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Genetic evidence for hybridization in red oaks (*Quercus* sect. *Lobatae*, Fagaceae)[1]

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- *Premise of the study:* Hybridization is pervasive in many plant taxa, with consequences for species taxonomy, local adaptation, and management. Oaks (*Quercus* spp.) are thought to hybridize readily yet retain distinct traits, drawing into question the biological species concept for such taxa, but the true extent of gene flow is controversial. Genetic data are beginning to shed new light on this issue, but red oaks (section *Lobatae*), an important component of North American forests, have largely been neglected. Moreover, gene flow estimates may be sensitive to the choice of life stage, marker type, or genetic structure statistic.
- *Methods:* We coupled genetic structure data with parentage analyses for two mixed-species stands in North Carolina. Genetic structure analyses of adults (including F_{ST}, R_{ST}, G'_{ST}, and STRUCTURE) reflect long-term patterns of gene flow, while the percentage of seedlings with parents of two different species reflect current levels of gene flow.
- *Key results:* Genetic structure analyses revealed low differentiation in microsatellite allele frequencies between co-occurring species, suggesting past gene flow. However, methods differed in their sensitivity to differentiation, indicating a need for caution when drawing conclusions from a single method. Parentage analyses identifed >20% of seedlings as potential hybrids. The species examined exhibit distinct morphologies, suggesting selection against intermediate phenotypes.
- *Conclusions:* Our results suggest that hybridization between co-occurring red oaks occurs, but that selection may limit introgression, especially at functional loci. However, by providing a source of genetic variation, hybridization could influence the response of oaks and other hybridizing taxa to environmental change.

Key words: Fagaceae; gene flow; genetic structure; hybridization; *Lobatae*; parentage; *Quercus*.

Hybridization is an important process in many plant taxa. It may lead to the formation of new species or to the collapse of existing taxa or affect levels of genetic variation, local adaptation, and the effectiveness of selection (Arnold, 1992; Allendorf et al., 2001; Aldrich et al., 2003b; Coyne and Orr, 2004; Riesberg and Willis, 2007; Hipp and Weber, 2008; Lorenzo et al., 2009). In oaks (*Quercus* L., Fagaceae), morphological data and controlled crosses have suggested widespread hybridization, leading some to question the utility of the biological species concept for this and other hybridizing taxa (Palmer, 1942; Burger, 1975; Rushton, 1993; Nixon, 2006). Others, however, argue that extensive hybridization in the wild is rare (Coyne and Orr, 2004). In the last few decades, genetic information has begun to contribute to the debate, but red oaks (section *Lobatae*), an important component of North American forests, have been largely neglected. Moreover, hybridization in natural populations can be challenging to measure. For instance, the choice of genetic markers and genetic structure measures can affect estimates of gene flow or population differentiation (Whittemore and Schaal, 1991; Ducousso et al., 1993; Hedrick, 1999; Craft and Ashley, 2006; Jost, 2008), while estimates of hybridization may vary depending on whether seeds, seedlings, or adult plants are examined. In this study, we use multiple measures of genetic structure, coupled with Bayesian parentage analysis of seedlings, to investigate current and past hybridization between co-occurring red oak species (*Q. rubra* L., *Q. velutina* Lam., *Q. coccinea* Münchh., *Q. falcata* Michx.) at two sites in North Carolina. Using multiple measures of gene flow allows us to investigate how the choice of life stage or genetic structure measure may affect conclusions about the extent of gene flow.

Many cases of putative natural hybridization of oaks have been identified since the 1800s (Rushton, 1993; Dodd and Afzal-Rafii, 2004). Deliberate crosses have confirmed that many oak species are interfertile (Cottam et al., 1982), although the success rate for interspecific crosses is typically lower than for intraspecific crosses (Rushton, 1993; Williams et al., 2001). Although in some cases hybrids have reduced fertility (Rushton, 1993), hybrid oaks generally appear healthy and produce viable seed and pollen (Salvini et al., 2009). However, this does not necessarily mean that hybridization is common in the wild because intermediate morphologies or similarity in chemical traits could reflect intraspecific variation or convergent evolution.

Species may exhibit four patterns with regard to hybridization: (1) complete reproductive isolation; (2) formation of a hybrid zone, where F1 hybrids are restricted to a narrow geographic area; (3) introgression over a more widespread area, but where parental types are still identifiable; or (4) the formation of a hybrid swarm, where most individuals exhibit intermediate morphologies and/or mixed genetic characteristics. Hybrid zones are most likely to be exhibited by species with strong differences in habitat requirement, where intermediate habitats exist between the parental ranges (Muller, 1952). In contrast, introgression may be facilitated when species co-occur in an area

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with smaller-scale environmental heterogeneity (Rushton, 1993; Valbuena-Carabana et al., 2007). Morphological data suggest that hybrid swarms are rare in oaks—taxa usually maintain fairly distinct suites of traits (Rushton, 1993; Coyne and Orr, 2004). However, genetic data indicate that complete isolation between closely related sympatric species may also be rare in *Quercus*.

Numerous studies have examined hybridization rates in white oaks (section *Quercus*) and, to a lesser extent, live oaks (section *Virentes*), using paternity analysis of seeds from known mother trees (Bacilieri et al., 1996; Streiff et al., 1999; Salvini et al., 2009) or measures of genetic differentiation between adults (Craft et al., 2002; Muir and Schlotterer, 2005; Craft and Ashley, 2006; Curtu et al., 2007; Valbuena-Carabana et al., 2007; Burgarella et al., 2009; Cavender-Bares and Pahlich, 2009). Estimates of hybridization rates, using both methods, range from over 25% to almost zero, with many in the 5–10% range. However, few studies have examined gene flow in red oaks, and none have estimated hybridization rates (Aldrich et al., 2003b; Dodd and Afzal-Rafii, 2004; González-Rodríguez et al., 2004; Tovar-Sánchez and Oyama, 2004; Hipp and Weber, 2008; Peñaloza-Ramírez et al., 2010). Red oaks are an important part of the North American flora, with ca. 200 species endemic to the continent, and there are indications that species barriers may be weaker than in white oaks (Guttman and Weigt, 1989; Kashani and Dodd, 2002; Aldrich et al., 2003b).

The choice of methods may greatly affect estimates of hybridization. For instance, the high allelic diversity of the microsatellite markers currently favored in population genetic studies can result in low estimates of population differentiation using traditional measures such as F_{ST}, even when populations have no alleles in common (Hedrick, 1999). Life stage can also influence conclusions about hybridization. In white oaks, many of the higher hybridization estimates (>7%) come from studies of seeds (Bacilieri et al., 1996; Streiff et al., 1999; Salvini et al., 2009), while the lowest estimates (<5%) all came from studies of adults (Craft et al., 2002; Muir and Schlotterer, 2005; Curtu et al., 2007; Burgarella et al., 2009). This is what one would expect if there selection against hybrids, but previous studies did not examine adults and juveniles in the same population.

In addition to affecting the taxonomic status of populations, the existence of interspecific gene flow has management implications. Forest managers interested in promoting certain traits could find their efforts unexpectedly helped or hindered by cryptic gene flow (Elena-Rossello et al., 1992). For instance, introgression from *Q. ellipsoidalis* could reduce the value of *Q. rubra* timber if hybrids share the knotty wood grain of *Q. ellipsoidalis* (Aldrich et al., 2003b). The ecological and genetic distinctness of rare species and subspecies can also be endangered when they hybridize with a common relative (Allendorf et al., 2001; Lorenzo et al., 2009). Finally, environmental perturbations, including changes in climate and land use, could affect both the opportunity for hybridization and the consequences of interspecific gene flow. For instance, in a region where summer temperature is increasing and/or precipitation is decreasing, introgression from more drought-tolerant relatives might introduce alleles useful for local adaptation. Dodd and Afzal-Rafii (2004) found that for *Q. wislizeni*, the level of introgression from two other species was correlated with the temperature and moisture of the site. It has even been suggested that hybridization, followed by backcrossing, may have played a role in the migration of some oak species following the last glacial period (Petit et al., 2003).

In this study, we measured the degree of neutral genetic differentiation between adults of co-occurring red oak species at two sites in North Carolina using eight different measures of genetic structure and compared these with parentage analyses of seedlings to investigate the occurrence of past vs. current interspecific gene flow. If species rarely or never hybridize, then we expect to observe a high degree of genetic differentiation between morphologically defined species groups. If, on the other hand, hybridization is, or has been, common, then genetic structure measures should show no significant differentiation between morphospecies and/or genetic structuring within stands that does not correspond to morphologically defined species. If hybridization is currently occurring, we would expect to find evidence of this in parentage analysis results, with many seedlings assigned parents belonging to two different morphospecies.

MATERIALS AND METHODS

***The focal species and study populations*—**We made use of two long-term research stands in North Carolina: one in the Blackwood Division of the Duke Forest in the Piedmont (35°58′N, 79°5′W, http://www.env.duke.edu/forest) and one at the Coweeta long-term ecological research (LTER) site in the Southern Appalachians (35°03′N, 83°27′W, http://coweeta.ecology.uga.edu). Both stands consist of ca. 80-yr-old secondary forest. These stands were established for prior forest dynamics studies (Clark et al., 2004; Ibáñez et al., 2007), and long-term demographic data are available for all trees within the original stand area. For the purpose of parentage and dispersal studies, an additional 40–60 m border area was surveyed for oaks, regularizing the borders of the mapped stands (which were originally nonrectangular) and increasing total area to 12 ha at Duke Forest and 7.5 ha at Coweeta. All trees >2 m tall were tagged and measured. Originally, 2-m^2 plots (70 at Coweeta, 124 at the larger Duke Forest site) were established in cross-shaped transects at both sites (Clark et al., 2004; Ibáñez et al., 2007). At Duke Forest, where the seedling layer is sparse, 79 census plots of 1 m^2 and 70 of 7 m^2 were added to increase sample size for an initial dispersal analysis (Moran and Clark, 2011). No plot was <30 m from the edge of the mapped stand. The parentage and dispersal model, described below, accounts for differences in plot size and for the distance between adults and plots.

Three red oak species are present at each site: *Quercus rubra* (northern red oak), *Q. velutina* (black oak), and *Q. falcata* (southern red oak) at Duke Forest, and *Q. rubra*, *Q. velutina*, and *Q. coccinea* (scarlet oak) at Coweeta. No recent phylogeny of the red oak clade included all four species. An early electrophoretic analysis (Guttman and Weigt, 1989) identified *Q. falcata* as a close relative of *Q. rubra*, while *Q. velutina* was placed in a separate clade along with *Q. marilandica* and *Q. nigra*. An analysis by Oh and Manos (2008) based on a nuclear locus (CRC), however, showed that *Q. falcata* and *Q. rubra* were not sister taxa: *Q. marilandica* was more closely related to *Q. falcata*, and *Q. nigra* was more closely related to *Q. rubra*. Manos and Stanford (2001) came to a similar conclusion, showing *Q. falcata* as more closely related to *Q. palustris* than to *Q. rubra*. AFLP markers seem to indicate that *Q. coccinea* and *Q. ellipsoidalis* are more closely related to one another than either is to *Q. velutina*, though there is some evidence of hybridization between these groups (Hipp and Weber, 2008). According to the Flora of North America, *Q. rubra* may hybridize with *Q. coccinea* and *Q. velutina*; *Q. falcata* with *Q. velutina*; and *Q. velutina* with all three other species (www.eFloras.org). Other sources have reported hybridization between *Q. rubra*, *Q. velutina*, and *Q. coccinea* (Jensen, 1977; Tomlinson et al., 2000), but we found no reports of hybridization between *Q. rubra* and *Q. falcata*. All four species are broadly distributed in eastern North America (Little, 1980), and species are frequently found growing side-by-side at both study sites (Appendix S2 (Fig S2.1), see Supplemental Data in online version of this article).

We identified trees using a whole-tree silvics approach, similar to that described by Aldrich et al. (2003b) (Fig. 1). "*Quercus rubra*" has large, smooth, thin leaves with 7–11 bristle-tipped lobes all the way around the margin; gray bark that in adult trees is deeply cracked and furrowed, giving the impression of dark and light stripes; and large acorns (1.5–2.8 cm) with shallow cups of closely overlapping scales. "*Quercus coccinea*" is similar, but the leaf lobes are narrower and deeper, divided nearly to the midvein; the pale stripes on the bark

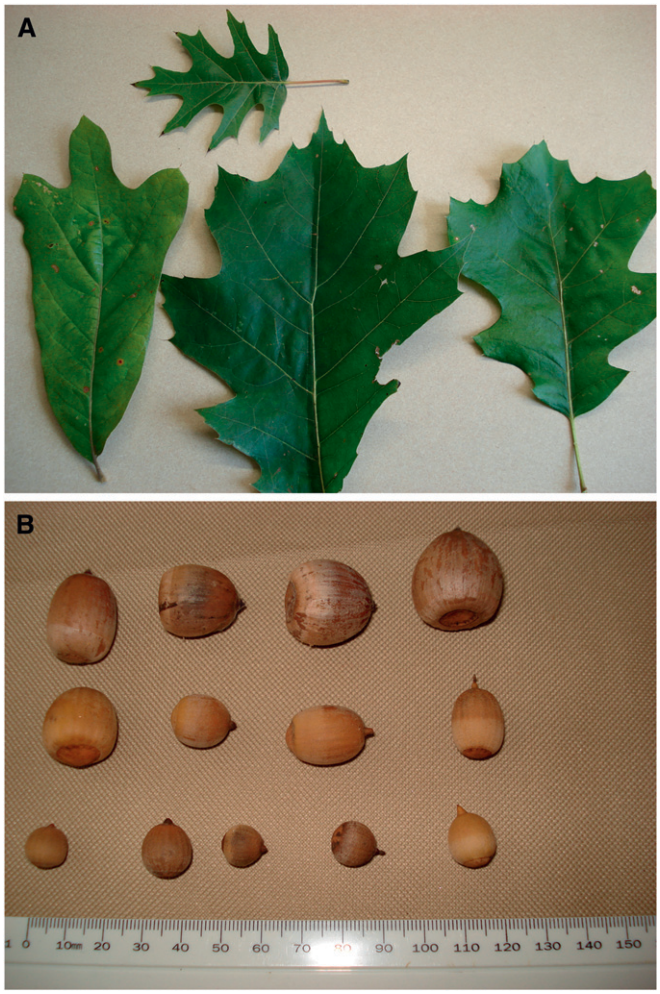

Fig. 1. (A) Adult leaves of *Quercus coccinea* (top), *Q. falcata* (left), *Q. rubra* (middle), and *Q. velutina* (right); and (B) acorns of *Q. rubra* (top row), *Q. velutina* (middle), and *Q. falcata* (bottom).

are less obvious; trees tend to retain persistent dead limbs; acorns are smaller and egg-shaped, 1.2–2.5 cm long; and the cup scales are short, pointed, and closely overlapping. "*Quercus velutina*" has thicker, pubescent leaves with shallower lobes than *Q. rubra*; the bark is blackish and deeply cracked and furrowed; acorns are elliptical (1.5–1.9 cm long) and lighter in color than those of *Q. rubra*; and the cup scales are long, loose, and pointed. Finally, "*Q. falcata*" has thick, pubescent leaves with brownish hairs underneath and three primary lobes near the tip; medium-gray ridged bark; small (1.2–1.5 cm), round, dark brown acorns; and short, pointed, and closely overlapping cup scales (Little, 1980). Nearly all adults could be easily identified according to these traits. Because bark and fruit traits are not visible for juveniles, seedlings were identified to species based on leaf traits alone.

***Data collection*—**All adults and all seedlings in the census plots (Table 1) were genotyped at six nuclear microsatellite loci: GA-1F07, GA-0E09, GA-0C11, GA-1J11, and GA-1F02 (Aldrich et al., 2002, 2003a), hereafter referred to as F07, E09, etc. Leaf samples were stored at −80°C before total genomic DNA extraction using a modified CTAB procedure (for details, see online Appendix S1). Alleles were manually scored using the program GeneMarker 1.4 (Softgenetics, State College, Pennsylvania, USA). Many individuals and loci were regenotyped to establish genotyping error rates (Appendix S3: Table S3.1). For genetic structure analyses, the best available genotype at each locus was used—for instance, if data indicated allelic dropout in one amplification, the heterozygous genotype was used instead.

TABLE 1. Sample sizes for *Quercus* taxa at Duke Forest and Coweeta.

Species	Duke Forest		Coweeta	
	Potential parents,	Sampled seedlings	Potential parents,	Sampled seedlings
Q. rubra	68	96	129	158
Q. velutina	22	85	15	13
Q. falcata	28	38	NA	NA
Q. coccinea	NA	NA	54	7

Note: NA = not applicable

***Genetic structure analysis*—**Many different methods have been developed to assess genetic differentiation between populations. The oldest and still most widely used are *F*-statistics (Wright, 1951) and their relatives (Φ_{ST}, G_{ST}). High values of F_{ST} (the reduction in heterozygosity between groups due to genetic drift) relative to F_{IS} (the reduction due to inbreeding) are expected for groups that are reproductively isolated (Wright, 1951). *R*-statistics (R_{ST}, R_{IS}) are similar measures developed specifically for microsatellites, based on the assumption of stepwise mutation rather than infinite alleles (Slatkin, 1995); they are interpreted in a similar manner to F_{ST} and F_{IS}. We calculated Φ_{ST} (Weir and Cockerham, 1984) and R_{ST} (Slatkin, 1995) for all loci together and each locus individually using the AMOVA option of the program GENALEX 6.1 (Peakall and Smouse, 2006); statistical significance was assessed using permutation (999 steps).

Observed heterozygosity for both stands was lower than expected under Hardy–Weinberg conditions. To investigate the possible influence of null alleles, we used the program FreeNA (http://www1.montpellier.inra.fr/URLB/). This program calculates F_{ST} based on Weir (1996), and a measure F_{ST}^{ENA}, which corrects for the influence of null alleles for all loci together (Chapuis and Estoup, 2007); 95% confidence intervals were constructed using a bootstrap procedure (1000 steps). The program also estimates both measures for each locus, but confidence intervals are not constructed.

While F_{ST} and its relatives remain popular, several authors have pointed out that using these measures for highly variable markers such as microsatellites can result in very low values even when strong population differentiation in allele frequencies is present (Hedrick, 1999, 2005; Gregorius et al., 2007; Jost, 2008). To address this, we used the web-based program SMOGD (Crawford, 2010), which calculates G_{ST} (Nei, 1973), G'_{ST} (Hedrick, 2005), and D (Jost, 2008). Unlike G_{ST}, the measure G'_{ST} ranges between 0 and 1 even for very high levels of heterozygosity (Hedrick, 2005). D improves on this further, as both G_{ST} and G'_{ST} will incorrectly report that a population is entirely differentiated at a locus when one subpopulation is fixed at allele A and all others are fixed at allele B (Gregorius et al., 2007). SMOGD estimates a value for each measure at each locus and constructs a 95% confidence interval using a bootstrap procedure (500 steps), but it does not estimate a value for all loci together. Unfortunately, no existing software package simultaneously corrects for high heterozygosity and for null alleles.

For each measure of differentiation, we compared estimates for the amount of differentiation between morphospecies within sites to the amount between sites for *Q. rubra* and *Q. velutina* and between *Q. falcata* at Duke Forest and *Q. coccinea* at Coweeta. Duke Forest and Coweeta are ca. 300 km apart. Gene flow between *Q. falcata* and *Q. coccinea* is expected to be near zero because these distant subpopulations are also different morphospecies; we expect to see statistically significant values of all differentiation measures. In wind-pollinated tree species, populations separated by a few hundred kilometers often exhibit low but significant genetic structure (Schwarzmann and Gerhold, 1991; Craft and Ashley, 2006; Sato et al., 2006). This is thought to be due to isolation by distance tempered by long-distance dispersal of pollen (Craft and Ashley, 2007; Ashley, 2010; Mimura and Aitken, 2010). If species are strongly reproductively isolated, then we would expect within-site between-species measures to be greater than between-site within-species measures and similar to the between-site between-species measure.

We also analyzed adult genotype data with the program STRUCTURE 2.2 (Pritchard et al., 2000), which uses Bayesian inference to evaluate the probability that sampled genotypes come from K distinct populations. We used the admixture model, which allows that individuals may derive portions of their genomes from more than one population. A run of 10 000 Markov chain Monte Carlo (MCMC) steps after an initial burn-in of 10 000 proved to be sufficient for parameter convergence. Because shared ancestry may introduce correlations in allele frequencies between populations, we ran the data using both correlated

and uncorrelated assumptions. Three runs were conducted for each site and for each set of correlation assumptions at values of K from one to five. The programs CLUMPP (Jakobsson and Rosenberg, 2007) and DISTRUCT (Rosenberg, 2004) were used to align replicates and produce consensus assignments (see Fig. 2). If species are largely isolated, we would expect STRUCTURE to distinguish three groups at each site, each group consisting mostly of individuals identified as a particular species.

***Parentage analysis*—**Finally, we examined the results of Bayesian parentage analyses of both populations (E. V. Moran and J. S. Clark, unpublished manuscript) to look for evidence of ongoing hybridization. Our model, fully described in Moran and Clark (2011) and summarized in online Appendix S3, estimates parentage and seed and pollen dispersal parameters, using as inputs genotype, location, and individual fecundity. Unlike earlier parentage and dispersal models, this model simultaneously takes into account (1) genotyping error and (2) ecological factors such as the distance between individuals and the fecundity of adults and (3) treats dispersal inside and outside the plot in a fully consistent manner.

Both mistyping error and allelic dropout are considered, allowing for the possibility that an apparent homozygote may actually be a heterozygote accounts for both allelic dropout and the potential influence of null alleles. Genotyping error rates were estimated by re-genotyping multiple loci in many individuals (Appendix S3: Table S3.1). The distribution of seedling census plots in each site is a relic of previous forest dynamics studies, but the model accounts for plot distribution and size by calculating a probability per square meter that a seed will be dispersed over distance d_{ik} between tree i and the plot in which seedling k is growing, multiplied by the area of the plot. The model also accounts for dispersal of seed and pollen from outside the stand by assuming that allele frequencies and fecundities are similar inside and outside the mapped stand and that a single dispersal kernel governs seed or pollen movement inside and outside. The model does not assume that the closest parent must be the mother, but mixes over uncertainty in the identity of the mother vs. father. Individuals of all species at each site were included as potential parents and offspring. All trees >10 cm dbh were considered potential parents. This is a conservative cutoff; red oaks with dbh <25 cm are rarely mature; however, trees 10–25 cm dbh do have a small probability of producing seed or pollen, particularly if located in a high-light site such as the edge of a canopy gap (Clark et al., 2010).

We identified as putative hybrids seedlings assigned parents of two different species. Because only leaf characters are available to assign seedlings to a species, this designation is less certain; consequently, seedlings of all species were considered together in this analysis. To take into account uncertainty in parentage assignment when estimating the frequency of hybridization, we calculated the proportion of putative hybrids for each of 300 random draws from the multinomial posterior parentage distributions.

RESULTS

***Genetic diversity*—**Allelic diversity was high at all loci, ranging from 16 to 33 alleles per locus, and most alleles were shared between species within a site (Appendix S1). However, observed heterozygosity was lower than expected under Hardy–Weinberg assumptions for all loci at both sites (Appendix S1: Table S1.2). Spatial genetic structure within populations was evident. An analysis using the r genetic correlation statistic in GENALEX revealed significant spatial correlation in genotypes on the scale of 0–40 m at Coweeta and 0–20 m and 40–50 m at Duke Forest (Appendix S1: Fig. S1.5) for adult trees. In seedlings, spatial genetic structure was evident at the 0–20 m scale at Coweeta and the 0–30 m and 50–70 m scale at Duke Forest (Moran and Clark, unpublished manuscript).

***F_{ST} and other differentiation measures*—**The various differentiation statistics differed substantially in their sensitivity to differences in allele frequencies between groups (Table 2). R_{ST} did not indicate significant differentiation between any subpopulations, even for groups between which gene flow should be negligible. Consistent with our expectation, all other measures agreed that the greatest genetic differentiation exists between *Q. falcata* at Duke Forest and *Q. coccinea* at Coweeta. Most measures showed low but significant genetic differentiation between *Q. rubra* at Duke Forest and Coweeta; for measures in the F_{ST} family between-site within-species differentiation was 16.2–20.4% of the between-site between-species differentiation, while G'_{ST} was 34.2% and D was 33.9% of the between-site between-species value. None of the measures showed statistically significant differentiation between *Q. velutina* at the two sites, most likely due to small sample sizes.

Estimates of differentiation between morphospecies within sites were generally of a similar magnitude or smaller than estimates of between-site differentiation in *Q. rubra* (Table 2). Of the various pairwise combinations, the strongest differentiation was found between *Q. velutina* and *Q. falcata* at Duke Forest (for all measures other than R_{ST}). Overall, however, more differentiation

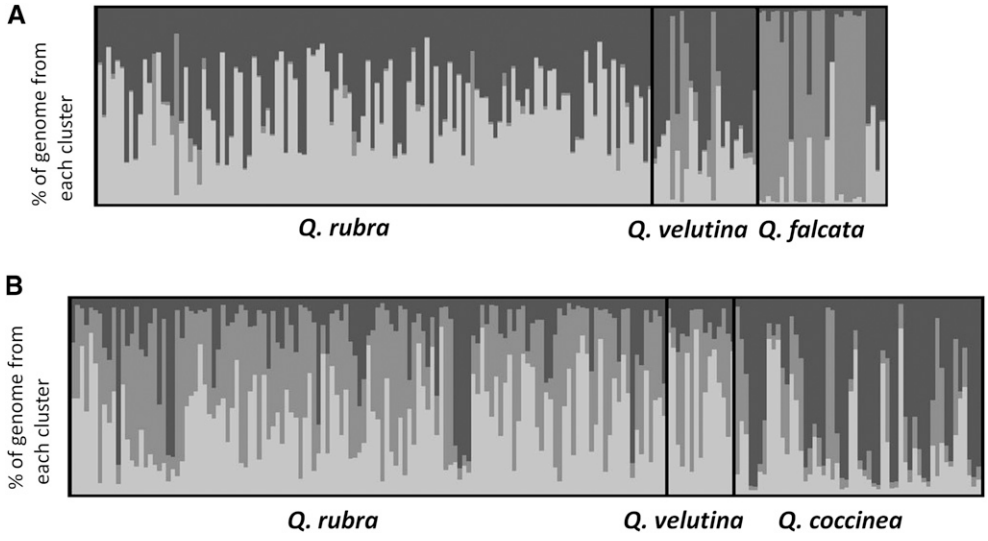

Fig. 2. Population structure for *Quercus* taxa at Duke Forest (A) and Coweeta (B) for $K = 3$, correlated allele frequencies. Vertical bars represent individuals. The proportion of the bar in each color represents the proportion of the genome attributed to genetic clusters 1 (light gray), 2 (gray), or 3 (dark gray).

TABLE 2. Measures of population differentiation between adults at the two sites and for all species together and pairwise within each site.

Comparison	Overall or by locus	ϕ_{ST}	R_{ST}	F_{ST}	F_{ST}^{ENA}	Mean G_{ST}	Mean G'_{ST}	Mean D
Two sites								
Qufa vs. Quco	**Overall**	**0.137***	–0.018	**0.107***	**0.105***	**0.07**	**0.632**	**0.611**
	Loci >0	5*	0	6+	5+	6*	6*	6*
Quru vs. Quru	**Overall**	**0.028***	0.001	**0.018***	**0.017***	**0.012**	**0.216**	**0.207**
	Loci >0	6*	1*	6+	6+	6*	6*	6*
Quve vs. Quve	**Overall**	0.005	0.021	0.003	0.004	0.022	0.336	0.324
	Loci >0	0	0	3+	4+	3*	3*	3*
Coweeta								
All	**Overall**	**0.006***	–0.014	**0.004***	**0.005***	**0.017**	**0.217**	**0.204**
	Loci >0	2*	0	5+	5+	6*	6*	6*
Quru vs Quve	**Overall**	0.005	–0.024	0.005	0.007	**0.015**	**0.247**	**0.237**
	Loci >0	1*	0	3+	3+	4*	4*	4*
Quve vs Quco	**Overall**	–0.012	–0.026	–0.006	–0.003	**0.015**	**0.23**	**0.22**
	Loci >0	0	0	2+	2+	4*	4*	4*
Quru vs Quco	**Overall**	**0.011***	0.001	**0.008***	**0.007***	**0.009**	**0.153**	**0.146**
	Loci >0	4*	1*	5+	5+	5*	5*	5*
Duke Forest								
All	**Overall**	0.00	0.006	–0.0001	0.001	**0.013**	**0.185**	**0.175**
	Loci >0	0	1*	2+	4+	6*	6*	6*
Quru vs Quve	**Overall**	–0.001	0.005	–0.0001	0.0002	**0.009**	**0.165**	**0.159**
	Loci >0	1*	1*	2+	3+	4*	4*	4*
Quve vs Qufa	**Overall**	0.012	–0.003	**0.0104***	**0.013***	**0.021**	**0.252**	**0.238**
	Loci >0	1*	0	5+	4+	4*	4*	4*
Quru vs Qufa	**Overall**	–0.002	0.015	–0.002	–0.001	**0.007**	**0.131**	**0.129**
	Loci >0	0	0	2+	2+	4*	4*	4*

Notes: An asterisk indicates statistically significant differentiation ($\alpha = 0.05$), while boldface print indicates differentiation at four or more loci. For each estimate, either the number of loci that had statistically significant differentiation (*) or the number that had an estimated value >0 (+) is shown below the overall estimate. Qufa = *Quercus falcata*, Quco = *Q. coccinea*, Quru = *Q. rubra*, and Quve = *Q. velutina*.

between morphospecies was evident at Coweeta than at Duke Forest, though genetic structure measures disagreed about which species pairs were most differentiated. None of the measures showed strong differentiation between *Q. rubra* and *Q. falcata* at Duke Forest.

Structure—If the three species at each site are reproductively isolated, then we would expect STRUCTURE to identify clusters of genetically similar individuals corresponding to species identities. STRUCTURE analyses did not support this hypothesis.

Within each site, heterozygote frequencies did not conform to Hardy–Weinberg equilibrium, indicating either the existence of population substructure (due to the presence of genetically isolated groups, inbreeding, and/or spatial genetic structure) or the presence of null alleles. The difference in likelihood between any $K > 1$ was insignificant; values of K between 3 and 5 (the highest value tested) were generally favored. Assumptions of correlated allele frequencies between species led to slightly smaller numbers of "populations" being favored than if allele frequencies were independent. Whether allele frequencies were assumed to be correlated, genetic clusters did not correspond closely to morphological species, and most individuals were inferred to be of mixed ancestry (Fig. 2; Appendix S2: Fig. S2.3). At Duke Forest, individuals identified as *Q. rubra* or *Q. velutina* mostly fell between populations 1 and 3 (when $K = 3$). Individuals identified as *Q. falcata* were assigned primarily to population 2, as were a few *Q. velutina*, but some *Q. falcata* clustered with *Q. rubra* and *Q. velutina* between populations 1 and 3. At Coweeta, differentiation between species was also low, though there was some tendency for individuals of *Q. coccinea* to cluster together (Fig. 2). The somewhat greater differentiation of *Q. falcata* at Duke Forest and *Q. coccinea* at Coweeta are consistent with the fact that *Q. falcata* vs. *Q. velutina* and *Q. rubra* vs. *Q. coccinea* showed statistically significant differentiation for multiple differentiation statistics tested (Table 2). The STRUCTURE results were similar for both seedlings and adults (Appendix S2: Fig. S2.2, S2.3).

Parentage—At both sites, a large proportion of seedlings were estimated to have one or both parents outside the plot based on genotypic differences between adults and seedlings (Moran and Clark, 2011). At Coweeta, 22.9% of seedlings were assigned two in-plot parents, while at Duke Forest only 16% were assigned two in-plot parents. Of the seedlings with two assigned parents at Coweeta, 65.9% of the time parents belonged to the same morphospecies, while 34.1% of the time, parents were of different morphospecies, making the seedling a putative hybrid. Of the seedlings with two assigned parents at Duke Forest, 62.9% of the time parents belonged to the same morphospecies, while 37.1% of seedlings were putative hybrids. When uncertainty in parentage was taken into account by repeated draws from the posterior distribution for parentage, the proportion of putative hybrids among seedlings with two in-plot parents ranged from 26.5–50% at Coweeta and 31.5–62.7% at Duke Forest.

DISCUSSION

Only a handful of studies to date have examined hybridization in red oaks and, to our knowledge, this is the first to both compare eight different measures of genetic differentiation in adults to address past gene flow and to employ parentage analysis to

estimate current hybridization rates. Our results suggest that gene flow occurs between co-occurring red oak species, even when these species remain morphologically distinct. All genetic structure analyses indicate low genetic differentiation between species within sites and between sites within species relative to between-species between-site differentiation, and genetic clusters identified by STRUCTURE did not correspond with morphologically defined species, all of which suggest past gene flow between species. However, the measures of genetic structure differed greatly in their sensitivity to differences in allele frequencies, suggesting that caution is needed when drawing conclusions from any single differentiation statistic. Parentage results suggest that >20% of seedlings may have parents belonging to two different morphologically defined species, indicating high current hybridization rates. We conclude that there is little evidence of strong reproductive isolation between the oak species at either site. Given the morphological differentiation between species, this suggests that selection may limit introgression at loci responsible for functional traits.

When populations are strongly isolated, STRUCTURE can identify the correct number and composition of populations using as few as 5–7 microsatellite loci (Pritchard et al., 2000; Craft et al., 2002; Salvini et al., 2009). Our results, showing low differentiation between co-occurring species, are consistent with those of Aldrich et al. (2003b) for red oaks in Indiana, which were based on 15 microsatellite loci. However, it should be noted that similarities in allele frequencies can sometimes result from shared ancestry (Muir and Schlotterer, 2005), and the fact that many alleles were shared between *Q. falcata* at Duke Forest and *Q. coccinea* at Coweeta (Appendix S1: Fig. S1.1–S1.4) and that some of these alleles are common in both populations (e.g., 163 at F02 and 222 at C19) suggest that shared ancestry does have some influence. A larger number of microsatellite loci might have enabled STRUCTURE to identify more subtle differentiation between groups. Indeed, several of the other genetic structure measures did detect statistically significant differentiation between co-occurring species. However, statistical significance does not necessarily indicate biological significance. A baseline for zero gene flow is therefore needed. Because gene flow between sampled *Q. falcata* and *Q. coccinea* would require both hybridization and long-distance dispersal, and so is likely to be near zero, we used the differentiation between them as our baseline. Between-site within-species differentiation was 16.2–34.2% of this baseline (depending on the statistic used), which is consistent with the connectivity provided by long-distance pollen dispersal. Between-species within-site differentiation was on a similar or smaller scale (0–39.9%, depending on the statistic).

Another factor that must be considered when interpreting these results is the effect of lower-than-expected heterozygosity. Observed heterozygosity was lower than Hardy–Weinberg expectations for all loci at both sites. This heterozygote deficiency has been observed in a number of other tree populations, including oaks (e.g., Ducousso et al., 1993; Craft et al., 2002; Morand et al., 2002; Jones et al., 2006). There are several potential causes. Inbreeding, nonrandom mating, or population substructure can lead to a lack of heterozygotes. We found significant spatial correlation in genotype for both adults and seedlings at each site (Appendix S2: Fig. S2.4), which supports the idea that distance-dependent seed and pollen dispersal could lead to a Wahlund effect and to increased biparental inbreeding. Heterozygote deficiency has been detected in some *Q. rubra* populations using allozyme data as well as microsatellite markers (Ducousso et al., 1993). Moreover, one would expect inbreeding to result in consistent heterozygote deficiencies across loci, compared to the variable effects of null alleles (Morand et al., 2002), and this is indeed what we saw: Observed heterozygosity is between 62 and 89% of expected heterozygosity for all loci. However, one cannot discount the possibility of null alleles entirely unless controlled crosses are conducted or another analysis is done with improved primers (Morand et al., 2002). The F_{ST}^{ENA} measure was developed to correct for heterozygote deficiencies due to null alleles, but most commonly used measures of population differentiation assume that HW frequencies should apply within populations.

The seven population differentiation statistics provided very different results (Table 2), but when taken together, patterns emerged, including the much larger differentiation between *Q. falcata* and *Q. coccinea* relative to within-species between-site or between-species within-site pairs. As expected (Hedrick, 1999, 2005; Gregorius et al., 2007; Jost, 2008), F_{ST} and related statistics yielded relatively low estimates of differentiation, while G'_{ST} and D were more sensitive to differences in allele frequencies. In addition, R_{ST} failed to detect any differentiation, even between *Q. falcata* and *Q. coccinea*, which suggests that it is insufficiently sensitive for most applications. There was very little difference between the basic F_{ST} and the F_{ST}^{ENA} corrected for null alleles. However, given the greater sensitivity of G'_{ST} and D, it would be desirable to develop methods of calculating these statistics while taking into account the possibility of null alleles. STRUCTURE has the ability to group individuals by genetic similarity but, since it assumes HW genotype frequencies within subpopulations, heterozygote deficiencies due to inbreeding or null alleles may artificially inflate estimates of K. For our populations, STRUCTURE indicated $K > 1$ even though genetic groups did not correspond to morphologically defined species. These differences between methods suggest that it may be valuable to use more than one measure of population structure, particularly if the results are intended to guide management or conservation actions.

The relatively low differentiation between species within sites supports the inclusion of all individuals as potential parents and offspring in parentage analyses, as does the observation that seeds taken from known mother trees in these populations sometimes yield seedlings of a different morphospecies than the mother (E. Moran, personal observation; I. Ibáñez, University of Michigan, personal communication). The percentage of seedlings with two in-plot parents that belonged to different morphospecies was slightly higher at Duke Forest (37.1%) relative to Coweeta (34.1%). Uncertainty in parentage assignment and the small number of seedlings with two in-plot parents led to a high level of uncertainty in these percentages (range 31.5–62.7% at Duke Forest, 26.5–50% at Coweeta), but the general pattern is consistent with the lower three-species genetic structure observed at Duke Forest for adult trees. The lower rates and higher differentiation at Coweeta may be related to the low numbers of *Q. velutina* and *Q. coccinea* relative to *Q. rubra*, which would reduce the probability that *Q. rubra* is fertilized by heterospecific pollen. Given the numbers of each species, if mating were entirely random and all species produced seedlings in proportion to their adult abundance, we would expect hybridization rates of 57.6% at Duke Forest and 49.7% at Coweeta.

Some caveats regarding the parentage analyses must be mentioned. First, artificial hybridization studies suggest that interspecific pollination events have lower success, but we did not

have enough data to attach weights to this process in the parentage model (Moran and Clark, 2011). Second, repeated mutations can cause alleles to be identical in length even if not identical by descent, and related species may share alleles even if there has been no recent gene flow. The numbers of seedlings with two in-plot parents is low, which reduces confidence in hybridization estimates based on these individuals alone. Finally, parentage analysis of seedlings will only reflect the proportion of hybrids at this early life stage. On the other hand, not including heterospecifics as potential parents significantly increases the number of seedlings without identified parents within the mapped area. When *Q. falcata* individuals are excluded from the Duke Forest analysis, the proportion of seedlings with two identified in-plot parents declines from 16 to 8.5%, and the proportion with no plausible in-plot parent increases from 37 to 45%. Although not all seedlings assigned heterospecific parents are necessarily true hybrids, our results suggest hybridization rates over 20%, which is on the higher end of the range estimated in the white oak group, including 25% female *Q. pubescens* × male *Q. petraea* (Salvini et al., 2009), 6–22% *Q. pyrenaica* × *Q. petraea* (Valbuena-Carabana et al., 2007), 16% *Q. pubescens* × *Q. frainetto* (Curtu et al., 2007), 4.6% *Q. lobata* × *Q. douglasii* (Craft et al., 2002), <2% *Q. ilex* × *Q. suber* (Burgarella et al., 2009), and *Q. frainetto* × *Q. robur* (Curtu et al., 2007), and from 27–48% to almost zero for *Q. robur* and *Q. petraea* (Bacilieri et al., 1996; Streiff et al., 1999; Muir and Schlotterer, 2005).

Our results are consistent with the hypothesis that barriers to interspecific gene flow are lower in red oaks (Guttman and Weigt, 1989). Hybridization between North American red oak species has not been extensively studied (González-Rodríguez et al., 2004), so it is difficult to say how common the patterns we observed—indicating gene flow between multiple co-occurring species—may be in this group. Three studies of hybridization across zones of range overlap for mostly allopatric Mexican red oaks confirmed the existence of hybrid adults within the zones of sympatry (González-Rodríguez et al., 2004; Tovar-Sánchez and Oyama, 2004; Peñaloza-Ramírez et al., 2010). Studies within the United States also focused on adult trees. Aldrich et al. (2003b) found little differentiation in microsatellite allele frequencies between *Q. rubra*, *Q. shumardii*, and *Q. palustris* co-occurring in an old-growth forest in Indiana. Dodd and Afzal Rafii (2004) found that morphologically typical *Q. wislezeni* often showed evidence of introgression from *Q. parvula*, and *Q. agrifolia*. Finally, Hipp and Weber (2008) found that *Q. ellipsoidalis* shows evidence of introgression from *Q. velutina,* but not *Q. coccinea*.

Our results and the range of hybridization rates estimated by previous studies indicate the need for closer examination of interspecific gene flow in oaks and the mechanisms that maintain their distinct phenotypes in the face of gene flow. Our study identified a large fraction of putative hybrids among seedlings with in-plot parents. Whether they will survive to reproduce is uncertain, yet the low genetic differentiation between adults of co-occurring species suggests that individuals of mixed genetic background have attained canopy status in the past. In spite of this, trees at both sites could be easily classified according to traditional morphological traits. While the relationship between percentage admixture and morphology is often weak in oaks (Valbuena-Carabana et al., 2007), this suggests that there may be some selection against trees bearing intermediate combinations of traits (Anderson, 1948; Muller, 1952; Kimball et al., 2008). Wind-dispersed tree species often exhibit local adaptation in functional loci within species despite widespread gene flow in neutral loci (Mimura and Aitken, 2010). On the other hand, changes in the environment due to climate change or land use could favor introgression from species with, for instance, higher drought tolerance (Dodd and Afzal-Rafii, 2004). *Quercus velutina* and *Q. falcata*, for instance, both grow in warmer, drier habitats than *Q. rubra* (Little, 1980), so introgression from these species in the southern part of the range of *Q. rubras* range might aid its persistence under future climate conditions (IPCC, 2007). Common garden experiments could be used to assess the fitness of hybrids under different environmental conditions (Cavender-Bares and Pahlich, 2009). As genomic resources become increasingly available for oaks (Fagaceae Genomics Web, http://www.fagaceae.org/), comparison of species and population differentiation at neutral vs. functional loci will help to identify traits important in local environments. Such information may help answer the question of how plant species can remain relatively "good" species, maintaining distinct suites of morphological and ecological traits, despite introgression from other taxa.

Summary—Hybridization is an important process in plant evolution and ecology, affecting speciation (Arnold, 1992; Riesberg and Willis, 2007), coevolution and community composition (Boecklen and Spellenberg, 1990; Whitham et al., 1994), and local adaptation (Dodd and Afzal-Rafii, 2004). Oaks have long been considered a "worst-case scenario" (Coyne and Orr, 2004) of widespread hybridization, challenging the biological species concept, but interspecific gene flow can be challenging to measure. Using both genetic structure and parentage analyses, we found evidence of current and historical gene flow between co-occurring red oak species, though some genetic differentiation was evident. Such gene flow is consistent with the limited data available for other North American red oaks (Aldrich et al., 2003b; Dodd and Afzal-Rafii, 2004) and with estimates of hybridization frequency between white oaks. However, measures of genetic structure differed greatly in their sensitivity to differences in allele frequencies, and many may be sensitive to factors that affect heterozygote frequency (including inbreeding and null alleles), suggesting that caution is necessary when using these measures to make taxonomic distinctions or to guide management and conservation actions. The use of multiple measures or data from multiple life stages, as in this study, can help in detecting general patterns. Our results suggest that gene flow between co-occurring red oaks may be common, but that selection on functional loci may help maintain the suites of morphological traits that characterize many oak species (Dodd and Afzal-Rafii, 2004). If so, then interspecific gene flow may provide a useful source of genetic variation for hybridizing taxa as global change shifts the selective environment.

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