# Adaptation to heavy-metal contaminated environments proceeds via selection on pre-existing genetic variation.

**Kevin M. Wright 1*, Uffe Hellsten 2, Chenling Xu 3, Annie L. Jeong 4,5, Avinash Sreedasyam 6, Jarrod A. Chapman 2, Jeremy Schmutz 6, Graham Coop 3, Daniel S. Rokhsar 2,7, John H. Willis 4**

1. Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA, USA.
2. Department of Energy Joint Genome Institute, Walnut Creek, CA 94598, USA.
3. Department of Evolution and Ecology, University of California, Davis, CA, 95616, USA.
4. Department of Biology, Duke University, Durham, NC 27708, USA.
5. University Program in Genetics and Genomics, Duke University Medical Center, Durham, North Carolina, USA
6. Hudson Alpha Institute for Biotechnology, Huntsville, Alabama 35806, USA.
7. Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

* Corresponding Author: wright@fas.harvard.edu

**Across a species range, islands of stressful habitats impose similar selection pressures on isolated populations. It is as yet unclear, when populations respond to these selective pressures, the extent to which this results in convergent genetic evolution and whether convergence is due to independent mutations or shared ancestral variation. We address these questions investigating a classic example of adaptation by natural selection - the colonization of plant species to heavy metal contaminated soils. We use field-based reciprocal transplant experiments to demonstrate that mine alleles at a major copper tolerance QTL, *Tol1*, are strongly selected in the mine environment, but are neutral, or nearly so, in the off-mine environment. To identify scaffolds in genetic linkage with this locus, we assemble the genome of a mine adapted *M. guttatus* genotype and sequence near isogenic lines (NILs) homozygous for tolerant or non-tolerant alleles at *Tol1*. We identify genes with differential expression between NILs and differences in allele frequency between independent pairs of mine and off-mine populations to identify *Tol1* candidate genes. We identify a single gene, a multicopper oxidase, with large differences in expression between NILs and allele frequency between populations. Furthermore, we find patterns of genetic variation at *Tol1*, and four additional candidate adaptation loci, are consistent with selection acting upon beneficial haplotypes that predates the existence of the copper mine habitat. We estimate the age of selected *Tol1* haplotype to be at least 1700 years old and was at a frequency of 0.4-0.6% in the ancestral population when mining was initiated 150 years ago. These results suggest that adaptation to the mine habitat routinely occurs via selection on ancestral variation, rather than independent *de-novo* mutations or migration between populations.**

The most spectacular demonstrations of evolution often involve independent populations repeatedly evolving similar traits in response to similar environmental pressures (Endler 1977; Joron and Mallet 1998; Schluter, 2000). Convergent evolution of similar phenotypes suggests convergence in genotype, as may occur when selection operates on the same ancestral allele in independent populations or species (Colosimo *et al.* 2005; The Heliconius Genome Consortium, 2012). Additionally, there are multiple instances of the similar phenotypes evolving via independent genetic mutations at the same locus (Tishkoff *et al.* 2007, Karasov *et al.* 2010). When evolution occurs via selection on pre-existing genetic variation, adaptation to novel environments will proceed quite quickly. Conversely, when evolution proceeds via independent genetic mutations, adaptation will be limited by the waiting time for beneficial mutations to occur and go to high frequency. In order to understand the circumstances under which either of these evolutionary scenarios is likely to occur, and make predictions about how species will respond to rapid environmental changes such as global climate change, we investigate the genomic basis of a classic example of convergent phenotypic evolution.

Investigations into the repeated colonization of stressful soil environments by plants species have a long history in the development of evolutionary biology (Kruckeberg 1951; Turner 1969; Antonovics *et al.* 1971; Macnair 1987). With the initiation of the first, and still on-going, field experiments at Park Grass, UK,

scientists demonstrated that soil nutrient levels and pH impose strong selection on the local plant communities causing some plant species to rapidly evolve tolerance, whereas other species, despite ample migration opportunities, never colonize these stressful habitats (Brenchley 1958). This result has been replicated in investigations of plants colonizing heavy metal contaminated soils (Walley *et al.* 1974, Bradshaw 1984, Symeonidis *et al.* 1985; Al-Hiyaly *et al.* 1990, 1993). Interestingly, the only species to colonize contaminated soils also have tolerant individuals in populations unexposed to soil contamination. Whereas species that did not evolved tolerance, lack tolerant individuals in unexposed populations (*summarized in* Bradshaw, 1991). Given these results, Bradshaw concluded that tolerant species possessed the necessary genetic variation in ancestral populations, and species which remain non-tolerant lack the necessary genetic variation to adapt to novel environmental pressures (1991). However these results do not eliminate the possibility that tolerance was absent in the ancestral population and spread, by migration, to uncontaminated sites following adaptation. To distinguish between these two hypotheses, we need additional evidence on the source of adaptive genetic variation in natural populations.

Here, we investigate the recent and repeated adaptation of the annual wildflower, *Mimulus guttatus*, to copper contaminated sites near Copperopolis, CA (Macnair *et al.* 1993). Contaminated sites have 10-100 fold increase in the concentration of copper in the soil compared to uncontaminated sites (Allan and Sheppard 1971; Macnair et al. 1993; S. Table 1). Copper mining in this region of the Sierra Nevada foothills started in 1861 (Aubury 1908). Mine populations are located in close geographic proximity, within 40km of each other, and *M. guttatus* is very common in uncontaminated soils adjacent to mine sites (Allan and Sheppard 1973; Macnair *et al.* 1993). Copper tolerance is nearly fixed at four mine populations (99.77%, N =2796); at intermediate frequency (56.0%; N=1121) at eleven sites, mostly located within one mile of contaminated soil, and at low frequency (2.2%; N=1056;) in 11 populations located 1-10 miles from contaminated soil (Macnair *et al.* 1993). This suggests that the copper tolerance phenotype, measured as root growth in a hydroponic solution, is under strong selection in contaminated habitats. It remains to be determined whether each mine was colonized independently and whether the low frequency of tolerance at uncontaminated sites represents ancestral variation or back migration from the mines. Previously, we identified a single large effect QTL for copper tolerance, *Tol1*, in line from the Copperopolis mine population (Wright *et al.* 2013). It is unknown whether this same locus contributes to copper tolerance in other mine adapted populations. We use field based transplant experiments, population genomic sequencing, and coalescence modeling to address three key questions: Does *Tol1* affect fitness in mine habitat, and if so, is it the primary determinate of survival in this habitat? Did convergent phenotypic evolution in independent mine populations occur via convergent genetic evolution? Did mine alleles at the candidate adaptation loci evolve from new beneficial mutations or via selection on pre-existing standing variation?

**_Tol1_ affects fitness in copper mine habitat**

113 We directly measured whether the mine and off-mine genotypes are locally adapted
114 to their respective habitats using a reciprocal transplant experiment across multiple
115 years (S. Figure 1). We found that plants from the mine population have greater
116 fitness than off-mine genotypes in mine habitat, supporting the hypothesis of strong
117 selection in this environment (Figure 1; S. Table 2-9). In the off-mine habitat, mine
118 genotypes were also slightly favored compared to off-mine genotypes (Figure 1; S.
119 Table 2-9). This suggests that high fitness in the mine environment does not cause a
120 detectable trade-off in fitness in the off-mine environment. In order to measure
121 whether the copper tolerance locus affects fitness, we conducted a reciprocal
122 transplant experiment with introgression lines possessing alternate alleles at *Tol1*.
123 We constructed introgression lines by reciprocally backcrossing lines with mine and
124 off-mine alleles at the tolerance locus ($Tol1_{CC}$ or $Tol1_{MM}$) to mine ($CCC_{BC}$) and off-
125 mine ($MED_{BC}$ ) genotypes (S. Figure 2). In the off-mine habitat, we found no
126 significant effect of the *Tol1* locus in our $CCC_{BC}$ introgression lines, (95% CI: 0.14, -
127 0.36, $p$ = 0.68, S. Table 6 ) which suggests that tolerant alleles at *Tol1* are neutral (or
128 only weakly selected) in off-mine populations. Conversely, we found that mine
129 alleles at *Tol1* greatly increases the probability of survival to flowering in the mine
130 habitat (Figure 1; S. Table 2). The strength of selection (s) on *Tol1*, defined as the
131 relative fitness of each allele, was significantly stronger in the off-mine genomic
132 background ($MED_{BC}$ s=0.69) compared to the mine genomic background ($CCC_{BC}$
133 s=0.30). Additionally, the difference in fitness between parental lines, (s=0.90; S.
134 Table 2) is greater than the fitness effect of *Tol1*. These two results support the
135 hypothesis that additional loci contribute to fitness in the mine habitat. Our finding
136 that the *Tol1* mine allele does not impose a measureable fitness cost in the off-mine
137 habitat is consistent with either back-migration or ancestral variation as possible
138 scenarios explaining the low frequency of copper tolerant plants in this
139 environment. To distinguish between these two scenarios we need to measure
140 segregating variants at *Tol1* in mine and off-mine populations.
141
142 **Targeted assembly of *Tol1* region**
143 To accurately measure genetic variation at *Tol1*, we need to first improve the
144 assembly of this region. *Tol1* is located in a poorly assembled pericentromeric
145 region of linkage group 9 in the M *guttatus* reference assembly, notably
146 characterized by large amounts repetitive DNA and low recombination rates
147 (Wright *et. al.* 2013). We sought to map all genomic scaffolds linked to *Tol1* by
148 resequencing plants derived from our original Near Isogenic Line (NIL) mapping
149 population (Wright *et al.* 2013). To accomplish this, we sequenced two pools of
150 plants each composed of 100 plants homozygous for either tolerant (NIL_$Tol1_{TT}$) or
151 nontolerant (NIL_$Tol1_{NTNT}$) alleles at the genetic marker, *Sc84_37kb*, located 0.32 cM
152 from *Tol1* (Wright *et al.* 2013). We reason that that scaffolds in this region should,
153 except in the case of a rare recombination event, be fixed for different haplotypes in
154 the NIL_$Tol1_{TT}$ and NIL_$Tol1_{NTNT}$ pools. We estimate the recombination distance
155 between genomic scaffolds and the *Sc84_37kb* marker as the frequency of SNPs in
156 the two pools to used to define the two pools (S. Text). We identified eight scaffolds,
157 comprising 4.9 Mb, within a somewhat enlarged search space of 0.80 cM (S. Figure
158 3; S. Table 10). We also identified small portions of other scaffolds that are in tight

159 linkage to *Tol1* (data not shown), suggesting problems with the v1.1 reference
160 assembly and/or translocations between the reference and mapping lines. To
161 address these assembly issues we sequenced a Copperopolis inbred line, CCC52, to
162 220X coverage and constructed a *de novo* genome assembly (S. Text, S. Table 11).
163 We repeated our mapping effort using this *de novo* assembly and identified 53
164 CCC52 scaffolds, totaling 3.1Mb in length, within 0.80 cM of the *Sc84_37kb* marker
165 (S. Text, S. Table 12). While most of the scaffolds are homologous to reference
166 assembly *Tol1* linked scaffolds, eight of them are unique to the CCC52 assembly.
167 Using this improved assembly, we sought to identify genes that exhibit significant
168 differences in expression between NIL_*Tol1*$_{TT}$ and NIL_*Tol1*$_{NTNT}$ lines.
169
170 **Differential expression of *Tol1* link genes**
171 We measured gene expression in the NIL_*Tol1*$_{TT}$ and NIL_*Tol1*$_{NTNT}$ lines grown in a
172 full-strength Hoaglands solution and Hoaglands enriched with 1.25 ppm CuSO4. We
173 extracted mRNA from root tissue, sequenced each mRNA library to 140X coverage,
174 and aligned all reads to a *de novo* transcriptome assembly (S. Text). We find that the
175 majority of differentially expressed genes are due to allelic variation between the
176 NIL_*Tol1*$_{TT}$ and NIL_*Tol1*$_{NTNT}$ lines, the copper treatment had negligible effect on
177 gene expression. We identified 277 genes that are differentially expressed (DE)
178 (Benjamini & Hockberg adjusted p-value < 0.1 and $\log_2$ fold change > 1.0) between
179 lines in either treatment, 37 of these genes are located on the 53 *Tol1* linked
180 scaffolds (S. Figure 4, S. Table 13). Some *Tol1* link DE genes - including a heavy
181 metal ion transporter, a potassium ion transporter, and a multicopper oxidase –
182 function to maintain intracellular ion homeostasis and may underlie the copper
183 tolerance phenotype. Next, we sought to determine whether *Tol1* linked DE genes
184 exhibit large differences in allele frequency in mine/off-mine population
185 comparison.
186
187 **Identification of candidate adaptation loci at *Tol1***
188 We sought to identify loci at *Tol1* that may underlie adaptation to the copper mine
189 environment by measuring the genetic variation within and between two mine and
190 two offmine populations. In a classic hard selective sweep, selection drives a single
191 new beneficial mutation to near fixation, causing linked sites to hitchhike to high
192 frequency, leading to a local reduction in genetic variation and large differences in
193 allele frequency between the ancestral and derived populations (Maynard Smith &
194 Haigh 1974, Barton 2000, Berg and Coop 2015). Alternatively, selection on pre-
195 existing adaptive variants, which reside on multiple haplotypes, is predicted to
196 produce a much smaller change in allele frequency at surrounding sites because no
197 single haplotype goes to fixation (Innan and Kim 2004, 2008; Prezeworski *et al.*
198 2005). This event is sometimes termed a soft selective sweep (Messer and Petrov
199 2013). By comparing independent populations that have both adapted to similar
200 environments, we can distinguish between selection on newly derived beneficial
201 mutations or shared ancestral variation.
202
203 We sequenced pooled DNA from 20-31 natural isolates from two mine and two off-
204 mine populations to 34-72X genome-wide coverage (S. Text, S. Table 1, S. Figure 5).

205 Genome wide nucleotide diversity is similar across all populations, ($\pi$ = 0.0254 –
206 0.0268 ; S. Table 14). Genome-wide estimates of genetic differentiation between
207 populations, measured with $F_{st}$ and the *G* statistic (accounts for difference in
208 coverage among sites and populations, S. Text) are higher between mine/off-mine
209 compared to off-mine/off-mine population comparisons ($F_{st}$ M/OM= 0.07-0.14; $F_{st}$
210 OM/OM=0.02; S. Table 15). Elevated genome-wide estimates of differentiation
211 between mine/off-mine populations is caused by a few, large effect genomic regions
212 (ie. S. Figure 6) whereas the majority of the genome shows little differentiation.
213 These results suggest that the mine populations did not undergo a strong bottleneck
214 and/or gene flow readily occurs between populations in contrasting habitats.
215
216 We identified genomic regions with elevated difference in allele frequency at *Tol1*,
217 as well as at other sites across the genome. Differentiation at *Tol1* is not elevated
218 across all scaffolds in tight linkage to this locus (S. Figure 7, 8), as would be expected
219 from a single hard selective sweep (S. Text), instead we find multiple loci with
220 nearly fixed differences in allele frequency between mine/off-mine populations
221 surrounded by large regions of shared genetic variation. We focus on loci that
222 exceed the 0.5% genome-wide threshold of allelic differentiation in both mine/off-
223 mine population comparisons (allelic differentiation is measured using the *G*
224 statistic in 500bp bins, S. Text). We identify five distinct candidate adaptation loci,
225 harboring seven genes, within the 53 *Tol1* link CCC52 scaffolds (S. Table 16). The
226 signal of differentiation at these loci is relatively narrow (<20kb) with no significant
227 differentiation at physically adjacent regions. To identify which, if any, of these
228 genes maybe a *Tol1* candidate, we compared these genes to the 37 differential
229 expression genes from the RNA-seq experiment. We find, one gene, a multicopper
230 oxidase (MCO) is significantly differentiated in both experiments. MCO is located on
231 a small scaffold (*s47895 - 1.7kb*) that is absent from the reference genome.
232 Furthermore, we find that mine populations exhibit a 6X fold enrichment of aligned
233 reads at MCO (S. Table 17), suggesting a recent tandem duplication of this gene is
234 responsible for the 12X increase in expression observed in copper tolerant lines (S.
235 Table 13). This gene represents an interesting *Tol1* candidate, but additional
236 functional studies are required to determine whether it is responsible for the copper
237 tolerance phenotype. Building upon the results from our transplant experiment, that
238 *Tol1* accounts for some but not all of the variation in fitness in the mine
239 environment (Figure 1), we next sought to identify additional loci with a population
240 genetic signature of selection.
241
242 **Genome-wide survey of candidate adaptation loci**
243 We identified multiple genomic regions with elevated levels of differentiation
244 between mine and offmine populations. As with *Tol1*, we focused on candidate
245 adaptation loci that exceed the 0.5% genome-wide *G* statistic threshold in mine/off-
246 mine population comparisons. Some loci are unique to one mine/off-mine
247 population comparison (S. Table 18, 19), however, loci with the largest difference in
248 allele frequency, 0.5% and 0.1% outliers, are much more likely to be shared
249 between the mine/off-mine population comparisons than would be expected from
250 random chance (S. Table 20; S. Figure 9). We rank the loci by their degree

differentiation (the quantity of physically adjacent 0.5% outlier bins) in both mine/off-mine population comparisons and find that *Tol1*, although it has a large effect on fitness, ranks as the fifth most differentiated region of the genome (Table 1). The identify of the top candidate adaptation loci in our screen are largely robust to changes in the genome-wide *G* statistic threshold (1%, 0.5%, 0.1%; S. Table 21), clustering of adjacent outlier bins (5kb, 10kb, 100kb; S. Table 22), the minimum number of SNPs per bin and measures of population differentiation (5 or 10 SNPs; *G* or $F_{st}$; S. Table 23). Nearly all candidate adaptation loci are restricted to single genes which encode proteins that function to maintain intracellular ion homeostasis (copper ion transporter, iron-zinc ion transporter, transmembrane ferric reductase, and potassium ion transporter;Table 1) and, in some cases, they have been implicated in heavy-metal tolerance (ie. Hanikenne *et al.* 2008 ; Grennan *et al.* 2009). The young age of mine populations and narrow interval of divergence (<30kb) suggests that beneficial variants pre-date the establishment of mine populations.

**Selection on pre-existing genetic variation**

The narrow interval of genetic differentiation at each candidate adaptation locus is inconsistent with expectations from a hard selective sweep. Given the intense selection on *Tol1* (s=0.69, Figure 1), a hard selective sweep would cause the mine allele to reach near fixation in ~20 generations and reduce genetic diversity by >50% for ~3Mb from the selected site (S. Text). In contrast, we observe that for *Tol1* outlier loci, heterozygosity recovers to 50% of background within a maximum of 20kb (S. Table 22). This quick recovery, despite strong selection, strongly suggests that the selected allele at *Tol1* was segregating in the ancestral population prior to the establishment of the mine populations. If this is true, we predict that each mine population would share the same haplotype at the candidate loci. In order to assess this hypothesis for the *Tol1* region, and all other candidate adaptation loci, we measured genetic differentiation between mine populations.

We find that three loci in tight linkage to *Tol1* and four additional unlinked candidate adaptation loci exhibit regions of very low genetic differentiation between mine populations (Table 2; i.e. Figure 2, S. Figure 9-15). This is inconsistent with independent sweeps of new mutations, but rather with selection on shared standing variation. Mine populations share the same *core haplotype* at these loci (extending from 1-18kb; Table 2) and outside this core, genetic differentiation between mine populations rises dramatically to levels comparable to differentiation between mine and off-mine populations (Figure 2). This peak-valley-peak pattern is additional evidence that the *core haplotype* was present in the ancestral population and recombined onto multiple genomic backgrounds prior to the establishment of the copper mines (Roesti *et al.* 2014). How old must the *core haplotypes* be to have an opportunity to recombine onto different genomic backgrounds prior to the initiation of mining in the region?

**Age of core haplotypes**

296 We estimate the age of the *core haplotypes* using the rate at which genetic diversity
297 around the mine allele recovers to background levels (S. Text). We find that all *core*
298 *haplotypes* predate the establishment of the copper mines (Table 2). Not
299 surprisingly, the locus with the strongest signature of selection, MET, has the
300 youngest *core haplotype* (639 generations), but even this haplotype existed in the
301 ancestral Copperopolis population long before the establishment of the mine (150
302 years; Table 2). In contrast to the four unlinked *core haplotype* regions, we find
303 three tightly linked *core haplotypes* at *Tol1*. This suggests these haplotypes were in
304 linkage disequilibrium (LD) in the ancestral population, a hypothesis consistent
305 with the result that each scaffold maps to <0.05 cM of the same position (S. Table
306 12), and LD was maintained as they rose to high frequency in each mine population.
307 We estimate the age of the *core haplotypes* at the three *Tol1* linked genes to predate
308 the establishment of the copper mines by hundreds-thousands of years (Table 2).
309 The CDK and PUM haplotypes have been present in the ancestral Copperopolis
310 population for 1.7-2.7K generations, whereas the MCO haplotype is much older
311 (12.5K generations). Using the age of these haplotypes and the strength of selection
312 on *Tol1*, we calculate that the CDK and PUM haplotypes were at 0.4-0.6% and the
313 MCO at 4.5% frequency when the copper mines were first established (S. Table 24).
314 These values are inflated for MCO because it is on a small scaffold with few
315 informative SNPs (S. Figure 15), thus we consider the CDK and PUM estimates to
316 better reflect the age and frequency of the selected allele at *Tol1*.
317
318 **Conclusion**
319 In this study, we investigate a classic example of rapid adaptation, colonization of
320 heavy metal contaminated environments. We investigate the genetic control of a
321 major copper tolerance QTL, *Tol1*. This locus has a significant effect on fitness in the
322 mine environment (s = 0.69). We improve the assembly of the pericentromeric
323 region harboring *Tol1*. Next we use whole genome RNA expression measurements
324 and population genomic sequencing to identify candidate genes at this locus. We
325 identify a single gene, a multicopper oxidase, with large differences in expression
326 between tolerant and nontolerant lines and allele frequency between mine and off-
327 mine populations. Interestingly, three tightly linked genes at *Tol1*, including MCO,
328 possess *core haplotypes* that are strongly differentiated from the off-mine
329 populations and highly similar between mine populations. This pattern suggests
330 these *core haplotypes* predates the establishment of the mine populations. We
331 develop a novel method to estimate the age of these haplotypes and find that they
332 predate the establishment of the copper mines by 1000s of generations.
333 Furthermore, we estimate the frequency of this haplotype in the ancestral
334 population at the onset of mining (< 1%) is consistent with estimates of the
335 frequency of tolerant plants in current populations inhabiting uncontaminated sites
336 (Macnair *et al.* 1993). Looking throughout the genome we find that the top four
337 candidate adaptation loci exhibit a *core haplotype* pattern similar to what we
338 observe at *Tol1*. The function of the majority of these genes, to maintain intracellular
339 ion homeostasis, suggests they contributed to adaptation in these copper
340 contaminated environments. This work combining field-based estimates of
341 selection, population genomics, and theoretical analyses provides thorough support

342 for the classic hypothesis that rapid adaptation in natural populations primarily
343 proceeds via selection on standing genetic variation (Bradshaw 1991).
344
345

## Figures

**Figure 1 (A)** Mean probability of survive to flowering for all genotypes in mine plots in 2012 reciprocal transplant experiment (error bars = standard deviation). The probability a plant survives to flower, and the p-values testing whether there is a significant effect of genotype, is calculated from the predicted values using a logistic regression. The different genotypes are: parental mine, parental off-mine, F1 hybrid, and backcross lines (BC). Backcross lines are homozygous for Copperopolis mine allele at *Tol1* (red bars) or MED off-mine allele at *Tol1*, blue bars. **(B)** Mine plot, replicate 1. **(C)** Survival to flowering for all genotypes in offmine plots in 2012 reciprocal transplant experiment. Due to low germination, we have no data for the BC MED with off-mine allele at *Tol1*. **(D)** Off-mine plot, replicate 1.

**Figure 2** Genetic differentiation at top ranked candidate adaptation locus on *Scaffold8*. (**A**) Genetic differentiation measured with G statistic for individual SNPs . Arrows depict annotated genes from *M. guttatus* genome V1.1. – Black arrows are genes within *core haplotype* **(B)** ratio: $\pi_{Mine}$/ $\pi_{Off\text{-}mine}$ measured in 500bp windows. Orange dots/lines: Mine1 vs. Off-mine1. Blue dots/lines: Mine2 vs. Off-mine2. Green dots in **(A)**: Mine1 vs. Mine2. Green line in **(B)** ratio of pairwise π: $\pi_{Mine1+Mine2}$/ $\pi_{Mine\text{-}Off\text{-}mine}$ . Grey dashed line indicates genomic intervals with no SNP data.

**Supplemental Figure 1** Local adaptation of parental genotypes in the mine habitat. A. Experimental plot 1 at the copper mine, soil Cu is 142ppm. B. Experimental off-mine plot 1, soil Cu is 12.9ppm. C. Copperopolis parental line flowering on May12, 2012. D. Probability of survival to flowering of parental genotypes in two years of reciprocal transplant experiments. Mine genotypes orange=2007; red=2012. Off-mine genotypes: purple=2007; blue=2012. E. Map of experimental plots at Napoleon Mine, 15 km from Copperopolis, CA. Orange shaded region is approximate outline of mine. Orange diamonds are two mine plots, and blue triangles are two off-mine plots.

**Supplemental Figure 2** Generation of outbred BC lines of known genotype at *Tol1* locus. Backcrossing to Copperopolis parental lines, CCC, proceeded for four generations and backcross to Off-mine parental line, MED, proceeded for one generation. Afterwards heterozygous *Tol1* plants were identified with *Tol1* linked markers and selfed to produce plants homozygous for alternate alleles at *Tol1*. Independent backcross lines were then intercrossed to produce outbred plants of known genotype at *Tol1* locus.

**Supplemental Figure 3** Assembly of *Tol1* region.
Plot of the recombination distance between a focal gene and the *Sc84_37kb* marker (indicated with blue arrow). Recombination distance estimated using all informative SNPs within focal gene (S. Text). *Tol1* linkage threshold (recombination distance of 0.008, or 0.8cM) is denoted with blue line. Black arrows at top indicate approximate location of v1.1 scaffolds.

391 **Supplemental Figure 4** Differentially expressed transcripts in tolerant and non-
392 tolerant lines.
393 Plot of log2 fold changes over mean expression. Blue horizontal lines represent fold
394 change in expression of 1.0. Red dots indicate transcripts that have adjusted p-value
395 less than 0.1. Triangles indicate transcripts that exceed 10 or -10 log2 fold change in
396 expression. **A.** Difference in expression between NIL_*Tol1*$_{TT}$ and NIL_*Tol1*$_{NTNT}$ lines
397 in Hoaglands + Cu treatment. We identified 93 and 107 transcripts down- and up-
398 regulated respectively at an FDR of 0.1. **B.** Hoaglands treatment. We identified 119
399 and 105 transcripts to be down- and up-regulated respectively at an FDR of 0.1.
400
401
402 **Supplemental Figure 5** Map of Copperopolis, Calaverous Cty, CA, USA.
403 Red circles are mine populations and blue circles denote off-mine populations.
404 Population key: CCC – Copperopolis mine; MCN - McNulty mine; OM1 –Off-Mine1/
405 O'Byrnes Ferry Rd.; and OM2 Off-mine2/ Stage Coach Rd.
406
407 **Supplemental Figure 6** Scaffold wide plots of allelic divergence between
408 Copperopolis and Off-mine 1.
409 Plot of rank *G* statistic between Copperopolis and Off-mine 1 for scaffold 8. Each dot
410 is 500bp bin - minimum 10 SNPs per bin- sliding window step size of 100bp. Black
411 line is 0.5% genome wide threshold used to identify outlier bins.
412
413
414 **Supplemental Figure 7** Genetic Differentiation at *Tol1* Linked Reference Genome
415 Scaffolds
416 Order of scaffolds (reference genome v1.1) is same for S. Figure 3. Scaffold wide
417 plots of allelic divergence, genome wide rank *G* statistic, between (A) Copperopolis /
418 Off-mine 1, orange dots and (B) McNulty Mine / Off-mine 2, blue dots. Each dot is
419 500bp bin - minimum 10 SNPs per bin- sliding window step size of 100bp. Black line
420 is 0.5% genome wide threshold used to identify outlier bins.
421
422 **Supplemental Figure 8** Genetic Differentiation at *Tol1* Linked CCC52 Genome
423 Scaffolds.
424 Population comparisons same as presented in S. Figure 7. Scaffolds are in numerical
425 order from 1-53 according to name ID in S Table 12.
426
427 **Supplemental Figure 9** Plot of allelic divergence between Copperopolis-Off-mine1
428 and McNulty-Off-mine2.
429 Candidate adaptation loci exceed 0.1% (dark blue), 0.5% (blue), 1.0% (light blue)
430 rank G statistic threshold in both mine/off-mine comparisons. Allelic divergence
431 measured as average G score for SNPs within 500bp window, each dot is a single
432 500bp bin with data for both population comparisons.
433
434 **Supplemental Figure 10** Allelic divergence for candidate region: rank 2, scaffolds
435 148 and 198. These two scaffolds are adjacent in M. guttatus genome V. 2.0. (**A**)
436 Genetic differentiation measured with G statistic for individual SNPs . Arrows depict

437 annotated genes from *M. guttatus* genome V1.1. – Black arrows are genes within
438 *core haplotype* **(B)** ratio: $\pi_{Mine}$/ $\pi_{Off\text{-}mine}$ measured in 500bp windows. Orange
439 dots/lines: Mine1 vs. Off-mine1. Blue dots/lines: Mine2 vs. Off-mine2. Green dots in
440 **(A)**: Mine1 vs. Mine2. Green line in **(B)** ratio of pairwise π: $\pi_{Mine1+Mine2}$/ $\pi_{Mine\text{-}Off\text{-}mine}$.
441 Grey dashed line indicates genomic intervals with no SNP data.
442
443 **Supplemental Figure 11** Allelic divergence for candidate region: rank 3, scaffold 4.
444 Figure details are provided in legend of S Figure 9.
445
446 **Supplemental Figure 12** Allelic divergence for candidate region: rank 4, scaffold 1.
447 Figure details are provided in legend of S Figure 9.
448
449 **Supplemental Figure 13** Allelic divergence for *Tol1* link candidate locus CDK,
450 scaffold 7467, CCC52 genome. Figure details are provided in legend of S Figure 9.
451
452 **Supplemental Figure 14** Allelic divergence for *Tol1* link candidate locus PUM,
453 scaffold 10201, CCC52 genome. Figure details are provided in legend of S Figure 9.
454
455 **Supplemental Figure 15** Allelic divergence for *Tol1* link candidate locus MCO,
456 scaffold 47895, CCC52 genome. Figure details are provided in legend of S Figure 9.
457
458
459
460
461

461
462
463 **Tables**
464
465 **Table 1. Top ranked candidate adaptation loci**

| Rank | Scaffold | Length (kb) | N - Genes | N - Bins Exceed 0.5% | N - SNPs Fixed Diff. | Locus ID | Annotation |
|---|---|---|---|---|---|---|---|
| 1 | 8 | 32.9 | 10 | 97 | 70\65 | MET | Cytochrome P450; Methytransferase; Protease Inhibitor |
| 2 | 148/ 198 | 24.1 | 2 | 39 | 0\32 | FER | Transmembrane Ferric Reductase |
| 3 | 4 | 7.7 | 2 | 25 | 0\0 | ZNC | Zinc/Iron Ion Transporter |
| 4 | 1 | 8.1 | 1 | 19 | 0\5 | COP | Copper Ion Transporter |
| 5A | 84 | 1.8 | 1 | 11 | 0\0 | MND | **Tol1 Link** - Mandelate Racemase |
| 5B | 84 | 5.8 | 1 | 8 | 3\0 | CDK | **Tol1 Link** - Cyclin Dependent Kinase |
| 6 | 44 | 2.5 | 1 | 9 | 0\0 | SHD | Protein Binding, SH3 domain |
| 7 | 80 | 6.2 | 1 | 6 | 0\0 | DUF | Unknown Function |
| 8 | 115 | 0.9 | 1 | 5 | 0\0 | KIT | Potassium Ion Transporter |
| 9 | 47 | 2.1 | 0 | 5 | 0\0 | - | Intergenic |
| 10 | 51 | 0.8 | 1 | 4 | 0\0 | HSP | Heat Shock Protein, HSP90. |

466
467
468
469 Candidate adaptation loci are composed on 500bp bins that exceed 0.5% rank G
470 statistic threshold in both mine/off-mine population comparisons. Outlier bins are
471 grouped together using the 10kb clustering algorithm. Loci are ranked by the
472 number of outlier bins at a locus. The physical length of a candidate locus is defined
473 by the region of overlap between both mine-off-mine population comparisons. The
474 FER locus spans two adjacent scaffolds: 148 and 198. The number of SNPs with fixed
475 differences is presented for CCC vs. Off-mine1 \ MCN vs. Off-mine2 comparisons.
476 Gene annotation from *M. guttatus* genome v1.1, phytozome.net. Annotation of loci
477 that span multiple genes is restricted to genes with strongest signal of
478 differentiation.
479
480
481
482

482 **Table 2 –Age of core haplotypes at candidate adaptation loci.**
483

| Locus ID | Core Haplotype | | | Coalescence Time | | | | Annotation |
|---|---|---|---|---|---|---|---|---|
| | Scaffold | Length (kb) | Num. Genes | CCC | MCN | CCC/ OM1 | MCN/ OM2 | |
| MET | 8 | 19.6 | 4 | 639 | 1191 | 1901 | 5699 | Cytochrome P450; Methytransferase; Protease Inhibitor; Unknown Func. |
| FER | 148/198 | 7.06 | 1 | 1533 | 2022 | 2285 | 1725 | Transmembrane Ferric Reductase |
| ZNC | 4 | 2.4 | 1 | 17191 | 960 | 1622 | 1537 | Zinc/Iron Ion Transporter |
| COP | 1 | 1.27 | 1 | 5062 | 3119 | 3956 | 3931 | Copper Ion Transporter |
| ***Tol1* Linked Genes** | | | | | | | | |
| CDK | 7467* | 1.26 | 1 | 1768 | 7840 | 19121 | 15778 | Cyclin Dependent Kinase |
| PUM | 10201* | 8.51 | 1 | 2789 | 10162 | 19264 | 23900 | Pumilio-family, RNA binding |
| MCO | 47895* | 1.06 | 1 | 12543 | 90664 | 340560 | 56920 | Multicopper oxidase |

484
485
486 The length and age of the core haplotypes are estimated by modeling the rate at
487 which $\pi_{M1\text{-}M2}$ (pairwise π between mine populations ) increases with distance from
488 the selected site using the program, MSSEL, see supplemental text for additional
489 details. The length of the core haplotype is estimated using equation 1 in S. Text.
490 Coalesence time in presented in generations for within population (CCC; MCN) and
491 between population (CCC/OM1; MCN/OM2) analyses. *Scaffold ID corresponds to
492 CCC52 de novo assembly.
493
494

## References


Al-Hiyaly, S. A. K., McNeilly, T., & Bradshaw, A. D. (1990). The Effect of Zinc Contamination from Electricity Pylons. Contrasting Patterns of Evolution in Five Grass Species. New Phytologist, 114(2), 183–190.

Al-Hiyaly, S. A. K., McNeilly, T., Bradshaw, A. D., & Mortimer, A. M. (1993). The effect of zinc contamination from electricity pylons. Genetic constraints on selection for zinc tolerance. Heredity, 70, 22-32.

Allen, W. R., & Sheppard, P. M. (1971). Copper Tolerance in Some Californian Populations of the Monkey Flower, *Mimulus guttatus*. Proc. R. Soc. B., 177(1047), 177–196.

Antonovics, J., Bradshaw, A. D., & Turner, R. G. (1971). Heavy metal tolerance in plants. Adv. Ecol. Res., 7:1–85.

Aubury, L. E. (1905). The copper resources of California (No. 23). WW Shannon, Superintendent State Print.

Barton, N. H. (2000). Genetic hitchhiking. Phil. Tran. R. Soc. B., 355(1403), 1553-1562.

Berg, J. J., & Coop, G. (2015). A Coalescent Model for a Sweep of a Unique Standing Variant. Genetics. 201.

Bradshaw, A. D. (1984). The importance of evolutionary ideas in ecology and vice versa. Evolutionary ecology, 1-25.

Bradshaw, A. D. (1991). The Croonian Lecture, 1991. Genostasis and the limits to evolution. Phil. Tran. R. Soc. B., 333(1267), 289–305.

Brenchley, W. E. (1958). The Park Grass Plots at Rothamsted 1856-1949. Rep. Rothamsted Exp. Stn.

Colosimo, P. F., Hosemann, K. E., Balabhadra, S., Villarreal, G., Dickson, M., Grimwood, J., et al. (2005). Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. Science, 307(5717), 1928–1933.

Endler, J. A. (1977). Geographic Variation, Speciation and Clines (p. 268). Princeton University Press.

Grennan, A. K. (2009). Identification of genes involved in metal transport in plants. Plant Physiology, 149(4), 1623–4.

Hanikenne, M., Talke, I. N., Haydon, M. J., Lanz, C., Nolte, A., Motte, P., et. al. (2008). Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. Nature, 453(7193), 391–395.

Heliconius Genome Consortium. (2012). Butterfly genome reveals promiscuous exchange of mimicry adaptations among species. Nature, 487(7405), 94-98.

Innan, H., & Kim, Y. (2004). Pattern of polymorphism after strong artificial selection in a domestication event. Proc. Nat. Acad. Sci.101(29), 10667-10672.

Innan, H., & Kim, Y. (2008). Detecting local adaptation using the joint sampling of polymorphism data in the parental and derived populations. Genetics, 179(3), 1713-1720.

Joron, M., & Mallet, J. L. (1998). Diversity in mimicry: paradox or paradigm? Trends Eco. & Evol, 13(11), 461-466.

Karasov, T., Messer, P. W., & Petrov, D. A. (2010). Evidence that Adaptation in *Drosophila* Is Not Limited by Mutation at Single Sites. PLoS Genetics, 6(6), e1000924.

Kruckeberg, Arthur R. (1951) Intraspecific variability in the response of certain native plant species to serpentine soil. Amer. Jour. Botany (1951): 408-419

Macnair, M. R. (1987). Heavy metal tolerance in plants: A model evolutionary system. Trends Eco. & Evol, 2(12), 354–359.

Macnair, M. R., Smith, S. E., & Cumbes, Q. J. (1993). Heritability and distribution of variation in degree of copper tolerance in Mimulus guttatus at Copperopolis, California. Heredity, 71(5), 445–455.

Maynard-Smith, J., & Haigh, J. (1974). The hitch-hiking effect of a favourable gene. Genetics Research, 23(01), 23–35.

Messer, P. W., & Petrov, D. A. (2013). Population genomics of rapid adaptation by soft selective sweeps. Trends Eco. & Evol, 28(11), 659–69.

Prezeworski, M., Coop, G., & Wall, J. D. (2005). The signature of positive selection on standing genetic variation. Evolution, 59(11), 2312-2323.

Roesti, M., Gavrilets, S., Hendry, A. P., Salzburger, W., & Berner, D. (2014). The genomic signature of parallel adaptation from shared genetic variation. Molecular Ecology, 23(16), 3944-3956.

Schluter, D. (2000). The Ecology of Adaptive Radiation. Oxford, England: Oxford University Press.

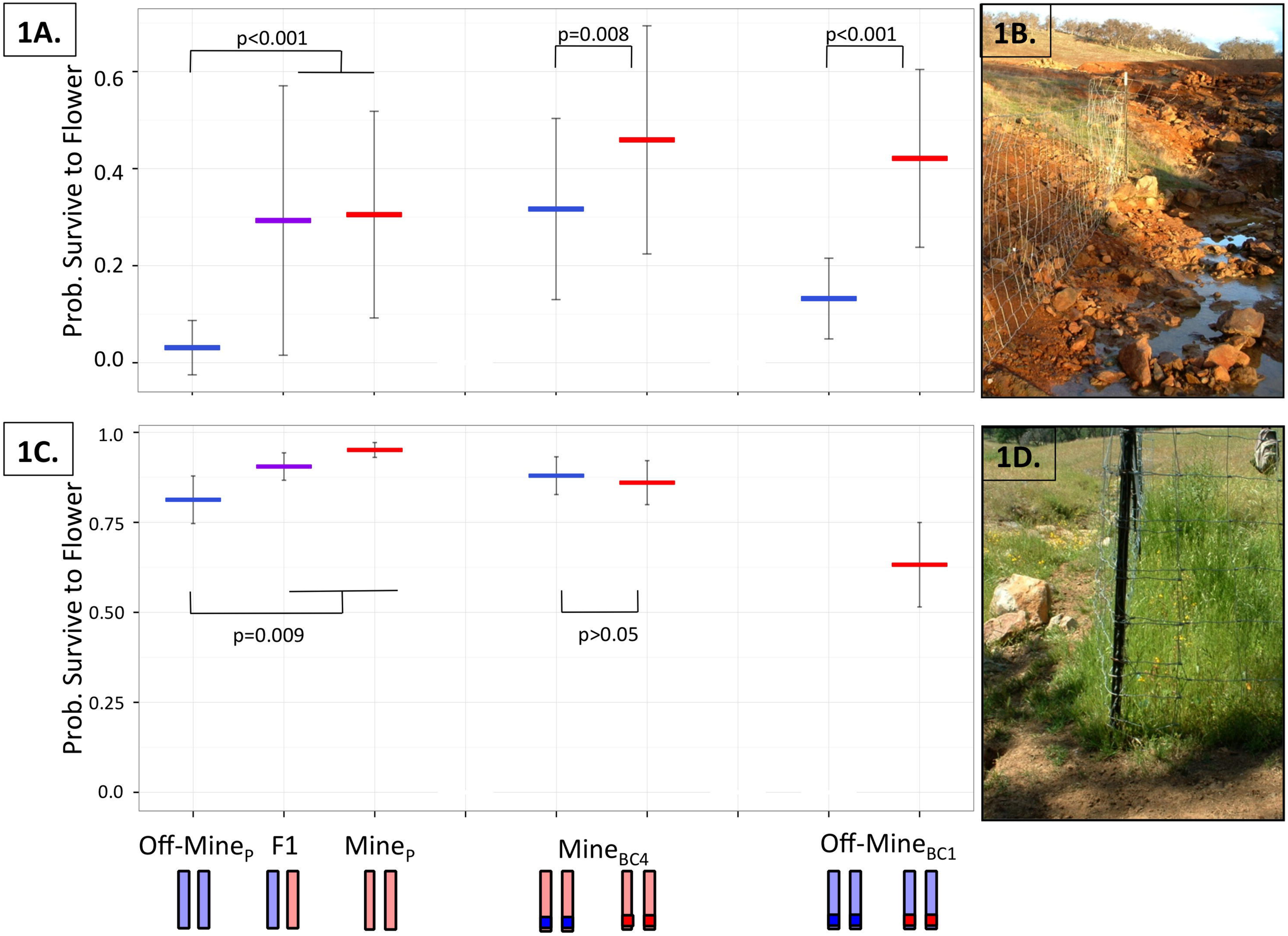
Symeonidis, L., McNeilly, T., & Bradshaw, A. D. (1985). Differential tolerance of three cultivars of Agrostis capillaries l. to cadmium, copper, lead, nickel and zinc. New Phytologist, 101(2), 309–315.

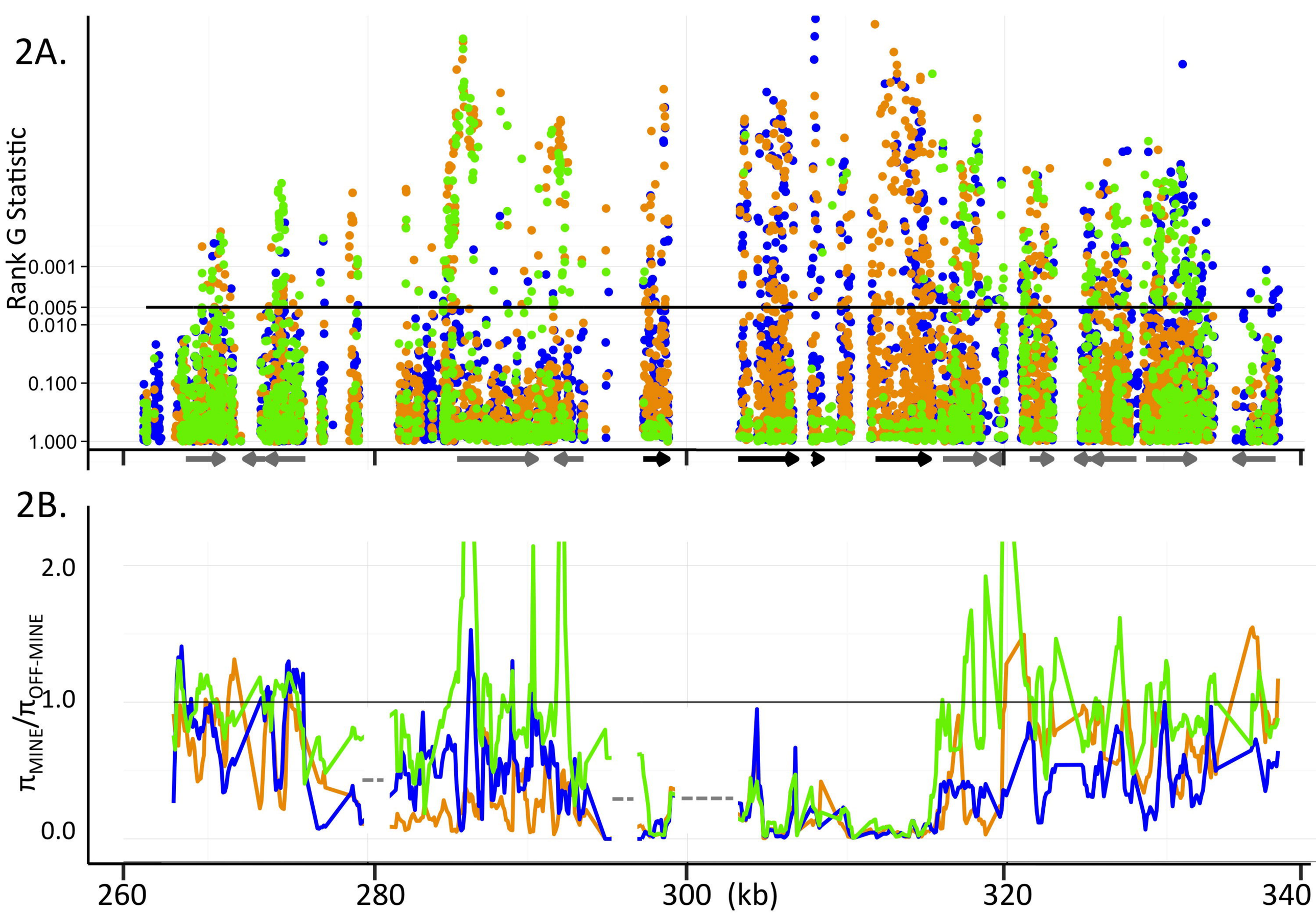
Tishkoff, S. A., Reed, F. A., Ranciaro, A., Voight, B. F., Babbitt, C. C., Silverman, J. S., … Deloukas, P. (2007). Convergent adaptation of human lactase persistence in Africa and Europe. Nature Genetics, 39(1), 31–40.

Turner, R. G. (1969). Heavy metal tolerance in plants. Vol. 9, 399-410. Blackwell Oxford.

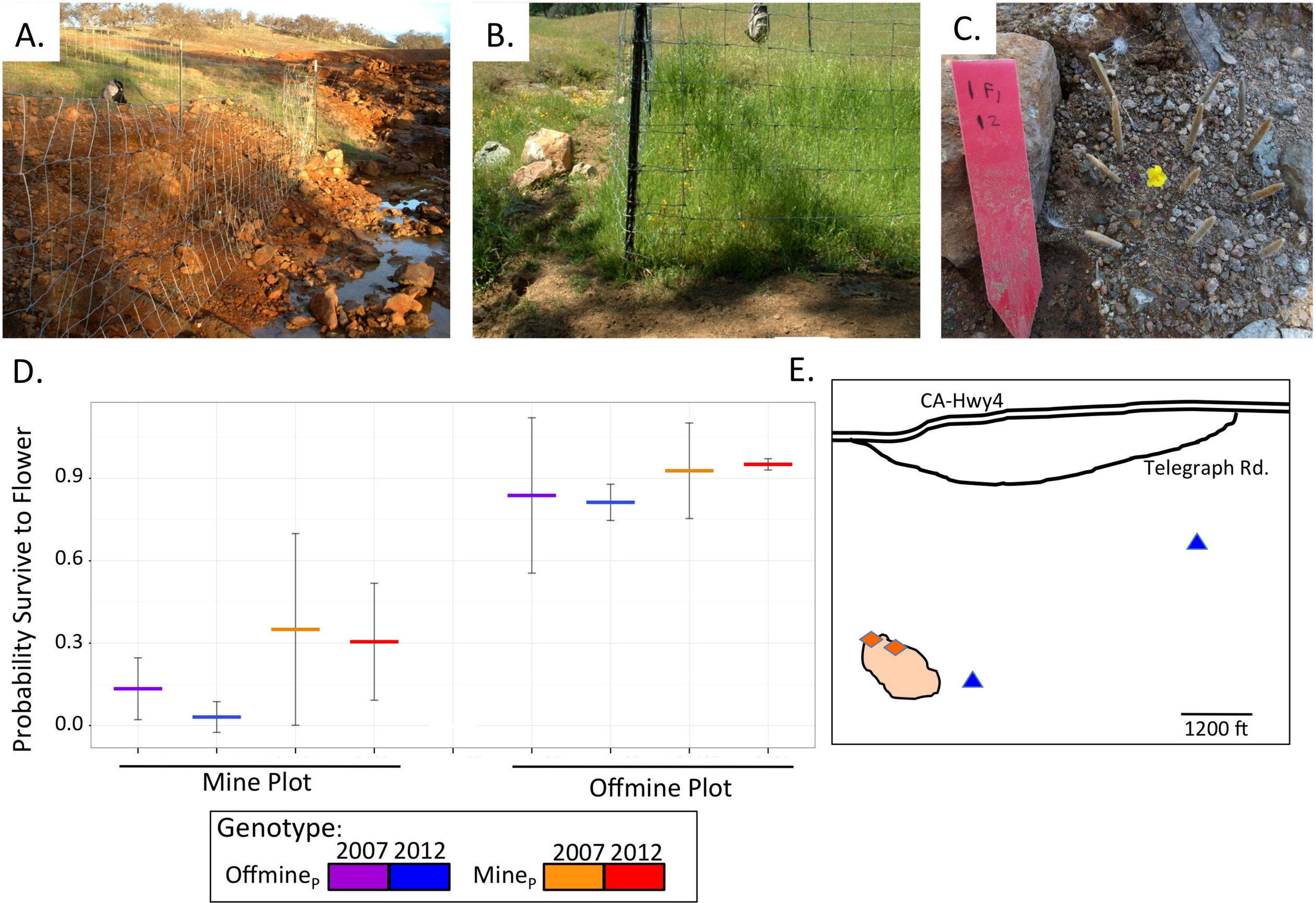
Walley, K. A., Khan, M. S. I., & Bradshaw, A. D. (1974). The potential for evolution of heavy metal tolerance in plants. I. Heredity, 32, 309-319.

Wright, K. M., Lloyd, D., Lowry, D. B., Macnair, M. R., & Willis, J. H. (2013). Indirect evolution of hybrid lethality due to linkage with selected locus in *Mimulus guttatus*. PLoS Biol, 11(2), e1001497.

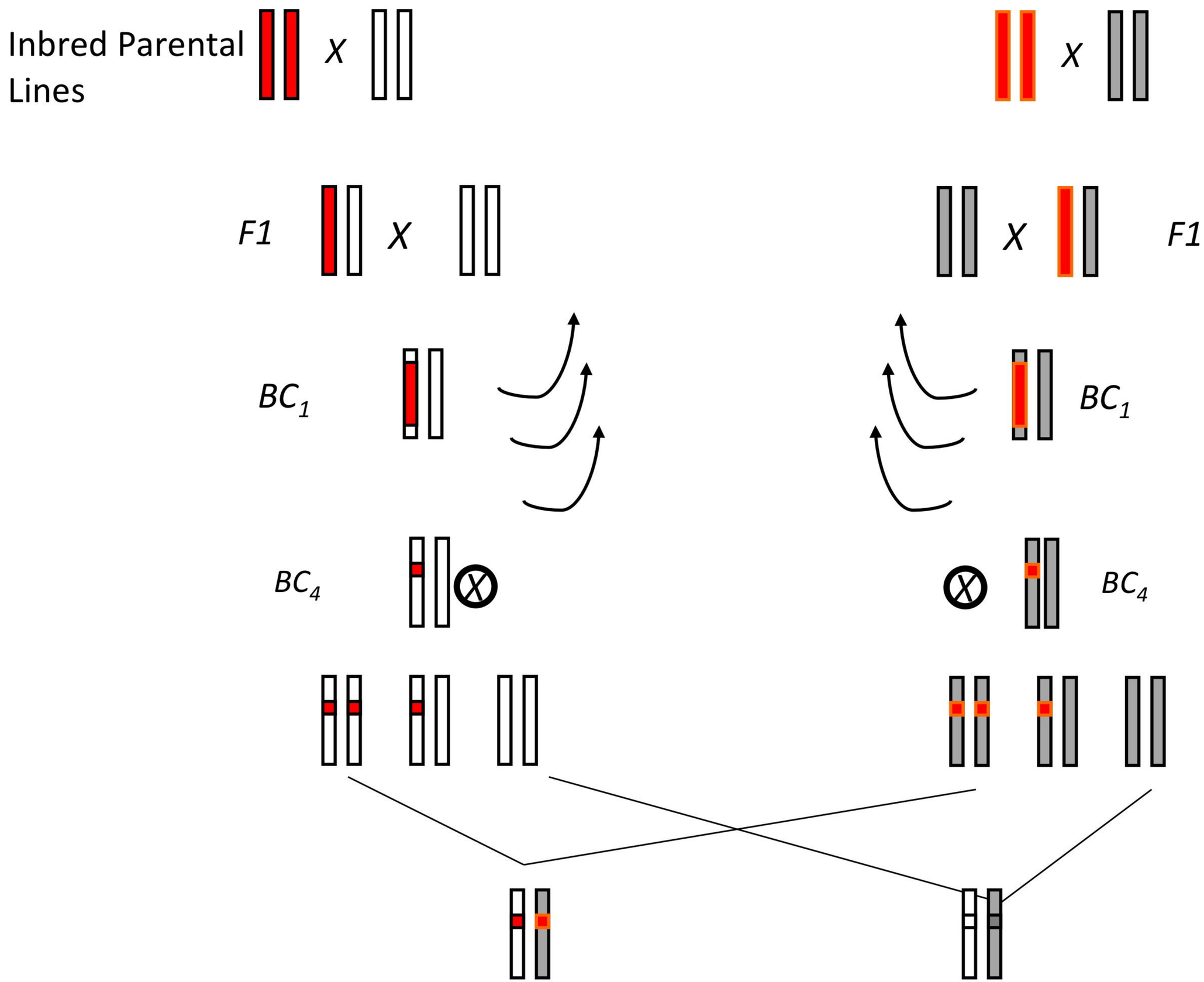

1A.
1B.
1C.
1D.
Prob. Survive to Flower
0.0
0.2
0.4
0.6
p<0.001
p=0.008
p<0.001
1.0
0.75
0.50
0.25
0.0
p=0.009
p>0.05
Off-Mine$_P$
F1
Mine$_P$
Mine$_{BC4}$
Off-Mine$_{BC1}$


2A.
Rank G Statistic
0.001
0.005
0.010
0.100
1.000
2B.
$\pi_{MINE}/\pi_{OFF\text{-}MINE}$
2.0
1.0
0.0
260
280
300 (kb)
320
340

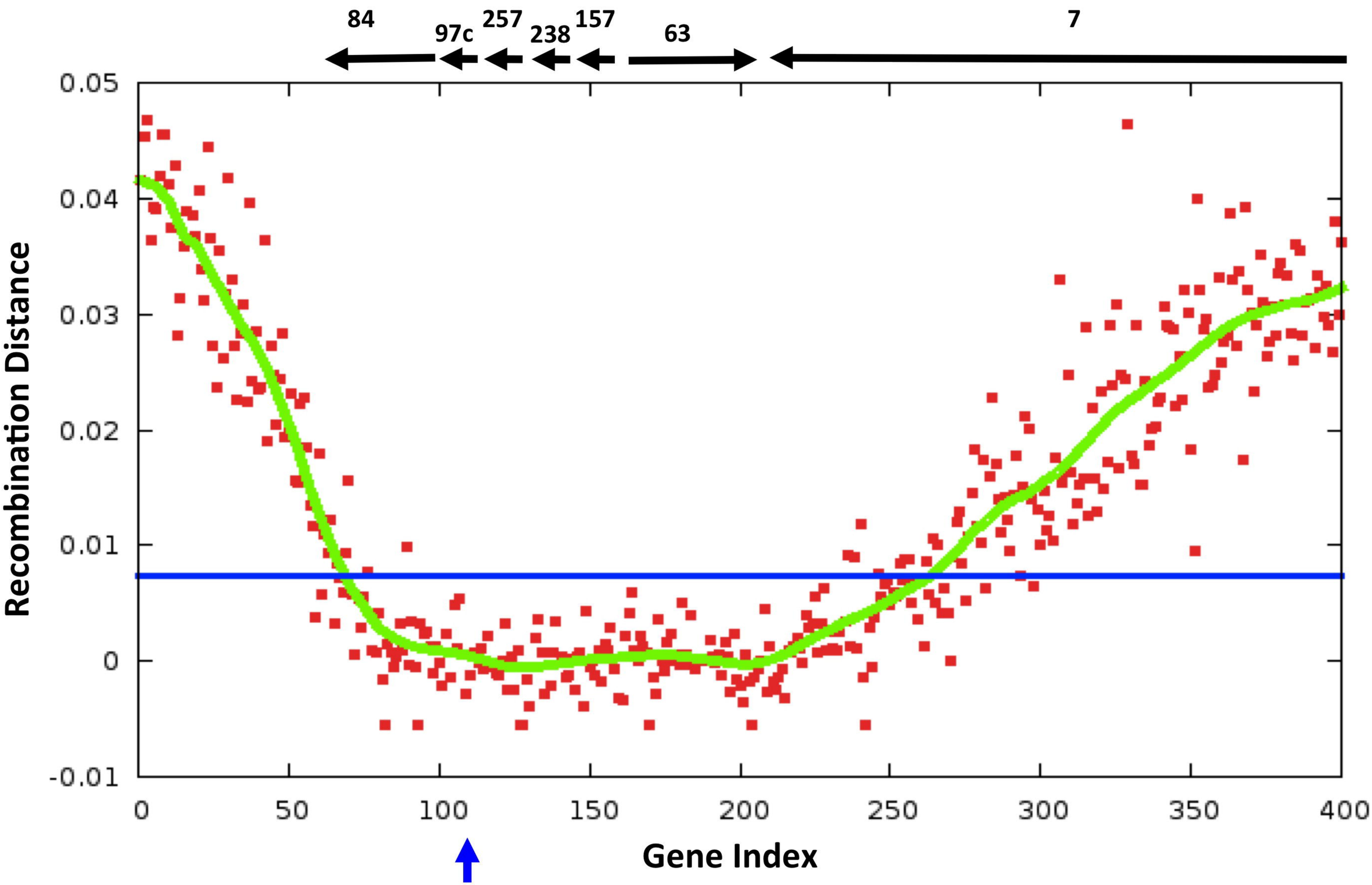
S Figure 1


A.
B.
C.
1 F1
12
D.
Probability Survive to Flower
0.9
0.6
0.3
0.0
Mine Plot
Offmine Plot
Genotype:
2007 2012
Offmine$_P$
2007 2012
Mine$_P$
E.
CA-Hwy4
Telegraph Rd.
1200 ft

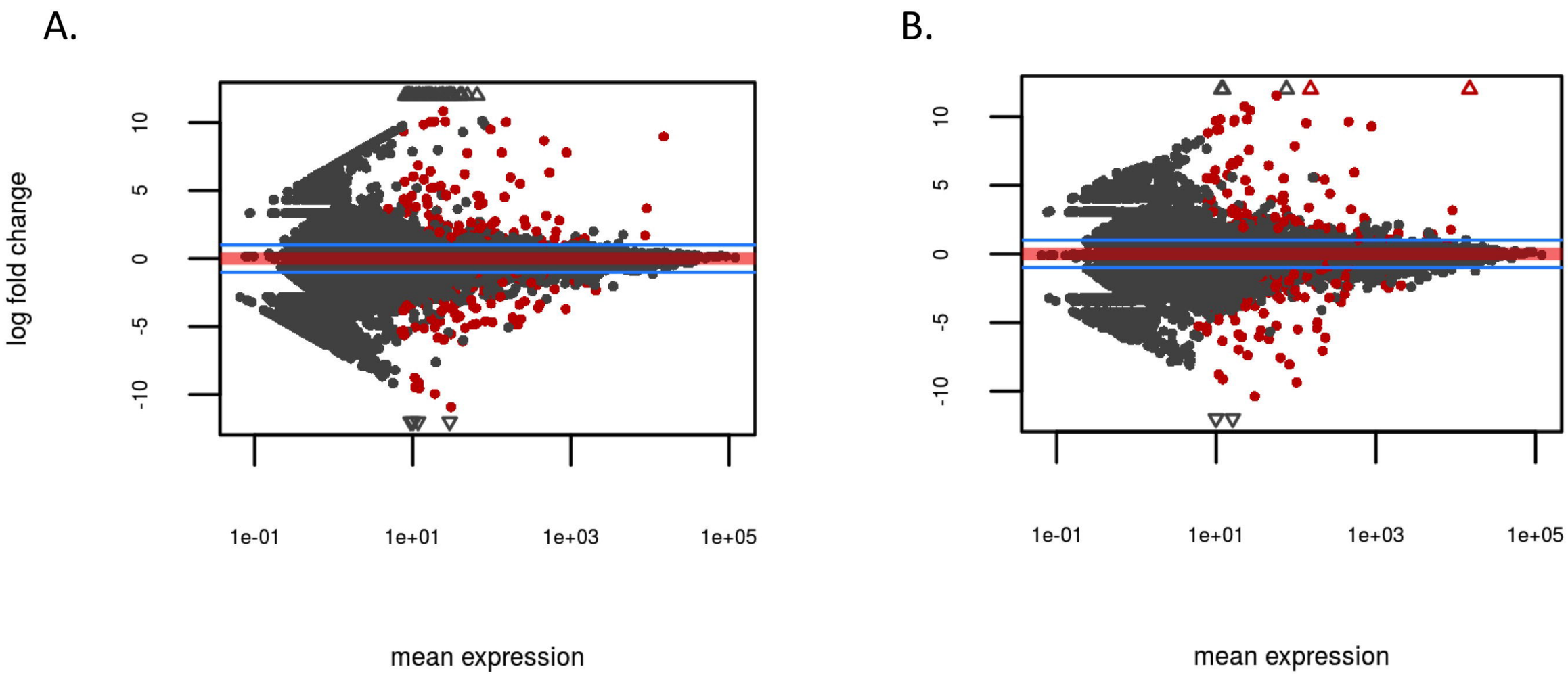
S Figure 2



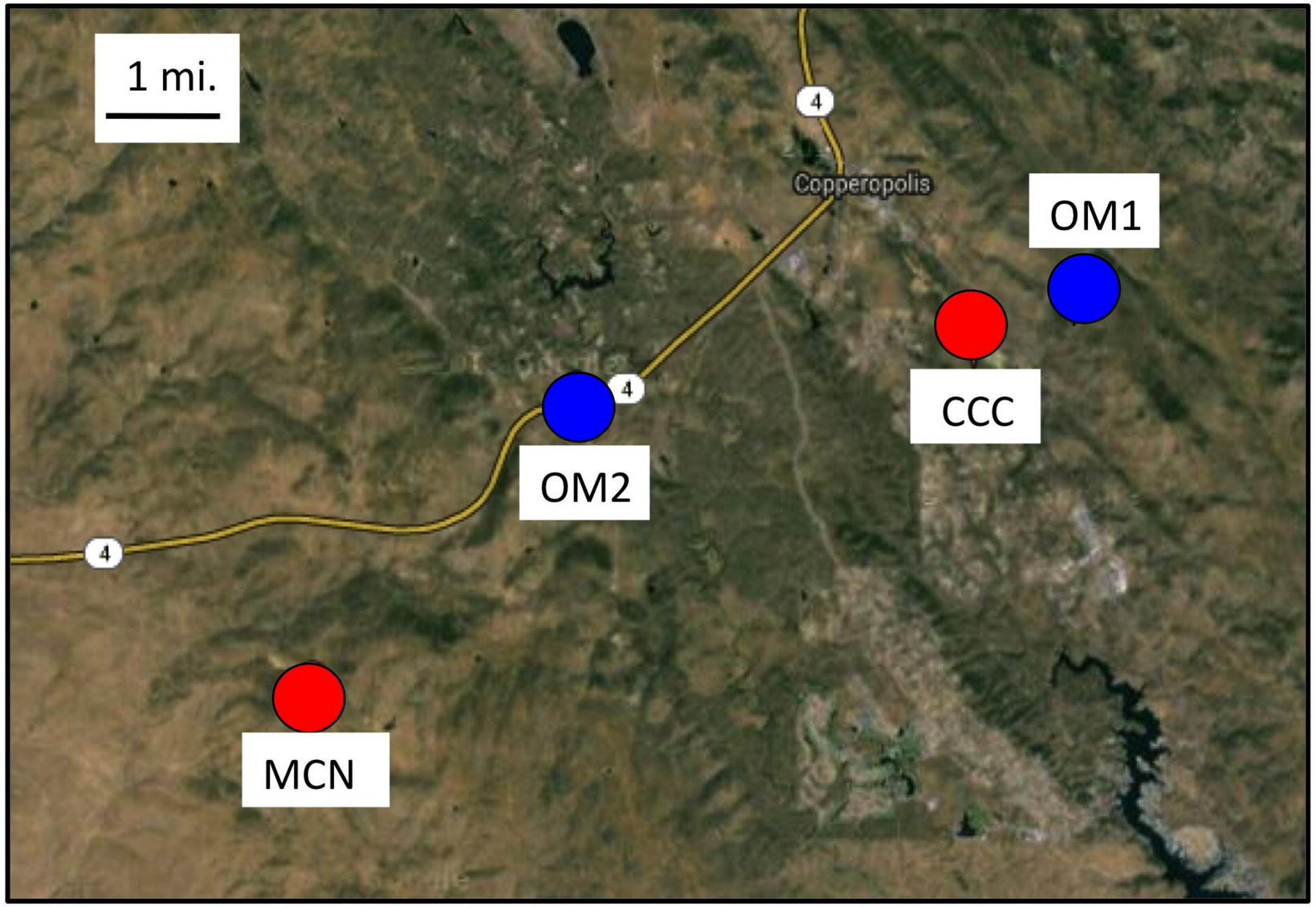
S Figure 3


84
97c
257
238
157
63
7
0.05
0.04
0.03
0.02
0.01
0
-0.01
Recombination Distance
0
50
100
150
200
250
300
350
400
Gene Index

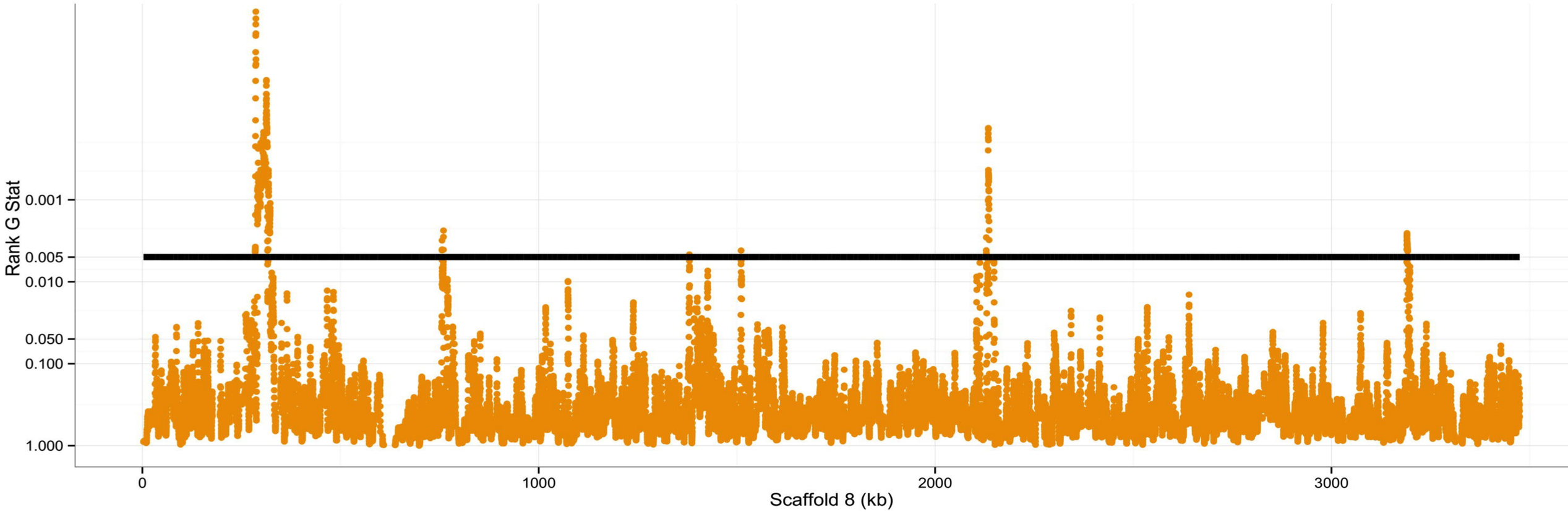
S Figure 4


A.
B.
log fold change
10
5
0
-5
-10
1e-01
1e+01
1e+03
1e+05
mean expression
mean expression

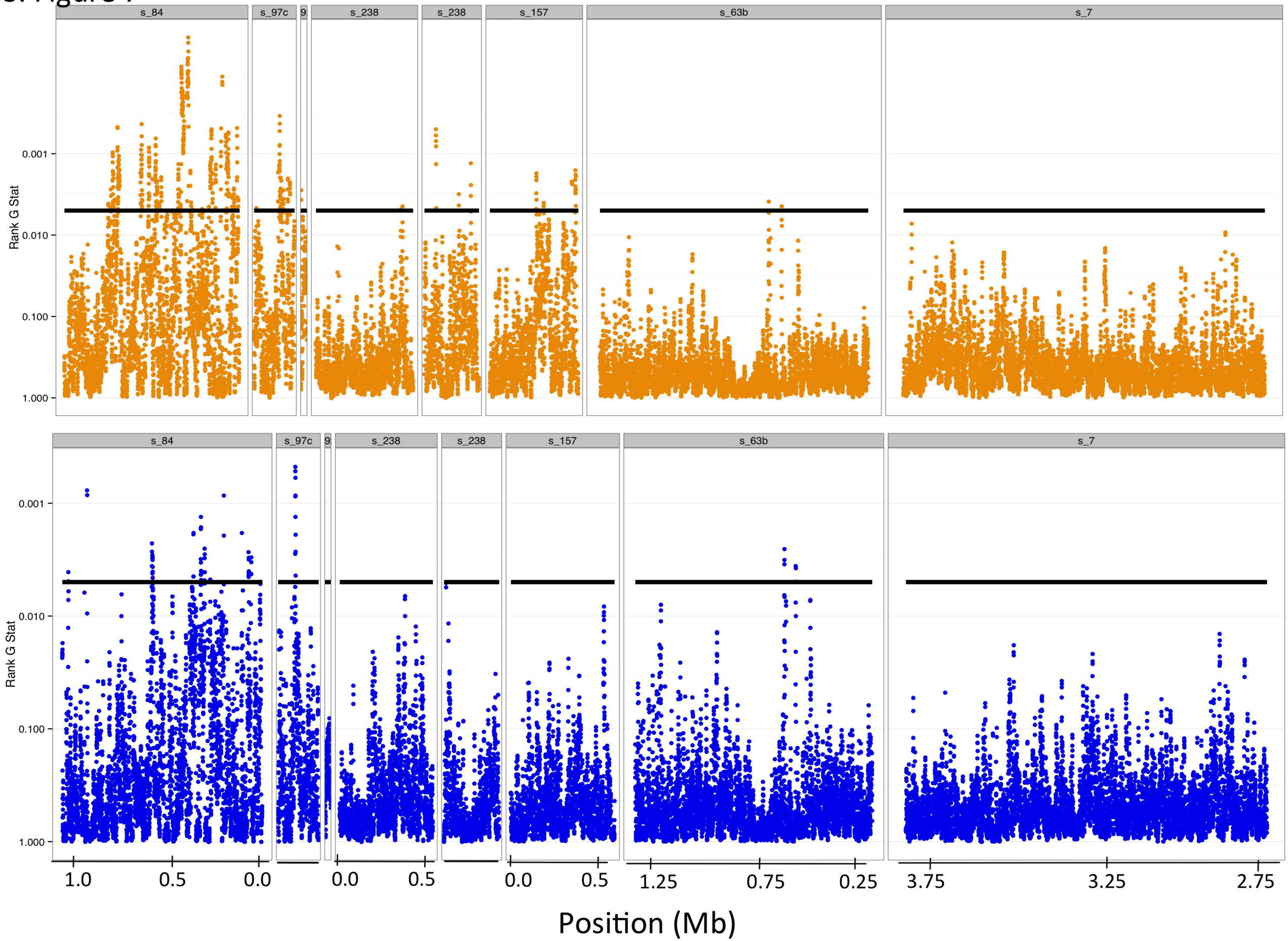
S Figure 5



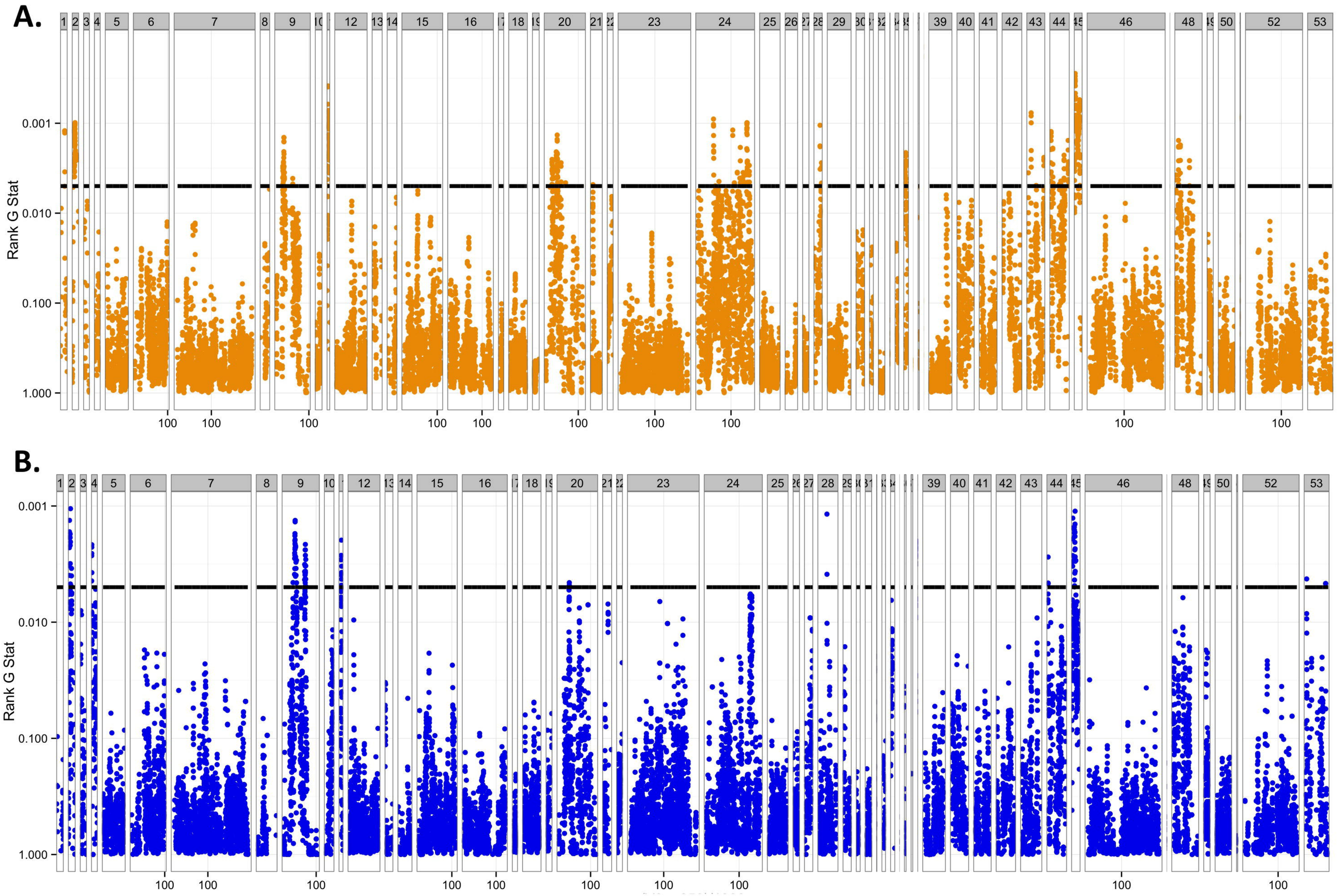
# S Figure 6


Rank G Stat
0.001
0.005
0.010
0.050
0.100
1.000
0
1000
2000
3000
Scaffold 8 (kb)

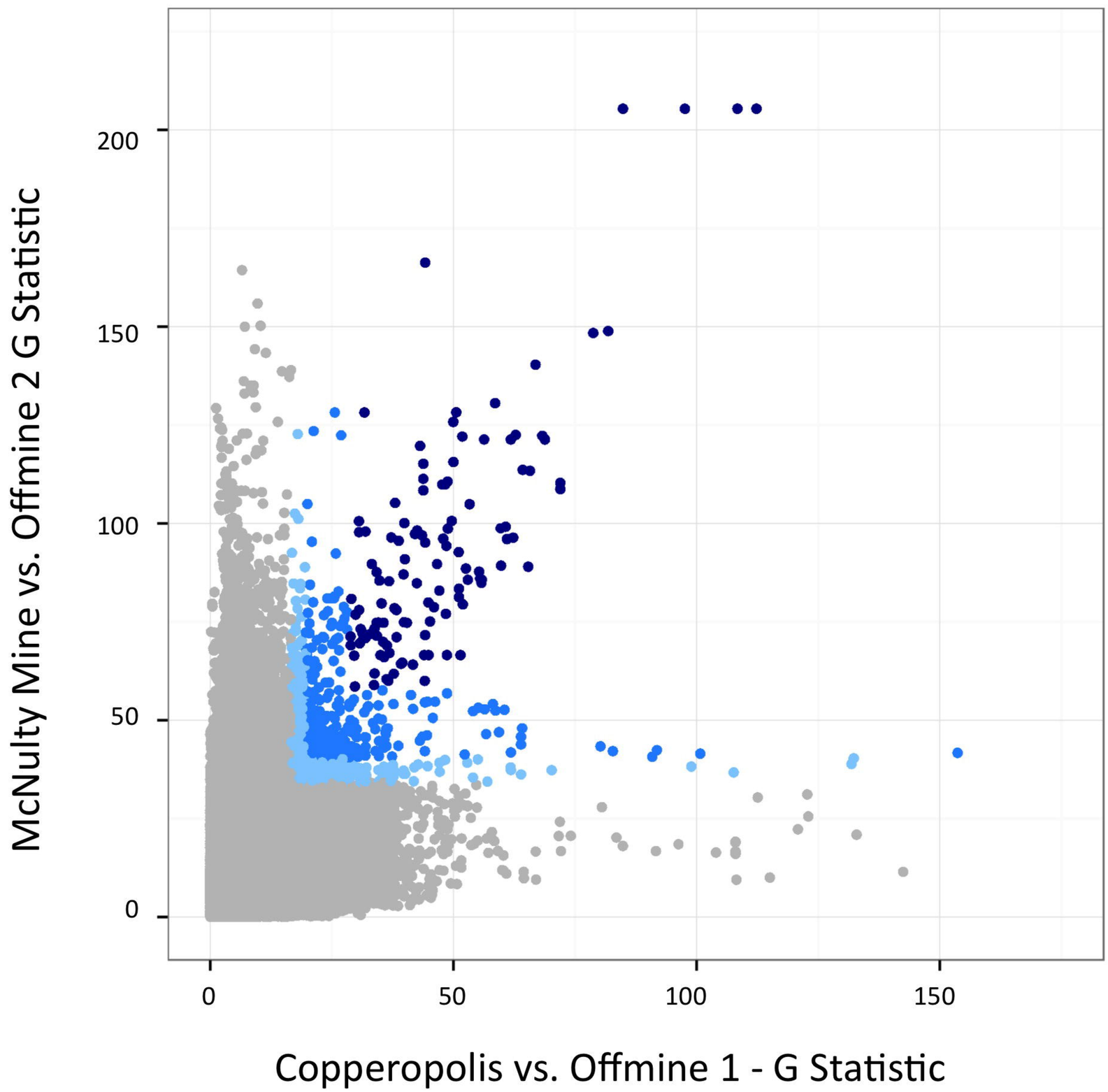
S. Figure 7


s_84
s_97c
9
s_238
s_238
s_157
s_63b
s_7
Rank G Stat
0.001
0.010
0.100
1.000
1.0
0.5
0.0
0.0
0.5
0.0
0.5
1.25
0.75
0.25
3.75
3.25
2.75
Position (Mb)

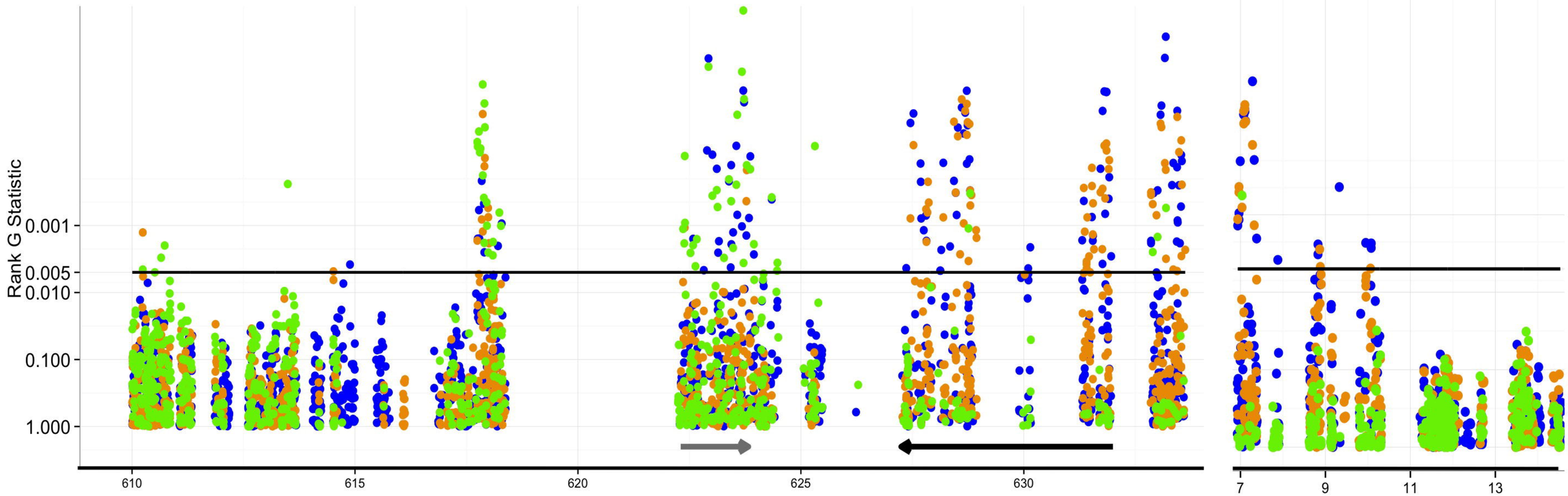
# S. Figure 8



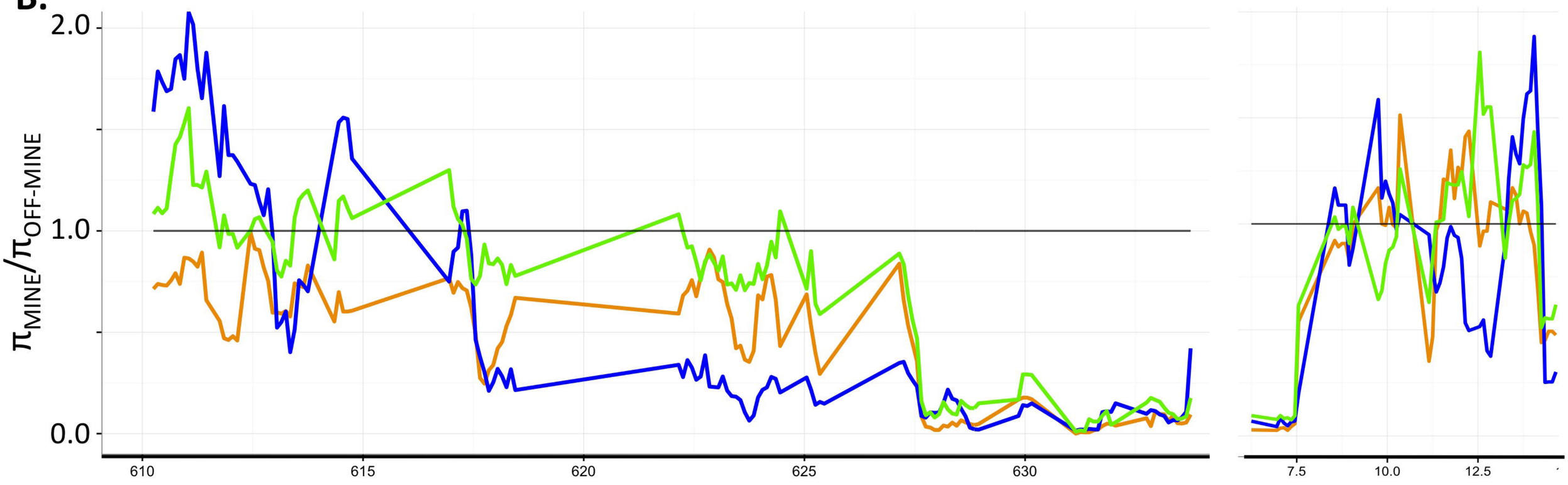
S Figure 9


McNulty Mine vs. Offmine 2 G Statistic
200
150
100
50
0
0
50
100
150
Copperopolis vs. Offmine 1 - G Statistic

**A.** S Figure 10

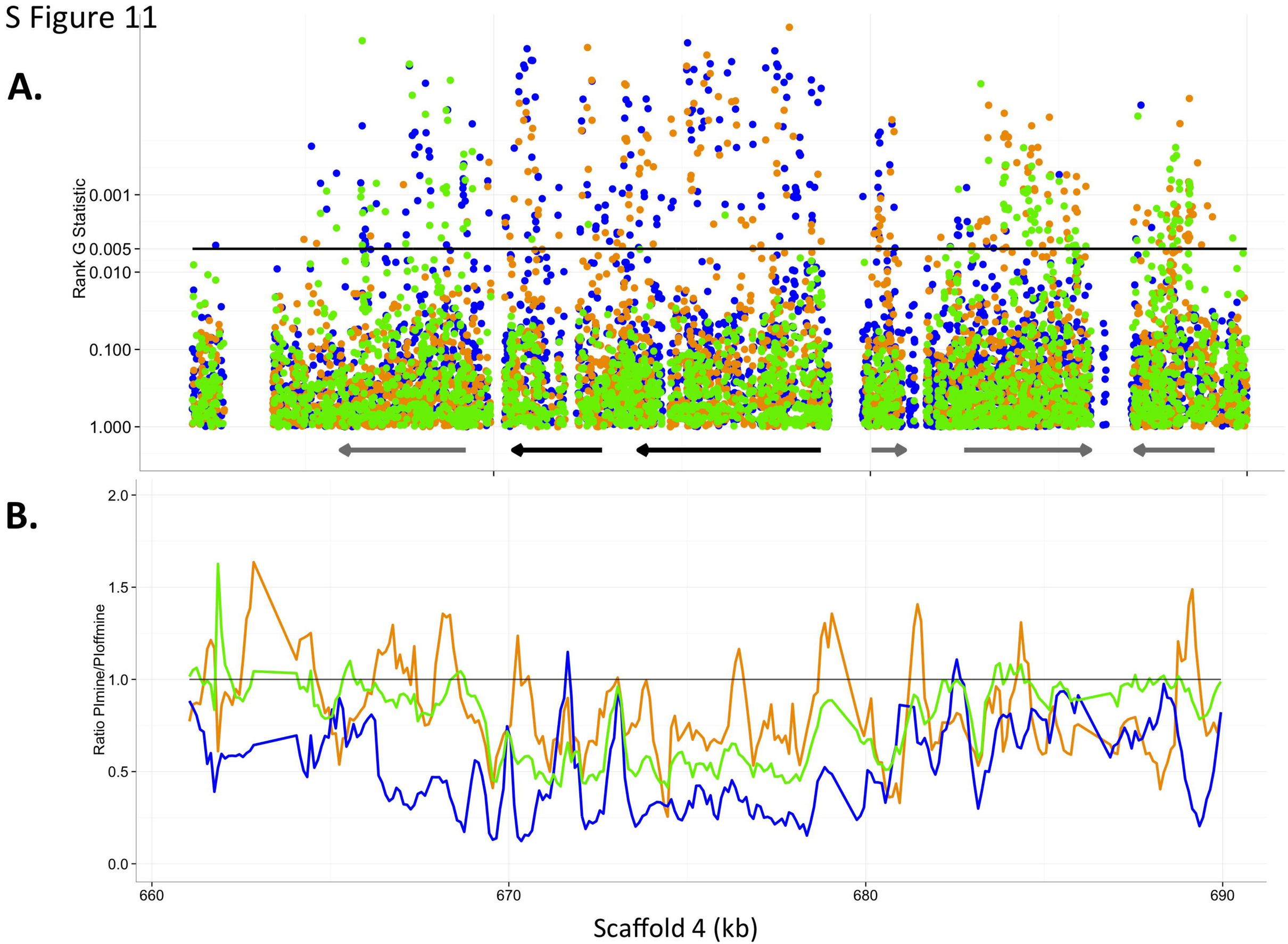



**B.**

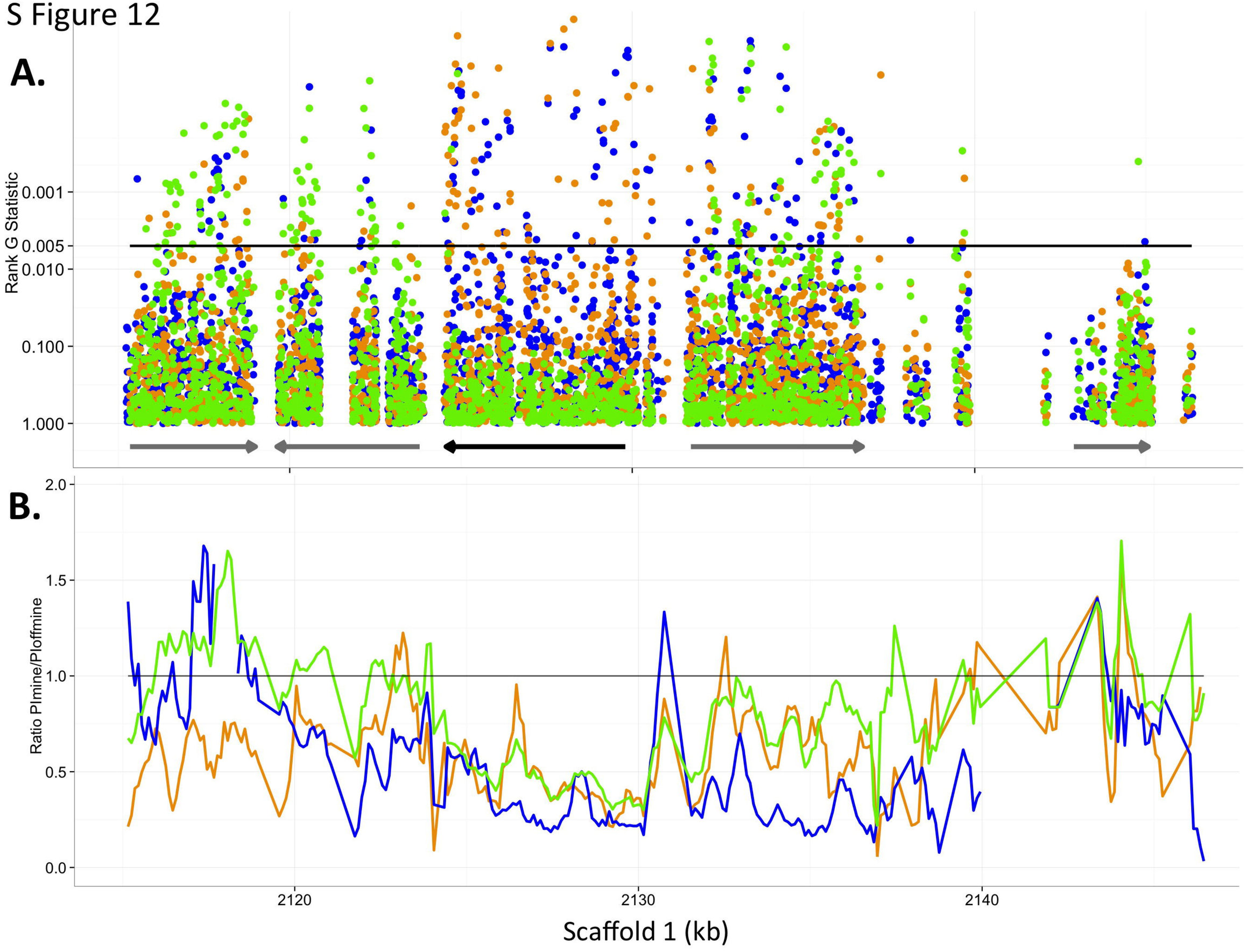



**Scaffold 148** **Scaffold 198**

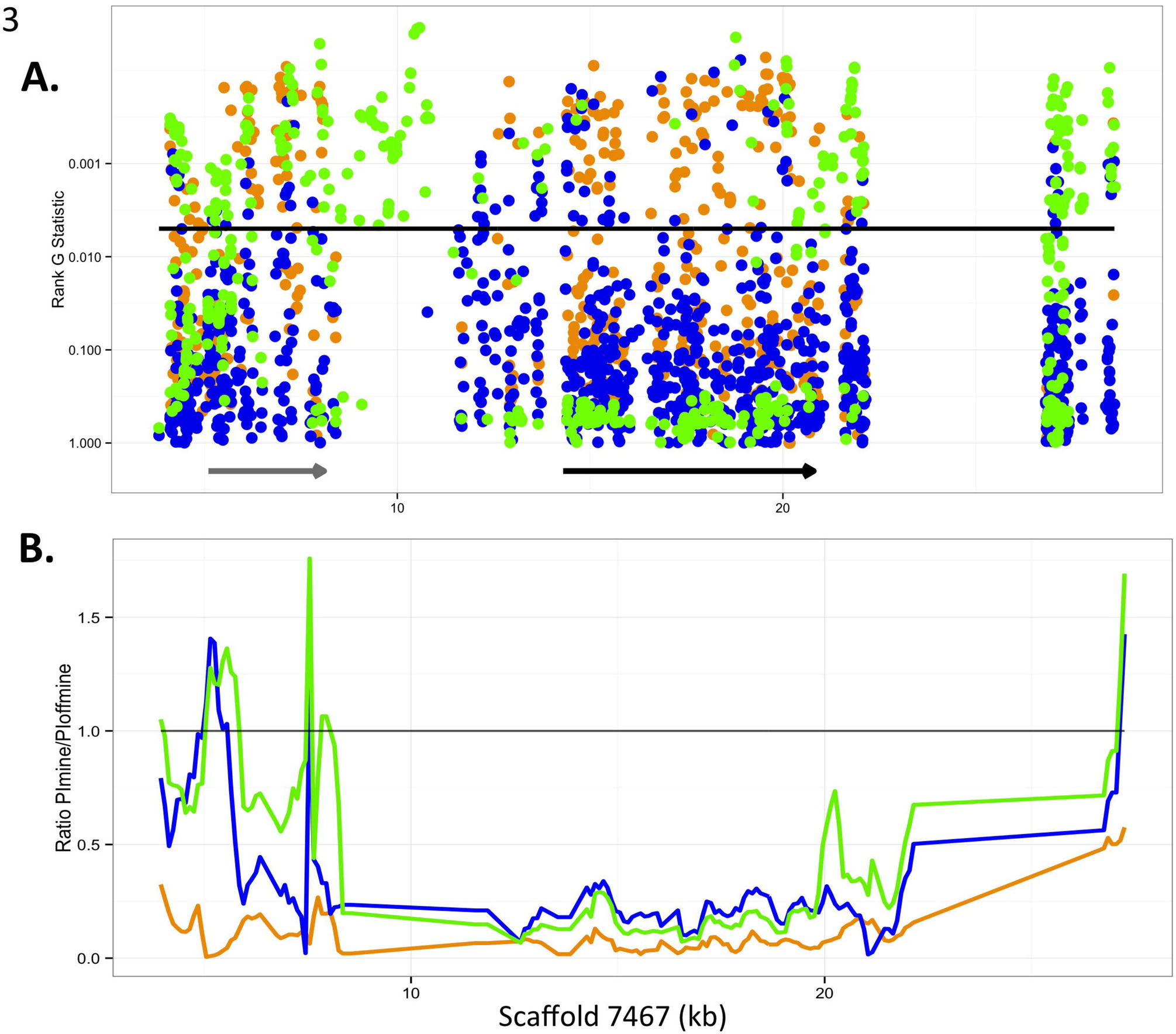
S Figure 11


A.
Rank G Statistic
0.001
0.005
0.010
0.100
1.000
B.
Ratio Plmine/Ploffmine
2.0
1.5
1.0
0.5
0.0
660
670
680
690
Scaffold 4 (kb)

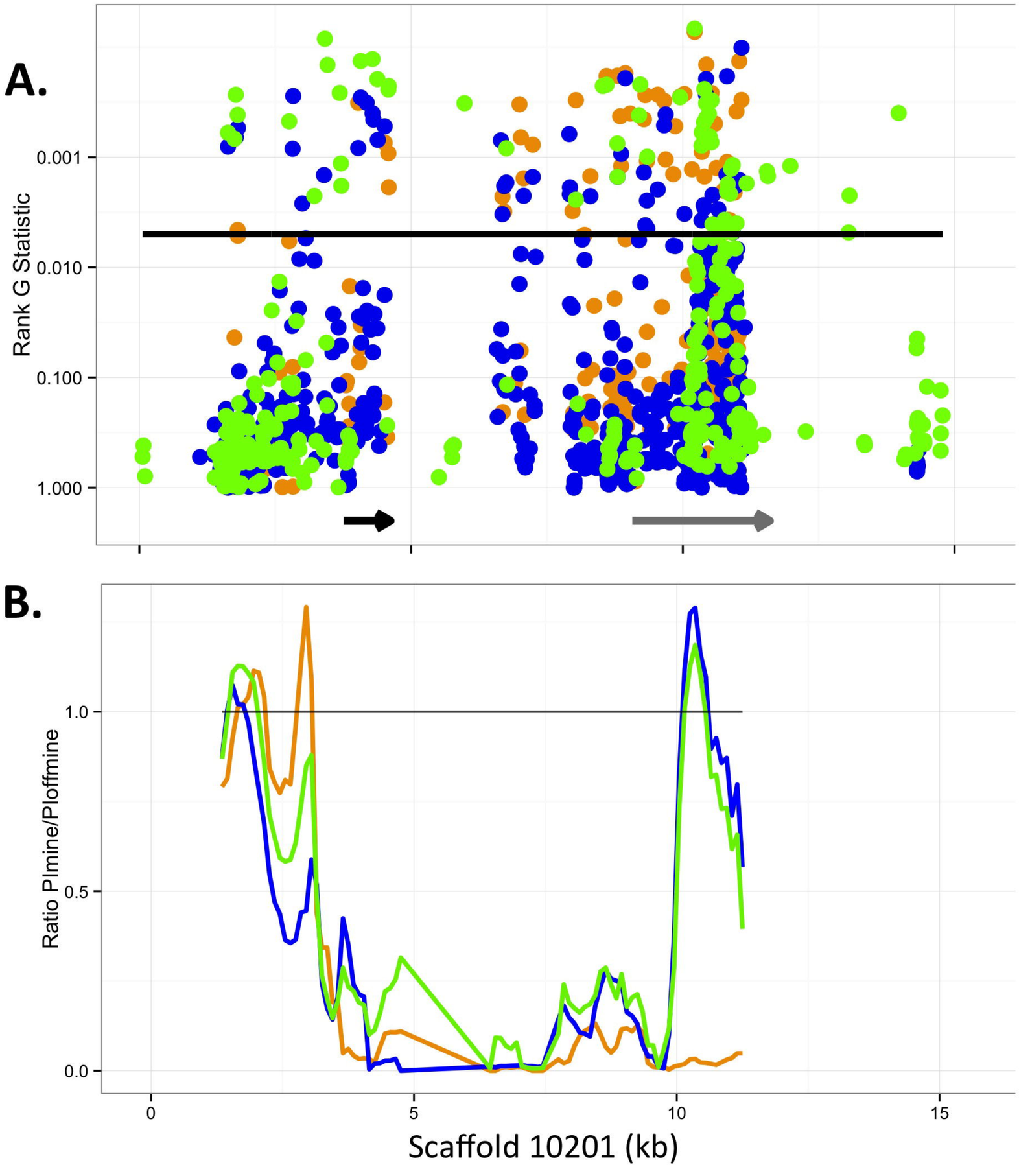
S Figure 12


A.
Rank G Statistic
0.001
0.005
0.010
0.100
1.000
B.
Ratio PImine/PIoffmine
2.0
1.5
1.0
0.5
0.0
2120
2130
2140
Scaffold 1 (kb)

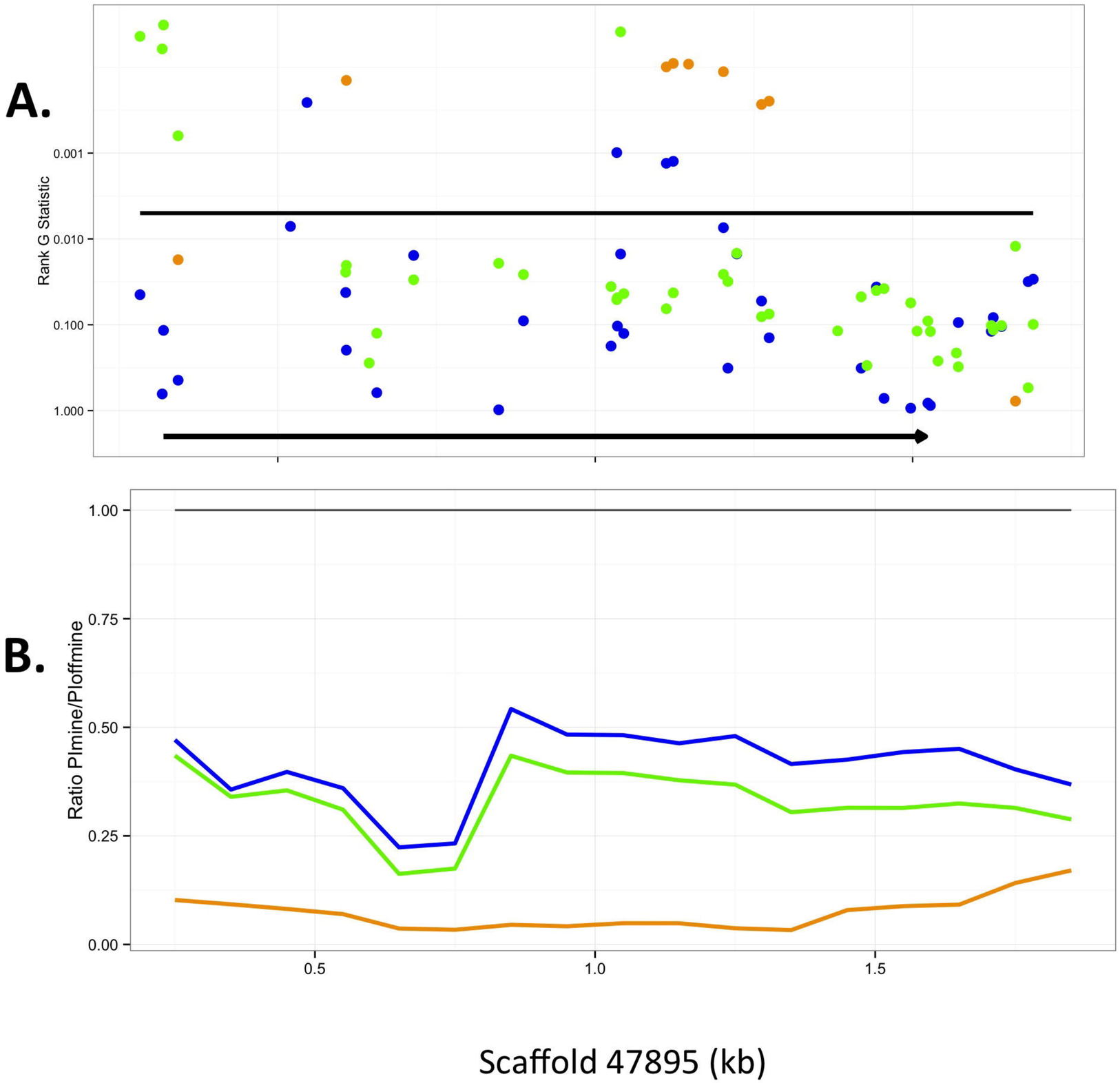
S Figure 13


A.
Rank G Statistic
0.001
0.010
0.100
1.000
10
20
B.
Ratio Plmine/Ploffmine
1.5
1.0
0.5
0.0
10
20
Scaffold 7467 (kb)

S Figure 14


A.
Rank G Statistic
0.001
0.010
0.100
1.000
B.
Ratio PImine/Ploffmine
1.0
0.5
0.0
0
5
10
15
Scaffold 10201 (kb)

S Figure 15


A.
Rank G Statistic
0.001
0.010
0.100
1.000
B.
Ratio PImine/PIoffmine
1.00
0.75
0.50
0.25
0.00
0.5
1.0
1.5
Scaffold 47895 (kb)