Accurate Estimation of Marker-Associated Genetic Variance and Heritability in Complex Trait Analyses

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ABSTRACT The emergence of high-throughput, genome-scale approaches for identifying and genotyping DNA variants has been a catalyst for the development of increasingly sophisticated whole-genome association and genomic prediction approaches, which together have revolutionized the study of complex traits in human, animal, and plant populations. These approaches have uncovered a broad spectrum of genetic complexity across traits and organisms, from a small number of detectable loci to an unknown number of undetectable loci. The heritable variation observed in a population is often partly caused by the segregation of one or more large-effect (statistically detectable) loci. Our study focused on the accurate estimation of the proportion of the genetic variance explained by such loci (\(p\)), a parameter estimated to quantify and predict the importance of causative loci or markers in linkage disequilibrium with causative loci. Here, we show that marker-associated genetic variances are systematically overestimated by standard statistical methods. The upward bias is purely mathematical in nature, unrelated to selection bias, and caused by the inequality between the genetic variance among progeny and sums of partitioned marker-associated genetic variances. We discovered a straightforward mathematical correction factor (\(k_M\)) that depends only on degrees of freedom and the number of entries, is constant for a given experiment design, expands to higher-order genetic models in a predictable pattern, and yields bias-corrected estimates of marker-associated genetic variance and heritability.

KEYWORDS heritability; linear mixed model; quantitative trait locus; single nucleotide polymorphism; average marginal variance; average semi-variance

Introduction

The introduction of chromosome-scale approaches for identifying and mapping quantitative trait loci (QTL) was an important milestone in quantitative genetics (Lander and Botstein 1989; Walsh 2001; Hill 2012; Churchill 2016). Through “interval mapping,” Lander and Botstein (1989) showed that a genome could be searched to identify QTL predicted by Mendelian genetics to underlie the quantitative genetic variation observed in a population. Their approach relied on genotyping individuals with a genome-wide framework of DNA markers. The emergence of increasingly powerful methods for identifying and genotyping DNA variants has facilitated genome-wide searches for markers linked to QTL by exploiting the historic recombination in populations through genome-wide association studies (GWAS) (Visscher et al. 2012, 2017). Because the genetic factors underlying quantitative traits are often exceedingly complex and difficult to resolve (Mackay 2001; Huang and Mackay 2016; Visscher and Yang 2016), the QTL discovered with these approaches often only explain a fraction of the heritable variation for a trait (Walsh 2001; Bernardo 2008; Hill 2012; Visscher et al. 2012; Bernardo 2016).

Building on the foundation of genome-wide QTL discovery, Lande and Thompson (1990) described how genotypes for DNA markers in linkage disequilibrium (LD) with statistically significant QTL could be combined with phenotypes in an optimum index for artificial selection in animals and plants. They showed that the index weights and predicted genetic gains (AG) from marker-assisted index selection were functions of narrow-sense heritability (\(h^2 = \sigma^2_A / \sigma^2_G\)) and the proportion of the additive genetic variance explained by marker loci in LD with the underlying QTL (\(p_A = \sigma^2_M / \sigma^2_A\)), where \(\sigma^2_A\) is the additive genetic variance, \(\sigma^2_M\) is the proportion of \(\sigma^2_A\) associated with statistically significant marker loci incorporated into the marker-assisted selection (MAS) index, and \(\sigma^2_G\) is the phenotypic variance (Neimann-Sorensen and Robertson 1961; Lande and Thompson 1990; Visscher et al. 2006). More generally, \(p = \sigma^2_M / \sigma^2_G\), where \(\sigma^2_G\) is the genetic variance among entries (individuals or families) and...
\( \sigma^2_M \) is the proportion of \( \sigma^2_G \) explained by the intra- and interlocus effects of marker loci. The interpretation of \( \sigma^2_M \) depends on the genetic or pedigree relationships among entries (Lynch and Walsh 1998), whereas the interpretation of \( \sigma^2_F \) depends on the marker effects specified in the model (Lande and Thompson 1990). Technically, \( H^2_M = \sigma^2_M / \sigma^2_F \) is the fraction of the phenotypic variance, similar to broad-sense heritability (\( H^2 = \sigma^2_A / \sigma^2_P \)), associated with a statistically significant subset of markers in LD with the underlying QTL.

The marker-assisted selection (MAS) approach envisioned by Lande and Thompson (1990) was ultimately supplanted by genomic selection, where breeding values are estimated by whole-genome regression (WGR) methods in populations genotyped with a dense genome-wide framework of DNA markers (Meuwissen et al. 2001; VanRaden 2008; Hill 2012, 2014; Visscher et al. 2019). When applying WGR methods, the additive effects for statistically significant and non-significant markers (the entire framework) are summed across the genome (Meuwissen et al. 2001), as opposed to identifying, selecting, and summing the additive effects of statistically significant markers only, as in the Lande and Thompson (1990) MAS index. WGR has, in addition, facilitated the development of the theory and methods for assumption-free estimation of genomic heritability and other genetic parameters (Visscher et al. 2006; VanRaden 2008; Visscher et al. 2008; de los Campos et al. 2015).

With the emergence of genomic prediction methods, the focus in quantitative genetic studies has heavily shifted away from QTL discovery per se towards genomic prediction and the estimation of genomic-estimated breeding values (GEBVs) (Meuwissen et al. 2001; Heffner et al. 2009; Piepho 2009; Daetwyler et al. 2010; de los Campos et al. 2013, 2015; Croassa et al. 2017; Lello et al. 2018; Damesa et al. 2019). Nevertheless, quantitative genetic studies typically entail a genome-wide search for large-effect (statistically significant) QTL in addition to genomic prediction using WGR methods (Mackay 2001; Walsh 2001; Rutkoski et al. 2014; de los Campos et al. 2015; Lello et al. 2018; Wang and Xu 2019; Lello et al. 2019; Rice and Lipka 2019).

Although the concepts of marker-associated genetic variance and marker heritability (\( H^2_M \)) are integral to the application genome-informed approaches in quantitative genetics, estimates of these parameters have rarely been reported (Landen and Botstein 1989; Lande and Thompson 1990; Lynch and Walsh 1998; Walsh 2001; Hill 2012; de los Campos et al. 2015). We discovered that \( p \) and \( H^2_M \) are systematically overestimated by standard statistical approaches. To explore this problem, we investigated the accuracy of two methods for estimating \( p \) and \( H^2_M \): average marginal variance (AMV) and average semi-variance (ASV) (Henderson 1953; Searle and Gruber 1971; Piepho 2019). As shown below, AMV estimators are identical to the well known ANOVA or method-of-moments estimators (Henderson 1953; Searle and Gruber 1971). On the surface, the estimation of \( p \) and \( H^2_M \) should be straightforward; however, when applying the Lande and Thompson (1990) index to a selection problem in sunflower (Tang et al. 2006), we observed AMV estimates of \( p \) and \( H^2_M \) that were greater than 1.0, the theoretical limit for both parameters. Here, we describe the root cause of the overestimation problem, identify the source of the bias, compare the accuracy of AMV and ASV estimators of \( p \) and \( H^2_M \), and show that ASV yields accurate estimates, whereas AMV yields systematically upwardly biased estimates of both ratios. Finally, we present a straightforward approach for bias-correcting AMV estimates of \( p \) and \( H^2_M \).

### Materials and Methods

#### Linear Mixed Models for ANOVA Estimation

The linear mixed models needed for ANOVA estimation of \( \sigma^2_G \) and \( \sigma^2_M \) are developed here. Consider an experiment where \( n_G \) entries (e.g., individuals or families) are phenotyped for a normally distributed quantitative trait, genotyped for one or more markers, and tested in a balanced completely randomized experiment design with \( r_G \) replications/entry, \( n_M \) marker genotypes/locus, and \( r_M \) replications/marker genotype. Here, we study balanced designs with a single explanatory marker locus (\( M_1 \)) for simplicity. Solutions for more complex genetic models and unbalanced data are shown in Appendices A, B, and C.

The LMM for estimating \( \sigma^2_G \) is:

\[
y_{jk} = \mu + G_j + \epsilon_{jk} \tag{1}
\]

where \( y_{jk} \) is the \( jk^{th} \) phenotypic observation, \( \mu \) is the population mean, \( G_j \) is the random effect of the \( j^{th} \) entry, \( \epsilon_{jk} \) is the random effect of the \( jk^{th} \) residual, \( G_j \sim N(0, \sigma^2_G) \), \( \epsilon_{jk} \sim N(0, \sigma^2_{\epsilon}) \), \( j = 1, 2, \ldots n_G \), and \( k = 1, 2, \ldots, r_G \). This is the classic LMM for estimating \( \sigma^2_G \) using ANOVA or REML methods (Table 1).

Suppose entries were genotyped for a single marker locus (\( M_1 \)) in LD with a QTL affecting the trait. The LMM for estimating the genetic variance associated with \( M_1 (\sigma^2_{M_1}) \) is:

\[
y_{ijk} = \mu + M_1i + G : M_1ij + \epsilon_{ijk} \tag{2}
\]

where \( y_{ijk} \) is the \( ijk^{th} \) phenotypic observation, \( M_1i \) is the random effect of the \( i^{th} \) marker genotype at locus \( M_1 \), \( G : M_1ij \) is the random effect of the \( ij^{th} \) entry nested in the \( i^{th} \) marker genotype, \( \epsilon_{ijk} \) is the random effect of the \( ijk^{th} \) residual, \( i = 1, 2, \ldots, n_M, j = 1, 2, \ldots, n_G \), and \( k = 1, 2, \ldots, r_G \). This is the classic LMM for estimating \( \sigma^2_{M_1} \) using ANOVA or REML methods (Table 1).

#### ANOVA Estimators of the Variance Components

Statistical analyses of LMMs (1) and (2) are needed to obtain estimates of \( \sigma^2_G, \sigma^2_M \), and \( \sigma^2_{\epsilon} \). ANOVA estimators of the between-entry (\( \sigma^2_G \)) and residual (\( \sigma^2_{\epsilon} \)) variance components for LMM (1) with balanced data are:

\[
\hat{\sigma}^2_G = \frac{MS_G - MS_e}{r_G} \tag{3}
\]

and

\[
\hat{\sigma}^2_{\epsilon} = \frac{MS_e}{r_G} \tag{4}
\]

where the mean sum of squares (MSSs) are defined in Table (1).

Similarly, for a single marker locus (\( M_1 \)), ANOVA estimators of the genetic variance associated with \( M_1 (\sigma^2_{M_1}) \), the entries nested in the marker locus (\( \sigma^2_{G|M_1} \)), and residual variance components for LMM (2) with balanced data are:

\[
\hat{\sigma}^2_{M_1} = \frac{MS_{M_1} - MS_{G|M_1}}{r_G r_{G|M_1}} \tag{5}
\]

and

\[
\hat{\sigma}^2_{G|M_1} = \frac{MS_{G|M_1} - MS_e}{r_G} \tag{6}
\]
\[ \hat{\sigma}_c^2 = MS_e \]

where \( n_{G,M1} \) is the number of entries nested in M1 and the MSs are defined in Table 1, \( E(\hat{\sigma}_{M1}^2) = \sigma_{M1}^2 \), and \( E(\hat{\sigma}_{G,M1}^2) = \sigma_{G,M1}^2 \). The residuals in LMMs (1) and (2) are identical when the data are balanced. Hence, for a single marker locus, the classic estimator of marker-associated genetic variance is:

\[ \beta_{M1} = \frac{\hat{\sigma}_{M1}^2}{\hat{\sigma}_G^2} \]  

(7)

and the estimator of broad-sense marker heritability on an entry-mean basis is:

\[ \hat{H}_{M1}^2 = \frac{\hat{\sigma}_{M1}^2}{\hat{\sigma}_G^2 + \hat{\sigma}_e^2} = \frac{\hat{\sigma}_{M1}^2}{\hat{\sigma}_G^2} \]  

(8)

where \( \hat{\sigma}_G^2 \) is the phenotypic variance on an entry-mean basis.

**Linear Mixed Models for AMV and ASV Estimation**

The phenotypic observations for AMV and ASV estimation of the variance components are the adjusted entry-level means (\( g_{ij} \)) from LMM (1), which are stored in an \( n_g \)-element vector (\( \tilde{g} \)). These are best linear unbiased estimates (BLUEs) of the entry means (Piepho et al. 2012; Piepho 2019). The LMM equivalent to (2) for the entry-level means analysis of the effect of a single marker locus (M1) is:

\[ \tilde{y}_{ij} = \mu + M_{ij} + G : M_{ij} + e_{ij} \]  

(9)

where \( \tilde{y}_{ij} \) is the phenotypic mean for the \( ij \)th entry, \( \mu \) is the population mean, \( M_{ij} \) is the random effect of the \( ij \)th marker genotype, \( var(M_{ij}) = \sigma_{M1}^2 \) and \( G : M_{ij} \) is the random effect of entries nested in M1, \( var(G : M_{ij}) = \sigma_{G,M1}^2 \), \( e_{ij} \) is the residual error, and \( var(e_{ij}) = r_{ij}^{-1} \sigma_e^2 \).

With the entry-mean vector (\( \tilde{g} \)) as input, the LMM equivalent to (9) is:

\[ \tilde{g} = 1_n \mu + g + e \]  

(10)

where \( 1_n \) is an \( n_g \)-vector of ones, \( \mu \) is the population mean, \( g \) is an \( n_g \)-vector of entry effects, \( g \sim N(0,g) \), \( e \) is an \( n_g \)-vector of residual effects, and \( e \sim N(0,e) \). The residual variance-covariance matrix (R) is estimated in the first stage of the two-stage ASV analysis (Piepho et al. 2012; Damesa et al. 2017, 2019). The structure of the entry variance-covariance matrix (G) depends on the model for marker locus effects (\( g \)). The \( g \) effect can be decomposed into the random effects of marker loci and entries nested in marker loci:

\[ g = g_1 + g_2 + \ldots + g_c = Z_1 u_1 + Z_2 u_2 + \ldots + Z_c u_c \]  

(11)

where the \( Z \) are design matrices and \( u_c \) are independent random effect vectors for \( c \) genetic factors in the model, e.g., marker loci, interactions between marker loci, and entries nested in marker loci with individual variance-covariance matrices \( G_c \). Hence, for a single marker locus M1, the c terms in (10) and (11) account for the genetic variation associated with M1 and the residual genetic variation associated with entries nested in M1 (\( G : M1 \)).

For LMM (10), the variance-covariance matrix on an entry-means basis is:

\[ var(\tilde{g}) = V = G + R = \sum_c (Z_c G_c Z_c') + R \]  

(12)

where \( \tilde{g} \) is a vector of adjusted entry-level means. AMV and ASV estimators of the variance components (\( \hat{g}_c \)) in LMMs (10) and (11) are shown in the results section, along with algebraic expressions of the biases of ASV estimators of \( p \) and \( H_{M1}^2 \) for different decompositions of \( g \) in (11).

**Biases of AMV and ASV Estimators of \( p \) and \( H_{M1}^2 \)**

True variance components in LMMs (1) and (2) are needed to estimate the biases of AMV and ASV estimators of \( p \) and \( H_{M1}^2 \). Here, we will use sample variances of realized effects to define true variances. Following Estaghi et al. (2013), the true variance for the between-entry (total genetic) effect in LMM (1), is:

\[ s_G^2 = (n_y - 1)^{-1} \sum_{i=1}^{n_y} (\tilde{g}_i - \bar{g})^2 \]  

where \( g = Z_{M1} u_{M1} + Z_{G,M1} u_{G,M1} \), \( \bar{g} \) is an \( n_x \)-element vector (\( \bar{g} \)) that is the phenotypic mean for the \( i \)th effect, \( i = 1,2,3,\ldots,n_y \) and \( \bar{g} \) is the mean of \( g \). We note that \( E(\hat{\sigma}_{G}^2) = E(\hat{\sigma}_{G}^2) = \sigma_G^2 \). The true variance for the residual effect in LMM (1) is:

\[ s_e^2 = (n_y - 1)^{-1} \sum_{i=1}^{n_y} (\tilde{e}_i - \bar{e})^2 \]  

where \( \bar{e} \) is the mean of \( \tilde{e} \), \( \bar{e} \) is the mean of \( e \). We note that \( E(\hat{\sigma}_{e}^2) = E(\hat{\sigma}_{e}^2) = \sigma_e^2 \).

The true variance for the effect of entries nested in M1 in LMM (2) is:

\[ s_{M1}^2 = (n_y - 1)^{-1} \sum_{i=1}^{n_y} (\tilde{m}_i - \bar{m})^2 \]  

where \( m = Z_{M1} u_{M1}, m_i \) is the effect of the \( i \)th genotype for marker locus 1, \( i = 1,2,3,\ldots,n_m \), and \( \bar{m} \) is the mean of \( m \) across entries. We demonstrate in the results that \( E(\hat{\sigma}_{M1}^2) \leq E(\hat{\sigma}_{M1}^2) = \sigma_{M1}^2 \). This inequality is at the heart of the problem we are studying and drives the overestimation of \( p \) and \( H_{M1}^2 \).

The bias of the AMV estimator of \( \sigma_{M1}^2 \) is:

\[ bias(\hat{\sigma}_{AMV}^2) = E[\hat{\sigma}_{AMV}^2] - s_{M1}^2 \]

where \( \hat{\sigma}_{AMV}^2 \) is the AMV estimate of \( \sigma_{M1}^2 \). Similarly, the bias of the ASV estimator is:

\[ bias(\hat{\sigma}_{ASV}^2) = E[\hat{\sigma}_{ASV}^2] - s_{M1}^2 \]

where \( \hat{\sigma}_{ASV}^2 \) is the ASV estimate of \( \sigma_{M1}^2 \). As shown in the results, the bias-correction factor (\( k_{M1} \)) is a constant determined by the independent variable structure. The relative bias, which is expressed as a proportion of the true value (\( s_{M1}^2 \)), is also constant for a given true value. The relative bias of the AMV estimator is:

\[ relBias(\hat{\sigma}_{AMV}^2) = \frac{bias(\hat{\sigma}_{AMV}^2)}{s_{M1}^2} \]

**Genetic Variance Estimation Bias**

where \( \hat{\sigma}_{AMV}^2 \) is the AMV estimate of \( \sigma_{M1}^2 \) in LMMs (10) and (11) shown in the results section, along with algebraic expressions of the biases of ASV estimators of \( p \) and \( H_{M1}^2 \) for different decompositions of \( g \) in (11).
\[ RB(\hat{\theta}_{AMV}^{\text{M1}}) = \frac{E[\hat{\theta}_{AMV}^{\text{M1}}] - \sigma_{M1}^2}{\sigma_{M1}^2} \]

and the relative bias of the ASV estimator is:

\[ RB(\hat{\theta}_{ASV}^{\text{M1}}) = \frac{E[\hat{\theta}_{ASV}^{\text{M1}}] - \sigma_{M1}^2}{\sigma_{M1}^2} \]

The biases and relative biases are determined through computer simulations.

**Simulation Experiments**

We used data to estimate the accuracy of AMV and ASV estimators of \( p \) and \( H_G^2 \). Plot-level phenotypic observations (\( y_{ijkt} \)) for LMMs (1) and (2) were simulated under 3 genotypes/locus and different \( G_G, r_G, n_G, \) and \( H_G^2 \). The phenotypic observations were simulated for 21 combinations of these variables with balanced or unbalanced data structures as shown in Table 2. As shown below, the bias is a mathematical constant for a given experiment design (fixed \( n_G, r_G, n_M, \) and \( r_M \)). Simulations were performed to verify that result and estimate the biases associated with AMV and ASV estimates of \( \sigma_{G}^2 \) for different independent variable structures (study designs) (Table 2). We simulated 1,000 samples (iterations) for every study design using standard statistical methods (Burton et al. 2006; Morris et al. 2019). The different effects (random normal variates) were summed according to LMMs (1) and (2) to obtain \( n = n_G \times n_M \) phenotypic observations in each sample for every study design.

The phenotypic observations for each sample were obtained by generating random normal variates for entries, marker loci, and residuals using the R function ‘rnorm()’ with known means and variances at the plot-level (R Core Team 2019). These plot-level observations were used to estimate the variance components in LMMs (1) and (2) and assess the accuracy of AMV and ASV estimators of \( p \) and \( H_G^2 \). In study designs 1-6, the true variances of marker loci (\( \sigma_{M1}^2 \)) and entries nested in marker loci (\( \sigma_{G1M1}^2 \)) were allowed to vary between samples such that the true marker heritability was between 0 and 1. Study designs 1-6 demonstrate how different experimental conditions, number of explanatory marker loci (\( n \)) and design balance, affect estimates of the variance explained by marker loci (Fig. 1; Table 2). In study designs 7-21, the true variances of the independent variables were fixed for all samples, which allowed us to estimate the bias and relative bias associated with the different estimators. Study designs 7-21 demonstrate how different independent variables, \( r_G \) and \( n_G \), and \( H_G^2 \) affect the estimates of bias and relative bias (Figs. 2-4; Table 2). The variance components were estimated using REML in the ‘lm4::lmer()’ v1.1-21 (Bates et al. 2015) package in R v3.6.0 (R Core Team 2019).

**Results**

**ANOVA Estimators**

Through genetic analyses of quantitative phenotypes in plant populations, we discovered that the sum of REML estimates of \( \sigma_{G}^2 \) and \( \sigma_{GGM}^2 \) from LMM (2) were greater than the REML estimate of \( \sigma_{G1M1}^2 \) from LMM (1) (Table 4). Intuitively, we expected \( \sigma_{M1}^2 \) and \( \sigma_{GGM}^2 \) to sum to \( \sigma_{G}^2 \); however, our analyses show that estimates of variance components from the individual LMMs are not corrected for differences in the number of observations for different factors in the models. While the estimation of \( p = \sigma_{M1}^2 / \sigma_{G}^2 \) seems straightforward, we found that this parameter is...
where \( \hat{\sigma}_G^2 \) is the ANOVA estimator of \( \sigma_G^2 \) from LMM (1), \( \hat{\sigma}_{G:1}^2 \) and \( \hat{\sigma}_{G:M1}^2 \) are ANOVA estimators of \( \sigma_{G:1}^2 \) and \( \sigma_{G:M1}^2 \) from LMM (2), and \( k_{M1} \) is the coefficient hidden in the expected mean squares of the two LMMs. While the sums of squares are additive (\( SS_G = SS_{M1} + SS_{G:M1} \)), the mean squares are not. This is where the problem arises with estimating \( p \) and \( H^2_M \) as functions of the ANOVA estimators from LMMs (1) and (2). The 'hidden' coefficients \( (k_M) \) are the source of the bias in ANOVA estimators of \( \sigma_M^2 \) (Tables 1-3).

To define the \( k_M \) algebraically, we equated ANOVA estimators of the variance components from LMM (1) with those from LMM (2). Here we show the solution for a model with a single marker locus (M1) with balanced data. Solutions for more complex genetic models and unbalanced data are shown in Table 1 and Appendices A, B, and C. From LMM (1) and equation (3), the ANOVA estimator of the between entry variance component is:

\[
\hat{\sigma}_G^2 = \frac{MS_G - MS_e}{r_G} = \frac{SS_G/d_{FG} - SS_e/d_{Fe}}{r_G} = \frac{1}{d_{FG}}SS_G - \frac{1}{d_{Fe}}SS_e (14)
\]

where \( SS_G \) and \( SS_e \) are sums of squares for the between entry and residual effect in LMM (1) and \( SS_G = SS_{M1} + SS_{G:M1} \). After substituting \( SS_{M1} + SS_{G:M1} \) for \( SS_G \) in (14), we obtained:

\[
(14) \text{ cont.} = \frac{1}{d_{FG}} (SS_{M1} + SS_{G:M1}) - \frac{1}{d_{Fe}} SS_e
= \frac{1}{d_{FG}} [d_{FG}(\hat{\sigma}_G^2 + r_{G:1}\hat{\sigma}_{G:1}^2 + r_{G:M1}\hat{\sigma}_{G:M1}^2)] - \frac{1}{d_{Fe}} d_{Fe}\hat{\sigma}_e^2
= \frac{df_{FG}}{d_{FG}} \hat{\sigma}_G^2 + \frac{df_{GM1}}{d_{FG}} \hat{\sigma}_{G:1}^2 + \frac{df_{GM1}}{d_{FG}} \hat{\sigma}_{G:M1}^2
= \frac{df_{GM1}}{d_{FG}} \hat{\sigma}_{M1}^2 + \frac{df_{GM1}}{d_{FG}} \hat{\sigma}_{G:M1}^2
= k_{M1}\hat{\sigma}_{M1}^2 + \hat{\sigma}_{G:M1}^2 (15)
\]

From (15), the sum of ANOVA estimators of \( \sigma_{M1}^2 \) and \( \sigma_{G:M1}^2 \) from LMM (1) is greater than the ANOVA estimator of \( \sigma_G^2 \) from LMM (2):

\[
\hat{\sigma}_{M1}^2 + \hat{\sigma}_{G:M1}^2 > \hat{\sigma}_G^2 = k_{M1}\hat{\sigma}_{M1}^2 + \hat{\sigma}_{G:M1}^2 (16)
\]

Hence, \( \hat{\sigma}_{M1}^2 + \hat{\sigma}_{G:M1}^2 \) overestimates \( \hat{\sigma}_G^2 \) by \((1-k_{M1})\hat{\sigma}_{M1}^2\).
where \( u_{G:M1} \) is a vector of random effect nested in M1 effects. Hence, from (14) and (15), the sum of AMV estimators of \( \sigma^2_{G1} \) and \( \sigma^2_{G:M1} \) is greater than the ANOVA estimator of \( \sigma^2_G \):

\[
\hat{\theta}_{AMV}^{G:M1} + \hat{\theta}_{AMV}^{G} \geq \sigma^2_G
\]

**Average Semi-Variance Estimators**

The average semi-variance or average variance of differences among observations leads to a definition of the total variance that provides a natural way to account for heterogeneity of variance and covariance among observations (Piepho 2019; Schmidt et al. 2019a). ASV can be defined for any variance-covariance structure in a generalized LMM and allows for missing and unbalanced data. The ASV measure of total variance is half the average total pairwise variance of a difference between entries:

\[
\hat{\theta}^{ASV} = (n_G - 1)^{-1} \text{tr}(VP_{nc})
\]

where \( P_{nc} = I_{nc} - n_G^{-1} I_{nc} \), is an \( n_G \times n_G \) identity matrix, and \( I_{nc} \) is an \( n_G \times n_G \) unit matrix (Piepho 2019). \( \hat{\theta}^{ASV} \) accounts for the variances and covariances of the phenotypes. By comparison, the sample variance of the adjusted entry-level means (\( \bar{y}_c \)) is:

\[
s^2_{\bar{y}} = (n_G - 1)^{-1} \sum_{i=1}^{n} (\bar{y}_i - \bar{y}_c)^2
\]

where \( \bar{y}_c = n_G^{-1} \sum_{i=1}^{n} \bar{y}_i = n_G^{-1} T_{nc} \bar{y}_c \). Interestingly, the expected value of \( s^2_{\bar{y}} \) is:

\[
E(s^2_{\bar{y}}) = \frac{(n_G - 1)^{-1} E(\bar{y}^T P_{nc} \bar{y})}{n_G - 1} = \hat{\theta}^{ASV}
\]

(Estaghvirou et al. 2013). The ASV measure of total variance can be decomposed into independent sources of variance (e.g., genetic and residual) according to LMM (12):

\[
\text{tr}(VP_{nc}) = \text{tr}(GP_{nc}) + \text{tr}(RP_{nc}) = \sum_c \text{tr}(Z_c G Z_c^T P_{nc}) + \text{tr}(RP_{nc})
\]

Thus,

\[
\hat{\theta}^{ASV}_c = \sum_c \hat{\theta}^{ASV} + \hat{\theta}^{ASV}_{\bar{y}}
\]

where \( \hat{\theta}^{ASV}_{\bar{y}} = (n_G - 1)^{-1} \text{tr}(Z_c G Z_c^T P_{nc}) \) is the variance explained by the \( c \)th genetic factor (\( u_c \)), genetic factors are marker loci, interactions between marker loci, and entries nested in marker loci, and \( \hat{\theta}^{ASV} = (n_G - 1)^{-1} \text{tr}(RP_{nc}) \) is the residual variance.

Equation (17) can be decomposed into the expected values of the sample variances of the random effects:

\[
E(s^2_{\bar{y}}) = \frac{(n_G - 1)^{-1} E(\bar{y}^T P_{nc} \bar{y})}{n_G - 1} = (n_G - 1)^{-1} \sum_c E(u_c^T Z_c^T P_{nc} Z_c u_c) + E(e^T P_{nc} e)
\]

\[
= \sum_c E(s^2_{\bar{y}}) + E(s^2_e)
\]
Simulations

The systematic biases of AMV estimators of \( p \) and \( H_M^2 \) were verified by analyses of simulated samples for 21 different study designs and parameter combinations (Figures 1-4; Supplemental Table 2). As predicted by (15) and (18), ASV estimates were unbiased, whereas AMV estimates were upwardly biased across simulation studies. The magnitude of the bias increased as \( H_M^2 \) increased but was proportionally constant (Figure 1). For a single locus with balanced data, for example, \( H_M^2 \), and bias increased as the effect of the QTL increased (Figure 1A). For experiments with multiple loci, the bias of the ANOVA estimators of marker-marker interactions was less than that for individual marker loci as a function of the underlying bias-correction coefficients (\( k_M \)). This suggests that as the number of observed levels of marker-marker interaction approaches the number of entries (\( n_C \)), the value of \( k_M \) approaches 1.0.

The bias was greater for unbalanced than for balanced data (Fig. 1). As shown for an F2 population segregating 1 AA : 2 Aa : 1 aa for a single marker locus, AMV and ASV estimates of \( H_M^2 \) were significantly more variable for unbalanced than balanced data (Fig. 1). The effect of unbalanced data was more extreme for the F2 simulation (Fig. 1D) than for simulations where 10-33% of the observations were missing for markers with roughly equal numbers of replications/marker genotype (Fig. 1E-F). The increased dispersion of AMV and ASV estimates for the F2 simulation (Figure 1D) relative to estimates for the other simulations (Figure 1A-C and E-F) resulted from lower precision of the variance component estimates. Even though the ASV estimates are unbiased, sampling variances among the simulated F2 samples were larger and yielded a small percentage of \( H_M^2 \) estimates slightly outside the theoretical range (Figure 1D). For the other simulations (Fig. 1A-C and E-F), none of the ASV estimates exceeded 1.0.

The relative biases were not affected by the number of replications of entries or the number of entries, although the precision of \( \sigma_M^2 \) estimates increased as \( n_C \) increased (Figures 2-3). Similarly, the relative biases were not affected by \( H_M^2 \), however, estimate precision was strongly affected by \( H_M^2 \) (Figure 4). These simulations show that the relative biases of the AMV estimators of \( \sigma_M^2 \) and \( H_M^2 \) are mathematically constant for a given experiment and study design and that the bias-correction we applied yielded unbiased estimates of \( \sigma_M^2 \), \( p \), and \( H_M^2 \) (Figures 1-4).

Case Study #1: Three Marker Loci With Slightly Unbalanced Genotypic Data

We estimated the marker-associated genetic variances for three loci (\( B, P \), and \( HYP \)) segregating in a sunflower RIL mapping population (Tang et al. 2006) using LMM (13) (Table 4). Uncorrected (AMV) and bias-corrected (ASV) REMI estimates of the marker-associated genetic variances for \( HYP \), \( B \times P \), and \( B \times P \times HYP \) were zero (Table 4). \( HYP \) was retained in the model because the marker-associated variance for \( B \times HYP \) was non-zero. The AMV estimate of \( \sigma_M^2 \) (17.85) was greater than the ASV estimate of \( \sigma_M^2 \) (9.24) for the full three-locus model, as predicted by (15) and (18). Similarly, the AMV estimate of marker heritability (0.79) was nearly two-fold greater than the ASV estimate of marker heritability (0.41) for the full three-locus model. The AMV estimate of the contribution of the \( B \) locus to marker heritability (0.51) was double that of the ASV estimate (0.25). Hence, AMV yielded inflated estimates of the contributions of these marker loci to the heritable variation for the trait under study in this population (seed oil concentration).

The ASV (bias-corrected AMV) estimate for the full three-locus model suggests that the intra- and interlocus effects of these loci explained 43.0% of the genetic variation (\( \hat{p} = 9.24/21.61 = 0.43 \)), as opposed to 79.0% for the upwardly biased AMV estimate (\( \hat{p} = 17.85/21.61 = 0.82 \)) (Table 4).

Case Study #2: A Single Marker Locus With Highly Unbalanced Genotypic Data

The application of the bias-correction is illustrated here for highly unbalanced genotypic data. Variance components were estimated for two SNP markers (AX493 and AX396) in LD with a gene (Fw1) affecting resistance to Fusarium wilt in a strawberry GWAS population (\( n_C = 564 \)) that was genotyped with a genome-wide framework of SNP markers (Pinrot et al. 2018). Both SNP markers were highly significant with \( \log_{10} p \)-values of 6.61 \times 10^{-31} \) for AX493 and 2.95 \times 10^{-222} \) for AX396. Genotype frequencies were highly unbalanced for both markers with a scarcity of AA homozygotes (2.8%) for AX396 (16A A : 177A a : 371aa) and 1 : 2 : 1 genotypic ratio for AX493 (141AA : 282Aa : 141aa). The \( k_M \) for these data (\( k_{AX493} = 0.62 \) and \( k_{AX396} = 0.47 \)) were calculated as shown in Appendix B.

The AMV estimate of \( H_M^2 \) for AX396 exceeded 1.0, a telltale sign of \( k_M \)-bias (Table 4). AMV estimates of \( \sigma_M^2 \) and \( H_M^2 \) for both SNP markers were double or nearly double their ASV estimates (Table 4). The bias-corrected ASV estimate for AX396 suggested that the causal locus (Fw1) explained approximately 62% of the genetic variance for resistance to Fusarium wilt (\( \hat{p}_M = 0.62 \), versus a non-sensical 133% for the biased AMV estimate (\( \hat{p}_M = 1.33 \)). Even with bias correction, the sum of ASV estimates of \( \sigma_M^2 \) and \( H_M^2 \) for AX396 was slightly greater than the ASV estimate of \( \sigma_M^2 \). This result was consistent with the pattern we observed for highly unbalanced marker genotypic data in our simulation studies where a certain fraction of bias-corrected estimates exceeded the theoretical limit for heritability because of decreased precision (Figure 1). The \( k_M \)-bias problem would not necessarily have been detected in the analysis of AX396 because the \( p \) and \( H_M^2 \) estimates fell within the acceptable range (0.1), e.g., (\( \hat{\theta}_{ASV, AX396} + \hat{\theta}_{ASV, AX396} / \hat{\theta}_p \) = 0.71). This estimate was nevertheless still upwardly biased (Table 4).

Discussion

We originally suspected that selection bias was the culprit behind the overestimation of \( p \) and \( H_M^2 \) (Beavis et al. 1994; Xu 2003; Bernardo 2004); however, the \( k_M \)-bias problem is purely statistical in nature and operates in combination with selection bias to inflate estimates of the importance (statistical contributions) of QTL to the heritable variation in a population (Table 3; Appendices A, B, and C). Although the concept of marker heritability was introduced early in the application of genome-informed approaches in quantitative genetics, \( \sigma_M^2 \), \( p \), and \( H_M^2 \) have not been widely estimated (Lande and Thompson 1990; Utz et al. 2000; Walsh 2001; Bernardo 2004; Visscher et al. 2006; Bernardo 2008; Visscher et al. 2008; Mackay et al. 2009; Manolio et al. 2009; Cockram and Mackay 2018; Schmidt et al. 2019b; Wang and Xu 2019). Our results show that the overestimation of \( p \) and \( H_M^2 \) is greatest for large-effect QTL, which were the initial focus of our study (Figure 1). As shown in the empirical examples, the overestimation problem would only be obvious in practice for cases where estimates of \( p \) and \( H_M^2 \) exceeded 1.0, as was the case in the two empirical examples we examined (Table 4). The two-fold difference between ASV estimates of \( H_M^2 \) for the two
SNP markers in the strawberry example illustrate the challenge with accurately estimating the proportion of the heritable variation associated with a marker. Because neither SNP appears to be a causal variant, the difference in the $H_M^2$ estimates stems from historic recombination between the causal locus (Fru1) and the SNP marker loci (AX936 was significantly more predictive than AX493). Regardless, the contributions of both loci were overestimated by AMV estimates of $H_M^2$.

Genomic heritability is routinely estimated in GWAS and genomic prediction studies where one of the goals is to accurately estimate the proportion of the genetic variance associated with thousands of DNA markers, in addition to estimating the 'missing' heritability (Vischer et al. 2006, 2008; VanRaden 2008; de los Campos et al. 2015). As shown by de los Campos et al. (2015), the mathematical relationship between heritability (estimated using family or pedigree relationships) and genomic heritability (estimated using genotypic relationships) is complex and not completely clear (Legarra et al. 2009). However, the proposed definition of marker heritability can be linked to genomic heritability, as described in Schmidt et al. (2019a), through the VanRaden (2008) definition of genomic relatedness. We used different terms to distinguish these parameters, one of which is directly estimated from statistically significant marker genotypes (marker heritability) and another of which is estimated from genetic relationship matrices (genomic heritability). For practical purposes, $H_M^2$ is the proportion of the heritability associated with one or more statistically significant markers, as opposed to the proportion associated with a genome-wide sample of both non-significant and statistically significant markers (Vischer et al. 2006, 2008; de los Campos et al. 2015).

Variance component ratios similar to $p$ and $H_M^2$ are used in diverse fields of study, most commonly to estimate the fraction of the total variance of a dependent variable associated with a specific factor or independent variable (Searle 1995; Sun et al. 2010; Piepho 2019; Schmidt et al. 2019a,b). These parameters include repeatability, intra-class correlation, and coefficient of determination (Hill and Nicholas 1974; Utz et al. 2000; Schmidt et al. 2019a,b; Piepho 2019). The mathematical definitions and interpretations of these parameters are analogous to heritability (Robertson 1959; Piepho and Möhring 2007; Benfey and Mitchell-Olds 2008; Sun et al. 2010; Isik et al. 2017; Schmidt et al. 2019a,b).

We suspect that biases similar to those described for marker heritability could be hidden in the estimators of these parameters, a problem that warrants further study. The approach we described appears to have applications in fields of study other than genetics to obtain unbiased estimates of the relevant parameters.

**Abbreviations**

AMV: average marginal variance; ANOVA: analysis of variance; ASV: average semi-variance; GEBV: genome estimates breeding value GWAS: genome-wide association study; LD: linkage disequilibrium; MAS: marker assisted selection; QTL: quantitative trait locus; REML: restricted maximum likelihood; SNP: single nucleotide polymorphism; WGR: whole-genome regression.

**Author’s Contributions**

MJF, WCB, and SJK conceived the overall project. MJF performed the analyses. MJF, HPP, WCB, and SJK wrote the paper.

**Competing Interests**

The authors declare no competing interests.

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Appendices

Appendix A. ASV Estimators of Marker-Associated Genetic Variances for Two Loci With Balanced Data

Here we develop the ASV estimator of the variance explained by markers for an experiment where $n_G$ entries are phenotyped for a normally distributed quantitative trait in a completely randomized experiment design, the data are balanced, and the variance components for the random effects of two marker loci (M1 and M2) are estimated using LMM (2):

$$y_{hijk} = \mu + M_1 h + M_2 i + (M_1 \times M_2)_{hi} + G : (M_1 \times M_2)_{hij} + \epsilon_{hijk}$$  \hspace{1cm} (20)$$

where $y_{hijk}$ is the $hijk$-th phenotypic observation, $\mu$ is the population mean, $h = 1, 2, 3, i = 1, 2, 3, j = 1, 2, \ldots$, $n_G$, $k = 1, 2, \ldots$, $r_G$, $M_1$ is the random effect of marker locus 1 with $var(M_1) = \sigma^2_{M_1}$, $M_2$ is the random effect of marker locus 2 with $var(M_2) = \sigma^2_{M_2}$, $(M_1 \times M_2)_{hi}$ is the random effect of the interaction between marker loci 1 and 2 with $var((M_1 \times M_2)_{hi}) = \sigma^2_{M_1M_2}$, $G : (M_1 \times M_2)_{hij}$ is the random effect of entries nested in marker loci with $var(G : (M_1 \times M_2)_{hij}) = \sigma^2_{G(M_1 \times M_2)}$ and $\epsilon_{hijk}$ is the residual effect with $var(\epsilon_{hijk}) = \sigma^2_{\epsilon}$.

The entry-mean or ASV model for the analysis of two loci is:

$$\hat{y}_{hij} = \mu + M_1 h + M_2 i + (M_1 \times M_2)_{hi} + G : (M_1 \times M_2)_{hij} + \hat{\epsilon}_{hij}.$$

where $\hat{y}_{hij}$ is the entry-mean, $\hat{\epsilon}_{hij}$ is the residual with $var(\hat{\epsilon}_{hij}) = \sigma^2_{\epsilon}$, and the other variables are as defined for (20).

The variance explained by the marker effect ($\hat{\theta}$) using the ASV approach (Piepho 2019) is:

$$\hat{\theta}^{ASV}_{M_1} = (n_G - 1)^{-1} \frac{\hat{\sigma}^2_{M_1} tr(Z_{h_{M_1}} Z^T_{h_{M_1}} P)}{\sigma^2_{M_1}}$$  \hspace{1cm} (21)

$$= \frac{(n_G - 1) n_{G(M_1 \times M_2)} n_{G(M_1 \times M_2)} \hat{\sigma}^2_{M_1}}{\sigma^2_{M_1}}$$  \hspace{1cm} (22)

$$= \frac{(d_f M_1 + d_f M_2) n_{G(M_1 \times M_2)} \hat{\sigma}^2_{M_1 \times M_2}}{d_f G}$$

where $Z_{h_{M_1}} = I_{r_{G(M_1 \times M_2)}} \otimes 1_{r_{G(M_1 \times M_2)}}$.

Finally, the variance explained by the random effect of entries nested in marker loci ($\hat{\theta}^{ASV}_{G(M_1 \times M_2)}$) is:

$$\hat{\theta}^{ASV}_{G(M_1 \times M_2)} = (n_G - 1)^{-1} \frac{\hat{\sigma}^2_{G(M_1 \times M_2)} tr(Z_{h_{G(M_1 \times M_2)}} Z^T_{h_{G(M_1 \times M_2)}}, P)}{\sigma^2_{G(M_1 \times M_2)}$$

$$= \frac{n_G - 1}{n_G - 1} \frac{\hat{\sigma}^2_{G(M_1 \times M_2)}}{\sigma^2_{G(M_1 \times M_2)}}$$

$$= \frac{\theta^2_{G(M_1 \times M_2)}}{\sigma^2_{G(M_1 \times M_2)}}$$

From (21), the coefficient for bias-correcting AMV estimates of $\sigma^2_{M_1}$ (the genetic variance explained by M1) is:

$$k_{M_1} = \frac{d_f M_1 n_{G(M_1 \times M_2)} n_{G(M_1 \times M_2)}}{d_f G}$$

This definition of $k_{M_1}$ is equivalent to the definitions in Table 3 with $r_G$ factored out. Similarly, from (22), the coefficient for bias-correcting AMV estimates of $\sigma^2_{M_2}$ (the genetic variance explained by the interaction between M1 and M2) is:

$$k_{M_2} = \frac{d_f M_2 n_{G(M_1 \times M_2)} n_{G(M_1 \times M_2)}}{d_f G}$$

The ASV definition of the total genetic variance is:

$$\theta^{ASV}_{G} = \theta^{ASV}_{M_1} + \theta^{ASV}_{M_2} + \theta^{ASV}_{G(M_1 \times M_2)} + \theta^{ASV}_{G(M_2 \times M_2)}$$

The ANOVA estimators of the variance components for LMM (1) with balanced data are previously defined in (3) and (4). The ANOVA estimators of $\sigma^2_{M_1}$, $\sigma^2_{M_2}$, $\sigma^2_{G(M_1 \times M_2)}$, and $\sigma^2_{G(M_2 \times M_2)}$ for LMM (20) are:

$$\hat{\sigma}^2_{M_1} = \frac{MS_{M_1} - MS_{M_1 \times M_2}}{r_G n_{G(M_1 \times M_2)} n_{M_1}}$$  \hspace{1cm} (24)$$

and

$$\hat{\sigma}^2_{M_2} = \frac{MS_{M_2} - MS_{M_1 \times M_2}}{r_G n_{G(M_1 \times M_2)} n_{M_2}}$$  \hspace{1cm} (25)$$

and

$$\hat{\sigma}^2_{G(M_1 \times M_2)} = \frac{MS_{G(M_1 \times M_2)} - MS_{M_1 \times M_2}}{r_G n_{G(M_1 \times M_2)}}$$  \hspace{1cm} (26)$$

and

$$\hat{\sigma}^2_{G(M_2 \times M_2)} = \frac{MS_{G(M_2 \times M_2)} - MS_{M_2}}{r_G n_{G(M_2 \times M_2)}}$$  \hspace{1cm} (27)$$

where $MS_{M_1}$ is the expected mean sum of squares for marker locus 1, $MS_{M_2}$ is the expected mean sum of squares for marker locus 2, and so on for the other expected mean sum of squares from the Table 1.

Thus, the total genetic variance can be estimated by substituting the bias-corrected ANOVA estimators (24 - 27) into (23):
When the entries are adjusted means (BLUEs), the ASV definition of marker heritability is:

$$\hat{\sigma}_G^2 = \frac{d\hat{\sigma}_{M1}}{df_G} (MS_{M1i} - MS_{M1M2}) + \frac{d\hat{\sigma}_{M2}}{df_G} (MS_{M2i} - MS_{M1M2}) + \frac{d\hat{\sigma}_{M1M2}}{df_G} (MS_{M1M2i} - MS_{M1M2}) \times (MS_{M1M2i} - MS_{M1M2}) + \frac{1}{r_G} (MS_{M1M2i} - MS_G)
$$

Substituting the appropriate values for (28) yields the bias-corrected estimates ($H_{M1i}$).

**Appendix B. ASV Estimator of the Marker-Associated Genetic Variance for One Locus with Unbalanced Data**

ASV estimators of $\sigma^2_{M1}$ and $\sigma^2_{M2}$ are developed here for unbalanced data. As before, the phenotypic observations are entry-means ($\bar{y}_{hi}$) and the LMM for the entry-mean analysis is (9). In vector notation, (9) can be written as:

$$\bar{y} = \mu + (\bar{y}_{hi} 1_{gM1i}) uM1 + (\bar{y}_{hi} 1_{gM2i}) uM2 + (\bar{y}_{hi} 1_{gM1M2i}) uM1M2 + \bar{e}_i$$

where $n_{gM1i}$ is the number of entries nested in the $h$th marker genotype, $n_G = \sum_h n_{gM1i}$, and observations are sorted by markers and entries within markers. The vectors $uM1, uM2, uM1M2$ and $G : M1h$ hold the random effects $M1h, M2h, M1h \times M2h, M1h \times M2h$ with $var(uM1) = I_{M1} \sigma^2_{M1}$, $var(uM2) = I_{M2} \sigma^2_{M2}$, $var(uM1M2) = I_{M1M2} \sigma^2_{M1M2}$, and $var(uG:M1M2) = I_{G:M1M2} \sigma^2_{G:M1M2}$, respectively. The proportion of the genetic variance explained by the marker locus is:

$$\hat{\sigma}_{M1M2}^2 = (n_G - 1)^{-1} \hat{\sigma}_{M1M2}^2 \bar{tr}(Z_{M1M2} Z_{M1M2}^T)$$

where $P = I_{M1} - n_{g1} 1_{gM1i}, Z_{M1i} = \otimes_h 1_{gM1i}$ and $SS_{gM1} = \sum_h (n_{gM1i} - n_{gM1})^2$. For the balanced case, $SS_{gM1} = 0$ and $n_G / n_{M1} = n_{gM1}$. The residual genetic variance associated with the entry nested in $M1$ genotypes is:

$$\hat{\sigma}_{G:M1}^2 = (n_G - 1)^{-1} \hat{\sigma}_{G:M1}^2 \bar{tr}(Z_{G:M1} Z_{G:M1}^T)$$

Hence, the general form for $k_{M1}$ is:

$$k_{M1} = \frac{n_G - n_{g1}}{df_G} \sum_{k} n_{gM1k} \hat{\sigma}_{G:M1k}^2$$

**Appendix C. ASV Estimator of the Marker-Associated Genetic Variance for Two Loci with Unbalanced Data**

ASV estimators for an experiment with two loci are developed here for unbalanced data. As before, the phenotypic observations are entry-means ($\bar{y}_{hi}$) and the LMM for the entry-mean analysis is (20). In vector notation, (20) can be written as:

$$\bar{y} = \mu + (\bar{y}_{hi} 1_{gM1i}) uM1 + (\bar{y}_{hi} 1_{gM2i}) uM2 + (\bar{y}_{hi} 1_{gM1M2i}) uM1M2 + \bar{e}_i$$

where $n_{gM1M2i}$ is the number of entries nested in the $h$th marker genotype, $n_{G} = \sum_h n_{gM1M2i}$, and observations are sorted by markers and entries within markers. The vectors $uM1, uM2, uM1M2$ and $G : M1h$ hold the random effects $M1h, M2h, M1h \times M2h, M1h \times M2h$ with $var(uM1) = I_{M1} \sigma^2_{M1}$, $var(uM2) = I_{M2} \sigma^2_{M2}$, $var(uM1M2) = I_{M1M2} \sigma^2_{M1M2}$, and $var(uG:M1M2) = I_{G:M1M2} \sigma^2_{G:M1M2}$, respectively. The proportion of the genetic variance explained by the marker locus is:

$$\hat{\sigma}_{M1M2}^2 = (n_G - 1)^{-1} \hat{\sigma}_{M1M2}^2 \bar{tr}(Z_{M1M2} Z_{M1M2}^T)$$

where $P = I_{M1} - n_{g1} 1_{gM1i}, Z_{M1i} = \otimes_h 1_{gM1i}$ and $SS_{gM1} = \sum_h (n_{gM1i} - n_{gM1})^2$. For the balanced case, $SS_{gM1} = 0$ and $n_G / n_{M1} = n_{gM1}$. The residual genetic variance associated with the entry nested in $M1$ genotypes is:

$$\hat{\sigma}_{G:M1}^2 = (n_G - 1)^{-1} \hat{\sigma}_{G:M1}^2 \bar{tr}(Z_{G:M1} Z_{G:M1}^T)$$

From (29), the coefficient for bias-correcting AMV estimates of $\sigma^2_{M1}$ (the genetic variance explained by $M1$) is:
where

\[ Z \] is the matrix of observations, and \( n \) is the number of entries nested in the

interaction between

Similarly, from (30), the coefficient for bias-correcting AMV estimates of \( \sigma^2_{M1 \times M2} \) (the genetic variance explained by the interaction between \( M1 \) and \( M2 \)) is:

\[
\hat{\theta}^{\text{ASV}}_{G:M1 \times M2} = \frac{n_G - n_G^{-1} \sum_i n_i^2 \hat{G}_{G:M1 \times M2}}{d_f G} \tag{31}
\]

where \( P = I_{n_G} - n_G^{-1} \sum_i n_i^2 \hat{G}_{G:M1} \otimes 1_{G:M1 \times M2} \). The variance explained by the interaction between marker loci 1 and 2 (\( u_{M1 \times M2} \)) using the ASV approach is:

\[
\hat{\theta}^{\text{ASV}}_{M1 \times M2} = \frac{n_G - n_G^{-1} \sum_i n_i^2 u_{M1 \times M2}}{d_f G} \hat{G}_{M1 \times M2} \tag{32}
\]

Similarly, the variance explained by the random effect of entries nested in marker loci (\( u_{G:M1 \times M2 \times M3} \)) is:

\[
\hat{\theta}^{\text{ASV}}_{G:M1 \times M2 \times M3} = \frac{n_G - n_G^{-1} \sum_i n_i^2 \hat{G}_{G:M1 \times M2 \times M3} \text{tr}(Z_{G:M1 \times M2 \times M3} Z_{G:M1 \times M2 \times M3}^T P)}{d_f G} \tag{33}
\]

Finally, the variance explained by the random effect of entries nested in marker loci (\( u_{G:M1 \times M2 \times M3} \)) is:

\[
\hat{\theta}^{\text{ASV}}_{G:M1 \times M2 \times M3} = \frac{n_G - n_G^{-1} \sum_i n_i^2 \hat{G}_{G:M1 \times M2 \times M3} \text{tr}(Z_{G:M1 \times M2 \times M3} Z_{G:M1 \times M2 \times M3}^T P)}{d_f G} \tag{34}
\]

where \( P = I_{n_G} - n_G^{-1} \sum_i n_i^2 \hat{G}_{G:M1 \times M2 \times M3} \otimes 1_{G:M1 \times M2 \times M3} \).
Table 1 Analyses of variance for LMMs (1) and (2) with balanced data where \( r_G \) replicates of \( n_G \) entries (individuals or families) are phenotyped for a normally distributed quantitative trait, replicates are arranged in a completely randomized experiment design, and parameters are estimated for one marker locus (M1) or two marker loci (M1 and M2). Sources of variation, degrees of freedom (df), mean squares (MS), and expected mean squares (E[MS]) are shown for ANOVA estimates of \( \sigma_G^2 \) for LMM (1) and ANOVA estimates of \( \sigma_M^2 \) for marker locus M1, \( \sigma_M^2 \) for marker locus 2, and \( \sigma_{M1-M2}^2 \) for the interaction between marker loci M1 and M2 using LMM (2). ANOVA breakdowns are shown for a single marker locus (M1) or two marker loci (M1 and M2). The residuals are identical for the three analyses.

| Source | \( df^a \) | MS | E[MS] \( b \) |
|--------|-----------|----|---------------|
| Entry (G) | \( d_{fg} = n_G - 1 \) | \( MS_G = \frac{SS_G}{df_G} \) | \( \sigma_e^2 + r_G \sigma_G^2 \) |
| M1 | \( d_{fM1} = n_{M1} - 1 \) | \( MS_{M1} = \frac{SS_{M1}}{df_{M1}} \) | \( \sigma_e^2 + r_G \sigma_G^2 + r_G \sigma_{M1}^2 \) |
| M2 | \( d_{fM2} = n_{M2} - 1 \) | \( MS_{M2} = \frac{SS_{M2}}{df_{M2}} \) | \( \sigma_e^2 + r_G \sigma_G^2 + r_G \sigma_{M2}^2 \) |
| M1 \( \times \) M2 | \( d_{fM1 \times M2} = (n_{M1} - 1)(n_{M2} - 1) \) | \( MS_{M1 \times M2} = \frac{SS_{M1 \times M2}}{df_{M1 \times M2}} \) | \( \sigma_e^2 + r_G \sigma_G^2 + r_G \sigma_{M1}^2 + r_G \sigma_{M2}^2 \) |
| G \( \times \) M1 | \( d_{fG \cdot M1} \) | \( MS_{G \cdot M1} = \frac{SS_{G \cdot M1}}{df_{G \cdot M1 \times M2}} \) | \( \sigma_e^2 + r_G \sigma_G^2 \) |
| Residual | \( d_{fe} = n_G(r_G - 1) \) | \( MS_e = \frac{SS_e}{df_e} \) | \( \sigma_e^2 \) |

\( ^a \) \( n_G = \) number of entries, \( n_{M1} = \) number of genotypes for marker locus 1, \( n_{M2} = \) number of genotypes for marker locus 2, \( n_{G:M1} = \) number of entries nested in genotypes for marker locus 1 and 2, \( n_{G:M2} = \) number of entries nested in genotypes for marker locus 1 and 2, and \( n_f = \) number of independent observations.

\( ^b \) \( \sigma_G^2 = \) genetic variance among entries, \( \sigma_{M1}^2 = \) genetic variance explained by marker locus 1, \( \sigma_{M2}^2 = \) genetic variance explained by marker locus 2, \( \sigma_{M1 \times M2}^2 = \) genetic variance explained by the interaction between marker locus 1 and 2, \( \sigma_{G:M1}^2 = \) residual genetic variance among entries nested in marker locus 1, and \( \sigma_{G:M1 \times M2}^2 = \) residual genetic variance among entries nested in marker locus 1 and 2.
Table 2 Simulation Study Designs and Variables. One thousand samples were simulated for each of 21 study designs with different marker heritabilities ($H^2_M$), $n_M = 3$ genotypes/marker locus, one to three marker loci ($m$), $r_M$ replicates/marker genotype, $n_G$ entries, $r_G$ replicates/entry, and $n$ total observations. The number of replications/marker genotype for study design 4 was equivalent to the expected number for the segregation of a co-dominant DNA marker in an $F_2$ population (1 AA : 2 Aa : 1 aa). The $r_M$, $n_G$, and $r_G$ for study designs 5 and 6 varied because 10% and 33% of the data were randomly deleted (treated as missing).

| Study Design | $m$ | $r_M$ | $n_G$ | $r_G$ | $n$ | $H^2_M$ Range |
|--------------|-----|-------|-------|-------|-----|----------------|
| 1            | 1   | 900   | 540   | 5     | 2,700 | (0.0, 1.0)     |
| 2            | 2   | 900   | 540   | 5     | 2,700 | (0.0, 1.0)     |
| 3            | 3   | 900   | 540   | 5     | 2,700 | (0.0, 1.0)     |
| 4            | 1   | 675:1,350:675 | 540   | 5     | 2,700 | (0.0, 1.0)     |
| 5            | 1   | $\leq$900 | $\leq$540 | $\leq$5 | 2,430 | (0.0, 1.0)     |
| 6            | 1   | $\leq$900 | $\leq$540 | $\leq$5 | 1,800 | (0.0, 1.0)     |
| 7            | 1   | 600   | 900   | 2     | 1,800 | 0.50           |
| 8            | 1   | 1,500 | 900   | 5     | 4,500 | 0.50           |
| 9            | 1   | 3,000 | 900   | 10    | 9,000 | 0.50           |
| 10           | 1   | 6,000 | 900   | 20    | 18,000| 0.50           |
| 11           | 1   | 15,000| 900   | 50    | 45,000| 0.50           |
| 12           | 1   | 750   | 450   | 5     | 2,250 | 0.50           |
| 13           | 1   | 1,500 | 900   | 5     | 4,500 | 0.50           |
| 14           | 1   | 3,000 | 1,800 | 5     | 9,000 | 0.50           |
| 15           | 1   | 6,000 | 3,600 | 5     | 18,000| 0.50           |
| 16           | 1   | 12,000| 7,200 | 5     | 36,000| 0.50           |
| 17           | 1   | 750   | 450   | 5     | 2,250 | 0.05           |
| 18           | 1   | 750   | 450   | 5     | 2,250 | 0.25           |
| 19           | 1   | 750   | 450   | 5     | 2,250 | 0.50           |
| 20           | 1   | 750   | 450   | 5     | 2,250 | 0.75           |
| 21           | 1   | 750   | 450   | 5     | 2,250 | 0.95           |

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Figure 1 Accuracy of AMV and ASV Estimates of Marker Heritability. AMV and ASV estimates of $H^2_M$ are shown for 1,000 segregating populations simulated for different numbers of entries ($n_G$ individuals or families), five replications/entry ($r_G = 5$), true marker heritabilities ranging from 0 to 1, and one to three marker loci with three genotypes/marker locus ($n_M = 3$). True marker heritabilities are shown on the x-axis. Uncorrected and bias-corrected estimates of $H^2_M$ are shown on the y-axis, where $\hat{H}^2_M$ are the uncorrected AMV estimates (red highlighted observations), and $\hat{H}^2_M^*$ are the bias-corrected ASV estimates (blue highlighted observations). AMV and ASV estimates of $H^2_M$ from simulations are shown for: (A) one locus with balanced data for $n_G = 540$ entries (study design 1); (B) two marker loci ($M_1$ and $M_2$) with balanced data for $n_G = 540$ (study design 2); (C) three marker loci ($M_1$, $M_2$, $M_3$, $M_1 \times M_2$, $M_1 \times M_3$, $M_2 \times M_3$, and $M_1 \times M_2 \times M_3$) with balanced data for $n_G = 540$ (study design 3); (D) an $F_2$ population segregating 1:2:1 for one marker locus with $r_M = 675$ for both homozygotes, $r_M = 1,350$ for the heterozygote, and $n_G = 540$ (study design 4); (E) one locus for $n_G \leq 540$ entries where 10% of the phenotypic observations are randomly missing (the data are unbalanced) (study design 5); and (F) one locus for $n_G \leq 540$ entries where 33% of the phenotypic observations are randomly missing (the data are unbalanced) (study design 6).
Figure 2 Effect of $c_0$ on the Bias of $\delta_{M}$ and $\delta_{M}$ Estimates of $\delta_{M}$. Phenotypic observations were simulated for 1,000 progeny located in a single genetic background, and $c_0 = 2.5, 10, 20$, or 30. The relative bias $[\delta_{M}/\hat{\delta}_{M}]$ was identical for different $c_0$.

Table 3 Coefficients ($k_4$) for calculating bias-corrected $\delta_{M}$ estimates.

| Source | $k_4$ | Variance ($\sigma^2$) | Uncorrected | Bias-Corrected |
|--------|-------|-----------------------|-------------|---------------|
| $M_1$  | $k_4$ | $\sigma^2_1$ | $\hat{\sigma}^2_1$ | $\sigma^2_1 - \hat{\sigma}^2_1$ |
| $M_1$  | $k_4$ | $\sigma^2_2$ | $\hat{\sigma}^2_2$ | $\sigma^2_2 - \hat{\sigma}^2_2$ |
| $M_1$  | $k_4$ | $\sigma^2_3$ | $\hat{\sigma}^2_3$ | $\sigma^2_3 - \hat{\sigma}^2_3$ |

$\hat{\sigma}^2_4 = \sigma^2_1 - \hat{\sigma}^2_1$, $\hat{\sigma}^2_5 = \sigma^2_2 - \hat{\sigma}^2_2$, $\hat{\sigma}^2_6 = \sigma^2_3 - \hat{\sigma}^2_3$, and $\hat{\sigma}^2_7 = \sigma^2_4 - \hat{\sigma}^2_4$, $\hat{\sigma}^2_8 = \sigma^2_5 - \hat{\sigma}^2_5$, $\hat{\sigma}^2_9 = \sigma^2_6 - \hat{\sigma}^2_6$, and $\hat{\sigma}^2_10 = \sigma^2_7 - \hat{\sigma}^2_7$. The relative bias $[\hat{\sigma}/\hat{\sigma}]$ was identical for different $c_0$. The relative bias $[\hat{\sigma}/\hat{\sigma}]$ was identical for different $c_0$. $\hat{\sigma}$ is the number of replications of marker genotypes. $\sigma$ is the number of replications of marker genotypes. $\hat{\sigma}$ is the number of replications of marker genotypes. $\sigma$ is the number of replications of marker genotypes.
Table 4 Uncorrected and bias-corrected estimates of marker-associated genetic variances ($\sigma^2_M$) and heritabilities ($H^2_M$) for three marker loci ($B$, $P$, and $HYP$) segregating in a sunflower recombinant inbred line (RIL) population ($n_G = 146$) with balanced data (Tang et al. 2006) and two marker loci (AX396 & AX493) segregating in a strawberry genome-wide association study (GWAS) population ($n_G = 565$) with unbalanced data (Pincot et al. 2018). The marker loci in the sunflower study were associated with QTL affecting seed oil content. The genetic variances associated with those loci were estimating using LMM (13). The marker loci in the strawberry study were associated with a locus ($Fw1$) affecting resistance to Fusarium wilt. The genetic variances associated with those loci were estimated using LMM (2). The coefficients ($k_M$) for bias-correcting AMV estimates of $\sigma^2_M$ were found as described in Table 3 for balanced data and Appendix B for unbalanced data.

| Case Study       | Source (G) | $k_M$ | Variance Component | Uncorrected | Bias-Corrected |
|------------------|------------|------|--------------------|-------------|----------------|
| Tang et al. (2006) | Entry (G) | $\sigma^2_G$ | $\sigma^2_M$ | 21.61 | 0.95 | 21.61 | 0.95 |
|                  | $M + G : M$ | $\sigma^2_B + \sigma^2_G:B \times P \times HYP$ | $\sigma^2_M$ | 30.76 | 1.35 | 22.15 | 0.98 |
|                  | $M$ | $\sigma^2_B + \sigma^2_G:B \times P \times HYP$ | $\sigma^2_M$ | 17.85 | 0.79 | 9.24 | 0.41 |
|                  | $B$ | $\sigma^2_B$ | $\sigma^2_M$ | 11.57 | 0.51 | 5.59 | 0.25 |
|                  | $P$ | $\sigma^2_P$ | $\sigma^2_M$ | 1.26 | 0.06 | 0.60 | 0.03 |
|                  | $HYP$ | $\sigma^2_HYP$ | $\sigma^2_M$ | 2.9 | 0.13 | 1.41 | 0.06 |
|                  | $B \times P$ | $\sigma^2_B \times P$ | $\sigma^2_M$ | 0.21 | 0.01 | 0.17 | 0.01 |
|                  | $B \times HYP$ | $\sigma^2_B \times HYP$ | $\sigma^2_M$ | 1.89 | 0.08 | 1.46 | 0.06 |
|                  | $P \times HYP$ | $\sigma^2_P \times HYP$ | $\sigma^2_M$ | 0.00 | 0.00 | 0.00 | 0.00 |
|                  | $B \times P \times HYP$ | $\sigma^2_B \times P \times HYP$ | $\sigma^2_M$ | 0.00 | 0.00 | 0.00 | 0.00 |
|                  | $G : B \times P \times HYP$ | $\sigma^2_G:B \times P \times HYP$ | $\sigma^2_M$ | 12.91 | 0.57 | 12.91 | 0.57 |
| Residual ($\epsilon$) | $\sigma^2_\epsilon$ | $\sigma^2_M$ | $\sigma^2_\epsilon$ | 2.07 | – | 2.07 | – |
| Pincot et al. (2018) | Entry (G) | $\sigma^2_G$ | $\sigma^2_M$ | 3.30 | 0.98 | 3.30 | 0.98 |
|                   | $M + G : M$ | $\sigma^2_G:AX493 + \sigma^2_G:AX493$ | $\sigma^2_M$ | 4.01 | 1.20 | 3.45 | 1.03 |
|                   | AX493 (M) | $\sigma^2_G:AX493$ | $\sigma^2_M$ | 1.48 | 0.44 | 0.93 | 0.27 |
|                   | $G : AX493$ | $\sigma^2_G:AX493$ | $\sigma^2_M$ | 2.53 | 0.75 | 2.53 | 0.75 |
| Residual ($\epsilon$) | $\sigma^2_\epsilon$ | $\sigma^2_M$ | $\sigma^2_\epsilon$ | 0.48 | – | 0.48 | – |
| Pincot et al. (2018) | Entry (G) | $\sigma^2_G$ | $\sigma^2_M$ | 3.30 | 0.98 | 3.30 | 0.98 |
|                   | $M + G : M$ | $\sigma^2_G:AX396 + \sigma^2_G:AX396$ | $\sigma^2_M$ | 4.77 | 1.42 | 2.39 | 0.71 |
|                   | AX396 (M) | $\sigma^2_G:AX396$ | $\sigma^2_M$ | 4.47 | 1.33 | 2.09 | 0.62 |
|                   | $G : AX396$ | $\sigma^2_G:AX396$ | $\sigma^2_M$ | 0.30 | 0.09 | 0.30 | 0.09 |
| Residual ($\epsilon$) | $\sigma^2_\epsilon$ | $\sigma^2_M$ | $\sigma^2_\epsilon$ | 0.48 | – | 0.48 | – |
Figure 3 Effect of $n_G$ on the Bias of AMV and ASV Estimates of $\sigma^2_{M1}$. Phenotypic observations were simulated for 1,000 populations segregating for a single marker locus with three genotypes ($n_{M1} = 3$), five replications/entry ($n_{M1} = 5$), and $n_G = 450, 900, 1,800, 3,600$, or $7,200$ entries/population (study designs 12-16). The marker locus was assumed to be in complete linkage disequilibrium with a single QTL with marker heritabilities ($H^2_{M1}$) equat to 0.50. (A) Distribution of the relative biases of AMV estimates of $\sigma^2_{M1}$ for different $n_G$. The relative bias ($RB[\theta_{AMV}^{M1}] = 0.499$) was identical across the variables tested. (B) Distribution of the relative biases of ASV estimates of $\sigma^2_{M1}$ for different $n_G$. The relative bias ($RB[\theta_{ASV}^{M1}] = 0.00$) was identical across the variables tested.
Figure 4 Effect of $H^2_{M1}$ on the Bias of AMV and ASV Estimates of $\sigma^2_{M1}$. Phenotypic observations were simulated for 1,000 populations segregating for a single marker locus with three genotypes ($n_{M1} = 3$), five replications/entry ($n_{M1} = 5$), and $n_G = 450$ entries/population. The marker locus was assumed to be in complete linkage disequilibrium with a single QTL with marker heritabilities ($H^2_{M1}$) ranging from 0.05 to 0.95 (study designs 17-21). (A) Distribution of the relative biases of AMV estimates of $\sigma^2_{M1}$ for different $H^2_{M1}$. The relative bias ($RB[\theta^{AMV}] = 0.496$) was identical across the variables tested. (B) Distribution of the relative biases of ASV estimates of $\sigma^2_{M1}$ for different $H^2_{M1}$. The relative bias ($RB[\theta^{ASV}] = 0.0$) was identical across the variables tested.