Core ideas:

1) Morphological nut characteristics are under highly polygenic control in American and American-European interspecific hazelnuts.

2) Best linear unbiased predictors allow for accurate prediction of morphological nut characteristics.

3) Marker density and training population design must be tailored to the sample population for which predictions are being made.

Title: COMPOSITE INTERVAL MAPPING AND GENOMIC PREDICTION OF NUT QUALITY TRAITS IN AMERICAN HAZELNUTS AND AMERICAN-EUROPEAN INTERSPECIFIC HYBRIDS

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ABSTRACT

The native, perennial shrub American hazelnut (*Corylus americana*) is cultivated in the Midwestern U.S. for its significant ecological benefits, as well as its high-value nut crop.

Perennial crops are a long-term breeding effort, and would benefit from the use of genetic data in selection to reduce breeding cycle time. In addition, high-throughput phenotyping methods are essentially to the efficient and accurate screening of large breeding populations. This study reports novel advances in both of these domains, for American (*C. americana*) and interspecific hybrids between European (*C. avellana*) and American hazelnuts. Two populations of hazelnuts, one composed of *C. americana* and one composed of *C. americana* x *C. avellana* hybrids, were phenotyped over the course of two years in two locations using a digital imagery-based method for quantifying morphological nut and kernel traits. This data was used to perform composite interval mapping (CIM) using a recently released genetic map, and genomic prediction using a newly-available chromosome-scale reference genome for *C. americana*. Multiple QTL were detected for all traits analyzed, with an average total R^2 of 52%. Genomic prediction exhibited high accuracy, with an average correlation coefficient between genotypic values and phenotypic observations of 0.78 across both environments. These results suggest that incorporating genetic data in selection is a tenable method for improving genetic gain for highly-polygenic traits in hazelnut breeding programs.

Keywords

Corylus americana, *Corylus avellana*, hazelnut, quantitative genetics, genomic prediction, linkage mapping, composite interval mapping, digital phenotyping

INTRODUCTION

Hazelnuts (*Corylus* spp.) are a globally significant nut crop, with annual production of over 1.3 million tons, produced across 34 countries (FAOSTAT, 2023). Currently, this cultivation is limited to areas with climatic conditions approximating the Mediterranean region in which the dominant cultivars of *C. avellana* were bred (Mehlenbacher and Molnar, 2021). The narrow range of cultivars has limited U.S. production of hazelnuts to the Willamette Valley in Oregon, where over 95% of U.S. acreage is situated (USDA Economic Research Service, 2025). In this context, the native species *C. americana*, which is endemic and well-adapted to much of the eastern U.S., represents a valuable source of genetic diversity for expanding hazelnut production. American hazelnut can exhibit vigorous growth in coldenvironments, and robust resistance to endemic pathogens such as the eastern filbert blight (EFB, *Anisogramma anomala*) (Molnar et al., 2018; Revord et al., 2020).

The genetic improvement of hazelnut is limited by its long generation time. A seedling hazelnut will not typically flower under field conditions for at least three years, and many of the most agronomically important traits (e.g., total kernel yield and bush architecture) require additional time to accurately phenotype. Mature plant yields will often not be fully apparent for a decade, and multi-year harvest data are necessary to precisely quantify such traits. Methods that can make parental and progeny selection decisions more efficient are therefore extremely valuable for a perennial species such as hazelnut, by accelerating this traditional breeding process.

Quantitative genetic tools are essential in this regard. The identification of QTL is essential to the implementation of marker-assisted selection, while genomic prediction can dramatically accelerate genetic gain for polygenic traits (Würschum, 2012; Heslot et al., 2015). The recent

development of genomic resources for *C. americana* have made both of these approaches accessible to breeding programs. First, a genetic map constructed using an F_1 progeny family descended from a cross between Oregon and Midwestern hazelnut varieties 'Jefferson' and 'Eric4-21', respectively, now permits linkage map-based approaches to detecting QTL (Brainard et al., 2023). Such interspecific populations are highly relevant to Midwest breeding efforts, as *C. avellana* remains a valuable source of diversity for nut quality-related traits. The use of linkage mapping populations is well-suited to such populations, where the contrasting phenotypes of parental varieties frequently produces high degrees of segregation in progeny families. In addition, chromosome-scale genome assemblies for the *C. americana* selections 'Rush' and 'Winkler' also now allow for the efficient identification of polymorphisms for use in genomic selection models (Brainard et al., 2024). In this study, a diverse collection of wild *C. americana* was utilized to test such a method. Diversity panels such as this are useful for assessing highly polygenic traits, and developing methods for American hazelnut are appropriate, as this native species not only has potential as a crop for the Upper Midwest, but is also a useful source of traits such as resistance to Eastern Filbert Blight, and cold hardiness.

This study uses both of these tools, in combination with a novel digital imagery-based phenotyping pipeline for precisely quantifying morphological characteristics of inshell hazelnuts and kernels. Recent developments in contour analysis of digital images has led to numerous methods for quantifying the sizes and shapes of plant structures (Hameed et al., 2018). In this study, we present the design and demonstration of such a platform that quantifies the size and shape features of hazelnuts, and validate its performance using two experimental populations: a diversity panel composed of a wild population of *C. americana* sourced from the Wisconsin

Department of Natural Resources, and three F_1 interspecific biparental progeny families generated by the University of Minnesota hazelnut breeding program.

MATERIALS AND METHODS

Plant Materials

Two populations of seedling hazelnut plants were used in this study. One is composed of 473 wild *C. americana* seedlings sourced from the Wisconsin Department of Natural Resources and planted in 2016 at a farm outside of Barneveld, Wisconsin (43.043266 N, -89.926068 W). This population was treated as a "diversity panel", and used to fit and validate genomic prediction models that could be used in introgressing valuable genetic diversity from *C. americana* into Midwest breeding programs. The second is 258 plants which comprise three F_1 families descended from crosses between clonal varieties sourced from the University of Minnesota and Oregon State University, specifically: Eric4-21 x Jefferson ("Eric\Jeff"), Gibs5-15 x OSU-919-031 ("Gibs\OSU"), and Gibs5-15 x York ("Gibs\York"). These were planted at a University of Minnesota research farm in Rosemount, MN (44.695784 N, -93.079196 W) in 2015 and 2016. These represented the biparental families that were used to test both genomic prediction in breeding populations that include *C. avellana*, as well as perform F_1 linkage mapping.

Phenotyping

Morphological characteristics of in-shell hazelnuts and shelled kernels were used as the trait data for this study. This was collected primarily using an adapted version of the digital imagery acquisition and analysis pipeline reported by (Brainard et al., 2021) (Fig. 1). In brief, bushes

were completely harvested by hand in August, 2020 and August 2021. Harvested clusters were dried in the greenhouse and husked. A sample of 30 in-shell nuts were randomly sampled per bush, and weighed in bulk. These nuts were arranged on a 6x5 grid with a QR code, and a Nikon 5600 DSLR camera tethered to a desktop computer was used to acquire a single image. The OpenCV Python library was used to isolate each in-shell nut, and produce a binary mask by applying a fixed HSV threshold to each pixel. An ellipse was then fit to each binary mask, and the length of its major and minor axis was calculated by converting pixel length to physical distance using a scale bar embedded in each image. Circularity of the nut was calculated as the ratio of these two lengths. Each nut was individually cracked, and the kernel was then returned to the grid, preserving the original arrangement of the nuts. A second photo was acquired, and the same traits were calculated. Finally, the kernels were weighed in bulk. This allowed for both a volumetric and gravimetric estimation of the percent kernel for each nut sampled. Python scripts for the image acquisition, processing, phenotyping, and file management are available at: https://github.com/shbrainard/hazelnut-phenotyping. Phenotypic measurements were averaged for each individual plant. The prcomp R function was used to perform PCA on this phenotypic data, and the ggplot2 function autoplot was used to visualize the relationship between these traits.

Sequencing

Roughly 1 cm^2 of leaf tissue was sampled from each bush in May, 2021, immediately following budbreak. Tissue was sampled into 96-well Qiagen Collection Microtubes (Qiagen N.V., Venlo, The Netherlands), and lyophilized using a Labconco 18 L freeze dryer set to 0.004mBar for 72 hours. Freeze-dried tissue was then macerated. DNA extraction, quantification, library

preparation, and sequencing was performed at the University of Wisconsin-Madison Biotechnology Center. Libraries were prepared for genotyping-by-sequencing using a double digestion with the restriction enzymes *NsiI* and *BfaI* following the methodology described by (Elshire et al., 2011). This combination was pre-selected based on an analysis of k-mer distributions of various enzyme digestions, where *NsiI/BfaI* was observed to maximize the k-mer diversity of the library. Illumina adapters and sample-specific barcodes were then annealed. Samples were multiplexed, and paired-end 150-bp sequence data was generated using an Illumina NovaSeq 6000, with an average of 10 million reads per sample. Trimming and demultiplexing of raw Illumina reads was performed using a custom Java application https://github.com/shbrainard/gbsTools. Reads were aligned to the *C. americana* genome for 'Winkler' (Brainard et al., 2024).

SNP calling

Haplotype-based markers were called using Stacks 2 (Rochette et al., 2019), which can identify multi-allelic markers using the phased nature of multiple indels or SNPs that appear within a single 150-bp paired-end read. Because such markers cannot be directly filtered for depth, the parameter 'gt-alpha' was increased to 0.01 as a method for ensuring genotype quality. Markers were then filtered for linkage disequilibrium using bcftools (prune plugin with a window size of 10kb, and r^2 filter of 0.95, retaining one site per window based on highest allele frequency). This generated a set of 78,079 markers, with an average of ~7,000 per chromosome.

Biallelic SNPs were called using the TASSEL GBSv2 pipeline (Bradbury et al., 2007). 1,161,144 called SNPs were first filtered to exclude sites with minor allele frequency < 0.05,

more than one alternate allele, or greater than 10% missingness across all samples, which reduced the number of SNPs to 878,306. This set was then further reduced to include only SNPs where the 80th percentile of allele depth across all samples was > 8, leaving 102,096 sites. Next, filtering was performed for linkage disequilibrium ($r^2 < 0.75$, with the same selection criteria above), which retained 53,696 SNPs. Finally, markers were then subset to only include those which were retained in both the *C. americana* diversity panel in Wisconsin and interspecific biparental populations in Minnesota, leaving 44,961 SNPs. All filtering was performed using bcftools (Danecek et al., 2021).

Linkage map construction and composite interval mapping

Since the interspecific biparental populations in Minnesota were constructed from controlled crosses between known hazelnut varieties, it was possible to build a genetic map by using the R package onemap (Margarido et al., 2007) (https://github.com/augusto-garcia/onemap). This map was previously reported in (Brainard et al., 2023). Briefly, markers called using Stacks 2 were first filtered to include only those of segregation types A1, A2 and B3.7 (following the notation of (Wu et al., 2002)), such that only markers with either three or four alleles remained. In these segregation types, the four possible alleles present in a biparental cross between two outbred diploid parents are represented as *a*, *b*, *c*, and *d*. A1 segregation types are loci for which the parental genotypes are *ab* x *cd*, A2 represents *ab* x *ac* crosses, and B3.7 represents *ab* x *ab*. Next, markers for which more than 5% of all samples had no called genotype were removed and two-point recombination frequencies were calculated for all possible phase configurations between all remaining markers using maximum likelihood. Maximum likelihood estimates were able to fully resolve phase between pairs of markers, due to the fully-informative segregation

types that were used. A hierarchical clustering algorithm was utilized to construct linkage groups, and markers were ordered and phased within these groups by using a Hidden Markov Model with an error rate of 0.05. Recombination frequencies were finally converted to genetic distances using the Kosambi mapping function (Kosambi, 1943). Finally, this map was imported into the R package fullsibQTL (Gazaffi et al., 2020) (https://github.com/augusto-garcia/fullsibQTL) which was used to perform composite interval mapping. Cofactors were selected to minimize the Akaike Information Criteria, and composite interval mapping was employed to perform a single QTL scan for each of the phenotypes measured using the digital image analysis protocol described above. Permutation tests were calculated to empirically estimate a LOD threshold representing a 0.05 genome-wide *p*-value. Additive and dominance effects were estimated for QTL exceeding this threshold using the function cim_char(). Least squares estimation was used to calculate the percent variance explained both for both these QTL individually, and collectively, using the function *r2_ls*. This R^2 is calculated using the residual sums of squares (RSS) for the null and full models:

$$R^2 = \frac{RSS.null - RSS.full}{RSS.null}$$

Calculation of GEBVs

In order calculate genomic-estimated breeding values from the biallelic SNP dataset described above, the R package StageWise (Endelman, 2023) (https://github.com/jendelman/StageWise) was used to compute variance components and best linear unbiased predictors (BLUPs) of additive genetic value. This software is designed to perform two-stage analysis, by first computing best linear unbiased estimators (BLUEs) for each genotype using a specified experimental design. Since the genotypes in both populations were comprised of unreplicated

seedlings, a fixed-effects linear model was used to first compute BLUEs for each genotype where:

$$Y_{ij} = \mu + G_i + Y_j + \varepsilon_{ij}$$

G_i represents the ith genotype effect, Y_j the jth year effect, and ε_{ij} the residual variance (which included the $G * Y$ effect), with $\varepsilon_{ij} \sim N(0, \sigma_\varepsilon^2)$). Models were fit for each trait independently, and then passed to the function *Stage2* in order to estimate genotypic values for each trait individually. Additive genetic values are here assumed to follow a multivariate normal distribution with a variance-covariance matrix proportional to the G matrix (VanRaden, 2008), where a centered marker of allele dosages **W** is normalized by the product of the major and minor allele frequencies (*p* and *q*) for the kth marker:

$$\boldsymbol{G} = \frac{\boldsymbol{W}\boldsymbol{W}^T}{2\sum_k p_k q_k}$$

A baseline for the reliability of genomic estimated genetic values was obtained by predicting the genetic value for all samples, using the full phenotypic and genotypic datasets available. This form of marker-assisted prediction provides a point of comparison for testing different parameters of the prediction model.

Reliabilities were calculated as both the mean r^2 returned by StageWise (here denoted as "Stagewise- r^2"), which is the squared correlation between the true and predicted values assuming the model is correct. This is a model parameter that is proportional to the prediction error variance (PEV) of the BLUP. Correlation coefficient between the predicted genotypic values and BLUEs for each genotype (calculated on the basis of the observed phenotypes) are also reported (here denoted as "Pearson-r^2").

Marker density evaluation

In order to assess the impact of number of SNPs used in the estimation of the variance-covariance "G" matrix by StageWise, the input marker matrix was randomly downsampled to simulate densities ranging from 500 to 45,000 markers. The resulting G matrix was then subtracted from G matrix calculated using the complete set of markers in order to estimate the ability of smaller marker sets to accurately capture the degree of relatedness between all individuals in each population.

Validation analyses were performed to assess the impact of marker density on the reliability of the predictions. Marker density was varied from 500 to 45,000, in 100 marker increments, in order to model the impact of the number of SNPs on prediction accuracy. This was assessed directly by calculating the difference between the G matrix calculated with the full set of markers, and the reduced set, as well as by modeling trait prediction accuracy at different marker densities. At each marker density, twenty random subsets of the marker matrix were produced, with even distribution of markers across the 11 chromosomes.

Population relatedness

The impact of the degree of relatedness between the training and validation population when performing marker-based selection was also assessed, using a leave-one-out cross-validation approach. In the *C. americana* population, this was done by first sorting the population by the degree of relatedness of each individual in the population to the validation individual, then using a 100-indivdual subset of the whole population as the training population, which was progressively designed to be less related to the validation individual (i.e., the 1st-100th most

related individuals, the 2nd-101st most related individuals, the 3rd-102nd most related individuals, etc.). Once an asymptotically-minimal prediction accuracy was identified, a secondary analysis was performed, by progressively expanding the training population starting from the 20th-121st most-related individual, adding in increasing numbers of less-related individuals. This allowed for an assessment of whether training population size could compensate for a lack of training population relatedness.

In the interspecific biparental populations, the known pedigrees of the families provided an alternative method for assessing the impact of relatedness on accuracy. These analyses were performed for each of the three families individually, assessing prediction accuracy by predicting the genotypic value each member of each of the three full-sib families, using each of the three families as the training population.

RESULTS

Morphological inshell and kernel characteristics

Histograms for six traits, inshell height and width, kernel height and width, and percent kernel, measured both volumetrically and gravimetrically, are shown in Fig. 2. In general, the interspecific biparental populations, being composed of crosses with *C. avellana* pollen parents from the Oregon State University breeding program, have larger inshell and kernel dimensions. However, the distribution of percent kernel was observed to be either nearly identical (when measured gravimetrically) or larger in the *C. americana* diversity panel (when measured volumetrically from the digital images).

Fig. 3 presents PCA biplots for the *C. americana* population (Fig. 3A), interspecific biparental populations (Fig. 3B) and the combined set of all individuals (Fig. 3C), illustrating the close correlation between inshell and kernel traits, and their relative independence from both measures of percent kernel.

Composite interval mapping

The use of multialleic markers in the onemap pipeline produced a high-quality genetic map. Map quality was confirmed by assessing inflation. Given the relatively small size of hazelnut chromosomes (with an average physical size of 29.78 Mb), a given linkage group should not be longer than 100 cM (under normal biological assumptions and a standard mapping function). Inflated map lengths in excess of this upper bound are nevertheless frequently observed by not controlling for genotyping errors, which increase estimates of recombination frequency. Using multi-point regression when converting genetic distances to the final genetic map counteracts this tendency. The linkage groups in our final map are no longer than 89.9 cM, with an average length of 66.3 cM, and a total length of 729.6 cM.

Single QTL scans were performed for each of six phenotypic traits. LOD profiles across the genetic map are shown in Fig 4. Table 1 summarizes the results of the QTL identified via composite interval mapping for each of the six traits. For the two inshell traits, four QTL were identified (with only one shared in common between both traits), with an average total R^2 of 52%. For the two kernel-specific measures, three QTL were detected for height, while four were detected for length (with no overlap between the two). In both cases, lower total R^2 was observed, with an average of 28.6%. The two methods for measuring percent kernel similarly

were associated with three (gravimetric) and four (volumetric) unique QTL, with a higher total average R^2 of 41.1%

Genomic prediction accuracy

Table 1 presents the heritabilities calculated by StageWise when BLUPs are calculated with all phenotypic and genotypic data for each population. This is equivalent to the proportion of variance attributable to the genotypes.

Two measures of predication accuracy are presented in Table 2. These are the average StageWise-r^2 across all individuals in each population, and the Pearson-r^2, calculated as the correlation between predicted genetic values and phenotypes for all individuals in each population. In the *C. americana* diversity panel, the average StageWise-r^2 across all traits was 0.56, while the average Pearson-r^2 was 0.88. In the interspecific biparental populations, these two measures were closer to one another: average StageWise-r^2 was 0.68, and the average Pearson-r^2 was 0.67.

Effect of marker density on prediction accuracy

The results of the marker density analysis are shown in Fig 5., where the y-axis represents the absolute value of the sum of the matrix resulting from the subtraction of the downsampling-derived G matrix. In both populations, an asymptotic minimum in the difference between the matrices is reached between 5,000 and 10,000 markers.

A similar pattern was observed for average StageWise-r^2 values, where there was a rapid increase in mean StageWise-r^2 as marker density increased, until an asymptotic maximum StageWise-r^2 was attained between 5,000 and 10,000 markers (Supp. Fig. 1) , further suggesting that a marker density in this range will most efficiently estimate relatedness between individuals. When all individuals were used in the calculation of BLUPs, the difference was relatively minor, representing an increase of only ~0.04 in the case of the *C. americana* diversity population. In the interspecific biparental populations, the difference is almost entirely negligible: while the exponential relationship is evident, the difference between the minimum and maximum reliability is ~0.01.

Impact of relatedness on prediction accuracy

Results from the comparison of variably-related training populations are shown in Fig 6. Prediction accuracy quickly drops as the most closely related individuals are removed from the training population, leading to asymptotically-minimal accuracies when the twenty most closely related individuals are removed. This reduction in accuracy represented a nearly 55% reduction in relatedness (measured by the average of the coefficients of the corresponding elements of the G matrix). However, even at maximal relatedness, prediction accuracy in the *C. americana* diversity population was relatively low, with maximum Pearson-r^2 values of ~0.45, and an average of 0.3.

The ability to compensate for reduced prediction accuracy by increasing the size of the training population was subsequently assessed. The results of this analysis are shown in Fig. 6. For the two measures of percent kernel (which also exhibited the lowest prediction accuracy in general),

this increase in population size had no impact on prediction accuracy, while for inshell height, the reduction in relatedness associated with increasing population size led to a reduction in prediction accuracy. Finally, for kernel height, kernel length, and inshell length, prediction accuracy slightly increased, however all of these changes in prediction accuracy represented less than 0.25% in total variation. This indicates that if the 20 most-closely related individuals are absent from the training population design, this cannot be substantially offset by simply including more weakly-related individuals. Importantly, the average degree of relatedness in the *C. americana* panel is relatively low, and thus this observation is not necessarily generalizable to all other training population designs.

In the interspecific biparental populations, similar results were observed when reducing the relatedness of the training population, and keeping the total size of the population constant at 100 individuals Supp. Fig. 2. However, there are limitations to this approach for these populations. Because of the structured nature of the full-sib populations, correlations between the genotypic values and phenotypic data across all individuals primarily reflects this structure. These results are shown in Table 3. Interestingly, marker-based prediction led to low (near-zero or negative) Pearson-r^2 values, when the training population represented the same interspecific biparental cross from which the sample being validated was drawn. For each of the families, however, there was one other biparental population which led to positive prediction accuracies, although these maximum Pearson-r^2 values were significantly lower than the maximum values observed in the diversity panel.

DISCUSSION

Morphological nut traits exhibit high degree of polygenicity

The composite interval mapping results reported above demonstrate clearly that the traits measured here related to physical characteristics of inshell nuts and kernels are highly polygenic. On average, the identified QTL individually explained only 11% of the phenotypic variation for a given trait. While for certain traits the total explained variance was much higher, the average total R^2 for all QTL associated with a trait was only 40.5%, with the unexplained variance ranging from 44.5% - 80.2%. A low total R^2 was particularly apparent for kernel traits, which was also reflected in the prediction accuracy for these traits when evaluating genomic prediction models using the interspecific biparental populations in Minnesota .

To our knowledge, this is the first study to ever map kernel and nut quality traits in an interspecific *C. americana* x *C. avellana* family. Several studies have performed association analysis in *C. avellana* populations of similar size. (Baytar et al., 2024) used similar GBS marker data, and evaluated both kernel and nut size, as well as percent kernel. However, they found only a single QTL for percent kernel. Another GWAS utilized a low density SSR map and only found one QTL for kernel weight and none for percent kernel (Ozturk et al., 2017). Most comparable to these results was a linkage mapping study performed using GBS markers and a relatively large F_1 population derived from Italian *C. avellana* cultivars (Torello Marinoni et al., 2018). This analysis included phenotypic data on both nut and kernel size, identifying one QTL for kernel size (on LG 5) and four for nut size (on LGs 1, 2, 4, and 6). However, none of these QTL explained more than 10% of the total phenotypic variance.

In light of these results, the CIM analyses reported here represent a significant advance in our understanding of the genetic control of these physical nut characteristics, which is likely attributable both to the significant variation observed in these interspecific crosses, the high resolution of the genetic map used, and the precision and scale of the phenotyping method utilized. Nevertheless, it appears likely that this study was underpowered to detect the numerous remaining QTL associated with these traits. This is likely not due to a limitation in the number of markers used, since the resolution of the map was greater than the number of expected recombination events present in the population. Indeed, it is likely that the relatively small size of the mapping population used in this study is primarily responsible for reduced statistical power. Building larger F_1 populations would therefore be useful in attempting to more accurately assess the true genetic architecture of these traits, however, the size of F_1 progeny families are limited by *Corylus* biology. Multiparent QTL mapping would, in particular, be a valuable approach (e.g., via a diallel mating design), as this would enable more precise estimation of dominance effects. However, the present results point strongly towards many QTL influencing these traits, and the absence of any single large effect QTL. As will be discussed below, this motivates relying upon genomic prediction models as the preferred method for utilizing marker information in applied breeding programs.

Discrepancies between C. americana diversity panel and interspecific biparental populations

While the correlation coefficient-based estimates of prediction accuracy in the interspecific biparental populations were nearly identical (but slightly less than) the mean Pearson-r^2 values, correlation coefficients in the *C. americana* diversity population were dramatically higher than

the mean Pearson-r^2 values. This difference is so great that while mean Pearson-r^2 values were higher in the interspecific biparental populations than in the *C. americana* diversity population (higher for every trait other the kernel-specific measurements), correlation coefficients were much higher in the *C. americana* diversity population than in the interspecific biparental populations. Possible interpretations of these discrepancies, and how they should inform the interpretation of these two metrics of reliability, are provided below. It should be noted at the outset that because best linear predictors are designed to maximize the correlation between the true and predicted genotypic values (and the covariance of the true and predicted values is equal to the variance of the true values, reliability of the model is equivalent to broad sense heritability as computed in a completely randomized design (Endelman, 2023). Correlation coefficients derived from a leave-one-out cross-validation approach can introduce bias by assuming the observed phenotypic data represents the "true" value of an individual (Zhou et al., 2017). However, in general, it is clear that marker-assisted selection generated strikingly high reliabilities, particularly for populations composed of unreplicated seedling genotypes. Except for the kernel-specific traits in the interspecific biparental populations, both measures of reliability were higher than 0.5. To our knowledge, this study represents the first reported evaluation of genomic selection in any *Corylus* species, but these results are comparable to assessments of prediction accuracy for morphological characteristics in other tree nut crops. For example, (Nishio et al., 2018) reported prediction accuracies of 0.4 for the weight of chestnuts using a GWAS model (BayesB).

There are numerous differences between the *C. americana* diversity population and the interspecific biparental populations in Minnesota which likely underlie the differences in trait

heritabilities observed for these two locations. While spatial variation within the field locations was not apparently greater in either the *C. americana* diversity population or the interspecific biparental populations (Supp. Fig. 3), the fact that both trials were composed of unreplicated seedling genotypes certainly lowered heritabilities, and unavoidably increased the relative size of environmental variance components, potentially to differing degrees.

In addition, the populations themselves were obviously markedly different. While the interspecific biparental populations were substantially smaller, they also exhibited much higher degrees of average relatedness both within and across biparental populations. The *C. americana* diversity population location was composed of wild-collected accessions with no known shared pedigree. The interspecific biparental populations, on the other hand, was composed of full-sib crosses in which maternal parents were both drawn from the Minnesota breeding program, and paternal parents were selected form the Oregon breeding program. As a result, in most training populations designed to test prediction accuracy in the interspecific biparental populations, the average coefficients of the respective G matrix were roughly an order of magnitude higher than in the *C. americana* diversity panel.

Excessive shrinkage within the interspecific biparental populations

The cross-validation results analyzing the impact of relatedness on prediction accuracy in the interspecific biparental populations are striking, and are consistent across traits. It is not immediately clear why prediction accuracies were consistently negative when the training population was composed of the same full sib family as the individual for which the prediction was being made. This is particularly surprising, given the relatively high accuracies (which for

certain traits, exceeded the asymptotic maximum prediction accuracy seen in the *C. americana* diversity population) when the prediction population selected is **not** the full sib family from which the given sample is drawn. Two important features of this analysis are likely impacting these results. First, there is substantial shrinkage, much greater than that seen in the *C. americana* diversity population, apparent across traits when performing these cross-validation analyses Supp. Fig. 4. With small population sizes, and unreplicated genotypes, it has been previously reported that shrinkage-based estimators can lower prediction accuracy for unphenotyped individuals (Endelman and Jannink, 2012; Zhao et al., 2013).

Second, and relatedly, the population sizes for these comparisons across families is small, ranging from 75-81 individuals being used in the given prediction populations. This is much smaller than is typically considered in studies assessing factors influencing genomic prediction accuracy, where significant drop-offs in accuracy are frequently observed when population size drops below 100 (Tayeh et al., 2015; Zhang et al., 2017; Sverrisdóttir et al., 2018; Edwards et al., 2019). As such, these were also smaller training populations than those used to analyze the impact of relatedness in the *C. americana* diversity population. Generating larger biparental families is biologically challenging in hazelnut, so breeding programs should adopt a strategy of ensuring biparental families are interconnected through shared parentage, such that higher effective numbers can be achieved in training populations.

Mean r^2 values vs. correlation coefficients as measures of reliability

The average of the r^2 values returned by the StageWise package and the Pearson correlation coefficients between the genotypic values estimated by StageWise and the BLUEs represent two

intuitive methods for estimating reliability. The high values for reliability reported here (>0.5 in nearly all cases) suggest relatively large broad-sense heritabilities i.e., large additive genetic variance components, for morphological nut traits. This suggests that applying genomic prediction methods within selection programs is realistic and tenable. There are, however, several caveats to be aware of in evaluating these measures of reliability.

Any correlation-based measure of reliability will be inflated to a degree that is proportion to the correlation between population structure and the traits of interest (Werner et al., 2020). The mean r^2 of the BLUPs returned by StageWise should in principle be less sensitive to this artificial inflation, as they are individual measures of correlation across replicates of each specific genotype (Endelman, 2023). At the same time, these correlation coefficients will only ever be accurate measures of true reliability to the degree that the BLUEs are taken as a form of "ground-truthed" data (Rincent et al., 2012). In the case of unreplicated seedling genotypes, where environmental variance components cannot be explicitly included in the model, this assumption is clearly not entirely justified (Waldmann, 2019).

Application of genomic prediction within hazelnut breeding programs

There are numerous practical considerations that will impact the use of genomic prediction in the various stages of a specific hazelnut breeding program. In particular, the per sample cost of genotyping will be a determining factor influencing the number of plants for which predictions can be made. In addition, important traits may express themselves relatively quickly (e.g., for disease screens which can be performed in a greenhouse environment), or take over a decade before a phenotype is available (e.g., for mature plant architecture, or multi-year cumulative

yield). This will significantly impact the benefit of genomic-based selection, relative to phenotypic selection.

In general, however, several conclusions are evident from the data presented here. First, prediction accuracies that are obtained when phenotypic data is not masked for individuals in the training population are clearly high enough to warrant including genetic marker information in the selection of parents. The set of parental candidates is typically relatively small, and breeders frequently have limited access to replicated trial data for all individuals with which crosses might realistically be made. At the same time, some phenotypic data will be available before a cross can physiologically be performed, and thus the inclusion of genotypic information would in this context be used to perform genomic-assisted selection (Jannink et al., 2010). In addition, in mature breeding programs, close relatives of potential parents may have substantial phenotypic information available, which can be used to improve the training population. The improvements in trait prediction offered by such an approach will generally be warranted, given the progressively declining cost of sequencing.

Second, in many applied contexts, genomic prediction is not simply used in a marker-assisted fashion, but also to perform marker-based prediction, where only genotypic data is available for some individuals in the population. In these cases, a so-called “training population” is phenotyped and genotyped, allowing genotypic values to be predicted for un-phenotyped individuals. The use of purely genomic-based prediction at the seedling stage, performed to determine which subset of progeny families to advance into field trials, will necessarily be more limited. Phenotypic data will be largely absent for such individuals. While there may be cases

in which phenotypic data exists for half-sib or full-sib families created from crosses using the same parents, any training population will be to some degree removed from the individuals for which predictions are being made. In addition, while the size of any specific progeny family will be limited, a breeding program as a whole could easily generate thousands of seedlings each year, genotyping all of which would likely represent a prohibitive expense. Instead, a prudent balancing act should be made between resources dedicated to genotyping, and field trials used to grow out new progeny each year, such that the number of progeny generated each year can in fact be genotyped. Even in the absence of phenotypic data, and thus reduced prediction accuracies, genotyping at the seedling stage will improve genetic gain, and allow for a more informed preservation of genetic variance within the breeding program (Endelman, 2025).

In sum, these results provide a promising basis for further developing genomic selection models for use in hazelnut breeding programs. This study represents the first such model developed for *Corylus*, and despite the limited degree of replication, the prediction accuracies obtained for nut quality traits suggest this method can substantially improve the rates of genetic gain for polygenic traits. We hope future studies will expand upon this work, validating models with more robust experimental designs to minimize better minimize environmental variances, and validate predication accuracies for a greater diversity of traits.

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SUPPLMENTAL MATERIAL

Supplementary Figure 1. The impact of marker dilution is illustrated in terms of its impact on prediction accuracy. **A:** results from the *C. americana* diversity panel; **B:** results from the interspecific biparental populations. As in Figure 5, asymptotic prediction accuracy is reached between 5,000 and 10,000 markers.

Supplementary Figure 2. Progressive dilution of the training population is shown for the interspecific biparental populations in Minnesota. The analysis is similar to what is shown in Fig. 6, however in this case, prediction accuracy is largely a function of correctly prediction F_1 population identity, and not the genetic value of individual samples, due to the significant degree of population structure present when all three biparental families are combined.

Supplementary Figure 3. Spatial variation across the field sites for **A:** kernel length in the Wisconsin *C. americana* diversity panel; **B:** kernel height in the Wisconsin *C. americana* diversity panel; **C:** kernel length in the Minnesota interspecific biparental populations; **D:** kernel

height in the Minnesota interspecific biparental populations. No clear structured spatial variation is observed within sites, or any degree of relatively greater or lesser variation between sites.

Supplementary Figure 4. Violin plots comparing observed phenotypic values with the genetic values predicted using BLUPs. **A:** Shrinkage is observed in the *C. americana* diversity panel, and this effect increases as the training population because less related to the sample. **B:** Much more extreme shrinkage is observed in the biparental populations across all traits, despite the similar training population size.

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CONFLICT OF INTERETS

The authors declare no conflict of interest.

DATA AVAILABILITY

Digital imagery (both raw and binary masks) which were used for phenotyping, genetic map using multialleic makers, ordered linkage groups for the "Eric\Jeff" F_1 population, input marker and phenotype files for use in fullsibQTL, as well as input marker and phenotype files for use in StageWise, are available via DataDryad [temporary link for peer review: http://datadryad.org/share/DdbiZHYdCypYeb2d7gCfkZC8edWZ4jpWjfmRZcyitpw].

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FIGURES

Figure 1. Digital image acquisition and processing pipeline. Staged images of subsamples of 30 inshell nuts (**A**) and kernels (**B**) were acquired using a tethered DSLR camera. QR codes were scanned, with identifying information used for file management. Blue boxes containing individual nuts or kernels were then cropped, with the dimensions of each box being used as a

scale for converting pixel to spatial resolution. RGB indexing was used to create a binary mask of each nut or kernel, with bounding boxes fit to each mask to obtain phenotypic measures.

Figure 2. Histograms for six traits measured using digital imagery. Blue distributions correspond to the Wisconsin diversity population of *C. americana*, while red distributions correspond to the interspecific biparental populations in Minnesota. Length and height measurements for inshell (**A**, **B**) and kernel (**C**, **D**) illustrate the larger size of nuts from the interspecific biparental populations in Minnesota, the paternal parents of which are commercial *C. avellana* cultivars. Slightly greater variance is also observed in the kernel measurements from the interspecific biparental populations. In contrast, higher percent kernel (measured volumetrically) was observed in the *C. americana* diversity population in Wisconsin (**E**), while the two populations had nearly identical distributions for percent kernel when measured by weight (**F**).

Figure 3. PCA biplots of kernel and nut morphological characteristics, specifically inshell length, inshell height, kernel length, kernel height, percentage of kernel by volume, and percentage of kernel by weight. Parallel vectors indicate a positive correlation between the two traits, while vectors oriented 180 degrees from each other indicate negative correlation, and vectors at right angles reflect independence between the relevant traits. In all analyses, kernel height and length, and inshell height and length, were tightly positively correlated with each other, while volumetric and gravimetric measures of percent kernel were more loosely positively correlated. **A:** In the *C. americana* diversity population in Wisconsin, inshell and kernel traits were also somewhat positively correlated, and uncorrelated with percent kernel metrics. **B:** In

the interspecific biparental populations in Minnesota, the positive correlation between inshell and kernel measures was substantially reduced, to such a degree that percent kernel metrics approached a negative correlation with inshell traits. **C:** When both populations were assessed together, correlations appeared much more similar to the *C. americana* population in Wisconsin (likely due to its larger size), with a slight weakening of the positive correlation between the two measure of percent kernel.

Figure 4. LOD profiles of the composite interval mapping results in the Eric\Jeff interspecific biparental F_1 population for six morphological nut traits. **A**: inshell length; **B:** inshell height; **C:** kernel length; **D:** kernel height; **E:** percent kernel (measured volumetrically); **F:** percent kernel (measure gravimetrically).

Figure 5. Assessment of the impact of marker density on pairwise estimates relatedness within both the *C. americana* diversity panel, and the interspecific biparental populations. First, the **G** matrix was calculated with the complete marker set, and then for each number of markers along the *x*-axis, a **G** matrix was calculated again using StageWise. These matrices were then subtracted to give a scalar difference, representing the deviation in total estimates of relatedness at varying levels of marker density. **A:** results for the *C. americana* diversity panel in Wisconsin; **B:** results for the interspecific biparental populations in Minnesota.

Figure 6. The impact of training population design on prediction accuracy in the *C. americana* diversity panel in Wisconsin. **A:** The impact of decreasing the relatedness of the training population to the predicted sample. Declining prediction accuracy is observed as the most

related individuals are removed from the training population, until an asymptotically minimum prediction accuracy is reached once the twenty most-closely related individuals have been removed. **B:** The impact of increasing population size on prediction accuracy, when these twenty most-20 individuals have been removed. Population size is increased from 100 to 275 individuals, with marginal increases in prediction accuracy observed for four traits (inshell and kernel height, inshell and kernel length), while prediction accuracy for both measures of percent kernel were unaffected.

TABLES

Table 1. Results of composite interval mapping results in the Eric\Jeff interspecific biparental F_1 population for six morphological nut traits. For each QTL which exceeded the permutation-test determined LOD threshold, the position is reported in cM along the respective linkage group. In addition, additive effects of both alleles, and dominance effects are reported. R^2 values for the individual QTLs, as well as the complete model are also given.

Inshell height

LG	Pos (cM)	α_p	α_q	δ_{pq}	R^2 (%)
1	17.346	-0.473	-0.294	0.457	19.239
2	6.234	0.016	-0.027	-0.429	13.592
8	9.623	0.068	0.490	-0.182	9.884
11	0.012	-0.093	0.435	-0.223	10.809
All	-	-	-	-	48.513

Inshell length

LG	Pos (cM)	α_p	α_q	δ_{pq}	R^2 (%)
1	17.062	-0.400	-0.254	0.386	19.517
2	3.564	-0.047	0.027	-0.573	19.066
6	38.255	-0.359	0.090	-0.272	7.574
8	32.854	-0.038	0.351	-0.300	9.723
All	-	-	-	-	55.472

Kernel height

LG	Pos (cM)	α_p	α_q	δ_{pq}	R^2 (%)
1	51.295	0.101	-0.262	-0.201	7.976
2	12.013	0.063	-0.186	-0.246	9.129
8	34.421	-0.167	0.397	-0.196	7.310
All	-	-	-	-	19.809

Kernel length

LG	Pos (cM)	α_p	α_q	δ_{pq}	R^2 (%)
4	9.422	0.152	-0.075	0.151	8.926
9	29.042	0.243	0.515	-0.440	8.076
10	4.213	-0.081	-0.080	0.290	7.279
11	18.915	-0.111	-0.084	-0.242	7.237
All	-	-	-	-	37.344

Percent kernel (gravimetric)

LG	Pos (cM)	α_p	α_q	δ_{pq}	R^2 (%)
6	27.213	0.021	-0.006	0.012	20.323
8	52.938	0.006	0.043	-0.017	10.622
9	35.709	0.002	-0.004	0.022	8.259
All	-	-	-	-	37.143

Percent kernel (volumetric)

LG	Pos (cM)	α_p	α_q	δ_{pq}	R^2 (%)
1	17.005	0.003	0.009	-0.0181	17.082
3	71.099	-0.002	-0.016	0.0007	7.434
6	32.960	-0.023	-0.025	0.0002	14.203
9	7.961	0.006	0.011	0.0134	12.035
All	-	-	-	-	45.142

Table 2. Heritabilities of six morphological nut traits in the *C. americana* diversity population in Wisconsin and the interspecific biparental families in Minnesota.

Trait	*Wisconsin*	*Minnesota*
Inshell height	0.585	0.718
Inshell length	0.510	0.654
Kernel height	0.678	0.301
Kernel length	0.527	0.347
Percent kernel (grav.)	0.615	0.553
Percent kernel (vol.)	0.513	0.542

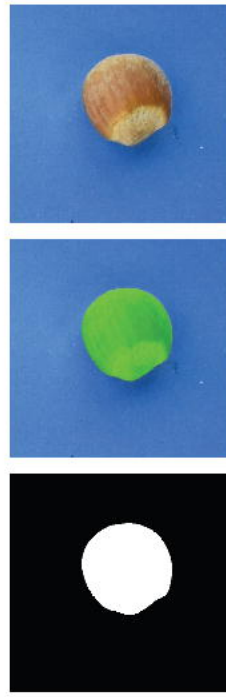
Trait	Wisconsin – StageWise-r^2	Wisconsin – Pearson-r^2	Minnesota – StageWise-r^2	Minnesota – Pearson-r^2
Height (in-shell)	0.55	0.90	0.88	0.87
Length (in-shell)	0.51	0.85	0.84	0.75
Height (kernel)	0.62	0.93	0.44	0.51
Length (kernel)	0.53	0.83	0.48	0.42
% kernel (grav.)	0.61	0.92	0.74	0.75
% kern (vol.)	0.53	0.87	0.71	0.73

Table 3. Impact of relatedness on prediction accuracy in the interspecific biparental populations in Minnesota. For each of the three F_1 families, leave-one-out validation was performed using the entirety of each of the three families, with average Pearson-r^2 values being calculated. The prediction population which generated the highest prediction accuracy is highlighted.

Sample population	**Prediction Population**	**Prediction accuracy**
Gibs\York	Eric\Jeff	-0.128
Gibs\York	**Gibs\OSU**	**0.281**
Gibs\York	Gibs\York	-0.0545
Eric\Jeff	Eric\Jeff	-0.723
Eric\Jeff	**Gibs\OSU**	**0.404**
Eric\Jeff	Gibs\York	-0.0219
Gibs\OSU	**Eric\Jeff**	**0.245**
Gibs\OSU	Gibs\OSU	-0.638
Gibs\OSU	Gibs\York	0.220

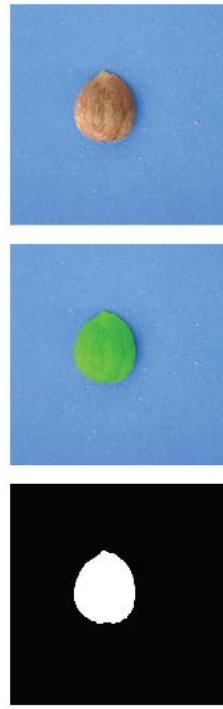
A

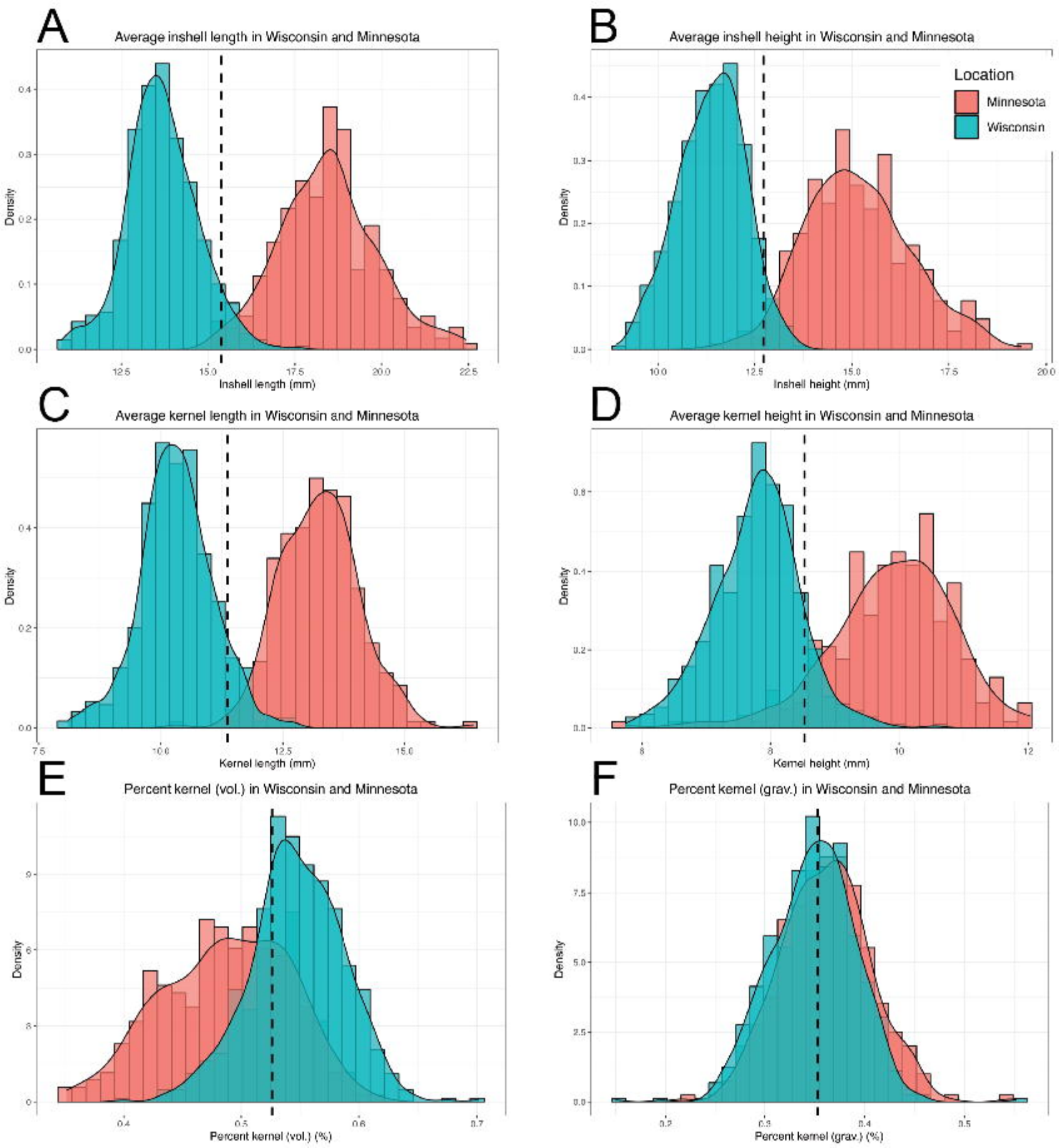
B



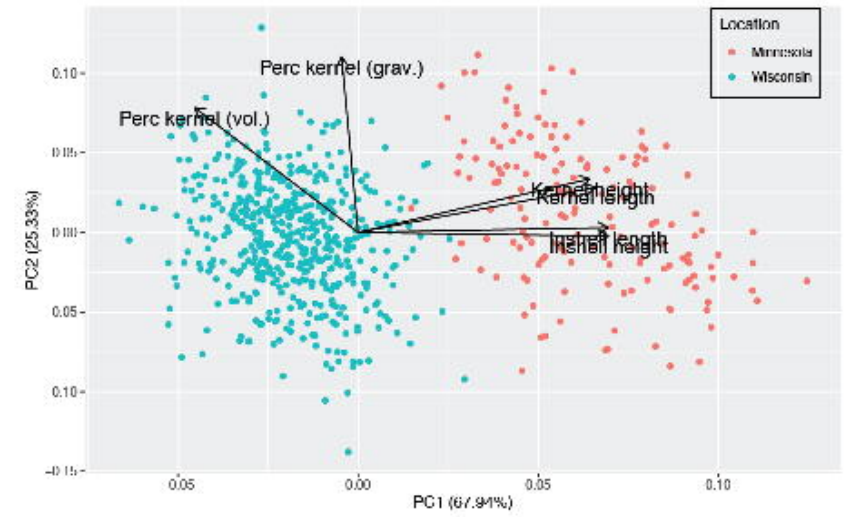
C

D

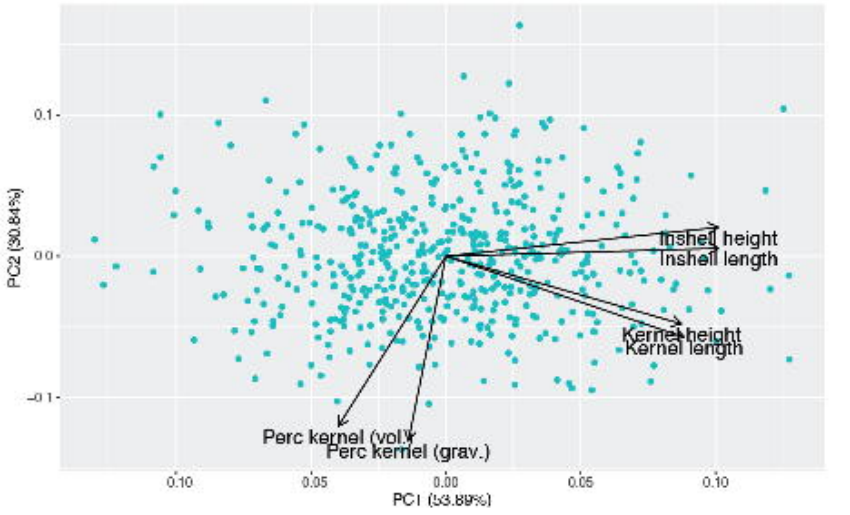



A
Average inshell length in Wisconsin and Minnesota
Density
Inshell length (mm)
B
Average inshell height in Wisconsin and Minnesota
Location
Minnesota
Wisconsin
Density
Inshell height (mm)
C
Average kernel length in Wisconsin and Minnesota
Density
Kernel length (mm)
D
Average kernel height in Wisconsin and Minnesota
Density
Kernel height (mm)
E
Percent kernel (vol.) in Wisconsin and Minnesota
Density
Percent kernel (vol.) (%)
F
Percent kernel (grav.) in Wisconsin and Minnesota
Density
Percent kernel (grav.) (%)

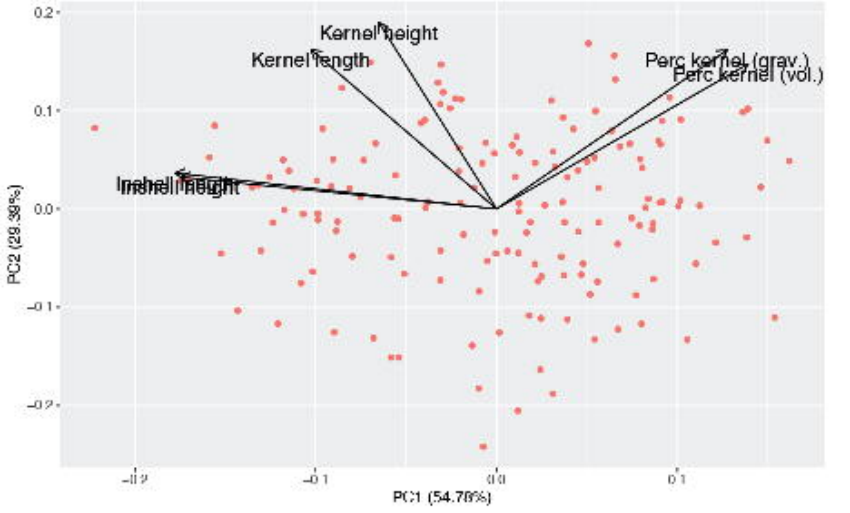
A

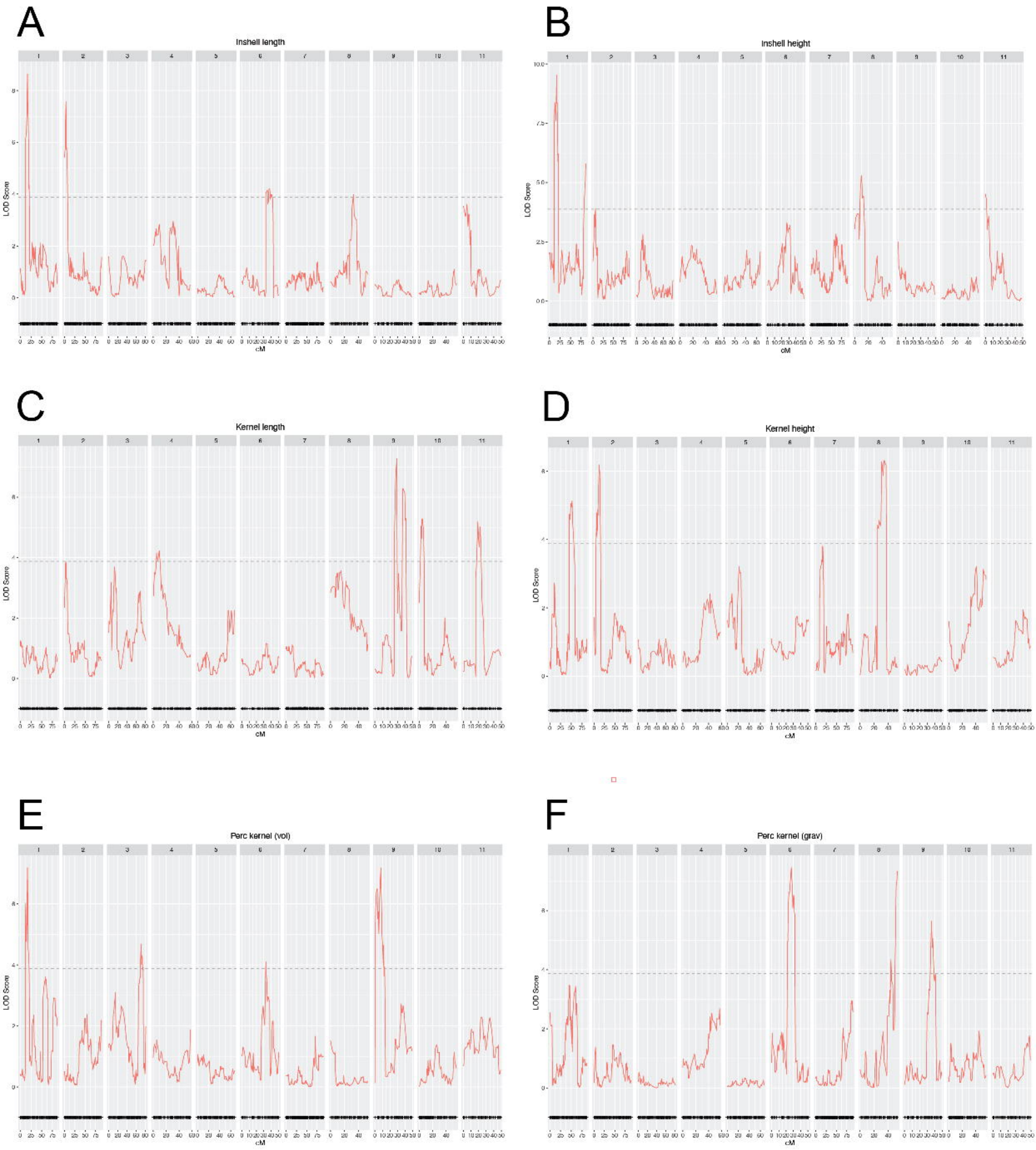

Location
Minnesota
Wisconsin
Perc kernel (grav.)
Perc kernel (vol.)
Kernel height
Kernel length
Inshell length
Inshell height
PC2 (25.33%)
PC1 (67.94%)

B

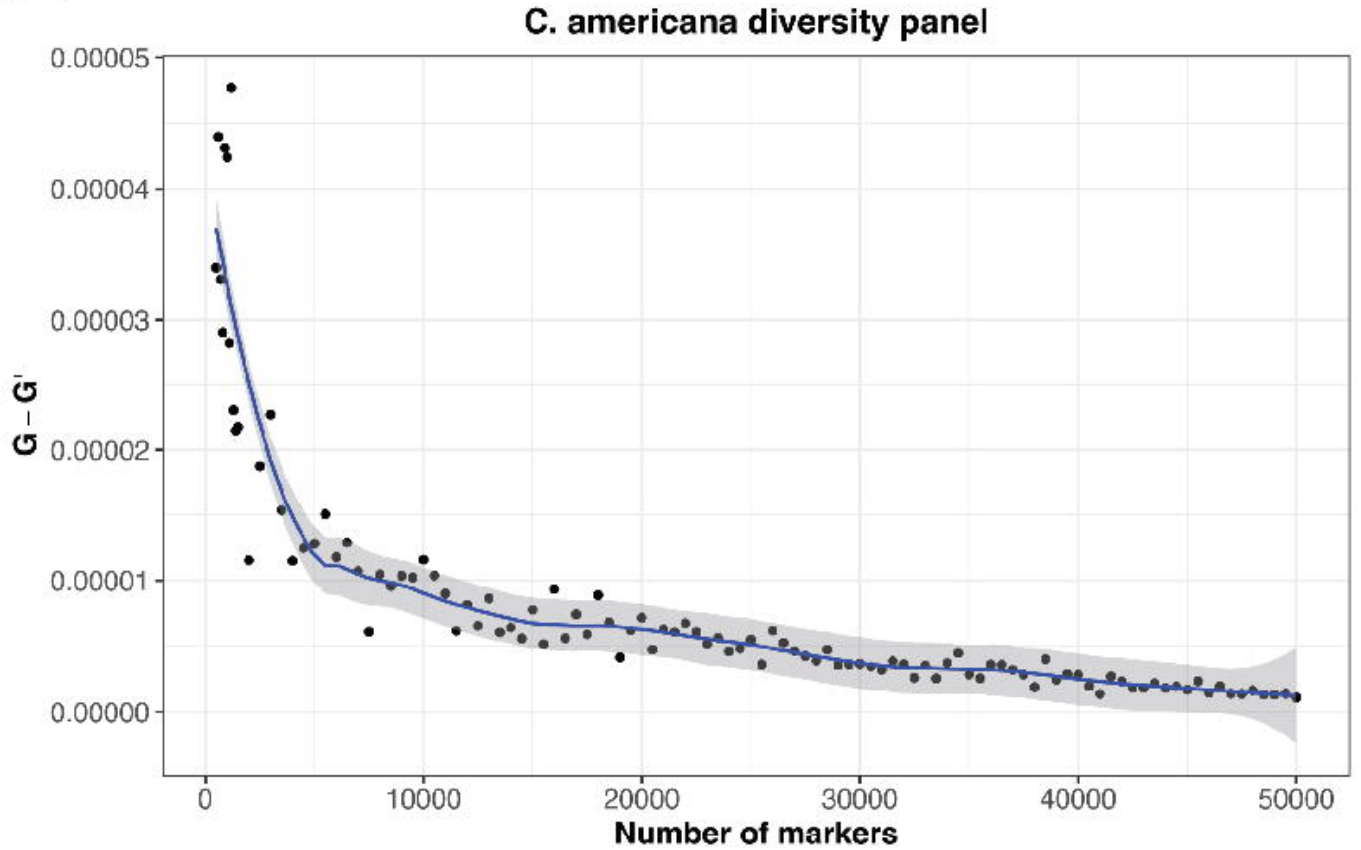

Inshell height
Inshell length
Kernel height
Kernel length
Perc kernel (vol.)
Perc kernel (grav.)
PC2 (30.84%)
PC1 (53.89%)

C

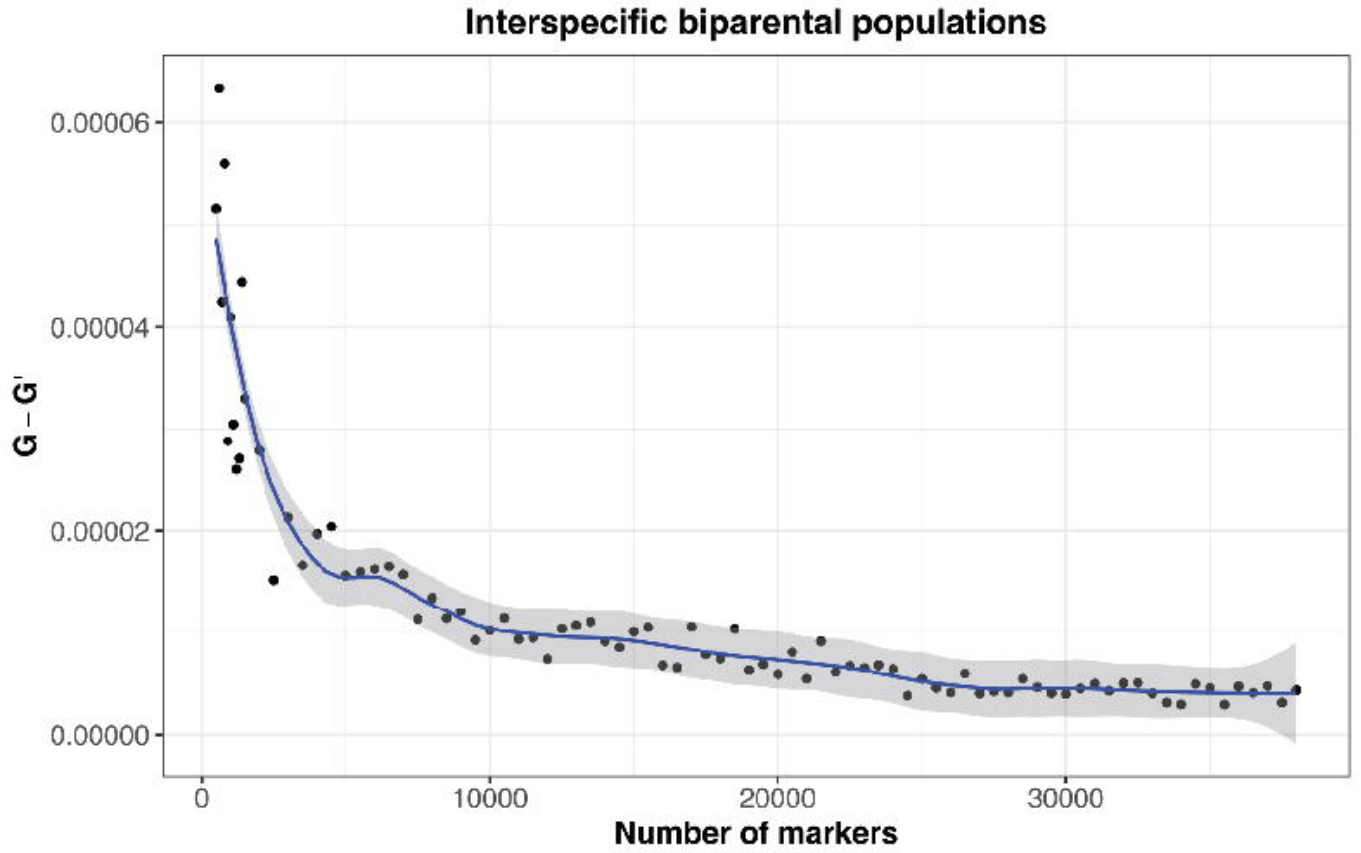

Kernel height
Kernel length
Perc kernel (grav.)
Perc kernel (vol.)
Inshell height
Inshell height
PC2 (29.39%)
PC1 (54.76%)


A
Inshell length
B
Inshell height
C
Kernel length
D
Kernel height
E
Perc kernel (vol)
F
Perc kernel (grav)
LOD Score
cM

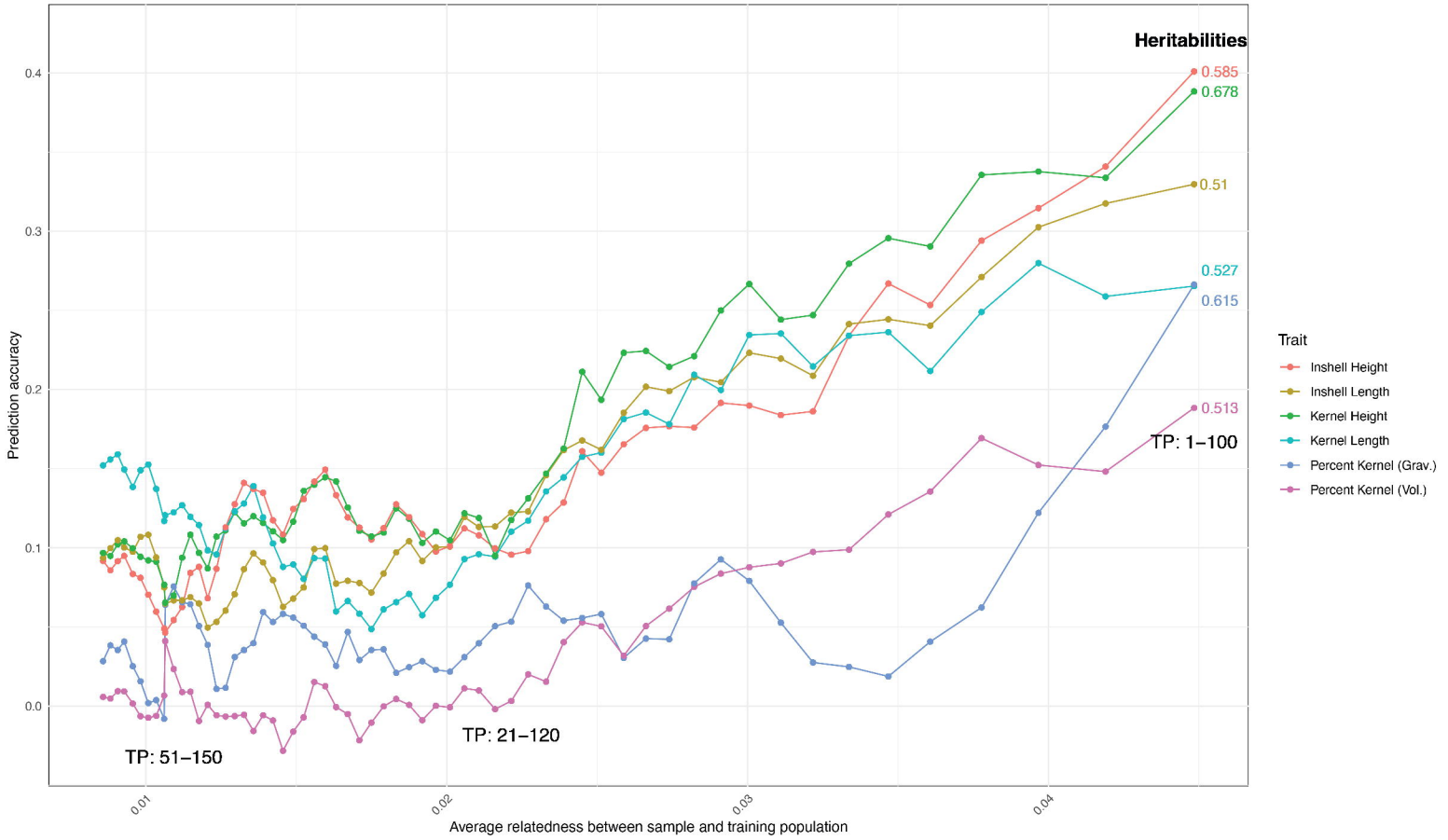
A


C. americana diversity panel
0.00005
0.00004
0.00003
0.00002
0.00001
0.00000
G – G'
0
10000
20000
30000
40000
50000
Number of markers

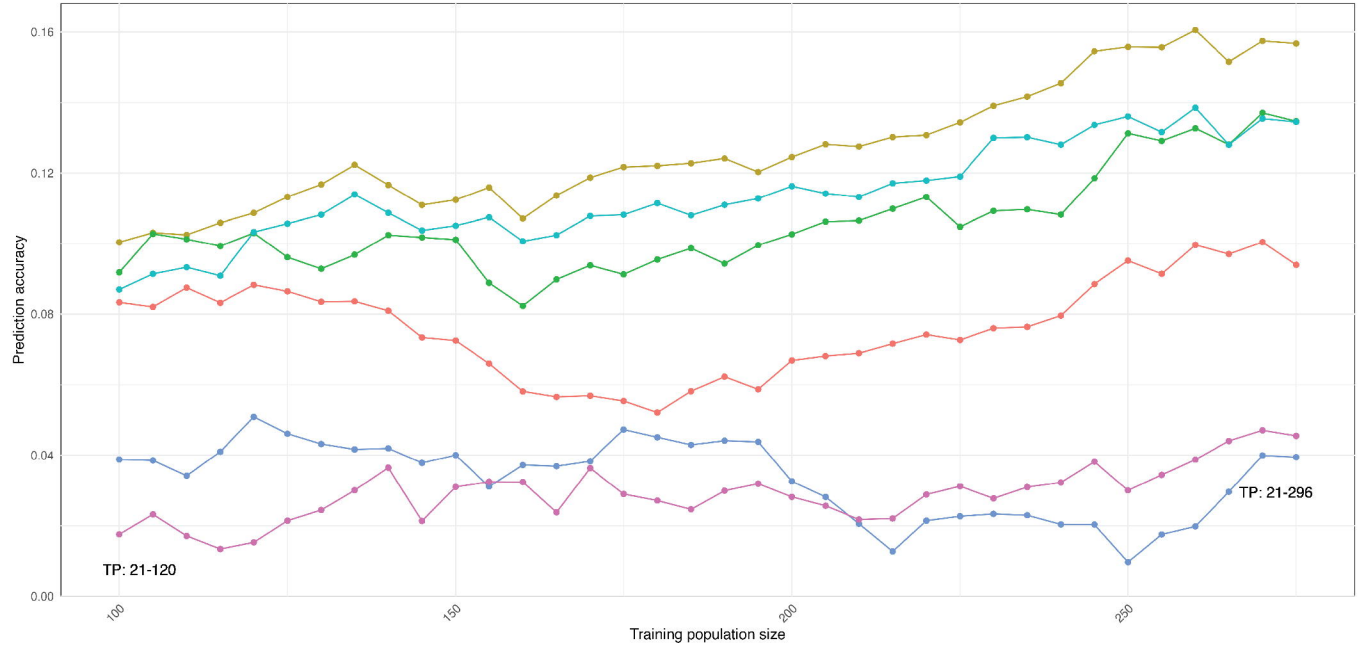
B


Interspecific biparental populations
0.00006
0.00004
0.00002
0.00000
G – G'
0
10000
20000
30000
Number of markers

A

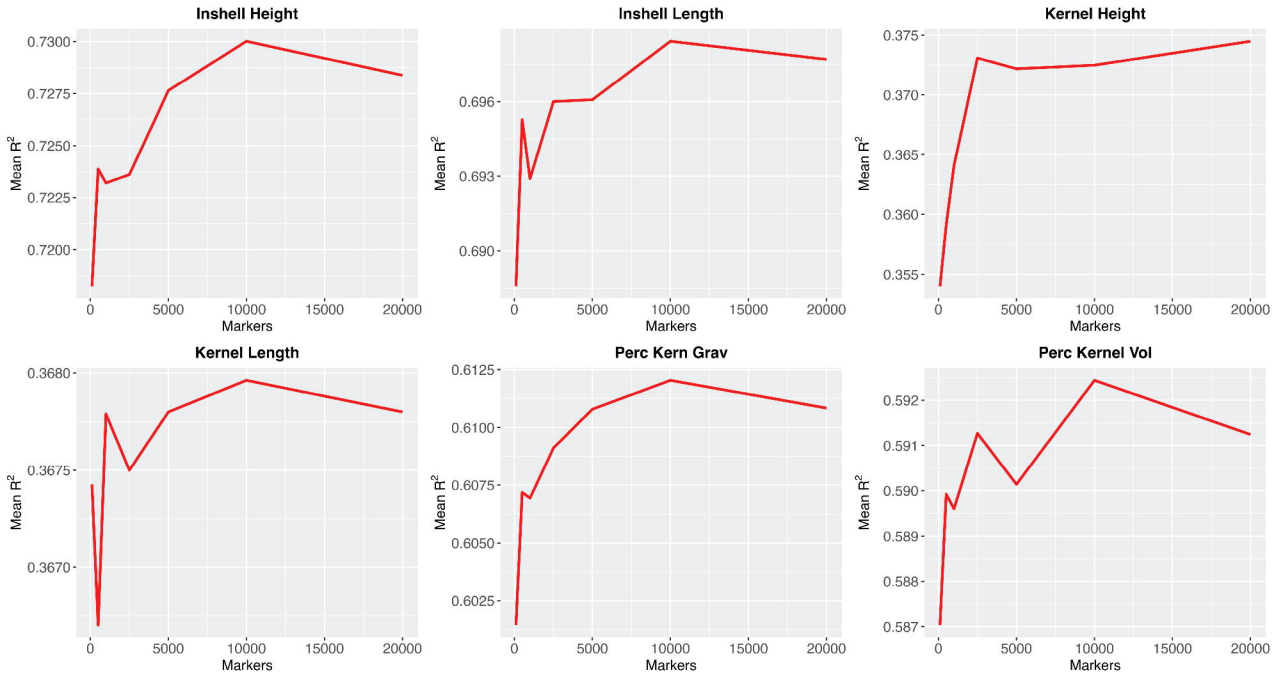

Heritabilities
0.585
0.678
0.51
0.527
0.615
0.513
TP: 1–100
TP: 21–120
TP: 51–150
Prediction accuracy
Average relatedness between sample and training population
Trait
Inshell Height
Inshell Length
Kernel Height
Kernel Length
Percent Kernel (Grav.)
Percent Kernel (Vol.)

B


TP: 21-296
TP: 21-120
Prediction accuracy
Training population size

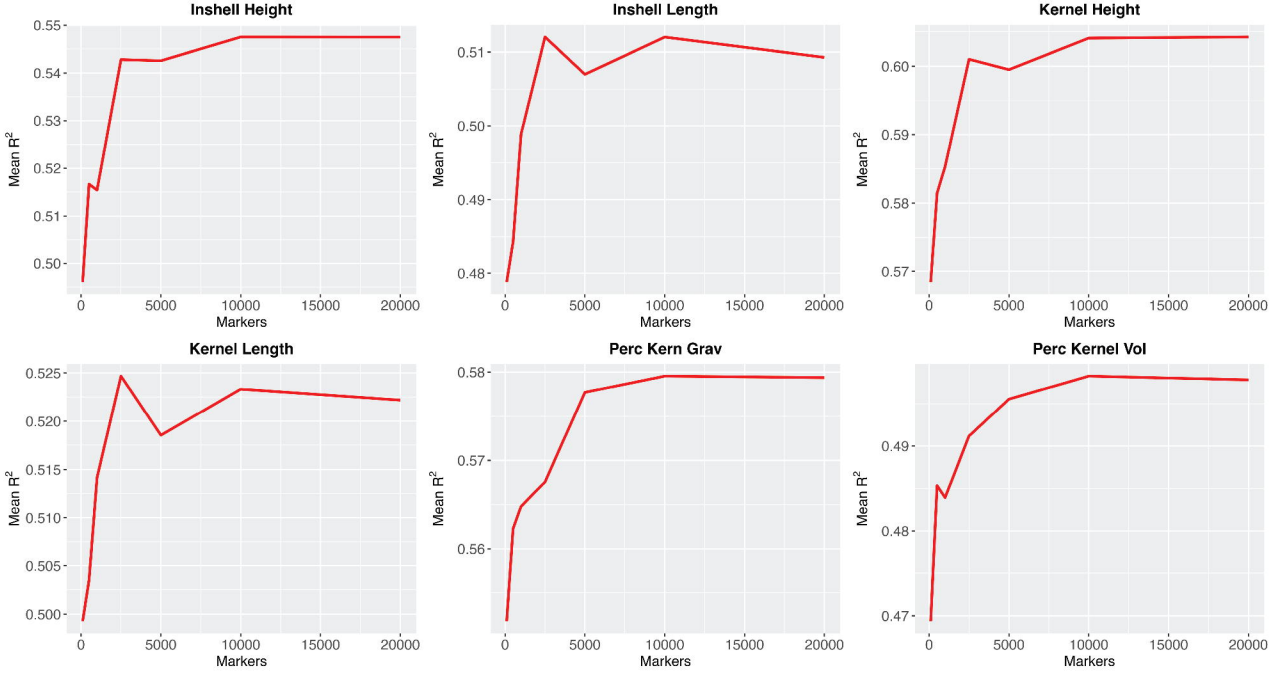
A

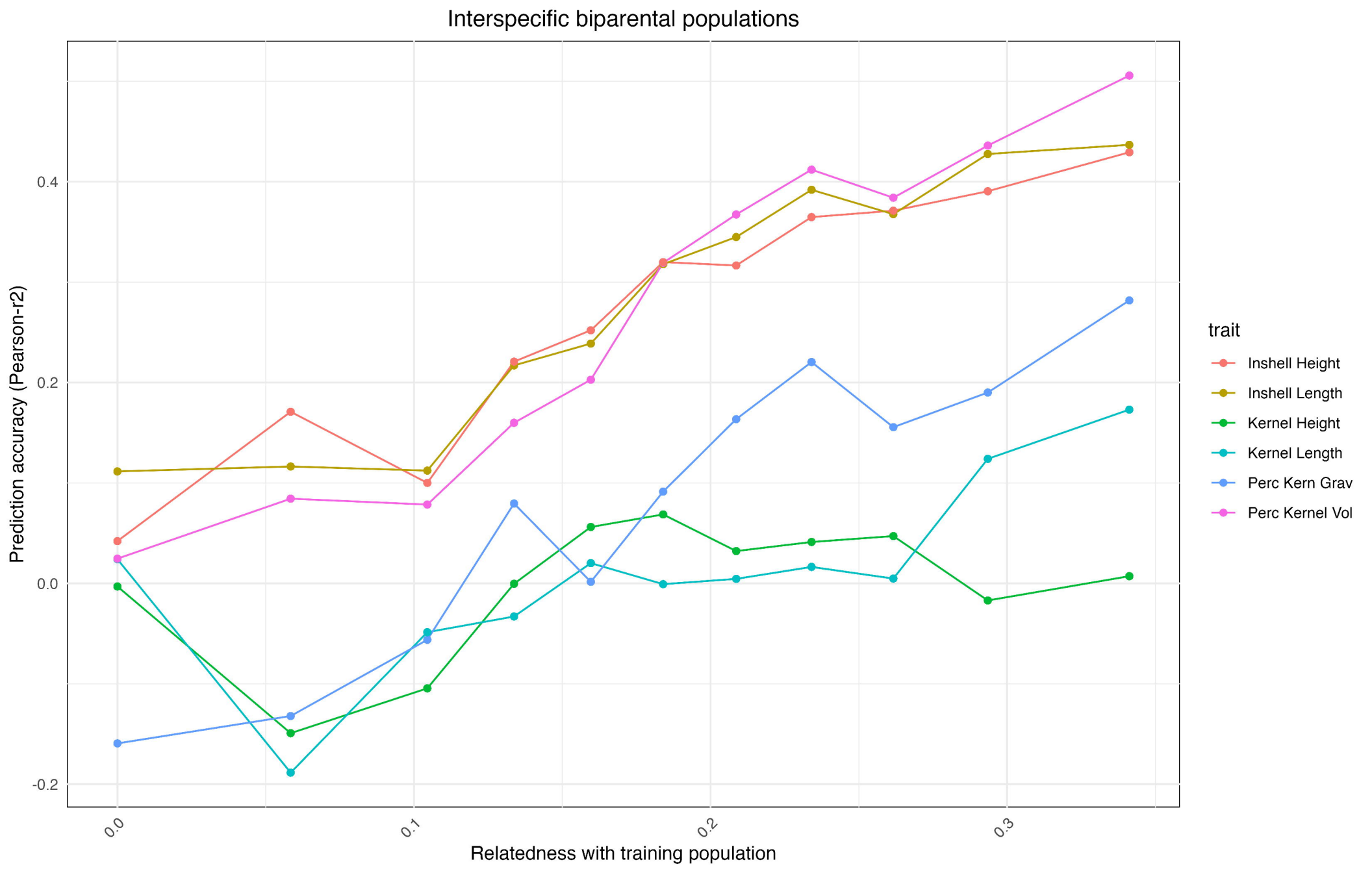
C. americana diversity panel

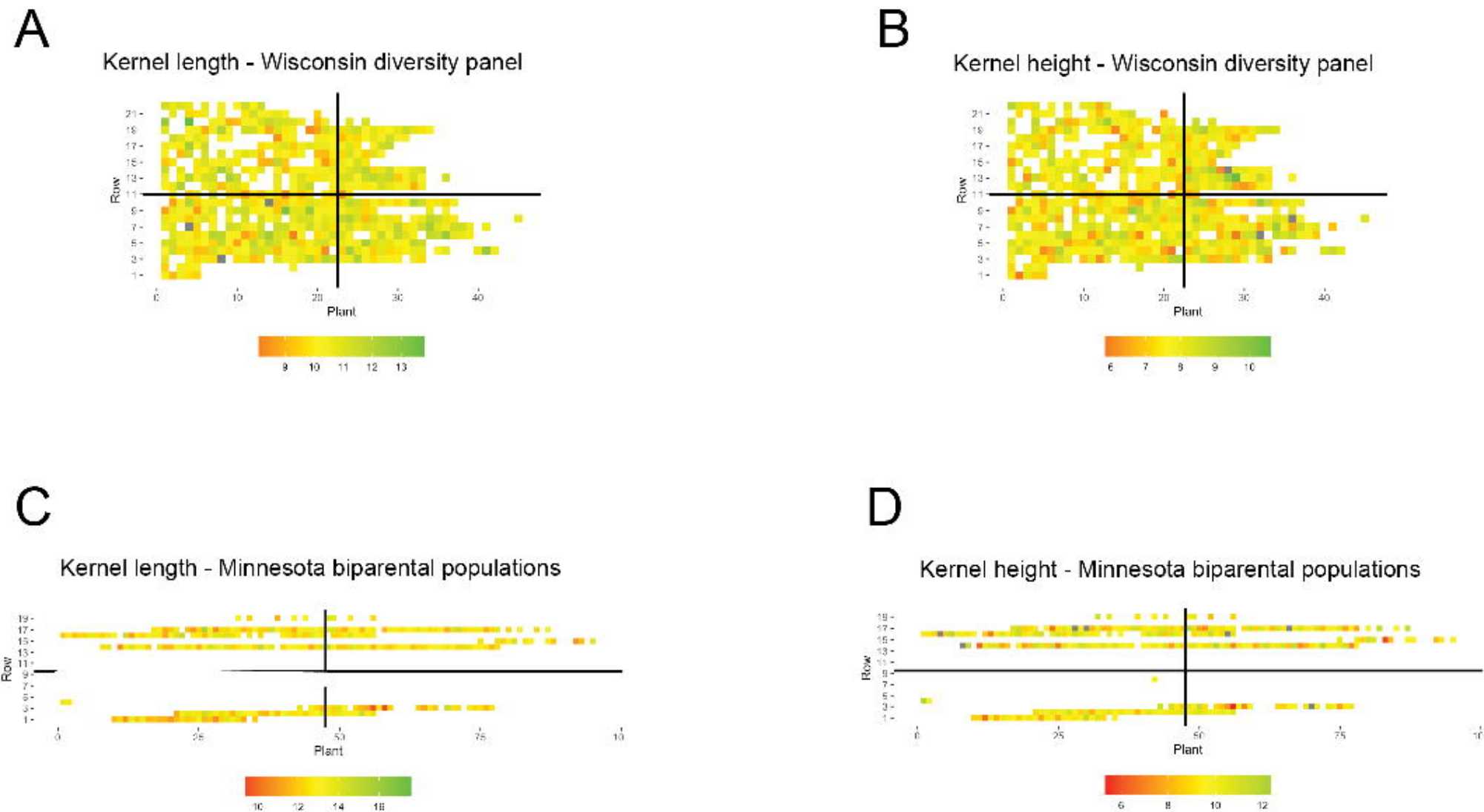


B

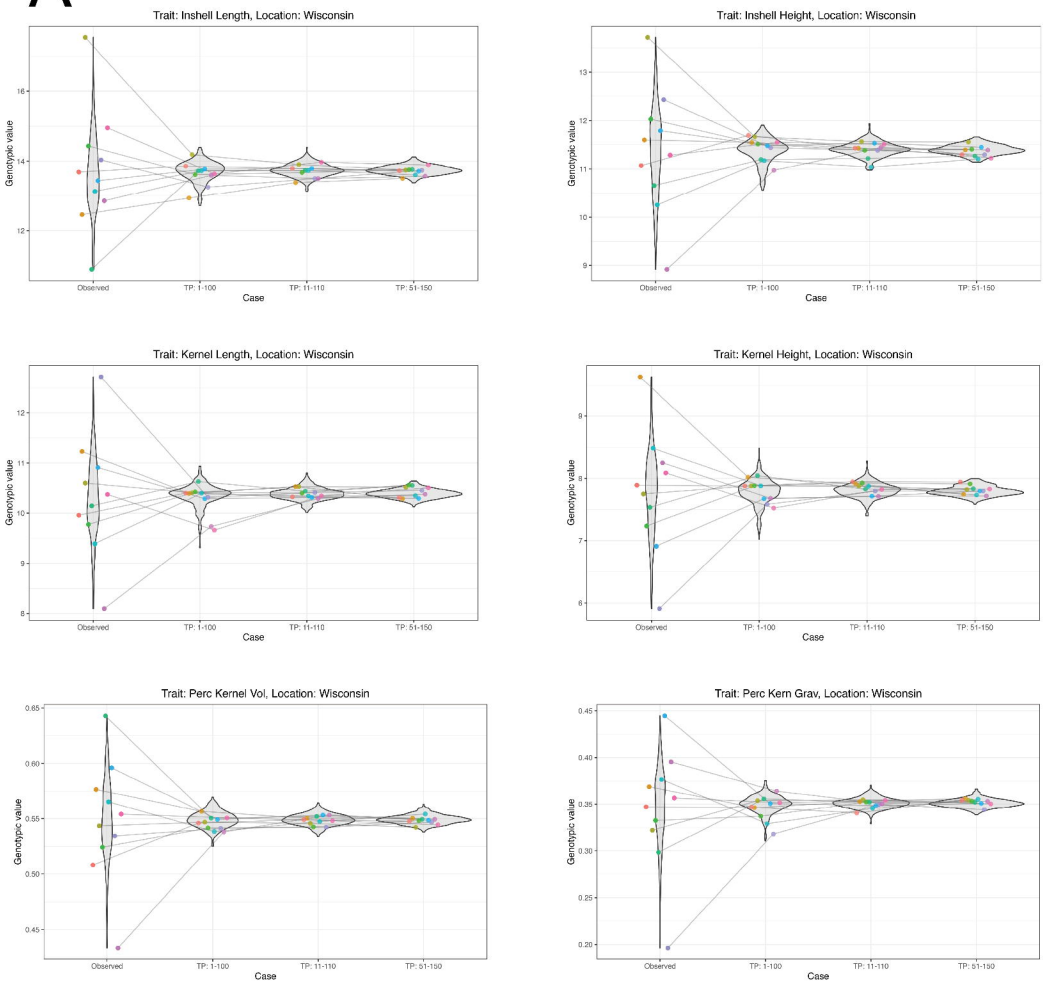
Interspecific biparental populations



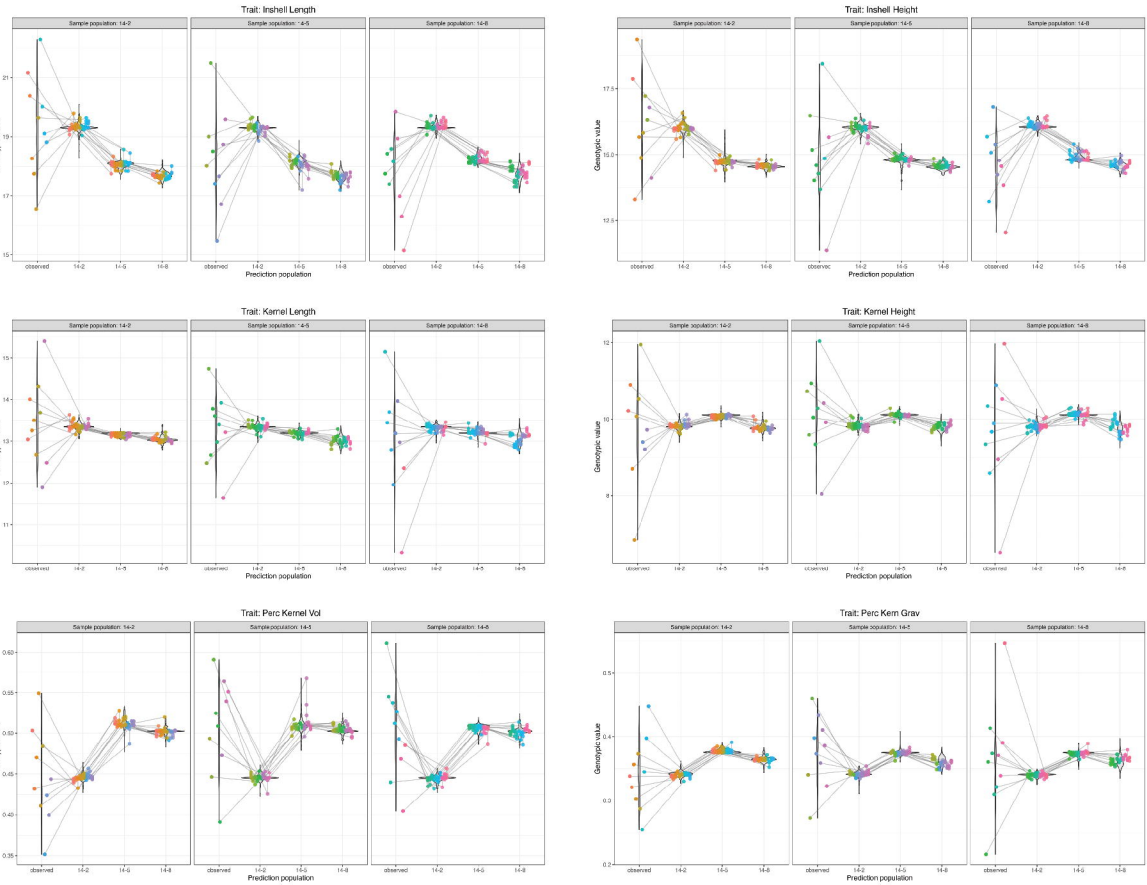

Interspecific biparental populations
Prediction accuracy (Pearson-r2)
0.4
0.2
0.0
-0.2
0.0
0.1
0.2
0.3
Relatedness with training population
trait
Inshell Height
Inshell Length
Kernel Height
Kernel Length
Perc Kern Grav
Perc Kernel Vol


A
Kernel length - Wisconsin diversity panel
Row
Plant
B
Kernel height - Wisconsin diversity panel
Row
Plant
C
Kernel length - Minnesota biparental populations
Row
Plant
D
Kernel height - Minnesota biparental populations
Row
Plant

A


Trait: Inshell Length, Location: Wisconsin
Trait: Inshell Height, Location: Wisconsin
Trait: Kernel Length, Location: Wisconsin
Trait: Kernel Height, Location: Wisconsin
Trait: Perc Kernel Vol, Location: Wisconsin
Trait: Perc Kern Grav, Location: Wisconsin
Genotypic value
Case
Observed
TP: 1-100
TP: 11-110
TP: 51-150

B


Trait: Inshell Length
Trait: Inshell Height
Trait: Kernel Length
Trait: Kernel Height
Trait: Perc Kernel Vol
Trait: Perc Kern Grav
Sample population: 14-2
Sample population: 14-5
Sample population: 14-8
Genotypic value
Prediction population
observed
14-2
14-5
14-8