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Chloroplast DNA variation of northern red oak (*Quercus rubra L.*) in Indiana

JEANNE ROMERO-SEVERSON[1,*], PRESTON ALDRICH[2], YI FENG[1], WEILIN SUN[1] and CHARLES MICHLER[2]

[1]*Hardwood Tree Improvement and Regeneration Center, Department of Forestry and Natural Resources, Purdue University, 1159 Forestry Bldg, West Lafayette, IN 47907, USA;* [2]*Hardwood Tree Improvement and Regeneration Center, USDA Forest Service, Purdue University, 1159 Forestry Bldg, West Lafayette, IN 47907, USA;* [*]*Author for correspondence*

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Abstract. Chloroplast DNA (cpDNA) variation was examined in 48 northern red oaks at 14 sites representing contrasting glacial histories and age structures within the state of Indiana in the United States. PCR-RFLP of three intergenic regions revealed five haplotypes. Haplotype I was common to seven sites and was the most frequent (17 trees). Haplotype II was common to five sites and was nearly as frequent as haplotype I (16 trees). Haplotypes III, IV and V were equally infrequent and did not occur together. Genetic diversity resided among rather than within populations (G_{ST} = 0.73 ± 0.14). This preliminary survey shows that cpDNA diversity will be a useful tool for the investigation of ancient seed dispersal patterns in northern red oak.

Introduction

The native range of northern red oak (*Quercus rubra* L.) extends from northern Ontario to southern Alabama and east from Nebraska to the Atlantic coast. Pin oak (*Quercus palustris* Muenchh.), northern pin oak (*Quercus ellipsoidalis* E.J. Hill), black oak (*Quercus velutina* Lam.) schumard oak (*Quercus schumardii* Buckl.), scarlet oak (*Quercus coccinea* Muenchh.), southern red oak (*Quercus falcata* Michx.) and cherrybark oak (*Quercus pagoda* Raf.) occur within this range. These red oaks (*Quercus* subgenus *Erythrobalanus* section rubrae) are economically the most important of the hardwood species native to North America. None of these species is well characterized with respect to genetic diversity or population substructure.

Glacial retreat in Europe resulted in strong phylogeographic structure within the European white oaks (Petit et al. 2002). Matrilineal chloroplast DNA polymorphisms (Dumolin et al. 1995) have permitted the detection of these ancient seed dispersal patterns in contemporary oak forests. The Wisconsinian glacier retreat in North America (Dyke and Prest 1987) may have had a similar effect on North American oaks. Detection of postglacial seed dispersal patterns in red oaks will permit us to distinguish the effect of postglacial recolonization from the effects of recent human disturbance.

Table 1. Haplotypes detected in intergenic regions amplified by the CD, FV and TC primers anchored in conserved chloroplast coding regions. Matching numbers within the same row indicate the same PCR-RFLP pattern.

				Haplotypes				
Locus	Primer	End	Coding region	I	II	III	IV	V
CD	trn**C**	5′	tRNA-Cys(GCA)	2	2	2	2	1
	trn**D**	3′	tRNA-Asp(GUC)					
FV	trn**F**	5′	tRNA-Phe (GAA)	1	2	3	1	1
	trn**V**	3′	tRNA-Val (UAC)					
TC	trn**T**	5′	tRNA-Thr(GGU)	1	1	1	2	1
	psb**C**	3′	psbC [psII 44 kd protein]					

The objective of this study was to detect chloroplast polymorphism in *Q. rubra*, the dominant red oak species. Using PCR-RFLP to detect variation, we examined 48 samples from putative old growth and secondary growth stands in glaciated and unglaciated regions in Indiana, a state located in the middle of the native range.

Materials and methods

DNA extraction and PCR-RFLP

Leaf collections were refrigerated or lyophilized. Acorns were stored at 4 °C or −20 °C. Amplifiable DNA was extracted using QIAGEN Dneasy Plant Mini Kits with the following modifications: for leaf samples, 75 mg of chopped fresh leaf sample or 20 mg lyophilized power was placed into a 2 ml micro centrifuge tube with 30 µl of 0.5 mm zirconia/silica beads, one 6 mm cylindrical bead, 800 µl AP1 buffer and 10 µl 2-mercapto-ethanol. RNase was not added at this time. The tube was then loaded on a FastPrep bead mill (Bio 101, model FP120-120) and shaken 40 s three times. The tube was placed on ice three minutes between times. For acorns, 200 mg of fresh or frozen cotyledon was added to same mix and shaken for 15 s twice. One µl RNase A (100 mg/ml) was added to the DNA after elution from the membrane and the sample was placed in a 37 °C water bath for at least 30 min.

A subset of the Indiana samples was initially screened by restriction digest of 10 intergenic cpDNA fragments amplified by PCR with pairs of consensus chloroplast primers anchored in highly conserved chloroplast coding regions (Demesure et al. 1995). The three intergenic regions that were amplifiable and polymorphic in this subset were then used to screen all of the samples in this initial study. The locus names CD, FV and TC are taken from the last letter of the names of the primers used to amplify the region in which the polymorphism was detected (Table 1).

PCR reactions were performed in a Programmable Thermal Controller (MJ Research, Inc.). The TC primer program includes 3 min denaturation at 95 °C; 35 cycles of 30 sec at 94 °C; 1 min annealing at 51.5 °C, and 4 min extension at 70 °C. The FV primer program includes 1 min denaturation at 95 °C; 36 cycles of 30 sec at 94 °C; 30 sec annealing at 57 °C and 3 min extension at 70 °C. The CD

primer touchdown program includes 1 min denaturation at 95 °C; 4 cycles of 30 sec at 94 °C; 30 sec at 67 °C with −2 °C by cycle; and 3 min at 70 °C; followed by 36 cycles of 30 sec at 94 °C; 30 sec at 57 °C; and 3 min at 70 °C with +1 sec by cycle. Each of the three amplified fragments was digested by a mix of seven restriction enzymes: *Alu* I, *Bam* HI, *Eco* RI, *Hha* I, *Msp* I, *Hae* III, and *Rsa* I. Restriction fragments were electrophoresed in 2.5% TreviGel (Trevigen) and stained with ethidium bromide. Alleles were detected visually by differences in restriction fragment length patterns (Figure 1). Once polymorphisms were detected within an intergenic region, standard samples for each polymorphism were run in every gel to insure correct allele identification.

Study sites

Acorns or leaves were collected from 14 locations in August and September 2000. Two to seven trees at least 50 meters apart were sampled at each site. The Indiana DNR classifies four sites as old growth forests (forests containing trees at least 150 years old). The Purdue University forest at the Davis Farm has remained undisturbed since 1916 and minimally disturbed since 1856 (Parker et al. 1985). The site contains many red oaks 150 years old or older. The nature preserves are protected sites that have not, to our knowledge, experienced recent cycles of cutting and replanting and may include some older trees. The state parks and state forests contain secondary growth and may have experienced deliberate plantings and

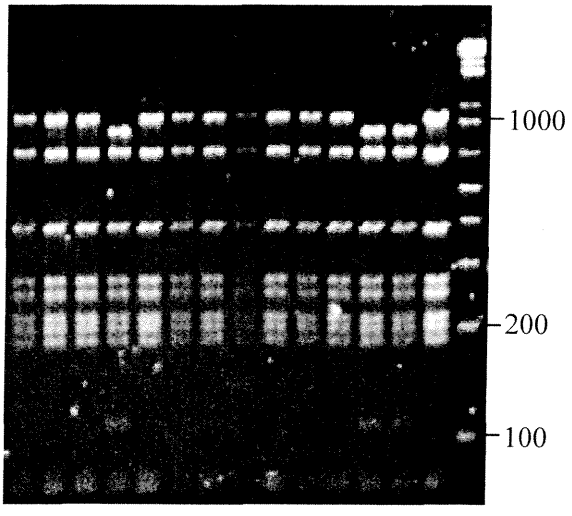

Figure 1. PCR-RFLP fingerprint pattern for the TC fragment. Lanes 4, 12, and 13 show the pattern detected at the Donaldson's Woods and Spring Mill sites (polymorphism 2 in haplotype IV, Tables 1 and 2). All other TC fingerprint patterns were identical to those seen in lanes 1–3, 5–11 and 14. Band size standards are in base pairs.

limited harvesting. Six sites were south of the southernmost advance of the Wisconsinian ice sheet.

Parent-progeny test

Sixteen acorns were harvested from each of two northern red oak trees growing 50 meters apart on the Purdue campus. Work done for another study had shown that these two trees had different chloroplast haplotypes. Acorn haplotypes for the CD, FV and TC loci were detected as described above.

Estimation of differentiation

Haplotype frequencies were used to compute the differentiation measure G_{ST} (Pons and Petit 1995) using the software HAPLODIV available at http://www.pierroton.inra.fr/genetics/labo/Software/. HAPLODIV also calculates the minimum sample size per population that gives the smallest variance for G_{ST}, given the number of populations examined. The average sample size per site (4.10) was larger than the minimum sample size (2.42) for the ten populations that had at least three samples.

Results

We detected two polymorphisms for TC, three for FV and two for CD (Table 1). Although we did not identify which restriction sites were polymorphic, the RFLP patterns produced by the restriction enzyme mix appeared to be the result of a polymorphism at a single restriction site (Figure 1). The data show five haplotypes, two of which (I and II) account for 69% of the data. The two trees chosen for the parent-progeny test had different haplotypes (I and II). All of the acorns tested had the haplotype of the mother tree. Haplotype III was found only at the Charles Deam and Pioneer Mothers site within the Hoosier National Forest (Figure 2). Haplotype IV was found only at Spring Mill and Donaldson's Woods, within 30 km of the Hoosier National Forest. Haplotypes III and IV occurred south of the southernmost advance of the Wisconsinian ice sheet in Indiana. Haplotype V was limited to the Indiana Dunes, Rocky Hollow and Pine Hills.

G_{ST} is a measure of population differentiation based on gene frequencies and does not depend on the number of observed populations. The number of observed populations influences the estimate of variance. Ten populations (sample size ≥ 3) were included in the calculations.. The optimum sample size that minimizes the variance of the estimate for G_{ST} (given 10 populations) is 2.42 samples per population, assuming populations of equal size (Table 3). Given this calculation, the mean sample size (4.10) seemed sufficient to give a preliminary estimate of G_{ST}, even with moderately unequal samples sizes (harmonic mean =3.76). Most of the genetic variation resided among rather than within populations ($G_{ST} = 0.73 \pm 0.14$).

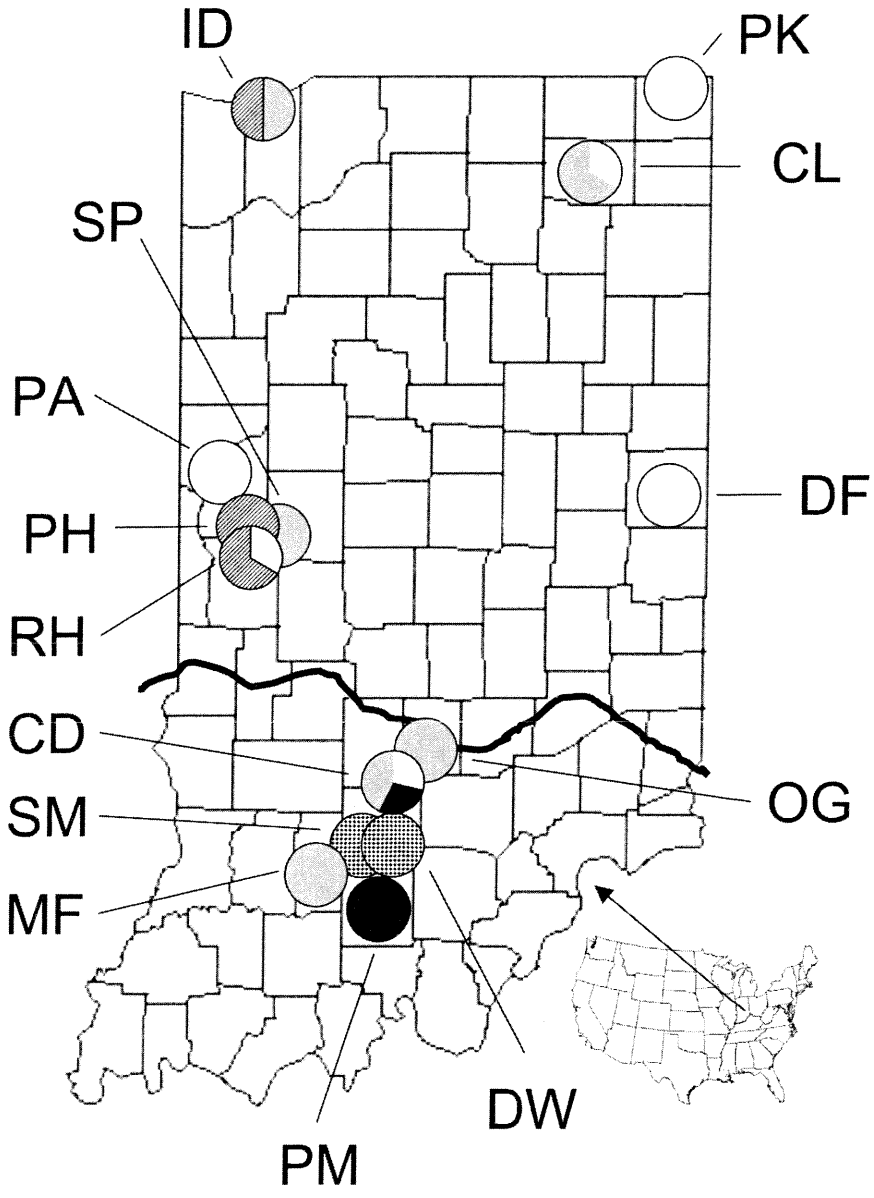

Figure 2. Geographic distribution and frequencies of haplotypes per site. Haplotypes I, II, III, IV and V are represented as white, gray, black, dotted and striped sectors, respectively. Site abbreviations are listed in Table 1. The irregular black line indicates the southernmost advance of the Wisconsinian ice sheet.

Discussion

PCR-RFLP of three intergenic regions in the chloroplast can detect polymorphisms

Table 2. Site locations, management type, Wisconsinian glaciation (GI), documented old growth status (OG) and haplotypes detected for northern red oaks.

						Haplotypes					
Place name	County	Code	Management	GI	OG	I	II	III	IV	V	Total
Davis Farm	Randolph	DF	Purdue Forest	+	+	5	0	0	0	0	5
Rocky Hollow	Parke	RH	Nature Preserve	+	+	1	0	0	0	2	3
Donaldson's Woods	Lawrence	DW	Nature Preserve	-	+	0	0	0	3	0	3
Pioneer Mothers	Orange	PM	National Forest	−	+	0	0	4	0	0	4
Pine Hills	Montgomery	PH	Nature Preserve	+	+	0	0	0	0	1	1
Indiana Dunes	Porter	ID	Nature Preserve	+		0	1	0	0	1	2
Chain O'Lakes	Noble	CL	State Park	+		1	2	0	0	0	3
Portland Arch	Fountain	PA	Nature Preserve	+		3	0	0	0	0	3
Potowotomi	Steuben	PK	Nature Preserve	+		5	0	0	0	0	5
Ogle Hollow	Brown	OG	Nature Preserve	−		0	3	0	0	0	3
Charles Deam	Monroe	CD	National Forest	−		2	3	2	0	0	7
Martin Forest	Martin	MF	State Forest	−		0	5	0	0	0	5
Spring Mill	Lawrence	SM	State Park	−		0	0	0	2	0	2
Shades	Parke	SP	State Park	+		0	2	0	0	0	2
TOTAL						17	16	6	5	4	48

Table 3. Diversity analysis for unordered alleles. Standard errors shown in parenthesis.

Number of populations	10
Mean sample size	4.10
Harmonic mean	3.76
Optimum sample size/pop.	2.42
h_S	0.209 (0.107)
h_T	0.779 (0.070)
G_{ST}	0.731 (0.143)

among populations of northern red oak. The parent-progeny test suggested that inheritance of the chloroplast is maternal, an observation consistent with previous studies on the European pedunculate oak *Quercus robur* (Dumolin et al. 1995). The chloroplast genome, when inherited through the maternal parent, serves as a seed-specific marker. The distribution of cpDNA polymorphisms can then be used to infer the pattern of post-glacial oak recolonization of the north central United States and Canada.

We initiated the Indiana study to explore north-south differences in diversity, choosing sites that we thought less likely to have experienced extensive human-mediated disturbance that might cloud relictual patterns from the most recent ice age. Haplotypes I and II occurred in both northern and southern sites, while haplotypes III, IV and V were associated with old growth sites (Donaldson's Woods, the Pioneer Mothers and Rocky Hollow). While the observed distribution of haplotypes is not inconsistent with north-south and stand age differences in diversity, too few populations were examined to permit firm conclusions Based on these preliminary results, we are examining more sites representing contrasting glacial histories and age structures.

The southern haplotypes occur in stands with different management histories. We

found haplotype IV in the Donaldson's Woods and in Spring Mill State Park, an area of secondary growth surrounding the Donaldson's Woods. Similarly, we detected haplotype V in two places within the Hoosier National Forest, in the Pioneer Mothers old growth stand (15 ha) and in the larger Charles Deam Wilderness (5286 ha). We sampled on a larger spatial scale in the Deam Wilderness, which increased our chances of detecting multiple haplotypes. Jenkins and Parker (2000) have shown that the Charles Deam land cover in 1939 consisted of agriculture or old fields (33%), open or grazed forest (25.6%) and closed canopy forest (41.4%). Haplotype V may have persisted in the open and closed canopy fragments and naturally re-established as the forest regenerated. All the northern red oak at the Pioneer Mothers and the Davis Farm (a northern site) was sampled and mapped in 2002 and will be evaluated for chloroplast haplotype in 2003. This investigation will shed light on the appropriate sample size and spatial scale for accurate estimations of haplotype frequencies within northern red oak populations.

Genetic variation in nuclear genome is likely to be correlated with genetic diversity in the matrilineal chloroplast genome (Kremer et al. 2002). We are now using nuclear microsatellite markers (Aldrich et al. 2002) to investigate this relationship in *Quercus rubra*. We are also initiating detailed comparisons of *Q. rubra* with other species within *Erythrobalanus* to clarify the chloroplast and nuclear genetic boundaries of the respective gene pools.

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