Genetic diversity of butternut (*Juglans cinerea*) and implications for conservation

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Abstract: The management of threatened and endangered species can be improved by understanding their patterns of genetic diversity and structure. This paper presents the results of the first analysis of the population genetics of butternut (*Juglans cinerea* L.) using nuclear microsatellites. Butternut once was an economically and ecologically valuable hardwood, but now its numbers are rapidly decreasing throughout its native range in eastern North America. By genotyping butternut trees ($n = 157$) from five populations at seven highly polymorphic loci, we determined that the remaining genetic diversity for the species is considerably higher than previously estimated (mean $H_E = 0.75$). Populations were nearly genetically homogenous ($F_{ST} = 0.025$), with no evidence of isolation by distance. These results indicate that butternut retains a large amount of genetic diversity, and that it is not too late to implement strategies to conserve local butternut populations.

Résumé : La gestion des espèces menacées et en voie de disparition peut être effectuée de façon plus judicieuse par la compréhension des patrons de diversité génétique et de sa structure. Les auteurs ont analysé pour la première fois la génétique des populations du noyer cendré (*Juglans cinerea* L.) au moyen de loci nucléaires de microsatellites. Le noyer cendré était jadis une espèce feuillue précieuse du point de vue économique et écologique avant de voir ses effectifs diminuer rapidement partout dans son aire de distribution naturelle dans l'est de l'Amérique du Nord. Les auteurs ont déterminé les génotypes des noyers cendrés ($n = 157$) de cinq populations pour sept loci hautement polymorphes. La diversité génétique résiduelle de l'espèce est apparue considérablement plus élevée que les estimations antérieures (H_E moyenne = 0,75). Les populations étaient quasi homogènes génétiquement ($F_{ST} = 0,025$), ne présentant aucun indice d'isolement par la distance. Ces résultats indiquent que le noyer cendré a conservé une diversité génétique élevée et qu'il n'est pas trop tard pour mettre en application des stratégies visant à conserver des populations locales de cette espèce.

[Traduit par la Rédaction]

Introduction

Butternut (*Juglans cinerea* L.) is a short-lived tree species being extirpated throughout most of its native range (Fig. 1) by an epiphytotic of butternut canker disease (Nielsen et al. 2003; Schultz 2003). Dramatic declines in butternut populations over the past few decades have resulted in the listing of butternut as endangered in Canada (Nielsen et al. 2003) and as a regional forester sensitive species in the USA. The disease was reported first in Wisconsin in 1967 (Renlund 1971), but it is believed to have spread from the southeastern USA (Nesom 2005). The causal agent of butternut canker, the fungus *Sirococcus clavigignenti-juglandacearum* Nair, Kostichka & Kuntz, was not described as a new spe-

cies until 1979 (Nair et al. 1979). The origin of the fungus is unknown; the genetic uniformity of the pathogen, however, probably indicates that it is a recently-introduced exotic species (Renlund 1971; Ostry 1997; Furnier et al. 1999). The fungus causes branch and stem cankers that ultimately girdle and kill host trees. Although butternut is susceptible to damage from other diseases and pests (Rink 1990), butternut canker is the greatest threat to the species' existence. By the 1990s, 91% of the butternut trees in Wisconsin were diseased (Schultz 2003), with similar infection trends reported across the species' range (Nielsen et al. 2003; Ostry and Woeste 2004; Schultz 2003) and the greatest losses occurring in the southern USA (Thompson et al. 2006). In Canada, butternut canker was first detected and confirmed in Ontario and Quebec in 1991 (Davis et al. 1992) and in New Brunswick in 1997, where it was thought to have been present for at least 7 years (Harrison et al. 1998).

Butternut grows best on well-drained rich (often riparian) soils. It is typically found in highly dispersed populations of a few widely scattered individuals in mixed-hardwood forests. Butternut was also historically planted on farmsteads throughout its range to provide a convenient source of nuts (Nesom 2005). Butternut's soft, tan heartwood is used for cabinet work, furniture, instrument cases, paneling, and turnery, and its sweet oily nuts are valued by humans for their

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Fig. 1. Map showing native range of butternut and approximate locations of sampled populations. For details concerning sample sites, see Table 1. Image from Little (1971).
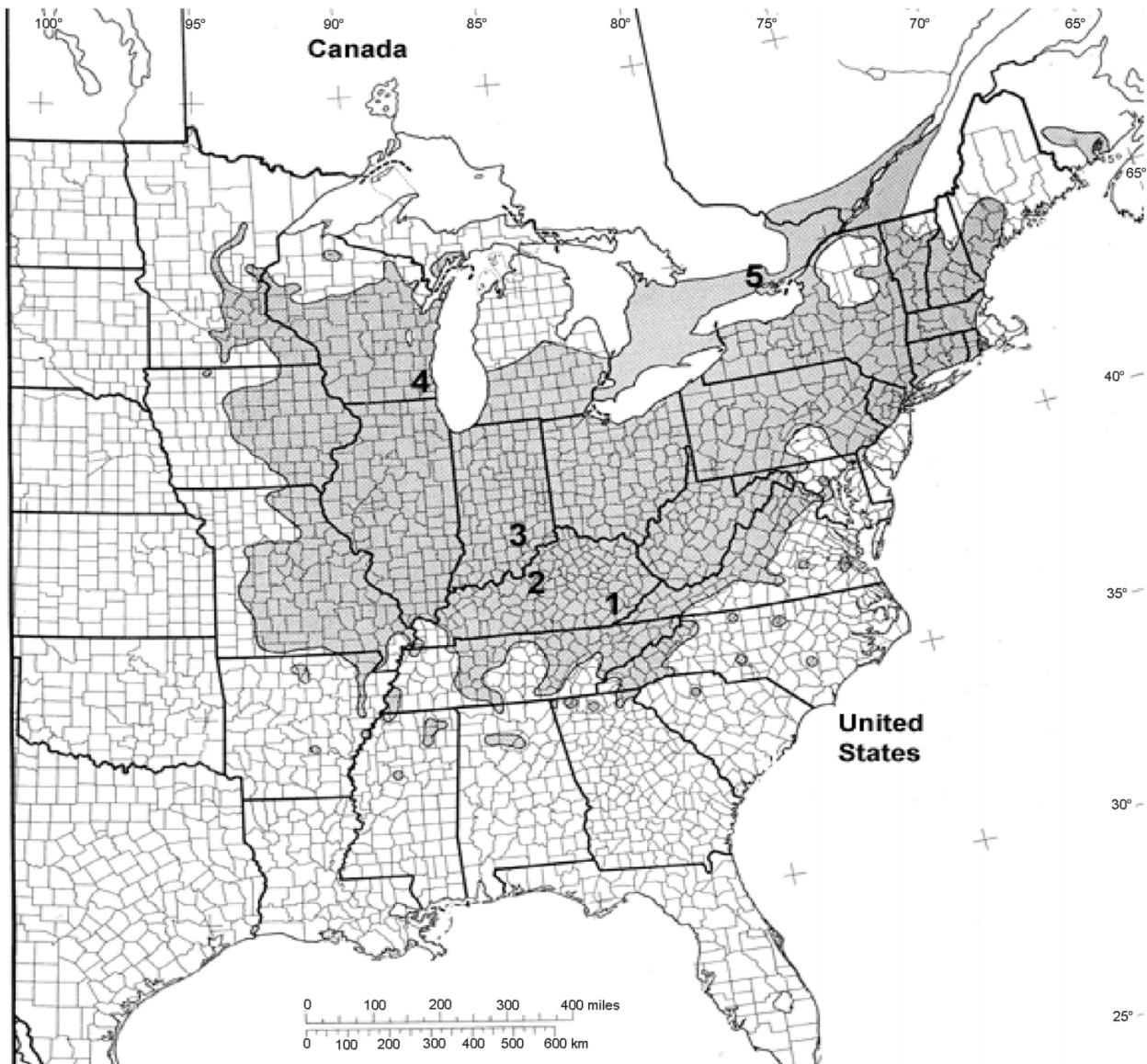
flavor as well as by wildlife as a source of mast (Ostry and Pijut 2000). Butternut bark, roots, and husks have been traditionally used for medical purposes (e.g., as a laxative; Krochmal and Krochmal 1982), and the husks were used by Native Americans, early settlers, and the Confederate Army to make orange- or yellow-brown dye (Peattie 1950). Native Americans and early settlers also extracted oil from crushed butternuts and collected butternut sap to make syrup (Goodell 1984).

Efforts to conserve butternut are ongoing and have included analyses of genetic diversity based on allozyme loci (Morin et al. 2000) and breeding programs for the development of disease-resistant individuals (Michler et al. 2005). Research based on allozyme loci reported low levels of genetic diversity partitioned within and among butternut populations in the northern portion of the species range (Morin et al. 2000). In this paper, we present the results of the first PCR-based analysis of genetic parameters for butternut pop-

ulations. Microsatellite markers often have greater resolution (i.e., higher levels of polymorphism) than allozyme markers, and thus provide more statistical power to analyze population differentiation and disequilibrium (Estoup et al. 1998). To gain insight into whether recent demographic changes in butternut populations are reflected in measures of population genetic parameters such as allelic diversity (AR) and inbreeding, we will compare values obtained for butternut using microsatellites with those observed by Morin et al. (2000) in butternut populations unaffected by canker and with recently published estimates for black walnut (*Juglans nigra* L.; Victory et al. 2006), a sympatric congener with reproductive and life history traits similar to butternut (Rink 1990).

Materials and methods

Plant material

Leaf samples were collected from 157 mature butternut

Table 1. Collection site locations and characteristics of sampled *Juglans cinerea* populations.

Population	Location	Coordinates	No. of trees	Estimated stand age (years)	DBH (cm)[a]	Height (m)[b]
1 (DBNF)	Daniel Boone National Forest, Kentucky, USA	37°N, –83°W	36	40–50	33.45±12.27	16.04±7.62
2 (MACA)	Mammoth Cave National Park, Kentucky, USA	37°N, –86°W	32	40–50	35.23±10.26	19.61±4.70
3 (HNF)	Hoosier National Forest, Indiana, USA	38°N, –86°W	44	30–40	25.81±6.81	17.83±4.23
4 (WI)	Whitewater, Wisconsin, USA	43°N, –89°W	36	45	28.52±9.40	Data not available
5 (Ontario)	Havelock, Ontario, Canada	44°N, –77°W	9	Uneven aged 10–40	Data not available	Data not available

[a]Mean ± SD.

[b]Mean ± SD.

trees found in five discrete and geographically distant locations throughout the species' native range (Table 1; Fig. 1). Samples were acquired using a line tied to a 12 oz (1 oz = 28.4 g) weight fired into tree canopies using a sling shot (BigShot, Sherrill Inc., Greensboro, North Carolina). A small number of leaves were shaken from the canopy of each tree and placed into labeled sealed bags and stored on ice until they were brought to Purdue University where they were freeze-dried. Each tree was photographed and its location fixed with a geographic positioning system (Garmin GPS II Plus, Garmin International Inc., Olathe, Kansas). Stand age was estimated for all populations based on age–diameter relationships described in Clark et al. (2008). Stand age for the Wisconsin (WI) population was known to be about 45 years, and the trees in the Havelock, Ontario, population were uneven aged, ranging in size from saplings to trees 55 cm diameter at breast height (dbh).

DNA extraction, quantification, amplification, and genotyping

Genomic DNA was extracted from freeze-dried leaves as described by Victory et al. (2006). All samples were quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, Delaware). Seven previously characterized microsatellite loci were chosen for genotyping because we had already verified that these loci produced consistent and clear results (Ross-Davis and Woeste 2007). To assure that there were no obvious problems with allele dropouts, null alleles, and scoring, the conformation of each locus to Hardy–Weinberg genotypic proportions was tested and verified using FSTAT software, by permuting alleles among individuals within populations 700 times and using the global F_{IS} estimate across populations as a test statistic (one-tailed test). HW-QuickCheck software, which uses an exact test (one-tailed) and 17 000 random permutations (Kalinowski, In Press), was also used. PCR conditions and genotyping were performed as described in Victory et al. (2006), except products were separated using an ABI 3730 sequencer (Applied Biosystems, Foster City, California). Fragment length data were collected using Gene Scan Analysis version 3.1 (Perkin Elmer, Waltham, Massachusetts) software, and genotypes were assigned with the aid of Genotyper version 2.5 (Perkin Elmer) software. Previously published black walnut genotypes (Victory et al. 2006) were initially used as size standards to calibrate allele sizes, then two butternut samples with previously assigned allele sizes were amplified with the rest of the samples in each PCR plate as allele scoring standards. In each amplification plate, one well without DNA but containing all other PCR reagents was amplified with the rest of the samples and used

as a negative control. For other details concerning the scoring of loci, see Ross-Davis and Woeste (2007). Genotypes were scored for each individual from at least two independent amplifications, and any samples that could not be definitively scored were reamplified and genotyped until the allele sizes could be definitively determined.

Genetic analysis

FSTAT version 2.9.3.2 (Goudet 1995) software was used to estimate global F statistics (F_{IS} (individuals relative to their subpopulations), F_{IT} (individuals relative to the total sampled population), and F_{ST} (the divergence of subpopulations in the total sample)) within and among populations, heterozygosity estimates (H_E (expected), H_O (observed), H_S (mean), H_t (expected under Hardy–Weinberg equilibrium for the total population)), and the number of total and private alleles per locus. FSTAT was used to calculate allelic richness, which was adjusted by rarefaction to a common population size of nine, the smallest population sampled. F_{IS} estimates for each population were tested for significant departure from expectation ($\alpha = 0.05$, two-tailed test) based on 700 randomizations within populations using FSTAT. SPAGeDi (Hardy and Vekemans 2002) software was used to estimate the coefficient of gene differentiation (G_{ST}), H_E corrected for sample size, and the standard distance, D_S, which is an unbiased estimator useful for small sample sizes (Nei 1978). Population migration rate, N_m, was estimated using Genepop (Raymond and Rousset 1995), based on the method of Barton and Slatkin (1986). Tests for bottlenecks were performed using Bottleneck version 1.2.02 (S. Piry, G. Luikart, and J.-M. Cornuet. www.montpellier.inra.fr/URLB/bottleneck/bottleneck.html) with the two phase model (TPM) under a range of conditions, including the default settings (variance = 30, probability = 70%) and variance set to 12% with probability at 70%, 80%, and 90% (Cornuet and Luikart 1996). A Mantel test of association between genetic and geographic distance was performed using Genepop (Raymond and Rousset 1995).

Results

The overall multilocus heterozygosity ($H_t = 0.762$; Nei 1977) and the mean number of alleles per locus, termed AR, (AR = 12.4; Table 2), indicated that the sampled butternut populations, which spanned the center of the species' range, were genetically diverse. The number of alleles per locus ranged from 6 (WGA 090) to 20 (WGA 004), with the number of private alleles within populations ranging from 1 (WGA 090 and WGA 221) to 6 (WGA 004). The Hoosier National Forest (HNF) population had 10 private alleles,

Table 2. Total number of genotypes, allele size range, number of alleles, allelic richness (adjusted to a common population size of $n = 9$), and private alleles observed for each locus surveyed in sampled *Juglans cinerea* populations.

Locus	No. of genotypes	Allele size range (bp)	No. of alleles	Allelic richness	No. of private alleles
WGA 004	155	225–273	20	7.9	6[a]
WGA 090	156	126–144	6	3.9	1[b]
WGA 142	155	161–199	10	4.8	4[c]
WGA 148	157	232–264	13	6.6	4[d]
WGA 204	156	168–200	15	7.1	4[e]
WGA 221	157	227–247	8	5.5	1[f]
WGA 256	157	205–241	15	8.1	3[g]
Mean	156.1	—	12.4		3.3

[a]Size 239 bp in DBNF population; size 263 in MACA population; sizes 227, 259, 267, and 271 in HNF population.

[b]Size 144 bp in MACA population.

[c]Sizes 161 and 183 bp in DBNF population; size 199 in MACA population; size 185 in HNF population.

[d]Size 232 bp in DBNF population; sizes 250 and 262 in MACA population; size 264 in HNF population.

[e]Sizes 192 bp in DBNF population; size 168 in MACA population; size 196 in HNF population; size 200 in WI population.

[f]Size 247 in HNF population.

[g]Size 205 in MACA population; sizes 239 and 241 bp in HNF population.

more than any other population. Mean within-population allelic richness (adjusted to a common sample size) ranged from 4.7 (for the Havelock, Ontario population) to 6.5 (for the HNF population). The Daniel Boone National Forest (DBNF), Mammoth Cave National Park (MACA), and WI populations had mean allelic richnesses of 6.2, 6.2, and 5.1 alleles per locus, respectively. Sixty-six of the 87 alleles (76%) were uncommon, occurring with an overall frequency of ≤0.10. Private alleles, all of which occurred at within-population frequencies of <0.02, accounted for 35% of the uncommon alleles; ergo, 65% of the uncommon alleles were found in more than one population. Slatkin (1985) proposed that the frequency of rare alleles could be used to estimate N_m. The estimate of N_m for the populations in our study was relatively high (8.19, corrected for population sizes), where $N_m > 1$ is considered sufficient to counteract the effects of drift and local directional selection, even in small populations (Ellstrand 1992).

H_O within populations ranged from 0.68 (for the WI population) to 0.83 (for the Havelock, Ontario population), with an overall H_O of 0.76 (Table 3). H_E values significantly differed from H_O values across populations at a small number of loci (Table 3), but in general, H_O values were nearly the same as those expected under Hardy–Weinberg equilibrium (HWE). Thus, the butternut populations we sampled approximated random mating. Accordingly, overall F_{IS} estimates were low and not significantly different from zero (–0.002 ± 0.0126; SE based on jackknife). Values of F_{IS} (fixation within populations) ranged from –0.148 (for the Havelock, Ontario population) to 0.022 (for the MACA population), but the value of F_{IS} was not significantly different than zero for any population (Table 3). The overall negative value of F_{IS}, although statistically not significantly different from zero, appears to reflect a strong influence of the Havelock, Ontario population. Overall F statistics (±SE based on jackknife; $F_{IT} = 0.023 ± 0.013$ and F_{ST}, a measure of population differentiation, $= 0.025 ± 0.008$) indicated low levels of genetic structure among sampled populations (Table 4). The 95% confidence interval (based on 700 bootstraps) for F_{IT} included zero, indicating no discernable trend toward inbreeding locally relative to the entire

studied population; the 99% confidence interval for F_{ST} did not include zero (0.007–0.045), indicating a small but statistically significant amount of differentiation among populations. G_{ST} is the ratio of the diversity among populations to the total diversity. G_{ST} is used as a measure of population differentiation that, unlike F_{ST}, is based on a fixed effects model and is not sensitive to differences in sample size among populations (Pons and Petit 1995). The mean pairwise G_{ST} for the populations in our study was $0.023 ± 0.010$.

The average genetic distance between a population and all other populations was estimated as the mean of the pairwise D_S (Nei 1978) between a population and the other (four) populations (Table 5). The WI population was most distant genetically from the others (0.136 ± 0.03 SE), and the HNF population was the least distant (0.044 ± 0.02 SE); the Havelock, Ontario population, which was geographically the most remote from the other populations, was intermediate among the populations for genetic distance (0.107 ± 0.03 SE). Populations that were more separated geographically tended to have larger D_S values as well, perhaps reflecting a trend toward isolation by distance, but the Mantel test for correlation between physical and genetic distance ($r = 0.605$; $p = 0.064$) was not significant.

Discussion

It is instructive to compare these results with those obtained by Morin et al. (2000) and Victory et al. (2006). Morin et al. (2000) investigated the genetic diversity of 9 butternut populations using 12 allozymes, while Victory et al. (2006) investigated the genetic diversity and structure of 43 black walnut populations using 12 nuclear microsatellites. Data from black walnut is relevant because black walnut is a sympatric congener with a mating system similar to that of butternut, although modern reports indicate that butternut historically has never been as common as black walnut (Rink 1990). Morin et al. (2000) concluded that the genetic diversity of butternut was low, probably throughout the species' entire range, and that butternut had a relatively homogeneous gene pool; only their sample from Vermont

Table 3. Observed (H_O) and expected (H_E) levels of heterozygosity and F_{IS} estimates for each sampled *Juglans cinerea* population for each microsatellite locus.

	Locus							
	WGA 004	WGA 90	WGA 142	WGA 148	WGA 204	WGA 221	WGA 256	Mean
DBNF population								
H_E	0.83	0.59	0.78	0.83	0.81	0.67	0.89[a]	0.77
H_O	0.81	0.67	0.75	0.84	0.94	0.64	0.78[a]	0.78
F_{IS}	0.025	–0.136	0.040	–0.009	–0.161	0.047	0.124	–0.004
MACA population								
H_E	0.85	0.56[a]	0.72	0.82[a]	0.79	0.75	0.88	0.77
H_O	0.84	0.45[a]	0.7	0.85[a]	0.77	0.73	0.86	0.75
F_{IS}	0.005	0.194	–0.010	–0.060	0.027	–0.012	–0.009	0.022
HNF population								
H_E	0.89	0.54	0.76	0.74	0.83	0.77	0.87	0.77
H_O	0.93	0.58	0.77	0.63	0.84	0.78	0.88	0.77
F_{IS}	–0.050	–0.071	–0.011	0.159	–0.015	0.028	0.022	0.000
WI population								
H_E	0.79	0.52	0.77	0.48	0.71	0.82	0.82	0.70
H_O	0.83	0.42	0.71	0.56	0.64	0.78	0.83	0.68
F_{IS}	–0.053	0.205	–0.092	–0.163	0.098	0.044	0.000	0.003
Havelock, Ontario population								
H_E	0.81	0.53	0.75	0.74	0.79	0.63	0.80	0.72
H_O	1.00	0.44	0.78	1.00	1.00	0.78	0.78	0.83
F_{IS}	–0.231	0.158	–0.037	–0.358	–0.274	–0.244	0.026	–0.148
Mean								
H_E[b]	0.84	0.55	0.74	0.76	0.80	0.77	0.86	0.75
H_O	0.88	0.51	0.76	0.77	0.84	0.74	0.83	0.76
F_{IS}	–0.061	0.070	–0.022	–0.086	–0.065	–0.027	0.033	–0.002

[a]Significant at $\alpha = 0.05$.

[b]Heterozygosity estimates corrected for differences in sample size among populations (Nei 1978).

Table 4. Overall F_{IT} and F_{ST} statistics, where means and SEs were obtained by jackknifing over loci and 95% confidence intervals were obtained by bootstrapping over loci.

			95% confidence limits	
	Mean	SE	Lower bound	Upper bound
F_{IT}	0.023	0.013	0.00	0.047
F_{ST}	0.025	0.008	0.010	0.040

was genetically distinct from the Canadian butternut populations they sampled. They also reported an overall excess of heterozygotes within populations. Fjellstrom and Parfitt (1994) found that, leaving aside an unusual sample from North Carolina butternut had an F_{ST} value of 0.071 and that butternut was less genetically variable than the other 12 *Juglans* spp. they examined using RFLPs. Victory et al. (2006) described the population genetics of black walnut as marked by high levels of pollen flow, low levels of genetic structure, and high levels of genetic diversity, with a small heterozygote deficit; these results are typical of other temperate forest tree species (Heuertz et al. 2004; Muir et al. 2004). We found that parameters of (neutral) genetic diversity for butternut had high values for all populations. The microsatellite loci we examined had large numbers of alleles and high levels of gene diversity ($H_E = 0.79$), similar to those of black walnut sampled in the same region (black walnut

$H_E = 0.79$). Levels of H_O in our samples of butternut ranged from 0.68 to 0.83 (mean = 0.76), and were comparable to those for black walnut (0.590–0.916, mean = 0.781; Victory et al. 2006).

Our results showed that the butternut populations we sampled exhibited low but significant levels of genetic structure, levels comparable to and even slightly higher than values observed in black walnut ($F_{ST} = 0.025$ vs. 0.017 in black walnut), although our estimates of gene differentiation among butternut populations ($G_{ST} = 0.023$) were still somewhat lower than those for other wind-pollinated species (e.g., G_{ST} in conifers is about 0.073; Hamrick et al. 1992). Perhaps genetic drift due to isolation has enhanced levels of genetic differentiation among populations of butternut relative to those of black walnut, a much more abundant species. It might be argued that we observed higher levels of genetic diversity than Morin et al. (2000) because most of our samples were from populations in the USA and not from populations at the northern edge of the range of butternut. Morin et al. (2000) recognized the possibility that southern butternut populations might be more genetically diverse than those in Canada, and Fjellstrom and Parfitt (1994) also found that their most southern source (North Carolina) had an unusually high heterozygosity. Although the value of F_{ST} for butternut reported here (0.025) was considerably lower than previous estimates (Morin et al. 2000, $F_{ST} = 0.078$; Fjellstrom and Parfitt 1994, $F_{ST} = 0.071$),

Table 5. Nei's (1978) standard distance (D_S) on upper right diagonal and geographic distance (km) on lower left diagonal.

| Geographic distance (km) | D_S | | | | |
	DBNF population	MACA population	HNF population	WI population	Havelock, Ontario population
DBNF population	—	0.033	0.021	0.158	0.075
MACA population	241	—	0.004	0.105	0.064
HNF population	338	258	—	0.072	0.080
WI population	853	789	580	—	0.207
Havelock, Ontario population	1175	1256	1175	1159	—

Morin et al. (2000) pointed out that if a single population (Vermont) was omitted from their analysis, their resulting estimate ($F_{ST} = 0.029$) became similar to the value we estimated. It is not clear why this Vermont population appeared so different from the others sampled by Morin et al. (2000). It has been suggested (D. Stone, personal communication) that the unusually genetically diverse butternut samples from North Carolina analyzed by Fjellstrom and Parfitt (1994) may have included hybrids with Japanese walnut (*Juglans ailantifolia* Carr.). If hybrids were included in Fjellstrom and Parfitt's samples from North Carolina, it might explain why this population was so different from the others they sampled, and a similar explanation might account for the divergence of the Vermont population sampled by Morin et al. (2000) from the rest of their samples. We believe, however, that the high level of genetic diversity we observed for butternut (relative to the Canadian samples of Morin et al. (2000) and those of Fjellstrom and Parfitt (1994) which were from Illinois, Vermont, and North Carolina), was most likely due to the increased power of microsatellites to detect heterozygosity, rather than a cline in genetic diversity from south to north. Diversity measures for the population we sampled from Canada were comparable to those from the rest of the species' range (Table 3), and although a gradual loss of allele number was noted for black walnut in more northern populations (Victory et al. 2006), the effect was quite small. The relationship between gene flow, migration, and F_{ST} is complex (Ellstrand 1992; Whitlock and McCauley 1999); without more extensive data for the particular butternut populations sampled in our study it would be hard to support any conclusions about differences in evolutionary processes in the Canadian versus the USA butternuts.

Like Morin et al. (2000), we found that our Canadian butternut population showed considerable heterozygote excess ($F_{IS} = -0.148$), setting it apart from the USA populations in our study. Victory et al. (2006) reported a small heterozygote deficit in their study of black walnut, although in general, mature trees often show an excess of heterozygosity as a consequence of selection. In our study, however, the trees from the Canadian population were similar in age to those sampled in the USA (Table 1), so while tree age does not fully explain the observed excess of heterozygotes in Canadian butternut populations, it is not clear what does. Morin et al. (2000) suggested the possibility of negative assortative mating; other contributing factors may have included selection, sampling across populations, and the relatively small number of populations sampled in both studies. In light of the slightly positive F_{IS} values we observed in the MACA and WI populations, we performed tests for bottlenecks. We found that only the Havelock, Ontario population showed a significant departure ($p = 0.039$) from mutation–drift equilibrium as determined by the Wilcoxon test, a result attributable to the previously described heterozygote "excess." In general, the values for H_O that we observed did not deviate greatly from H_E values, which is typical of large naturally outbreeding populations. However, in several instances (i.e., population × locus combinations) we observed levels of H_O slightly but significantly lower than expected under HWE. These deviations might have been caused by sampling across populations (Wahlund effect), inbreeding, positive assortative mating, or the presence of null alleles. Given the overall picture of random mating in the sampled populations, the data do not indicate an overriding role for any of these factors in our analysis; however, because neighborhood size has not been determined for butternut (or black walnut), Wahlund effects cannot definitively be ruled out, especially in the Kentucky populations where the steep, hilly terrain separated clusters of sampled trees. Only 3 of the 14 locus and population combinations from the DBNF and MACA populations deviated significantly from Hardy–Weinberg expectations. Because the deviations were relatively small, few in number, different in direction, and inconsistent (across loci), we believe that the observed deviations from HWE were probably due to sample size effects rather than any systematic cause such as a Wahlund effect. Morbidity and mortality from butternut canker was high at most of the sample sites, but our results do not support a strong pattern of assortative mating based on disease resistance. Evidence of selection for canker resistance at the neutral genetic markers we used would be an unlikely outcome in any case, since most of the trees we sampled were mature, and it probably resulted from matings that predated the current epiphytotic.

In general, despite the rapid population declines caused by butternut canker disease, the measures of genetic diversity and inbreeding we observed compared favorably with black walnut, an abundant species. This conundrum was particularly evident for the WI population, which is believed to be nearly even aged and to have originated from a small number of founders (probably 5–10) that were present in a pasture that was excluded from grazing (with a fence) in the early 1960s. Although natural regeneration based on a small number of parents might be expected to result in a population bottleneck, the F_{IS} estimate for this population was not extreme and there was no statistical evidence that the WI population was bottlenecked based on the methods of Cornuet and Luikart (1996). The results from the WI population are important because the number of individuals capable of reproduction in most butternut subpopulations may

become quite small in the future as mortality from butternut canker and age take their toll. The heterodichogamous mating system of butternut may reinforce the Allee effect caused by the loss of local populations to disease (Wagenius et al. 2007). The best explanation for why we did not see the negative genetic consequences of a founder event based on a small population in the WI population is gene flow. The area around the WI population contains highly fragmented forests separated by agricultural fields. Although we searched the vicinity of this population for other butternuts and did not find any, if the reproductive biology of butternut is similar to that of black walnut, a species for which pollination distances greater than 1 km are not unusual (Robichaud 2007), then pollen gene flow may have increased the genetic diversity of the WI population well beyond what was present in the small number of founders. Our estimate of N_m for butternut ($N_m = 8.19$) indicated considerable gene flow among contemporary butternut populations, although there are no previous estimates of N_m for butternut or black walnut for comparison.

The frequency and distribution of rare alleles can provide insights into the population dynamics and conservation genetics of a species (Barton and Slatkin 1986). Many butternut alleles were uncommon (those with a frequency ≤0.10), a trend previously observed in black walnut (77% of all butternut alleles were uncommon, 90% of all alleles in black walnut), and over 1/3 of the uncommon alleles in butternut were found in only one population, a much higher proportion than was reported for black walnut (11%) (Victory et al. 2006). This may indicate that butternut has lower levels of pollen gene flow than the exceptionally high levels found in black walnut (Victory et al. 2006). Nearly all the private alleles in black walnut were from the extreme ends of the allele size distribution, but not in butternut, where only 72% of the private alleles were at the end of the allele size distribution. This finding may indicate that local populations have lost alleles that were more common at an earlier time when gene flow and, presumably, population sizes, were higher. There were no private alleles in the Havelock, Ontario population, but this was probably because of our small sample size from this population. The MACA and HNF populations had nine private alleles each, despite their relative proximity to one another. Variance among populations in the loss of alleles can reflect numerous population genetic processes, such as drift and selection, but whatever the cause, the implications for conservation are critical. Allele loss may be a more sensitive assay of the loss of genetic variation in a population than is loss of heterozygosity, and the retention of alleles is important for the long-term adaptation and survival of a species (Allendorf 1985; Fuerst and Maruyama 1985). The butternut populations we sampled appeared to have a lower allelic richness than was observed for black walnut (Victory et al. 2006), which may be important because allelic richness can be more sensitive than gene diversity to recent reductions in population size (Jump and Peñuelas 2006). But a multitude of factors — including microsatellite source bias, differences in mutation rates, and potential differences in the ecology and reproductive biology of butternut and black walnut — make the importance of differences in allelic richness between the two species difficult to evaluate.

Unfortunately there are no common garden experiments for butternut from which to draw conclusions concerning local or regional adaptation and genetic diversity. It is likely that adaptive genetic variation is present in butternut, given its large native range. Decisions concerning butternut conservation and captive breeding will probably have to be based on molecular data, disease response phenotypes, and practical experience. There is no good evidence that butternut has low adaptive potential, a concern raised by Morin et al. (2000), but a more thorough sampling of populations, including the regenerating cohort, is needed to determine if inbreeding or Allee effects are beginning to affect butternut.

If verified by further study, the difference in F_{IS} estimates between the Canadian and the USA butternut populations observed by Morin et al. (2000) and reaffirmed to a certain extent by our findings could point to the need for particular conservation strategies for butternut populations at the northern edge of the species range. For example, if historically high rates of gene flow from the center of butternut's range to the northern edge (Fig. 1) resulted in negative assortative mating, then the rapid demographic collapse of butternut in the central USA (Schultz 2003) could permit more rapid adaptive evolution of populations on the margin (Hendry et al. 2002). Alternatively, the heterozygote excess observed in the Canadian populations may be due to gene flow from the exotic Japanese walnut, which is planted as a nut tree in both the USA and Canada. Hybrids between butternut and Japanese walnut are well documented (Bixby 1919), and the hybrids produce large numbers of seeds and copious amounts of pollen (Bixby 1919). Large reductions in gene flow, caused by demographic collapse of the USA butternut populations because of the butternut canker epiphytotic, and increases in the relative proportion of hybrid seeds and pollen could produce a scenario in which Canadian populations of butternut undergo relatively rapid genetic shifts.

In black walnut, nuts from a single tree are typically sired by a large number of males (males = No. of nuts harvested / 3), most of which are not nearby (Robichaud 2007). If butternut is similar to black walnut in this respect, it would mean that a strategy of taking large samples of nuts from a single tree might be an effective means of capturing local genetic diversity. This strategy might not be effective, however, in areas where populations are small and isolated, owing to the effects of canker disease and poor regeneration. Because large butternut populations are now becoming rare in the USA, there is need for studies that will reveal the level of reproductive connectivity of scattered butternut individuals. There is a great need to shed light on the ability of gene flow to overcome some of the genetic effects of the butternut canker epiphytotic. The relatively low F_{ST} values observed in this study indicate that small populations (<50 individuals) can maintain a large percentage of the overall genetic diversity of the species, but the loss of local populations to butternut canker disease and poor regeneration could rapidly lead to reduction in gene flow, increased isolation, loss of rare alleles, and increased genetic drift. The window of opportunity to sample a large number of butternut populations for conservation and reintroduction is closing rapidly. A broadscale sampling, rather than a narrow focus on a few

populations that appear to contain members that have canker resistance, would better serve butternut's long-term survival.

Acknowledgments

References

Allendorf, F.W. 1985. Genetic drift and the loss of alleles versus heterozygosity. Zoo Biol. **5**: 181–190. doi:10.1002/zoo.1430050212.

Barton, N.H., and Slatkin, M. 1986. A quasi-equilibrium theory of the distribution of rare alleles in a subdivided population. Heredity, **56**: 409–416. PMID:3733460.

Bixby, W.G. 1919. The butternut and the Japan walnut. Am. Nut J. **10**: 76–83.

Clark, S.L., Brosi, S.L., Schlarbaum, S.E., and Grissino-Mayer, H.D. 2008. Dendrochronology of two butternut (*Juglans cinerea* L.) populations in the southeastern United States. For. Ecol. Manage. In press.

Cornuet, J.M., and Luikart, G. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics, **144**: 2001–2014. PMID:8978083.

Davis, C.N., Myren, D.T., and Czerwinski, E.J. 1992. First report of butternut canker in Ontario. Plant Dis. **76**: 972.

Ellstrand, N.C. 1992. Gene flow among seed plant populations. New For. **6**: 241–256.

Estoup, A., Rousset, F., Michalakis, Y., Cornuet, J.-M., Adriamanga, M., and Guyomard, R. 1998. Comparative analysis of microsatellite and allozyme markers: a case study investigating microgeographic differentiation in brown trout (*Salmo trutta*). Mol. Ecol. **7**: 339–353. doi:10.1046/j.1365-294X.1998.00362.x. PMID:9561790.

Fjellstrom, R.G., and Parfitt, D.E. 1994. Walnut (*Juglans* spp.) genetic diversity determined by restriction fragment length polymorphisms. Genome, **37**: 690–700.

Fuerst, P.A., and Maruyama, T. 1985. Considerations on the conservation of alleles and of genetic heterozygosity in small populations. Zoo Biol. **5**: 171–179. doi:10.1002/zoo.1430050211.

Furnier, G.R., Stoltz, A.M., Mustaphi, R.M., and Ostry, M.E. 1999. Genetic evidence that butternut canker was recently introduced into North America. Can. J. Bot. **77**: 783–785. doi:10.1139/cjb-77-6-783.

Goodell, E. 1984. Walnuts for the northeast. Arnoldia, **44**: 3–19.

Goudet, J. 1995. FSTAT Version 1.2: a computer program to calculate F-statistics. J. Hered. **86**(6): 485–486. Version 2.9.3.2 available from www2.unil.ch/popgen/softwares/fstat.htm [accessed 23 August 2007].

Hamrick, J.L., Godt, M.J.W., and Sherman-Broyles, S.L. 1992. Factors influencing levels of genetic diversity in woody plant species. New For. **6**: 95–124.

Hardy, O.J., and Vekemans, X. 2002. SPAGeDi: a versatile computer program to analyze spatial genetic structure at the individual or population levels. Mol. Ecol. Notes, **2**: 618–620. doi:10.1046/j.1471-8286.2002.00305.x.

Harrison, K.J., Hurley, J.E., and Ostry, M.E. 1998. First report of butternut canker caused by *Sirococcus clavigignenti-juglandacearum* in New Brunswick, Canada. Plant Dis. **82**: 1282. doi:10.1094/PDIS.1998.82.11.1282B.

Hendry, A.P., Taylor, E.B., and McPhail, J.D. 2002. Adaptive divergence and the balance between selection and gene flow: lake and stream sticklebacks in the Misty system. Evolution, **56**: 1199–1216. PMID:12144020.

Heuertz, M., Hausman, J.-F., Hardy, O.J., Vendramin, G.G., Frascaria-Lacoste, N., and Vekemans, X. 2004. Nuclear microsatellites reveal contrasting patterns of genetic structure between western and southeastern European populations of the common ash (*Fraxinus excelsior* L.). Evolution, **58**: 976–988. PMID: 15212379.

Jump, A.S., and Peñuelas, J. 2006. Genetic effects of chronic habitat fragmentation in a wind-pollinated tree. Proc. Natl. Acad. Sci. U.S.A. **103**: 8096–8100. doi:10.1073/pnas.0510127103.

Kalinowski, S.T. In press. HW-QuickCheck: an easy-to-use computer program for checking genotypes for agreement with Hardy–Weinberg expectations. Mol. Ecol. Notes. Available from www.montana.edu/kalinowski/HW-Check/HW-Check_Home.htm [accessed 23 August 2007].

Krochmal, A., and Krochmal, C. 1982. Uncultivated nuts of the United States. USDA For. Serv. Agric. Info. Bull. 450.

Little, E.L. 1971. Atlas of United States trees. Vol. 1. Conifers and important hardwoods. USDA For. Serv. Misc. Publ. 1146.

Michler, C.H., Pijut, P.M., Jacobs, D.F., Meilan, R., Woeste, K.E., and Ostry, M.E. 2005. Improving disease resistance of butternut (*Juglans cinerea*), a threatened fine hardwood: a case for single tree selection through genetic improvement and deployment. Tree Physiol. **26**: 121–128.

Morin, R., Beaulieu, J., Deslauriers, M., Daoust, G., and Bousquet, J. 2000. Low genetic diversity at allozyme loci in *Juglans cinerea*. Can. J. Bot. **78**: 1238–1243. doi:10.1139/cjb-78-9-1238.

Muir, G., Lowe, A.J., Fleming, C.C., and Vogl, C. 2004. High nuclear genetic diversity, high levels of outcrossing and low differentiation among remnant populations of *Quercus petraea* at the margin of its range in Ireland. Ann. Bot. (Lond.), **93**: 691–697. doi:10.1093/aob/mch096. PMID:15087303.

Nair, V.M.G., Kostichka, C.J., and Kuntz, J.E. 1979. *Sirococcus clavigignenti-juglandacearum*: an undescribed species causing canker on butternut. Mycologia, **71**: 641–646. doi:10.2307/3759076.

Nei, M. 1977. *F*-statistics and analysis of gene diversity in subdivided populations. Ann. Hum. Genet. **41**: 225–233. doi:10.1111/j.1469-1809.1977.tb01918.x. PMID:596830.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, **76**: 379–390.

Nesom, G. 2005. Plant Guide: Butternut. USDA Natural Resources Conservation Service. Available from plants.usda.gov/plantguide/pdf/pg_juci.pdf [accessed 23 August 2007].

Nielsen, C., Cherry, M., Boysen, B., Hopkin, A., McLaughlin, J., and Beardmore, T. 2003. COSEWIC assessment and status report on the butternut (*Juglans cinerea*) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa, Ont.

Ostry, M.E. 1997. Butternut canker in North America 1967–1997. *In* Foliage, Shoots and Stems Diseases of Trees: Proceedings IUFRO Working Party 7.02.02 21, 26 May 2007, Sopron, Hungary. Inf. Rep. LAU-X-122.

Ostry, M.E., and Pijut, P.M. 2000. Butternut: an underused resource in North America. Horttechnology, **10**: 302–306.

Ostry, M.E., and Woeste, K.W. 2004. Spread of Butternut Canker in North America, Host Range, Evidence of Resistance within Butternut Populations and Conservation Genetics. *In* Black Walnut in a New Century, Proceedings of the 6th Walnut Council Research Symposium, Lafayette, Indiana, 25–28 July 2004. *Edited by* C.H. Michler, P.M. Pijut, J.W. van Sambeek, M.V. Coggeshall, J. Seifert, K. Woeste, R. Overton, and F. Ponder, Jr. USDA For. Serv. North Central Research Station, St. Paul, Minn. Gen Tech. Rep. NC 243. pp. 114–120.

Peattie, D.C. 1950. A natural history of trees of eastern and central North America. Houghton Mifflin, Boston, Mass.

Pons, O., and Petit, R.J. 1995. Estimation, variance and optimal sampling of gene diversity. I. Haploid locus. Theor. Appl. Genet. **90**: 462–470. doi:10.1007/BF00221991.

Raymond, M., and Rousset, F. 1995. GENEPOP (Version 1.2): population genetics software for exact tests and ecumenicism. J. Hered. **86**: 248–249. Available from genepop.curtin.edu.au/ [accessed 19 March 2008].

Renlund, D.W. 1971. Forest pest conditions in Wisconsin: annual report 1971. Wisconsin Department of Natural Resources, Madison, Wisc.

Rink, G. 1990. *Juglans cinerea* L. Butternut. *In* Silvics of North America. Vol. 2. Hardwoods. *Edited by* R.M. Burns and B.H. Honkala. USDA For. Serv. Agric. Handb. 654, Washington, D.C. pp. 386–390. Available from www.na.fs.fed.us/spfo/pubs/silvics_manual/volume_2/juglans/cinerea.htm [accessed 23 August 2007].

Robichaud, R. 2007. Pollen gene dispersal in black walnut across a heterogeneous landscape in Central Indiana. Ph.D. dissertation. Purdue University, West Lafayette, Ind.

Ross-Davis, A.L., and Woeste, K.E. 2007. Microsatellite markers for *Juglans cinerea* L., and their utility in other Juglandaceae species. Conserv. Genet. www.springerlink.com/content/0835125506p20482/ [accessed 23 August 2007].

Schultz, J. 2003. Conservation assessment for butternut or white walnut (*Juglans cinerea* L.). USDA For. Serv. Eastern Region, Milwaukee, Wisc.

Slatkin, M. 1985. Gene flow in natural populations. Annu. Rev. Ecol. Syst. **16**: 393–430.

Thompson, L.M., van Manen, F.T., Shlarbaum, S.E., and DePoy, M. 2006. A spatial modeling approach to identify potential butternut restoration sites in Mammoth Cave National Park. Restor. Ecol. **14**: 289–296. doi:10.1111/j.1526-100X.2006.00131.x.

Victory, E.R., Glaubitz, J.C., Rhodes, O.E., and Woeste, K.E. 2006. Genetic homogeneity in *Juglans nigra* (Juglandaceae) at nuclear microsatellites. Am. J. Bot. **93**: 118–126. doi:10.3732/ajb.93.1.118.

Wagenius, S., Lonsdorf, E., and Neuhauser, C. 2007. Patch aging and the *S*-Allee effect: Breeding system effects on the demographic response of plants to habitat fragmentation. Am. Nat. **169**: 383–397. doi:10.1086/511313.

Whitlock, M.C., and McCauley, D.E. 1999. Indirect measures of gene flow and migration: $F_{ST} \neq 1(4N_m + 1)$. Heredity, **82**: 117–125. doi:10.1038/sj.hdy.6884960. PMID:10098262.