between PDSI and longleaf pine growth, especially during July to November. Overall, it appears that longleaf pine growth is strongly related to drought severity and responds positively to precipitation, but its response to high temperatures is weak.

### *Water-use efficiency and climate adaptation*

Climate models predict an increased frequency of extreme climatic events, increased temperatures, and decreased water availability for the Southeastern United States (Carter et al., 2014). There is interest in restoring longleaf pine for its potential to be an adaptation strategy for climate change in the U.S. Southeast (Diop et al., 2009). Studies have shown longleaf pine suffers less wind damage than other *Pinus* species (Johnsen et al., 2009) and it is assumed to possess higher drought tolerance, as it is commonly found on well-drained, sandy sites.

One main strategy being developed to adapt forests to decreased water availability is planting species and genotypes that use less water for biomass production and can better cope with a decrease in water availability (Marguerit et al., 2014). Longleaf pine trees with good growth and moderate water loss, i.e. high water-use efficiency (WUE), would be potentially better adapted to future climates. At the leaf level, WUE is the ratio of the rates of net CO<sub>2</sub> assimilation (photosynthesis) and transpiration as estimated by stomatal conductance (Pérez-Harguindeguy and Díaz, 2013). This ratio can be scaled to the whole-tree level as plant biomass production per unit of water loss via transpiration. The diffusion of water from plants is 1.6 times faster than that of CO<sub>2</sub>, thus, intrinsic WUE is given by:

$$WUE_i = (c_a - c_i) / 1.6 \quad [1]$$

where  $c_a$  and  $c_i$  are the mole fractions of CO<sub>2</sub> in ambient air and the sub-stomatal cavity, respectively (Pérez-Harguindeguy and Díaz, 2013).

WUE is negatively correlated with  $\Delta^{13}\text{C}$ , a measure of the ratio of stable isotopes  $^{13}\text{C}/^{12}\text{C}$  resulting from isotope fractionation of  $^{13}\text{C}$  by plants. Photosynthetic enzymes

discriminate against  $^{13}\text{C}$  during photosynthesis, so that  $^{13}\text{C}$  in leaves is less than that found in the atmosphere. For C3 plants, discrimination of  $^{13}\text{C}$  is given by:

$$\Delta^{13}\text{C} = a + (b-a) c_i/c_a \quad [2]$$

where  $\Delta^{13}\text{C}$  is photosynthetic  $^{13}\text{C}$  discrimination,  $a = 4.4\text{‰}$  and  $b=27\text{‰}$  (Farquhar et al., 1982). That is, the rate of diffusion of  $^{13}\text{CO}_2$  across stomatal pores is lower than that of  $^{12}\text{CO}_2$  by a factor of 4.4‰ and the rate of fixation of  $^{13}\text{C}$  by rubisco is lower by a factor of 27‰. An increase in photosynthetic capacity or a decrease in stomatal conductance will lead to decreased discrimination of  $^{13}\text{C}$ , i.e. higher WUE.

Carbon isotope discrimination by plants is regarded as a reliable proxy for intrinsic WUE of photosynthesis as evidenced by numerous empirical studies (Kruse et al., 2012). The  $\Delta^{13}\text{C}$  signature is imprinted on photoassimilates, which are translocated from the source leaves to the cambial tissue and to wood constituents, storing the  $\Delta^{13}\text{C}$  signature in tree-rings (G. D. Farquhar, J. R. Ehleringer, 1989). Cellulose, lignin, and hemicellulose are not transferred between annual growth rings, thus, differences observed in the  $\Delta^{13}\text{C}$  signature across tree rings represent the physiological changes related to growing conditions at the time they were formed (Harlow et al., 2006). Secondary, more mobile, compounds present in wood cores are typically removed to avoid variation introduced by their slightly different isotopic composition (Wilson and Grinsted, 1977). Solvent-extractable compounds are called mobile extractives and include resin, lipids, organic acids, terpenes, phenols and waxes (Harlow et al., 2006) and can vary between 0% and 20% of wood content in longleaf pine (So and Eberhardt, 2013).

### *Genetic variation of $\Delta^{13}C$*

Significant genetic variation of  $\Delta^{13}C$  has been observed in a range of C3 cereal species as well as many tree species (Condon et al., 2006; Cregg and Zhang, 2000). Carbon isotope discrimination has been successfully used as a criterion in cereal breeding programs, such as wheat, to select individuals with higher WUE, as indicated by significant correlations between  $\Delta^{13}C$  and yield (Condon et al., 2006). Merchuk and Saranga (2013) suggest improved WUE may not only be beneficial in drought prone environments, but may also increase productivity in favorable conditions. Results from these studies suggest that there is sufficient variation in  $\Delta^{13}C$  to be useful in breeding and selection.

In order to successfully breed and select for traits of interest in a plant population, the trait must exhibit a significant amount of genetic variance (Zobel and Talbert, 1984). The ratio of total genetic variance to phenotypic variance is defined as broad-sense heritability ( $H^2$ ) and includes additive, dominance, epistatic, and maternal/paternal effects. Narrow-sense heritability ( $h^2$ ) only includes the proportion of additive variance to total phenotypic variance and is commonly calculated for traits of interest to determine their suitability for selection in breeding programs. Both are important measures used to quantify the amount of genetic variation in a plant population and determine the impact of environmental variation on plant phenotype. Genetic variation in  $\Delta^{13}C$  and its relationship to growth has been studied in a variety of tree species to investigate the feasibility of selecting for high WUE. Family heritability estimates for  $\Delta^{13}C$  in trees vary greatly ranging from  $h^2 = 0.09$  in loblolly pine (Baltunis et al., 2008) to  $h^2 = 0.72$  in hoop pine (*Araucaria cunninghamii* Aiton ex A.Cunn.) (Prasolova et al., 2001). The relationship between carbon isotope discrimination and tree

growth has been studied in some species. Marguerit et al. (2014) found moderate narrow-sense family heritability of  $\Delta^{13}\text{C}$  ( $h^2=0.29$ ) in maritime pine (*Pinus pinaster* Ait.) and their results indicated that higher WUE is not genetically linked to slower growth. Thus, they concluded it may be possible to select genotypes for both high WUE and growth. Xu et al. (2000) found strong, negative genetic correlations ( $r = -0.83$  to  $-0.96$ ) between  $\Delta^{13}\text{C}$  and tree growth, particularly tree height, in clones of the F1 hybrid between slash pine (*Pinus elliottii* Engelm.) and Caribbean pine (*Pinus caribaea* Morelet). Furthermore, Johnsen et al. (1999) found  $\Delta^{13}\text{C}$  to be moderately to highly heritable at a family level ( $h^2 = 0.54$ ) and strongly, negatively correlated with growth ( $r = -0.97$ ) in a 7 x 7 diallel of 22 year-old black spruce (*Picea mariana* Mill.), across sites with varying water availability. However, in a previous study of a subset of four families from the same diallel, a negative correlation between  $\Delta^{13}\text{C}$  and height was only present on the driest sites of the experiment (Flanagan and Johnsen, 1995). Similarly, a relationship between longleaf pine height and  $\Delta^{13}\text{C}$  at age 5 was observed ( $r = -0.55$ ), but only on the experimental site that had not been irrigated and had the lowest productivity (Johnsen et al., 2015). Further research is needed to determine the relationship between water use efficiency and growth in longleaf pine, especially in older trees.

### *Objectives*

The objectives of this study are to assess genetic variation of growth traits and water-use efficiency (WUE), as well as growth-WUE relationships in mature longleaf pine trees. We hypothesized that mean diameter at breast height (DBH) and height would vary significantly by family. We also predicted there would be significant genetic variation in WUE among families, as estimated by  $\Delta^{13}\text{C}$  measured from wood cores. Finally, we

hypothesized there would be a negative relationship between growth rates and  $\Delta^{13}\text{C}$  during early, intermediate, and mature growth stages.

## **MATERIALS AND METHODS**

### *Study site*

The longleaf pine trees in this study were grown at the U.S. Forest Service Harrison Experimental Forest (HEF) near Saucier, Mississippi, USA. (30.65N, 89.04W, elevation 50 m) as part of a genetic experiment established in 1960 (Snyder and Namkoong, 1978). The HEF spans 1,662 hectares within the De Soto National Forest and is representative of about 12.5 million hectares of land with similar soil properties and topography of the U.S. Southeast (Adams et al. 2004). The climate in this region is temperate-humid subtropical where annual temperatures range from -7 to 37°C and mean annual precipitation is 1651 mm, distributed relatively evenly throughout the year (Adams et al. 2004). Soils at the HEF are well-drained, fine-sandy loams of the Ruston and McLaurin series and are generally low in mineral nutrients.

### *Experimental design*

Snyder and Namkoong (1978) established a 13-parent, partial diallel, field experiment in 1960 to study trait inheritance patterns and parent-progeny correlations. Longleaf pine trees aged 19-29 years old were randomly selected from an open-pollinated population near Gulfport, Mississippi and crossed to produce 78 full-sib families. The seeds were germinated in the spring of 1960. One-year-old seedlings grown in milk cartons were used for planting, where first-year survival averaged 94%. Eight seedlings per family were planted on sites that

had been bull-dozed, plowed, and disked at a spacing of 3.7 meters at the vertexes of equilateral triangles. After planting, the site was mowed periodically and was sprayed with Bordeaux mixture three times a year for the first three years for prevention of brown-spot needle blight (*Mycosphaerella dearnessii* M.E. Barr).

The experimental design of the portion of the genetic test analyzed here is a randomized, complete-block design and includes a partial diallel of six parents to form nine full-sib families planted in four blocks (Table 1). Due to tree mortality since the experiment was established, we selected families for which there were at least three living trees per cross in each of the experimental blocks. The total number of trees sampled was 107 due to mortality of one tree in family 30 x 39. Tree DBH and height was measured using a diameter tape and a clinometer, respectively, at ages 7, 17, 30, and 40 with the exception of height for year 30. Growth after year 40 was not included in this study to avoid including any confounding effects from Hurricane Katrina, which impacted the region in 2005.

#### *Tree core sampling and preparation*

Trees were cored at approximately 1.2 meters above ground level using a 40.64 cm long, 5.15 mm diameter increment borer bit. Cores were extracted during October 2014 and November 2015 by US Forest Service technicians at the HEF and were 54 and 55 years-old at the time of sampling. The cores were taken west to east from the tree bole. After extraction, the cores were placed in plastic beverage straws, labeled with a Sharpie with the tree identification number and immediately put on in ice. The cores were stored in a freezer at -30°C until they were shipped on ice to the USDA Forest Service Southern Research Station (SRS) in Research Triangle Park, North Carolina, USA.

Segments corresponding to ages 7-17, 18-30, and 31-40 were cut from both halves of each tree core with a razor blade by counting back the tree rings from year 54 or 55 depending on when the cores were taken. The two segments were combined to form a sample for each time period for every tree. Each sample was dried overnight in a 105°C oven prior to extraction. Mobile extractives were removed from the wood core samples using a Soxhlet extractor with a 150 mL 2:1 solution of toluene and ethyl alcohol for 6 hours. The resin-extracted wood samples were re-dried in a 105°C oven and ground using a Wiley mill with a 1 mm mesh screen. The homogenized ground wood tissue was kept dry in a 60°C oven before it was prepared for isotope ratio mass spectrometry (IRMS) by weighing out a sample (1.5 mg ± 0.15) and enclosing it in a tin capsule.

#### *Carbon isotope discrimination*

Carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) was calculated from carbon isotope values of the ground wood core samples as follows:

$$\delta_p(\text{‰}) = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) * 1000 \quad [3]$$

$$\Delta = \frac{\delta_a - \delta_p}{1 + \delta_p} \quad [4]$$

where  $R_{\text{sample}}$  and  $R_{\text{standard}}$  are the  $^{13}\text{C}/^{12}\text{C}$  content of the sample and the international Vienna Pee Dee Belemnite (PDB) standard respectively,  $\delta_a$  is the  $\delta^{13}\text{C}$  for source atmospheric  $\text{CO}_2$  (-7.9 ‰), and  $\delta_p$  is the  $\delta^{13}\text{C}$  for the plant tissue (G. D. Farquhar, J. R. Ehleringer, 1989; J.R. Ehleringer, 1989). The units are expressed in units of per mil (‰), parts per thousand. WUE is negatively correlated with  $\Delta^{13}\text{C}$  so values closer to zero indicate higher WUE. The samples were analyzed using a Carlo Erba Elemental Analyzer with zero-blank autosampler at the

Duke Environmental Stable Isotope Laboratory in Durham, North Carolina. Accuracy and precision of  $\delta^{13}\text{C}$  in wood measurements was tested by making repeated measurements of the U.S. National Bureau of Standards NBS-22 (graphite) with a known  $\delta^{13}\text{C}$  value of -29.9 ‰ on the PDB scale (Qi et al., 2003) and at least 2 internal standards (sucrose, cellulose, acetanilide, cyclohexanone, and USGS 40). The final number of  $\Delta^{13}\text{C}$  values included in our analysis was 320 (three per tree), when accounting for one missing sample. Precision of  $\Delta^{13}\text{C}$  values reported is +/- 0.2 per mil at 1 standard deviation relative to NBS-22.

### *Statistical analysis*

Carbon isotope discrimination values, DBH, and height were analyzed using a linear mixed model. Repeated measurements (within-subject) were taken from each tree so a first-order autoregressive covariance structure for correlated errors was included in the model. Type III sums of squares were used and hypotheses were tested at  $\alpha = 0.05$ . The following model, hereafter referred to as the full model, was fit to the data to test for differences in response variables among families, ages, experimental blocks, and their interactions:

$$Y_{ijkl} = \mu + B_i + F_j + A_k + FA_{jk} + \epsilon_{ijkl} \quad [5]$$

where:

$Y_{ijkl}$  is the  $l^{\text{th}}$  observation in the  $i^{\text{th}}$  block in the  $j^{\text{th}}$  family and the  $k^{\text{th}}$  age/period;

$\mu$  is the grand mean;

$B_i$  is the random effect of block,  $i= 1, 2, 3, 4$  with expectations  $\sim N(0, \sigma^2_B)$ ;

$F_j$  is the fixed effect of family,  $j= 1,2,\dots,9$ ;

$A_k$  is the fixed effect of age,  $k=1, 2, 3, 4$  or period,  $k= 1, 2, 3$ ;

$FA_{jk}$  is the interaction effect between family and age/period, and

$\epsilon_{ijkl}$  is the error term with expectations  $N(0, \sigma^2 \epsilon)$ .

This model was fitted for measured DBH and height as well as their growth rates per year and  $\Delta^{13}\text{C}$ . Growth rates during ages 7-17, 18-30, and 31-40 for DBH and 7-17, 18-40, and 7-40 for height were calculated by subtracting the corresponding field measurements from each other and dividing the total growth by the appropriate number of years.

Assumptions of normality were verified visually using histograms and Q-Q plots of the residuals as well as comparing the mean and median of  $\Delta^{13}\text{C}$ , DBH, and height. Model terms with significant p-values were further tested using the Tukey-HSD multiple comparison test to control the experiment-wise error rate ( $\alpha = 0.05$ ). One tree in family 26 x 32 was excluded from all analysis after it was visually assessed as an outlier, leaving the final number of sample trees at 106. We estimated Pearson product-moment correlations between traits as the standard. Analyses were carried out using SAS 9.4 © 2014 (SAS Institute Inc., Cary, NC, USA).

## RESULTS

### *Height and DBH*

Mean DBH and height for all trees in this experiment at ages 7, 17, 30, 40 showed a sigmoidal growth trend for all families (Figures 1 and 2, respectively). Height and DBH at age 40 exhibited a moderate, positive correlation ( $r = 0.44$ ,  $P < 0.001$ ). There were significant age, family, and age\*family effects for DBH (Table 2). Some families, such as 21 x 29 and 29 x 30 tended to have smaller mean DBH at all ages (Figure 3). Changes in family ranks of mean DBH primarily took place between ages 7 and 17, when all but 3 families changed in

rank by an average of 2 and up to 5 places (Figure 4). For example, by age 17 the highest ranked family dropped 4 places and continued to drop one more spot by age 40. Mean DBH ( $\pm$  standard error) growth rates during age periods 7-17, 18-30, and 31-40 were 1.1 ( $\pm$  0.02), 0.4 ( $\pm$  0.01), and 0.3 ( $\pm$  0.01) cm/year, respectively, and were all significantly different from each other ( $F=1538.64$ ,  $P<0.001$ ). DBH at age 40 was significantly correlated with DBH at age 7, 17, 30 where the Pearson's correlation coefficients were  $r = 0.35$ ,  $0.83$ , and  $0.95$ , respectively ( $P<0.001$ ).

There was a significant age effect for height of trees, but the family and family\*age terms were not statistically significant when data was fitted to the full model (Table 2). Family ranks of mean height changed substantially – all but 2 families changed in rank from ages 7 to 17 and all changed from ages 17-40 (Figure 5). Mean height growth rate during ages 7-17 was 0.9 m/year and was significantly greater than during ages 18-40 when mean growth rate was 0.4 m/year ( $F=1623.52$ ,  $P<0.001$ ). Height at age 40 was moderately correlated with height at age 17 ( $r = 0.23$ ,  $P=0.02$ ), but not with height at age 7.

#### *Carbon isotope discrimination*

The range of observed  $\Delta^{13}\text{C}$  values for all trees and time periods was 17.2 to 20.2‰. There were significant age, family, and family\*age effects for  $\Delta^{13}\text{C}$  (Table 2). Mean  $\Delta^{13}\text{C}$  values ( $\pm$  SE) during age periods 7-17, 18-30, and 31-40 were 18.7 ( $\pm$  0.04), 18.9 ( $\pm$  0.04), and 19.3 ‰ ( $\pm$  0.05), respectively. Families 21 x 39, 29 x 32, and 32 x 39 consistently had the lowest mean  $\Delta^{13}\text{C}$  (‰) during all age periods (Figure 6). Mean  $\Delta^{13}\text{C}$  (‰) generally increased from early to late growth stages, although differences between growth periods were not statistically significant within all families. Family ranks of mean  $\Delta^{13}\text{C}$  remained

relatively constant across time (Figure 7).  $\Delta^{13}\text{C}$  for periods 7-17 and 18-30 were significantly correlated to  $\Delta^{13}\text{C}$  during ages 31-40 ( $r = 0.62$  and  $0.79$  respectively,  $P < 0.001$ ). No relationship between  $\Delta^{13}\text{C}$  and DBH or height growth rates was observed (Figures 8 and 9, respectively). Mean  $\Delta^{13}\text{C}$  for all time periods was not significantly correlated with either DBH or height at age 40.

## DISCUSSION

In order to improve the seed and seedling stock available for restoration of longleaf pine ecosystems, fundamental knowledge of genetic variation of this species is needed. In this study, we assessed genetic variation of DBH, height, and  $\Delta^{13}\text{C}$  as a proxy for water-use efficiency. Genetic variation was identified in this subset of families from the full diallel established Snyder and Namkoong (1978). Differences in mean DBH of longleaf pine were significant for families tested. We found DBH at age 7 to be a fair predictor of DBH at maturity for longleaf pine ( $r = 0.35$ ). However, the correlation between DBH at age 17 and 40 increases by more than two-fold to  $r = 0.83$  in 10 years. Furthermore, the majority of rank changes in family mean DBH occurred from age 7 to 17. Therefore, although DBH age 7 is a fair predictor of DBH at maturity in these longleaf pine families, the most genetic gain in DBH could be achieved by using measurements from age 17 or beyond for selection criteria. Variation in mean height was not significant when comparing family means. This may indicate environmental factors may play a significant role in height growth. Furthermore, height at maturity (age 40) was only correlated with height at age 17 and not at age 7 and family rankings of mean height were highly variable. However, DBH at age 40 is moderately

correlated with height at age 40 ( $r = 0.44$ ) indicating DBH is a good indicator of total tree biomass. Longleaf pine breeding programs focusing on growth may want to use DBH as the main selection criterion for growth rather than height.

Carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) varied significantly among these longleaf pine families. Although the absolute differences in family means were not large, very small standard errors were associated with  $\Delta^{13}\text{C}$  measurements. Minor differences in carbon isotope discrimination can be highly heritable and strongly related to growth (Johnsen et al., 1999).  $\Delta^{13}\text{C}$  during ages 7-17 and 18-30 were significantly correlated to  $\Delta^{13}\text{C}$  during ages 31-40, indicating it may be possible to select trees with higher WUE based on  $\Delta^{13}\text{C}$  values as early as age 7. Ranking of families based on  $\Delta^{13}\text{C}$  were relatively stable as the trees aged. The top four performing families remained among top positions, while the lesser performing families had some rank changes between periods. Our results indicate  $\Delta^{13}\text{C}$  is genetically controlled in this sample of longleaf pine, and suggest there could be potential for this trait to be utilized in future longleaf pine tree improvement efforts.

An age-related increase in  $\Delta^{13}\text{C}$  was observed when comparing early, intermediate, and mature growth stages of longleaf pine.  $\Delta^{13}\text{C}$  was lowest during ages 7-17, which is also when growth rates were the greatest. However, we did not find  $\Delta^{13}\text{C}$  to be correlated with DBH or height growth rates. These results indicate that it may be possible to select for higher WUE (lower  $\Delta^{13}\text{C}$ ) without affecting growth in longleaf pine. However, higher WUE may not necessarily provide longleaf pine trees with a significant growing advantage.

Alternatively, higher WUE may be more important under water-limited conditions where improved WUE may result in less tree mortality. We also found that DBH and height growth

rates were also decreasing significantly with age. This trend was observed across all families and may be indicative of an overall change in physiology as the trees age and the canopy closes. Longleaf pine trees do not tolerate competition well and canopy closure typically occurs around age 17 in longleaf pine stands (Haywood, 2007). This age corresponded to a decrease in DBH and height growth in the families included in this study. Assuming that stomatal conductance and precipitation remained relatively constant, increasing  $\Delta^{13}\text{C}$  may be attributed to decreased photosynthetic rates. Canopy closure would reduce light availability and lead to a decline in net carbon assimilation rates in the trees.

Genetic variation in DBH and  $\Delta^{13}\text{C}$  was identified in this longleaf population. These results are consistent with observed patterns in genetic diversity of longleaf pine in other studies (Schmidting & Hipkins 1998). This provides further evidence that longleaf pine is suitable for tree improvement focusing on increasing growth. It is important to note that Stine et al. (2002) report in an earlier analysis of this same progeny test that the ratio of non-additive to additive genetic variance in this population of longleaf pine was higher than observed for other southern yellow pines. Due to the low number of families used, as well as their genetic structure, heritability for  $\Delta^{13}\text{C}$  was not calculated for this study. However, our study indicates that a significant amount phenotypic variation observed in  $\Delta^{13}\text{C}$  is due to genetic differences, including additive genetic variance. These results may indicate broad-sense heritability estimates would be high in this population. Baltunis et al. (2008) found family heritability estimates of  $\Delta^{13}\text{C}$  in loblolly pine varied across sites, where  $h^2 = 0.14$ ,  $0.20$ , and  $0.09$  in Florida, Georgia, and across sites respectively. Narrow-sense family heritability of  $\Delta^{13}\text{C}$  in maritime pine was estimated as  $h^2 = 0.29$  across site with varying

water availability (Marguerit et al., 2014). Based on these results, it is likely that heritability of  $\Delta^{13}\text{C}$  of longleaf is within these ranges and there is sufficient additive genetic variance of this trait for tree improvement or selection of adapted genotypes.

Genetic variation in water-use efficiency is a result of either increased photosynthetic capacity or a decrease in stomatal conductance. In some species, variation of WUE is due to differences in photosynthetic capacity (Baltunis et al., 2008; Johnsen et al., 1999), while in others, stomatal conductance is the driver of observed differences (Aitken et al., 1995; Grossnickle and Fan, 1998; Olivas-Garcia et al., 2000). Water-use efficiency is a complex physiological trait as many factors contribute to photosynthetic capacity and stomatal conductance of trees and genetic variation of  $\Delta^{13}\text{C}$  is ultimately a result of climatic, evolutionary, morphological, molecular, etc. differences. Genomics studies will provide further insight into the patterns and mechanisms of genetic diversity of photosynthetic capacity and stomatal conductance for different species. For example, 14 single nucleotide polymorphisms (SNPs) associated with carbon isotope discrimination in loblolly pine have been identified in an association genetics population (Cumbie et al., 2011).

The observed genetic variation in water-use efficiency in longleaf pine is a possible indication of the adaptive capacity of this tree species. Trees in the genus *Pinus* have been found to use a drought avoidance strategy, where water use is decreased quickly under water-limited conditions and maximized in adequate water environments (Martínez-Vilalta et al., 2004). Our study provides further evidence that restoration of longleaf pine ecosystems may be a successful adaptation strategy in the U.S. Southeast in response to climate change. Although a comparison of species did not find that longleaf has more a more conservative

water use strategy (Samuelson et al., 2012), the fact that there was significant genetic variation of WUE may indicate that longleaf pine may be well adapted to drought conditions, or at least contain substantial pre-adaptive genetic variation. Similarly, a previous study in maritime pine seedlings found variation in  $\Delta^{13}\text{C}$  response to drought, indicating differences in phenotypic plasticity in response to water availability (Aranda et al., 2009).

Although a relationship between growth and water-use efficiency has been observed in other conifer species (Baltunis et al., 2008; Marguerit et al., 2014; Xu et al., 2000), such relationship was not observed in this population of longleaf pine. A previous study found a significant relationship between  $\Delta^{13}\text{C}$  and longleaf pine tree height at age 5, but only in low productivity sites in Virginia that had not been irrigated (Johnsen et al., 2015). The Gulf Coast receives the most precipitation in the native range of longleaf pine with up to 1750 mm per year (Burns and Honkala, 1990). It may be possible that a relationship between WUE and growth is only present in low quality and/or drier sites where longleaf pine is more likely to be water limited.

Although efforts were made to analyze a representative sample of longleaf pine families from the full diallel, it is important to address the limitations of our sample size. The limited number of samples in our analysis prevents us from estimating genetic parameters such as heritability of traits of interests and genetic correlations among those traits. Using foliar  $\delta^{13}\text{C}$  instead of wood  $\delta^{13}\text{C}$  would have allowed for a larger sample size, but foliage of these trees was inaccessible due to their spacing and height. Moreover, foliage sampling would have allowed us to measure WUE only at the present time, whereas sampling the wood cores allowed the analysis across time. Regardless, the results of our study indicate

that further exploration of genetic variation of carbon isotope discrimination in longleaf pine would be productive. Additionally, wood extraction is a time-consuming processes so time limitations reduced the number of samples that could be included in this study. It is also important to note that the degree to which wood cores should be extracted is an area of debate. We decided to use extractive-free wood because  $\delta^{13}\text{C}$  were found to correlate well with cellulose  $\delta^{13}\text{C}$  values (Harlow et al., 2006). The precision of isotopic values of  $\delta^{13}\text{C}$  values in extractive-free wood is sufficient to elucidate significant ecological trends in carbon isotope discrimination in longleaf pine. Removing mobile extractives is also a more feasible methodology for processing high number of tree cores and is more likely to be used in future studies allowing for direct comparisons. Here we were interested in examining genetic variation in carbon isotope discrimination across varied stages of tree development, but not in relationship to variation in rainfall. An increase in resolution in future studies could be achieved by identifying drought and/or water-limited years and the corresponding tree rings to measure  $\Delta^{13}\text{C}$  during water limited periods. Such studies would provide insight into how WUE responds to water stress and its relationship to growth and/or mortality in longleaf pine.

Harris et al. (2006) stresses the importance of considering future climate scenarios in restoration practices and proposes to focus on the balance between restoring historical ecosystems and attempting to build resilient systems for the future. Given the great interest and investment in longleaf pine restoration, it is important to plant the appropriate genetic material to increase the success of restoration efforts. Tree improvement efforts of longleaf pine are limited when compared to other commercially important species such as loblolly

pine. In addition to understanding genetic variation of growth traits, it is important to understand genetic variation in physiological adaptive traits such as water-use efficiency in longleaf pine. Seedling stock being currently being planted in newly established longleaf plantations may be poorly adapted to current and predicted climate conditions, which would be detrimental to restoration efforts. It will be important for land managers to have access to knowledge on which genotypes of longleaf pine are best suited for climate-related stressors such as drought.

## CONCLUSION

The main findings of our study are that there is genetic variation in growth and water-use efficiency as estimated by  $\Delta^{13}\text{C}$  in longleaf pine. However, carbon isotope discrimination and tree growth were not found to be correlated. We did find that WUE decreased for all families as the tree aged. This study is the first to assess  $\Delta^{13}\text{C}$  in mature longleaf pine and provides evidence for continuing to characterize patterns of genetic variation of carbon isotope discrimination. In addition to increasing our fundamental knowledge of the physiology of this species, we provide some insights into growth trends in longleaf pine that could be useful in tree improvement programs. Our results indicate that, because there were family differences in growth traits, it may be possible to select for high growth and WUE without affecting the other. In this scenario, breeding for high growth would not impact the physiological adaptive capacity of longleaf pine.

Identifying genetic variation in longleaf pine is important considering how many plantings of this species are being planted in the U.S. Southeast. Our study begins to address

some of the physiological processes under genetic control that may indicate longleaf pine ecosystems' adaptive capacity. Understanding the physiological basis of ecological adaptation of longleaf pine in current and future climates will be necessary to ensure the vitality of this ecosystem in the future. However, more studies are needed to evaluate the effects of climate-induced stress such as drought and high temperatures on longleaf pine.

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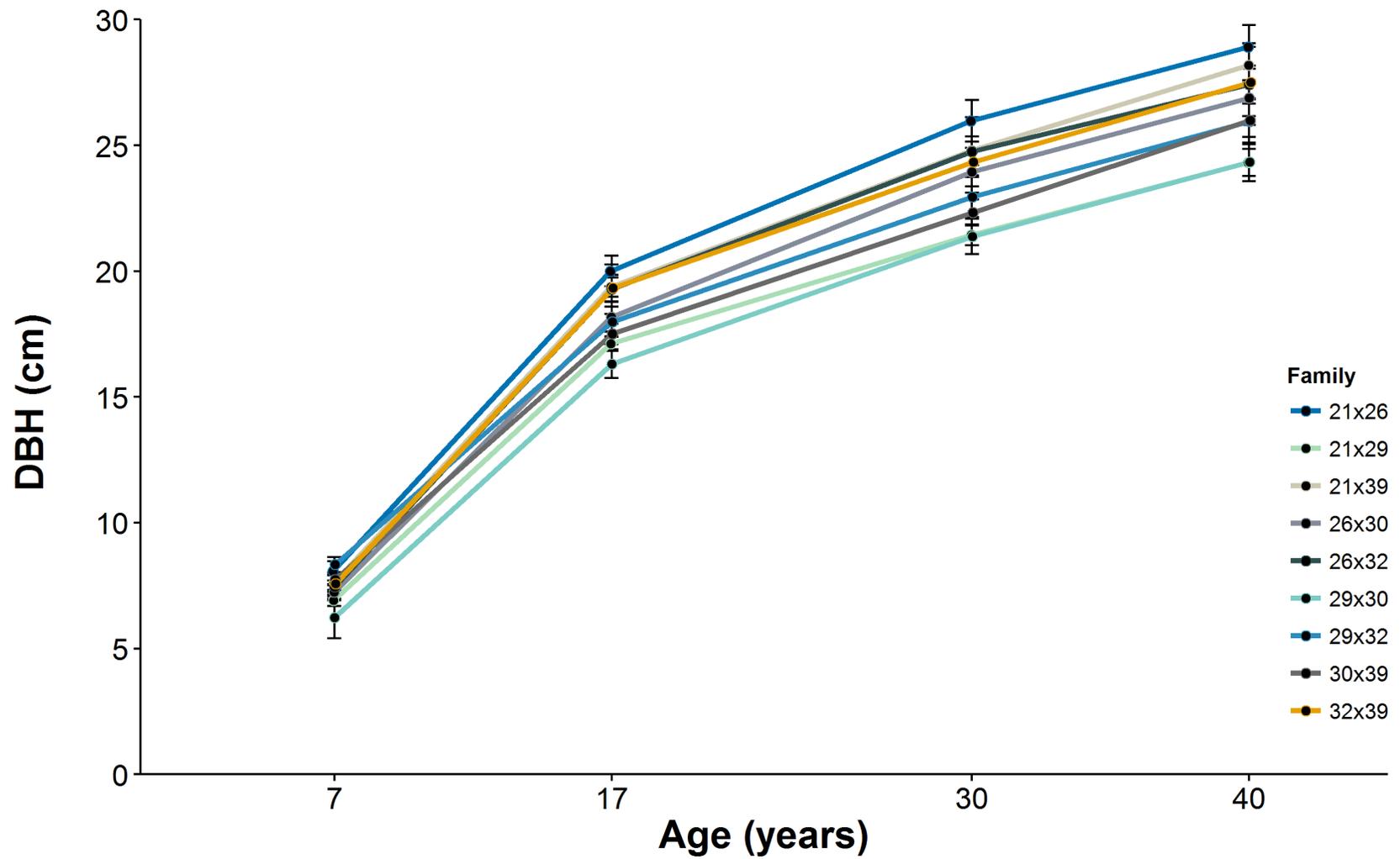
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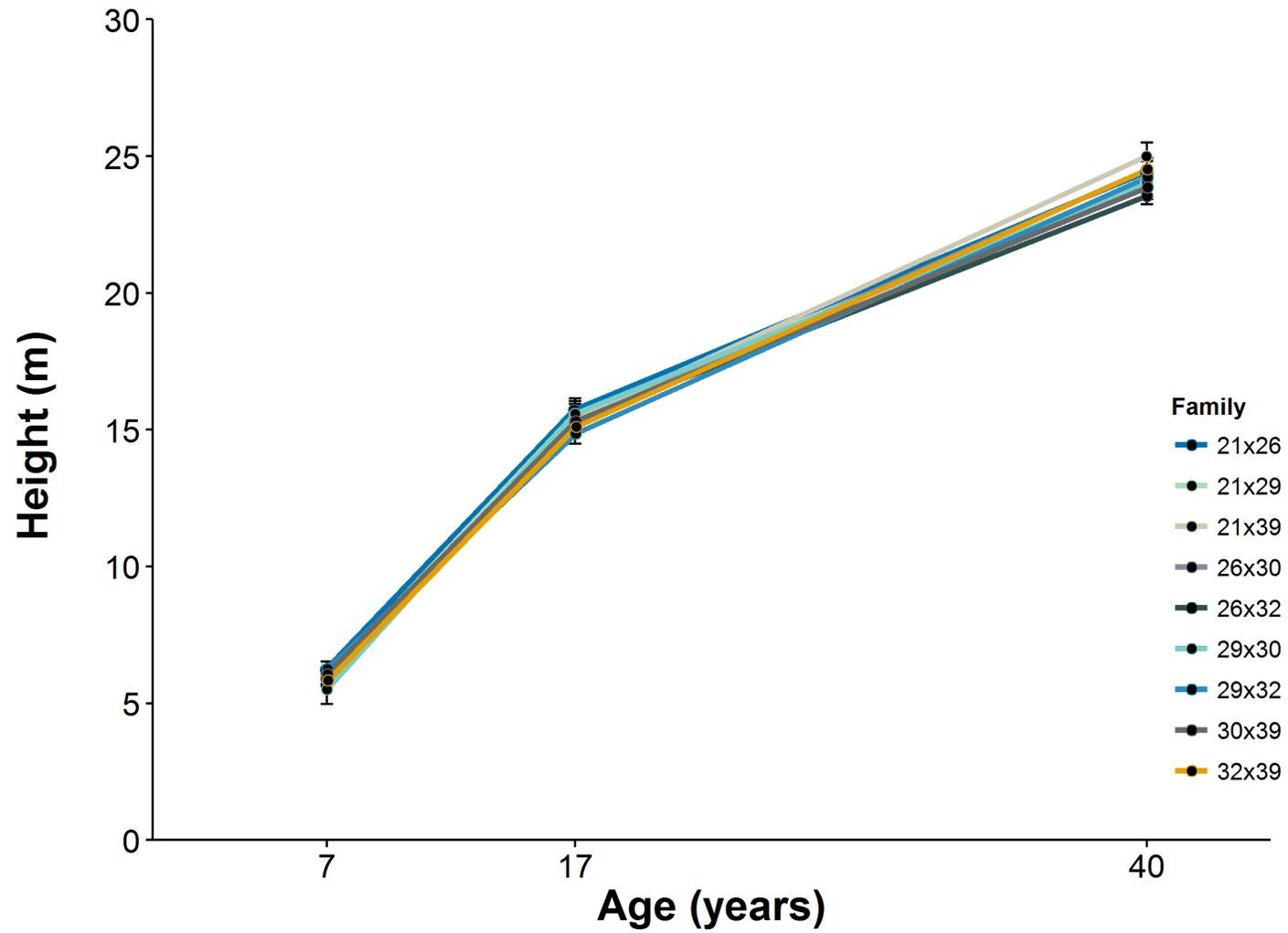
## TABLES & FIGURES

**Table 1.** Crossing design of the nine longleaf pine families included in our study. They are a subset of a larger progeny test established by the USDA Forest Service at the Harrison Experimental Forest in 1960.

	Female Parents				
Male Parents	21	26	29	30	32
26	X				
29	X				
30		X	X		
32		X	X		
39	X			X	X



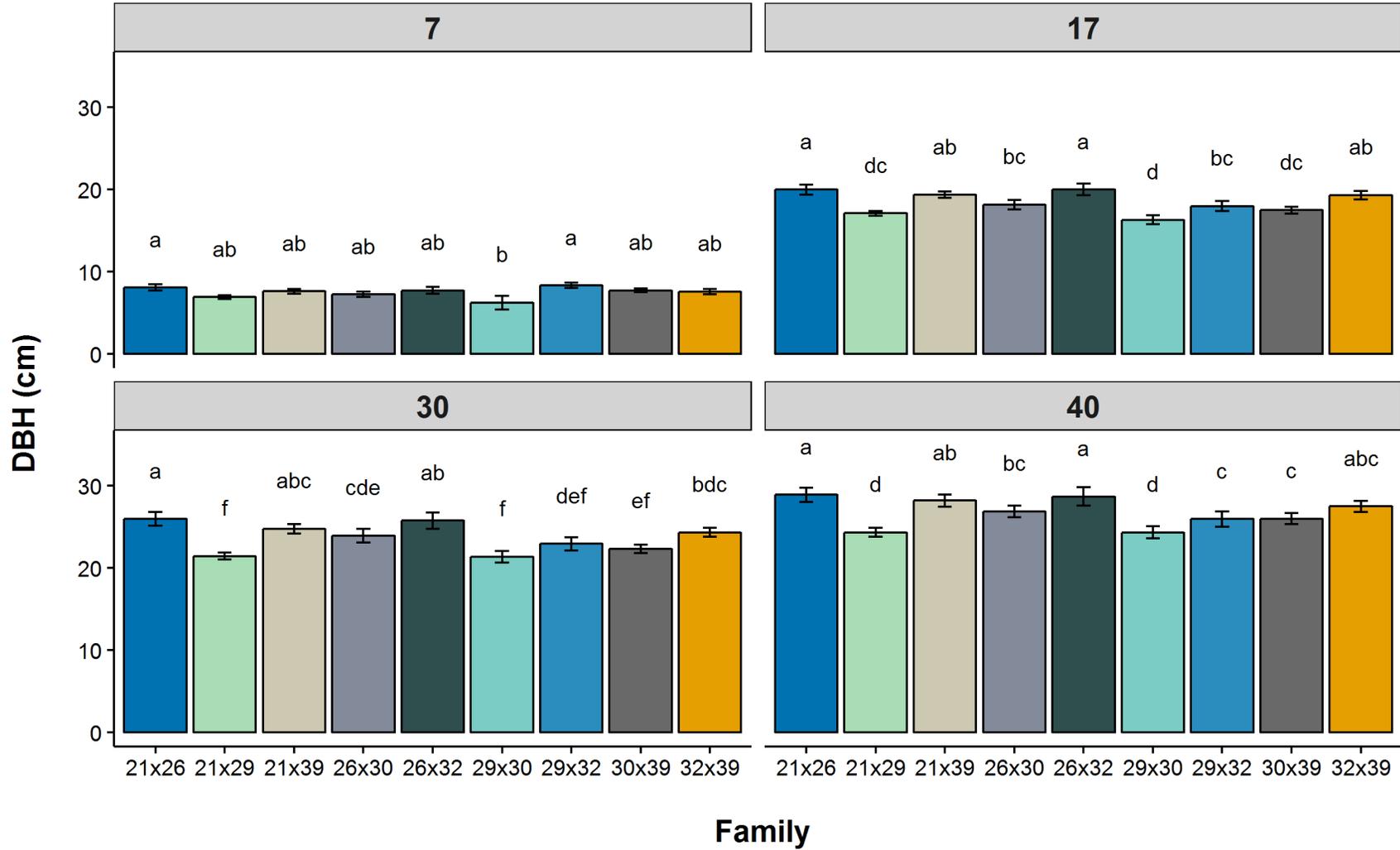
**Figure 1.** Family mean diameter at breast height (DBH) at ages 7, 17, 30, and 40. Lines for each data point represent the standard error of the mean.



**Figure 2.** Family mean height at ages 7, 17, and 40. Lines for each data point represent the standard error of the mean.

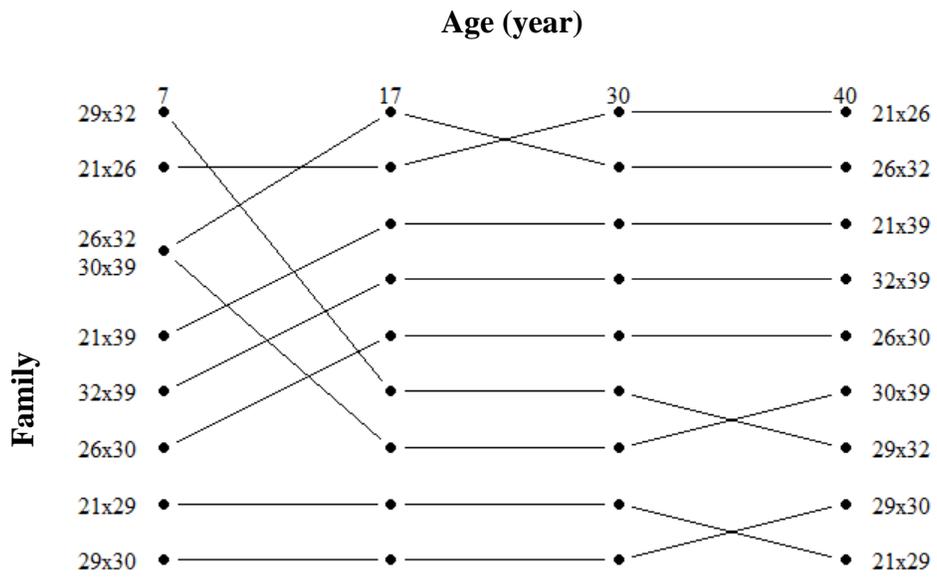
**Table 2.** F-test probability values for DBH, height, and  $\Delta^{13}\text{C}$  in longleaf pine testing the effects of age, family and their interactions.

<b>Variable</b>	<b>Numerator DF</b>	<b>Denominator DF</b>	<b>F</b>	<b>Pr</b>
<b>DBH</b>				
Age	3	385	4426.51	<0.001
Family	8	385	8.15	<0.001
FxA	24	385	2.98	<0.001
<b>Height</b>				
Age	2	288	8324.38	<0.001
Family	8	288	0.82	0.582
FxA	16	288	1.1	0.3563
<b><math>\Delta^{13}\text{C}</math></b>				
Period	2	287	116.41	<0.001
Family	8	287	8.55	<0.001
FxA	16	287	1.73	0.0403

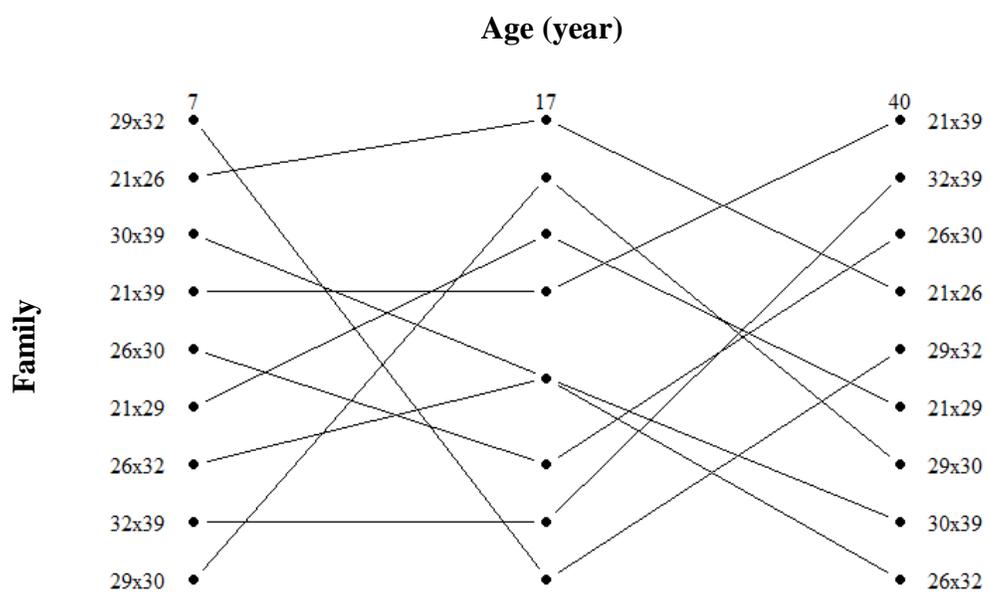


**Figure 3.** Family mean DBH for all families at ages 7, 17, 30, and 40. Lines on each bar represent the standard error of the mean.

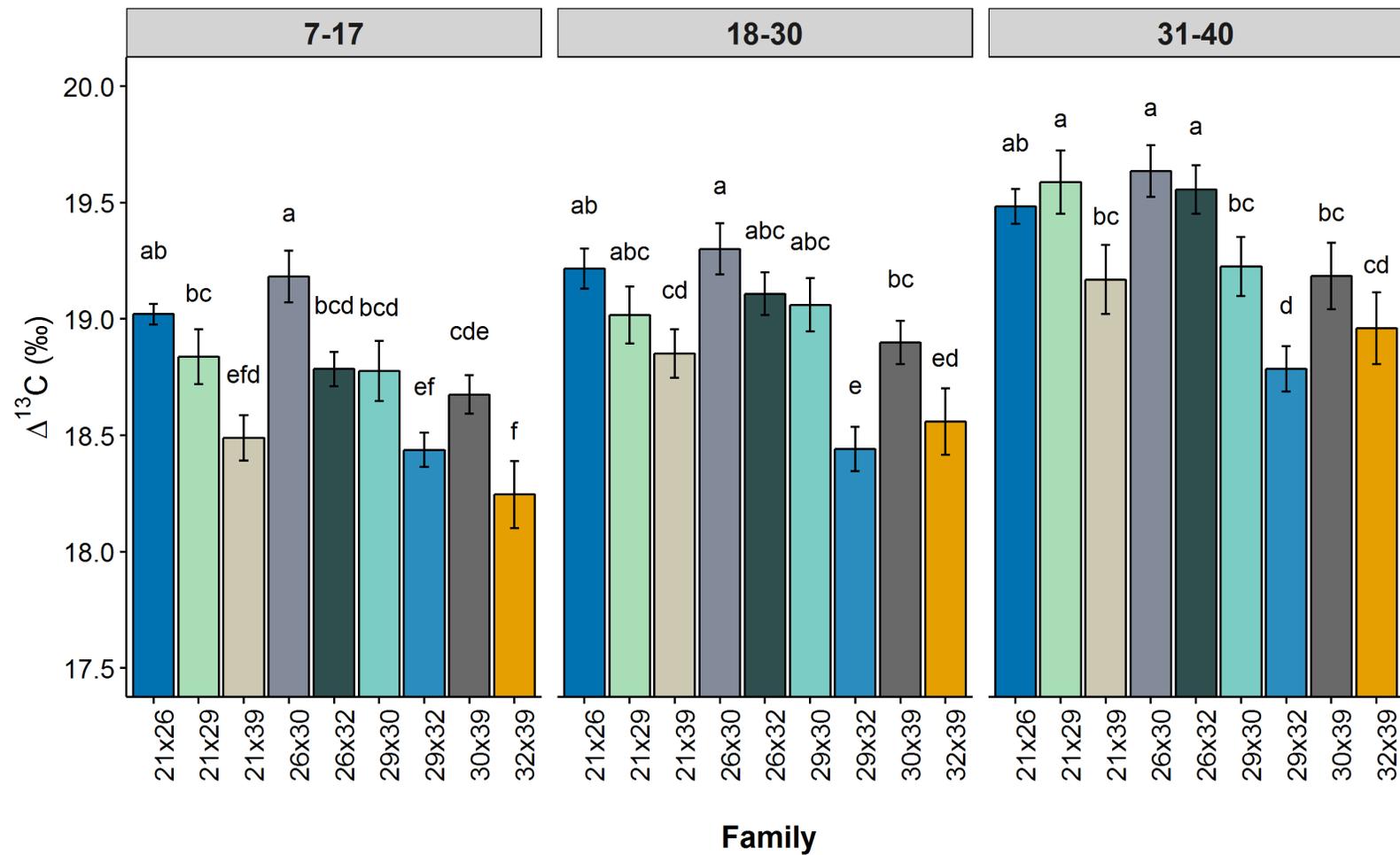
Families with the same letter are not significantly different as indicated by Tukey HSD post-hoc test ( $\alpha=0.05$ ).



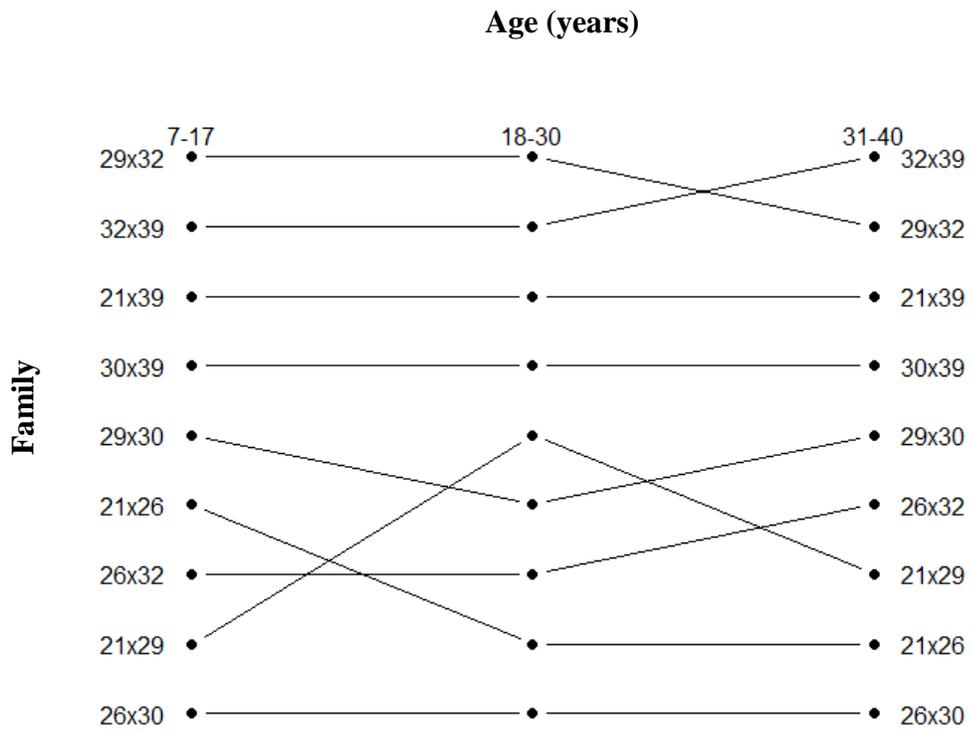
**Figure 4.** Longleaf pine families ranked from largest to smallest mean DBH at ages 7, 17, 30, and 40.



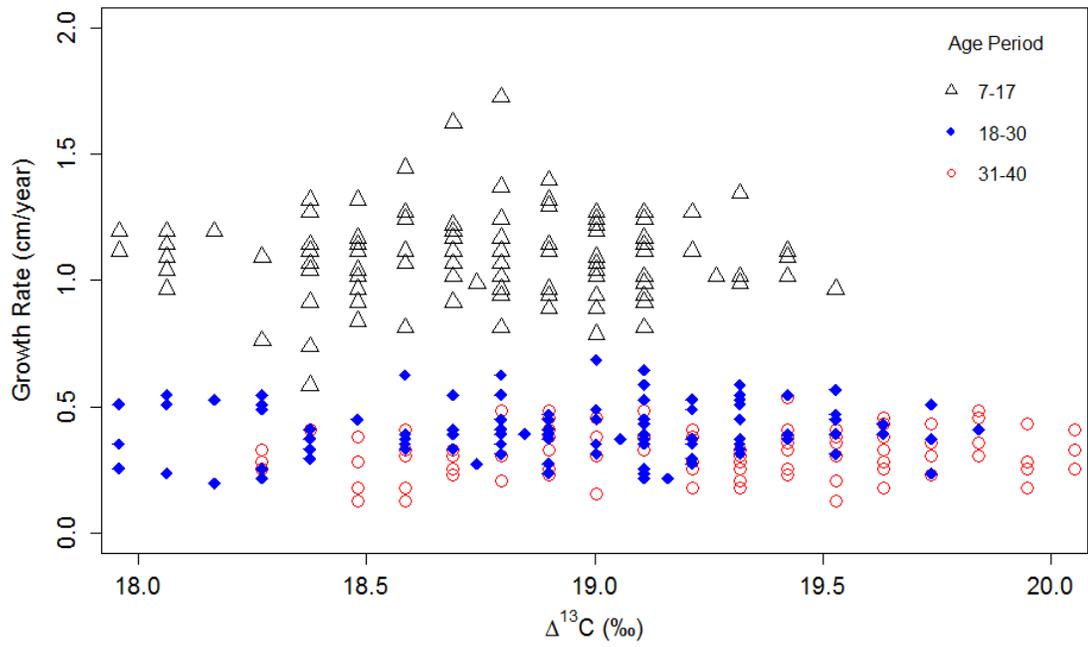
**Figure 5.** Longleaf pine families ranked from largest to smallest mean height at ages 7, 17, and 40.



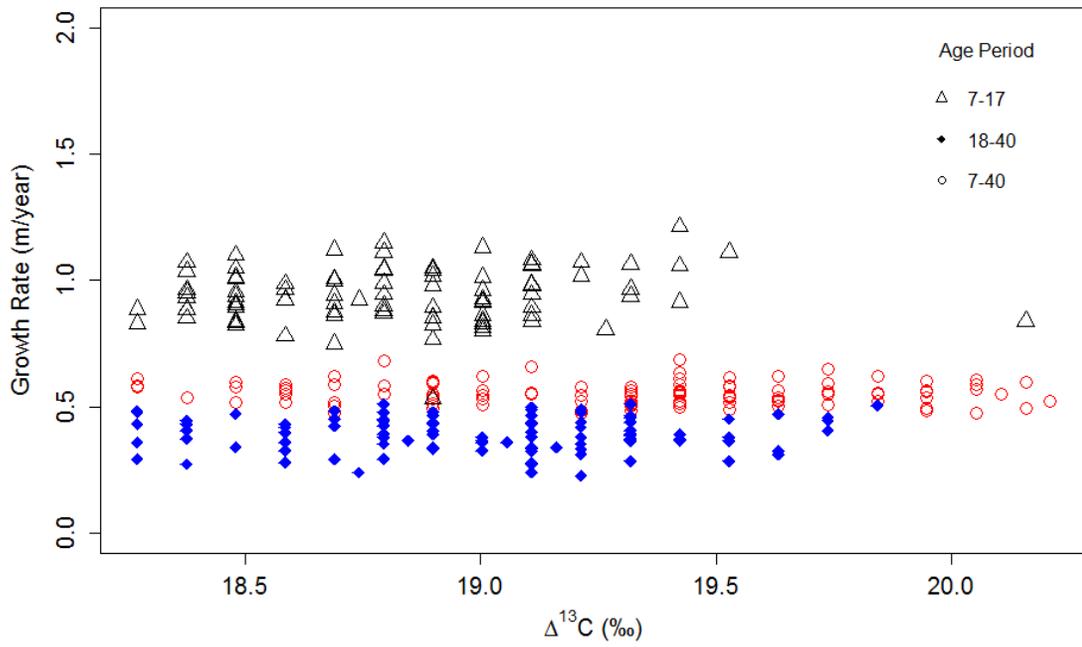
**Figure 6.** Mean ( $\pm$  SE)  $\Delta^{13}\text{C}$  (‰) varied significantly among families during early, intermediate, and late growth ages. Means with the same letter within each time period are not significantly different as indicated by Tukey HSD post-hoc test ( $\alpha=0.05$ ).



**Figure 7.** Longleaf pine families ranked from lowest to highest mean  $\Delta^{13}\text{C}$  (‰), i.e. highest to lowest water-use efficiency, for age periods 7-17, 18-30, 31-40.



**Figure 8.** The correlations between  $\Delta^{13}\text{C}$  (‰) and DBH growth rates (cm/year) were non-significant for all time periods.



**Figure 9.** The correlations between  $\Delta^{13}\text{C}$  (‰) and height growth rates (m/year) during years 7-17, 18-40, and 7-40 were non-significant.