Interchromosomal Linkage Disequilibrium Analysis Reveals Strong Indications of Sign Epistasis in Wheat Breeding Families

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Abstract

Additive gene action is assumed to underly quantitative traits, but the eventual poor performance of elite wheat lines as parents suggests that epistasis could be the underlying genetic architecture. Sign epistasis is characterized by alleles having either a beneficial or detrimental effect depending on the genetic background, which can result in elite lines that fail as parents in certain parental combinations. Hence, the objective of this study were to test the existence of sign epistasis and examine its consequences to wheat breeding. The presence of sign epistasis is expected to distort the allele frequency distribution between two interacting genes compared to neutral sites, creating strong linkage disequilibrium (LD). To test this hypothesis, analysis of interchromosomal LD in breeding families was performed and detected 19 regions in strong disequilibrium, whose allele frequency distribution matched the sign epistasis prediction and falsified the competing hypothesis of additive selection. To validate these candidate interactions while avoiding the biases of a circular analysis and the confounding effects of genetic drift, two independent sets of populations were analyzed. Genetic drift was attributed to creating the sign epistasis patterns observed in eleven interactions, but there was not sufficient evidence to reject the sign epistasis hypothesis in eight interactions. Sign epistasis may explain the poor performance of elite lines as parents, as crossing lines with complementary allelic combination re-establishes epistatic variance in the offspring. Reduction in the effective population size in certain crosses may also occur when unfavorable sign epistatic combinations are deleterious. The potential existence of di-genic and higher order epistatic interactions in elite germplasm can tremendously impact breeding strategies as managing epistasis becomes imperative for success.

Introduction

The infinitesimal model (Fisher, 1918) proposes that additive effects play a primary role in genetic inheritance, while relegating dominance and epistasis to lesser importance (Turelli, 2017). However, the eventual occurrence of elite lines performing poorly as parents suggests that epistasis might be the underlying genetic architecture. Sign epistasis, a significant form of gene action in evolutionary genetics (Wright, 1932), is characterized by alleles exhibiting either advantageous or detrimental effects depending on the allelic state of the interacting gene (Weinreich et al., 2005; Poelwijk et al., 2007). Under the influence of sign epistasis, genotypes can adapt to environmental pressures in various combinations of alleles, often leading to similar adaptation (Wright, 1988). In breeding, it implies the existence of elite genotypes with different frameworks of favorable allelic combinations. Hence, crossing contrasting genotypes causes the favorable allelic combinations to segregate in the offspring and results in mean performance lower than the midparent value. This clash of epistatic interactions between parental lines may justify the eventual poor performance of elite lines as parents.

Establishing the importance and extent of epistasis in wheat breeding by modeling epistatic variance has tremendous challenges because of the lack of orthogonality with additive effects (Tessele et al., 2024; Raffo et al., 2022; Vitezica et al., 2017). However, the existence of sign epistasis interactions under selection may leave detectable signatures in the genome. The influence of sign epistasis in each locus closely resembles the effects of balancing selection, which maintains genetic diversity at sites under selection and can augment diversity at linked loci (Lewontin and Hubby, 1966; Hudson and Kaplan, 1988; Charlesworth et al., 1997; Takahata and Satta, 1998). The polymorphism flanking the loci under balancing selection can span over large or short regions, depending on local recombination frequency and strength of LD (Charlesworth et al., 1997; Wiuf et al. 2004; Charlesworth, 2006), the age of the advantageous polymorphism and the selection intensity (Kreitman and Rienzo, 2004; Tian et al., 2002). Besides affecting each locus independently, sign epistasis also impacts the relationship between interacting genes because selection acts on allelic combinations rather than on individual alleles. In a two-way interaction, the preferential selection of favorable allelic combinations distorts the allele frequency distribution of interacting genes compared to two neutral sites, creating strong disequilibrium that can be regarded as a selection signature.

Although selection for sign epistasis may leave signatures in the genome, the mere existence of LD does not imply sign epistasis. For instance, short-range linkage disequilibrium (SRLD) primarily arises due to random genetic drift or common ancestry of chromosomal blocks (Koch et al., 2013). SRLD are perpetuated under low recombination, making it virtually impossible to disentangle from an eventual sign epistasis signature. Long-range linkage disequilibrium, however, suggests that a countervailing force to recombination maintains strong LD between

distant sites, which is expected to be even more prominent in interchromosomal sites because the independent assortment of chromosomes recombines physically unlinked loci in every meiotic event. If sign epistasis is pervasive in wheat, breeding programs may perpetuate interchromosomal LD by continuously selecting favorable interactions. Detecting true selection signatures of epistasis (Lewontin et al., 1960) is challenging as other confounding effects can also create long-range LD, as population admixture (Maccaferri et al., 2005; Chao et al., 2010), genetic drift (Koch et al., 2013), artificial selection (Flint-Garcia et al., 2005; Joukhadar et al., 2019), or structural variation in chromosomes (Zhao et al., 2022). Disentangling signatures of epistasis from genetic artifacts may be possible through examining the allele frequency of sites in strong LD, as additive selection is expected to create a different allele frequency distribution than epistasis. Population structure can be minimized by analyzing breeding families independently. Analyzing interactions independently from the base population used for discovery may reduce the bias steaming from genetic drift and prevent statistical inference on a circular analysis (Kriegeskorte et al., 2009).

The main hypothesis is that sign epistasis exists and has major implications to wheat breeding. The objectives were to: (i) validate the existence of loci in abnormally strong interchromosomal linkage disequilibrium; (ii) test if selection for sign epistasis creates the observed strong interchromosomal LD; (iii) verify the existence of and disentangle confounding genetic artifacts that could likewise create strong interchromosomal LD.

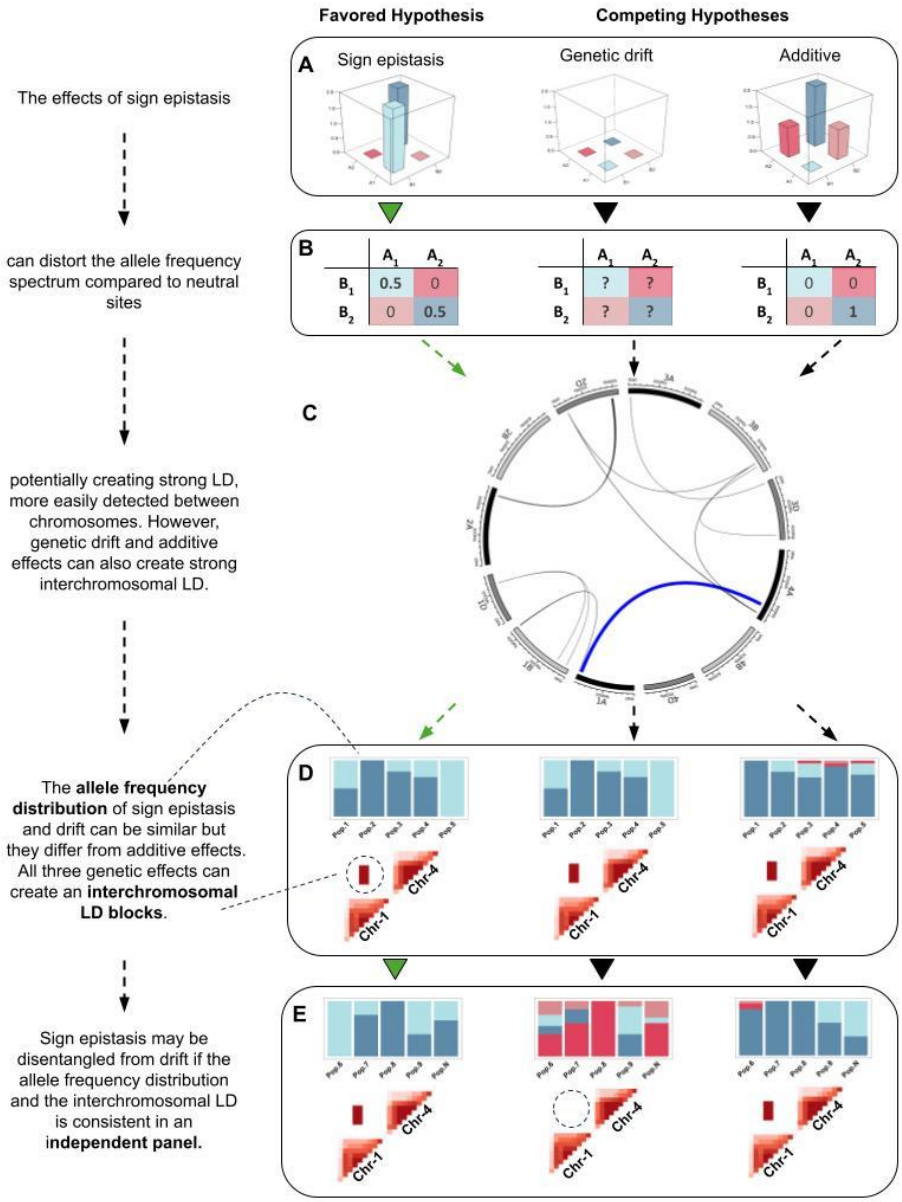
Material and Methods

Plant Material and Genomic Data

The dataset consisted of genomic information on experimental lines from the Kansas State Hard Red Winter Wheat breeding program for the years 2011 through 2021, which were categorized based on the last stage of the breeding pipeline reached by each experimental line. The four stages in the breeding pipeline were early, preliminary, advanced, and elite yield trials, named IPSR, PYN, AYN and EYN, respectively. All experimental lines were genotyped in the IPSR stage using genotype-by-sequencing (GBS) (Poland et al. 2012). As part of the genotyping pipeline of the K-State wheat breeding program, markers with a MAF <0.01, more than 20% missing values and more than 20% of heterozygotes were filtered, as described in Jarquin et al (2017). The R package 'rrBLUP '(Endelman, 2011) was used for marker imputation, where missing markers were imputed with the mean value among all lines for that marker and resulted in a total of 55,148 SNPs.

Validating the existence of strong interchromosomal linkage disequilibrium

106 This exploratory stages sought to validate the existence of interchromosomal linkage
107 disequilibrium in breeding germplasm. To test this hypothesis, experimental lines from the EYN
108 stage of the breeding program from 2010 to 2021 were considered. A linkage disequilibrium
109 analysis (r^2) focusing only on interchromosomal interactions was conducted and identified
110 pairwise markers in strong linkage disequilibrium. Validating the existence of strong
111 interchromosomal LD in wheat enabled further investigation into the hypothesis of selection
112 signatures of sign epistasis (Figure 1).
113



114
115 **Figure 1 Selection of sign epistasis interactions can create interchromosomal LD**. **A** depicts the
116 levels of adaptation of two genes (A and B) under the effects of sign epistasis, drift and additive.
117 **B** shows the expected allele frequency distribution under an ideal scenario for each genetic effect.

118 C illustrates strong linkage disequilibrium between pairwise interchromosomal sites, where inner
119 links connect sites in strong LD. D represents the allele frequency distribution in five populations
120 for two genes in strong LD and the respective heatmap of intra- and inter-chromosomal LD for
121 each genetic effect. E represents the allele frequency and LD values in an independent panel from
122 D, which highlights the consistency of sign epistasis contrasting to the randomness of genetic
123 drift.
124
125 *Minimizing the influence of population structure and genetic drift*
126 Historical data from 25 breeding families was used for the discovery of selection
127 signatures indicative of sign epistasis (Figure 1C). These were large families tested in the early
128 yield trial stage of the breeding pipeline, with sizes ranging from 20 to 45 genotypes. As a data
129 quality measure, a principal component analysis was conducted for each of the families to identify
130 and exclude any outlier genotypes detected in the two-dimensional plot defined by the first two
131 principal components to mitigate the potential introduction of population structure resulting
132 from genotyping or seed processing errors.
133 An interchromosomal linkage disequilibrium (r^2) analysis was conducted only on markers
134 with MAF>0.3 to reduce the total number of interactions to screen for and to increase the
135 likelihood of detecting interactions with allele frequency characteristic of sign epistasis (Figure 1A
136 and B). To circumvent the effect of population structure, the interchromosomal LD analysis was
137 conducted in each family individually and all interactions presenting $r^2 > 0.9$ were retained.
138 Although this approach controls population structure, it was highly influenced by genetic drift
139 within families. To address the genetic drift effect, the combined data of the 25 families was used
140 to run another interchromosomal LD analysis, and all interactions presenting $r^2>0.8$ were
141 selected. Interactions selected in both analyses and observed in a minimum of two families were
142 deemed worthy of further investigation.
143 In parallel, a control interaction was utilized to serve as a null hypothesis. The control
144 interaction consisted of two randomly selected single nucleotide polymorphisms (SNPs) with MAF
145 greater than 0.3 and found in chromosomes 1D (S1D_463434894) and 2B (S2B_163607648). To
146 establish a null control for the candidate three-way interaction, a third SNP was sampled from
147 the genome, which was found in chromosome 5A (S5A_298295448). The analytical procedures
148 applied to assess the candidate interactions were also applied in the evaluation of the control
149 interaction.
150
151 *The Balancing Selection Nature of Sign Epistasis and Interchromosomal Linkage Blocks*
152 A sign epistatic interaction under selection, besides the characteristic intrachromosomal

153 haplotype block, may also create an "interchromosomal block" (Figure 1D). To investigate this
154 hypothesis, 40 markers flanking each candidate sign epistatic marker were used to run a linkage
155 disequilibrium analysis, and the resulting intra and interchromosomal LD values were used to
156 construct heatmaps of 2D LD plots.
157
158 *Allelic Frequencies to Differentiate Additive and Sign Epistasis*
159 Selection for sign epistasis is expected to change allele frequencies differently than
160 additive selection (Figure 1B). To empirically test this hypothesis, the allele frequencies of each
161 candidate interaction was computed, and the patterns were analyzed (Figure 1D). Since these
162 families were genotyped in the $F_{5:6}$ generation using GBS, heterozygous loci were still observed.
163
164 *Independent Family and Breeding Program Validation*
165 To minimize the effects of genetic drift and to avoid a circular analysis, 15 distinct
166 validation families were selected to reanalyze the heatmaps of 2D LD plots and the distribution
167 of allele frequencies in order to validate candidate interactions (Figure 1E). Although independent
168 from the discovery set, the total number of families analyzed was relatively small and prone to
169 the effects of genetic drift. To diminish the confounding effects of drift in small populations, data
170 from a time span of 10 years in the wheat breeding program was also utilized to further validate
171 the consistency of candidate interactions. The breeding program dataset was divided sequentially
172 from early to elite yield trials and allele frequencies were calculated for each stage. In addition,
173 the combined data of the breeding program was used to falsify candidate interactions if the
174 opposite combination (relative to the most common allelic combination) had less than 10% of the
175 total allelic combinations.
176
177 *Software and Packages*
178 The linkage disequilibrium analysis was performed using the software PLINK (Purcell et
179 al., 2007). The R software packages "factoextra" (Kassambara et al., 2017), "circlize" (Gu and Gu,
180 2022), and "gaston" were utilized to perform the principal component analysis, to plot the SNPs
181 presenting strong interchromosomal LD, and to plot the heatmap of 2D LD plots, respectively.
182 The package "ggplot2" was used to plot allele frequency counts (Wickham, 2016). In addition,
183 built-in functions in R were used during data management and to calculate allele frequencies.
184
185 **Results**
186 *Detection of loci in strong interchromosomal linkage disequilibrium*
187 The goal of the discovery stage was to pinpoint a manageable number of interactions in

strong LD while minimizing the likelihood of identifying artifacts of population structure, genetic drift, or additive selection. In total, 269 pairwise SNPs, which represent 19 interacting genomic regions, passed the set thresholds, and were considered candidate selection signatures (Figure 2). Wheat subgenome B had the most candidate interactions with 15, followed by A and D with 13 and 10, respectively. The chromosomes with the most candidate interactions were 1B and 6B, with four interactions each. In total, 14 out of the 19 interactions were intergenomic, and most involved subgenomes B and D. Subgenome A and B had candidate intragenomic interactions, which were absent in subgenome D. In addition, a candidate of a three-way selection signature was also observed in chromosomes 2D, 3B and 4A (Figure 2). A total of seven candidate interactions had opposed allelic combinations fixed in different families, and 12 were fixed for one allelic combination while the opposed allelic combination was observed in segregating families (Supplemental Table 1).

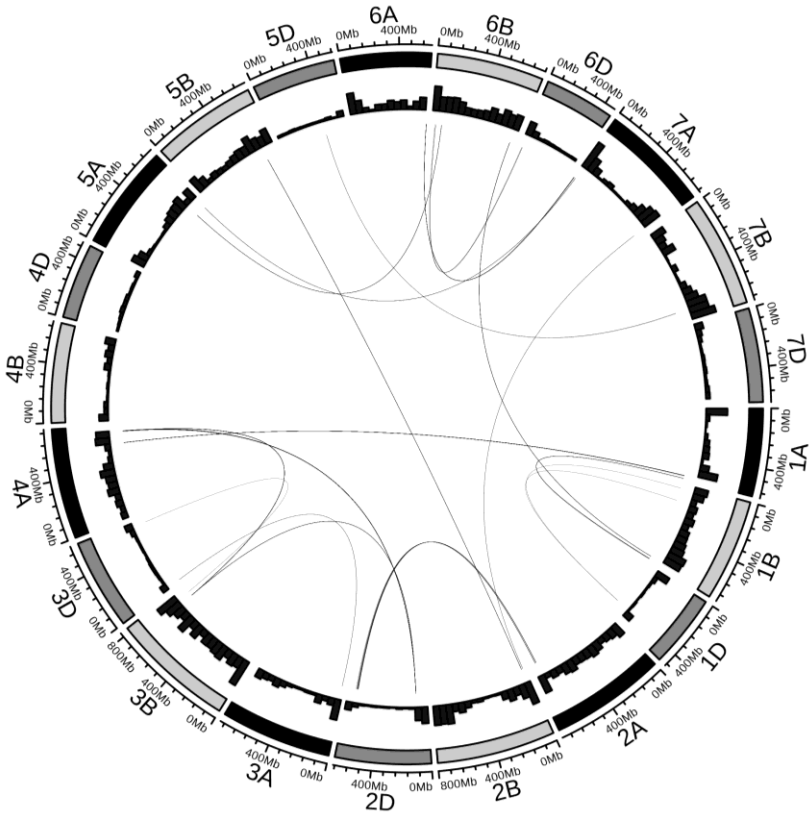


Figure 2 Candidate selection signatures of sign epistasis. The inner links represent regions (SNPs) in strong interchromosomal LD following the discovery approach previously described. The outer histogram plots represent the marker density in each chromosome. The outermost bar represents the 7 chromosomes and 3 subgenomes of wheat.

207 *Putative balancing selection associated with sign epistasis*

208 The analysis of linkage drag was performed for each of the 19 candidate selection
209 signatures of sign epistasis. The results for the candidate interaction involving chromosomes 1A
210 and 4A reveals the presence of "interchromosomal linkage blocks" associated with the candidate
211 selection signature (Figure 3B). These blocks are primarily attributed to a sizable linkage block
212 located on chromosome 1A and a smaller block situated on chromosome 4A. The control
213 interaction, however, displayed some level of intrachromosomal LD on chromosome 2B but no
214 “interchromosomal LD block” was observed (Figure 3B).

215 The remaining interactions presented “interchromosomal linkage blocks” of variable
216 sizes, as the number of flanking SNPs in strong LD with the candidate interaction was variable
217 (Supplemental Figures 1-18). For example, candidate interactions 5D-7B, 2D-3B, 1BS-1D and 1BL-
218 1D were in intrachromosomal LD with a few SNPs, resulting in very small “interchromosomal LD
219 blocks”.

220

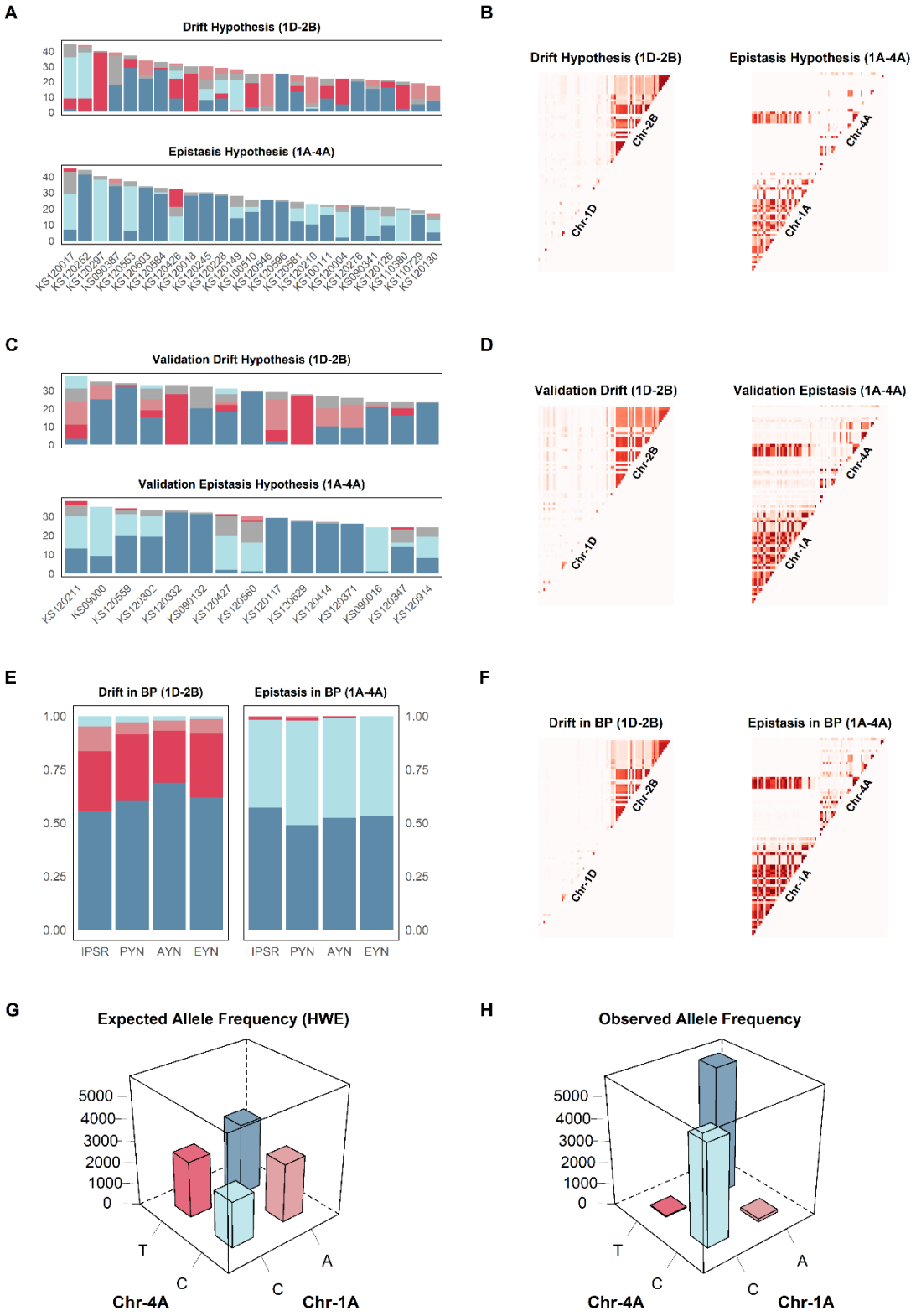


Figure 3. Testing independent breeding panels for sign epistasis. Allele frequency distribution (left column) and heatmaps of 2D LD plots (right column) of the candidate epistatic interaction involving segments of chromosomes 1A and 4A and the drift competing hypothesis involving segments of chromosomes 1B and 2D. **A** and **B** represents the data from the discovery panel. **C** and **D** displays data from the validation panel. **E** and **F** consists of data from the last four stages

227 of the breeding pipeline. G displays the expected allele frequency distribution in the entire
228 breeding program under Hardy-Weinberg Equilibrium and H shows the observed allele frequency
229 distribution.

230

231 *Allele frequencies disentangle epistasis from additive selection*

232 The allele frequencies of candidate interaction 1A-4A in the discovery stage reveals many
233 families nearly fixed for the A/T and C/C allelic combinations (Figure 3A). Families segregating for
234 these two alleles exhibited a high frequency of opposed allelic combinations, suggesting that
235 selection is actively favoring both the A/T and C/C allelic combinations. Off-combinations
236 consistently presented very low frequency in most families. The absence of a predominant allelic
237 combination in high frequency among segregating populations weakens the competing
238 hypothesis of additive selection. The allele frequency of the control interaction shows a variable
239 predominance of allelic combinations in different families, which is suggestive of genetic drift in
240 unselected loci (Figure 3A).

241 The candidate interactions 6B-6D, 3B-3D, 2B-5B had a similar distribution of opposing
242 allelic combinations as interaction 1A-4A (Supplemental Figures 1-A, 3-A, 4-A). Candidate
243 interactions 2A-2D, 2D-3B, 2D-4A, 3B-4A and 5A-7A had a common pattern where one of the
244 contrasting combinations predominated in most families, followed by the opposed combination
245 as the second most frequent, and followed by heterozygotes (Supplemental Figures 5-A, 8:11-A).
246 However, for interactions 5A-6B, 1B-6B, 5D-7B, 6A-7A, 3A-3D, 1A-1B, 1BL-1D,1BS-1D, 2B-7A and
247 6BL-6D, the most common allelic combination was found at a very high frequency in most
248 families, and the opposing combination was observed in only a few families (Supplemental Figures
249 2-A, 6-A, 7-A, 12:18-A). In all cases, the off-combinations were observed at very low frequencies
250 or absent in many families.

251 The allele frequencies of the candidate three-way epistatic interaction indicates the
252 predominance of the C/C/C alleles, which was fixed in many families (Supplemental Figure 19-A).
253 In segregating families, the opposing allelic combination (A/T/T) was found in high frequency,
254 suggesting that both combinations could be under positive selection. Off-combinations were less
255 common than heterozygotes in most families.

256

257 *Validation Families Support Sign Epistasis Hypothesis*

258 The patterns of LD in the validation families remained consistent with the discovery
259 families for interaction 1A-4A, where a long linkage block on chromosome 1A and a small block
260 on 4A created the characteristic “interchromosomal linkage block” (Figure 3-D). The frequency of

261 the allelic combinations in the validation families presented a similar pattern as the discovery
262 families (Figure 3-C). Six populations are fixed for the A/T combination, and the remaining 11 are
263 segregating with both A/T and C/C as the most frequent combinations. The frequency of off-
264 combinations was also very low. The random interaction (1D-2B) exhibited different allelic
265 combinations dominating different families as in the discovery families, highlighting the influence
266 of genetic drift in loci likely not under selection.

267 Interaction 2B-5B (Figure 4-C) had a similar pattern of allelic combination as interaction
268 1A-4A, where the validation families had an allele frequency pattern similar to the discovery
269 families. The interchromosomal linkage analysis for interactions 1B-6B, and 5D-7B did not present
270 the characteristic "interchromosomal LD block" (Supplemental Figure6-D and 7-D) because most
271 families were fixed for the major allelic combination (Supplemental Figure 6-C and 7-D).

272 On the allele frequency analysis, the candidate interactions 5A-7A, 1BL-1D, 1BS-1D, 2B-
273 7A and 6B-6Db (Supplemental Figures 11-C, 15:18-C) had at least one family with an off-
274 combination as the most frequent allelic combination. This suggests that these loci are not under
275 selection, and the sign epistasis hypothesis was rejected in these candidate interactions. For
276 interaction 6B-6D*a*, family KS120427 had an off-combination as the most frequent allelic
277 combination. However, the abnormal high frequency of heterozygotes, which could have a
278 biological meaning or simply be the consequence of genotyping errors, prevents drawing any
279 definitive conclusions about the interaction (Supplemental Figure 1-C). Similarly, for interaction
280 3B-3D, the family KS090132 was fixed for an off-combination but the remaining families
281 presented allelic frequencies matching the sign epistasis hypothesis (Supplemental Figure 3-C). It
282 is probable that the parental lines of population KS090132 were fixed for the off-combination,
283 which prevents observing the favorable candidate combinations. Thus, this interaction was kept
284 for further analysis.

285 For the remaining interactions, a consistent pattern emerged where one allelic
286 combination predominated in the majority of families, either fixed or at a very high frequency,
287 while the opposing allelic combination was observed in a few other families at varying frequencies
288 (Supplemental Figure 2-C, 5-C, 8:10-C, 12:14-C). The candidate three-way interaction was
289 segregating in few populations, with the allelic combination C/C/C as the most frequent, followed
290 by the opposed allelic combination (A/T/T) and a small occurrence of off-combinations
291 (Supplemental Figure 19-C). The remaining families were fixed for the C/C/C allelic combination.

292

293 *Breeding Program Validation*

294 To further test if the 12 non-falsified interactions are not the result of strong genetic drift
295 effects in small population sizes, data from 10 years of the breeding program was used to

296 calculate the frequency of allelic combinations. The results for interaction 1A-4A (Figure 1-E)
297 reveal that off-combinations were present at low frequencies in the early stages of yield trials
298 (IPSR). The frequency of off-combination declined with successive generations of selection,
299 ultimately leading to complete elimination of off-combinations in the EYN. Notably, the two
300 opposing allelic combinations, A/T and C/C, exhibited a balanced frequency across all breeding
301 stages analyzed. For the control interaction, an inconsistent pattern of allele frequencies was
302 observed across breeding stages.

303 The same pattern was observed for interactions 6B-6Da, 5A-6B, and 6A-7A (Supplemental
304 Fig 1-E, 2-E, and 12-E), which exhibited no or very few off-combinations at the EYN stage.
305 However, the latter two candidate interactions had the opposite combination with less than 10%
306 of the total allelic combinations, suggesting that the detection of these interactions may have
307 arisen due to genetic drift. Consequently, the sign epistasis hypothesis for the 5A-6B and 6A-7A
308 interactions was rejected. Similar allele frequencies were observed for interactions 3A-3D and
309 1A-1B, leading to the rejection of these combinations as well (Supplemental Figure 13-E and 14-
310 E).

311 The six remaining interactions (3B-3D, 2B-5B, 2A-2D, 2D-3B, 2D-4A, and 3B-4A) displayed
312 a high frequency of both opposing allelic combinations but showed minor variations in off-
313 combinations across the breeding stages (Supplemental Figures 3:5-E, 8:10-E). Interactions 3B-
314 3D, 2B-5B, and 2A-2D had an average of 94% of genotypes with the two favorable contrasting
315 allelic combinations, at an average ratio of 4:1 between the most common allelic combination
316 and its opposed combination. The interactions 2D-3B, 2D-4A, and 3B-4A comprised the candidate
317 three-way sign epistasis interaction and had the two favorable interactions in very high
318 frequencies across the four breeding stages (Figure 19-E). The C/C/C combination added up to
319 almost 88% of the genotypes across the breeding stages while the A/T/T represented a little over
320 10%. The remaining 6 off-combinations summed up to less than 2% of the interactions.

321

322 ## Discussion

323 *Strong interchromosomal LD could be created from selection of epistatic interactions*

324 The poor performance of elite lines as parents is hypothesized to be related to epistatic
325 effects. Although estimating epistatic variance is difficult because of non-orthogonality with
326 additive effects (Tessele et al., 2024; Raffo et al., 2022; Vitezica et al., 2017), selection signatures
327 of epistasis are expected to exist in breeding programs. In this study, 25 breeding families were
328 utilized to identify 19 regions in strong interchromosomal LD which are hypothesized to be
329 selection signatures of epistasis. Interchromosomal LD patterns were also observed in tetraploid
330 wheat (Laido et al 2014) and common beans (Rossi et al., 2009; Diniz et al., 2018), but no

331 inferences regarding the genetic mechanism leading to interchromosomal LD were drawn.

332 The allele frequency distribution of candidate interactions resembled that expected
333 under the influence of sign epistasis, which falsifies the additive hypothesis. Similar results were
334 also observed in wild barley, where the cross of plants based on spatial distance resulted in co-
335 adapted gene complexes fixed in locally abundant genotypes (Volis et al., 2011). In two diverse
336 populations of barley, several generations of natural selection resulted in two out of the 16
337 potential combinations of gametes (4^2) as the most frequent, which presented perfectly
338 complementary gametic types (Clegg et al. 1972), suggesting natural selection acted on
339 coadapted (Dobzhansky, 1970) multilocus units.

340 As an attempt of disentangling epistasis from drift, an independent set of 15 validation
341 families was utilized to further test the consistency of the candidate interactions, which falsified
342 seven interactions. As the strength of genetic drift effects is expected to be especially
343 predominant in smaller populations (Charlesworth et al., 2003; Charlesworth, 2009; Kliman et al.,
344 2008; Rothammer et al. 2013), the assembly of allelic combinations resembling selection for sign
345 epistasis in the discovery stage could have been a consequence of genetic drift. Random genetic
346 drift has also been attributed to the emergence of apparent selection signatures between pairs
347 of SNPs in simulation studies (Id-Lahoucine et al., 2019; Vilas et al., 2012).

348 To mitigate the impact of genetic drift, a significantly larger dataset spanning a decade of
349 breeding records was analyzed, leading to the rejection of four interactions. Among the remaining
350 eight interactions, 2A-2D, 2D-3B, 2D-4A, and 3B-4A had a single allelic combination representing
351 close to 90% of the total allelic combinations, contradicting the assumption of comparable
352 adaptability between opposing allelic combinations (Wight, 1932). However, this observed
353 phenomenon may relate to founder effects (James, 1971), where a restriction in allele frequency
354 within the founder germplasm results in the spread of one of allelic combination in the breeding
355 program. In that sense, the balanced frequency of opposed allelic combinations in interactions
356 1A-4A, 6B-6D, 3B-3D, and 2B-5B within an elite breeding program is surprising, considering that
357 recycling lines and extensive utilization of good parental lines is expected to favor one epistatic
358 combination over generations. For this reason, it is expected that the number and extent of sign
359 epistatic interactions should be much larger when considering different breeding programs, as a
360 function of founder effects, aka founder germplasm.

361

362 *Sign Epistasis Reduces the Effective Population Size of Breeding Populations*

363 The near-complete recovery of opposed allelic combinations as observed for interaction
364 1A-4A suggests artificial selection alone may not be the primary driver of distorted allele
365 frequencies. Instead, it is possible that natural selection is responsible for recovering favorable

366 allelic combinations, as observed in barley and wild barley populations (Volis et al., 2011; Clegg
367 et al. 1972), or through a nearly lethal response to off-combinations, similar to hybrid necrosis in
368 wheat (Tsunewaki, 1960). Anecdotal evidence on this hypothesis has been observed in single seed
369 descent families in the Kansas State wheat breeding program. In this speed breeding pipeline, the
370 quantity of seed recovered after three generations of inbreeding is often significantly lower than
371 expected based solely on poor levels of germination. This could be an indication that a varying
372 numbers of epistatic interactions, as interactions 1A-4A, 6B-6D, and 3B-3D, segregate in the
373 families and plants homozygotes to off-combinations may experience a severe setback on
374 performance which eventually leads to premature death or reproductive incapability. Populations
375 with a higher number of segregating epistatic interactions, if additionally segregating to
376 interactions 2B-5B, 2A-2D, and 2D-3B, for instance, may consequently end up with fewer viable
377 plants. One alternative to overcome this challenge is to constrain the genetic diversity of the
378 breeding program with the fixation of one of the interacting alleles, converting epistatic variance
379 into additive variance (Hill et al., 2008; Technow et al., 2021). In the candidate interaction 1A-4A,
380 fixing the A allele in chromosome 1A causes the allele in chromosome 4A to behave additively,
381 where the T allele is always positive, and C is always negative (Figure 3 A, C, and E). This ensures
382 that the selection pressure is constant for the alleles in the interacting locus and increases the
383 probability of having one favorable combination fixed.
384
385 *Sign Epistasis Creates a Rugged Surface on the Fitness Landscape*
386 The possible existence of di-genic sign epistasis, as observed in interaction 1A-4A (Figure
387 4A), implies that transitioning from one superior combination to another requires two allelic
388 substitutions, passing through a transitional state of inferior performance (Wright, 1988; Wade,
389 2002). The fitness landscape of the potential tri-genic sign epistasis interaction reported in this
390 study has a valley between the two optimum allelic combinations (Figure 4B). And, if extended to
391 higher order epistasis and more gene interactions (Figure 4C), it indicates that different
392 combinations of alleles can present similar levels of adaptation to the same environmental
393 conditions (Wright, 1988). The entire field of allelic combinations and respective levels of
394 adaptation can be greatly simplified through the fitness landscape metaphor (Figure 4D), as
395 proposed in the shifting balance theory (Wright, 1932), where hills and valleys represent high and
396 low levels of adaptation, respectively. The fitness landscape, as a metaphor, should not be taken
397 literally (Wade, 2002), as it is inadequate to represent higher order epistatic interactions (Wright,
398 1932). It does, however, provides a powerful simplified representation of complex genetic
399 systems and how populations navigate the rugged surface during evolution. The concept of
400 evolution under the shifting balance theory has striking similarities with the structure of breeding

programs (Technow et a., 2021) and could be leveraged to better understand epistasis in the face of managing breeding germplasm pools.

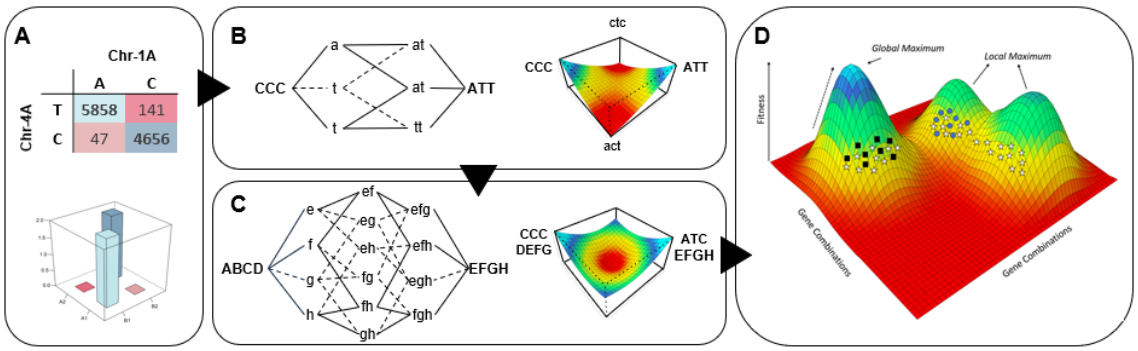


Figure 4. Rugged fitness landscapes are formed from sign epistasis interactions. A The allele frequency of interaction 1A-4A across the breeding program indicates a di-genic sign epistasis interaction. **B** The potential three-gene sign epistasis interaction involving chromosomes 2D, 3B and 4A creates a valley of adaptation between the two optimum allelic combinations. **C** Combining a hypothetical four- and the potential three-gene sign epistasis interaction creates a fitness landscape with regions of high, intermediate, and low adaptation. Factual representation of the fitness landscape is difficult because of the elevated complexity of the genetic system. **D** Rugged fitness landscape formed by several sign epistasis interactions with variable levels of gene interactions. This is an extreme simplification of a highly complex, multi-dimensional genetic system. Adaptations from Wight (1932).

Breeding Strategies on a Rugged Fitness Landscape

A rugged surface on the fitness landscape has implications for breeding programs as the strategy used to manage the germplasm pool can lead to higher or lower levels of epistatic variance in breeding families. The operation of a closed breeding program inevitably reduces the genetic diversity in the germplasm pool and, under the influence of drift and selection, leads to the fixation of alleles involved in sign epistatic interactions. This process converts epistatic variance into additive variance and increases the potential of adaptation of the breeding program (populations) to local environments (Wade and Goodnight, 1998), as less complex genetic systems have higher heritability and respond better to selection. This can be visualized in Figure 4D as the *black squares* or *blue circles* genotypes, located in independent hills and assumed to comprise separate breeding programs. Alternatively, operating a breeding program with a large genetic base may reintroduce epistatic variance when recycling lines from within the breeding program, which can be depicted as the *white star* genotypes in Figure 4D. The cross of parental

429 lines that belong to different adaptive peaks leads to a phenomenon called F_2 breakdown
430 (Whitlock et al., 1995), where the mean performance of the population is lower than the
431 midparent value. This is a consequence of disrupting, in the offspring, the contrasting epistatic
432 interactions that were homozygous in each parental line. Breeding families with high levels of
433 epistatic variance present a low correlation between early and late generations (Upadhyaya and
434 Nigam, 1998; Humphrey et al., 1969), indicating a limited response to early generation selection.
435 If late generation selection is adopted, the quick pace of inbreeding and the commonly small
436 population size of breeding families causes both favorable and unfavorable combinations to be
437 fixed, reducing the probability of assembling optimal allelic combinations.

438 One common feature of breeding programs is the exchange of germplasm, where elite
439 genotypes are anticipated to transmit superior allelic combinations from one breeding pool to
440 another (Wright, 1932; Technow et al., 2021). The success of introducing germplasm on a rugged
441 fitness landscape scenario depends on having both parental lines on the same adaptive peak, that
442 is, sharing the same founding allelic combinations, otherwise, F_2 breakdown may also be observed
443 in breeding families. For navigating on multiple adaptive peaks, broad genetic base programs may
444 be more suitable for introducing external germplasm as there is more than one set of founding
445 allelic combinations that could match the germplasm to be introduced. Although normally used
446 as a strategy to introduce a few alleles in the background of a recurrent line (Hospital, 2005),
447 backcrossing can be leveraged by closed breeding programs to recover the epistatic framework
448 of the recurrent line. Backcrossing constraints epistatic variance, increasing additive variance and
449 the response to selection in breeding populations. For a comprehensive discussion of the
450 implications of sign epistasis from the perspective of the fitness landscape metaphor, view
451 Tessele et al. (2024) and Holland (2001).

452

453 ## Conclusion

454 To expand the understanding of how epistasis could be responsible for the poor
455 performance of elite lines as parents and be of critical importance to breeding, the existence of
456 selection signatures of sign epistasis in wheat families was investigated in this study. In total, 19
457 candidate interactions were detected, whose allelic patterns agreed with the sign epistasis
458 predictions and refuting the competing additive hypothesis. To validate the candidate
459 interactions while avoiding the biases of a circular analysis and the confounding effects of random
460 genetic drift, two independent sets of materials were analyzed and falsified 11 interactions. There
461 was not sufficient evidence to reject the sign epistasis hypothesis for eight candidate interactions
462 with the current dataset and analysis. The possibility existence of sign epistasis could be
463 responsible for a reduction in the effective population size in crosses with contrasting sign

epistasis combinations, and for the eventual failure to recycle elite lines in the breeding program as epistatic variance is reestablished in the offspring. The potential presence of a rugged fitness landscape underscores the importance of adequately managing epistatic variance within breeding programs for efficiently developing elite germplasm.

Conflict of Interest

None declared.

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Data Availability

Marker information of discovery and validation populations in HapMap format is included in files 'Discovery-Populations.hmp.txt' and 'Validation-Populations.hmp.txt'. The code to run the PCA analysis to eliminate outliers within families is found in 'PCA.R'. The code used to calculate linkage disequilibrium is found in 'C-Linkage_Disequilibrium_Loops.bach'. The code used to calculate the 2D-LD plots in discovery and validation families and the control plot is found in the `*_2D_LDplot.R' files. The 'Circleze.R' file has the code used to plot interchromosomal LD with chromosomes displayed circularly. Supplemental material is available at Figshare. The raw genotypic data, available at Figshare, will be deposited at NCBI if/upon the manuscript is accepted for publication.

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