

Utilizing evolutionary conservation information to improve prediction accuracy in genomic selection

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ABSTRACT Genomic selection (GS) has gained popularity recently as the availability of genome-wide markers has increased. Current methods for GS weigh all the available SNPs equally in model training, without considering their functional differences. Genetic variations detected at evolutionary conserved sites tend to be deleterious and, thus, may be more informative for GS. To utilize this kind of information as a prior into the GS model, we proposed a method to put more weight on evolutionarily constrained sites. As a proof-of-concept, a half diallel population based on 12 diverse inbred lines was used, from which seven phenotypic traits were collected. Some of these traits show high levels of heterosis. After sequencing the 12 founder lines, about 14 million SNPs were discovered and the SNPs were used to identify 502,913 haplotype blocks shared through identity by descent (IBD). A five fold cross-validation experiment was conducted using the summary statistics of the SNP conservation scores, which were computed by evaluating sequences similarity of multiple divergent species, in the IBD blocks as explanatory variables. The results show that the prediction accuracies are significantly better than shuffled data with randomly assigned conservation scores. This study demonstrates the importance of incorporating evolutionary information in GS and its potential use in plant breeding.

KEYWORDS genomic selection; diallel; GERP; maize

The phenomenon of heterosis has been observed for many species across taxa, from yeast (Shapira *et al.* 2014) to vertebrates (Gama *et al.* 2013). Although heterosis has been broadly applied for food production and has been extensively studied by many researchers, the underlying mechanism controlling for it remains elusive. Recent studies indicated that the complementation of the deleterious alleles, which fit the classical dominance genetic model, may play an important role in determining heterosis (Charlesworth and Willis 2009). Deleterious alleles were arisen from new mutations during meiosis. In maize, about 90 new mutations were generated per meiosis (Clark *et al.* 2005), majority of which were deemed to be deleterious according to empirical estimates (Joseph and Hall 2004). In a natural outcross population, the negative effects on fitness of these deleterious

alleles make them subject to be selected against. Therefore, deleterious alleles were mainly maintained in a low frequency (Eyre-Walker and Keightley 2007).

In maize, the total number of mildly deleterious mutations is substantial because of the exponential growth of population size after domestication. The modern breeding probably aims to remove these deleterious mutations and pyramiding beneficial alleles for agronomical important traits. In practice, the relatively homogeneous maize germplasm pool was artificially divided into different heterotic groups (van Heerwaarden *et al.* 2012). It enabled the improvement of germplasm pools to be conducted in a parallel fashion, and therefore, facilitated the breeding efficiency. By doing this, the maize yield has been steadily improved since the early 20th century (citation). However, the removing of deleterious mutations in low recombination regions or in tightly linked regions is less effective. Studies indicated that residual heterozygosity correlates negatively with recombination (Gore *et al.* 2009; McMullen *et al.* 2009) and the low recombination is effective over long period of time (Had-drill *et al.* 2007). As a consequence, the deleterious alleles would

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be accumulated in the pericentromeric region and the vigorous performance could only be realized by combining two sets of non-deleterious or beneficial alleles in repulsion state, thus lead to pseudo-overdominance. Recent QTL study identified loci controlling for heterosis are enriched in centromeric regions (Lari pe et al. 2012), which partly support this pseudo-overdominance hypothesis.

To study the relationship between deleterious variants, especially those with minor effect or rare in the population, and their contribution to heterosis, a diallel population was employed (). This diallel population enabled us to investigate the different modes of inheritance of the deleterious variant in a hybrid population with super dense SNP markers; while only relative little sequencing efforts need to be conducted on founder lines. Our previous study observed that an excess of deleterious SNPs were enriched in GWAS set (Mezmouk and Ross-Ibarra 2014). In that study, deleterious variants were defined as nonsynonymous mutations. Clearly, deleterious variants are not limited to coding regions. Here, we expanded the characterization of deleterious variants to genome wide by using genomic evolutionary rate profiling (GERP) (Cooper et al. 2005). GERP scores were obtained by computing the rejected substitutions subtracted by the neutral rate after multiple sequence alignment of a set of related species (Davydov et al. 2010). **Sofina's GWAS results..** Because most of the deleterious alleles are rare, their effects are difficult to be detected by traditional GWAS approach. To overcome it, a genomic selection approach was conceived, which enable us to estimate the combined effects of all the possible deleterious variants simultaneously. As the results, genomic selection with GERP score incorporated in the prediction model show better performance than circular shuffled data for some phenotypic traits and their heterosis transformations. This study demonstrates the importance of incorporating evolutionary information in genomic selection and its potential use in plant breeding.

Materials and Methods

Plant Material and Phenotypic Data

Twelve maize inbred lines were selected and crossed in a partial diallel fashion without considering reciprocal effects (CITE). The experimental design includes the 66 F1 hybrids, the 12 inbred parents, and 2 current commercial check hybrids grown in an incomplete block design with 3 replications; hybrids and inbreds were grouped separately. The test was grown at Urbana, IL in 2009, 2010, and 2011. Plots consisted of 4 rows, with all observations taken from the inside 2 rows to minimize effects of shading and maturity differences from adjacent plots. Both inbred lines and the 66 resulting hybrids were field evaluated. Phenotypic data was collected for plant height (PHT, in cm), ear height (EHT, in cm), days to 50% silking (DTS), days to 50% pollen shed (DTP), anthesis-silking interval (ASI, in days), grain yield adjusted to 15.5% moisture (adj GY, in bu/A), and test weight (TWT, in pounds) (CITE).

Best Linear Unbiased Estimation (BLUE) of the genetic effects were calculated with ASReml-R (CITE) following the linear model [check the following mixed linear model?](#):

$$y_{ijkl} = \mu + \zeta_i + \delta_{ij} + \beta_{jk} + \alpha_l + \zeta_i \cdot \alpha_l + \varepsilon$$

where y_{ijkl} is the phenotypic value of the l^{th} genotype evaluated in the k^{th} block of the j^{th} replicate within the i^{th} environment; μ , the overall mean; ζ_i , the fixed effect of the i^{th} environment; δ_{ij} , the fixed effect of the j^{th} replicate nested in the i^{th} environment;

β_{jk} , the random effect of the k^{th} block nested in the j^{th} block; α_l , the fixed genetic effect of the l^{th} individual; $\zeta_i \cdot \alpha_l$, the interaction effect of the l^{th} individual with the i^{th} environment; ε , the model residuals.

Heterosis for each hybrid was then estimated by both best- and mid-parent heterosis (BPH and MPH, respectively):

$$MPH_{ij} = \hat{G}_{ij} - \frac{1}{2}(\hat{G}_i + \hat{G}_j)$$

$$BPH_{min,ij} = \hat{G}_{ij} - \min(\hat{G}_i, \hat{G}_j)$$

$$BPH_{max,ij} = \hat{G}_{ij} - \max(\hat{G}_i, \hat{G}_j)$$

where \hat{G}_{ij} , \hat{G}_i and \hat{G}_j are the genetic values of the hybrid and its two parents i and j . BPH_{min} was used instead of BPH_{max} for days to anthesis.

Sequencing of Founder Lines and SNP Callings

DNA from the twelve inbred lines was CTAB extracted (Doyle and Doyle 1987) and Covaris sheared for Illumina library preparation. The DNA libraries were then sequenced to an average coverage of 10X (**say where the sequencing was done?**).

Raw paired reads (reverse and forward for each sequence), were trimmed for adapter contamination with Scythe package (<https://github.com/vsbuffalo/scythe>) which calculate the probability of having a contamination given the adapter sequence, the number of mismatches and sequence quality. The reads were then trimmed for quality and sequence length (≥ 20 nucleotides) with Sickle package (<https://github.com/najoshi/sickle>).

Read pairs, kept after filtering, were mapped to the maize B73 reference genome (AGPv2) with bwa-mem (Li and Durbin 2009). Reads, with mapping quality (MAPQ) higher than 10 and with a best alignment score higher than the second best one, were kept for further analyses.

Single nucleotide polymorphisms (SNPs) were called with mpileup from samtools utilities (Li et al. 2009). To deal with paralogy, which is a major problem in maize sequence mapping (Chia et al. 2012), all SNPs were filtered to a) be heterozygote in less than 3 inbred lines, b) have a mean minor allele depth over all genomes of at least 4, c) have a mean depth over all individuals lower than 30 and d) have missing/heterozygote alleles in less than 6 inbred lines (allelic information for at least 15 hybrids in the partial diallel design).

Haplotype identification and SNP annotation

All missing alleles were then imputed with BEAGLE package (Browning and Browning 2009) and identity by descent regions (IBD) between the 12 inbred lines were identified with BEAGLE's fastIBD method (Browning and Browning 2011). The pairwise IBD region starts and ends were used to delimit haplotypic blocs where several inbred lines shared a homogenous haplotypes.

The SNPs were annotated as synonymous and non-synonymous with the software polydNdS from the analysis package of libsequence (Thornton 2003) using the first transcript of each gene in B73 5b filtered gene set. Deleterious effects of amino acid changes were then predicted with both SIFT (Ng and Henikoff 2003, 2006) and MAPP (Stone and Sidow 2005) software packages as described by (Mezmouk and Ross-Ibarra 2014).

Genomic evolutionary rate profiling (GERP) (CITE), which estimates the evolutionary constraint by quantifying substitution deficits after multiple genome alignments, was obtained from [cite eli2015](#) for AGPv2.

Association mapping

SNP association with heterosis (BPH and MPH) was tested assuming dominance/recessivity of the reference allele or assuming overdominance where only the heterozygote alleles are expected to be significant. For each SNP, root mean square error were used to select the best fitting model. Haplotype association with heterosis were tested comparing the heterozygote alleles to all homozygote ones all confounded.

Genomic-enabled prediction with GERP score

A haplotype based genomic selection (GS) strategy was conceived by using the IBD blocks as the explanatory variables containing conservation information. The reference genome sites with GERP score >0 were considered as conserved sites and genomic variations at these sites were deemed as deleterious. To incorporate the conservation information into IBD blocks, SNPs falling into a given IBD block were added up using their GERP scores as the conservation estimates of the IBD block. This estimation was calculated using a python script `gerpIBD` (<https://github.com/RILAB/pvpDiallel>) with additive and dominant models. Under the additive model, 2 x GERP score was assigned to the homozygous loci with non-reference SNP calls; 1 x GERP score was assigned to the heterozygous loci; and 0 was assigned to the homozygous loci with reference SNP calls. Under the dominant model, 1 x GERP score was assigned to both the homozygous loci with non-reference SNP calls and heterozygous loci; 0 was assigned to homozygous loci with reference SNP calls.

seven phenotypic traits were trained with the conservation statistics in IBD block as genotype. A Bayesian-based approach, BayesC [CITE](#), was employed for the GS experiments. To conduct predict, the diallel population was randomly divided into training and validation sets for 10 times according to a 5-fold cross-validation method. First, the BayesC model was trained independently on each of the training set. Second, the prediction accuracy was obtained by comparing the predicted and observed phenotypes on the corresponding validation set.

In addition, the GERP scores were circularly shuffled. The cross-validation experiments using the circularly shuffled data were conducted on the same training and validation sets.

Data Access

GENETICS is committed to the open access to all primary data (see [Genetics](#), 184: 1). Please indicate where data can be found (supplemental files, public repository, or published with another paper).

Results

- Genetic values, heritability, heterosis and combining ability
- SNP calls and annotation; distribution of deleterious mutations along the genome
- correlation between complementation at deleterious SNPs with heterosis and SCA for the different
- IBD region size and general statistics
- IBD correlation with heterosis and SCA
- GWAS results at a SNP level and then comparison with haplotypic results
- Analyses of the significant haplotypic bloc to see if there is any pattern

Genetic values, heritability and heterosis of the half diallel population

A half diallel population was created using 12 maize inbred lines (Figure [S1a](#)). Two of them are important public inbreds, B73 and Mo17. And the other ten of them are proprietary inbreds (LH1, LH123HT, LH82, PH207, 4676A, PHG39, PHG47, PHG84, PHJ40, PHZ51) that have expired from Plant Variety Protection (PVP) and represent the lineage of key heterotic germplasm pools used in present-day commercial corn hybrids. This diallel facilitated the creation of F1 hybrids that are adapted to the U.S.. The set is diverse enough to facilitate a broad sweep of the heterotic sub-groups that comprise U.S. commercial germplasm. From this population, phenotypic data was collected for plant height (PHT, in cm), ear height (EHT, in cm), days to 50% silking (DTS), days to 50% pollen shed (DTP), anthesis-silking interval (ASI, in days), grain yield adjusted to 15.5% moisture (GY, in bu/A), and test weight (TWT, in pounds).

The best linear unbiased estimators (BLUEs) for genotypes of the seven traits were derived from mixed linear models (Table [S1](#)). In the models, all fixed effects, including the genotype ones, were significant (Wald test p -value <0.05) for all traits except ASI for which the effect of the replicates within environments were not significant. As shown in the Figure [S1b](#), the BLUE values were normally distributed (normality test P values > 0.05). The broad sense heritability of the traits ranged from 0.65 for ASI to 0.95 for PHT (Table [1](#)) [Sofiane, could you add heritability of all the traits to the table 1](#). The levels of heterosis measured by percent high-parental heterosis (pHPH) and percent mid-parental heterosis (pMPH) of these traits are relative low except for GY (pMPH = 120%+23, pHPH = 130% + 23%). Because the selected inbred lines are commercial relevant and fairly elite in performance, it is expected that the hybrids exhibit relative low hybrid vigor than hybrids created from non-elite inbreds. Finally, general and specific combining ability (GCA and SCA) respectively were estimated following ([Falconer and Mackay 1996](#)). The general combining ability (GCA) varied according to the traits, generally, B73, LH123HT and PHG39 are the three inbred lines that combining relative better than others (Table [S2](#)).

Genomic sites under evolutionary constraint

In this study, all twelve inbreds were sequenced to an average depth of $\sim 10\times$. Reads were mapped to the maize B73 reference genome (AGPv2) with `bwa-mem`. After filtering of depth, heterozygosity and missingness, 13.8 million SNPs were kept for further analysis, including 1.9 million SNPs in genic regions and 361,280 in protein coding regions. We estimated the allelic error rate by first comparing our genotype calls to those of 41,292 overlapping SNPs on the maize SNP50 bead chip ([van Heerwaarden et al. 2012](#)); we then compared our SNP alleles for B73 and Mo17 with the 10,426,715 SNP previously identified in HapMap2 ([Chia et al. 2012](#)); finally, we compared our SNPs to 180,313 overlapping SNPs identified through genotyping by sequencing (GBS) ([Romay et al. 2013](#)). The comparisons showed 99.12% allele similarity with shared SNPs previously identified.

Evolutionary constraint information for genomic sites was obtained by computing the rejected substitution rate relative to the neutral rate after multiple genome alignment, or genomic evolutionary rate profiling (GERP) ([Davydov et al. 2010](#)). This approach could estimate the GERP score at a base-pair level, extending the definition of deleterious variants to intergenic regions compared to previous approaches, such as SIFT ([citation](#)) or MAPP ([citation](#)). In B73 reference genome AGPv2, a

Table 1 Phenotypic values, heterosis and heritability

Trait	Heritability ^a	MPH%	HPH%	Notes
ASI	82%	1	82%	anthesis-silking interval
DTP	65%	3	82%	days to 50% pollen shed
DTS	73%	2	82%	days to 50% silking
EHT	73%	2	82%	ear height
GY	73%	2	82%	grain yield adjusted to 15.5% moisture
PHT	73%	2	82%	plant height
TW	73%	2	82%	test weight

^a This is an example of a footnote in a table. Lowercase, superscript italic letters (a, b, c, etc.) are used by default. You can also use *, **, and *** to indicate conventional levels of statistical significance, explained below the table.

total of 86,006,888 sites (4.2% of the genome) [how this compared to human](#), were detected having GERP scores >0 ; and these sites were determined as evolutionary constraint sites. Genomic variants occurred on them were potentially deleterious. From genome-wide of view, generally, genomic sequences are evolutionarily conserved near the telomeric regions and the conserveness dropped toward centromeric regions (Figure S2); there are some exceptions, such as the long arm of chr4, chr5 and chr1.

Although genomic sites with GERP >0 were evolutionary constraint, they are not immune to mutation. Indeed, in our diallel population, N SNPs were detected at sites with GERP score >0 . Consistent with previous study (())[eli](#), the minor allele frequency (MAF) was negatively correlated with the mean GERP scores (correlation P value =). This observation indicated the putative deleterious alleles tend to be purged and maintained in a low frequency in the population.

[how many in genic and how many in non-genic](#)

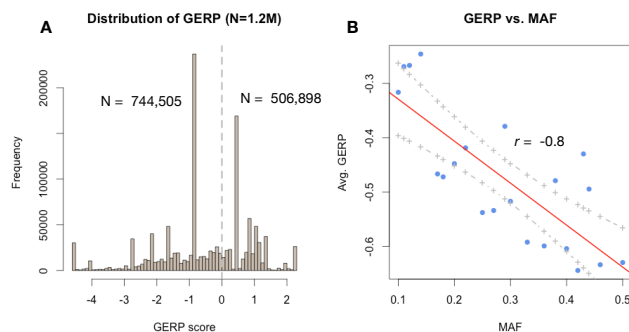


Figure 1 GERP distribution of SNPs and relationship between GERP and minor allele frequency. GERP scores were obtained for ~1.2 million (~10%) SNPs. The spikes of the MAF distribution. Of these, 506,898 (42%) were under evolutionary constraint and considered as deleterious variants. **(B)** Mean GERP scores were calculated for each bin (bin size = 0.01) of minor allele frequency (MAF). It shows that variants at conserved sites are maintained at low frequency. The red line and grey lines define the regression and its 95% confidence interval.

Supplementary Figure and a little bit story of the conserved

regions.

IBD region size and general statistics

The phenotypic effects of the genetic load of these potentially deleterious alleles have not been explored. To estimate their phenotypic effects and their contributions to heterosis, we conceived a genomic selection approach to predict their joint effects in the population. However, because SNPs with high GERP score are negatively correlated with the MAF (Figure and citation), they become less useful due to statistical limitation (citation). To capture the information carried by these potentially deleterious sites, we conducted the genomic-enabled prediction with the SNP's GERP score.

Identity by descent was estimated with fasibd. Average size 44,980 bp (36 to 10,320,000 bp) Figure 1.

SNPs in IBD regions IBD: ranged from 1 to 370,152, with the mean of 10,167 and median 4162. Number of SNPs in the IBD blocks: 1-1600, mean 22.83 and median 9.

To estimate the phenotypic effects of the genetic loads, we conceived the Genomic selection approach to evaluate whether incorporating the GERP score would potentially improve the prediction accuracy. However, the population in this study is relative small with only 66 individuals. The 20k conserved SNPs are highly colinear with others. Simple SNP based analysis would suffer a lot from so called big p small n problem. To allviate this problem, we employed a haplotype-based approach for genomic selection, where IBD blocks were considered as a haplotype (citation).

With pairwise comparisons, IBD regions were chopped into 55,000 IBD blocks. These IBD blocks have the average size of 44,980 bp (ranged from 36 to 10,320,000 bp, Figure S3).

For IBD blocks >1 -kb and contains at least one deleterious alleles (SNP variation with GERP >0), they were used for the analysis. Two modes, additive and dominant, were used to code the IBD blocks as the measurement of the conserveness of haplotypes.

Evolutionary conservation information improved prediction accuracies

With a 5-fold cross-validation approach, the prediction accuracies of the real data and circularly shuffled data were compared. As shown in Figure 2, for traits *per se*, prediction accuracies were

significantly ($FDR < 0.05$) improved for ASI and PHT when incorporating GERP score information in the IBD blocks under the additive model; prediction accuracy was significantly improved for ASI under the dominant model. For heterosis (measured by BPH and pBPH), incorporation of GERP scores improved BPH of GY under the additive model and improved BPH of DTP, DTS and TW under the dominant model; and the method improved predictions for pBPH of TW under the additive model and pBPH of GY under the dominant model (Figure 2 C-F, Supplementary table N). In general, the average prediction accuracies are higher using the additive model ($r = 0.81, 0.49$ and 0.29 for trait *per se*, BPH and pBPH) than the dominant model ($r = 0.70, 0.42$ and 0.24). And the prediction accuracies decreased for predicting heterosis transformations (BPH and pBPH) as compared to predictions for traits *per se*.

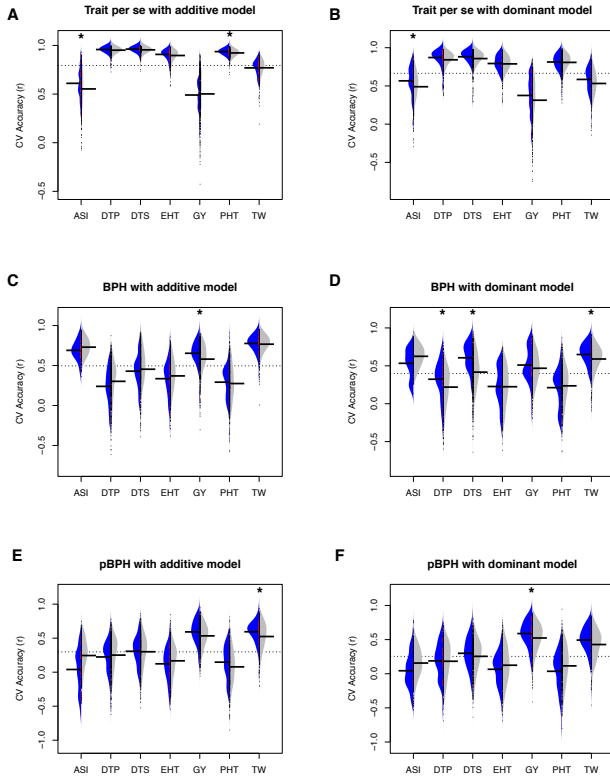


Figure 2 Beanplots of cross-validation accuracies. Cross-validation experiments were conducted using SNPs with positive GERP score and circular shuffled data from the same set of the genic for traits *per se* (A, B), HPH (C, D) and pHPH (E, F) under additive (A, C, E) and dominant (B, D, F) models. Accuracies from the real data were plotted on the left side of the bean (blue) and permutation results plotted on the right (grey). Horizontal bars on beans indicate mean accuracies. The grey dashed line indicates the overall average accuracy. Stars indicate significantly improved cross-validation accuracies with $FDR < 0.05$.

To rule out the possibility that genic SNPs are more conserved than intergenic ones. We elected the SNPs in genic regions and did the circular shuffling to random assign GERP scores to the same set of the selected SNPs. By doing this, the relatedness of the SNPs were kept the same, the difference were

considered came from the GERP scores.

MAF by categories!

As the results, for traits *per se*, model prediction accuracies were significantly improved for ASI, DTS and PHT when incorporating GERP score information under the additive model. Prediction accuracies were significantly improved for ASI, DTP, GY and TW under the dominant model. In general, the average prediction rates are higher using the additive model ($r = 0.8$) than the dominant model ($r = 0.7$). For heterosis, incorporation of GERP scores only improved grain yield prediction and only under a dominant model (Figure 3).

A Bayesian-based approach (BayesC) (Habier et al., 2011) was employed for the genomic selection (GS) experiments. To estimate predict accuracies, the diallel population was randomly divided into training and validation sets for 10 times using a 5-fold cross-validation method. Circular permutations were used both considering all SNPs or considering only genic SNPs to control for differences between genic and nongenic regions.

For traits *per se*, model prediction accuracies were significantly improved for ASI, DTS and PHT when incorporating GERP score information under the additive model. Prediction accuracies were significantly improved for ASI, DTP, GY and TW under the dominant model. In general, the average prediction rates are higher using the additive model ($r = 0.8$) than the dominant model ($r = 0.7$). For heterosis, incorporation of GERP scores only improved grain yield prediction and only under a dominant model.

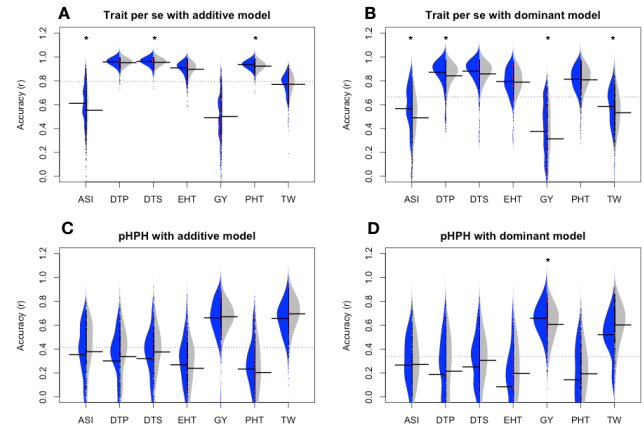


Figure 3 Beanplots of cross-validation accuracies. Cross-validation experiments were conducted using genic SNPs and circular shuffled data from the same set of the genic SNPs for traits *per se* (A, B) and pHPH (C, D) under additive (A, C) and dominant (B, D) models. Accuracies from the real data were plotted on the left side of the bean (blue) and permutation results plotted on the right (grey). Horizontal bars on beans indicate mean accuracies. The grey dashed line indicates the overall average accuracy. Stars indicate significantly improved cross-validation accuracies.

The top predictors are enriched in centeromeic regions

1. It capture high order of interactions.

2. Explanatory variables came from the centeromic regions.

TO DO: 0. preparing all the traits (per se, HPH, MPH, pHPH and pMPH) 1. comparing same no. of random SNPs vs. GERP SNPs without considering their SCORE. 2. comparing MAF in

different categories 3. training all the data and get the idea of significant ones.

Discussion

- how do results match with heritability and heterosis?
- do we support deleterious model of Mezouk et al.?

Schmitt: How to explain the prediction difference? - First, broad sense heritability of the traits are different. Second, from the simulation we learned that different traits may be controlled by different proportion of additive, dominant and even recessive gene actions. Our naive model only built the pure additive and pure dominant effects in. For the more complicated cases, the models may not work very well.

In this study, more than 500,000 deleterious SNPs were identified in elite maize lines including in noncoding regions of the genome. Majority of them were maintained in a low frequency, which is consistent with the previous observation [Eli PNAS, 2015](#) and indicates the deleteriousness of the variants in the conserved sites. A genomic selection pipeline was developed, which utilized evolutionary conservation information in the model. Cross-validation results suggested prediction accuracies for some traits could be significantly improved by incorporating GERP scores.

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Supporting Information

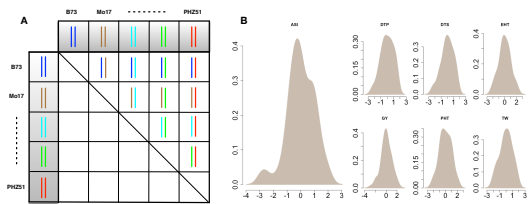


Figure S1 Diallel experimental design and distribution of phenotypic data. (A) Twelve maize inbred lines were selected and crossed in a half diallel. Ten of these (LH1, LH123HT, LH82, PH207, 4676A, PHG39, PHG47, PHG84, PHJ40, PHZ51) are proprietary inbreds that have expired from Plant Variety Protection (PVP) and represent the lineage of key heterotic germplasm pools used in present-day commercial corn hybrids. Two of them are important public inbreds, B73 and Mo17. (B) Phenotypic data were collected for anthesis-silking interval (ASI, in days), days to 50% pollen shed (DTP), days to 50% silking (DTS), ear height (EHT, in cm), grain yield adjusted to 15.5% moisture (GY, in bu/A), plant height (PHT, in cm), and test weight (TW, in pounds). Analyses were carried out on the traits per se as well as percent high parent heterosis (pHPH).

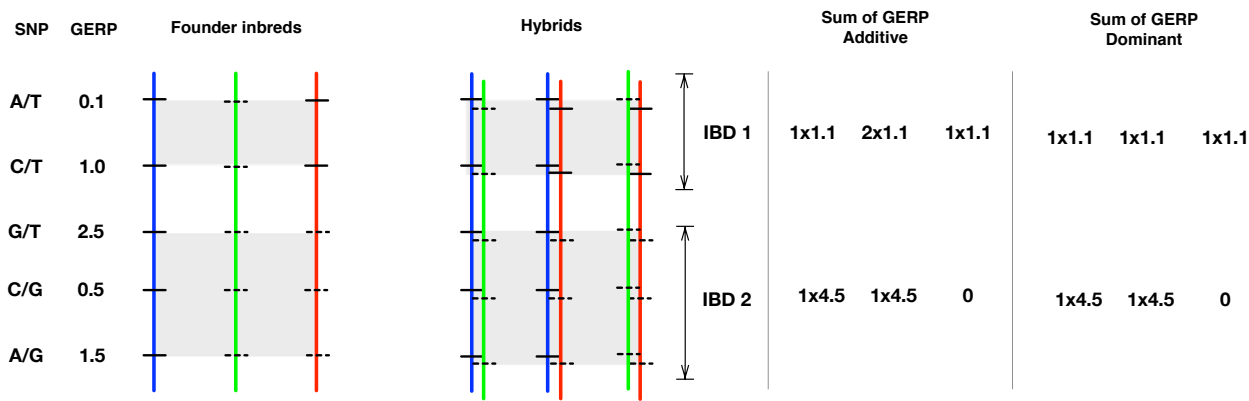


Figure S2 Incorporation of conservation information into IBD blocks. Regions of the genome that are identical by descent (IBD) among the 12 inbreds were identified using Beagle (Browning and Browning 2009). The GERP scores of SNPs in an IBD block were summed under both additive and dominant models. Under the additive model, 2 x GERP score was assigned to genotypes homozygous for the non-reference allele, 1 x GERP score was assigned to heterozygotes, and 0 was assigned to the homozygous reference genotype. Under the dominant model, 1 x GERP score was assigned to both genotypes with a nonreference allele and 0 to the homozygous reference genotype.