DOI: 10.1534/genetics.105.042952

Marker-Based Quantitative Genetics in the Wild?: The Heritability and Genetic Correlation of Chemical Defenses in Eucalyptus

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Manuscript received March 7, 2005
Accepted for publication August 19, 2005

ABSTRACT

Marker-based methods for estimating heritability and genetic correlation in the wild have attracted interest because traditional methods may be impractical or introduce bias via $G \times E$ effects, mating system variation, and sampling effects. However, they have not been widely used, especially in plants. A regression-based approach, which uses a continuous measure of genetic relatedness, promises to be particularly appropriate for use in plants with mixed-mating systems and overlapping generations. Using this method, we found significant narrow-sense heritability of foliar defense chemicals in a natural population of *Eucalyptus melliodora*. We also demonstrated a genetic basis for the phenotypic correlation underlying an ecological example of conditioned flavor aversion involving different biosynthetic pathways. Our results revealed that heritability estimates depend on the spatial scale of the analysis in a way that offers insight into the distribution of genetic and environmental variance. This study is the first to successfully use a marker-based method to measure quantitative genetic parameters in a tree. We suggest that this method will prove to be a useful tool in other studies and offer some recommendations for future applications of the method.

MARKER-based methods for estimating quantitative genetic parameters in wild populations have attracted interest for two principal reasons: (1) experimental methods may be impractical or unfeasible in some systems; and (2) traditional methods may introduce bias through genotype-environment interactions, mating system variation, or sampling effects. The genetic basis of quantitative trait variation is usually studied using known pedigrees and controlled environments, most often for the purpose of plant or animal breeding (Lynch and Walsh 1998). However, when the aim is instead to measure parameters pertaining to evolution in wild organisms, it becomes desirable to describe genetic variation in quantitative traits in natural populations. While traditional methods are good for making predictions about potential breeding gains in domesticated organisms, the results may not accurately describe the organism in its natural state.

These problems are particularly relevant for long-lived trees and for those with mixed-mating systems and restricted dispersal. Adult traits may take years to develop, so that common-garden trials are time-consuming and expensive. Estimates of genetic parameters from even well-designed common-garden or laboratory experiments may not accurately reflect the parameters affecting trait evolution in the field. Environmental factors, such as stress and competition, can change the observed genetic effects in experiments, compared with natural conditions, because genotype × environment interactions can affect additive genetic variances and covariances (Donohue *et al.* 2000; Mutikainen *et al.* 2000; Orians *et al.* 2003; Osier and Lindroth 2004; Sgro and Hoffmann 2004). Statistical models for open-pollinated trials often assume a common pollen pool and a constant degree of relatedness within families, which may be incorrect for species with correlated paternity or local spatial genetic structure, for example. Furthermore, collecting available seed may produce a biased sample in species with variable reproduction, particularly where flowering time is under genetic control.

Two strategies have been used for marker-based estimation of variance components in natural populations: the application of traditional mixed models to sibships reconstructed from marker data (Lynch and Walsh 1998) and of a regression-based method that does not assume predefined classes of relationship (Ritland 1996b). Ritland's method uses a modified linear regression of phenotypic similarity on relatedness to estimate heritability. Whereas standard least-squares regression requires the predictor variable (in this case relatedness) to be known without error, this obstacle can be overcome by estimating its "actual" variance, as distinct from its statistical variance (Ritland 1996b). Sibship reconstruction methods tend to involve maximum-likelihood estimators of relationship (Mousseau *et al.* 1998; Thomas *et al.*

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2000; Milligan 2003) or Markov chain Monte Carlo procedures (Thomas and Hill 2000, 2002; Thomas *et al.* 2002). Ritland's (1996b) alternative uses method-of-moments estimators of relatedness, which vary continuously and are more appropriate for species with mixed-mating systems (Queller and Goodnight 1989; Ritland 1996a; Lynch and Ritland 1999; Wang 2002).

Despite the promise of marker-based methods, serious obstacles remain. Variation in the degree of relatedness is essential and this may limit the method's applicability to populations with strong genetic structure. To date, Ritland's (1996b) method has been used only to study quantitative inheritance in two plant species. Significant heritabilities were found in an annual herb, *Mimulus guttatus*, for several traits, including fitness-related characters (Ritland and Ritland 1996). Klaper *et al.* (2001) used allozyme and microsatellite markers to study the heritability of resistance characters in the clonal tree, *Quercus laevis*. Covariance between phenotypic similarity and relatedness suggested that several foliar phenolic compounds were heritable. However, the lack of significant actual variance in relatedness prevented estimation of quantitative genetic parameters, despite prior knowledge of population structure and close attention to experimental design. Although an association between relatedness and phenotypic similarity was shown, Ritland's method has still not been applied successfully in a long-lived tree.

Eucalyptus, the predominant tree genus in much of Australia, consists of >700 species and supports a diverse group of herbivores. Both insect and vertebrate herbivores of Eucalyptus are deterred by a range of secondary metabolites in Eucalyptus leaves, most importantly the formylated phloroglucinol compounds (FPCs), *e.g.*, sideroxylonal (Lawler *et al.* 1998; Wallis *et al.* 2002; Moore and Foley 2005). Second, the concentration of the volatile terpenoid 1,8-cineole is highly correlated with sideroxylonal, allowing it to act as an indicator of palatability (Lawler *et al.* 1999). Finally, tannins can also play a role in deterring vertebrate herbivores of Eucalyptus (Marsh *et al.* 2003b).

In this study, we used Ritland's method to infer quantitative inheritance of the chemical characteristics of *Eucalyptus melliodora* (yellow box) leaves that are relevant to herbivores. Ontogenetic changes in defense chemical phenotypes are known to occur in Eucalyptus and the adult traits were of interest. As *E. melliodora* is not a commercial forestry species, no preexisting experimental plantings were available, and therefore standard quantitative genetic approaches were not practical. However, this species was considered to be a good candidate for applying Ritland's method, since we expected to find strong fine-scale spatial genetic structure, resulting from restricted pollen and seed dispersal (Skabo *et al.* 1998), which should produce significant actual variance of relatedness. Our objectives were: (1) to estimate the heritability of four chemical components in the foliage and the genetic correlations between them, (2) to evaluate alternative statistical testing procedures, and (3) to investigate the effect of spatial scale on parameter estimates. Given our findings, we draw general conclusions about the broader applicability of this method and offer recommendations for future applications.

MATERIALS AND METHODS

Study species and sample collection: *E. melliodora* (yellow box) is widespread in southeastern Australia, but the yellow box/red gum (*E. blakeleyi*) grassy woodland vegetation type is an endangered community due to clearing for agriculture and development. *E. melliodora* is a mass-flowering tree and pollination is largely by insects (Moncur and Boland 1989). There is evidence for inbreeding depression in *E. melliodora*, as several measures of seed production are reduced in isolated paddock trees compared to intact woodland trees (Burrows 2000).

The study population was located at Mulligan's Flat, part of Canberra Nature Park, which is one of the largest remaining patches of yellow box/red gum grassy woodland. The woodland is a mosaic of patches dominated by different, unrelated, species, probably determined by factors such as soil and topology. The area chosen for this study had a high proportion of *E. melliodora* (~95% of all mature trees), whereas the surrounding woodland contained few trees of this species. In a 220 × 270-m area, 259 *E. melliodora* trees >9 cm in diameter at breast height (DBH) were mapped and sampled. Intertree distances were measured using a SONIN sonar device and the program INTERPNT (Boose *et al.* 1998) was used to generate a map of the trees. Branches were cut from the canopy and leaf samples collected for microsatellite and chemical analysis. Samples destined for DNA extraction and measurement of 1,8-cineole were stored at −20°, and the remaining leaves were freeze dried for 3–4 days and ground to pass through a 1-mm sieve in a Tecator cyclone mill.

Analysis of foliar defense chemistry: Four foliar chemical characteristics were measured that have previously been shown to be important determinants of feeding in insect and or vertebrate herbivores; foliar nitrogen was chosen as an indicator of foliar protein, and sideroxylonal and cineole are major antifeedant chemicals. The capacity of leaves to bind polyethylene glycol (PEG) is a good indicator of those tannins that affect feeding by vertebrates on Eucalyptus (Marsh *et al.* 2003a) and so PEG-binding capacity of the foliage was quantified rather than by nonspecific measures such as "total phenolics." All chemical analyses were performed by near-infrared reflectance spectroscopy (NIRS). This method relies on establishing the relationship between the near-infrared spectra (700–2500 nm) of a subset of the samples and reference chemical values. These relationships are then used to predict the concentration of the desired analyte in the whole sample set. The calibration equations were derived from a subset of ground, dried *E. melliodora* samples chosen to maximize spectral variation. Details of the laboratory and NIRS procedures are given elsewhere (Wallis *et al.* 2002; Moore *et al.* 2004).

Microsatellites: Many microsatellites have been developed in Eucalyptus and cross-species amplification has been reported (Byrne *et al.* 1996; Ottewell *et al.* 2005). We tested a panel of 30 loci developed in *E. globulus* (G. Moran, personal communication; Steane *et al.* 2001), *E. nitens* (Byrne *et al.* 1996), *E. sieberi* (Glaubitz *et al.* 2001), and *E. leucoxylon* (Ottewell *et al.* 2005) on up to 95 randomly chosen *E. melliodora* samples.

TABLE 1

Cross-transferred loci amplified in *Eucalyptus melliodora*

	Eg65	Eg91	EL13	Emcrc8	Emcrc11	Es54	Mean
N	232	232	232	232	232	232	232
N_a	9	16	19	24	18	27	18.8
H_o	0.737	0.776	0.931	0.931	0.905	0.884	0.861
H_e	0.703	0.803	0.917	0.940	0.903	0.895	0.860
F	−0.048	0.034	−0.015	0.010	−0.002	0.013	−0.002

The number of individuals included in the analysis, the number of alleles, the observed and expected heterozygosity, and the inbreeding coefficient are given for each locus, as well as the average across loci.

We applied strict criteria for the selection of the final set of loci. The loci had to (1) be mapped to different linkage groups (Marques *et al.* 2002; Thamarus *et al.* 2002), (2) amplify consistently, (3) display scorable variation, and (4) show no evidence for null alleles. The six loci chosen showed observed and expected heterozygosities consistent with random mating and were highly variable, with 9–27 alleles per locus and a total of 113 across loci (Table 1). While the number of loci was at the lower end of the recommended range, the allelic variability, rather than the number of loci, is the key to success with this method (Ritland 1996b, 2000).

DNA was extracted using the method of Byrne *et al.* (1993), modified to include 5% w/v PEG 6000 and 5% w/v PVP-40 in the extraction buffer. Loci were fluorescently labeled using M13 tailed primers (Schuelke 2000), except for EMCRC11, which was amplified with fluorescent nucleotides because amplification with M13-tailed primer failed. Ten-microliter reactions were carried out according to Schuelke (2000), using TaqGold (Perkin Elmer, Norwalk, CT). Fluorescent nucleotide reactions contained 1.5 mm $MgCl_2$, 0.2 μm dNTP, 0.2 mg ml^{-1} bovine serum albumin, both primers at 0.2 μm, and fUTP (R110, R6G, or TAMRA; Perkin Elmer) and the thermal profile followed Steane *et al.* (2001). Fragments were separated on an ABI377 automatic sequencer and sizes were determined using GENESCAN and GENOTYPER fragment analysis software (Applied Biosystems, Foster City, CA). Only the 232 individuals scored for all six loci were included in the heritability analysis. Heterozygosity and inbreeding coefficients were calculated using GenAlEx 6 (Peakall and Smouse 2005).

Heritability model: We estimated the heritability of foliar sideroxylonal, cineole, PEG-binding capacity, and nitrogen using Ritland's (1996b) regression method. Ritland proposed that narrow-sense heritability could be estimated by regressing a measure of phenotypic similarity (Z_{ij}, based on phenotypic covariance) on a marker-based estimate of relatedness. For this study, the full multiple-regression heritability model described in Ritland (1996b) was used initially:

$$Z_{ij} = 2h^2 r_{ij} + (H - h^2)\Delta_{ij} + b_f^2 f_{2ij} + b_e d_{ij} + a_e + e_{ij}. \quad (1)$$

The explanatory variables for individuals i and j are: r_{ij}, multilocus relatedness; Δ_{ij}, four-gene relatedness (as defined in Lynch and Ritland 1999; equivalent to h in Ritland 1996a; equivalent to r_2 in Ritland 1996b); f_{2ij}, a measure of shared individual inbreeding coefficients (as defined in Ritland 1996b); and d_{ij}, Euclidean spatial distance. The parameters estimated are: h^2, narrow-sense heritability; H, broad-sense heritability; b_f^2, inbreeding depression (the variance of a trait value resulting from inbreeding); b_e, the decrease of environmental correlation per meter; and a_e, the intercept.

Significant actual variance of the explanatory variables is required for parameter estimates to be nonzero. Therefore, explanatory variables were dropped from the model if the estimates of their actual variances were not significantly greater than zero, on the basis of the bootstrap percentile test. Spatial distance was left in even when b_e was not significant, since its variance was positive and collinearity between relatedness and spatial distance rendered the standard error estimates unreliable unless further measures were taken (see below). We used the matrix formulation of the linear model in (1) to estimate heritabilities and genetic correlations (see Ritland 1996b).

Genetic covariances and correlations: Genetic covariances and correlations between traits can be estimated using a linear model for the covariance between traits (Ritland 1996b; Lynch and Walsh 1998). The matrix formulation of the model produces an estimate that can be easily transformed into genetic covariance and correlations (Ritland 1996b). The sign of the genetic correlation is the same as the genetic covariance, as the denominator, $\sqrt{\hat{h}^2(x)\hat{h}^2(y)}$, is positive by definition. Since the heritability estimates in some bootstraps and permutations may be negative, the genetic covariance was used for testing the sign of the correlation. Phenotypic correlations between chemical traits were calculated using GenStat version 8 (VSN International, Hemel Hempstead, UK).

Relationship estimators: Lynch and Ritland's (1999) estimates of r_{ij} and Δ_{ij} were used and shared inbreeding, f_{2ij}, was estimated using Ritland's (1996b) formula. Ritland's earlier estimators for r_{ij} and Δ_{ij} are more appropriate for less variable markers, such as allozymes. Wang's (2002) estimators are more efficient for highly variable markers, but the optimal weighting scheme is not amenable to Ritland's (1996b) method for estimating the actual variance of relatedness.

Lynch and Ritland's (1999) estimates are based on the probability of the genotype of one individual in a pair, given the other genotype, yielding different estimates depending on which individual is taken as the reference individual. Symmetric versions were calculated by averaging the two possible locus weights and single-locus estimates for each locus before combining them in the multilocus estimates for each pair. This approach produced a single weight for each locus, which could be used for estimation of actual variance.

Parameter estimates, errors, and tests: All heritability and genetic correlation analyses were done using a Fortran77 program developed by Kermit Ritland for use in Ritland and Ritland (1996), modified by R. L. Andrew to accept microsatellite data, to calculate Lynch and Ritland's (1999) relatedness estimators and perform alternative tests. The original program performed bootstraps, but we developed additional permutation and resampling options to improve statistical testing. The nonindependence of individuals in the sample violates the key assumption of bootstrap tests. Bootstrapping individuals tends to overestimate sampling error by 10–100% (Thomas *et al.* 2002). Bootstraps were carried out across individuals to give conservative estimates of standard errors. When the same individual occurred twice in a bootstrap sample, the comparison between identical individuals was excluded; however, we repeated the bootstrap tests with the inclusion of these identical comparisons to test whether this approach altered the distribution of relatedness values. Jackknife tests require the same assumption of independent sampling and we performed jackknifing on both loci, which do vary independently, and trees, which again gave a conservative test. Kermit Ritland's recently released MARK 3.0 (Ritland 2004) does not use the full heritability model or a symmetric version of the Lynch and Ritland (1999) relatedness estimators, but does allow estimation of heritability and genetic correlation estimation via regression or likelihood, with bootstrap testing of estimates.

Permutation tests enable testing of a hypothesis by creating a null distribution, with which a sample parameter is compared. For this study, parameter estimates were tested against a null hypothesis of no spatial or genetic effects by permuting the quantitative trait values across the genotypes and spatial positions. This method maintains the covariance of relatedness and spatial distance, the actual variance of relatedness, and the variance of spatial distance, but unfortunately removes the covariance of phenotypic similarity with distance. However, removing the covariance between phenotypic similarity and distance has a positive effect on the heritability estimator, if both $\mathrm{Cov}(\hat{r}_{ij}, d_{ij})$ and $\mathrm{Cov}(d_{ij}, Z_{ij})$ are negative:

$$\hat{h}^2 = \frac{\mathrm{Cov}(\hat{r}_{ij}, Z_{ij})\mathrm{Var}(d_{ij}) - \mathrm{Cov}(d_{ij}, Z_{ij})\mathrm{Cov}(\hat{r}_{ij}, d_{ij})}{2(\hat{\mathrm{V}}\mathrm{ar}(r_{ij})\mathrm{Var}(d_{ij}) - \mathrm{Cov}(\hat{r}_{ij}, d_{ij})^2)}. \quad (2)$$

Hence, the upper confidence limit is biased upward and so this is a conservative test of the null hypothesis of no genetic effects. Likewise, the permuted b_e estimate is biased downward, giving a conservative test of this parameter; however, the estimate of a_e is biased downward and should not be tested in this way. Alternative approaches, such as permuting the microsatellite genotypes over the spatial and phenotypic data, alter the crucial variances. The distance cutoffs would include a random sample of the genotypes, rather than a structured sample, and the actual variance of relatedness in the permutations would be the population mean, which could differ substantially from the unpermuted sample. Permutations were not used to test parameters in the full model, because the direction of the bias is harder to predict when more variables are included.

The effect of sampling scale on additive genetic variation was investigated by repeating the analysis with distance cutoffs ranging from 10 to 310 m. For each cutoff, pairs whose spatial distance exceeded this limit were excluded from the regression model. Distance classes with lower limits were also used to compare partitioning of phenotypic variance with increasing distance. A 60-m distance cutoff was chosen for comparison, on the basis of examination of the decrease in relatedness and actual variance of relatedness with increasing distance. This distance also described the extent of positive spatial autocorrelation in the data set (results not shown), as estimated by the method of Smouse and Peakall (1999), using GenAlEx 6 (Peakall and Smouse 2005).

Collinearity among explanatory variables can inflate standard errors around partial regression slopes. Strong collinearity between r_{ij} and d_{ij} led us to develop a model free from spatial effects, by using residuals from the regression of phenotypic similarity on spatial distance, instead of the raw phenotypic similarities. Both the preliminary regression and heritability estimation were carried out on the subset of pairwise comparisons within the chosen distance cutoff.

RESULTS

Quantitative traits: The foliar sideroxylonal, cineole, and nitrogen concentrations reported in Table 2 are similar to those previously reported for *E. melliodora* in the same woodland (Wallis *et al.* 2002). Sideroxylonal and cineole were significantly correlated ($r = 0.781$, $P < 0.0001$), while nitrogen was slightly correlated with sideroxylonal ($r = 0.204$, $P = 0.0009$) and cineole ($r = 0.358$, $P < 0.0001$). PEG-binding capacity (PEGBC) was negatively correlated with nitrogen ($r = -0.419$, $P < 0.0001$) and marginally with cineole ($r = -0.101$, $P < 0.0625$).

TABLE 2

Mean and standard deviation of four leaf chemical characteristics in *Eucalyptus melliodora*

Trait	Range	Mean	SD
Sideroxylonal	5.8–32.5	17.9	5.3
Cineole	3.1–11.6	7.2	1.8
PEGBC	10.4–19.7	13.9	1.3
Nitrogen	11.4–16.9	13.6	0.9

All values are measured in milligrams per gram of dry matter.

Heritability estimates: Mean pairwise relatedness (r_{ij}) was significantly positive with distance cutoffs up to 80 m (Figure 1A), mean four-gene relatedness (Δ_{ij}) was positive, but not significant, at all cutoffs, and the mean inbreeding correlation (f_{2ij}) was negative, but not significant, at all cutoffs. Actual variance in relatedness was highly significant at all distance cutoffs (Figure 1B), but Δ and f_2 had no significant actual variance according to the bootstrap tests.

Using the full model and the entire data set without a distance cutoff, unrealistic parameter estimates were obtained. Estimates of $H - h^2$ and b_f^2 were outside the

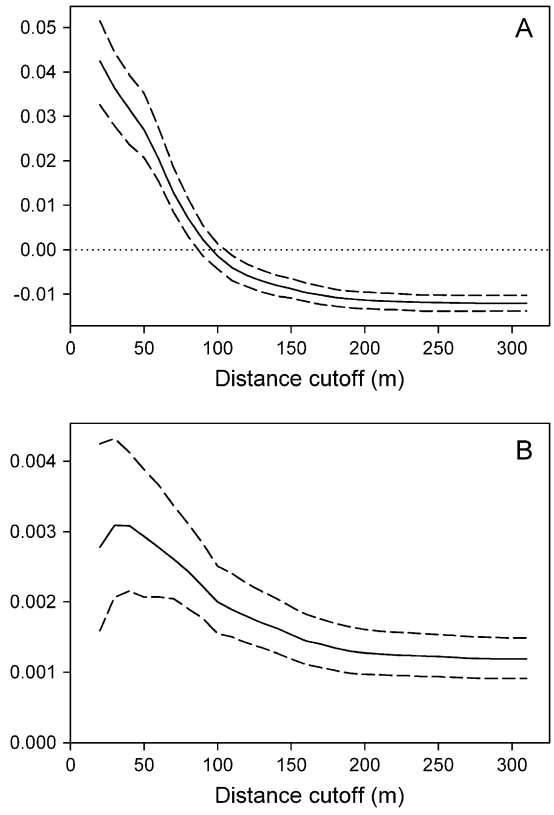

Figure 1.—Mean relatedness (A) and estimated actual variance of relatedness (B) with increasing distance cutoff. The maximum distance cutoff was 310 m. The dashed lines represent a 95% confidence envelope estimated by bootstrapping trees. Note that the distance cutoffs are cumulative.

TABLE 3

Parameter estimates for sideroxylonal using full and reduced heritability models

Independent variables	h^2		$H - h^2$		b_f^2		b_e		a_e	
	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE
r, Δ, f_2, d, 1	0.56	(15.18)	−2.85	(89.27)	17.58	(346.73)	−0.0001	(0.0078)	0.051	(1.944)
r, f_2, d, 1	0.61	(15.25)			17.27	(272.29)	−0.0001	(0.0075)	0.026	(1.711)
r, Δ, d, 1	0.49	(38.93)	−3.25	(65.23)			−0.0001	(0.0102)	0.048	(17.95)
r, d, 1	0.55*	(0.44)					−0.0001	(0.0003)	0.019	(0.022)
r, 1	0.59**	(0.37)							0.010	(0.011)
r	0.54**	(0.32)								

No distance cutoff was used. Standard errors were estimated from bootstraps and asterisks indicate significance based on bootstrap percentiles (*$P < 0.1$, **$P < 0.05$).

parameter space (from zero to one) regardless of the model used (Table 3). The four- and five-term models did not produce significant parameter estimates for any trait, but the covariance between phenotypic similarity for nitrogen concentration and f_2 was positive in 95% of the bootstraps, suggesting that there may be an effect of inbreeding on foliar nitrogen. Removing spatial distance from the model increased the heritability estimate for sideroxylonal and dropping the intercept term decreased it (Table 3).

Using the model for joint estimation of h^2 and b_e with all trees included, concentrations of sideroxylonal and nitrogen were significantly heritable according to the permutation tests, but those of cineole and PEGBC were not (Table 4). The linear environmental correlation, b_e, was significant for cineole ($P < 0.001$) and PEGBC ($P < 0.05$) and the intercept term was significant for each trait (Table 4). According to bootstrap tests, only nitrogen was significantly heritable and b_e was significant for cineole only. Including identical comparisons reduced the bootstrap standard errors of the heritability estimates, but produced a strong bias in the estimates of heritability and actual variance of relatedness (not shown), confirming that this method alters the genetic structure of the resampled data sets and is therefore inappropriate. Jackknifing individuals gave results similar to those of bootstraps (excluding identical comparisons). Jackknifing loci, on the other hand, produced smaller standard errors, but they are potentially unreliable, being based on only six loci (Figure 2).

Applying a distance cutoff altered the results of both heritability and genetic correlation estimation. A cutoff of 60 m, chosen because of the scale of spatial genetic structure (Figure 1), produced different estimates from those involving all comparisons (Figure 3). Heritability estimates were much higher for sideroxylonal and cineole and were significant according to bootstrap tests ($P < 0.05$) as well as permutation tests (Table 4). Bootstrap standard errors were approximately the same as when no distance cutoff was used, but the locus jackknife gave a larger standard error with a 60-m cutoff. On the basis of bootstrap distributions (excluding identical comparisons), the effect of spatial distance and the intercept of the environmental correlation were not significant, but according to the permutation tests, they were highly significant for foliar sideroxylonal ($P < 0.01$) and a_e was significant for PEG-binding capacity. Heritability estimates for nitrogen remained high for all distance cutoffs, while heritability of sideroxylonal fluctuated with increasing cutoffs and that for cineole decreased with cutoffs >50 m (Figure 3). The strongest contrast was seen in cineole: with a 60-m distance cutoff

TABLE 4

Estimates of heritability for leaf chemical traits in *Eucalyptus melliodora*

Trait	No cutoff			60-m cutoff		
	h^2	b_e	a_e	h^2	b_e	a_e
Sideroxylonal	0.554**	−0.00009	0.018*	*0.890***	−0.0026**	0.124**
Cineole	0.345	*−0.00124****	*0.136****	*0.723***	0.0008	0.022
PEGBC	0.224	−0.00023*	0.026*	−0.276	0.0002	0.085*
Nitrogen	*1.033****	−0.00007	0.028**	*0.770***	0.0014	−0.056

The model for joint estimation of heritability and environmental correlation was used and standard errors were estimated from bootstraps (1000 bootstraps excluding identical comparisons) and locus jackknifes. Population estimates in italics are significant at the 0.05 level from bootstrap percentiles and asterisks indicate significance levels from permutation tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

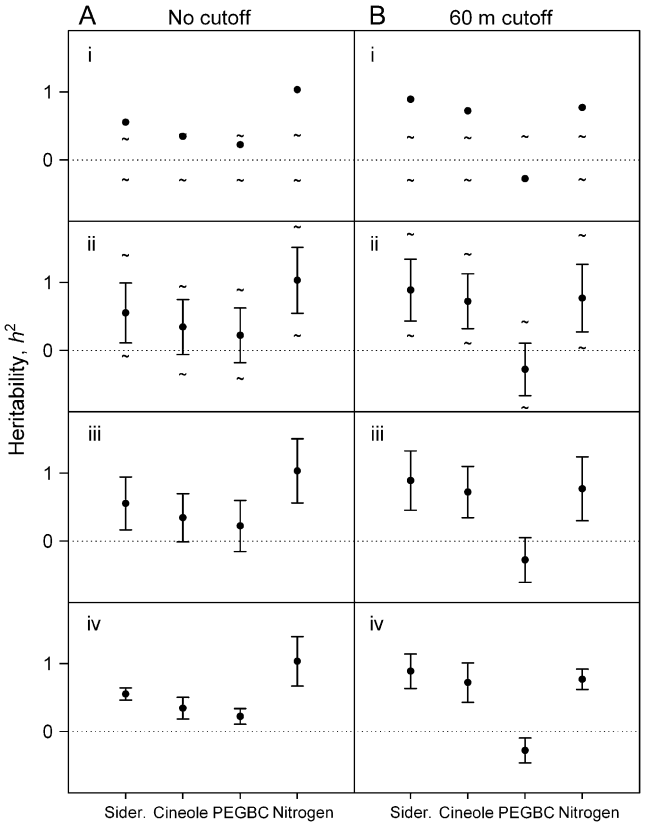

FIGURE 2.—Summary of heritability estimates for chemical traits without a distance cutoff (A) and with a 60-m cutoff (B). The results of testing by (i) permutation, (ii) bootstrapping individuals, (iii) jackknifing individuals, and (iv) jackknifing loci are shown. The population estimates of heritability are shown by solid circles. The 90% confidence intervals are shown for the permutation and bootstrap distributions, indicated by ~. The 95th percentile of permutation results and the 5th bootstrap percentile were used for one-sided tests ($\alpha = 0.05$). For the bootstraps and jackknifes, the error bars represent the estimated standard error of heritability.

h^2 was highly significant, but it steadily decreased as increasingly distant pairs of trees were included and b_e became significant (Table 4).

Preliminary regression: Performing a preliminary regression to eliminate spatial covariance in the phenotypic similarities and excluding spatial distance from the heritability model gave us conservative estimates of heritability. When all trees were included, the slope of the preliminary regression was significantly negative for all traits, while only the slope for sideroxylonal was significant with a distance cutoff of 60 m (Table 5). The preliminary regression procedure gave lower heritability estimates for the chemical traits, with smaller standard errors and narrower null distributions from permutations, but did not substantially alter the significance of the heritability estimates (Table 6).

Genetic correlations: With no distance cutoff, genetic covariances were significant and positive between sideroxylonal, cineole, and nitrogen, but none of these traits was genetically correlated with PEGBC (Table 7).

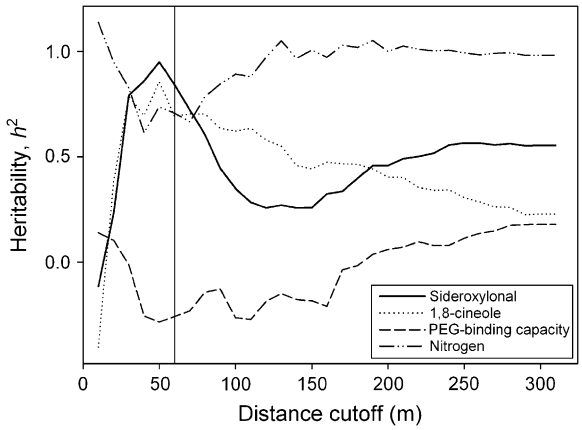

FIGURE 3.—Estimates of heritability for sideroxylonal, 1,8-cineole, PEG-binding capacity, and nitrogen with increasing distance cutoffs. The 60-m vertical line shows the distance cutoff used in Figure 2.

With a 60-m distance cutoff, these correlations were stronger and PEGBC was also genetically correlated with nitrogen.

DISCUSSION

Quantitative genetics of chemical defenses in *E. melliodora*: Using marker-based methods, we found significant narrow-sense heritability of the foliar defense compounds sideroxylonal and cineole and of total foliar nitrogen in a natural *E. melliodora* population. A range of statistical tests supported these conclusions, despite large bootstrap standard errors in some cases. This is the first study to demonstrate additive genetic control of forymylated phloroglucinol compounds within populations. Our results for cineole are congruent with common-garden studies in Eucalyptus, which have shown that it is highly heritable (DORAN and MATHESON 1994). The genetic correlation of sideroxylonal with cineole is further evidence of common regulatory mechanisms, despite no obvious biosynthetic link between the two

TABLE 5

Preliminary regression slopes of phenotypic similarity on spatial distance

Trait	No cutoff	60-m cutoff
Sideroxylonal	*−0.00035***	*−0.00458****
Cineole	*−0.00140****	−0.00085
PEGBC	*−0.00033***	0.00042
Nitrogen	*−0.00054***	−0.00035

Phenotypic similarity (Z_i) is a variance-standardized measure, and spatial distance is measured in meters. Slopes in italics are significant at the 0.05 level from bootstraps, excluding identical comparisons. Asterisks indicate significance levels calculated from permutations (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

TABLE 6

Heritability estimates with spatial effects eliminated

Cutoff	Trait	h^2 Estimate	h^2 SE
60 m	Sideroxylonal	*0.789***	(0.397)
None	Sideroxylonal	0.465**	(0.370)
None	Cineole	0.290	(0.335)
None	PEGBC	0.189	(0.351)
None	Nitrogen	*0.868***	(0.419)

A preliminary regression of phenotypic similarity was performed on spatial distance and the residuals were used in the final model instead of the raw phenotypic similarity. Estimates in italics are significant at the 0.05 level from bootstraps, excluding identical comparisons. Asterisks indicate significance levels calculated from permutations ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

compounds (Ghisalberti 1996). PEG-binding capacity has not been subjected to genetic analysis previously. Although variation in individual tannins may be heritable in trees (Whitham *et al.* 2003), our results suggest that the component visible to herbivore pressure is not.

The heritability of sideroxylonal and cineole indicates that there is potential for natural selection to occur as a result of herbivory. Insect and vertebrate herbivores can cause almost 100% defoliation of palatable eucalypts (Edwards *et al.* 1993), a serious cost when leaves could otherwise persist for several years. Our failure to detect additive genetic variation in PEG-binding capacity may reflect a stronger selective advantage conferred by PEG-binding tannins. Given the high heritability of sideroxylonal and its effectiveness as a deterrent against vertebrate and invertebrate herbivores, it is surprising that undefended trees persist in the population. However, heritable plant defenses are found in many systems, despite the apparent impact of herbivores on fitness, suggesting that other factors commonly mitigate the expected decrease in genetic variation (James and Newcombe 2000; Agrawal *et al.* 2002; Fornoni *et al.* 2003). Genetic trade-offs, patchy selection, and diffuse selection could all play a role in the maintenance of additive genetic variance in eucalypt defenses.

TABLE 7

Genetic correlations of chemical traits in *Eucalyptus melliodora*

Trait 1	Trait 2: Sideroxylonal	Cineole	PEGBC	Nitrogen
Sideroxylonal		*0.942****	−0.124	*0.857****
Cineole	0.847*		0.019	*0.987****
PEGBC	−0.212	−0.324		0.252*
Nitrogen	0.507**	*0.940***	−0.199	

Correlations were estimated with no distance cutoff (below diagonal) and with a 60-m cutoff (above diagonal). Tests for significance were performed using bootstraps and permutations of the covariances. Correlations in italics are significant at the 0.05 level, using bootstraps excluding identical comparisons, and asterisks indicate significance levels calculated from permutations ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

The effect of scale on the parameter estimates was striking, indicating that the distribution of genetic and environmental variance is complex. While the heritabilities of sideroxylonal, cineole, and nitrogen were similar at a fine scale, their behavior with increasing distance cutoffs differed dramatically (Figure 3). Spatially autocorrelated environmental effects would be expected to give increasing environmental variance, lowering heritability, with increasing distance cutoff. This pattern was seen for cineole. Sideroxylonal heritability fluctuated, indicating that the additive genetic variance or the environmental variance for this trait is patchily distributed. This suggests that, although sideroxylonal and cineole are genetically correlated, nongenetic effects act independently on the two traits. Clearly, the fine-scale spatial structure of both genotypic and phenotypic variation in natural populations warrants further investigation (R. L. Andrew, unpublished results).

A role for marker-based quantitative genetics in the wild: This study is the first successful application of Ritland's (1996b) regression method for genetic marker-based estimation of quantitative genetic parameters in a natural tree population. These results are important because they demonstrate that marker-based heritability methods can be applied to adult traits in a long-lived, mixed-mating tree species, for which traditional quantitative genetic techniques are frequently impractical.

Ritland's and other marker-based methods have been tested most thoroughly in animal systems and pedigree-based approaches have been found to be superior (Thomas *et al.* 2002; Coltman 2005). This result is consistent with theory. In obligate outbreeders, the proportion of related individuals in a large population is low. Because the actual variance of relatedness is therefore low, it cannot be estimated accurately using markers, which leads to unreliable estimates of heritability. Moreover, pedigrees for outbreeders are relatively simple, and even partial pedigrees may provide more accurate information on the proportion of genes shared than marker-based estimates of relatedness (Kruuk *et al.* 2000; Pemberton 2004).

Our results demonstrate that Ritland's approaches to marker-based quantitative genetics in the wild should not be abandoned because they are unsuccessful in obligate outbreeders. In contrast to the animal systems where Ritland's method has been tested, long-lived plants with mixed-mating systems have much more complex pedigrees because of selfing and biparental inbreeding across generations. This makes the shared genetic background difficult to encapsulate in discrete relationship classes, such as "half-sibling" and "parent-offspring," traditionally used in quantitative genetics. Furthermore, the level of variation in relationships is

greater in mixed-mating plants because of these complex pedigrees and can be controlled by taking advantage of fine-scale spatial genetic structure.

In addition to providing heritability estimates that are relevant to the ecological conditions and selective forces experienced in the wild, marker-based methods may offer additional benefits that are not often recognized, possibly due to the bias in evolutionary quantitative genetics toward animals and domesticated or easily propagated plants. They can be used to avoid common violations of the assumptions of certain models that are used in traditional common-garden experiments. Common-garden experiments with open-pollinated families assume a constant level of relatedness between progeny that is often unrealistic, due to variable outcrossing rates and correlated paternity. Marker-based heritability estimators can incorporate variable levels of relatedness resulting from complex pedigrees on a pairwise basis (Ritland 1996b). In addition to heritability, phenotypic similarity between relatives is affected by dominance, shared environments, and shared inbreeding levels. Ritland's (1996b) regression method enables these to be considered by extending the model. Where reproduction is spatially or temporally variable, as is often the case in forest trees, sampling seed at one time could give an unrepresentative snapshot, whereas marker-based methods can include the whole population, which may comprise several generations. These advantages do not apply to non-marker-based approaches to quantitative genetics in natural populations, such as regressing progeny phenotypes on those of wild parents (see Coyne and Beecham's 1987 Appendix by Lande) and the refinement by Riska *et al.* (1989), which require progeny to be raised in laboratory or common-garden environments.

While Ritland's (1996b) regression method is a major advance in studying quantitative genetic variation in natural populations, further methodological improvements may enhance the benefits of this approach. Our method allows least-squares estimation despite measurement error in the predictor variable; however, appropriate statistical testing is difficult to achieve. Resampling is unsatisfactory due to the nonindependence of samples, while permutation tests can be designed to give conservative tests of significance, but not confidence intervals for estimates. By performing a preliminary regression of phenotypic similarity on spatial distance and using the residuals to estimate heritability, we were able to eliminate the confounding spatial effects. These "corrected" estimates had slightly smaller standard errors, but were conservative as estimates of heritability. While we have addressed some of the statistical issues here, room for improvement remains.

Recommendations for future applications of Ritland's method for estimating quantitative genetic parameters in the wild: This method promises to be particularly useful where traditional, common-garden methods are impractical, such as studying adult traits in long-lived trees. However, we recommend that marker-based approaches be tried in more plant species and verified by comparison with common-garden techniques.

The choice of species may be a key factor in the success or otherwise of Ritland's method. Given our results, fine-scale spatial genetic structure appears to be a good indication that actual variance in relatedness may be found. This is more likely in plants with restricted pollen and seed dispersal and overlapping generations. Ritland's method is most likely to succeed in populations in which strong fine-scale spatial genetic autocorrelation has already been demonstrated or where there is an expectation of strongly restricted dispersal. The fine-scale spatial genetic structure in *E. melliodora* was also particularly strong, giving us a better chance of detecting significant actual variance of relatedness than that in *Q. laevis* (Klaper *et al.* 2001). It has been suggested that sampling design might be important; however, resampling in clusters from the study population provided no benefits over randomly resampling or intensively resampling the same number of individuals (results not shown). The biggest contribution to actual variance of relatedness is likely to be made by closely related individuals and intensive sampling will maximize the number of comparisons.

Undoubtedly, the availability of sufficient markers is also a key requirement. The marker system used in our study had 50% more alleles than the markers used by Klaper *et al.* (2001), which may help explain why they did not detect significant actual variance of relatedness. With a powerful marker system, it may even be feasible to estimate inbreeding depression and dominance effects.

The spatial scale of the analysis is critical to the results of this method and we therefore recommend repeating the estimation with a range of distance cutoffs. Expecting a single heritability value for a trait may oversimplify the variation in a natural population. We should instead select our scales carefully with respect to factors such as dispersal and selective pressures and consider what the range of values tells us about the distribution of various causes of variation. The maximum heritability estimate may be more comparable with measurements made in common-garden experiments, which are designed to minimize environmental variance. On the other hand, estimates obtained at larger sampling scales might be more suitable for predicting long-term responses to selection, particularly since selection itself may be patchy. One of the key differences between traditional quantitative genetic studies and marker-based studies in natural populations may be that what is lost in precision by studying genetic variation in the wild can be gained in insight into the distribution of natural genetic and phenotypic variation.

In summary, the conditions for successful application of Ritland's method are likely to be: (1) appropriate choice of species, with the likelihood of strong spatial

genetic structure; (2) a highly informative marker system; (3) statistical testing using a range of methods; and (4) consideration of estimates at several spatial scales. As we have shown, this approach can be successful and offers a useful tool for evolutionary biologists working on wild organisms.

The authors thank Kermit Ritland for freely providing access to his Fortran code and advice on methodology; Miranda Ebbers, Nadine Scholtz, Michelle Michie, Daniel Ebert, Ben Moore, and Saul Cunningham for assistance in various aspects of the field and laboratory work; and Kym Ottewell and Gavin Moran for their unpublished primer sequences. We also thank the anonymous reviewers for their helpful comments. This work was funded by grants from the Australian Research Council and the Rural Industries Research and Development Corporation (RIRDC) to W.J.F. and by a scholarship from RIRDC and the Australian National University to R.L.A.

LITERATURE CITED

Agrawal, A. A., A. Janssen, J. Bruin, M. A. Posthumus and M. W. Sabelis, 2002 An ecological cost of plant defence: attractiveness of bitter cucumber plants to natural enemies of herbivores. Ecol. Lett. **5:** 377–385.

Boose, E. R., E. F. Boose and A. L. Lezberg, 1998 A practical method for mapping trees using distance measurements. Ecology **79:** 819–827.

Burrows, G. E., 2000 Seed production in woodland and isolated trees of *Eucalyptus melliodora* (yellow box, Myrtaceae) in the south western slopes of New South Wales. Aust. J. Bot. **48:** 681–685.

Byrne, M., G. F. Moran and W. N. Tibbits, 1993 Restriction map and maternal inheritance of chloroplast DNA in *Eucalyptus nitens*. J. Hered. **84:** 218–220.

Byrne, M., M. I. Marquezgarcia, T. Uren, D. S. Smith and G. F. Moran, 1996 Conservation and genetic diversity of microsatellite loci in the genus *Eucalyptus*. Aust. J. Bot. **44:** 331–341.

Coltman, D. W., 2005 Testing marker-based estimates of heritability in the wild. Mol. Ecol. **14:** 2593–2599.

Coyne, J. A., and E. Beecham, 1987 Heritability of two morphological characters within and among natural populations of *Drosophila melanogaster*. Genetics **117:** 727–737.

Donohue, K., E. H. Pyle, D. Messiqua, M. S. Heschel and J. Schmitt, 2000 Density dependence and population differentiation of genetic architecture in *Impatiens capensis* in natural environments. Evolution **54:** 1969–1981.

Doran, J. C., and A. C. Matheson, 1994 Genetic parameters and expected gains from selection for monoterpene yields in Petford *Eucalyptus camaldulensis*. New For. **8:** 155–167.

Edwards, P. B., W. J. Wanjura and W. V. Brown, 1993 Selective herbivory by Christmas beetles in response to intraspecific variation in *Eucalyptus* terpenoids. Oecologia **95:** 551–557.

Fornoni, J., P. L. Valverde and J. Nunez-Farfan, 2003 Quantitative genetics of plant tolerance and resistance against natural enemies of two natural populations of *Datura stramonium*. Evol. Ecol. Res. **5:** 1049–1065.

Ghisalberti, E. L., 1996 Bioactive acylphloroglucinol derivatives from *Eucalyptus* species. Phytochemistry **41:** 7–22.

Glaubitz, J. C., L. C. Emebiri and G. F. Moran, 2001 Dinucleotide microsatellites from *Eucalyptus sieberi*: inheritance, diversity, and improved scoring of single-base differences. Genome **44:** 1041–1045.

James, R. R., and G. Newcombe, 2000 Defoliation patterns and genetics of insect resistance in cottonwoods. Can. J. For. Res. **30:** 85–90.

Klaper, R., K. Ritland, T. A. Mousseau and M. D. Hunter, 2001 Heritability of phenolics in *Quercus laevis* inferred using molecular markers. J. Hered. **92:** 421–426.

Kruuk, L. E. B., T. H. Clutton-Brock, J. Slate, J. M. Pemberton, S. Brotherstone *et al.*, 2000 Heritability of fitness in a wild mammal population. Proc. Natl. Acad. Sci. USA **97:** 698–703.

Lawler, I. R., W. J. Foley, B. M. Eschler, D. M. Pass and K. Handasyde, 1998 Intraspecific variation in *Eucalyptus* secondary metabolites determines food intake by folivorous marsupials. Oecologia **116:** 160–169.

Lawler, I. R., J. Stapley, W. J. Foley and B. M. Eschler, 1999 Ecological example of conditioned flavor aversion in plant-herbivore interactions: effect of terpenes of *Eucalyptus* leaves on feeding by common ringtail and brushtail possums. J. Chem. Ecol. **25:** 401–415.

Lynch, M., and K. Ritland, 1999 Estimation of pairwise relatedness with molecular markers. Genetics **152:** 1753–1766.

Lynch, M., and B. Walsh, 1998 *Genetics and Analysis of Quantitative Traits*. Sinauer, Sunderland, MA.

Marques, C. M., R. P. V. Brondani, D. Grattapaglia and R. Sederoff, 2002 Conservation and synteny of SSR loci and QTLs for vegetative propagation in four *Eucalyptus* species. Theor. Appl. Genet. **105:** 474–478.

Marsh, K. J., W. J. Foley, A. Cowling and I. R. Wallis, 2003a Differential susceptibility to *Eucalyptus* secondary compounds explains feeding by the common ringtail (*Pseudocheirus peregrinus*) and common brushtail possum (*Trichosurus vulpecula*). J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. **173:** 69–78.

Marsh, K. J., I. R. Wallis and W. J. Foley, 2003b The effect of inactivating tannins on the intake of *Eucalyptus* foliage by a specialist *Eucalyptus* folivore (*Pseudocheirus peregrinus*) and a generalist herbivore (*Trichosurus vulpecula*). Aust. J. Zool. **51:** 31–42.

Milligan, B. G., 2003 Maximum-likelihood estimation of relatedness. Genetics **163:** 1153–1167.

Moncur, M. W., and D. J. Boland, 1989 Floral morphology of *Eucalyptus melliodora* A. Cunn. ex Schau. and comparisons with other eucalypt species. Aust. J. Bot. **37:** 125–135.

Moore, B. D., and W. J. Foley, 2005 Tree use by koalas in a chemically complex landscape. Nature **435:** 488–490.

Moore, B. D., I. R. Wallis, J. T. Wood and W. J. Foley, 2004 Foliar nutrition, site quality, and temperature influence foliar chemistry of tallowwood (*Eucalyptus microcorys*). Ecol. Monogr. **74:** 553–568.

Mousseau, T. A., K. Ritland and D. D. Heath, 1998 A novel method for estimating heritability using molecular markers. Heredity **80:** 218–224.

Mutikainen, P., M. Walls, J. Ovaska, M. Keinanen, R. Julkunen-Tiitto *et al.*, 2000 Herbivore resistance in *Betula pendula*: effect of fertilization, defoliation, and plant genotype. Ecology **81:** 49–65.

Orians, C. M., S. Lower, R. S. Fritz and B. M. Roche, 2003 The effects of plant genetic variation and soil nutrients on secondary chemistry and growth in a shrubby willow, *Salix sericea*: patterns and constraints on the evolution of resistance traits. Biochem. Syst. Ecol. **31:** 233–247.

Osier, T. L., and R. L. Lindroth, 2004 Long-term effects of defoliation on quaking aspen in relation to genotype and nutrient availability: plant growth, phytochemistry and insect performance. Oecologia **139:** 55–65.

Ottewell, K. M., S. C. Donnellan, G. F. Moran and D. C. Paton, 2005 Multiplexed microsatellite markers for the genetic analysis of *Eucalyptus leucoxylon* (Myrtaceae) and their utility for ecological and breeding studies in other *Eucalyptus* species. J. Hered. **96:** 445–451.

Peakall, R., and P. E. Smouse, 2005 *GenAlEx 6: Genetic Analysis in Excel., Population Genetic Software for Teaching and Research*. Australian National University, Canberra, Australia.

Pemberton, J., 2004 Measuring inbreeding depression in the wild: the old ways are the best. Trends Ecol. Evol. **19:** 613–615.

Queller, D. C., and K. F. Goodnight, 1989 Estimating relatedness using genetic markers. Evolution **43:** 258–275.

Riska, B., T. Prout and M. Turelli, 1989 Laboratory estimates of heritabilities and genetic correlations in nature. Genetics **123:** 865–871.

Ritland, K., 1996a Estimators for pairwise relatedness and individual inbreeding coefficients. Genet. Res. **67:** 175–185.

Ritland, K., 1996b A marker-based method for inferences about quantitative inheritance in natural populations. Evolution **50:** 1062–1073.

Ritland, K., 2000 Marker-inferred relatedness as a tool for detecting heritability in nature. Mol. Ecol. **9:** 1195–1204.

Ritland, K., 2004 *MARK—Genetic Marker Analysis Program*. University of British Columbia, Vancouver, BC, Canada.

Ritland, K., and C. Ritland, 1996 Inferences about quantitative inheritance based on natural population structure in the yellow monkeyflower, *Mimulus guttatus*. Evolution **50:** 1074–1082.

Schuelke, M., 2000 An economic method for the fluorescent labeling of PCR fragments. Nat. Biotechnol. **18:** 233–234.

Sgro, C. M., and A. A. Hoffmann, 2004 Genetic correlations, tradeoffs and environmental variation. Heredity **93:** 241–248.

Skabo, S., R. E. Vaillancourt and B. M. Potts, 1998 Fine-scale genetic structure of *Eucalyptus globulus* ssp. *globulus* forest revealed by RAPDs. Aust. J. Bot. **46:** 583–594.

Smouse, P. E., and R. Peakall, 1999 Spatial autocorrelation analysis of individual multiallele and multilocus genetic structure. Heredity **82:** 561–573.

Steane, D. A., R. E. Vaillancourt, J. Russell, W. Powell, D. Marshall *et al.*, 2001 Development and characterisation of microsatellite loci in *Eucalyptus globulus* (Myrtaceae). Silvae Genet. **50:** 89–91.

Thamarus, K. A., K. Groom, J. Murrell, M. Byrne and G. F. Moran, 2002 A genetic linkage map for *Eucalyptus globulus* with candidate loci for wood, fibre, and floral traits. Theor. Appl. Genet. **104:** 379–387.

Thomas, S. C., and W. G. Hill, 2000 Estimating quantitative genetic parameters using sibships reconstructed from marker data. Genetics **155:** 1961–1972.

Thomas, S. C., and W. G. Hill, 2002 Sibship reconstruction in hierarchical population structures using Markov chain Monte Carlo techniques. Genet. Res. **79:** 227–234.

Thomas, S. C., J. M. Pemberton and W. G. Hill, 2000 Estimating variance components in natural populations using inferred relationships. Heredity **84:** 427–436.

Thomas, S. C., D. W. Coltman and J. M. Pemberton, 2002 The use of marker-based relationship information to estimate the heritability of body weight in a natural population: a cautionary tale. J. Evol. Biol. **15:** 92–99.

Wallis, I. R., M. L. Watson and W. J. Foley, 2002 Secondary metabolites in *Eucalyptus melliodora*: field distribution and laboratory feeding choices by a generalist herbivore, the common brushtail possum. Aust. J. Zool. **50:** 507–519.

Wang, J. L., 2002 An estimator for pairwise relatedness using molecular markers. Genetics **160:** 1203–1215.

Whitham, T. G., W. P. Young, G. D. Martinsen, C. A. Gehring, J. A. Schweitzer *et al.*, 2003 Community and ecosystem genetics: a consequence of the extended phenotype. Ecology **84:** 559–573.

Communicating editor: J. Bergelson