

# Estimating heritability of disease resistance and factors that contribute to long-term survival in butternut (*Juglans cinerea* L.)

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**Abstract** For most wild species affected by exotic pests or pathogens, the relative importance of heritable genetic differences in determining apparent variation in disease resistance is unknown. This is true in particular for butternut, a North American hardwood affected by butternut canker disease and undergoing demographic contraction. Little is known about site effects on butternut decline, in part because long-term monitoring data are lacking. We collected detailed disease phenotypes and multilocus microsatellite genotypes for all surviving individuals in a large natural population of butternut in 2003 ( $n=302$ ) and 2012 ( $n=113$ ). Two analytical methods, correlations between pairwise phenotypic similarity and pairwise relatedness, and estimation of among-family variance, both indicated weak heritability of disease-related traits and no heritability for overall tree health in the population. Additionally, an analysis

of spatial data collected in 2001 ( $n=341$ ) and 2012 ( $n=113$ ) demonstrated that drier, upland sites contribute to increased likelihood of survival. We conclude that genetic differences among wild butternut individuals contributed little to observed variance in survival over 10 years but fine-scale site differences were useful predictors of butternut mortality.

**Keywords** *Ophiognomonia clavignenti-juglandacearum* · Butternut canker disease · Heritability · Forest genetics · Butternut

## Introduction

Contact between long-isolated congeneric trees in temperate East Asia, North America, and Europe has resulted in the spread of pests and diseases that have devastated naïve species (Simberloff 2000; Aukema et al. 2010; Santini et al. 2013). The emergence of new forest tree diseases (Brasier et al. 1999; Paoletti et al. 2005; Gross et al. 2014) indicates that introduced diseases will continue to threaten forest ecosystems. Affected species could be restored by breeding for disease resistance (Shurtleff 1980; Ingwell and Preisser 2010), but this strategy has sometimes proved impracticable. Breeding programs for American elm (*Ulmus americana* L.) affected by Dutch elm disease and American chestnut (*Castanea dentata* (Marsh.) Borkh.) affected by chestnut blight have mostly relied on interspecific crosses with resistant congeners because adequate resistance was not found within the afflicted species (Griffin et al. 1983; Smalley and Guries 1993; Jacobs 2005). On the other hand, American beech (*Fagus grandifolia* Ehrh.) appears to possess heritable resistance to the introduced scale insect that initiates beech bark disease (Koch et al. 2010), some North American pines (*Pinus* spp.) have simply inherited resistance to white pine blister rust (Devey et al.

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Michael E. Ostry is retired from his position at the Forest Service.

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1995; Kinloch 2003), and European ash (*Fraxinus excelsior* L.) shows genetic variability in resistance to ash dieback (McKinney et al. 2011). In other species, such as oaks (*Quercus* spp.) and tanoaks (*Lithocarpus* spp.) affected by sudden oak death (Hayden et al. 2011) and butternut (*Juglans cinerea* L.) affected by butternut canker disease, the potential for genetic disease resistance remains unclear.

Narrow-sense heritability ( $h^2$ ), the proportion of phenotypic variance explained by additive genetic variance (Holland et al. 2003), is typically estimated in tree breeding programs by planting several genetically similar groups of trees—half-sibs or full-sibs—and measuring a trait of interest (McKeand and Beineke 1980); in disease resistance breeding, the trait is reaction upon exposure to the pathogen (Carson and Carson 1989). Narrow-sense heritability is calculated as the additive genetic variance—e.g., four times the family variance for half-sibs divided by the overall phenotypic variance (Rink and Kung 1995; Lynch and Walsh 1998). Since molecular markers allow genetic relationships between individuals to be estimated in the absence of pedigree information (Queller and Goodnight 1989; Blouin et al. 1996), heritability can be estimated in wild populations (Ritland 1996, 2000; Blouin 2003; Coltman 2005). Ritland's heritability estimation method (1996) has been applied with limited success to populations of perennial herbaceous plants (Van Kleunen and Ritland 2005; Castellanos et al. 2011) and trees (Klaper et al. 2001; Andrew et al. 2005; Bessega et al. 2008). In this method, heritability is estimated as the slope of the regression of pairwise phenotypic similarity onto pairwise relatedness, or  $h^2 = C_{ZR}/2V_r$ , where  $C_{ZR}$  is the covariance of phenotypes and  $V_r$  the variance of relatedness. Wild populations of trees are a difficult case for this method (Klaper et al. 2001) because they typically contain mostly unrelated trees and low  $V_r$ . When adequate variance is present, estimates of heritability can be made (Andrew et al. 2005), and although their accuracy is low when compared with  $h^2$  estimates derived from known pedigrees (Rodríguez-Ramilo et al. 2007; Bessega et al. 2008), marker-derived estimates can generally discriminate between heritable and non-heritable traits (Coltman 2005).

Butternut is a hardwood tree in the walnut family (Juglandaceae) that formerly occurred in scattered populations throughout a large part of eastern North America (Rink 1990). A stem canker disease of butternut caused by the ascomycete fungus *Ophiognomonia clavigignenti-juglandacearum* (*Oc-j*) (= *Sirococcus clavigignenti-juglandacearum*) (Broders and Boland 2011) was first reported in the late 1960s (Renlund 1971), although it was most likely introduced decades prior in some areas (Broders et al. 2012, 2014). Cankers generally initiate in small crown branches, spreading to main limbs and the trunk (Nair et al. 1979; Ostry and Woeste 2004) and causing mortality either when cankers coalesce to girdle the stem or when secondary decay results in breakage or windfall. Incidence of canker disease is near 100 % in most of

butternut's range and mortality rates are as high as 90 % (Schultz 2003), but monitoring has revealed that a few trees in areas of high disease incidence have survived and remained vigorous or, in rare cases, largely free of cankers. Most of these survivors grow among other trees killed by canker disease, and some share an uncommon dark bark phenotype (Ostry and Woeste 2004; Ostry and Moore 2008). If there is a genetic basis for the survival of these trees, they could be collected and propagated to produce resistant breeding populations of butternut (Michler et al. 2005).

Efforts to estimate  $h^2$  for butternut canker resistance using traditional methods have met with mixed success (Ostry and Moore 2008; McKenna et al. 2011): differences in canker length have been observed between pairs of families, but not between putatively resistant and susceptible families. This result indicates that resistance may not be heritable in butternut, but the inoculation technique used in these studies also caused cankers on species that are not commonly observed with cankers in the wild; thus, the inoculations may have circumvented natural resistance mechanisms (Ostry and Moore 2008; McKenna et al. 2011). Molecular techniques offer a possible alternative to inoculation studies for the investigation of disease resistance in butternut. Butternut populations tend to have low levels of relatedness, but some genetic structure due to short seed dispersal distances and brief, rapid demographic increases under appropriate conditions (Hoban et al. 2014; Parks et al. 2014). Using marker-based techniques and genetic and phenotypic data collected in 2003 and 2012, we investigated the following questions: (1) Is disease resistance heritable in a wild population of butternut? (2) Does genetic background have an effect on its long-term survival?

Since phenotypic variation results from genetic and environmental variation, it is likely that local environment causes some of the differences in disease severity and survival observed among butternuts. In American beech (Latty et al. 2003) and North American five-needled pines (White et al. 2002; Dunlap 2012), site variation has been associated with variation in the effects of beech bark disease and white pine blister rust, respectively. If there are critical environmental effects on butternut canker disease morbidity and mortality, managers need this information to plan restoration plantings of the species. Using spatial and phenotypic datasets collected in the same stand in 2001 and 2013, we investigated whether differences in soil type and topography affected canker symptoms and survival in butternut.

## Materials and methods

### Study sites

The primary study population, known as Slocum's Woods (SLOW), occurs on private land in the Rock River watershed near Whitewater, WI. This population, observed by

researchers since the early 1990s, has been sampled as a part of many studies (Ross-Davis et al. 2008; Hoban et al. 2014) and is the source of several “resistant” accessions in butternut germplasm collections (McKenna et al. 2011). SLOW is a 15-ha mixed hardwood woodlot (UTM NAD83 Zone 16N: 351721 E 4743347 N). The butternut population on the site once numbered more than 500 individuals (Table 1), which are believed to have originated from a small number of mother trees. It has previously been established that introgression from Japanese walnut is not present in SLOW (Woeste, unpublished data), so variation in disease resistance is not due to past hybridization events. In 2012, 152 living and dead trees were present (Table 1). The site is mostly level, with a few areas of steep (15–25 %) but short (5–10 m) slopes. The lower, northern part of the site is made up of deep silt loams (Dresden and Colwood silt loams, Hebron loam; Fig. 1) overlaying lacustrine sand and a water table about 2 m below the surface, with ephemeral pools observed on several visits. The southern part of the site is more elevated, consisting of Whalan loam overlaying a limestone outcrop to depths of 1 to 2 m (Natural Resources Conservation Service Soil Survey Staff 2013). A smaller population (28 living and 10 dead butternut with many young trees) was discovered on the Kettle Moraine State Forest (KMSF) 16 km east of SLOW (UTM NAD83 Zone 16N: 362745 478739). This population was also phenotyped and genotyped but due to its small size was of limited use. A random sample of 55 Great Smoky Mountains National Park (GSMNP) butternuts (Parks et al. 2014) was genotyped as an out-group for relatedness and population genetic analyses.

### Field data collection

Butternut leaves were sampled for DNA extraction at SLOW in summer 2003 and May 2012 and at KMSF in September

2012. Leaves collected in 2003 were lyophilized and stored until genotyping in summer 2013. GPS points (UTM, NAD 1983) were recorded using a Garmin GPSMap 76 at SLOW in winter 2001, spring/summer 2012, and winter 2013 and at KMSF in September 2012 (Table 1). Locations were recorded multiple times to avoid inaccurate estimates due to dense summer canopy. To capture as much variation as possible in disease symptoms, several assessments of disease severity were performed using previously established criteria (Ostry et al. 1996; Parks et al. 2014) at the same time as leaf sampling in SLOW and KMSF. Tree health was assessed by counting total number of cankers below breast height (1.5 m), number of cankers within 10 cm of the soil line (basal cankers), estimating the percentage of main sun-exposed crown limbs killed by cankers, and visually estimating the percentage of the trunk circumference girdled by cankers. Canker severity and loss of crown vigor were rated using qualitative scales from 0 (no cankers, healthy crown) to 3 (rapidly expanding cankers, dead crown) with six total categories. Bark color was visually rated on a six-point scale with 1 being the lightest and 6 the darkest bark observed in SLOW, the population with the greatest bark color variability. Diameter (diameter at breast height (DBH)) and presence/absence of epicormic branching were recorded for each tree.

### Phenotypic data analysis

We determined that a synthetic disease severity index (DI) would be best suited to heritability analysis. We used principal components analysis (PCA) to identify the main axes of variability in the disease symptom data and derive an index (Muir and McCune 1987; Arbaugh et al. 1998). PCA was performed by standardizing all variables to mean=0 and standard deviation=1 and processing with PROC PRINCOMP in SAS 9.3 (SAS Institute Inc., Cary, NC, USA). The index value was calculated for individual trees by multiplying each standardized disease-related value by its weight in the first principal component and adding these values.

**Table 1** Summary of SLOW butternut samples

Sampling year	Population			Data recorded				
	Living trees	Dead trees	Found 2012 <sup>a</sup>	GPS locations	Disease index <sup>b</sup>	Bark color	Qualitative health assessment	DNA samples <sup>c</sup>
2001	341	200	141	X		X	X	
2003	302	n <sup>d</sup>	93			X	X	X
2012	113	39	n/a	X	X	X		X

“X” indicates a data type recorded in the indicated sampling year

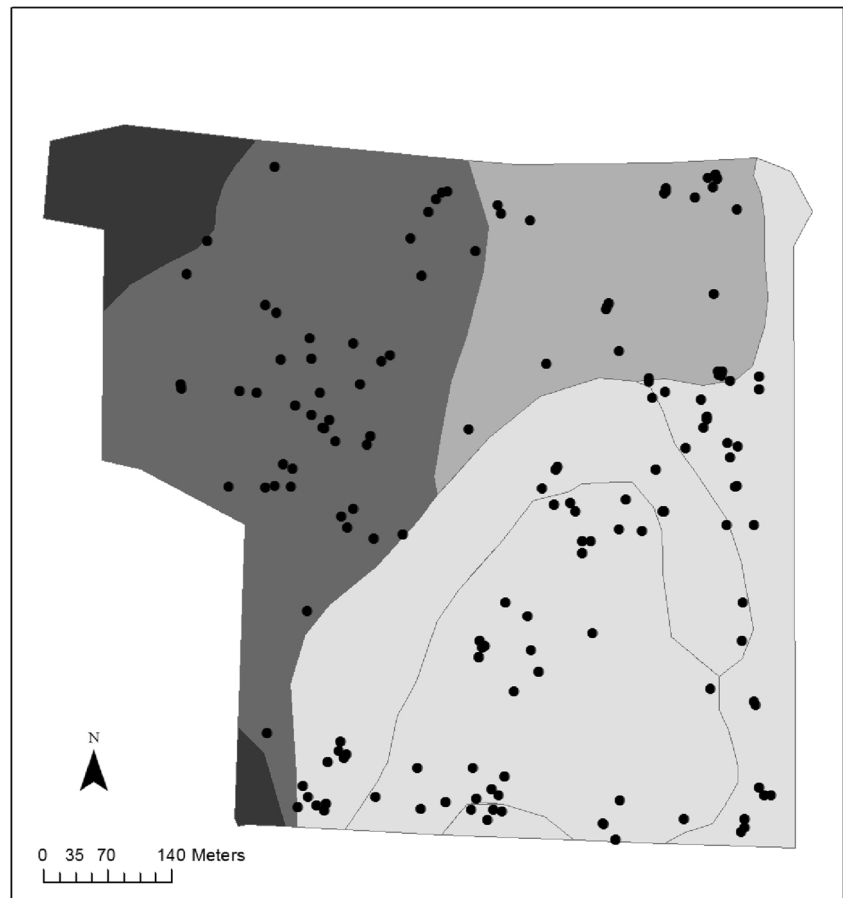
<sup>a</sup> Trees from the 2012 dataset identified in the older dataset by spatial proximity (2001) and genetic identity (2003)

<sup>b</sup> Disease index derived from PCA of six disease symptom measurements





<sup>c</sup> Number of trees sampled for DNA is equal to the number of living trees

<sup>d</sup> Dead trees were not included in the 2003 census

**Fig. 1** Map of SLOW showing major soil classes and locations of 152 living and dead butternuts sampled in 2012 (*black dots*)



#### Soil Type

	Dresden silt loam
	Hebron loam
	Whalan loam
	Colwood silt loam

## Genotyping

DNA was extracted from fresh leaves using a modified CTAB buffer and phenol-chloroform extraction protocol (Doyle and Doyle 1987) as described in Ross-Davis et al. (2008). DNA was extracted from lyophilized leaves (summer 2003 samples) using the same buffer and extraction method, but with an initial extraction step using phenol alone instead of 25:24:1 phenol:chloroform:isoamyl alcohol. Previously published simple sequence repeat (SSR) loci ( $n=17$ ) (Robichaud et al. 2006; Hoban et al. 2008; Ross-Davis et al. 2008), along with two new primer pairs generated using Roche 454 sequencing (Table A.1), were used to genotype individuals sampled in 2003. Two additional previously published markers (total  $n=21$ ) were used to genotype individuals sampled in 2012 (Table A.2). PCR methods were as described in Parks et al. (2014) for the

15 loci used in those studies. PCR conditions for the remaining six loci can be found in Table A.1. One negative control well was included on each sample plate, along with duplicate samples of three individuals as allele standards when genotyping samples collected in 2012. When samples collected in 2003 were genotyped, three individuals from the 2012 sample were used as standards. Genotypes were scored using Genemapper software v.3.7.1 (Applied Biosystems). Genotypes were checked for errors and null allele frequencies were estimated using Micro-Checker (van Oosterhout et al. 2004). Error rate was manually estimated by rescoring a random sample of genotypes and comparing with original scores. Allele frequencies and allelic richness were calculated in GenAlEx (Peakall and Smouse 2012), and allelic richness was subjected to rarefaction using HPRARE (Kalinowski 2005) to account for differences in population size.

## Genetic structure and relatedness

GenALEX was used to calculate Lynch and Ritland's (1999) estimate of pairwise relatedness ( $r_{ij}$ ). ML-Relate (Kalinowski et al. 2006) was also used to derive marker-based relatedness estimates. Geneland was used to identify groups of genetically similar trees in SLOW. The 2003 genetic dataset was submitted to Geneland with the following specifications: spatial model, false; null allele model, true; iterations, 50,000; and pop min=1 and pop max=30 for 10 independent runs. The spatial model was not used because most of the trees sampled in 2003 did not have spatial coordinates. For the 2012 dataset, settings were the same except pop min=1 and pop max=20, and spatial model=true. Individuals were assigned to a consensus group of relatives if they were assigned to the same cluster in five or more independent runs.

## Heritability analysis

Ritland's regression method of heritability estimation (Ritland 1996, 2000) was performed using the software SPAGeDi (Hardy and Vekemans 2002; 2007). We estimated heritability using a version of Ritland's method recommended by Thomas et al. (2000) in which a "pair's phenotypic similarity is regressed against their estimated relationship." Pairwise phenotypic similarity,  $u_{ij}$ , was calculated as

$$u_{ij} = (u_i - \hat{u})(u_j - \hat{u})/s^2(u)$$

where  $u_i$  and  $u_j$  was an individual phenotypic value (bark color and DI in this case) and  $\hat{u}$ , the estimated population mean, was the dependent variable (Ritland 1996). SPAGeDi was used to calculate Lynch and Ritland's relatedness estimator (Lynch and Ritland 1999) along with the estimators of Queller and Goodnight (1989) and Wang (2002) and true variance or relatedness.

Mantel tests, an alternative that does not depend on variance of relatedness, were performed to identify correlations between the pairwise matrices of  $r_{ij}$  (calculated using ML-Relate; Kalinowski et al. 2006 and GenALEX) and  $u_{ij}$  for all disease phenotypes collected using PASSaGE (Rosenberg and Anderson 2011). Mantel tests were also used to estimate correlations between spatial distance, relatedness, and disease-related traits of interest. Significance was determined by 999 permutations of the data.

One potential limitation of both the Ritland regression method and Mantel tests results from the inability of marker-based methods to identify distant relatives or even to accurately identify close relatives (Csillery et al. 2006). The most distant relationship marker-based methods can reliably identify, with 20 polymorphic markers, is half-sibling or equivalent ( $r_{ij} \sim 0.25$ ) (Blouin et al. 1996). Therefore, individuals more distantly related than half-sibling sharing identical-by-

descent alleles of large phenotypic effect would not contribute to the signal of heritability, since  $r_{ij}$  would be  $\sim 0$ .

We devised a method for relating genotypic and phenotypic similarity that relies on constructing "families" of close relatives for each individual and comparing phenotypic similarity within the families. For each individual, other trees from the population were counted as half-sibs if  $r_{ij}$  was above a threshold, using a custom Perl script to sort and extract individuals from the pairwise  $r$  matrix. We tested thresholds from 0.15 to 0.35 at intervals of 0.05. Individuals with fewer than two relatives were not included in the analysis. The R functions `aov` and `lmer` (from the `lme4` package) were used to determine the proportion of variance in bark color and disease resistance explained by differences among and within groups of related trees. This analysis did not fully satisfy the assumptions of general linear models because the data points were not strictly independent; some individuals contributed to many "half-sib families." Our analysis was intended to identify whether or not a signal of heritability might have been missed by analysis with the Ritland method, rather than estimating narrow-sense heritability per se. To test the method on a trait with established heritability, we derived marker-based half-sib families from a black walnut progeny test ( $n=325$ ; Zhao et al. 2013) genotyped for 12 microsatellites using the same methods described above and calculated the among- and within-family variance for 5-year height growth.

As a second method to look for among-family variance, the population genetic clustering program Geneland (Guillot et al. 2005, 2008) was used to group related trees as an alternative to using  $r$  values. Geneland was run for 30,000 MCMC iterations with 1000 burn-in, K set from 1 to 30, spatial model false, null allele model true. Results from 10 independent runs were used to cluster genotypes; if an individual was placed in the same cluster in 5 or more of the runs, it was included as a cluster member for analysis. Fifteen clusters, or families, were identified (modal K from runs ranged from 12 to 17); posterior probability of individual assignment to a cluster ranged from 0.18 to 0.26, and individuals not grouped in any cluster were deleted from analysis. We used a matrix of pairwise relatedness as the numerator relationship matrix in an unequal slopes random coefficient model using PROC GLIMMIX in SAS to estimate breeding values of families (the so-called animal model) (Blonk et al. 2010; Kruuk 2004). Soil type and the soil type times Geneland family group interaction were the covariates in the model. Family group was the random coefficient. We used the `type=Lin(1)` to read in the covariance matrix for the coancestry coefficients. Disease index was best modeled with a log-normal distribution and a log link function. We output the predicted values and used these to identify families significantly different than the overall mean for disease index.

## Site effects on mortality

GPS coordinates, DBH, health, and descriptive data collected in a 2001 survey of the SLOW population were used to identify trees that had survived from 2001 to 2012 (Ostry et al. unpublished data). Of the 152 individuals in the 2012 dataset, 141 were identified in the 2001 dataset: 101 of these were still alive (Table 1). To assess the impact of local environment on tree mortality, a soil map and UTM data were used to determine which trees were growing in the shallower soils on the southern part of the site and which trees were growing closer to the water table on the northern part of the site. Different microsites were delineated using a soil map (Natural Resources Conservation Service). Tree locations and soil classes were visualized in ArcMap 10 (ESRI 2011). Bark color was assessed as a binary variable in the 2001 study. Bark color (light/dark) and soil type (shallow over limestone/deep over sandy substrate) were used as predictors in a logistic regression (SAS: PROC LOGISTIC). Diameter (2001) was included as a quantitative covariate to account for the possible attrition of larger, older trees at the site.

## Results

### Disease phenotype assessment

At both the KMSF and SLOW sites, a wide range of disease phenotypes were observed: many trees were severely cankered and declining, many appeared vigorous despite obvious stem cankers, and a few were healthy and mostly free of cankers. At SLOW, DBH of butternut showed an approximately normal distribution with a mean of 32.14 cm and standard deviation of 10.87 cm. Number of cankers below DBH also showed a nearly normal distribution, while most other traits (limbs cankered, percent circumference cankered, number of basal cankers) showed moderately left-skewed distributions, with the mode occurring at a low severity and a long right tail of more severe symptoms. Bark color had a left-skewed distribution with 22 % more trees having light bark than dark. In the PCA, the first principal component (PC1) accounted for 50 % of the variance and the first and second together 73 % (Tables B.1 and B.2). PC1 was used as a disease index because it weighted most phenotype measures similarly, and high values of PC1 corresponded to the most diseased trees in the stand. The disease index values computed for individual trees followed a roughly bimodal distribution. Bark color was negatively correlated with disease index (individual trees with darker bark tended to be healthier, with lower DI scores) (Figure B.1):  $r=0.39$ ,  $p=0.001$ , as calculated using a Mantel test of the matrices of pairwise individual similarity of bark color and disease index (Table A.3).

## SSR genotyping and relatedness

SSR genotypes indicated high heterozygosity across all populations and loci. Allele numbers per locus ranged from 8 to 24, and heterozygosity for most loci was near 0.7, typical for outcrossing forest trees (Tables A.4–A.7). Allelic richness (with rarefaction) varied between the three genotyped populations, ranging from a mean of 6.5 across loci (KMSF) to 9.7 (GSMNP). Likely, null alleles were detected for six of 21 loci genotyped: estimates of their frequency ranged from 0.038 to 0.1297 (Tables A.4–A.7). These loci were not excluded from any analyses because at such low frequencies, they were not likely to bias parentage assignment or measures of differentiation (Oddou-Muratorio et al. 2009) and because both Geneland and ML-Relate accommodate null alleles. Including GSMNP butternuts as an out-group had a negligible effect on the mean level of  $r_{ij}$  estimated for SLOW (0.02 with GSMNP,  $-0.009$  without). KMSF had a much higher mean relatedness (0.125) because most of the young trees were the offspring of two large individuals.

## Heritability analysis

In SLOW, the actual variance of relatedness (Lynch and Ritland 1999) computed by SPAGeDi was not significantly different from zero. No inference could be made from the regression using this variance estimate.  $\text{Var}(r_{ij})$  computed using Wang's (2002) estimator and Queller and Goodnight's (1989) estimator were small but non-zero, based on standard errors of the estimates (Table 2). Estimates of the slope of the regression lines computed using these relatedness measures were non-significant for both disease index and bark color (Table 2).

In the 2012 SLOW population, significant but low correlations were found using Mantel tests between the matrices of pairwise relatedness (computed using ML-Relate) and pairwise similarity in number of basal cankers, number of total cankers below DBH, and the percentage of limbs cankered (Table 3). Relatedness and Euclidean distance (Table A.3) were more strongly correlated ( $r=-0.21$ ,  $p<0.01$ : Table A.3). Disease index similarity was not correlated with distance.

When marker-based half-sib families were derived for the 2003 and 2012 SLOW populations, the proportion of variance accounted for among groups was trivial compared to the variance within groups, even when the  $r_{ij}$  threshold was increased to only include closely related trees (Fig. 2). When the same technique was applied to black walnut height data, the proportion of among-family variance was higher (as expected) and close to estimates obtained when individuals were grouped in half-sib families based on shared mother tree. When mean phenotypic (disease index and bark color) similarity was

**Table 2** Estimates of true variance of relatedness (Ritland 1996) computed using SPAGeDi (Hardy and Vekemans 2007) and regression coefficients of bark color and disease resistance in SLOW butternuts sampled in 2012 ( $n=113$ )

Estimator	Variance of $r_{ij}$ (S.E.) <sup>a</sup>	Disease index $h^2$ (S.E.) <sup>b</sup>	Bark color $h^2$ (S.E.)
Queller and Goodnight (1989)	0.00527 (0.00231)	-0.0011 (0.00634)	-0.0052 (0.00743)
Lynch and Ritland (1999)	0.00065 (0.00285)	0.00330 (0.002)	0.00081 (0.00167)
Wang (2002)	0.00424 (0.00170)	0.00007 (0.01098)	0.00027 (0.0085)

<sup>a</sup> All standard errors computed by jackknifing over loci

<sup>b</sup> Slope estimate for the regression of pairwise phenotypic similarity on pairwise relatedness

calculated for all trees with  $r_{ij} > 0.20$ , it was not significantly different from 0.

We evaluated variance among 15 families identified by the software Geneland. “Family” means for disease index ranged from 9.46 (where 10=dead) to 5.45. A single family which is comprised of 20 individuals had a breeding value for DI significantly lower than the population mean ( $P > t = 0.0213$ ) based on best linear unbiased prediction (BLUP), when a log-normal distribution was used to model disease index. The fixed effect of soil was not a significant covariate ( $P > t = 0.446$ ).

**Spatial analysis of mortality**

The dataset from 2001 included 541 butternuts in SLOW. Of these, 200 were dead. In 2012, there were 113 living and 39

**Table 3** Mantel tests of relatedness matrices (Lynch and Ritland 1999; Kalinowski et al. 2006) and pairwise phenotypic similarity matrices for SLOW butternuts

Phenotype measure	Correlation	$p$ value <sup>†</sup>
Lynch and Ritland $r_{ij}$		
Disease index	0.01232	0.15
Bark color	0.00832	0.228
Number of basal cankers	0.02481	0.041*
Number of cankers below DBH	0.01669	0.13
Percent of trunk cankered <sup>a</sup>	0.00725	0.282
Percent scaffold limbs dead	0.03442	0.014*
Canker containment <sup>a</sup>	0.00297	0.397
Crown vigor <sup>a</sup>	0.00587	0.303
Epicormic branching	0.01133	0.186
ML-Relate $r_{ij}$		
Disease index	0.00684	0.268
Bark color	0.00247	0.388
Number of basal cankers	0.03371	0.006**
Number of cankers below DBH	0.02199	0.046*
Percent scaffold limbs dead	0.02822	0.028*
Epicormic branching	0.00832	0.24

<sup>a</sup> Mantel test not performed because results were far from significant in the first set of tests

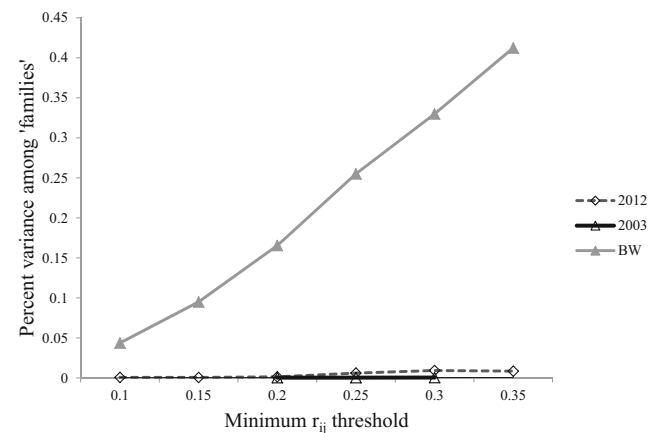
<sup>†</sup> Right-tailed  $p$  value (999 permutations); significance with \* $\alpha=0.05$ , \*\* $\alpha<0.01$

dead butternuts (Table C.1). Using GPS position, diameter, and other descriptors noted (number of trunks, metal tag numbers), 141 of 152 trees from 2012 were identified in the 2001 dataset. Mortality differed among trees on different soil classes and with different bark color scores. The number of trees without cankers declined from 90 in 2001, to 37 in 2003, to 1 in 2012 (Table C.2). Light- and dark-barked trees were randomly distributed with respect to soil type ( $\chi^2_1=0.82$ ). In the logistic regression, both dark bark and “upper” soil class were significant predictors of survival (Table 4), which was verified by Wald chi-squared scores (bark color,  $p=0.0013$ ; soil type,  $p=0.0023$ ). DBH was a non-significant predictor of survival.

**Discussion**

**Heritability analysis**

When  $r_{ij}$  was calculated for trees in the 2012 SLOW survey, we found the variance of relatedness was extremely low or indistinguishable from 0. This outcome was surprising, as



**Fig. 2** Proportion of among-group variance in disease severity (point estimates calculated using R function lmer) for marker-based half-sib families constructed using SSR loci in 2003 ( $n=264$ ) and 2012 ( $n=113$ ) SLOW butternut populations, and for 5-year height in a genotyped black walnut (BW) progeny test ( $n=325$ ). Families were constructed for each individual by including all individuals above an  $r_{ij}$  threshold derived from microsatellite genotypes, i.e., each individual’s closest relatives. In 2012 disease severity was estimated using a PCA-derived index of disease severity; in 2003 it was estimated using a three-point scale

**Table 4** Binomial logistic regression results modeling probability of survival 2001–2012 in SLOW butternuts as a function of soil type, bark color, and diameter

Coefficient	Estimate	Std. error	Z value	p value
Intercept	-1.4947	0.4025	-3.714	0.0002
Soil type <sup>a</sup>	0.8382	0.2614	3.206	0.0013
Bark color <sup>b</sup>	0.7804	0.2487	3.137	0.0017
DBH	-0.0086	0.0338	-0.254	0.7997

<sup>a</sup> Lower-elevation soils=0, higher-elevation soils=1

<sup>b</sup> Light bark=0, dark bark=1

accounts of the population's origin suggested a small number of founders. Our data were consistent with 10–15 founder trees. Only about 5 % of  $r_{ij}$  values were greater than 0.2, a value which indicates half-sib or closer relationships. According to Ritland (1996), the lower bound for non-zero variance of relatedness is near 13 % half-sibs and 87 % unrelated in a population. The variance of relatedness we found using Wang's (2002) and Queller and Goodnight's (1989)  $r_{ij}$  estimators was small but within the range seen in other plant studies (Castellanos et al. 2011). The lack of significant variance of relatedness in natural populations has constrained applications of Ritland's method (Klaper et al. 2001; Rodriguez-Ramilo et al. 2007), and, even when estimation of heritability is possible, the results are often not accurate (Thomas et al. 2002; Coltman 2005; Bouvet et al. 2008) but can provide a general signal of heritability (Bessega et al. 2008). Bouvet et al. (2008) used Wang's  $r_{ij}$  estimator alongside Lynch and Ritland's to estimate heritability in shea tree (*Vitellaria paradoxa*) and found estimates of  $\text{var}(r_{ij})$  were higher with the former, as was also the case among the SLOW butternuts. The results of the regression analysis did not support the hypothesis that bark color and disease resistance are heritable in wild populations of butternut.

Correlations between genotypic and phenotypic similarity computed by Mantel tests do not allow any inference on the actual value of heritability but can indicate whether or not there is any genetic basis for a trait (Bessega et al. 2008; Barbour et al. 2009). When among-family variance was calculated using  $r_{ij}$ -derived families in SLOW, almost all the variation in disease index and bark color was within groups of relatives, rather than among groups: the test dataset of black walnut height data showed a signal of heritability (increasing among-family variance as  $r_{ij}$  threshold increased) despite being based on fewer marker loci and accordingly less-accurate estimates of relatedness.

Although there was apparently no genetic basis for differences in bark color or overall disease index in SLOW or KMSF, percent crown limbs dead, number of cankers below DBH, and number of basal cankers showed low but significant correlations with relatedness. *Oc-j* infections start in the

upper crown on the thin bark of upper twigs and small branches. From there, they spread via conidia to the main branches and then to the bole (Tisserat and Kuntz 1984). The significant correlations we observed for canker-related traits may indicate there is a genetic basis for resistance to canker initiation on the main branches and bole, but not for the ability of individual trees to maintain vigor under high disease pressure, which the disease index was intended to measure. The PCA-derived disease index and the individual measures used to construct it were all life-history traits, which tend to have lower heritability than morphological or physiological traits, partly because they are more difficult to accurately measure but mainly because environmental variance can overwhelm limited genetic variance for life-history traits (Lynch and Walsh 1998). Only additive genetic variance contributes to narrow-sense heritability, so it is possible that non-additive variance or genotype-environment interactions contributed to the variation in disease resistance observed at SLOW, but these potential sources of variation would not be useful in a butternut breeding program, which would rely on selection of progeny from open-pollinated seed orchards.

The computed breeding values for DI/survival among the SLOW butternuts were close to zero, which is consistent with the other heritability analyses. Soil was not a good predictor of the DI/survival phenotype, but this was probably because there were many missing values for this variable (no GPS points were included in the 2003 data). The one genetic cluster with a breeding value different from zero consisted of a group of related trees that occurred entirely on steep slopes and elevated, rocky soils on the southern edge of the site. It seems likely that the significant value for this family was due more to its unusual and favorable location (and thus a genotype-environment interaction that was confounded with genotypic effects in the heritability estimate) than any additive genetic variance for canker resistance. In the small KMSF population, young trees ( $n=8$ ) in an open area were uniformly healthy despite the large difference in the health (DI=7.52 vs. 1.172) of their most likely mother trees as determined by Cervus (Kalinowski et al. 2007) (Table A.9).

Darker bark color was significantly associated with longer survival and reduced severity of disease symptoms, but no significant correlation between bark color and relatedness was observed. Heritability has been successfully estimated for defensive traits of wild trees using similar techniques (Andrew et al. 2005). Dark-barked trees occur throughout the range of butternut, but the phenotype is poorly understood and it has no clear association with environmental factors or stem diameter. Given its close correspondence with apparent tree health and long-term survival, it appears that bark color has an important association with disease resistance regardless of its non-heritable phenotype in this study. Because *Oc-j* spores initiate infections through wounds in bark, it is possible dark bark trees present elevated levels of defensive



compounds (Ostry and Moore 2013) or novel defensive compounds not found in more common light-barked trees. It is possible that bark color is a polygenic trait with heritability too low to be detected by marker-based methods—the result of interactions among many individual bark compounds and physical properties such as thickness—or that it arises as a result of epigenetic or yet-unidentified environmental factors.

The methods used to estimate the heritability in this study are imprecise, but we were interested in detecting a *signal* of heritability, which these methods are capable of, rather than an estimate. Our conclusion—that the heritability of butternut canker disease resistance in wild butternut is negligible and of little value to breeders—arises not only from these results but also from a lack of experimental evidence that resistant butternut parents can transmit resistance to their offspring, or even to grafted clones. Of course, limited inference can be drawn from the characteristics of a single population, but SLOW is an important source of resistant breeding material in the northern part of butternut's range and the long-term monitoring data collected there are unique.

### Spatial effects on survival

The butternut population of SLOW declined more than 60 % between 2001 and 2012. Butternut is short-lived for a hardwood tree, with a lifespan of less than 100 years, so it is possible that some attrition was due to age. The non-significant logistic regression coefficient for DBH as a predictor of mortality, where DBH was a proxy for age, indicated that age-related mortality was not likely an important contributor to the population collapse. Butternut canker disease was probably not the only factor explaining the precipitous decline in numbers at SLOW. In the 2001 survey, 147 trees, 27 % of the total population, were listed as “broken.” This breakage may have been due to a severe thunderstorm with 90–110-mph winds that struck Rock County, WI, where Slocum's Woods is located, in the summer of 2000 (National Weather Service 2011). Butternut has brittle wood and is generally vulnerable to wind damage (Schultz 2003). In addition, butternut canker could predispose butternut to mortality from natural disturbances, as has been observed in populations of American beech affected by beech bark disease (Papaik et al. 2005).

In addition to increasing the susceptibility of trees to wind damage, butternut canker disease can increase vulnerability to common forest diseases and decay fungi, such as *Armillaria* root rot, which can cause decline, extensive basal decay, and premature windthrow (Williams et al. 1986). *Armillaria* is commonly observed on trees affected by butternut canker disease (Schultz 2003; Schmalz and Bergdahl 2006) and was observed at SLOW. Because *Armillaria* grows most successfully in moist, sandy soils (Morrison 1976; Rizzo and Whiting 1998), it could cause more extensive damage to cankered

butternut on the sandier, lower, wetter soils at SLOW. Butternut plantings sited on higher, drier sites in Indiana tend to have considerably lower rates of natural canker infection than those on nearby, lower, moister sites (McKenna et al. 2013). Microsite variation seems to be a factor in butternut canker disease progression, as it is in American beech affected by beech bark disease (Latty et al. 2003). Despite the clear difference in mortality between the two microsites (dry, shallow, and deep, moist soils) at SLOW, there was no significant difference in disease index in 2012 between trees growing on the two main soil classes, but a significant difference was observed in the number of basal cankers, the disease symptom most likely to cause mortality in butternut. Furthermore, canker prevalence tended to be higher in 2001 on wetter soils (81 % had cankers) than on drier parts of the site (62 %), while more trees were classified as suffering from dieback on the lower parts of the site (22 vs. 18 %). This indicates that disease progression was slower on the upper parts of the site, even if today all the trees have some cankers.

Dark bark was a predictor of survival in the spatial longitudinal analysis; it was significant in the genetic longitudinal analysis as well, but was not a meaningful predictor when included with the mean survival of a tree's closest relatives. The value of bark color as a predictor may have been obscured by the scoring of bark color in 2003 as light, moderate, and dark instead of a simple light/dark dichotomy used in 2001. The simpler light/dark dichotomy seems to capture most of the difference in disease resistance between bark types.

### Conclusions

The results of this study contribute to a body of evidence that indicates, despite the apparent health of individual butternut trees in disease-affected populations, there is little genetic basis for resistance to canker disease in the species. It is likely that there is some heritable variation in disease morbidity and mortality, but it is equally unlikely that this variation is great enough to form the basis of a tree improvement program with only pure butternut as a source of disease resistance. Hybrids with Japanese walnut, on the other hand, appear to be more resistant to canker disease (McKenna et al. 2011), and they could form the basis for a hybrid breeding program in the mold of the American Chestnut foundation's hybrid disease resistance development in American chestnut (Michler et al. 2005). Bark color appears to be an indicator of disease resistance but one with very low heritability. The biochemical bases and the effects of bark compounds on growth of *Oc-j* are important research questions currently under investigation (Ostry and Moore 2013). Diseased butternut adults can give rise to healthy, vigorous offspring if proper conditions for regeneration are present, and butternut is more likely to survive on upland sites. For butternut management in the

immediate future, this implies that plantings of butternut on open, well-drained sites may be a viable strategy for preserving the species as established natural populations in closed-canopy forests continue to decline. The problems of butternut canker disease and a lack of suitable sites for regeneration near surviving adults are nearly equal threats to the survival of this valuable North American hardwood species.

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**Data archiving statement** Microsatellite genotypes for SLOW 2003, SLOW 2012, KMSF, and GSMNP butternuts are archived at TreeGenes under accession number TGDR046. The two microsatellite loci used in this project and not previously published (JC092 and JC242) are archived at GenBank under the accession codes KR119067 and KR119068, respectively. Canker disease phenotypes and GPS coordinates of trees may be requested for scientific purposes from the corresponding author.

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