

Heritability of Phenolics in *Quercus laevis* Inferred Using Molecular Markers

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Studies of quantitative inheritance of phenotypes do not generally encompass the range of environmental conditions to which a population may be exposed in a natural setting and are rarely conducted on long-lived species due to the time required for traditional crossing experiments. We used a marker-based method to estimate relatedness with microsatellite markers in a natural population of a long-lived oak, then used this inferred relatedness to examine quantitative genetic variation in the concentration of foliar phenolics. Estimating heritability using this method requires both significant relatedness and variance in relatedness over distance. However, this population did not show significant variance of relatedness, so only the presence of heritability, and its ranking among traits and environments, could be estimated. Seven foliar phenolics showed a significant relationship between phenotypic similarity and relatedness. The significance of this relationship varied among individual phenolic compounds, as well as by season. Genetic factors appeared to have a more measurable influence on the production of secondary compounds early in the season. After leaf expansion, covariance of relatedness and phenotypic variance appear to become less significant. Therefore heritability may vary seasonally for these traits.

The interaction of genetics and environment is important for determining plant resistance to herbivores (Fritz 1990; Madrox and Cappuccino 1986). Several studies have examined the effects of genetic and environmental variability on defensive characters of plants such as secondary compounds (Oluwatosin 1999; Zangerl and Berenbaum 1990), and physical structures of the plant or leaf (Van Dam et al. 1999), but these studies have been primarily of agricultural or annual, short-lived species that are easily manipulated. In these studies the additive genetic variance, the portion of variance that may be acted on by selection, is calculated by crossing individuals within a common garden and measuring the phenotypic covariance between offspring and parents (Falconer and Mackay 1996). Calculating heritability in this manner is difficult for long-lived organisms that do not reproduce regularly and where the phenotype of interest may not develop fully for many years. In addition, heritability can also change with environment. Since natural populations exist within variable environments, it is necessary to estimate heritability within natural landscapes to under-

stand the potential for natural selection to operate on phenotypic traits.

Natural populations harbor significant genetic variation for resistance characters (Zangerl and Berenbaum 1990). However, additive genetic variance for such defensive traits has not been estimated for long-lived species under natural field conditions. Instead, studies are conducted in common gardens which may not simulate the natural environment. Since the expression of defensive factors depends on the environment that the plant experiences (Maddox and Cappuccino 1986), realistic field conditions are desirable when measuring heritabilities for these traits.

Molecular markers provide new tools for ecological and genetic studies of evolutionary processes (Cruzan 1998). Allozymes have been used for many years to describe differences in genetic structure among individuals and within and among populations (Berg and Hamrick 1995; Hamrick et al. 1989). Newer markers such as microsatellites and random amplified polymorphic DNA (RAPD) provide more detailed genetic information due to either the increased variability of loci or the greater numbers of loci available (Ashley and Dow 1994; Cruzan 1998). These mark-

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ers have been used successfully to estimate levels of relatedness among individuals for paternity exclusion (Dow and Ashley 1998), studies of mating systems, and studies of seed dispersal and seedling establishment in natural populations (Dow and Ashley 1996).

Ritland (1996a) developed a new use of molecular markers for estimating natural heritabilities. In this method, relatedness among individuals in natural populations is estimated and then correlated with phenotypic similarities, ultimately to estimate heritability for quantitative traits. This method has been used successfully to calculate the heritability of floral traits in natural monkey flower populations (Ritland and Ritland 1996) and to measure heritability of mating behavior in chinook salmon (Mousseau et al. 1998).

In this article we used this method to determine the relationship between genetic relatedness and the production of several phenolic compounds in the foliage of turkey oak (*Quercus laevis*). Phenolic compounds influence both the preference and performance of insect herbivores (Dudt and Shure 1994; Roth et al. 1997) and are affected by both genetic (Lindroth and Hwang 1996) and environmental factors (Bryant et al. 1987; Horner et al. 1993; Shure et al. 1998). Yet there is debate over which of these factors causes the greatest proportion of variation and ultimately whether genetic variation and heritability of these compounds are important in natural populations. Production of these phenolics has been shown to have a strong genetic component in other plant species (Desphande and Campbell 1992; Mueller-Harvey and Dhanoa 1991; Nurmi et al. 1996; Orians and Fritz 1995) and their heritability has been calculated in common garden experiments for several crop species (Grayer et al. 1992; Oluwatosin 1999; Vaillancourt et al. 1986). In oaks, budburst phenology has also been shown to influence the susceptibility of individual trees to herbivore attack (Hunter 1992). We applied Ritland's model to investigate the heritability of several phenolic compounds that have been found to vary between individuals within a natural population of turkey oak at the Savannah River Site near Aiken, South Carolina (Klaper and Hunter 1997) using both microsatellite and allozyme markers to determine relatedness.

Materials and Methods

Study Population

We chose 200 *Q. laevis* trees from a naturally occurring turkey oak population at

the Savannah River Site near Aiken, South Carolina. *Q. laevis* is an endemic long-lived species found on sandy upland soils in the southeastern United States (Christensen 1988). Trees reproduce clonally by several new ramets sprouting from stumps, and sexually through wind pollination and acorns. Therefore a population may contain several clones of the same genotype, in close proximity, as well as many genotypes from sexual efforts, creating a population of varying degrees of genetic relatedness. Berg and Hamrick (1993, 1995) mapped 4000 trees from this 160m × 160 m site on an *x,y* grid. The *x*-axis is approximately the east-west direction of the plot and the *y*-axis is the north-south direction. Trees were also genotyped by Berg and Hamrick using nine polymorphic allozyme markers. We randomly chose 10 trees each of 20 "genotypes" (defined as identical for 9 polymorphic allozyme markers) to obtain a subsample of the population with varying levels of relatedness at different distances. Trees chosen from each genotype were scattered across the plot and did not occur more than two to a clump.

Microsatellite Genotypes

We further characterized the genotypes of these trees using five polymorphic microsatellite markers [SSRQ8 and SSRQ16 from *Q. macrocarpa* (Dow and Ashley 1996; Dow et al. 1995) and Qzag 9, Qzag 15, and Qzag 110 from *Q. petraea* (Steinkellner et al. 1997)] to increase the accuracy of our estimates of relatedness. Leaves were collected from each tree in April 1998 and flash frozen in liquid nitrogen in the field. Samples were placed in a -80°C freezer until analysis. A small sample of leaf material (approximately 0.1 g) was ground with mortar and pestle in liquid nitrogen and DNA was extracted using a Qiagen DNeasy plant mini kit (Qiagen Inc., Santa Clarita, CA). Recovery was fairly low (1–20 ng/μl) but allowed for clean amplification. DNA was amplified using a 7.5 μl polymerase chain reaction (PCR) mix that included 10 μM fluorescently labeled primers, 2.5 μM dNTPs, 0.3 U Amplitaq Gold, 2 ng DNA, and 25 mM MgCl₂. We were able to multiplex all three Qzag primers using TET to label Qzag 15 and Qzag 110 and FAM to label Qzag 9. SSRQ8 and 16 (labeled with FAM and HEX, respectively) were amplified separately and then run within the same lane on the acrylamide gel. PCR amplification for all but SSRQ16 consisted of a 12-min denaturation/activation step for the Amplitaq Gold (95°C), then 30 cycles

of 1 min denaturation (92°C), 1 min annealing (55°C), and 30 sec extension (72°C), finishing with a 7 min final extension period (72°C). SSRQ16 required 38 cycles with a 59°C annealing temperature. Samples were run on a 12 cm, 4.2% acrylamide gel solution with an ABI 377 DNA sequencer for 1.5 h. Fragments were analyzed by size using a Perkin-Elmer Genescan 500 TAMRA size standard and GeneScan ABI 377 software (Perkin-Elmer, Foster City, CA). Fragments from the various primers ranged from 100 to 270 bp with a total of 72 alleles for all primers combined.

Relatedness and Heritability

In Ritland's (1996a) approach for estimating heritability, relatedness (*r*) between pairs of trees is first estimated via a weighted method of moments estimator. Pairwise relatedness is defined as the average frequency when two homologous alleles from two separate individuals are identical by descent. This estimator can be described with the Kronecker operator δ as follows (see Ritland 2000). At a single locus, let the first individual have alleles A_i and A_j and the second A_k and A_l . If alleles A_i and A_k are the same (e.g., the same band or sequence), then $\delta_{ik} = 1$, while if different, $\delta_{ik} = 0$, etc. Noting that there are four pairwise comparisons, the estimator of pairwise relatedness is

$$\hat{r} = \frac{[(\delta_{ik} + \delta_{il})/p_i] + [(\delta_{jk} + \delta_{jl})/p_j] - 1}{4(n - 1)} \quad (1)$$

where *n* is the number of alleles at the locus and p_i is the estimated frequency of allele A_i . An efficient multilocus estimate is the sum of locus-specific estimates, each weighted proportionally by $(n - 1)$. Now, to estimate heritability, we first define a "phenotypic similarity," $Z = (Y - u)(Y' - u)/V$, where *u* and *V* are the phenotypic mean and variance, and the *Y*s are the trait measures on each individual. Heritability is then estimated as the covariance of *Z* with relatedness *r* (C_{Zr}), divided by the actual variance of relatedness (V_r) estimated across all pairs of individuals:

$$\hat{h}^2 = \frac{C_{Zr}}{2V_r} \quad (2)$$

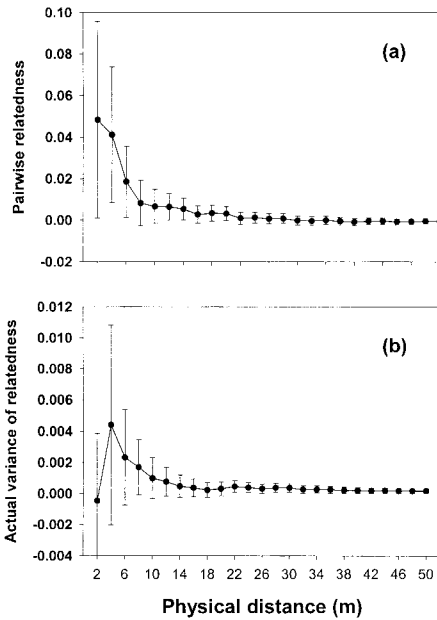


Figure 1. (a) Mean pairwise relatedness and (b) mean actual variance of relatedness (estimator) in a natural population of *Quercus laevis* (amounts are means within given distances, not means at given distances). Standard errors, based on the bootstrap method, are indicated by vertical bars.

Briefly the estimated actual variance of relatedness is calculated as

$$\text{var}(r_{ij}) = \text{avg} \left[\frac{\left(\sum_k w_k \hat{r}_{ij,k} \right)^2 - \sum_k w_k^2 \hat{r}_{ij,k}^2}{1 - \sum_k w_k^2} \right] - \hat{r}^2. \quad (3)$$

In this equation, r_{ij} is the relatedness estimate for pair ij , which is calculated at each locus k , and w_k is the weight used at each locus equal to the expected heterozygosity for locus k . The variance is the average over all pairs and \hat{r} is the average pairwise relatedness in the population. This estimation of the actual variance of relatedness is described in more detail in Ritland (1996a) and Ritland (2000), and requires at least two marker loci.

We calculated relatedness between pairs of trees using both allozyme and microsatellite markers. To determine the spatial scale of genetic structure we plotted relatedness versus distance, and of the actual variance of relatedness versus distance (Figure 1). In this figure, mean pairwise relatedness *within* a given distance was found, as opposed to mean pairwise relatedness *at* a given distance. The number of pairwise comparisons, and the consequential product of the number of individuals times each of the two related-

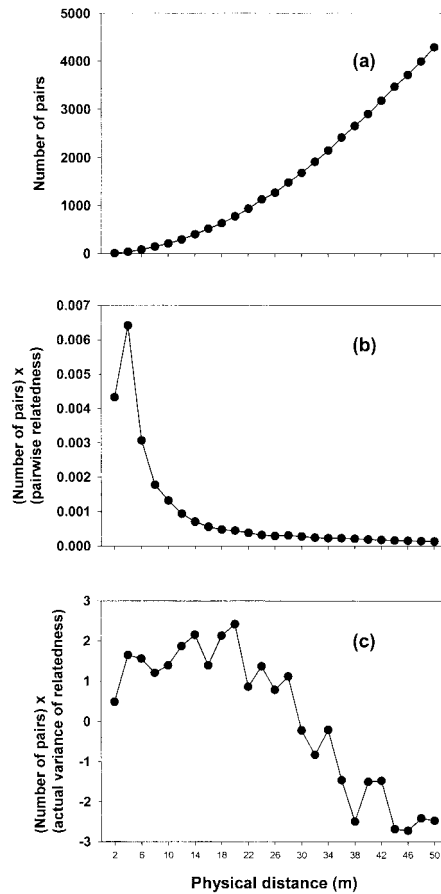


Figure 2. (a) The number of pairs included in the analyses for a given distance, (b) the number of pairs times the mean pairwise relatedness, and (c) the number of pairs times the mean actual variance of relatedness. The latter two plots indicate an optimal distance of approximately 20–25 m, within which all pairwise comparisons are made for heritability estimation.

ness quantities, was also plotted as a function of distance (Figure 2). Relatedness declines with physical distance, and the number of pairwise comparisons in relation to relatedness is greatest at a distance of 20–25 m. We restricted comparisons of relatedness and phenotypic similarity to distances within 20–25 m to increase the number of paired comparisons while choosing a sample distance with relatively large relatedness values (see Ritland 1996b; Ritland and Ritland 1996).

We then used the above method to infer the quantitative inheritance of phenolics measured in foliar tissue samples collected in April and May 1997. Errors of estimates were determined by resampling the data 100 times in a bootstrapping procedure, wherein individuals were resampled (pairwise comparisons between identical individuals from the original dataset were

omitted). For a full review of bootstrapping see Manly (1998), and for this procedure in reference to calculating errors for relatedness see Ritland (2000).

Measurements of Oak Phenotype

In 1997 a total of 15 leaves were haphazardly removed from throughout the canopy of each sample tree. A 1 cm diameter sample was taken from each leaf and placed immediately into 100% methanol in the field. Oak leaf chemistry changes rapidly during leaf expansion (Feeny 1970; Schultz et al. 1982), so we sampled in both April (during expansion) and May (at the end of expansion). An internal standard, chrysin, was added to each sample and phenolic compounds were separated and quantified by high-performance liquid chromatography (HPLC) using a C-18 reverse-phase Sephadex column with a 47-min cycle and a 10 to 100% methanol gradient. Twenty different phenolic compounds were separated and identified using known standards and ultraviolet (UV)-spectra, and 13 of these showed significant variation among individual trees. Compounds fell into six different categories: quercetin glycosides, p-coumarylquercetin glycosides, p-coumarylkaempferol glycosides, kaempferol-3-rutinocide, and three unknowns, possibly p-coumaric isoflavones (Table 1).

On April 8, 1997, during leaf expansion, we also measured the lengths of 10 buds per tree distributed across at least three branches using a vernier caliper. This single-point estimate has proven to be a reliable estimator of oak budburst phenology in previous studies (e.g., Hunter 1992).

Results

Relatedness among trees was low and declined sharply from 0–15 m, dropping to zero by 30 m (Figure 1a). Variance in relatedness was also low and declined over a similar distance (Figure 1b). Standard errors are indicated by the vertical bars in the figure; although these often overlapped with zero, the regression of relatedness upon distance within the 0–25 m range was significantly negative (results not shown). Between 20 and 30 m the number of pairs of trees for comparison was relatively high (Figure 2a), and together with the relatedness estimates (Figure 2b,c), all pairs within 20–25 m seemed the optimal region to calculate the covariance (C_{zr}) between relatedness (r) and phenotypic similarity (Z) (Table 1).

To calculate heritability using Equation

Table 1. Number of positive covariances from 100 bootstraps of relatedness values and phenotypic variance using Ritland (1996)

Date	Trait	Positive covariances 25 m	Positive covariances 20 m
April 1997	Budburst	36	23
	Quercetin glycoside-1	95	85
	Paracoumaric isoflavone-1	73	79
	Paracoumaric isoflavone-2	76	47
	Unknown 1	74	63
	Unknown 2	57	45
	Unknown 3	44	35
	Kaempferol 3-glycoside	85	76
	Quercetin glycoside-4	8	6
	May 1997	Quercetin glycoside-1	52
Paracoumaric isoflavone-1		51	51
Paracoumaric isoflavone-2		56	59
Unknown 1		66	68
Kaempferol-3-rutinocide		38	39
Quercetin glycoside-4		62	68
Paracoumaryl quercetin glycoside-2		55	33
Paracoumaryl kaempferol glycoside-5		48	40
Paracoumaryl kaempferol glycoside-6		76	70
Paracoumaryl kaempferol glycoside-7		85	83

Covariance was calculated at both 20 m and 25 m to get an estimate of differences at these two cutoffs. The greater the distance, the more pairs were included in the procedure. Calculations were done for two sampling dates.

2, the population sample in question must show significant relatedness and variance in relatedness. Our mean relatedness from 100 bootstraps for pairs at 25 m was 0.000485 (SD = 0.00411) and the mean variance in relatedness 0.000703 (SD = 0.000912). Since relatedness of sibs is 0.125 and of first cousins is 0.052, individuals in our sample showed very low relatedness and insignificant variance of relatedness. Variance of relatedness values of zero lead to an inoperable function in Equation 2. Therefore we were not able to do a complete heritability calculation. Instead, we examined the number of positive C_{zr} values for 100 bootstraps. In general, if 90% of the bootstraps give a positive covariance value then the trait is most probably heritable by additive genetic effects ($P = .10$). If 70% of bootstraps give a positive covariance value, the trait has a fairly high chance of being heritable.

Several compounds have positive covariances at the 70% significance level or above and the number of positive covariances generally increased from the 20–25 m distance class (Table 1). Five of eight phenolic compounds identified in April (quercetin glycoside-1, paracoumaryl isoflavone-1 and -2, kaempferol 3-glycoside, and one unknown phenolic) exhibited a 70% significance level for the number of positive covariances under bootstrapping. Quercetin glycoside-1 in April appeared to have a strong additive genetic component and was the only phenolic to reach a 95% significance level.

Season of measurement appeared to affect covariance values significantly and would be a factor to consider in future heritability calculations. With the exception of quercetin glycoside-4, additive genetic effects appeared to be stronger in samples that were taken during leaf expansion (April). Most of the samples in May, with the exception of paracoumaryl kaempferol glycosides-6 and -7, had less than 70% positive covariance values.

Similarity in chemical structure did not provide a reliable predictor of similarity in genetic influence. Compounds that differed only in their glycoside component were no more likely to have a similar covariance value than those that differed by the major phenolic component of the molecule. For example, May paracoumaryl kaempferol glycoside-7 and May paracoumaryl kaempferol glycoside-2 have 85 and 55 positive bootstrap values, respectively, despite the fact that their structure differs only by the glycoside or the sugar attached to the molecule (Table 1). Unlike seven of the phenolic characters, budburst phenology, did not appear to express significant additive genetic variance.

Discussion

This is the first study to investigate the heritability of the production of plant secondary compounds in a natural population using marker-based estimates of relatedness. Although we were unable to calculate heritability per se, we were able

to determine that 7 of the 13 phenolic compounds are likely to express additive genetic variation. Five of eight phenolic compounds in April and two of eight phenolics in May reached a 70% significance level for the number of positive covariances under bootstrapping. April quercetin glycoside-1 was the only compound to reach a 95% significance level for either sampling period.

Similarity of compound structure does not necessarily predict similarity in genetic variance of production. Quercetin glycosides in both April and May samples and May paracoumaryl kaempferol glycosides show large differences in the proportion of positive covariances. Orians et al. (1996), in a standard quantitative genetics progeny study of willows, also found differences in heritability values of phenolics that varied in the addition of side groups. In this study, salicortin had a heritability value of 0.20 versus 0.59 for 2'-cinnamoylsalicortin.

The data we have obtained also indicate that the strength of additive genetic effects may change over the period from budburst to leaf expansion (Table 1). Oak leaf phenolics change rapidly over this time period, therefore measurements of these compounds are greatly influenced by time since budburst or the phenology of the tree (Feeny 1970; Schultz et al. 1982). This study, as well as a previous study by Klaper (2000), indicate that budburst does not appear to be significantly influenced by additive genetic effects but by environmental variables. In the current study, budlength accumulated only 36 positive covariance values (Table 1) and Klaper (2000) found that the budburst phenology of *Q. laevis* varied at a much larger spatial scale than genetic relatedness. Although the environmental factor that drives variation in *Q. laevis* budburst phenology has yet to be established (Klaper 2000), the strong link between season and foliar chemistry suggests that heritability estimates for secondary compounds using this method or other methods should take season of measurement into consideration.

Our analyses indicate that experimental design is critical to the application of Ritland's model to plant phenotype. In our experiment, we chose trees based on previous allozyme information that indicated that highly related individuals were scattered across our 160 m × 160 m plot. Turkey oak reproduces both clonally by stump and root sprouts, and sexually via flowers. If we had sampled individuals in

a more clumped design, choosing two closely related and two other individuals growing in proximity to one another in patches around the plot, we would have had a greater chance of (a) obtaining a greater range of relatedness and (b) including more variation in relatedness over distance. This in turn would have allowed us to calculate heritability (Ritland 1996). Sampling a larger number of individuals also provides greater power for statistical analyses by increasing the chance of detecting heritabilities (Mitchell-Olds and Bergelson 1990). Our simple analysis is also lacking estimates of maternal effects (Rossiter 1998), gene by environment interactions (although by limiting analysis by distance, the chance of encountering significant genes by environment interaction is reduced), or dominance effects (Falconer 1981). However, despite the limitations, Ritland's (1996a,b) method does provide a novel way of investigating heritabilities in quantitative traits in long-lived species where crossing experiments are not feasible and effects of the natural environment are pervasive.

Ritland and Ritland (1996) used this method of heritability calculation to compare heritabilities between two populations of plants in different environments. Since the influence of additive genetic variation will change with the environment, these types of calculations in natural environments provide another key to examining the possibility for evolution by describing heritability within a setting where the organism naturally occurs, exposed to many varying environmental conditions, in contrast to a common garden where variation in environment is limited. For example, turkey oak is found in sandhill ecosystems across the southeastern United States. In the more northern range in South Carolina (near Columbia), the sites are more upland and dryer, and the distribution of trees is more widespread and parklike. At these sites *Brachys tessalatus*, a specialist leaf mining Buprestid beetle, occurs in outbreak densities and their larvae consume more than 70% of the foliage of each tree. Variation in beetle densities could lead to different levels of selection and therefore additive genetic variance for phenolic traits could differ among populations. The link between natural variation and herbivore variation could provide new insight into evolution of plant-insect interactions.

This study is the first to attempt to quantify variation in plant secondary compounds within a natural population of

long-lived species using this nonmanipulative marker-based experimental method. With the appropriate sampling regime this technique could be useful in quantifying additive genetic effects for phenotypic traits of interest, such as stress, disease, or insect resistance traits for other long-lived species, including economically important varieties.

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Received August 12, 2000

Accepted April 30, 2001

Corresponding Editor: Bruce S. Weir