

## Evidence of Relic Genotypes Persisting in the Texas Hybrid Swarm of *Quercus* Series *Virentes*

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### ABSTRACT

*Quercus* series *Virentes* (live oaks) are many independent populations of semi-evergreen trees common from the southeastern United States to Texas, California, and Mexico. Southern live oak (*Q. virginiana* Mill.) and Texas live oak (*Q. fusiformis* Small) are species valued in native and urban landscapes. In Texas, morphological similarities between the standards for these species have complicated classification of individual populations. Classification is further confounded by populations which interact reproductively. Hybrid progeny create opportunity for phenotypic and genotypic diverse *Virentes* series populations. A putative population of *Quercus* series *Virentes* located near Lake Alan Henry, TX was identified. This population is highlighted by a relic individual which appears to be the progenitor of the population. The phylogeny of this specimen was analyzed and compared to other Texas native oak species. Native *Quercus* accessions were sampled from known locations of *Q. fusiformis*, the hybrid swarm of *Q. virginiana* and *Q. fusiformis*, and other *Quercus* species. Genetic diversity analysis was conducted using 70 expressed sequence tags. Four genetic clusters were found which represent populations in different terrestrial regions of Texas. Results indicate the Lake Alan Henry, TX specimen, and a second individual located in Elmendorf, TX are relictual trees of *Quercus* series *Virentes* populations.

**Key words:** *Quercus fusiformis*, Texas live oak, adaptation, variation, hybrid, polymerase chain reaction

### INTRODUCTION

Hybridization of closely related species has a significant role in speciation and reshuffling of genetic and phenotypic variation (Denk et al. 2017; Hipp 2015; Muller 1961;

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Nixon 1993a, 1993b). Such events may have significant consequences on plant taxa population and species dynamics (Eaton et al. 2015; Hipp et al. 2017). Hybridization may limit identification of small numbers of individual hybrid trees within hybrid populations. However, hybridization may also be extensive, with large tree populations expressing hybrid characteristics (Hipp 2015; Rushton 1993). In addition, interspecific hybridization may occur in transition zones (hybrid swarms) between two or more pure populations (Rushton 1993). Hybrid individuals often possess intermediate morphology and may make up a substantial portion of the transition zone population (Jones and Seigler 1975).

In Texas, native live oaks (*Quercus* series *Virentes*) possess traits representative of two standards of speciation. *Quercus virginiana* (southern live oak) is one of the quintessential species associated with southeastern United States flora. In Texas, remnants of pure *Q. virginiana* individuals may be found isolated in east Texas (Arnold 2006; Correll and Johnston 1979; Gould 1969; Mueller 1951) (Figs. 1, 2), and this isolation is likely created by climatic barriers within the state (Lewis and Olivera 1979). *Q. virginiana* thrives in wet, warm environments. However, *Q. virginiana* tends to deteriorate in hot, dry environments with alkaline soils (environments similar to those found in the West Texas High Plains) (Carey 1992; Cavender-Bares et al. 2015). Texas live oak (*Q. fusiformis*) is native from the Black Land Prairies to the Brazos Valley (Arnold 2006; Correll and Johnston 1979; Gould 1969) (Figs. 1, 2), and exhibits greater drought and soil alkalinity tolerance when compared to *Q. virginiana* (Arnold 2006; Cavender-Bares et al. 2015; Muller 1951). Within Texas *Virentes* populations, defining characteristics often fall within standards of both species. Therefore, morphological similarities between taxonomic standards for *Q. fusiformis* and *Q. virginiana* have complicated classification of individual populations, and the lack of clear discrete standards of these closely related species renders classifying plants and populations difficult (Coder 2003; Mueller 1961). Even though *Q. fusiformis* and *Q. virginiana* exhibit phenotypic and habitat differences, they are not geographically and reproductively isolated. Therefore, *Virentes* species found between the two populations are considered to be a hybrid swarm (Small 1933) (Fig. 2).

Many hybrid swarms have been characterized, and in some cases, progeny given new species names (Eaton et al. 2015; Hipp 2015). This process often occurs before a hybrid status is completely known (Nixon 1993a, 1993b). *Quercus* represents a genus where species may maintain specific morphological characteristics even with extensive hybridization (Nixon 1984; Whittemore and Schaal 1991). For example, when *Q. fusiformis* hybridizes with other *Lepidobalanus* (white oak subgenera) species, there may be extensive genetic exchange (Nixon 2006). However, hybridized progeny will likely be phenotypically similar to *Q. fusiformis* (Whittemore and Schaal 1991). Often for *Quercus* species, defining morphological characteristics indicate a wide range of natural variation. Thus, other identifying criteria may be required (Rushton 1993). Morphological similarities between taxonomic standards for *Q. fusiformis* and *Q. virginiana* have complicated classification of individual and hybrid populations (Nixon 1984). Within Texas *Quercus* populations, defining species characteristics (plant size, leaf or acorn morphology, etc.) of hybrid progeny often fall within standards of hybrid species. Thus, difficulties when defining or characterizing population dynamics of *Quercus* hybrid swarm populations often occur (Coder 2003).

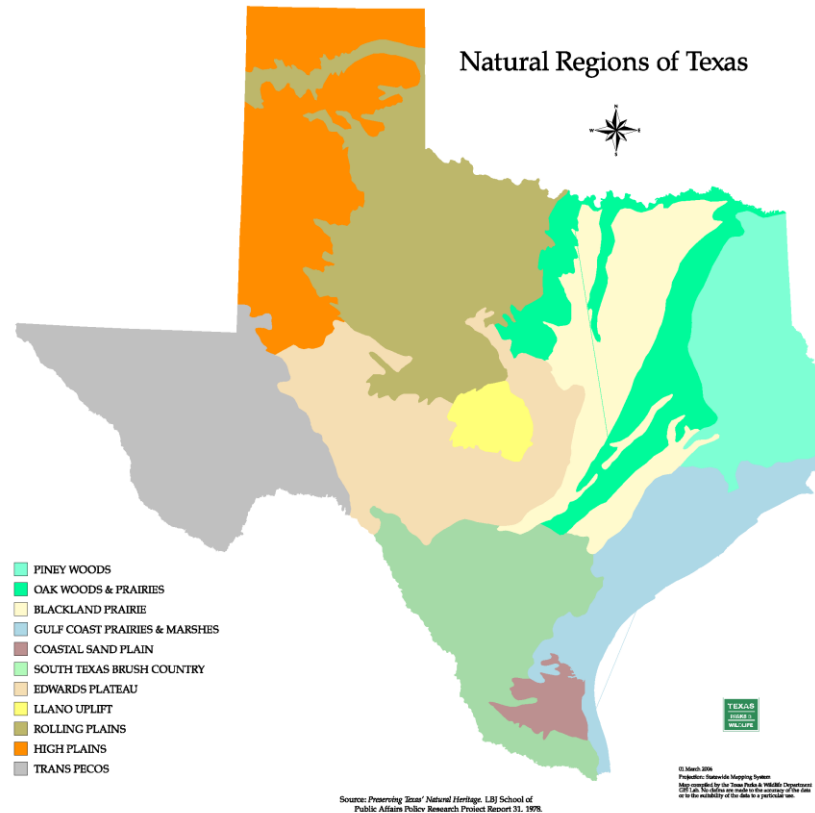


Figure 1. Natural growing regions within the state of Texas (adapted from Texas Parks and Wildlife Department 2021b).

Because *Quercus* species within a subgenera hybridize readily, barriers limiting reproductive fitness (establishment and migration) of progeny are key factors influencing population dynamics (Muller 1952; Rushton 1993; Stebbins et al. 1947). Most hybrid progeny able to endure and prosper are better adapted to their environment. However, many progenies perish (Hardin 1975). Thus, natural selection has resulted in hybrids with enhanced survivability (for example, greater drought tolerance or temperature tolerance) (Coder 2003; Hardin 1975). Fertile *Quercus* hybrids will only occur between members of the same subgenera (Sork 1993). For example, an out-crossing rate of nearly 100% has been found for *Q. rubra* (red oak) (Sork 1993). Similarly, members of the subgenera *Erythrobalanus*, such as *Q. velutina* (black oak) and *Q. coccinea* (scarlet oak), hybridize extensively (Guttman and Weight 1989). However, in most situations inter-specific hybridization results in few progenies within scattered clusters of intermediate individuals (Hardin 1975). Conversely, Hardin (1975) indicates in rare occasions hybridization may produce broadly distributed populations with extreme morphological variability.

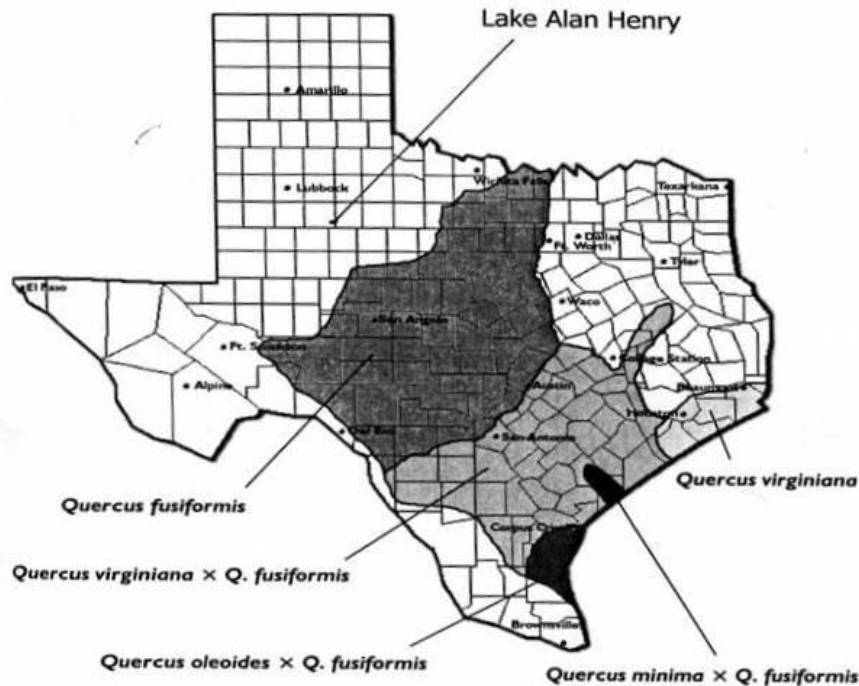


Figure 2. Live oak (*Virentes* series) complex within the state of Texas (Arnold 2006). Adapted from originals of Benny J. Simpson).

While *Virentes* populations are generally believed to extend from east Texas to the western edge of the Edwards Plateau, there are isolated populations which reside outside these parameters (Figs. 1, 2) (Correll and Johnston 1979; Gould 1969; Simpson 1988). South of Justiceburg, TX, a population of *Virentes* has been identified in the Lake Alan Henry Basin (Fig. 2) and is of interest for a number of factors. This native population is found within the Texas High Plains, which is considered a short grass prairie with few native tree and shrub species (Correll and Johnston 1979; Gould 1969; Texas Parks and Wildlife Department 2021a). In addition, this population is much further north and west than previously documented *Q. fusiformis* species (Correll and Johnston 1979; Gould 1969; Simpson 1988). Also, individual trees within this population measure over 12 m tall and may be nearly 25 m wide (Fig. 3).



Figure 3. Representative Texas *Virentes* series live oak from Lake Alan Henry, TX population.

Molecular techniques have previously determined effects of hybridization in native oak populations (Cavender-Bares et al. 2011; Cavender-Bares et al. 2015; Eaton, al., 2015; Nixon, 2006). Whittemore and Schaal (1991) exposed several oak species (*Q. alba* (white oak), *Q. macrocarpa* (bur oak), *Q. michauxii* (swamp chestnut oak), *Q. stellata* (post oak), and *Q. virginiana*) to organelle DNA analysis, and found historical information is preserved among the hybrid genome. In addition, Whittemore and Schall (1991) illustrate hybridization success may be relative to geography, especially isolation. Hipp et al. (2017) used restriction-site associated DNA sequencing of 146 *Quercus* species to determine North American oak evolution rates and species diversity. They concluded the two major American oak clades arose in what is now the boreal zone and radiated in parallel lines from eastern North America into Mexico and Central America (Hipp et al. 2017). Therefore, to better characterize genetic similarities of relictual oaks found in the Lake Alan Henry Basin with other native Texas *Quercus* species, a genetic analysis using DNA markers was used to examine heredity relationships of Lake Alan Henry and other native Texas *Quercus* accessions.

## MATERIALS AND METHOD

Over a two-year period, leaf tissue was collected from 48 native *Quercus* species throughout Texas. At each sample location GPS coordinate information was collected, and coordinates were inserted into ArcGIS software (ArcGIS Desktop 10.7.1, Ersi, Redlands, CA), and plotted to a preexisting Texas map (Fig. 4). To minimize sampling of half sib descendants which would result from pollen dissemination from a common parent (Saintagne et al. 2004), a 104 km minimum sampling distance was used between any two sampled species. The exception to this restriction were individual trees sampled in the Lake

Alan Henry region. Leaves from several *Quercus* species included in the red oak sub genera (Erythrobalanus) were sampled: *Q. rubra*, *Q. coccinea*, and *Q. shumardii* (Shumard oak). In addition, numerous species from the Lepidobalanus (white oak subgenera) were sampled: *Q. macrocarpa*, *Q. muehlenbergii* (chinquapin oak), *Q. gambelii* (gambel oak), *Q. havardii* (shinnery oak), *Q. stellata*, and *Q. polymorpha* (Monterey oak). These species served as outgroups (controls) for phylogenetic analysis. Leaf tissue samples were collected, placed in zipper storage bags, and immediately placed within a cooler containing dry ice. Prior to isolation of DNA, samples were stored in a -80 °C freezer.

Sample *Quercus* accession locations were from a number of natural Texas growing regions (Figs. 1, 2, and 4). Samples from individual trees were assigned letters and numbers which indicate where a sample was collected (letter), and the sample order (number) individual samples were collected from each location. For example, sample A-1 was collected from area A, and was the first sample collected within area A. Individual samples were generally collected from native populations in remote stands. Collection Round A was generally conducted in north central and east Texas. Round B was conducted in central and western Texas. Round C was primarily collected from the Lake Alan Henry population. A number of these accessions were given common names (Boat Ramp Tree, Brazos Oak) based upon geographic location. Round D specimens were collected in the southern portion of Texas, and Round E consisted of miscellaneous specimens collected from throughout the state.

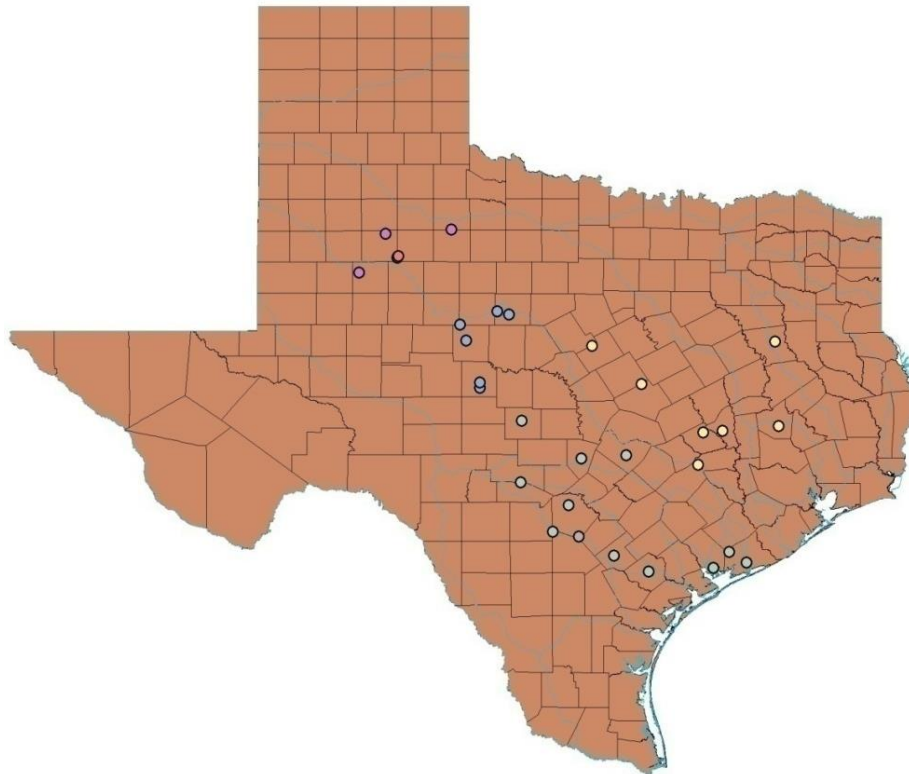


Figure 4. Symbols designating location of *Quercus* species collected within Texas.

Genome leaf DNA from each individual species accession was isolated using a cetyltrimethylammonium bromide method described for cotton (*Gossypium* spp.) (Paterson et al. 1993). Concentration and quality of DNA were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and adjusted to a working concentration of 10ng/μl. Preliminary primer tests were first initiated to find primers which responded to *Virentes*. Unfortunately, previously recorded research on live oak genetics were not discovered. Therefore, in order to successfully produce clear bands, 12 expressed sequence tag (EST) primers from the *Lepidobalanus* subgenera were selected (*Quercus* Portal 2020). Because *Virentes* species are members of the *Lepidobalanus* subgenera, the following primers were selected: contig\_7984, contig\_4073, contig\_10332, contig\_16270, contig\_2216, contig\_17748, contig\_12362, contig\_21724, contig\_4391, contig\_20633, contig\_9525, and contig\_15631. To optimize conditions for amplification, gradient polymerase chain reaction (PCR) was initially performed using an Eppendorf Mastercycler Ep Gradient (Eppendorf AG, Hamburg, Germany). PCR was performed in a total volume of 25 μl containing 30 ng of DNA, 1X PCR buffer, 10% v/v of pvp 10, 2.0 mM of BSA, 2.0 mM of MgCl<sub>2</sub>, 0.2 mM of dNTP, 0.5uM of primer, and 2U of Taq polymerase. PCR was conducted using a three-step process including a single denaturing step of 95 °C for 5 min followed by 40 cycles of 95 °C for 30 sec, 50.3 °C for 1 min, 72 °C for 30 sec, and a final extension step of 72 °C for 7 min. PCR product was separated using a 3.5% super fine agarose gel for 4 hrs at 80 volts. Gels were stained with ethidium bromide, and visualized using an AlphaImager HP (Alpha Innotech, San Leandro, CA). Each image was recorded and stored in tagged image file format. To create an un-weighted neighbor tree between accessions, polymorphic and monomorphic fragments were scored manually as present (1) or absent (0) and analyzed with Numerical Taxonomy and Multivariate Analysis system clustering software (NTSYS v.2.1) (Rohlf 2005). NTSYS v.2.1 software organizes clusters using the unweighted pair group method with arithmetic mean and identifies local homology between units in order of similarity using a stepwise procedure.

## RESULTS

EST analysis of collected accessions yielded 70 scored amplicons of 150 to 300 bp in length. Primers targeting each EST amplified an average of five for each individual. Each amplified fragment was polymorphic. Association of accessions on the basis of pairwise genetic distance separated into four main clusters (Fig. 5). The white oak subgenera (*Lepidobalanus*) cluster includes *Q. macrocarpa*, *Q. muehlenbergii*, *Q. gambelii*, and *Q. polymorpha* comprise the most unrelated outlier group (Fig 5). This group was followed by the red oak subgenera (*Erythrobalanus*) which includes *Q. rubra*, *Q. coccinea*, and *Q. shumardii*. *Q. havardii* and *Q. stelletta* are more closely related, and cluster into the third main grouping (Fig 5). Collected *Virentes* accessions group into a single main cluster. Accessions within this cluster are morphologically identified as members of the *Virentes* series.

Within the *Virentes* accessions group, five of 10 Lake Alan Henry Basin accessions (C-1, C-2, C-8, Brazos Oak Progeny, and Brazos Oak) split as the oldest outlier cluster (Fig 5). These five accessions are located within a Lake Alan Henry runoff zone, and are within 200 m of each other, and 500 m from the southwestern shore of Lake Alan Henry. Accessions C-1, C-2, and C-8 are of similar size and shape, while the Brazos Oak accession is noticeably larger and appears to be more mature (Fig. 6). Brazos Oak is the

unique individual of interest at Lake Alan Henry, while the Brazos Oak Progeny tree was collected from a population of trees near the Brazos Oak specimen. The 0.72 pair-wise genetic coefficient supports Brazos Oak's older age and putative relic classification (Fig. 5). Both Lake Alan Henry subgroups are separated by a single accession (the Elmendorf, TX oak). Based upon genetic relatedness and the tree's vast size and maturity, the Elmendorf oak also appears to be a relictual specimen (Fig. 7). The next cluster consists of the remaining Lake Alan Henry population. These accessions are approximately 400 m north of the Brazos Oak (C-4, C-6, and C-5), and 2,500 m northeast of the Brazos Oak (C-9 and Boat Ramp Tree), respectively. Accessions C-4, C-6, and C-5 are located along the Lake Alan Henry's southwestern shoreline in a separate runoff zone. This runoff zone combines with the previously mentioned C-1, C-2, and C-8 specimen runoff zone watershed into the lake.

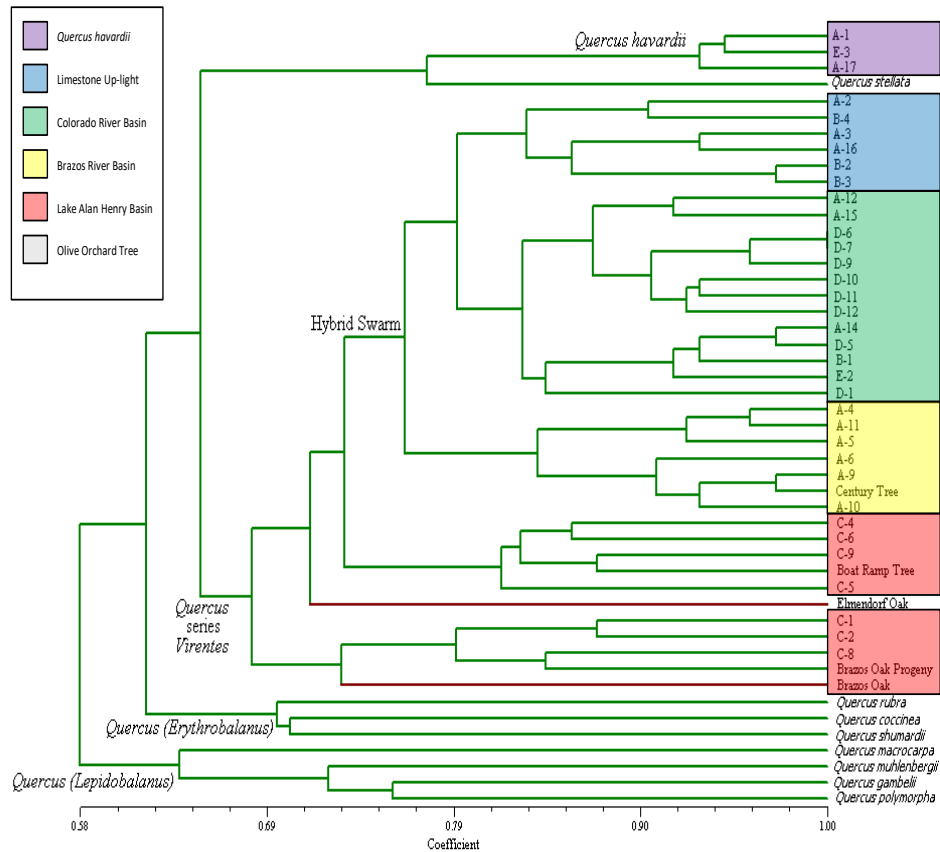


Figure 5. *Quercus* series *Virentes* heredity map representing accessions collected within Texas.





Figure 6. *Quercus fusiformis* 'Brazos Oak' (Lake Alan Henry, TX).



Figure 7. *Quercus* spp. accession (Elmendorf, TX).

The Hybrid Swarm cluster is defined by three close related splits associated with accession geographic location (Fig. 5). Split one separates accessions (A-4, A-11, A-5, A-6, A-9, Century Tree, and A-10). These specimens were collected in the eastern portion of Texas (Brazos River Basin). Division two separates accessions located in the Colorado River Basin (A-12, A-15, D-6, D-7, D-9, D-10, D-11, D-12, A-14, D-5, B-1, E-2, and D-1), and the Limestone Uplift region (A-2, B-4, A-3, A-16, B-2, and B-3).

## CONCLUSIONS AND DISCUSSION

The heredity map created for this study (Fig. 5) appears to represent clustered accessions appropriately. As a control, *Q. macrocarpa*, *Q. muehlenbergii*, *Q. gambelii*, *Q. stellata*, and *Q. havardii* (all from the *Lepidobalanus* sub genera) were added to the experiment to examine any like gene flow. Within the heredity map, the *Lepidobalanus* cluster is the initial group from which all individuals branch (Fig. 5). Since *Quercus* series *Virentes* are considered *Lepidobalanus* species (Nixon 2006; Denk et al. 2017), this genetic relationship is likely exhibited at the ~.58 coefficient. Interestingly, *Q. stellata* and *Q. havardii* do not group with the initial *Lepidobalanus*. but branch at the ~.62 coefficient where they share some relation with the *Quercus* series *Virentes* group. There is evidence *Q. stellata*, will cross with *Q. havardii*, and the two may be hybrids as a result of *Q. stellata*'s historic range (which extended more westerly than it does today) (Peterson and Boyd, 1998; Villena-Rodriguez, 1987). Also, *Erythrobalanus* sub genera (*Q. shumardii*, *Q. rubra*, and *Q. coccinea*) were included in the study, and seem to branch appropriately (~.64 coefficient). The species seem to exhibit a weak relationship with *Quercus* series *Virentes* (Fig. 5).

Within the *Quercus* series *Virentes* cluster, individuals group according to geographical constraints (Fig. 5). This group is labeled as the Hybrid Swarm (Fig. 2), and represents interspecific hybrids of *Q. virginiana* and *Q. fusiformis*. As stated previously, the goals of this study were not to identify solid genetic differences between *Q. virginiana* and *Q. fusiformis* (our intentions were to examine relationships within the Texas *Virentes* series). Individuals A-2, B-4, A-3, A-16, B-2, and B-3 are native to the Central Texas region known as the Llano Uplift, or the Limestone Uplift (Fig. 1). The Llano Uplift is a circular area of Precambrian rock consisting of granite and limestone located in central Texas (United States Department of Agriculture 2021). This genetic relation may be from the evolutionary success of the *Virentes* series ability to adapt to surrounding soils and climate (Cavender-Bares et al. 2011; Hipp et al. 2017). Because they may not be adapted to granite and limestone soils, other *Virentes* series genotypes in this region may not be as successful as these individuals. This group, based on geographic location, would be considered closely related genetically to *Q. fusiformis* (Fig. 5).

There also appears to be relationships between major river basins in Texas and genetic drift. Individuals within this cluster (A-12, A-15, D-6, D-7, D-9, D-10, D-11, D-12, A-14, D-5, B-1, E-2, and D-1) (Fig. 5) represent accessions considered to be located within the Colorado River Basin (Fig. 8) and represents a large majority of the Hybrid Swarm. Within this cluster A-14, D-5, B-1, E-2, and D-1 cluster further into a group located in the San Antonio River Basin (Fig. 8). Similar to accessions of the Colorado River Basin, individuals A-4, A-11, A-5, A-6, A-9, Century Tree, and A-10 share similar genetic relationships (Fig. 5), and are located on the Brazos River Basin (Fig. 8). However, because this region contains much of the Hybrid Swarm, classification of these individual accessions is difficult to decipher (Fig. 5). Clusters which have genetic relationships based on river basins are likely evidence accessions are geographically specific (Cavender-Bares et al. 2011; Cavender-Bares et al. 2015). In addition, it appears *Quercus* germplasm has unique unidirectional movement underlined by hydro forces that drive seed dispersal and propagules from upstream toward downstream populations (Liu 2006).

Branching from the Hybrid Swarm is the first cluster from the Lake Alan Henry location. This includes individual accessions C-4, C-6, C-9, Boat Ramp Tree, and C-5 (Fig. 5). These accessions show relation to the Hybrid Swarm at the ~.77 coefficient. Separated

from the entire population of the *Quercus* series *Virentes* cluster are accessions C-1, C-2, C-8, Brazos Oak (Fig. 6), and Brazos Oak Progeny (Figs. 3, 5). These accessions and the Brazos Oak tree are unique specimens for a number of reasons. First, these trees are *Quercus* series *Virentes* accessions located in the transition zone from the Rolling Plains to the High Plains, a region known to be a short grass prairie (Fig. 1). Native woody species within this area include small trees and shrubs such as *Prosopis glandulosa* (mesquite) and *Juniperus ashei* (Ashe or post cedar) (Simpson 1988; Texas Parks and Wildlife Department 2021a). However, since the arrival of Europeans to this region, native woody species have increased in cover and density (i.e., brush invasion) (Scifres 1980). Native trees the size of the Brazos Oak are considered relic species (species which represent ancient populations, or populations with older genetic material) (Mueller 1961). Except for the Lake Alan Henry population, the nearest known native *Virentes* species are located 130 km to the southeast (Simpson 1988). Because of the geographic isolation of these *Virentes* accessions, this population is likely the most northwestern *Q. fusiformis* population known. Based upon their morphological characteristics the Lake Alan Henry population is likely associated with *Q. fusiformis*. Leaves of Lake Alan Henry accessions are much more linear than they are wide. In addition, acorn tips are long and fusiform or acute, and are similar to other *Q. fusiformis* acorns found across Texas (Mueller 1961; Simpson 1988).

This population is located along the southwestern shore of Lake Alan Henry in a Spade and Veal fine sandy loam soil (United States Department of Agriculture 2021). *Q. fusiformis* responds well to fine sandy loam soil, while *Q. virginiana* tends to prefer a more clay to loam soil (Arnold 2006; Cavender-Bares et al. 2015; Mueller 1951). This soil profile likely encouraged growth of the Brazos Oak tree, especially considering the demanding environment in which the tree is located (Simpson 1988) (Fig. 6). The Brazos Oak accession is the largest specimen in the region. Drip-line diameter of the Brazos Oak is approximately 30 m, height of the Brazos Oak is nearly 12 m, and trunk diameter at breast height is approximately 441 cm (Fig. 6). One of the definitive characteristics of the Brazos Oak is the broad dome shaped canopy commonly associated with *Q. virginiana* trees in the southern United States (Fig. 6). Leaves of the Brazos Oak resemble narrow leaves commonly found on *Q. fusiformis*, rather than broader leaves of *Q. virginiana*. However, when compared to other *Quercus* series *Virentes* progeny in the area, leaves of Brazos Oak progeny have a greater number of points and lobes. Leaves of Brazos Oak also have a unique characteristic in that during times of extreme air temperature change (generally during the fall season), foliage color change occurs (Fig. 9).

When determining differences between *Q. virginiana* and *Q. fusiformis*, acorn morphology is frequently compared (Mueller 1951, 1952). Brazos Oak acorns are prototypical of *Q. fusiformis*. Brazos Oak acorn caps cover less than half the acorn, and the acorn itself is longer than it is wide (representative of *Q. fusiformis*). *Q. virginiana* acorns are characterized by the cup covering at least half of the acorn, while the nut itself is nearly as long as it is wide (Mueller 1961; Simpson 1988). Acorn cotyledons of *Quercus* series *Virentes* trees are fused, while other *Quercus* species cotyledons are separate. We determined Brazos Oak cotyledons are fused. Therefore, the Brazos Oak tree likely represents a true *Virentes* series specimen and indicates lack of hybridization between the Brazos Oak and non-*Virentes* *Quercus* species. These morphological and genetic characteristics suggest the Brazos Oak located near Lake Alan Henry, TX is a genetically pure *Q. fusiformis* specimen. Lack of hybridization between the Brazos Oak and other *Quercus* species is likely due to isolation and distance from inter-breeding species

(Cavender-Bares et al. 2011; Cavender-Bares et al. 2015). This is confirmed by Brazos Oak's growth characteristics, fused cotyledons, acorn, and leaf morphology.

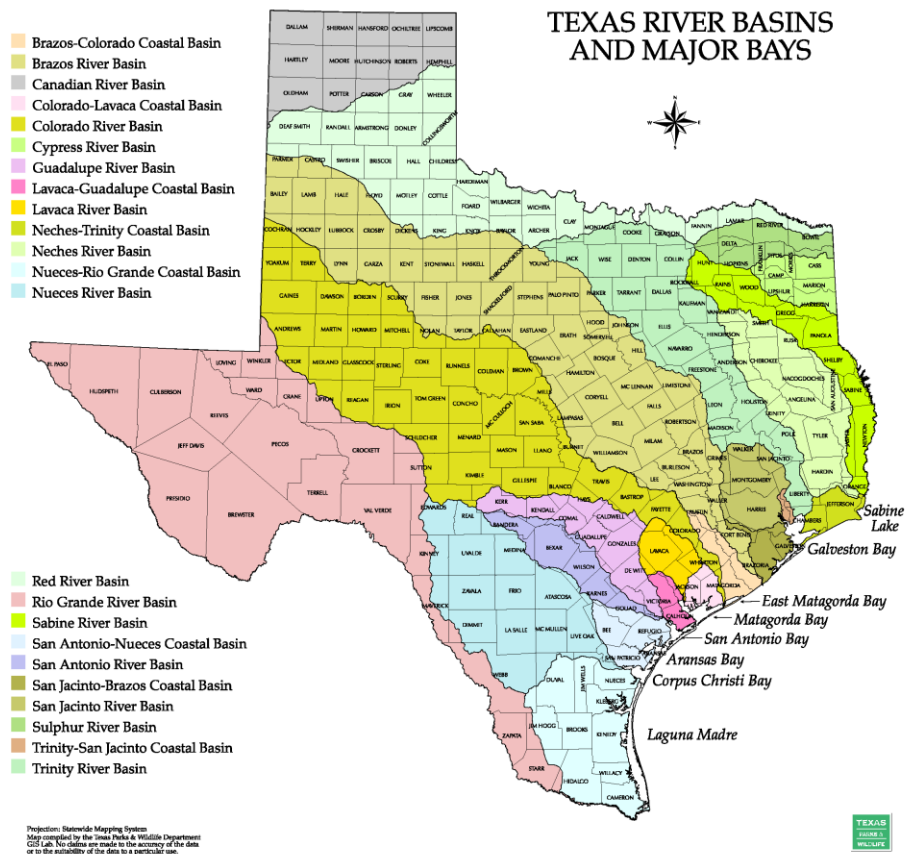


Figure 8. Major river basins within the state of Texas (adapted from Texas Parks and Wildlife Department 2021c).

Brazos Oak and the Elmendorf Oak separate from the Hybrid Swarm of collected *Quercus* series *Virentes* trees at approximately coefficient  $\sim.68$  and  $\sim.72$ , respectively (Fig. 5). These data suggest Brazos and Elmendorf oaks are two distinct, relic species (Mueller 1961). With regards to the Brazos Oak, this relic is likely a result of isolation from populations of other *Quercus* series *Virentes* species. Premature branching of the Brazos Oak from the *Quercus* series *Virentes* cluster (Fig. 5) indicates accessions considered to be in the Hybrid Swarm and the Brazos Oak. This is a relationship that is genetically older than collected individuals of *Quercus* series *Virentes* in Texas. A plant trademark has been issued for *Q. fusiformis* 'Brazos Oak' (#77828794). Current research is further investigating the genetic relationship of Brazos Oak, *Q. fusiformis*, and *Q. virginiana*. To help confirm relic classification of the Elmendorf Oak, future research is anticipated.



Figure 9. *Quercus fusiformis* 'Brazos Oak' fall foliage (Lake Alan Henry, TX).

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