

Genetic Differentiation of Two California Red Oak Species, *Quercus parvula* var. *shreveii* and *Q. wislizeni*, based on AFLP Genetic Markers¹

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Abstract

Oaks are renowned for posing problems in defining species boundaries. One example is the case of the interior live oak complex that is usually taken to include two varieties of *Quercus wislizeni* from the Coast Ranges of California and the Sierra Nevada, and *Q. parvula* var. *shreveii* from the central coast of California. The latter taxon was separated from *Q. wislizeni*, and was recognized in *The Jepson Manual*, but still is not fully accepted, since its morphological separation is ambiguous. In an attempt to test differentiation of these two taxa, molecular genetic analysis was carried out using Amplified Fragment Length Polymorphism (AFLP) markers. A total of 202 individuals from 19 populations including *Q. parvula* var. *shreveii*, *Quercus wislizeni*, *Q. kelloggii* and *Q. agrifolia* were analyzed. Four primer sets generated 311 polymorphic band classes. Although species-specific markers were observed between *Q. kelloggii* and other members of the red oak group in California, fixed genetic differences were not apparent among *Q. agrifolia*, *Q. parvula* var. *shreveii* and *Q. wislizeni*. Cluster analysis and principal coordinate analysis revealed low levels of genetic differentiation among coast live oak, interior live oak and shreve oak. Cluster analysis also suggested that *Q. agrifolia* populations are more similar to populations of *Q. parvula* var. *shreveii* than to coastal populations of *Q. wislizeni*. This may reflect the relatively short time since divergence of these species. On the other hand, repetitive hybridization and introgression may have acted as a homogenizing factor for these genetic markers. The relatively high genetic similarity between *Q. agrifolia* and *Q. parvula* var. *shreveii* may have implications for the management of Sudden Oak Death in the limited range of the latter taxon.

Introduction

Characteristics such as high levels of phenotypic plasticity (Jensen 1988, Jensen and others 1984, Tucker 1974) and genetic variation (Guttman and Weight 1988, Hokanson and others 1993, Kremer and Petit 1993, Manos and Fairbrothers 1987, Samuel and others 1995, Schnabel and Hamrick 1990, Zanetto and others 1993) contribute to the success of the genus *Quercus*. However these features pose difficulties in estimating genetic differentiation between species, genetic architecture of populations, and definitive ruling on taxonomic relationship among species

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(Guttman and Weight 1988, Hokanson and others 1993, Manos and Fairbrothers 1987).

Studies on European white oaks (subgenus *Quercus*) show that most genetic variation is found within population rather than between populations or between species. Kremer and Petit (1993) reported that 74 percent of isoenzyme diversity is found within populations, whereas variation between populations and species are 3 percent and 23 percent respectively. Other studies on genetic variation of European and north American white oaks (Guttman and Weight 1988, Muller-Starck and others 1993, Schnabel and Hammrick 1990, Zanetto and others 1993) not only demonstrated that most genetic variation is found within populations, it also showed high genetic identity between populations and species.

There are approximately 200 species of red oaks (*Quercus*, section Lobatae) (Jensen 1997, Vazques and Nixon 1999) restricted to the New World. Jensen (1997) included 35 species of red oaks in “Flora of North America,” north of Mexico.

Despite the rich diversity and wide distribution of red oaks in the U.S., relatively few molecular genetic studies have been conducted on their diversity and phylogeny. Similar to white oaks, most genetic variation in red oaks is contained within the population (though this level is higher for the red oaks, 98-99 percent than white oaks). Manos and Fairbrothers (1987) showed low estimates of genetic diversity between populations ($D_{st} = 0.001-0.021$) as compared to within population diversity ($H_S = 0.055-0.115$) in red oaks of New Jersey. They further showed minimal differentiation among five of the six species studied. In a similar study over a wide range and multiple species of subgenus Lobatae, Guttman and Weight (1989) found most red oak taxa to be similar in mean number of alleles per locus, percent polymorphic loci and mean heterozygosity. Schwarzman and Gerhold (1991) and Hokanson and others (1993) also reported low levels of genetic differentiation among populations and between species. Hokanson and others (1993) further found no species-specific markers for *Q. rubra* and *Q. ellipsoidalis* from the Apostle Islands, Wisconsin, despite high levels of polymorphism among the enzymes studied.

Studies on genetic variation or genetic differentiation of California red oaks are very limited (Dodd and others 1993a, Dodd and others 1993b, Dodd and others 1997, Millar and others 1990). Univariate and multivariate analysis of acorn fatty acids of *Q. agrifolia* and *Q. wislizeni* [classical name of *Q. wislizenii* is changed to *Q. wislizeni* according to the new International Code of Botanical Nomenclature (Greuter 1994)] significantly separated these two species from each other ($p < 0.0001$) (Dodd and others 1993). Among fatty acids identified, oleic acid and the percentage of unsaturated fatty acids were significantly higher in *Q. agrifolia* than in *Q. wislizeni*.

In a recent study, Dodd and others (1997) showed marked biochemical differences between *Q. wislizeni* from the Sierra Nevada and a population of *Q. parvula* from Palo Colorado canyon on the Central coast of California (type locality for *Q. parvula*). Sierran populations of *Q. wislizeni* showed low levels of the aliphatic hydrocarbon hentriacontane (C₂₉) while this pattern was reversed in populations of *Q. parvula*. This pattern was not very clear in coastal populations of *Q. wislizeni*, as they were more variable and includes individuals with *Q. parvula* biochemical characteristics. Dodd and others (1997) suggested that the variable pattern observed in the coastal populations of *Q. wislizeni* might be result of hybridization between *Q. wislizeni* and *Q. parvula*.

In view of morphological and chemical data supporting the differentiation of *Q. parvula* and *Q. wislizeni*, it was considered important to search for molecular genetic evidence (Amplified Fragment Length Polymorphism, AFLP markers) to substantiate species status and also to investigate the possible range of *Q. parvula*. Further, the genetic relationship of these taxa with other California black oaks (*Q. agrifolia* and *Q. kelloggii*) will be examined.

Materials and Methods

Experimental Material

Foliage was collected from 6-10 individuals from each of 11 populations of *Q. wislizeni* var. *wislizeni* and 4 populations of *Q. parvula* var. *shrevei* (hereinafter referred to as *Q. parvula*). The shrub form of *Q. parvula* was not considered in this analysis. Sampled individuals of the former species included 6 populations from the foothills of the Sierra Nevada and 5 populations from the coast ranges of California (table 1, fig. 1). Putative hybrid individuals were sampled if they were encountered in the population. Two control populations each of *Q. kelloggii* and *Q. agrifolia* were added to the analysis. These controls were collected from presumed pure populations (table 1, fig. 1). Within each population, foliage from mature trees at least 50 m. apart were collected randomly. Leaves were kept on ice and subsequently at 4°C until extraction.

Table 1—Location of populations sampled.

Pop code	Species	Location	County	Nearest city	Latitude	Longitude
W07	<i>Q. wislizeni</i>	Brickyard Rd. (Hwy 88)	Amador	Ione	32° 21'	120° 55'
W53 (K53)	<i>Q. wislizeni</i> ^{1,2}	Geysers Rd.	Sonoma	Geyserville	38° 45'	122° 50'
W55 (K55)	<i>Q. wislizeni</i> ^{1,2}	Orr Springs Rd.	Mendocino	Ukiah	39° 11'	123° 16'
W60 (K60)	<i>Q. wislizeni</i> ²	Covelo Rd. (Hwy 162)	Mendocino	Longvale	39° 34'	123° 25'
W66 (K66)	<i>Q. wislizeni</i> ^{1,2}	Highway 70	Butte	Oroville	39° 38'	121° 33'
W67	<i>Q. wislizeni</i>	Highway 36	Tehama	Red Bluff	40° 20'	122° 06'
W68 (K68)	<i>Q. wislizeni</i> ^{1,2}	Lake Shasta (Bailey Cove)	Shasta	Redding	40° 49'	122° 18'
W69 (K69)	<i>Q. wislizeni</i> ²	Highway 299	Shasta	Whiskey Town	40° 37'	122° 31'
W70 (K70)	<i>Q. wislizeni</i> ^{1,2}	Highway 3	Trinity	Douglas City	40° 37'	122° 57'
W72	<i>Q. wislizeni</i>	Highway 140	Mariposa	Midpines	37° 33'	120° 24'
W75	<i>Q. wislizeni</i>	Highway 4	Calaveras	Copperopolis	37° 59'	120° 39'
P71	<i>Q. parvula</i>	Big Creek reserve	Monterey	Lucia	36° 05'	121° 36'
P73	<i>Q. parvula</i>	Sycamore Canyon Rd.	Monterey	Big Sur	36° 13'	121° 47'
P78	<i>Q. parvula</i> ¹	Nacimiento-Fergusson Rd.	Monterey	Lucia	35° 09'	121° 28'
P79	<i>Q. parvula</i>	Coast Rd.	Monterey	Big Sur	36° 17'	121° 51'
K01	<i>Q. kelloggii</i>	Blodgett Reserve	Placer	Georgetown	38° 56'	120° 43'
K74	<i>Q. kelloggii</i>	Highway 299	Humboldt	Blue Lake	40° 54'	123° 55'
A03	<i>Q. agrifolia</i>	Marshall-Petaluma Rd.	Sonoma	Marshall	38° 10'	122° 54'
A04	<i>Q. agrifolia</i>	Petaluma-Point Reyes Rd.	Sonoma	Point Reyes Station	38° 05'	122° 48'

¹ Population where putative hybrids were observed and collected.

² Population where *Q. kelloggii* was collected in addition to target species. Code in parenthesis

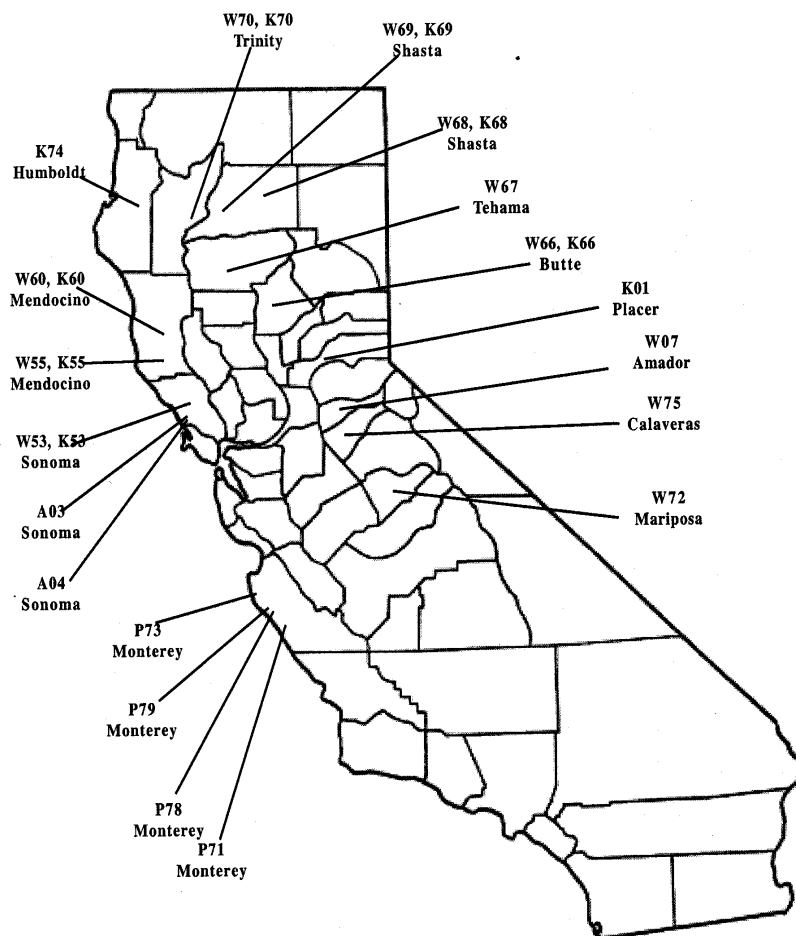


Figure 1—Map of sampled populations in California by county. (For more detail on locations see *table 1*).

DNA Extraction and AFLP Procedure

DNA was extracted from fresh foliage following a simplified Doyle and Doyle method (Cullings 1992). The amplified fragment length polymorphism (AFLP) method of Vos and others (1995) was performed following the protocol of Life Technologies (Rockville, MD, U.S.A.). DNA was restricted with *EcoRI* and *MseI* (1.25 U/μl of each) in a reaction buffer [10 mM tris-HCl (pH 7.5), 10 mM Mg-acetate and 50 mM K-acetate] in a total volume of 12.5 μl. The restriction reaction was carried out at 37°C for 2 hours followed by denaturation of restriction enzymes at 65°C for 10 min. Adapter ligation was conducted in the ligation buffer using T4 DNA ligase for 2 hours at 20°C. Subsequently, the ligation product was diluted 1:10 for primary amplification.

Primary amplification was carried out in a standard Polymerase Chain Reaction (PCR) cocktail containing 1.5 mM MgCl₂ and 0.5 mM of each of the primary amplification primers. For the primers, the *EcoRI* primer sequence was identical to the adapter sequence, whereas the *MseI* primer had an extra cytosine (“C”) as a

selective nucleotide (table 2). The PCR reaction was performed in a Techne Genius thermocycler for 20 cycles using the following cycling parameters: 30 s at 94°C, 60 s at 56°C and 60 s at 72°C. The primary amplification product was then diluted 1:50 for selective amplification. Selective amplification was carried out in a standard PCR reaction cocktail containing 2.5 mM MgCl₂ and 0.5 mM of the selective amplification primers. The PCR program had two cycles: 13 cycles of 30s at 94°C, 30 s at 65°C (annealing temperature was lowered 0.7°C at each cycle) and 60 s at 72°C, followed by 23 cycles of 30 s at 94°C, 30s at 56°C and 60 s at 72°C. Four sets of selective primers were used: 1/2, 3/5, 3/6 and 4/8. Selective amplification products were resolved on 6 percent polyacrylamide native gel. Gels were stained with Gelstar (FMC Bioproducts, Rockland, ME, U.S.A.) following manufacturer protocol and documented with a Kodak DC 120 digital camera. Bands were scored as present/absent using Gelcompar II software (Applied Maths, Kortrijk, Belgium).⁴

Statistical Analysis

AFLP scores were treated phenetically and any reference to genetics (e.g. molecular genetic markers) implies observed restriction length variation as revealed by AFLP methodology. UPGMA (Un-weighted pair-group method using arithmetic averages) cluster analysis (Sneath and Sokal 1973) was carried out on Jaccard's coefficient (Dunn and Everitt 1982). This coefficient is the ratio of number of positive matches to the total number of characters minus the number of negative matches. Jaccard's coefficient omits consideration of negative matches (Sneath and Sokal 1973). Cluster analysis is a process in which samples are partitioned into one or more groups (clusters) in a hierarchical order. UPGMA in particular calculates the average similarity or dissimilarity of a candidate sample to an extant cluster. Principal coordinate analysis and cluster analysis were carried out using NTSYS-pc (Rohlf 1993). Principal coordinate analysis is an ordination method in which samples are grouped together based on their similarities. The output of the analysis usually is plotted on a two dimensional graph, which help the researcher to inspect the possible relationship of the samples or specimens.

Results

Four primer sets generated a total of 311 band classes for all the individuals in the study, including putative hybrids. *Q. kelloggii* showed the least number of band classes followed by *Q. agrifolia*. Several markers also were observed to be fixed for *Q. kelloggii*. Principal coordinate analysis (Pcoor) of all individuals of *Q. agrifolia*, *Q. kelloggii*, *Q. parvula* and *Q. wislizeni* showed clear separation of *Q. kelloggii* from other red oaks of California (fig. 2). The remaining species showed three overlapping groups. Both *Q. parvula* and *Q. wislizeni* showed high variability, as it is evident by the spread of individuals of these species on Pcoor plot (fig. 2). This could have arisen from wider range of sampling for *Q. parvula* and *Q. wislizeni*, while *Q. agrifolia* populations were apart not more than 20 miles. However *Q. kelloggii* populations which were sampled over wide range showed a very close affinity to each other. UPGMA of all individuals was in agreement with the principal coordinate analysis (fig. 3). Two major clusters were observed. All black oak individuals fell in

⁴ Mention of trade names or products is for information only and does not imply endorsement by the U.S. Department of Agriculture.

one cluster and individuals of each population shared a common node. The second major cluster contained *Q. agrifolia*, *Q. parvula* and *Q. wislizeni*. Individuals of each population of coast live oak shared a common node, while individuals of shreve oak populations did not always branch from the same node. For example, individuals of population P71 branched from two different nodes. Except for the populations W07 and P71 which clustered with *Q. agrifolia* populations in the second major cluster, all other populations of *Q. parvula* and *Q. wislizeni* shared a common node. Similar to shreve oak, all individuals of some populations of interior live oak shared a common node while others (W53, W60, W66, W68 and W70) branched from different nodes. In this analysis, one *Q. agrifolia* and several *Q. parvula* and *Q. wislizeni* did not resolve with their respective populations and placed as basal branches to the dendrogram (unresolved). UPGMA of all individuals of *Q. parvula* and *Q. wislizeni* showed no major changes in the topology compared to previous analysis (data not shown). However, four individuals of population W69 clustered with population P71 and W07.

Lack of clear resolution of some individuals under study into their respective clusters or groups could have come from misidentification of bands. This could have occurred either at band selection or band class grouping stages. At the band selection stage, a band could have been misidentified either due to its weak signal or being obscured by a strong signal from an adjacent band. Band class grouping was performed by the “Gelcompar” algorithm and was set to minimize misclassification. Failure to identify a band (band selection) or misclassification of a band to a different class could potentially cause distortion of the data. To minimize this effect, a population was considered as an operational taxonomic unit (OTU). For these OTUs, a character (band class) was accepted as being present, if at least one individual of a population carried the band. Similarly, a band was considered missing when the marker was absent in all individuals of the population. Then UPGMA based on OTUs was performed. The resulting dendrogram consisted of 2 major clusters, the *Q. kelloggii* cluster and the cluster of all other live oak OTUs (fig. 4). This dendrogram also supported high phenetic similarity of coast live oak, interior live oak and shreve oak.

Discussion

The observed levels of taxon differentiation in genus *Quercus* varies depending on the choice of characters (Bruschi and others 2000, Chechowitz and others 1989, Hokanson and others 1993, Le Corre and others 1997). Based on morphological and biochemical characters, oaks can be separated into distinct species (Bruschi and others 2000, Dodd and others 1996.). However, allozyme (direct product of genes) and nuclear genome markers (e.g., RFLP, RAPD and AFLP) have frequently failed to allow clear resolution of species boundaries between closely related oaks (Schwarzman and Gerhold 1991, Le Corre and others 1997, Bruschi and others 2000). *Quercus parvula* and *Q. wislizeni* are not exceptions to this.

Using 16 quantitative and 14 subjectively scored morphological characters; Nixon (1980) clearly separated *Q. parvula* and *Q. wislizeni* from each other. However, his data failed to resolve the varieties of these species as separate groups. In the present study, AFLP markers fail to place all *Q. parvula* and *Q. wislizeni* populations into two separate clusters. However, *Q. parvula* populations P78, P79 and P73 constituted their own cluster except for P732 and not exclusive of W712 and

W536. Individuals of population P71 shared a common node. The latter (P71) is in the same cluster with W07 and some individuals from W69 as well as W723 and P732. The former populations (P78, P79, and P73) are in the same cluster as W75. Lack of clear resolution of red oak taxa into distinct groups (clusters) based on AFLP markers is consistent with other molecular genetic studies of red oaks (Manos and Fairbrothers 1987, Guttman and Wiegert 1989, Hokanson and others 1993). Manos and Fairbrothers (1987) and Guttman and Wiegert (1989) found no species-specific marker of any value for identifying most of the red oak species that they studied. The present study also demonstrated lack of species-specific markers among the evergreen oak species studied. Except for absence of several AFLP markers in *Q. kelloggii*, no species-specific marker could be found among these oaks. Moreover, several markers were fixed or nearly fixed for the California black oak, while no fixed markers were observed for the other species. Manos and Fairbrother (1987) and Guttman and Wiegert (1988) suggested the relatively short time since divergence and rapid speciation as causes for scarcity of molecular genetic markers in red oaks of the eastern United States.

In late Eocene strata, fossil foliage of species displaying characteristics of major groups of oaks (e.g., subgenus *Lobatae*) has been found. In the Miocene (5-13 mya) most oak species existing today were evidently present (Wolfe 1979). Fossils of *Q. kelloggii* ancestors were reported from the early Miocene fossil flora of California while the progenitor of *Q. wislizeni* and *Q. agrifolia* was described from the late Miocene fossil flora. Nixon (1980) postulated that the nearest relatives of a *Q. parvula* progenitor are likely to be found in central and southern Mexico, while ancestors of *Q. wislizeni* probably had close affinities to interior desert species of northern Mexico. Nixon (1980) suggested that the progenitor of *Q. parvula* was associated with “oak-pine-laurel” forest of the Tertiary and was restricted to the subtropical coastal forests of California. Nixon (1980) also suggested that the *Q. wislizeni* ancestor was found in interior woodland and savannah habitats. Alternatively, Axelrod (1983) proposed a common ancestor for interior live oak and shreve oak. Axelrod (1983) estimated that about 20 mya shreveoides, a progenitor of *Q. parvula*, first appeared in west-central Nevada and shifted westward to the Sierra Nevada. As climate became dryer in the Upper Miocene (13-12 mya), wislizenoides replaced shreveoides, at first in western Nevada, and later in the lower Sierra Nevada and the Inner Coast Ranges. According to Axelrod (1983), the *shrevei* line has contributed to mesic broad-leaved sclerophyll forest for fully 18.5 million years. *Q. wislizenoides*, on the other hand, has typified semiarid oak-grassland and woodland vegetation since the later Miocene. Axelrod (1983) further postulated that oaks of the *wislizeni* alliance are related to series *Peninsulares* of Baja California, which in turn is associated with series *Durifokiae* of the southwestern United States and northwestern Mexico.

Results of the present study are more easily reconciled with Axelrod's (1983) hypothesis of a common progenitor for *Q. parvula* and *Q. wislizeni* than with Nixon's (1980) idea. Both cluster analysis and principal coordinate analysis showed a high degree of similarity between these two species (figs. 6-12). Even though most *Q. parvula* populations shared a common dendrogram node, these populations were scattered within the same major cluster containing *Q. wislizeni*. Principal coordinate analysis of *Q. wislizeni* and *Q. parvula* also showed a close affinity between interior live oak and shreve oak, and was not able to segregate these two species into distinct groups. As in eastern red oaks (Guttman and Weigt 1989, Manos and Fairbrothers

1987), rapid speciation with minimal changes in AFLP markers may have occurred since divergence of *Q. parvula* and *Q. wislizeni* about 10-15 mya (Axelrod 1983).

The high similarity between *Q. parvula*, *Q. wislizeni* and *Q. agrifolia* could be explained by reticulation of characters due to frequent hybridization. It is plausible that during the last glaciation in late Pleistocene the distribution of California red oaks was constricted to refugia on the central and southern slopes of the coast ranges and western slopes of the southern Sierra Nevada. In these refugia, extensive hybridization and introgression conceivably could have occurred. After retreat of the glaciers, oaks may have expanded in distribution rapidly with minimal changes in AFLP neutral markers. However if this is the case, *Q. kelloggii*, which hybridizes with each of the evergreen oaks of California, should have shown a higher degree of similarity with other red oaks in this study. All the analyses showed a clear separation between *Q. kelloggii* and other red oaks. It is very difficult to assume absence of *Q. kelloggii* from these putative refugia; but uni-directional gene flow from California black oak to other evergreen red oaks could have occurred. Evidence of hybridization in black oaks of California will be discussed in Chapter 3.

The current study showed strong similarity among *Q. agrifolia*, *Q. parvula* and *Q. wislizeni*. However, Muller (1938) noted close affinity between *Q. parvula* and *Q. agrifolia*. He placed these species in the section *Agrifolae*, which includes *Q. wislizeni*. Considering the small number of coast live oaks used in this study, it is very difficult to draw any definitive conclusion about the genetic relationship of *Q. agrifolia* with *Q. parvula* and *Q. wislizeni* or the progenitor of *Q. agrifolia*. Nevertheless, it is possible that *Q. agrifolia* may have evolved from a *Q. parvula* like progenitor. Sampling more *Q. agrifolia* probably will show more genetic variation and possibly more separation between coast live oak and the other taxa.

Conclusion

The current study showed no diagnostic AFLP markers that would allow unequivocal discrimination between *Q. parvula* and *Q. wislizeni*. However, fossil records (Axelrod 1983) ecological preferences (Nixon 1980, Tucker 1997) and biochemical data (Dodd and others 1997) furnish enough evidence to consider these taxa as two separate species. Radiation over a relatively short time period, and rapid morphological divergence with minimal changes in nuclear genetic markers [e.g., allozyme (Millar and others 1990), AFLP (current study)] may provide the best explanation for the high similarity observed between shreve oak and interior live oak. Even though convergence of characters due to hybridization cannot be ruled out, the evidence presented here suggests a common progenitor for *Q. parvula* and *Q. wislizeni* (Axelrod 1983) and possibly *Q. agrifolia*. Unless a thorough phylogenetic analysis based on nuclear and organellar DNA sequences are conducted, the taxonomic status of *Q. parvula* and *Q. wislizeni* cannot be settled with confidence.

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