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The clonal structure of *Quercus geminata* revealed by conserved microsatellite loci

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Abstract

The scrub oak communities of the southeastern USA may have existed at their present locations for thousands of years. These oaks form suckers, and excavations of root systems suggest that clones may occupy very large areas. Resolution of the clonal nature of scrub oaks is important both to manage the tracts of this ecosystem that remain, and in conducting long-term ecological studies, where the study area must substantially exceed the area occupied by any single clone. Microsatellites were used to determine the genetic diversity of a dominant oak species within a 2-ha long-term experimental site on Merritt Island at the Kennedy Space Center. This area contains a long-term study of the effects of elevated CO_2 on the ecosystem. Conservation of seven microsatellite loci, previously identified in the sessile oak, *Quercus petraea*, was tested in two Florida scrub oak species, *Q. geminata* and *Q. myrtifolia*. Sequence analysis revealed that all seven microsatellite loci were conserved in *Q. geminata* and five loci were conserved in *Q. myrtifolia*. Six microsatellite loci were polymorphic in *Q. geminata* and these were subsequently used to investigate the clonal structure of the *Q. geminata* population. Twenty-one unique combinations of microsatellites, or haplotypes, occurred only once, whereas the remaining 26 individuals belonged to a total of seven different haplotypes. Trees with identical haplotypes were in close proximity, supporting the interpretation that they were clones. The results showed that there is significant genetic diversity within the 2-ha experimental site. Microsatellites provided a powerful and noninvasive tool for distinguishing individual genotypes and determining an adequate area for long-term ecosystem studies.

Keywords: genetic marker, microsatellite, *Quercus geminata*, *Quercus myrtifolia*, simple sequence repeat

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Introduction

Scrub oak-dominated communities have patchy distributions across the southeastern USA, including Florida (McDonald & Hamrick 1996). As in other woody ecosystems in which succession is arrested by recurring fires, trees regenerate from suckers from spreading roots. *Quercus geminata* and *Q. myrtifolia* are sclerophyllous evergreen trees that dominate these communities (Schmalzer & Hinkle 1992), which occur in unglaciated areas and have persisted for thousands of years (Webb 1990). A single clone in a community could therefore colonize and dominate a very large area. Because morphological characters of scrub oaks are highly variable with growth conditions (Godfrey 1988), the only means of determining the extent of clones has been to excavate root systems. Excavation experiments suggest that single clones can occupy 30–1000 m^2 (Guerin 1993). However, this may be a serious underestimate as the connecting root systems may have degenerated completely over thousands of years and apparently isolated individuals may be clones (Webb 1990).

The clonal nature of these ecosystems has important implications for long-term ecological research projects within this community type, as well as for the conservation of this habitat. For logistical reasons, long-term ecological experiments, such as CO_2 or temperature manipulations mimicking future global change, cannot sample the whole

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biome. Rather, such experiments are limited to sampling within a relatively small area, assumed to be 'representative.' This assumption is seriously compromised, however, if clones occupy large areas and sampling is unknowingly limited to one or few clones. A specific example is the long-term study of the effects of CO_2 enrichment on the scrub oak community at the Kennedy Space Center, Florida. This area, within the Merritt Island National Wildlife Refuge, is one of the best remaining examples of the scrub oak community that once occupied much of the southeastern USA. It is the only experiment that has studied the effect of future elevated CO_2 levels on a community from regrowth to canopy closure (Dijkstra *et al*. 2002). The area required to include a significant number of genetically distinct individuals is also important information in determining minimum areas for conservation of this natural system (McDonald & Hamrick 1996). Molecular markers, for the first time, allowed an accurate assessment of the extent of clones in this community.

In our study, microsatellite markers previously identified in *Q. petraea* (Steinkellner *et al*. 1997a, 1997b) were tested for conservation within two Florida scrub oak species, *Q. geminata* and *Q. myrtifolia*. Conserved markers were used to determine whether single *Q. geminata* clones exclusively occupy large areas, or if there is an intermingling of several clones at the scale of the current long-term ecological experiment within this community.

Materials and methods

Experimental site

The study site was located at the Merritt Island Wildlife Refuge on the east coast of central Florida, USA (28°38′ N, 80°42′ W), and was limited to a 2-ha area. Sixteen octagonal open-top chambers, dispersed throughout the 2-ha area, were previously established in order to expose areas (9.4 m^2) of scrub oak vegetation to elevated CO_2 concentrations. These served as 16 randomly placed sample plots for this study. Three *Quercus myrtifolia* and four *Q. geminata* individuals were initially sampled in order to test the utility of previously described microsatellite primers. Sixty-four *Q. geminata* trees, four individuals from each chamber, were then sampled for genotypic (clonal) analysis.

DNA extraction

Young *Q. geminata* and *Q. myrtifolia* leaves were collected, frozen in liquid N_2 and stored at –80 °C until DNA was extracted. Genomic DNA was extracted following a modified method of Doyle & Doyle (1990). Briefly, frozen leaf tissue (≈ 100 mg) was ground in liquid N_2 and incubated for 20–40 min at 56 °C in 0.6 mL CTAB extraction buffer with 5 μL β-mercaptoethanol. Each sample was then mixed with 0.4 mL chloroform and centrifuged for 5 min. Supernatant was incubated with 5 μL RNaseA (10 mg/mL; Sigma) at 37 °C for 1 h, and mixed first with 100 μL of 10% (w/v) CTAB and then 0.6 mL chloroform. After further centrifugation, the aqueous phase was transferred to a microfuge tube containing 0.5 mL ice-cold isopropanol and placed at –20 °C for 30–60 min. After 10 min centrifugation at 4 °C, the isopropanol was decanted and the pellet was washed first with 75% EtOH, then with 95% EtOH. The DNA was dried and resuspended in TE buffer. The amount of DNA was quantified by gel electrophoresis with a known standard.

Microsatellite loci

Seven microsatellite loci, defined from *Q. petraea* (Steinkellner *et al*. 1997a) and conserved among at least some *Quercus* species (Steinkellner *et al*. 1997b), were used in the analysis. These loci (*QpZAG1/2*, *QpZAG9*, *QpZAG15*, *QpZAG16*, *QpZAG36*, *QpZAG58*, *QpZAG110*) included an $(AG)_n$ or $(CT)_n$ dinucleotide repeat in *Q. petraea* (Fig. 1). Conservation of these microsatellite loci in *Q. geminata* and *Q. myrtifolia* was tested by sequencing the amplified fragment from at least one individual per species per locus. Fragments were amplified using polymerase chain reactions (PCRs) with *Taq* DNA polymerase (High Fidelity Taq, Roche Diagnostics) according to the manufacturer's general protocol. PCR products were fractionated in 1.0% agarose gels and desired fragments were excised and purified using a gel purification kit (Qiagen). Cycle sequencing was performed according to the kit manufacturer's instructions (ABI Prism® Big Dye™ Terminator kit, Applied Biosystems) and sequence analysis was performed on an automated sequencer (ABI 377, Applied Biosystems).

Fragment analysis

Haplotypes of each *Q. geminata* were scored using primers defined for the microsatellite loci described above. The primers were synthesized with 5′-fluorescent labels (6-FAM or HEX) (ABI, PE Corp.). PCR amplifications were performed under the following conditions: 94 °C initial denaturation for 5 min; 30 cycles of 94 °C denaturation for 45 s, 55 °C annealing for 45 s and 74 °C extension for 45 s; and 74 °C final extension for 5 min. Each PCR mixture contained ≈ 5 ng of DNA, 10 μM of each primer, 200 μM of each dNTP, 0.2 U of *Taq* DNA polymerase (Invitrogen Corp.), 1.5 mM $MgCl_2$, and 1× supplied Mg-minus buffer in a final volume of 15 μL. PCR products were resolved on a 5% denaturing polyacrylamide gel, and sizes were determined by automated fluorescent scanning detection (ABI Prism 377, GENESCAN analysis software, ABI).

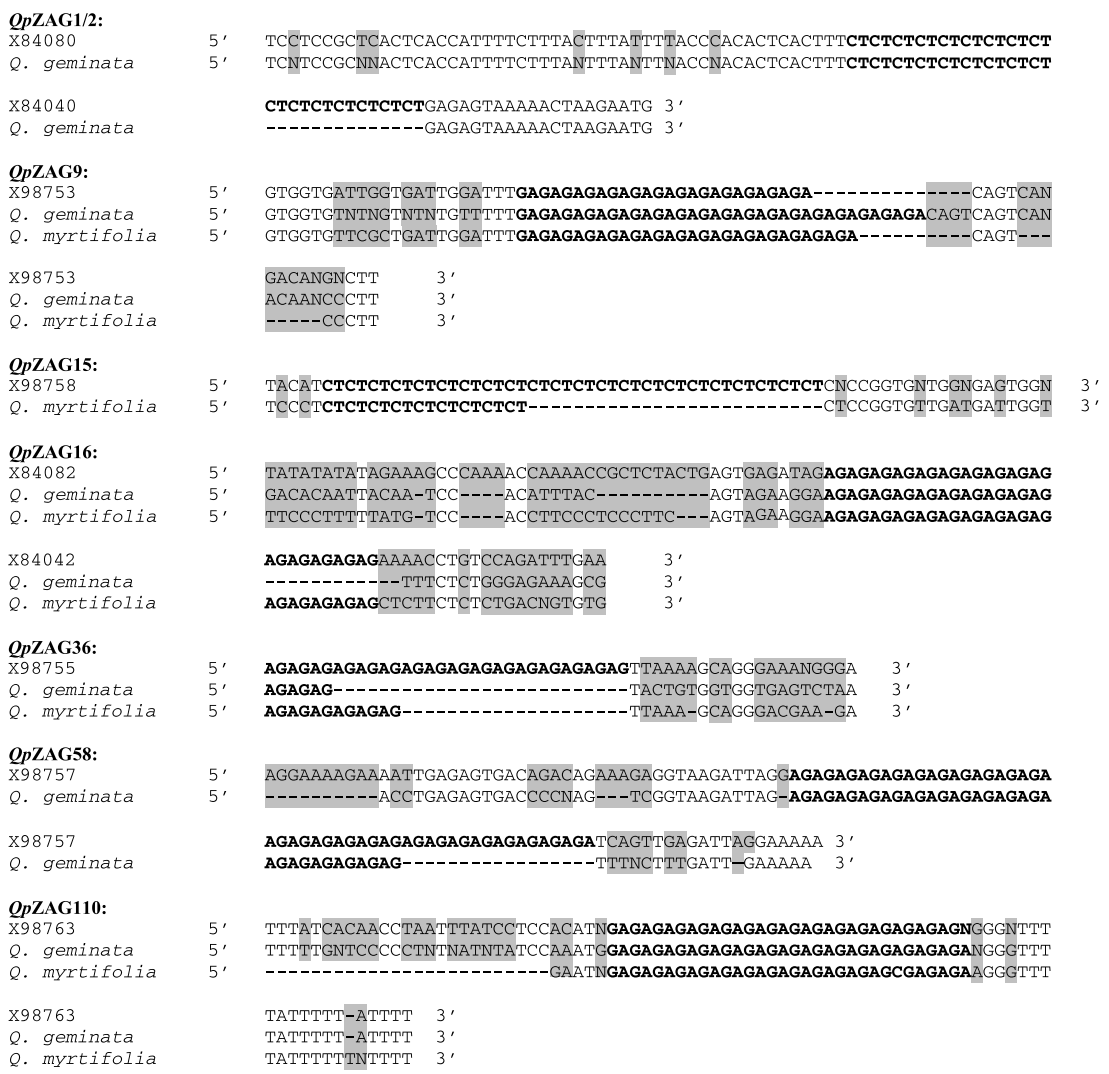

Fig. 1 Sequence alignments for microsatellite loci originally identified in *Quercus petraea* and conserved in *Q. geminata* and/or *Q. myrtifolia*. For each locus, the *Q. petraea* nucleotide sequence data is shown first, following its GenBank Accession no. The entire 5′-end of the region and the first 20 bases flanking the dinucleotide repeat are shown. Repeat regions are highlighted in bold and shaded nucleotides show differences in the flanking sequences of the repeat. Although *Q. geminata* contained a locus homologous with *QpZAG15*, sequence data were ambiguous and therefore are not shown.

Analysis of clonal structure and diversity

Samples for which at least five of the loci did not amplify were excluded from further analysis. For this reason, no individual from one of the 16 plots was included in the analysis. The number of unique *Q. geminata* haplotypes possible at the study site was computed using the equation:

$$N_g = \prod_{i=1}^{L} [a_i(a_i+1)]/2$$

where L is the number of loci, and a_i is the number of alleles at the locus i (Reusch *et al*. 1998). The clonal diversity at the site was calculated as P_d, the proportion of distinguishable genotypes (Ellstrand & Roose 1987). For each locus, allele frequencies, observed heterozygosity (H_O), expected heterozygosity (H_E), and effective number of alleles [$A_E = 1/(1 - H_E)$] were calculated according to Nei (1987). The likelihood that a haplotype occurred more than once by random chance was determined by calculating the expected probability (P_{gen}) of each six-locus haplotype:

$$p_{gen} = \prod_{b=1}^{k} (p_i)2\text{^}b$$

where k is the number of alleles each genotype carries, p_i is the frequency of the ith allele of the genotype, and h is the

Table 1 Allele frequencies at six microsatellite loci of 26 unique genotypes identified from 52 *Quercus geminata* plants

Locus	Allele size (bp)	Frequency
QpZAG1/2	99	0.250
	102	0.333
	104	0.250
	110	0.042
	114	0.042
	122	0.083
QpZAG9	254	0.438
	258	0.250
	260	0.062
	262	0.042
	264	0.062
	266	0.042
	268	0.083
	272	0.021
QpZAG15	105	0.043
	111	0.043
	113	0.217
	114	0.043
	116	0.043
	117	0.435
	121	0.174
QpZAG16	252	0.045
	254	0.091
	255	0.091
	256	0.182
	258	0.273
	259	0.091
	260	0.045
	262	0.045
	264	0.091
	268	0.045
QpZAG36	197	0.038
	195	0.962
QpZAG110	208	0.196
	210	0.652
	212	0.022
	214	0.130

number of heterozygous loci (Reusch *et al*. 1998). The unbiased estimator of Wright's inbreeding coefficient, *f*, was calculated according to Weir & Cockerham (1984) (GENEPOP Version 3.1d, Raymond & Rousset 1995). Deviation of genotypic frequencies from Hardy–Weinberg proportions was tested using a Markov-chain algorithm developed by Guo & Thompson (1992) (GENEPOP Version 3.1d, Raymond & Rousset 1995).

Results

All seven of the tested microsatellite primer pairs developed for *Quercus petraea* amplified homologous

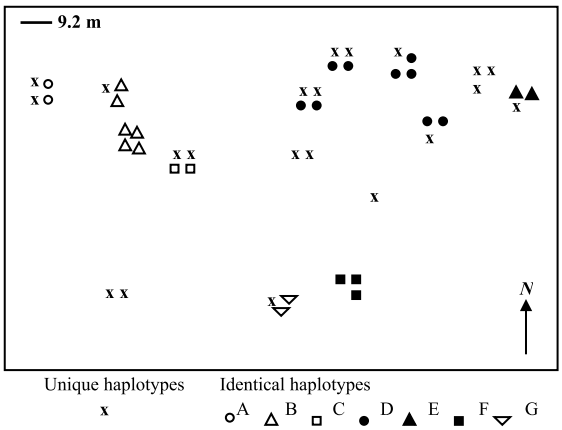

Fig. 2 Spatial distribution of *Quercus geminata* genotypes within the randomly distributed plots at the Smithsonian elevated CO_2 research site (≈ 2 ha). Marker combinations that occur only once are symbolized with x. Clones A, B, C, D, E, F and G defined in Table 2 are marked with symbols.

regions in *Q. geminata* and five of the primer pairs amplified fragments in *Q. myrtifolia* (Fig. 1). Sequence analysis of the microsatellite loci confirmed both similar flanking primer sequences and the presence of a repeat region. Both *QpZAG1/2* and *QpZAG58* amplified in both *Q. petraea* and *Q. geminata*, but not in *Q. myrtifolia*. Although the *QpZAG15* locus clearly amplified in *Q. geminata*, the sequence data were ambiguous.

In *Q. geminata*, all loci were polymorphic except *QpZAG58*, with the number of alleles varying from 2 to 10 (Table 1). The total number of alleles scored in 47 individuals over all 6 loci was 37. Twenty-eight *Q. geminata* marker combinations or haplotypes of a possible 3.49×10^7 six-locus combinations were distinguished at the site. The proportion of distinguishable genotypes, P_d, was 0.60 (i.e. 28/47). Twenty-one haplotypes occurred only once, whereas the remaining 26 individuals belonged to a total of 7 haplotypes. The number of plants with each of these seven haplotypes varied from two to nine. We assumed that plants with the same haplotype were clones; however, it is possible that six microsatellites were not enough to distinguish distinct individuals. Putative clones, inferred from the microsatellite data, were always found within the same chamber or in adjacent chambers within an ≈ 18-m radius (Fig. 2), supporting our conclusion that plants with the same haplotypes were clones. The likelihood that individuals were erroneously assigned to the same genotype was statistically small (all $P_{gen} < 0.002$; Table 2).

Calculations of genetic diversity within *Q. geminata* are given in Table 3. A high level of polymorphism was apparent, based on the number of observed alleles per locus (mean $A_O = 6$, Table 3); however, for all loci, the expected heterozygosity was greater than the observed heterozygosity.

Table 2 The haplotypes and number of clones per haplotype which occurred more than once within the experimental site. The spatial distribution of these haplotypes are shown in Fig. 2. The likelihood of a chance occurrence of each six-locus haplotype (P_{gen}) was calculated and was statistically small for each haplotype

Haplotype	No. clones per haplotype	P_{gen}
A	2	0.002
B	6	1.765×10^{-6}
C	2	2.684×10^{-7}
D	9	3.765×10^{-4}
E	2	1.637×10^{-5}
F	3	2.103×10^{-4}
G	2	5.026×10^{-5}

Table 3 Within-population genetic diversity in *Quercus geminata*. Alleles detected (A_O), effective allele number (A_E), observed and expected heterozygosities (H_O, H_E), and inbreeding coefficient (f) with the exact test of departure from Hardy–Weinberg genotypic proportions (*$P < 0.01$) indicated for each locus

Locus	A_O	A_E	H_O	H_E	F
QpZAG1/2	6	1.64	0.13	0.39	0.69*
QpZAG9	8	1.59	0.29	0.37	0.22*
QpZAG15	7	1.59	0.09	0.37	0.80*
QpZAG16	9	1.79	0.30	0.44	0.33*
QpZAG36	2	1.04	0	0.04	1.00*
QpZAG110	4	1.36	0.22	0.27	0.18
Mean	6.00	1.50	0.17	0.31	0.54*
SD	2.61	0.27	0.12	0.15	0.34

This caused the effective allele number (mean $A_E = 1.50$, Table 3) to be much smaller than the observed allele number at each locus. Five of six loci deviated from Hardy–Weinberg equilibrium (HWE; Table 3); however, these deviations were likely due to the small sample sizes.

Discussion

Conservation of microsatellite loci within closely related tree species, genera and families has been well documented (e.g. Steinkellner *et al*. 1997b; Lefort *et al*. 1999). We tested microsatellite markers developed for the deciduous oak, *Quercus petraea*, for utility on evergreen Florida scrub oak species, *Q. myrtifolia* and *Q. geminata*. Of the seven loci evaluated, five were conserved in *Q. myrtifolia* and all seven were conserved in *Q. geminata*. The original *Q. petraea* microsatellite repeat $(AG)_n$ or $(CT)_n$ was conserved in the two *Quercus* species tested in this experiment (Fig. 1). Previous tests of cross-species amplifications of these loci revealed broad conservation within the Fagaceae family (Steinkellner *et al*. 1997b).

Our results show that there are numerous *Q. geminata* genotypes present within the 2-ha long-term ecological elevated CO_2 research site at the Kennedy Space Center. Until now, it has not been clear how much, if any, diversity within species was present in this area. The presence of 21 individuals with unique genotypes suggests substantial recruitment of new genotypes from acorns. There are clones that appear in more than one chamber, which could potentially confound results of other ecological or physiological experiments, but only if sample numbers were very small ($\approx n < 4$).

Microsatellites have proven an extremely useful tool for revealing the clonal structure of populations (Reusch *et al*. 1998, 2000; Schilder *et al*. 1999), and were successful in the discrimination of *Q. geminata* genotypes in the 2-ha experimental site. The clonal structure of oak clumps has previously been revealed only by destructive excavations in order to assess roots (Guerin 1993); microsatellites provide a promising method of nondestructively assessing oak clones. Of the 47 *Q. geminata* plants examined in this analysis, we found 28 distinct genotypes. The microsatellite loci were highly multi-allelic, much more than typical isozyme loci, so the number of individuals genotyped (47) is a limited sample for all possible genotypes at a locus (Collevatti *et al*. 2001). This likely explains the deviations from HWE. However, microsatellites clearly distinguished a number of different genotypes within this long-term experimental site. A number of physiological and ecological studies have been performed within this community without any knowledge of the genetic diversity within the species (Li *et al*. 1999; Ainsworth *et al*. 2002; Dijkstra *et al*. 2002; Hymus *et al*. 2002). We now know that the limited sampling area of this experiment site contains significant genetic diversity within *Q. geminata*, and therefore results from prior studies are likely to be widely applicable to the species in its wider distribution in the southeastern USA.

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