

1 **Two synthetic 18-way outcrossed populations of diploid budding**
2 **yeast with utility for complex trait dissection**

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9 Short title: *18-way budding yeast synthetic populations*

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17 **Abstract**

18 Advanced generation multi-parent populations (MPPs) are a valuable tool for dissecting
19 complex traits, having more power than GWAS to detect rare variants, and higher
20 resolution than F₂ linkage mapping. To extend the advantages of MPPs in budding yeast,
21 we describe the creation and characterization of two outbred MPPs derived from eighteen
22 genetically diverse founding strains. We carried out *de novo* assemblies of the genomes
23 of the eighteen founder strains, such that virtually all variation segregating between these
24 strains is known and represent those assemblies as Santa Cruz Genome Browser tracks.
25 We discover complex patterns of structural variation segregating amongst the founders,
26 including a large deletion within the vacuolar ATPase *VMA1*, several different deletions
27 within the osmosensor *MSB2*, a series of deletions and insertions at *PRM7* and the
28 adjacent *BSC1*, as well as copy number variation at the dehydrogenase *ALD2*.
29 Resequenced haploid recombinant clones from the two MPPs have a median
30 unrecombined block size of 66kb, demonstrating the population are highly recombined.
31 We pool sequenced the two MPPs to 3270X and 2226X coverage and demonstrate that
32 we can accurately estimate local haplotype frequencies using pooled data. We further
33 down-sampled the poolseq data to ~20-40X and show that local haplotype frequency
34 estimates remain accurate, with median error rate 0.8% and 0.6% at 20X and 40X,
35 respectively. Haplotypes frequencies are estimated much more accurately than SNP
36 frequencies obtained directly from the same data. Deep sequencing of the two
37 populations revealed that ten or more founders are present at a detectable frequency for
38 over 98% of the genome, validating the utility of this resource for the exploration of the
39 role of standing variation in the architecture of complex traits.

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45 **Introduction**

46 A complete understanding of the genetic basis of complex traits is a goal shared by many
47 disciplines. Although much progress has been made in dissecting the genetic architecture
48 of complex traits such as adaptation, disease susceptibility, human height, and crop
49 performance, a major fraction of standing variation for most traits has remained
50 recalcitrant to dissection (Manolio *et al.* 2009). This is often referred to as the ‘missing’
51 heritability problem. Rapid progress in addressing the missing heritability problem seems
52 most likely in model systems that can be genetically and experimentally manipulated in a
53 controlled setting. In contrast to humans, in model genetic systems variants of subtle
54 effect can be validated via allele replacement experiments.

55

56 One of the mainstays of modern genetic mapping studies has been the use of pairwise
57 crosses between genetically diverged founder strains. Large segregating populations can
58 then be used to map phenotype to genotype. This approach, laid out in its modern form
59 for complex traits was initially described by (Lander and Botstein 1989) and reviewed in
60 (Flint and Mott 2001; Mackay 2001; Liti and Louis 2012) has proven to be especially
61 fruitful in budding yeast, such that mapped QTL tend to explain > 70% of the narrow-
62 sense heritability of most traits (Ehrenreich *et al.* 2010; Bloom *et al.* 2013, 2015, 2019;
63 Märtens *et al.* 2016). However, QTL mapping has suffered both from a lack of resolution
64 and a severe under-sampling of the functional variation potentially segregating in natural

65 populations. In this regard, association studies enjoy much finer mapping resolution and
66 sample a larger proportion of the variation present in a natural population (WTCCC 2007;
67 Visscher 2008). However, large-scale association studies are often under-powered to
68 detect rare alleles (Spencer *et al.* 2009), regions that harbor multiple causal sites in weak
69 LD with one another (Pritchard 2001; Thornton *et al.* 2013), rare or poorly tagged
70 structural variants (Hehir-Kwa *et al.* 2016) or variants that are poorly tagged more
71 generally. Furthermore, as GWAS studies grow to include tens of thousands of
72 individuals they can suffer from false positives from population stratification (Berg *et al.*
73 2019) or other experimental block artifacts associated with large scale projects
74 (Sebastiani *et al.* 2011; Chen *et al.* 2017).

75

76 Advanced generation multiparent populations (MPPs) consisting of recombinants derived
77 from several founder individuals have been proposed as a bridge between pairwise
78 linkage mapping and association studies in outbred populations (“The Collaborative
79 Cross, a community resource for the genetic analysis of complex traits” 2004; Macdonald
80 and Long 2007). MPPs are created by crossing several (inbred or isogenic) founder
81 strains to one another in order to maximize diversity, and then intercrossing the resulting
82 population for several additional generations to increase the number of recombination
83 events in the population. In many model systems Recombinant Inbred Lines (RILs) are
84 derived from the MPP via inbreeding. The resulting homozygous RILs are fine-grained
85 mosaics of the original founding strains that have been successfully used to dissect
86 complex traits in *Arabidopsis thaliana* (Kover *et al.* 2009; Huang *et al.* 2011), *Drosophila*
87 *melanogaster* (Macdonald and Long 2007; King *et al.* 2012a, 2012b), *Mus musculus*

88 (Aylor *et al.* 2011; Threadgill and Churchill 2012), *Saccharomyces cerevisiae* (Cubillos *et*
89 *al.* 2013), *Zea mays* (McMullen *et al.* 2009), *Caenorhabditis elegans* (Noble *et al.* 2019),
90 and several other systems (de Koning and McIntyre 2017). MPP RILs are a powerful
91 resource for dissecting complex traits due to increased mapping resolution relative to F2
92 populations and increased natural variation sampled by the founders. Furthermore, unlike
93 association studies, both rare alleles of large effect segregating among the founders as
94 well as allelic heterogeneity can be detected in MPP RILs (Long *et al.* 2014). Although
95 the majority of studies to date have studied RILs derived from MPPs, it is possible to
96 dispense with the creation and maintenance of RILs and sample the MPP directly (Mott
97 *et al.* 2000; Macdonald and Long 2007), and indeed early MPP efforts did not employ
98 RILs.

99
100 Despite the clear advantages of MPPs, only a single MPP has been described in budding
101 yeast (Cubillos *et al.* 2013), which is surprising as this species is ideally suited in many
102 other ways for the dissection of complex traits. Large population sizes can be maintained
103 in a controlled environment and a few rounds of meiosis results in recombination events
104 spaced at near genic resolution. The potential of MPPs in budding yeast was
105 demonstrated by Cubillos *et al.*, who crossed four genetically highly diverged strains and
106 intercrossed the resulting population for twelve generations to generate a highly
107 recombined population that has been shown to be capable of mapping complex traits to
108 high resolution (Cubillos *et al.* 2013, 2017). In order to expand the potential of budding
109 yeast to contribute to our understanding of complex traits we have developed two large,
110 highly outbred populations of budding yeast derived from a cross of 18 genetically

111 diverged founders. Like previous work, populations were intercrossed for 12 generations
112 to produce highly recombined mosaic populations that capture a large amount of the
113 standing variation present in *S. cerevisiae*. Here we describe the derivation of the
114 founders that allows the 18-way cross to be carried out, *de novo* PacBio assemblies of
115 each founder such that all variation segregating in the population is known, and the
116 characterization of ten haploid recombinant clones from each population to estimate the
117 size distribution of haplotype blocks in the MPPs. We further carry out deep short read
118 resequencing of the MPPs, estimate founder haplotype frequencies as a function of
119 location in the genome, and show that at Illumina sequencing coverages as low as ~20X-
120 40X haplotype frequencies can be accurately estimated. The MPPs and tools we derive
121 have great utility for dissecting complex traits in yeast.

122

123 **Materials and Methods**

124 *Strains and media*

125 All yeast strains used in this study came from heterothallic, haploid derivatives of a subset
126 of the SGRP yeast strain collection kindly provided by Gianni Liti (Cubillos *et al.* 2009). A
127 list of strains used, relevant genotypes (before and after our modifications), and their
128 geographical origins is shown in Table 1. Additionally, two mate-type testing yeast strains
129 were used (kindly provided by Ian Ehrenreich) that are selectively killed by the presence
130 of either *Mat a* or *Mat α* haploids, but not by diploids. For propagating plasmids,
131 *Escherichia coli* strain DH5 α was used according to the manufacturer's recommendations
132 (Invitrogen). Bacterial transformants were selected on LB agar, supplemented with
133 100ug/mL ampicillin ('LBamp') (Fisher). Nonselective media for growth and maintenance

134 of all yeast strains included rich media consisting of 1% yeast extract, 2% peptone, and
135 2% dextrose ('YPD') (Fisher). For solid media, 2% agar was added. Additionally, media
136 consisting of 1% yeast extract, 2% peptone, 2% glycerol and 2.5% ethanol ('YPEG') was
137 used to prevent the growth of *petite* mutants. For selecting yeast transformants, when
138 *Ura3MX* was the marker, synthetic complete drop-out uracil (Sc -Ura) plates were used
139 (Sunrise Scientific). When *KanMX*, *HphMX*, or *NatMX* were the markers used,
140 transformants were selected on YPD plates supplemented with 200ug/mL of G418,
141 300ug/mL of Hygromycin B ('hyg'), or 100ug/mL nourseothricin sulfate ('cloNAT'),
142 respectively. For counterselection of yeast that lost the *Ura3MX* marker, synthetic
143 complete media supplemented with 1mg/mL 5-FOA was used ('5-FOA'). Two types of
144 sporulation media were used in this study. Type 1 consisted of 1% potassium acetate,
145 0.1% yeast extract, and 0.05% dextrose ('PYD') to which ampicillin was added to a final
146 concentration of 50ug/mL, while type 2 consisted of 1% potassium acetate and a 1X
147 dilution of a 10X amino acid stock (composed of 3.7g of CSM -lysine (Sunrise Scientific)
148 supplemented w/10mL of 10mg/mL lysine in 1L total volume), pH adjusted to 7 ('PA7').
149 Just before use, ampicillin was added to PA7 to a final concentration of 100ug/mL.

150

151 *Modification of 24 haploid budding yeast strains to create founders for the synthetic* 152 *population*

153 The strains used in this study were modified by generating clean deletions of the *HO* gene
154 to recover the *HphMX* marker, followed by replacement of a pseudogene, *YCR043C*,
155 which is closely linked to the mating type locus, with either a *NatMX* cassette in *Mat a*

156 haploids or a *HphMX* cassette in *Mat α* haploids. This manipulation was carried out to
157 enable high-throughput selection of diploids.

158

159 The *HphMX* marker in *HO* was recovered via transformation with a URA3 cassette
160 flanked with direct repeats and selection on URA- plates followed by selection on 5-FOA
161 plates to recover URA3. The URA3 cassette was assembled from four fragments: a
162 pBluescript II KS(+) backbone linearized with EcoRV and gel purified (for propagation in
163 *Escherichia coli*), the *URA3* gene from *Candida albicans* with flanking 500bp direct
164 repeats from *Aschbya gossypii* (pAG61, addgene #35129), and a 450bp region directly
165 upstream of the *HO* gene, and a 390bp region directly downstream of the *HO* gene.
166 Primers pAG61_HO-F/R were used to amplify *URA3* and the flanking direct repeats, while
167 primers HO-US F/R and HO-DS F/R were used to amplify the regions flanking the *HO*
168 gene from strain DBVPG6765. Primers used in this study are listed in Table S1 and
169 included overhangs to allow for HiFi assembly. The four fragments were assembled using
170 the NEB HiFi Assembly Master Mix according to the manufacturer's recommendations
171 (NEB), transformed into chemically competent DH5α (Invitrogen), and recovered on LB
172 Amp plates. Recovered URA plasmid cassettes with *HO* flanking sequences were PCR
173 amplified from the plasmid template using primers HO-US-F and HO-DS-R, transformed
174 into all 24 haploid strains using a standard lithium acetate protocol, and plated onto Sc -
175 Ura plates. Single colonies were re-streaked onto Sc -Ura (X2), final colonies were tested
176 for the presence of the *KanMX4* marker and absence of *HphMX4* marker via *G418* and
177 *hyg* plating respectively. O/N cultures of successfully knocked-out transformants were
178 spread onto 5-FOA plates and grown for 2d at 30°C in order to select for cells that had

179 'popped out' the *Ura3* cassette. Single colonies were re-streaked onto 5-FOA plates (2X).
180 DNA was extracted (adapted from CSH handbook, p. 116) from the resulting colonies
181 and DNA amplicons spanning the *HO* locus were obtained and Sanger sequenced to
182 confirm the clean deletion of the *HO* gene.

183

184 To delete *YGR043C* in the 24 newly generated haploid *hoΔ Ura3::KanMX4* strains, oligos
185 were ordered from IDT that amplify the entire MX4 cassette, including the promoter and
186 terminator regions, and were tailed with 100 bases of homology to the regions
187 immediately upstream and downstream of the *YGR043C* CDS. Either pAG32 (addgene
188 #35122) or pAG25 (addgene #35121) were used as a template to generate knock-out
189 constructs that incorporate the *HphMX4* or *NatMX4* cassettes, respectively. PCR
190 reactions were cleaned up to remove unamplified circular plasmid template by gel
191 extraction followed by digestion with DpnI and a PCR cleanup reaction (Qiagen PCR
192 Purification kit). *Mat a* yeast were then transformed with the *cloNAT* resistance cassette,
193 while *Mat α* yeast were transformed with the *hyg* resistance cassette using the standard
194 lithium acetate protocol and selecting on YPD supplemented with cloNAT and G418 or
195 hyg and G418, respectively. This double selection with G418 was done to ensure that
196 cassette swapping had not occurred. To ensure *YGR043C* had been correctly replaced
197 in each strain, the region was amplified and Sanger sequenced. All 24 newly generated
198 strains were checked again for *HO* deletion using the HO-big-flank-F/R primers. The
199 strains were also checked to ensure they had maintained the correct barcodes originally
200 inserted in Cubillos et al throughout all the manipulation steps by amplifying the barcodes
201 using the barcode-check-F/R primer pair and Sanger sequencing the amplicons using the

202 M13(-47)F primer. As a final check, all 24 haploid strains were streaked onto YPD
 203 supplemented with hyg and cloNAT to ensure that none of the strains could grow on both
 204 antibiotics.

205

206 **Table 1.** An overview of the strains used in this study.

ADL*	NCYC	Isolate	Origin	Original genotype	Modified genotype**
A1	3597	DBVPG6765	Europe	<i>Mat a, ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat a, hoΔ, ura3::KanMX-Barcode, ygr043C::NatMX</i>
A2	3600	DBVPG6044	West Africa; wine	<i>Mat a, ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat a, hoΔ, ura3::KanMX-Barcode, ygr043C::NatMX</i>
A3	3607	YPS128	USA; soil beneath Quercus alba	<i>Mat a, ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat a, hoΔ, ura3::KanMX-Barcode, ygr043C::NatMX</i>
A4	3605	Y12	Japan; sake	<i>Mat a, ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat a, hoΔ, ura3::KanMX-Barcode, ygr043C::NatMX</i>
A5	3586	Yllc17_E5	France; wine	<i>Mat a, ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat a, hoΔ, ura3::KanMX-Barcode, ygr043C::NatMX</i>
A6	3591	BC187	USA; wine	<i>Mat a, ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat a, hoΔ, ura3::KanMX-Barcode, ygr043C::NatMX</i>
A7	3590	SK1	USA; soil	<i>Mat a, ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat a, hoΔ, ura3::KanMX-Barcode, ygr043C::NatMX</i>
A8	3598	L_1374	Chile; wine	<i>Mat a, ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat a, hoΔ, ura3::KanMX-Barcode, ygr043C::NatMX</i>
A9	3602	UWOPS03_461_4	Malaysia; nectar, Bertram palm	<i>Mat a, ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat a, hoΔ, ura3::KanMX-Barcode, ygr043C::NatMX</i>
A10***	3604	UWOPS05_227_2	Malaysia; stingless bee, near Bertram palm	<i>Mat a, ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat a, hoΔ, ura3::KanMX-Barcode, ygr043C::NatMX</i>
A11	3592	YJM978	Italy; vagina, clinical isolate	<i>Mat a, ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat a, hoΔ, ura3::KanMX-Barcode, ygr043C::NatMX</i>

A12	3594	YJM975	Italy; vagina, clinical isolate	<i>Mat a</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat a</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::NatMX</i>
B1	3622	DBVPG6765	Europe	<i>Mat α</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat α</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::HygMX</i>
B2	3625	DBVPG6044	West Africa; wine	<i>Mat α</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat α</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::HygMX</i>
B3	3632	YPS128	USA; soil beneath <i>Quercus alba</i>	<i>Mat α</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat α</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::HygMX</i>
B4	3630	Y12	Japan; sake	<i>Mat α</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat α</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::HygMX</i>
B5	3611	273614N	UK; fecal sample, clinical isolate	<i>Mat α</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat α</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::HygMX</i>
B6	3631	YPS606	USA; bark of <i>Quercus rubra</i>	<i>Mat α</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat α</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::HygMX</i>
B7	3624	L_1528	Chile; wine	<i>Mat α</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat α</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::HygMX</i>
B8	3614	UWOPS83_787_3	Bahamas; fruit, <i>Opuntia megacantha</i>	<i>Mat α</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat α</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::HygMX</i>
B9	3609	UWOPS87_2421	USA; cladode, <i>Opuntia megacantha</i>	<i>Mat α</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat α</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::HygMX</i>
B10***	3628	UWOPS05_217_3	Malaysia; nectar, Bertram palm	<i>Mat α</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat α</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::HygMX</i>
B11	3618	YJM981	Italy; vagina, clinical isolate	<i>Mat α</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat α</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::HygMX</i>
B12	3613	Y55	France; grape	<i>Mat α</i> , <i>ho::HygMX,ura3::KanMX-Barcode</i>	<i>Mat α</i> , <i>hoΔ</i> , <i>ura3::KanMX-Barcode</i> , <i>ygr043C::HygMX</i>

207 * These are the abbreviated names used throughout this manuscript. Note that all A strains are

208 *Mat a*, and all B strains *Mat α*.

209 ** Bold text indicates changes made from the original strain genotypes.

210 *** These two strains were excluded from subsequent experiments as they mate poorly with the
211 other strains.

212

213 *18-way crossing scheme, version 1*

214 A full diallele cross of eleven *Mat a* and eleven *Mat α* strains (excluding strains A10 and
215 B10) was carried out (with four strains in common). A schematic of the mating scheme is
216 shown in Figure 1A, while Table 1 lists the strains used in this study. Strains A1-A5 and
217 A6-A12 (excluding A10) were struck in horizontal rows onto two YPD plates each (total
218 of 4 YPD plates), then strains B1-B5 and B6-B12 (excluding B10) were each struck in
219 vertical rows onto two of the YPD plates such that each 'B' strains intersected with each
220 'A' strains. All 121 pairwise combinations of the 'A' and 'B' strains were thus represented
221 across the four YPD mating plates. Mating occurred O/N at 30°C after which diploids were
222 selected by replica plating onto YPD plates with hyg and cloNAT. A single colony from
223 each of the 121 crosses was then incubated O/N in YPD with hyg and cloNAT at 30°C at
224 180RPM. An equal volume of each culture and 30% glycerol was used to make frozen
225 stock that was then archived at -80°C. An equal volume from each diploid culture was
226 then combined to make the 18-way population, which was washed twice with PYD + amp
227 then split into two 1L flasks with 200mL total PYD + amp each. Sporulation was carried
228 out for 5d *en mass* at 30°C at 180RPM to complete the first round of out-crossing.

229

230 *Additional outcrossing in the 18-way cross, version 1*

231 Eleven additional cycles of mass sporulation followed by random mating were carried out
232 for a total of twelve rounds of outcrossing for both replicates (Table S3). After sporulation,
233 50mL of culture was spun down at 2000g for 2m and resuspended in 1mL of Y-PER.
234 Samples were transferred to a 1.5mL centrifuge tube and vortexed. Cells were washed
235 twice and resuspended in 500uL of ddH₂O with 5uL of 5U/uL zymolyase. The tubes were

236 shaken vigorously in the Geno Grinder 2000 at 750 shakes/minute for 45m. 500uL of
237 400um silica beads were then added to the samples, which were again put in the Geno
238 Grinder for 5m at 1500 shakes/minute. The supernatant was transferred to a fresh 1.5mL
239 centrifuge tube, washed once in YPD, resuspended in 500uL of YPD, and transferred into
240 50mL of YPD in a 1L flask. Mating was carried out O/N at 30°C at 40RPM. The next day,
241 mated cells were harvested, transferred to YPD with cloNAT, hyg, and amp, and
242 incubated O/N at 30°C at 180RPM. The next day, 7mL from the O/N culture was used to
243 make glycerol stock while 5mL was harvested, washed twice, and resuspended in 200mL
244 of PYD + amp. Sporulation was carried out for 5d at 30°C at 180RPM (see Table S2). If
245 the experiment had to be paused, 5mL of glycerol stock from the most recently completed
246 cycle was used to begin the next cycle of sporulation.

247

248 *18-way crossing scheme, version 2*

249 A full diallele cross of the same eleven *Mat a* and eleven *Mat α* founder strains used to
250 create 18F12v1 was again carried out. To initiate this process, an equal volume from
251 cultures containing each *Mat a* founder strain were mixed with each *Mat α* founder strain
252 in all 121 possible pairwise combinations in 24-well deep-well plates (hereafter '24DWP')
253 in a total volume of 1mL of YPD (no ampicillin added) (see Note S1). Mating was carried
254 out in liquid culture for 4-5h at 30°C at 50RPM, after which mating was verified by
255 checking for the presence of zygotes and/or shmooing under a microscope. At this point,
256 1mL of YPD supplemented with 200ug/mL cloNAT and 600ug/mL hyg was added to each
257 culture (the final concentrations of cloNAT and hyg were 100ug/mL and 300ug/mL,
258 respectively) to select for successfully mated diploids and incubated O/N at 200RPM at

259 30°C. After O/N selection, 140uL from each culture was combined w/140uL of 30%
260 glycerol to make frozen stock of each cross. The remaining cultures were harvested at
261 3000 RPM for 5m and the pellets were washed then resuspended in 4mL of PA7 + amp.
262 Sporulation was carried out for 6d at 30°C at 275 RPM in the 24DWP.

263

264 All 121 sporulating cultures were checked using a microscope to determine the amount
265 of sporulation that occurred; cultures were graded on a scale of 0-5 with 0 being no
266 sporulation and 5 being almost complete sporulation. Crosses that did not sporulate were
267 excluded from subsequent steps (see Table S2). After checking for sporulation, cultures
268 were harvested, washed, then resuspended in 500uL of spore isolation solution (hereafter
269 'SIS': 25U zymolyase + 10mM DTT + 50mM EDTA + 100mM Tris-HCl, pH 7.2 up to
270 500uL) and incubated for 1h at 30°C at 250 RPM to spheroplast cells. Cultures were then
271 harvested and resuspended in 1% Tween 20 to selectively lyse unsporulated cells.
272 Following this, cultures were again harvested and resuspended in 500uL of spore
273 dispersal solution (hereafter 'SDS': 1mg lysozyme + 5U zymolyase + 1% Triton X-100 +
274 2% dextrose + 100mM PBS, pH 7.2 up to 500uL). Cultures were transferred to Eppendorf
275 tubes with 500uL of 400um beads and bead milled using a Geno Grinder 2000 at 1500
276 strokes per minute for 5m to break up tetrads after which all cultures were placed at 4°C
277 O/N. The next day, all tubes were vortexed at high speed for 30s, the supernatant was
278 transferred to a 24DWP, 500uL of 100mM PBS, pH 7.2 was added back to the beads,
279 followed by vortexing for 30s and transferring to the same wells of a 24DWP to maximize
280 recovery of spores from the beads. Cultures were washed once in PBS, then
281 resuspended in 500uL of 100mM PBS, pH 7.2 and 100uL was transferred to a 96-well

282 clear plate to measure the OD630 of each culture in duplicate using a BioTek Synergy
283 HT plate reader. OD630 measurements were then used to normalize the density of
284 spores from each cross that were pooled together (see Note S2). The spore pool was
285 washed twice with 5mL of YPD, then resuspended in 12.5mL of YPD. This culture was
286 split in half and transferred to two 250mL flasks, each with 6.25mL of YPD, to establish
287 two replicate populations. Mating was carried out O/N at 30°C with gentle shaking at 40
288 RPM. The next day, 12.5mL of YPDach (with a 2x mix of ampicillin, cloNAT, and hyg)
289 was added to each culture to select for diploids. Cultures were incubated O/N at 200 RPM
290 at 30°C. This established replicate F₂ populations of the 18-way cross, version 2
291 (hereafter '18F2v2'). The following day, 7mL of the replicate populations were frozen
292 down at -80°C with an equal volume of 30% glycerol. The remaining volume was spun
293 down and used to initiate a second round of outcrossing.

294

295 *Additional outcrossing in the 18-way cross, version 2*

296 Eleven additional cycles of mass sporulation followed by random mating were carried out
297 for a total of twelve rounds of outcrossing for both replicates. As replicate 2 was treated
298 differently during a couple of cycles, replicate 1 was the population chosen for subsequent
299 analyses and, as such, will be the only replicate of version 2 described further. Each cycle
300 consisted of 3-6d of sporulation after which diploids were randomly mated for 3-4h. This
301 was followed by an O/N selection step in YPDach to enrich for mated diploids. After
302 selection, an aliquot of each population was frozen down at -80°C with the remaining
303 culture used to initiate the next cycle of outcrossing. Table S3 enumerates the days of
304 sporulation for each cycle as well as additional details regarding the culturing conditions

305 for both versions of the 18-way population. After each round of sporulation, cultures were
306 processed as detailed above with the following modifications: 5mL of SIS, 10mL of 1%
307 Tween 20, and 5mL of SDS were used to kill vegetative cells. Tetrads were disrupted by
308 bead milling at 1500 strokes/minute using the Geno Grinder 2000 for 25-45m. The
309 contents of the tubes were mixed thoroughly with a pipette to ensure maximal recovery
310 of cells from the bead slurry. The supernatant was then transferred to 50mL Falcon tubes,
311 after which 500uL of YPDa was added back to the tubes, which were then vortexed at the
312 highest setting briefly. The supernatant was transferred to the same 50mL Falcon tube.
313 Cultures were harvested, washed, then resuspended in 5mL of YPDa. At this point, cells
314 were carefully mixed by pipetting and then transferred to a 250mL Erlenmeyer flask with
315 7.5mL of YPDa. Spores were mated for 3-4h at 30°C at 40RPM, after which the presence
316 of shmoos and/or zygotes was checked under the microscope. 12.5mL of YPDach was
317 added to the mated cells, which were incubated O/N at 200RPM at 30°C. The next day,
318 cells were transferred to 50mL Falcon tubes, 7mL of culture was mixed with an equal
319 volume of 30% glycerol to make frozen stock, while the rest of the culture was spun down
320 at 3000RPM for 5m. Cultures were washed twice, resuspended in 25mL of PA7, then
321 transferred to a 250mL flask with 50uL of 100mg/mL ampicillin and sporulated at 30°C at
322 275RPM to initiate the next cycle of outcrossing. Following the twelfth cycle of sporulation
323 followed by random mating, cells were transferred to 1L flasks with 187.5mL of YPDach
324 and incubated O/N at 30°C at 200RPM. The following day, all 200mL of culture was mixed
325 with an equal volume of 40% glycerol and frozen down at -80°C in a combination of 2mL
326 cryotubes and 15mL Falcon tubes.

327

328 **Table 2.** Assembly statistics for the 18 sequenced founder strains.

Strain	Assembly size (Mb)	Assembly N50 (kb)	Assembly QV	Assembly BUSCO (complete)	Total PacBio data (Mb)	PacBio read N50 (kb)	Total Illumina data (Mb)	Type of Illumina reads	x PacBio coverage	x Illumina coverage
A5	12.13	757	48.8	0.990	737	11.10	3,396	PE100	61.4	283.0
A6	11.98	913	46.6	0.986	620	11.68	4,079	PE150	51.7	340.0
A7	12.62	901	66.2	0.990	3,979	5.97	2,775	PE100	331.6	231.3
A8	12.03	917	55.5	0.993	622	11.79	3,420	PE100	51.9	285.0
A9	12.53	571	53.0	0.993	5,845	5.10	2,769	PE100	487.1	230.7
A11	12.10	702	49.3	0.986	644	12.03	2,758	PE100	53.7	229.8
A12	12.00	795	45.5	0.993	613	11.83	3,563	PE100	51.1	296.9
B5	12.32	738	46.5	0.990	741	10.97	3,861	PE100	61.7	321.8
B6	12.10	772	44.9	0.990	401	11.67	2,906	PE100	33.4	242.2
B7	11.91	765	39.5	0.986	719	11.79	4,266	PE100	59.9	355.5
B8	11.99	802	66.0	0.990	741	12.00	3,054	PE100	61.8	254.5
B9	12.40	856	54.6	0.993	1,083	12.05	3,419	PE100	90.2	284.9
B11	12.12	789	55.0	0.990	765	12.13	2,236	PE100	63.8	186.3
B12	12.04	790	48.7	0.986	804	10.79	3,930	PE100	67.0	327.5
AB1	12.35	901	47.9	0.993	3,315	5.84	3,509	PE150	276.3	292.4
AB2	12.83	741	66.2	0.990	5,230	4.77	4,230	PE150	435.8	352.5
AB3	12.44	809	55.8	0.990	3,254	6.21	3,891	PE150	271.2	324.2
AB4	12.32	800	55.7	0.990	4,649	6.43	4,490	PE150	387.4	374.2

329 *Cells highlighted in blue represent founder strains that were previously sequenced using
 330 PacBio technology in (Yue *et al.* 2017)

331
 332

333 *Whole genome sequencing of the haploid founder strains*

334 All 18 founder strains were sequenced using a combination of PacBio long read and
 335 Illumina short read technology. PacBio sequencing data was available from a previous
 336 study for 6 of the 18 strains (founder AB1-AB4, A7, and A9) (Yue *et al.* 2017), which was
 337 downloaded and reassembled using our pipeline so that all assemblies are directly
 338 comparable. The remaining strains were struck out onto YPD plates for 3d at 30°C, after
 339 which a single colony was inoculated into 50mL of YPDamp and incubated at 30°C at
 340 200RPM O/N. DNA was extracted using the Qiagen G-tip DNA extraction kit. Purified

341 genomic DNA was sheared using 24-gauge blunt needles. The resulting sheared gDNA
342 samples were quality checked by a FIGE run at 134V O/N and concentrations were
343 measured using Qubit. Samples were considered acceptable if the majority of gDNA was
344 sheared to between 20kb and 100kb. In our hands carefully controlling the gDNA size
345 distribution results in longer N50 PacBio reads which gives better *de novo* assemblies
346 with less data. SMRTbell libraries were prepared and sequenced at the UCI Genomics
347 High Throughput Facility using a PacBio RSII machine. The details of PacBio library
348 creation for the purpose of *de novo* genome assembly are described in (Chakraborty *et*
349 *al.* 2016). The average per-site coverage of the six previously sequenced strains was
350 365x as compared to 59x for the twelve strains sequenced in our hands, while the average
351 PacBio read N50 for the previously sequenced strains was 5.73 kb as compared to
352 11.65kb for the strains sequenced by our lab.

353

354 Libraries for Illumina sequencing were made for all 18 founder strains. The same genomic
355 DNA that had been used to prep the SMRTbell libraries was used to make Illumina
356 libraries. Genomic DNA from the six remaining strains was prepared using the Qiagen G-
357 tip kit as above. All genomic DNA was sheared to ~300-400bp using the Covaris S220
358 Focused Acoustic Shearer with the following settings: peak incident power (w) of 140,
359 Duty Factor of 10%, Cycles per Burst of 200, Treatment time of 65s, temperature of 4°C,
360 and water 12. Illumina compatible libraries were prepared using the NEBNext Ultra II DNA
361 Library Prep kit along with the NEBNext Multiplex Oligos for Illumina (Index Primer Set 1)
362 as per the manufacturer's recommendations. Adaptor-ligated DNA was size-selected and
363 PCR-enriched for five cycles, followed by clean-up of the PCR reaction using AMPure XP

364 Beads as per the NEBNext Ultra II DNA Library Prep protocol. Sequencing was carried
365 out using the Illumina HiSeq4000 with PE100 or PE150 reads (see Note S3). The average
366 per-site coverage of the 18 founder strains was 290x with the lowest coverage being 186x
367 (founder B11) and the highest coverage at 374x (founder AB4).

368

369 *Genome assembly*

370 We assembled the PacBio reads using canu v1.7 (commit r8700; options:
371 corMhapSensitivity=high, corOutCoverage=500, minReadLength=500,
372 corMinCoverage=0, correctedErrorRate=0.105) (Koren *et al.* 2017). We generated hybrid
373 assemblies using the PacBio and Illumina reads for the twelve strains for which we
374 generated the PacBio reads. The PacBio reads from the six strains from (Yue *et al.* 2017)
375 were too short to assemble with DBG2OLC, the hybrid assembler we use (Ye *et al.* 2016).
376 The DBG2OLC hybrid assemblies were used to fill gaps in the corresponding canu
377 assemblies using quickmerge, following the two steps merging approach (Chakraborty *et*
378 *al.* 2016; Solares *et al.* 2018). The PacBio reads from Yue *et al.* were sequenced using
379 an older chemistry of Pacific Biosciences (P4-C2) than our PacBio reads (P6-C4), so they
380 required a different algorithm for optimal polishing than the assemblies created with the
381 P6-C4 reads. Hence, we polished the P4-C2 based assemblies twice using Quiver and
382 the P6-C4 based assemblies twice using Arrow (smrtanalysis v5.2.1). Finally, we polished
383 all assemblies twice with the paired end Illumina reads using Pilon (Walker *et al.* 2014).

384

385 *BUSCO assessment*

386 We estimated the number of fungi BUSCOs (n=290) in each polished assembly using
387 BUSCO v3.0.2 (Waterhouse *et al.* 2018) (Table 2). For the augustus gene prediction step
388 in BUSCO we used 'saccharomyces_cerevisiae_S288C' as the species option.

389

390 *QV estimate*

391 To estimate assembly error rate, paired end Illumina reads used in assembly polishing
392 were mapped to the final assembly using bowtie2 (Langmead and Salzberg 2012). SNPs
393 and small indels were identified using freebayes v0.9.21 (-C 10 -O -q 20 -z 0.10 -E 0 -
394 X -u -p 1 -F 0.75) (Garrison and Marth 2012a). To estimate the error rate, total bases due
395 to SNPs and small indels (e) and the total number of assembly bases (b) with read
396 coverage ≥ 3 were counted and qv was calculated as $-10 \times \log(e/b)$ (Koren *et al.* 2017)
397 (Table 2).

398

399 *Santa Cruz Browser Tracks*

400 Assembled genomes were aligned to one another and the *SacCer3* reference genome
401 using *ProgressiveCactus* (<https://github.com/ComparativeGenomicsToolkit/cactus>)
402 (Paten *et al.* 2011a, 2011b). Santa Cruz Browser Track Hubs were created using the
403 *hal2assemblyhub* script that is part of the *ProgressiveCactus* software
404 (<https://github.com/ComparativeGenomicsToolkit/Comparative-Annotation-Toolkit>). The
405 resulting SNAKE tracks are viewable at <http://bit.ly/2ZrreUd>. SNPs were identified
406 between the founder strains using a generic GATK pipeline, with SNPs functionally
407 annotated using SNPeff (Cingolani *et al.* 2012). Scripts to align the genomes and call
408 SNPs in the founders are available here: https://github.com/tdlong/yeast_resource.

409

410 *Analysis of structural variants*

411 We aligned each founder genome assembly to the s288c reference genome
412 (GCA_000146055.2) using MUMmer v4.0 (Marçais *et al.* 2018) (nucmer --maxmatch --
413 prefix founder.ref.fasta founder.fasta). To annotate the SVs, the delta alignment file for
414 each strain was then processed with SVMU (commit e9c0ea1) (Chakraborty *et al.* 2019).

415

416 *Whole genome sequencing of the two base populations*

417 The two base populations were deeply sequenced using Illumina technology. In total,
418 4mL of the 18F12v1 frozen stock was thawed at RT, pelleted at 3000 RPM for 5m, and
419 resuspended in 20mL of YPDamp. This was followed by incubation at 30°C for 3.5h at
420 275RPM. Genomic DNA was extracted using the Qiagen DNeasy kit. The genomic DNA
421 was sheared using the Covaris S220 as above and Illumina compatible libraries were
422 prepared using the NEBNext Ultra II DNA Library Prep kit as above. The NEBNext and
423 libraries were pooled and sequenced on the HiSeq4000 using PE100 reads. The
424 NEBNext libraries were sequenced at a mean per-site coverage of 3270x.

425

426 For 18F12v2, similarly to 18F12v1, 4mL of frozen stock was thawed at RT, pelleted at
427 3000RPM for 5m, and resuspended in 20mL of YPDamp, followed by incubation at 30°C
428 for 3.5h at 275RPM. Genomic DNA was extracted using the Qiagen G-tip kit. Nextera
429 libraries were prepped for 18F12v2 by following the standard Nextera protocol with slight
430 modifications. Tagmentation reactions were carried out in 2.5uL reactions for 10m at
431 55°C. Reactions were stopped by adding SDS to a final concentration of 0.02% followed

432 by incubation at 55°C for 7m. Samples were immediately transferred to ice. Limited cycle
433 PCR was carried out to add two unique barcodes to each library to enable dual index
434 sequencing. This avoids the problem of barcode switching when *N* i7 and *M* i5 barcodes
435 are used to create *MN* combinations. The KAPA HiFi Ready Mix (2X) was used in
436 conjunction with the KAPA forward and reverse primers to amplify tagmented libraries in
437 25uL total volume. Thermocycling parameters consisted of 3m at 72°C, 5m at 98°C,
438 followed by 15 cycles of 10s at 98°C, 30s at 63°C, and 30s at 72°C, with a hold of 72°C
439 for 5m at the end. PCR reactions were cleaned up using AMPure XP Beads (Beckman
440 Coulter, Inc.) and libraries quantified by Qubit. Sequencing was performed on the
441 HiSeq4000 using PE150 reads. 18F12v2 libraries received 2226x coverage.

442

443 *Whole genome sequencing of the first two meiotic generations of the second base*
444 *population*

445 For 18F1v2 and 18F2v2, ~1mL of frozen stock was thawed at RT, spun down at 7500
446 RPM for 5m in microcentrifuge tubes, resuspended in 1mL of YPDamp, transferred to a
447 250mL flask with 19mL of YPDamp and incubated at 30°C for 3.5h at 275RPM. Genomic
448 DNA from both samples was extracted using the Qiagen G-tip kit. Nextera libraries were
449 prepped by following the Nextera flex protocol using 1/5th reactions with slight
450 modifications. Limited cycle PCR was carried out using the KAPA HiFi Ready Mix (2X) as
451 detailed above to add barcoded Illumina-compatible adapters in 12.5uL reactions.
452 Thermocycling parameters consisted of 3m at 72°C, 3m at 98°C, followed by 12 cycles
453 of 45s at 98°C, 30s at 62°C, and 2m at 72°C, with a hold of 72°C for 1m at the end.
454 Proteinase K was added to each reaction (50ug/mL final concentration) to digest the

455 polymerase. Samples were incubated for 30m at 37°C and 10m at 68°C. Reactions were
456 cleaned up using the SPB beads provided with the Nextera flex kit. Sequencing was
457 performed as above using PE100 reads. 18F1v2 received 98x coverage while 18F2v2
458 received 73x coverage.

459

460 *Whole genome resequencing of recombinant haploid clones*

461 Ten haploid recombinant clones (5 of each mating type) were isolated from each of the
462 two base populations. 18F12v1-derived haploids were generated by sporulating an O/N
463 culture of the 18F12v1 population in 2mL of PA7 in a 10mL culture tube at 30°C for 3d.
464 Spore isolation and dispersal were carried out as detailed above for the creation of
465 18F12v2 with 15m of bead milling to disperse spores. Spores were plated at low density
466 onto YPD plates and incubated for 2d at 30°C. One of the YPD plates was then replica
467 plated onto four different plates: YPD with hyg, YPD with cloNAT, YPD with mate-type
468 tester 1, and YPD with mate-type tester 2. Five haploids of each mating type were
469 inoculated into YPD O/N. Genomic DNA was extracted using the Qiagen DNeasy kit and
470 Nextera libraries prepared as above. Libraries were sequenced on a HiSeq4000 using
471 PE100 reads to a mean per-site coverage of ~32x.

472

473 18F12v2-derived haploids were generated by sporulating an O/N culture of the 18F12v2
474 population in 4mL of PA7 in a 24 deep-well plate at 30°C at 275RPM for 3d. Spore
475 isolation and dispersal were carried out as detailed above for the creation of 18F12v2
476 with 20m of bead milling to disperse spores. Spores were plated at low density onto YPD
477 plates and incubated at 30°C for 3d. 96 single colonies were then transferred into a 96

478 deep-well plate with YPDamp using sterile toothpicks. After O/N incubation at 30°C,
479 200uL of culture from each well was transferred to a 96 shallow plate and pinned YPD
480 plates with either cloNAT, hyg, mate-type tester 1, or mate-type tester 2 using a 48-well
481 replicator tool. The source plate was covered with an adhesive membrane and stored at
482 4°C. The mate typing plates were incubated at 30°C for 2d, after which five haploids of
483 each mating type were transferred from the original source plate to 1.5mL eppendorfs
484 and genomic DNA was extracted using the Qiagen DNeasy kit. Nextera libraries were
485 prepared as above. Libraries were sequenced on a HiSeq4000 using PE150 reads to a
486 mean per-site coverage of ~60x.

487

488

489 *Haplotype calling in Illumina resequenced MPPs and recombinant haploid clones*

490 De-multiplexed fastq files were used in analyses. Detailed scripts/software versions to
491 reproduce our analysis are located at https://github.com/tdlong/yeast_resource.git.
492 Briefly, reads were aligned to the *sacCer* reference genome using *bwa-mem* and default
493 parameters (Li and Durbin 2009; Li 2013). We maintain two SNP lists, a set of known
494 SNPs in the strains obtained from a GATK pipeline that only considers the isogenic
495 founders, and a subset of those SNPs that are well-behaved (i.e., frequency of the REF
496 allele close to zero or one in all founder lines, pass GATK qualify filters, etc.). The list of
497 well-behaved SNPs that are polymorphic in the founders can be used to speed-up
498 subsequent steps, where we sometimes examine hundreds of samples, since only
499 variants polymorphic among the founders need be considered when working with
500 samples from a synthetic population (except when calling newly arising mutations).

501 *samtools mpileup* (Li *et al.* 2009; Li 2011) and *bcftools* (Narasimhan *et al.* 2016) are used
502 to query well-behaved known SNPs. We have no interest in calling genotypes, but
503 instead simply output the frequency of the REF allele in each sample at each location
504 (output=SNPtable). In a separate analysis *freebayes* (Garrison and Marth 2012b),
505 *vcfallelicprimitives* (<https://github.com/vcflib/vcflib>), and *vt normalize* (Tan *et al.* 2015) are
506 used to call all SNPs, and the SNPs not in our list of known SNPs considered candidate
507 new mutations.

508

509 We have developed custom software to infer the frequency of each founder haplotype at
510 each location in the genome in pooled samples using the SNPtable as input and the
511 *haplotyper.limSolve.code.R* script in the github archive. This same algorithm can also be
512 used without modification to infer genotypes in recombinant haploid clones. Briefly, we
513 slide through the genome in 1kb steps considering a 60kb window for each step. For all
514 SNPs in the window we calculate a Gaussian weight such that the 50 SNPs closest to the
515 window center account for 50% of the sum of the weights. We then consider F founders
516 and use the *lse* function of the *limSolve* package (*limSolve: Solving Linear Inverse*
517 *Models*, R package 1.5.1) (Meersche *et al.* 2009) in R to identify a set of F mixing
518 proportions (each greater than zero and summing to one) that minimize the sum of the
519 weighted squared differences between founder haplotypes and the observed frequency
520 of each SNP in a pooled sample. That is, for a N SNP window we call *lse* with the
521 following parameters: A=N*F matrix of founder genotypes, B=N*1 vector of SNP
522 frequencies in a pooled sample, E=F*1 vector of 1's, F=1, G=F*F identity matrix, H= F*1
523 vector of 0's, and Wa=N*1 vector of weights. Finally, for windows where the i^{th} and j^{th}

524 founders have near indistinguishable haplotypes, implying the sum of the two mixing
525 proportions are correct, but not individual estimates, we estimate the haplotype frequency
526 as half the sum of the two mixing proportions. This method of accounting for
527 indistinguishable haplotypes is regional and is generalized to more than two near identical
528 founders and multiple such sets.

529

530 *Validation of the haplotype caller*

531 In order to validate our haplotype-calling algorithm, we identified 70,478 SNPs private
532 to a single founder strain (excluding those present in founders merged due to high
533 sequence similarity). The haplotype caller was run on 18F12v2 using the full coverage
534 data (i.e., 2230X) or down-sampled 18F12v2 to simulate a more typical poolseq re-
535 sequencing depth (typical applications using the MPPs are likely to sequence hundreds
536 of experimental units to ~20-60X). For each private SNP, the frequency of the SNP in the
537 full coverage data was estimated and the founder harbouring that SNP identified. Since
538 the sequence depth of the non-downsampled population is 2230X, the frequency of each
539 private SNP is measured very accurately. We then infer the frequency of the founder
540 haplotype harbouring the private SNP at the position closest to the private SNP, in both
541 the full coverage and each downsampled population. The error rate associated with the
542 haplotype caller is the absolute difference between frequency of each private SNP and
543 the founder haplotype harbouring it.

544

545 In our examination of the relationship between haplotype and SNP frequency estimates
546 (Figure 4) we identified and removed 91 outlier SNPs among the 71,301 private SNPs.

547 These SNPs were identified as private SNPs whose frequency was more than 5%
548 different than the frequency of the haplotype harbouring it in the full dataset, while
549 exhibiting flanking private SNPs in the same founder whose frequency agree with the
550 founder frequency. We believe these outlier SNPs are cases where that particular SNP
551 in a pooled sample cannot be aligned to the reference genome very accurately. It is
552 noteworthy that it is more difficult to identify poorly performing SNPs that are not private
553 to a single founder, and such SNPs likely hurt haplotype inference methods.

554

555 *Delineating haplotype blocks in recombinant haploid clones*

556 The haplotype caller is primarily used to estimate the frequency of founder haplotypes at
557 different positions in the genome in a DNA pool from a segregating population, but it can
558 also be run on DNA obtained from a haploid or diploid clone. In a haploid clone the
559 haplotype caller should return a haplotype frequency of close to 100% for one of the
560 founder haplotypes for much of the genome, with sharp transitions between founder
561 states near recombination breakpoints. In depicting the haplotypic structure of haploid
562 clones we classify genomic regions at which the inferred haplotype frequency of a single
563 founder (or multiple indistinguishable founders) is less than 95% as having an 'unknown'
564 haplotype (these unknown intervals typically being associated with state transitions). We
565 also observe intervals in which several founders are indistinguishable from one another
566 (due to insufficient SNP divergence between the founders in these window). We could
567 sometimes resolve these intervals to a single founding haplotype when flanking
568 haplotypes were unambiguously called as derived from the same single founder. Custom
569 R scripts were used for these analyses as well as to calculate the length of haplotype

570 blocks in haploid clones. Haplotype block sizes were inferred by finding the positional
571 difference between the beginning and end of runs of the same haplotype.

572

573 *Data and reagent availability*

574 Strains and plasmids are available upon request. All genome sequencing data and
575 assemblies have been deposited into public repositories. Sequence data generated for
576 the two base populations (18F12v1, 18F12v2, 18F1v2, and 18F2v2) as well as the
577 recombinant haploid clones are available in the Short Reads Archive under the bioproject
578 PRJNA551443 in accessions SRX6465384 to SRX6465405 and SRX6983898 to
579 SRX6983899. All PacBio and Illumina data generated for the 18 founding strains is also
580 available in the Short Reads Archive under the bioproject PRJNA552112 in accessions
581 SRX6380915 to SRX6380944. Detailed scripts/software versions to reproduce our
582 analysis are located at https://github.com/tldlong/yeast_resource.git.

583

584 **Results and Discussion**

585 *Recovery of hyg^r and insertion of dominant selectable markers for high-throughput diploid*
586 *selection*

587 We further engineering a subset of the yeast SGRP resource strains (Cubillos *et al.* 2009)
588 to serve as founders for an 18-way synthetic population. We first recovered the *hyg^r*
589 marker used to delete the *HO* gene in the haploid SGRP strains. Previous work
590 (McDonald *et al.* 2016) replaced *YGR043C*, a pseudogene that is physically close to the
591 mating type locus, with dominant selectable markers in order to facilitate high-throughput
592 selection of diploids after mating. We echoed that approach here by replacing *YGR043C*

593 with *NatMX4* in 15 *Mat a* (or “A”) founders and with *HphMX4* in 15 *Mat α* (or “B” founders).
594 The presence of these cassettes confers resistance to the antibiotics nourseothricin and
595 hygromycin B, respectively, enabling the selection of doubly resistant diploids. All newly
596 engineered strains are given in Table 1.

597

598 *de novo assembly of high-quality reference genomes for the 18 founding strains*

599 We generated *de novo* genome assemblies for the founders used to create our MPPs
600 using a hybrid sequencing strategy detailed in (Chakraborty *et al.* 2016) that involves
601 using a combination of long-read (PacBio) and short-read (Illumina paired-end)
602 sequencing technology. The *de novo* assemblies allow us to reliably identify structural
603 variants while the overall assembly has a low per base pair error rate. We assembled
604 58.9X PacBio reads on average (33-90X) for 12 of the founder strains, and re-assembled
605 the other six strains using publicly available shorter length 364.9X PacBio reads on
606 average. Despite the different number and chemistry of PacBio reads used in assembling
607 the genomes, all of our assemblies are highly accurate (average $qv = 52.5$) and show
608 comparable contiguity. For example, the average contig N50 of our assemblies is ~800Kb
609 (N50 = 50% of the assembly is contained within sequences of this length or longer),
610 indicating that the majority of the chromosomes are represented as single contigs (Table
611 2). Examination of 290 conserved fungal single copy orthologs (Benchmarking Single
612 Copy Orthologs or BUSCO) show that completeness (~99%) of all our assembled
613 genomes is comparable to the reference S288C assembly (99%).

614

615 We aligned the assemblies to one another and represent them as Santa Cruz genome
616 browser tracks (<http://bit.ly/2ZrreUd>). These tracks have utility when looking for candidate
617 causative variants in small regions of genetic interest. The large amount of genetic
618 diversity sampled by the founders can be illustrated by zooming in on regions such as
619 that shown in Figure 2, which highlights the numerous alleles segregating at a gene
620 implicated in many genetic mapping studies in budding yeast, the highly pleiotropic *MKT1*.
621 *MKT1* influences several cellular processes including the DNA damage response,
622 mitochondrial genome stability, drug resistance, and post-transcriptional regulation of *HO*
623 (Dimitrov *et al.* 2009; Ehrenreich *et al.* 2010; Tkach *et al.* 2012; Kowalec *et al.* 2015a).
624 Studies have found that different alleles of *MKT1* can differentially affect several
625 phenotypes, including mitochondrial genome stability and drug resistance. Variation at
626 this gene amongst our founders includes ten nonsynonymous SNPs and thirty-four
627 synonymous SNPs. Of the ten nonsynonymous SNPs, six are predicted to change the
628 secondary structure of the protein. Taking into account only nonsynonymous SNPs, there
629 are seven different alleles segregating amongst the founders (all segregating in our
630 18F12v2 MPP).

631

632 The genome browser tracks are also useful for visualizing structural variants such as
633 those shown in Figure 3, which highlights a large (>1kb) deletion in the vacuolar ATPase
634 *VMA1* (Figure 3A) present in half of the founders. Previous work has shown that the
635 deleted region encodes a self-splicing intein, PI-SceI, a site-specific homing
636 endonuclease that catalyzes its' own integration into inteinless alleles of *VMA1* during
637 meiosis (Gimble and Thorner 1992). This selfish genetic element has been shown to

638 persist in populations solely through horizontal gene transfer and is present in many
639 species of yeast. Perturbation of *VMA1* itself has been shown to influence both replicative
640 and chronological lifespan, resistance to metals, as well as oxidative stress tolerance
641 (Kane 2007; Ruckenstein *et al.* 2014).

642

643 In addition to large deletions, copy number variants (CNVs) can also be found, such as
644 that shown in Figure 3B, in which the cytoplasmic aldehyde dehydrogenase, *ALD2*, is
645 duplicated in founder A5. Conversely, this gene has been deleted in founders B5 and B8.
646 *ALD2* has been shown to be involved in the osmotic stress response as well as the
647 response to glucose exhaustion (Navarro-Aviño *et al.* 1999). A more structurally complex
648 region was identified on Chromosome VII (Figure 3C) at which multiple different deletions
649 (ranging from ~50bp to >300bp) were found to occur at *MSB2*, an osmosensor involved
650 in the establishment of cell polarity (O'Rourke and Herskowitz 2002; Cullen *et al.* 2004).
651 Null alleles of *MSB2* have been shown to have decreased chemical resistance.

652

653 One of the most structurally complex regions we identify contains ~2kb of repetitive
654 sequence and is present on chromosome IV (Figure 3D and E as well FigureS1) at which
655 multiple different deletions (ranging from ~80bp to >500bp) as well as duplications occur
656 in multiple founders within the *PRM7* and *BSC1* genes. Due to the highly complex nature
657 of the variation present, this region is represented as a series of dot plots, with two
658 founders highlighted in the main text (Figure 3D and E). Dot plots of this region in all
659 founder strains are shown in FigureS1. A previous study demonstrated that although two
660 distinct genes (*PRM7* and *BSC1*) are present in S288C, a combination of small deletions

661 and point mutations in another yeast strain (W303) have caused the STOP codon to be
662 absent from *BSC1*, leading to the read-through transcription of a new gene that
663 encompasses sequence from both *PRM7* and *BSC1* as well as the intergenic region
664 between them (Kowalec *et al.* 2015b). This gene, *IMI1*, was shown to affect mtDNA
665 stability as well as intracellular levels of reduced glutathione (GSH).

666

667 The above regions highlight the utility of our *de novo* genome assembly approach, as
668 deletions and CNVs of this scale would be difficult to detect via the usual method of
669 aligning short reads to a reference genome. But if an investigator mapped a QTL to one
670 of these genes they would certainly want to know about the existence of the segregating
671 structural variation.

672

673 Despite the large amount of natural variation present amongst the founders in general,
674 some of the founders were found to be genetically very similar to one another (AB3/B6)
675 Figure S2 and shown in Table S4), having less than 200 pairwise SNP differences. This
676 lack of divergence makes this set of founders difficult to distinguish from one another for
677 much of the genome and as a result we collapse them for subsequent analyses (despite
678 that fact that a subset of these 200 differences could be functional). Three additional
679 founders (A11/A12/B11) were also found to be highly genetically similar to one another,
680 with, on average, less than 2,000 pairwise SNP differences. These differences are
681 concentrated in a small number of regions, making these three founders distinguishable
682 for these regions (but indistinguishable for much of the remainder of the genome). We
683 keep these strains separate for downstream analyses.

684

685 *Creation of two 18-way highly outcrossed populations*

686 MPPs created using multiple rounds of recombination can significantly increase the
687 resolution of genetic mapping studies by virtue of haplotypes sampled from these
688 population having a greater number of genetic breakpoints. Furthermore, multiple
689 founders results in high levels of standing variation present in the MPP. These two
690 features result in populations that more realistically mimic natural outbred diploid
691 populations, and samples more functional alleles and haplotypes from the species as a
692 whole than a two-way cross. With these goals in mind, we constructed a large, genetically
693 heterogenous population by crossing 18 different founder strains (each strain being
694 derived from the SGRP (Cubillos *et al.* 2009)). The 18 founder strains were chosen to
695 represent a broad swathe of the natural diversity of the species and belong to diverse
696 phylogenies, including: Wine/European, West African, North American, Sake, and
697 Malaysian (see Table 1). It is also noteworthy that founder strains A1-4 and B1-4 are the
698 same four strains using in (Cubillos *et al.* 2013), and were introduced into the population
699 as both *Mat a* and *Mat α* mating types. We created two versions of our 18-way MPP. In
700 both cases a full diallele cross was used to create all 121 unique diploid genotypes from
701 11 *Mat a* and 11 *Mat α* strains (see Figure 1A and B). All 121 diploid genotypes were
702 combined and the resulting population was taken through 12 rounds of sporulation
703 followed by random mating to break up linkage disequilibrium. Previous work has shown
704 that 12 rounds of random recombination breaks up haplotype blocks to the point where
705 additional outcrossing does not significantly decrease LD (Parts *et al.* 2011). For brevity,
706 the two different outcrossed populations will be referred to as 18F12v1 and 18F12v2,

707 respectively, throughout the rest of this manuscript. The version 1 MPP differed primarily
708 from version 2 in that the 121 diploid genotypes obtained from the diallele were directly
709 combined and sporulated *en masse* (version 1; Figure 1A) to create the MPP, as opposed
710 to being individually carried through sporulation and spore disruption before being
711 combined (version 2; Figure 1B). Furthermore, due to a technical artefact during the 12
712 rounds of outcrossing, 18F12v1 is cross-contaminated with the 4-way F12 population
713 from (Cubillos *et al.* 2013) which contains a functional URA3 gene. As a result, 18F12v1
714 MPP is of limited utility for experiments that require uracil auxotrophy, and 18F12v2 is the
715 current primary focus of work in our lab.

716

717

718 *Development of an algorithm for accurately inferring haplotype frequencies*

719 In QTL mapping experiments using MPPs it is often advantageous to map QTLs back to
720 founder haplotypes. In experiments derived from a two way cross between isogenic
721 founders genotyping SNPs accomplishes this, but with multiple founders parental
722 haplotypes have to be inferred in recombinant offspring (Mott *et al.* 2000). In a similar
723 manner when MPPs are used as a base population and genetic changes detected
724 following an experimental treatment it is often of value to examine changes in haplotype
725 frequency (as done in (Burke *et al.* 2014) and reviewed in (Barghi and Schlötterer 2019)).
726 We developed a sliding window haplotype caller that can be used in the situation when
727 the founder haplotypes are known and apply it to both single haploid clones and pools
728 consisting of millions of diploid individuals. This haplotype caller differs from other widely
729 used callers (Long *et al.* 2011; Kessner *et al.* 2013) in that it acknowledges that in some

730 windows pairs of founders are poorly resolved or indistinguishable and relies solely on
731 read counts at known SNP positions in both founders and recombinant populations.

732

733 To benchmark the haplotype-calling algorithm, we compared the frequency of SNPs
734 private to a single founder to the haplotype frequency of the same founder for the interval
735 closest to the SNP location in the 18F12v2 base population. Since this base population
736 is sequenced to 2226X we initially wished to look at the error in the haplotype estimate at
737 full coverage where the sampling variation on the SNP frequency estimate is quite low
738 (proportional to $1/\sqrt{2226}$ or $<2\%$). For the high coverage base population regions
739 showing large difference between SNP and haplotype frequency estimates likely
740 represent instances where the haplotype caller breaks down, since we attempted to
741 remove SNPs whose frequencies are poorly estimated. Figure S3, depicting the absolute
742 difference in SNP versus haplotype frequency differences, shows that haplotype and SNP
743 frequencies generally agree with one another with average and median error rates of
744 0.4% and 0.2%, respectively (below the sampling error of SNP frequency).

745

746 Of course, typical experiments employing these base populations will sample the
747 population following some treatment, and comparing haplotype frequencies in control
748 versus treated samples. Although the 18F12v2 base population is sequenced to 2226X,
749 it would be cost-effective if we could infer haplotype frequencies from pooled samples
750 sequenced to much lower coverage. To determine the accuracy of our haplotype
751 estimates as a function of sequencing coverage the 18F12v2 was down-sampled 50-fold
752 and 100-fold, which corresponds to poolseq datasets of $\sim 40X$ and $\sim 20X$ respectively. We

753 then estimated relative haplotype frequency error rates as a function of sequence
754 coverage (Figure S3b) and absolute error rates as a function of coverage and genomic
755 location (Figure S4). It is apparent that the error rate is an increasing function of
756 decreasing coverage, but for much of the genome the absolute error in haplotype
757 frequency estimate is actually lower than the binomial sampling errors associated with
758 directly estimating SNP frequencies at the same coverage (*i.e.*, at 20-40X coverage
759 binomial sampling errors on frequency are >10%). It is also apparent that the average
760 error rate is likely driven by a few regions where the haplotype caller struggles; these are
761 presumably regions with poor divergence between founders in the window examined.
762 Overall the mean (median) error rates on haplotype frequency estimates are low, 1.3%
763 (0.8%) at 20X and 1% (0.6%) at 40X, respectively.

764

765

766 **Table 3.** Mean haplotype frequencies in 18F12v1 and 18F12v2

Founder	18F12v1_frequency	18F12v2_frequency
AB1	4.4%	1.2%
AB2	3.8%	0.5%
AB3	10.6%	41.4%
AB4	5.8%	3.3%
A5	1.2%	14.0%
A6	0.8%	14.3%
A7	2.4%	0.4%
A8	1.2%	0.7%
A9	0.1%	0.5%
A11	9.9%	1.2%
A12	18.1%	1.7%
B5	27.1%	11.3%
B7	1.1%	1.0%
B8	0.5%	5.7%
B9	0.9%	0.8%

B11	9.8%	1.3%
B12	2.4%	0.6%

767

768 *Characterization of 18F12v1 and 18F12v2 base populations*

769 18F12v1 and 18F12v2 were subjected to high coverage whole-genome sequencing to
770 both characterize their population structure and to establish a baseline for future mapping
771 studies. Figures 5 and 6 show the inferred sliding window haplotype frequencies for
772 18F12v1 and 18F12v2, respectively, while Table 3 shows the mean per founder
773 haplotype frequencies genome-wide. One trend that is evident is that in both the 18F12v1
774 and 18F12v2 MPPs a small number of founders are over-represented. In order to identify
775 the origin of this bias, at least for 18F12v2, the first two meiotic generations of 18F12v2
776 were sequenced (Figures S5 and S6; Table S5). Despite having an initially more balanced
777 population after the first round of random mating, a few strains quickly became
778 disproportionately over-represented. One possible explanation for this is that a few
779 founding haplotypes were selected for early in the twelve rounds of intercrossing. Figure
780 S7 provides suggestive evidence that this may have been the case, as the frequency of
781 haplotypes derived from founder A5 increase genome-wide after the second round of
782 meiosis. The latter half of chromosome XIII (from founder A5) emphasizes this point as it
783 was very highly selected for initially. Another potential source of bias was the pooling
784 strategy, which was done using optical density as a proxy for cell numbers. This may have
785 resulted in an uneven distribution of founders in the initial pool. Nonetheless, after 12
786 rounds of random mating, deep sequencing of 18F12v2 revealed that haplotypes from all
787 founding strains were present in the population at a detectable frequency (Table 3).
788 Specifically, haplotypes from ten or more founders were detected as segregating in over

789 99% of the genome in 18F12v2 and close to 98% of the genome in 18F12v1.
790 Furthermore, 18F12v2 was verified to be auxotrophic for uracil, facilitating future
791 manipulations for downstream analyses.

792

793 *Characterizing the recombination landscape of 18F12v1- and 18F12v2-derived*
794 *segregants*

795 In order to further characterize 18F12v1 and 18F12v2, ten haploid segregants were
796 generated from each diploid population and subjected to whole-genome sequencing. The
797 complex structure of these populations is highlighted in Figure 7. The mean (median) size
798 of haplotype blocks in 18F12v1-generated segregants was 103kb (66kb) while the mean
799 (median) size of haplotype blocks in 18F12v2-generated segregants was 106kb (66kb)
800 (Figure S8). The mean number of discrete haplotype blocks in 18F12v1-generated
801 segregants was 106 as compared to 104 in 18F12v2-generated segregants. A previous
802 study (Cubillos *et al.* 2013) found that twelve rounds of meiosis in a yeast 4-way cross
803 resulted in a median block size of 23kb with 374 discrete haplotype blocks. Some of the
804 failure to obtain the smaller block sizes and more numerous discrete blocks of this
805 previous study may be due to undetectable recombination events occurring within
806 haplotypes over-represented in our populations. Another possibility is that, due to the
807 large number of founding haplotypes, recombination events were missed in regions at
808 which multiple founding strains are highly genetically similar. It is also possible that some
809 of the founding strains used in this study have relatively low natural recombination rates.

810

811 To highlight the diversity present in the two outbred populations, a close-up view of
812 inferred haplotypes in segregants derived from each population at chromosome X is
813 shown in Figure S9. Regions in which the founding haplotype is unknown tended to occur
814 at the transitions between haplotypes (see Note S4) and are a mean (median) length of
815 7.8kb (6kb) in 18F12v1-derived segregants and 7.5kb (6kb) in 18F12v2-derived
816 segregants. Also noticeable is, at least for this chromosome, the larger amount of
817 variation segregating in 18F12v2 (Figure S9B).

818

819 **Conclusion**

820 The paradigm of utilizing pairwise crosses to dissect the genetic basis of complex traits
821 has enjoyed much success in diverse model organisms. However, such studies typically
822 underestimate the standing variation present in natural populations and often lack the
823 resolution to pin-point causal variants to a small number of genes. Conversely,
824 association studies are typically underpowered to detect rare alleles, poorly tagged
825 variants, and regions with multiple causal sites in weak LD with one another. MPPs have
826 been proposed to bridge the gap between the above two approaches. Although MPPs
827 have been created in several model systems, only a single MPP has thus far been
828 described in budding yeast. By generating two large, highly outcrossed and genetically
829 heterogeneous populations of *S. cerevisiae* derived from eighteen different founder
830 strains, we have created a powerful resource that can be used in a variety of experimental
831 settings. For instance, these populations can be used in large-scale X-QTL mapping
832 experiments (Ehrenreich *et al.* 2010) to comprehensively dissect the genetic architecture
833 of complex traits as well as large-scale evolve and re-sequence experiments (Lang *et al.*

834 2011; Parts *et al.* 2011; Burke *et al.* 2014) to determine the mechanisms and course of
835 adaptation to diverse stimuli. Large number of recombinant haploid clones generated
836 from these populations can be used in complementary large-scale I-QTL studies (Bloom
837 *et al.* 2013; Wilkening *et al.* 2014). Due to the high levels of standing variation present,
838 these populations should also prove to be a powerful resource in evolutionary engineering
839 applications, as they are presumably capable of being evolved to carry out a plethora of
840 useful tasks.

841

842 The haplotype calling software generated in this study represents a useful resource for
843 the MPP community in general, as it enables highly accurate haplotype calling in poolseq
844 data at reduced coverage. The ability of the algorithm to deal with windows where all
845 founder haplotypes cannot be resolved will have utility in a subset of systems, including
846 our yeast populations. Candidate causal regions can be identified by comparing
847 haplotype frequencies at discrete intervals across the genome in control versus treatment
848 populations. Candidate regions can then be examined in the UCSC genome browser,
849 where genome-wide alignments of all founder strains have been posted. Structural
850 variants can be easily visualized in the browser as can nonsynonymous SNPs, thus
851 pointing investigators to potentially causal genes.

852

853 In conclusion, the populations generated in this study represent a novel resource that
854 brings together the power of QTL mapping, the resolution of association studies, and a
855 large amount of natural variation to a model system capable of teasing apart and directly
856 testing the molecular underpinnings of complex traits.

857

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863

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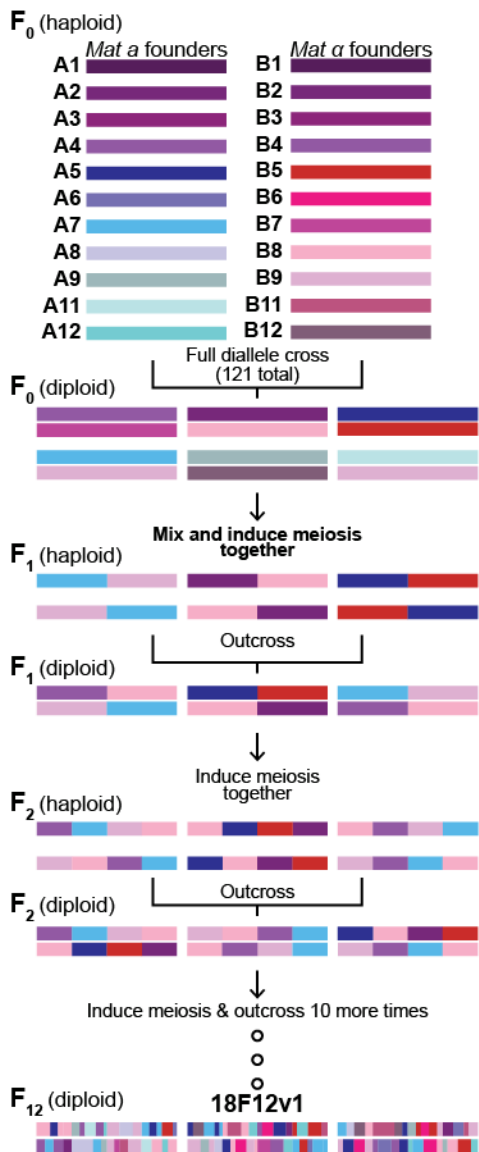
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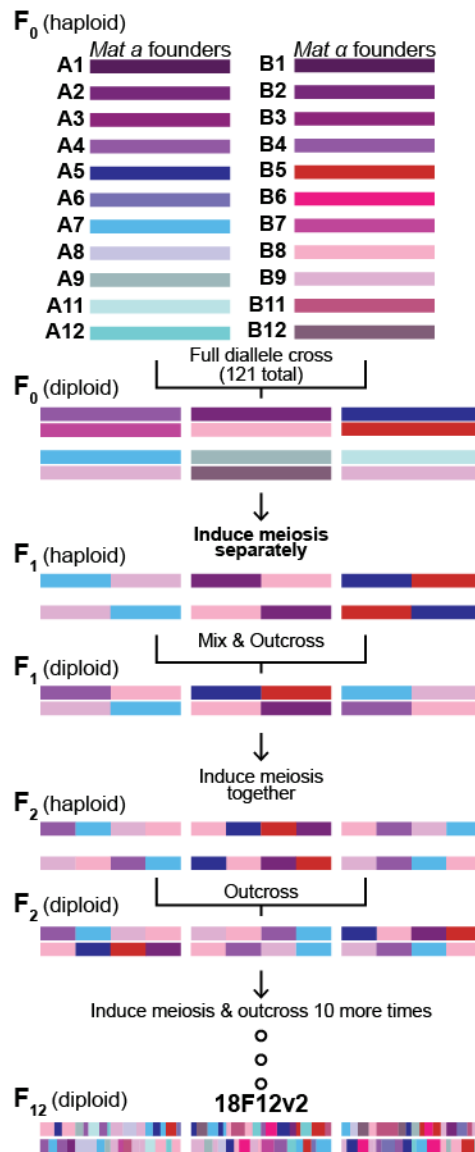
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1065 **Figure 1.** Schematic of the outcrossing process used to make the two 18F12 diploid
 1066 populations. Both populations were established by a full diallele cross of all 22 isogenic
 1067 haploid founder strains. A1/B1, A2/B2, A3/B3, and A4/B4 are different mating types of
 1068 the same strains and are the same strains used in (Cubillos *et al.* 2013). In **(A)**, all
 1069 pairwise crosses were mixed before the first round of sporulation. This is in contrast to
 1070 **(B)**, in which mixing did not occur until after an initial sporulation event. In both cases,

1071 mixed populations were taken through additional rounds of sporulation and random
1072 mating for a total of 12 meiotic generations.

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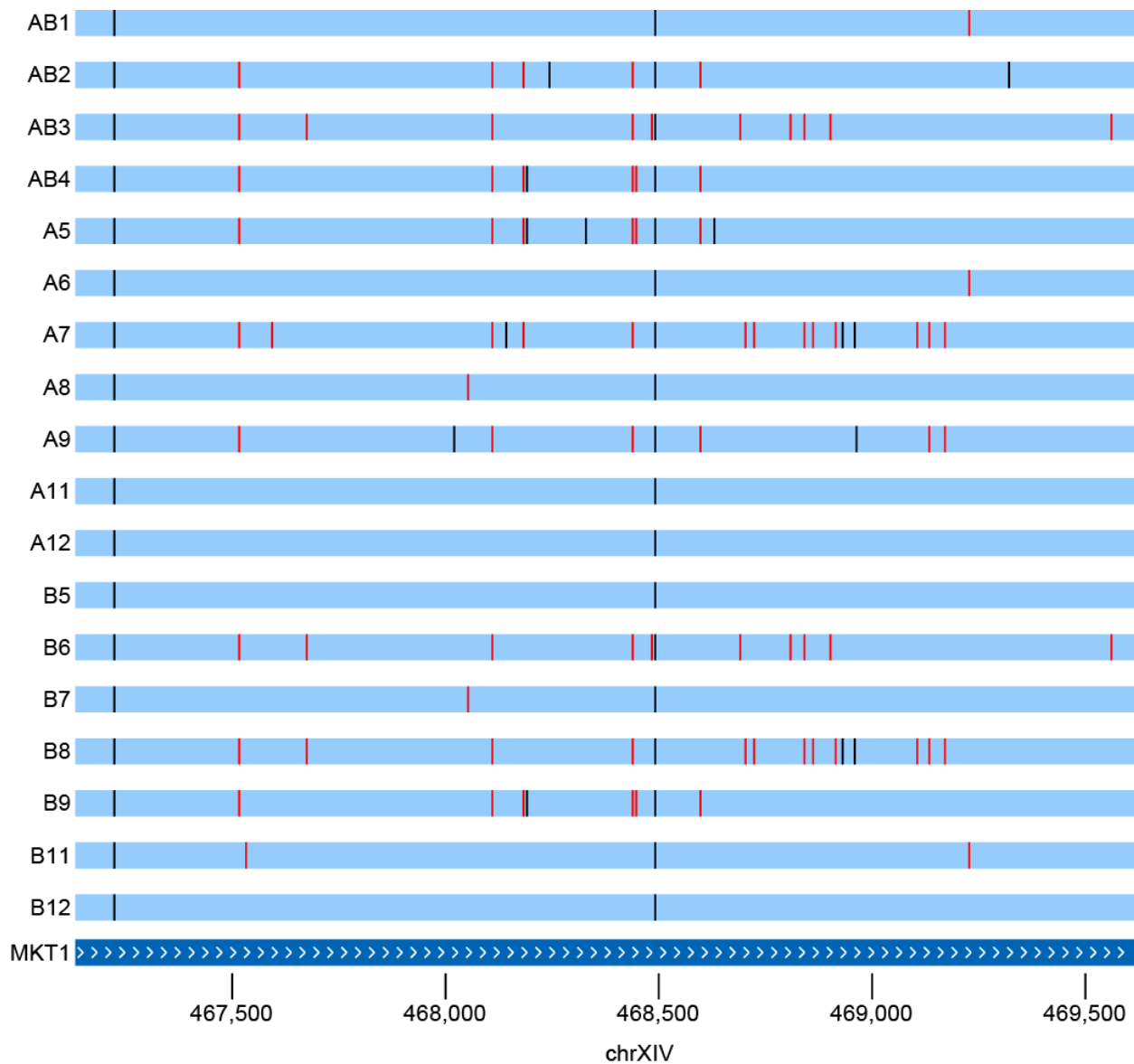
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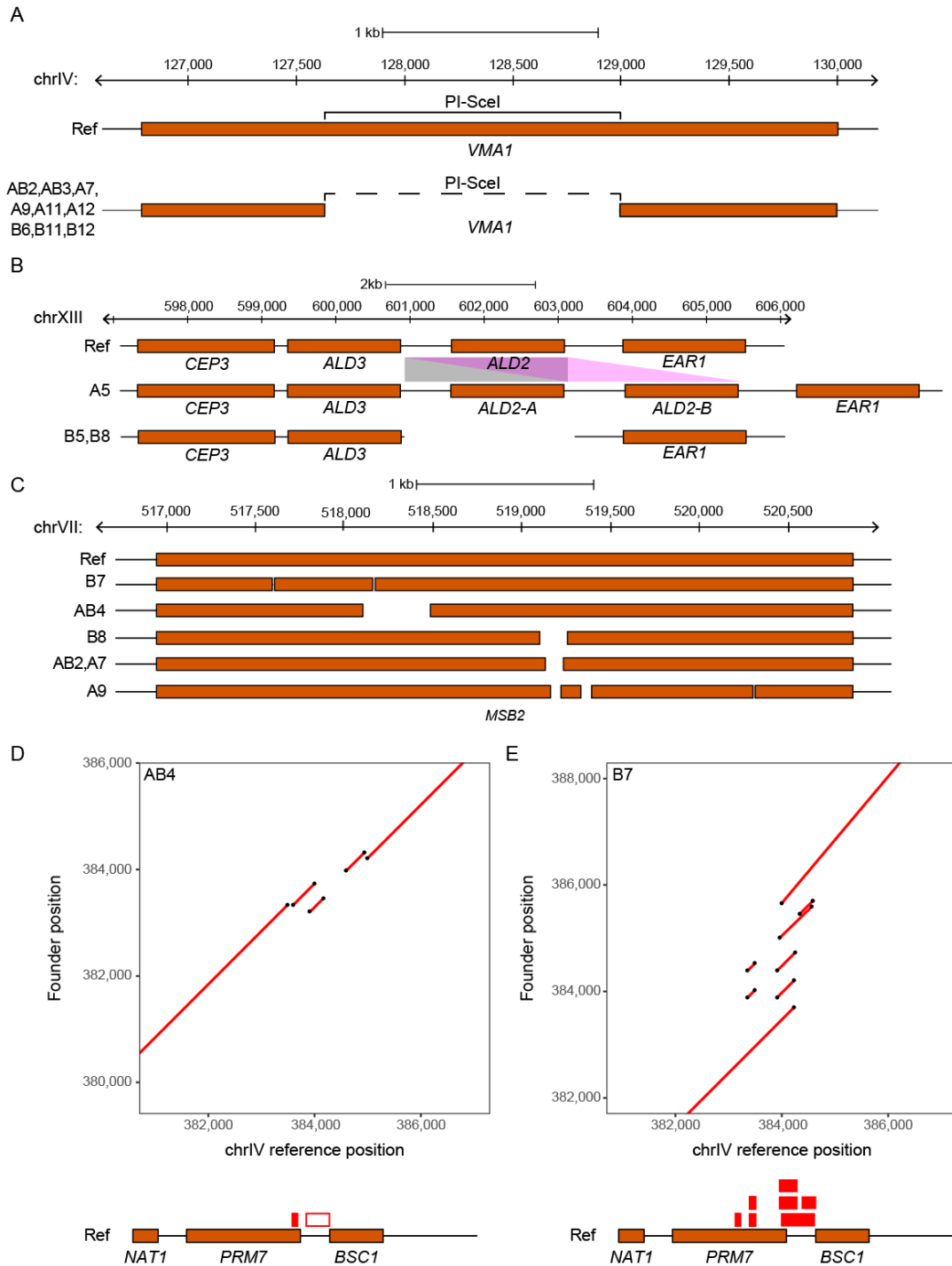
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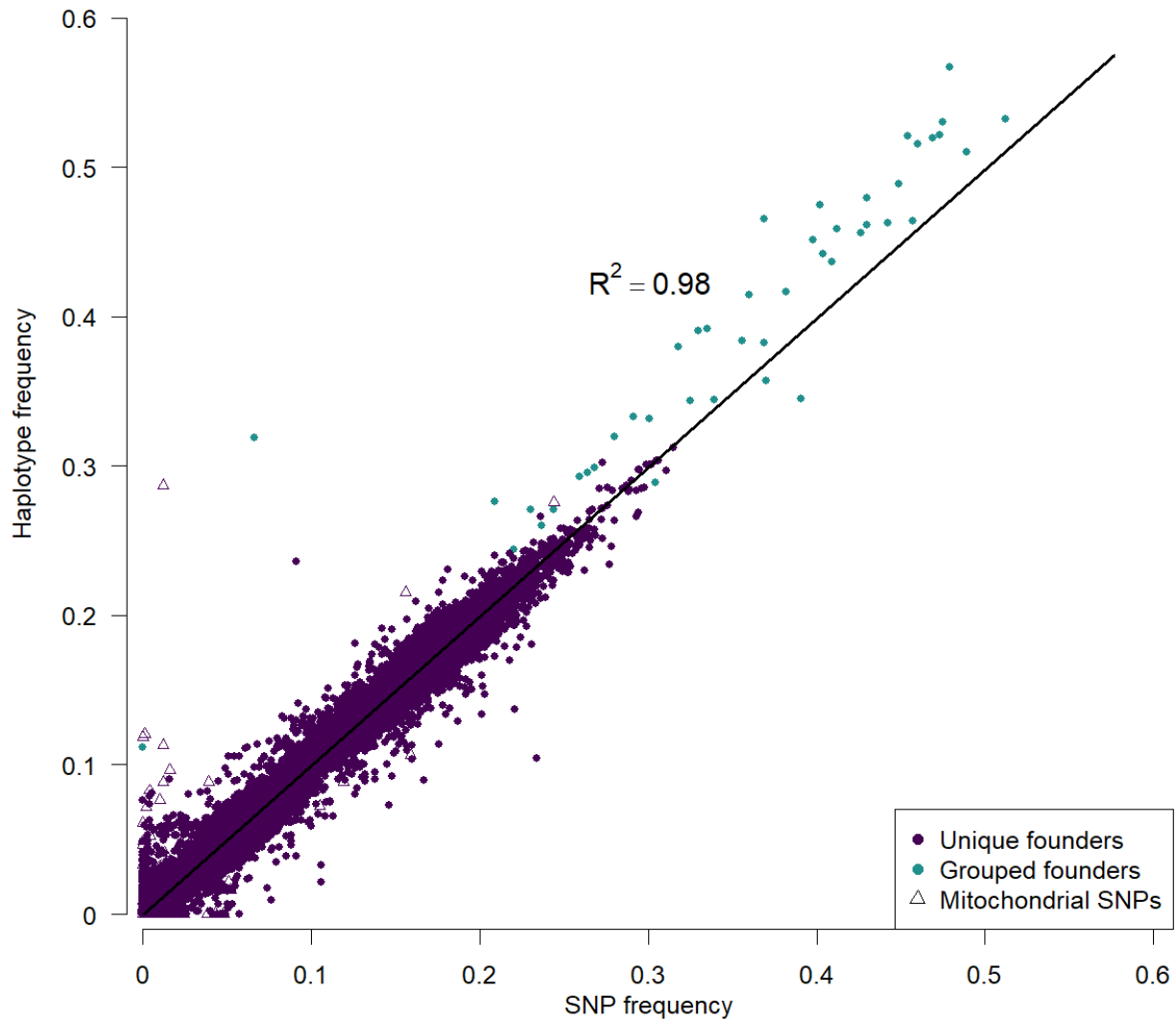
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1081 **Figure 2.** Many alleles of the highly pleiotropic *MKT1* gene are segregating amongst the
1082 founder strains, highlighting the potential of uncovering complex allelic series using
1083 populations derived from these strains. Seven of these alleles are differentiated by
1084 nonsynonymous SNPs, of which six are predicted to be segregating in 18F12v2.
1085 Vertical red lines are synonymous SNP differences from the reference S288C strain,
1086 and black bars nonsynonymous SNPs.
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1089 **Figure 3.** Combining contiguous long-read sequencing with accurate short-read data
1090 enables the detection of structural variants such as those depicted above. In **(A)**, a large
1091 (>1kb) deletion within a vacuolar ATPase (*VMA1*) is present in half of the strains used in
1092 this study. This deletion directly overlaps the self-splicing intein, PI-SceI. Copy number

1093 variants of *ALD2*, an aldehyde dehydrogenase, were detected (**B**) and include a
1094 duplication of this gene in founder A5 (represented as *ALD2-A* and *ALD2-B*) as well as
1095 its' deletion in founders B5 and B8. In (**C**), multiple deletions of different lengths in the
1096 osmosensor *MSB2* were detected in multiple founder strains. Dotplots of a structurally
1097 complex region on Chromosome IV are shown for founders AB4 (**D**) and B7 (**E**). These
1098 plots show alignments of regions from the founder strains (depicted on the y axis) with
1099 the corresponding region from the S288C reference strain (depicted on the x axis). The
1100 red boxes present above the genes in the reference strain map duplications (solid
1101 boxes) and deletions (empty boxes) detected in each founder strain to the
1102 corresponding reference sequence. In all panels, the reference strain S288C (aka 'Ref')
1103 is used to highlight the various arrangements of SVs present in the founder strains.
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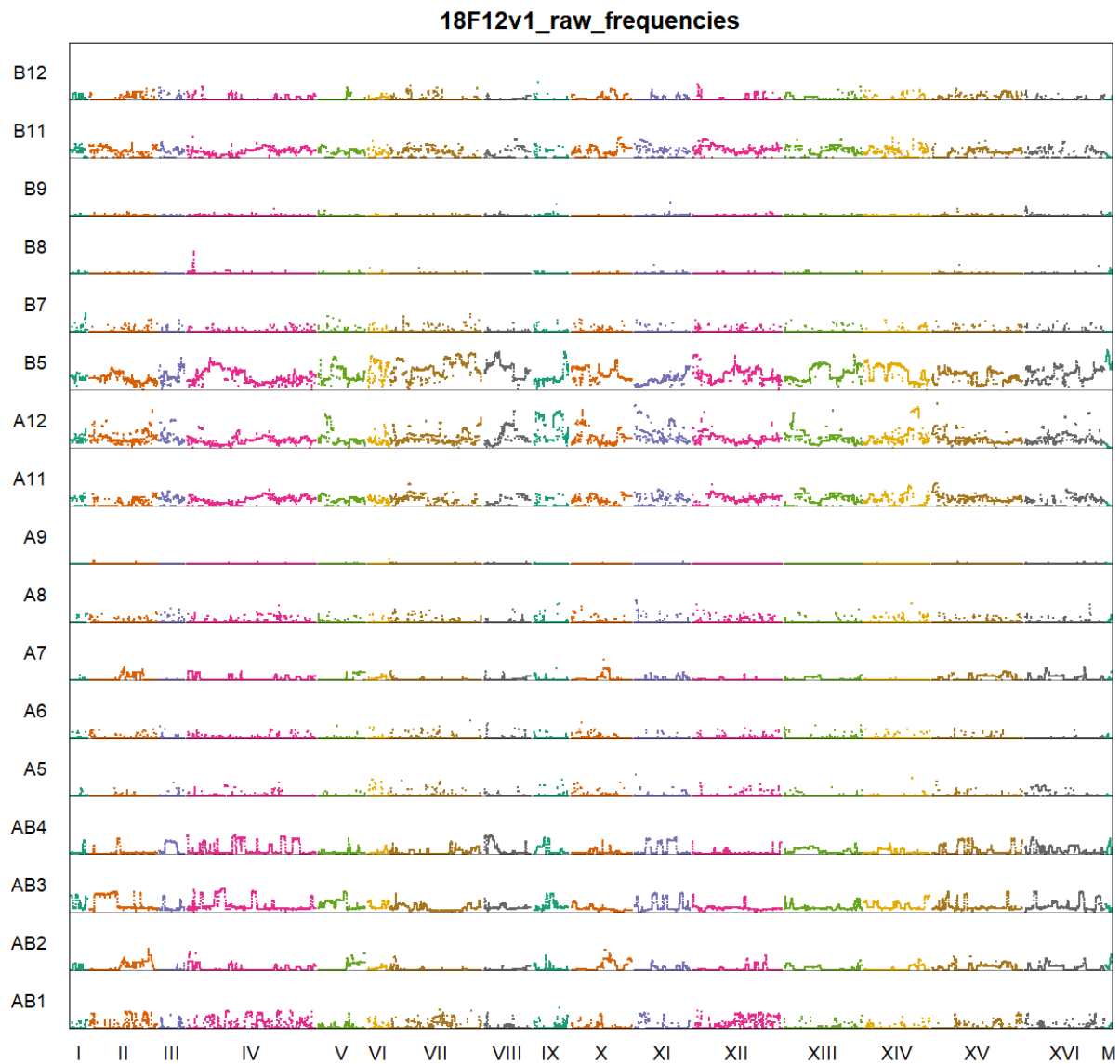
1107 **Figure 4.** The frequency of SNPs private to a single founder are highly correlated with
1108 the estimated haplotype frequencies at these SNPs in 18F12v2. As the frequency of a
1109 private SNP should be equal to the corresponding haplotype frequency, this measure
1110 provides a benchmark with which the accuracy of our haplotype caller can be
1111 measured. Cyan points represent founders that were pooled when estimating haplotype
1112 frequencies (“grouped founder”) due to the high degree of sequence similarity between

1113 their genomes. Triangles represent mitochondrial SNPs, which, together with SNPs
1114 private to pooled founders, represent the bulk of the major outliers. The coefficient of
1115 determination was calculated by regressing haplotype frequency onto SNP frequency,
1116 excluding SNPs from grouped founders and mitochondrial SNPs.

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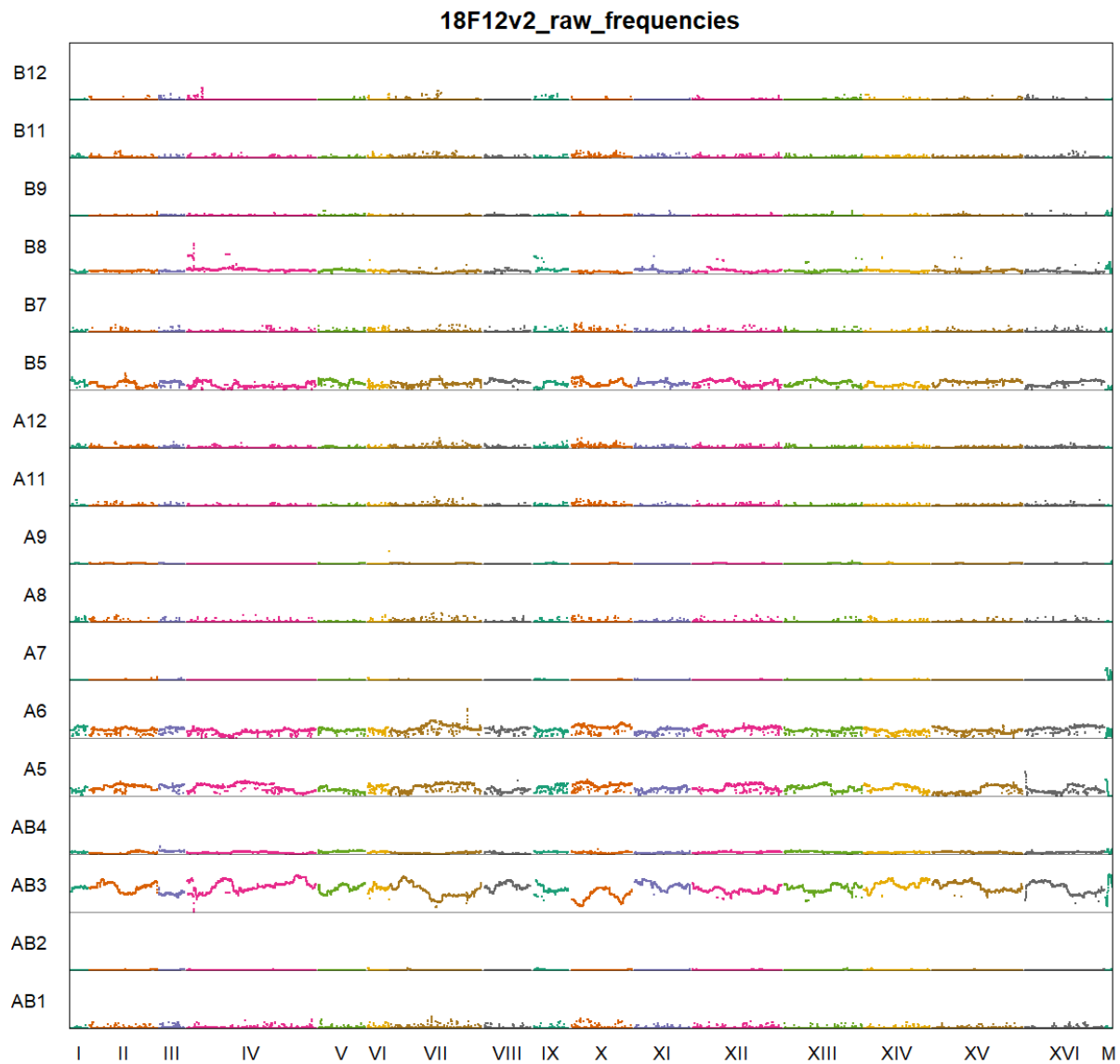
1121 **Figure 5.** Genome-wide haplotype frequencies for 18F12v1.

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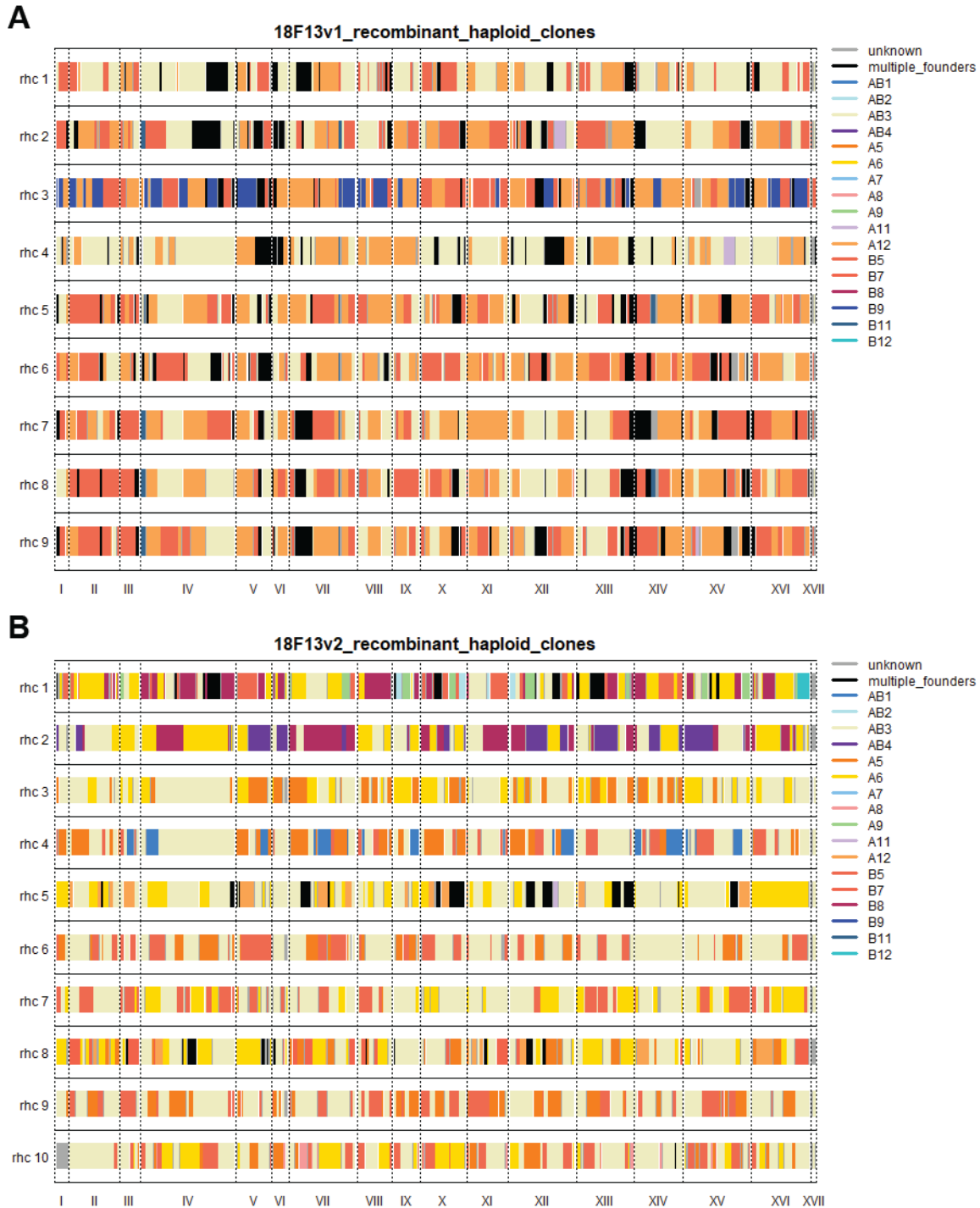
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1127 **Figure 6.** Genome-wide haplotype frequencies for 18F12v2.



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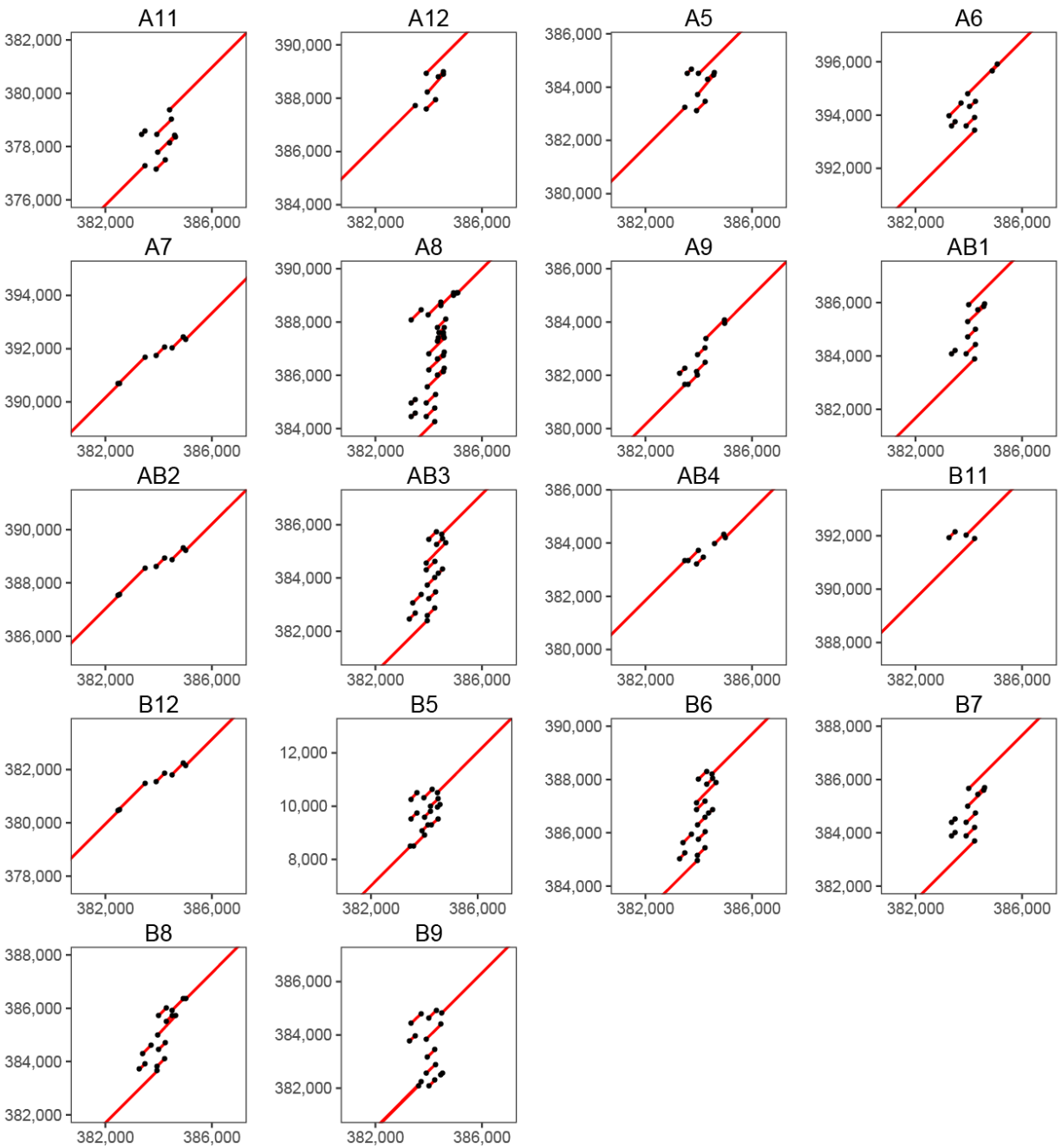
1129 **Figure 7.** Haploids derived from 18F12v1 (**A**) and 18F12v2 (**B**) were isolated and

1130 sequenced, providing a glimpse into the recombinogenic landscape and haplotype

1131 diversity present within these populations.

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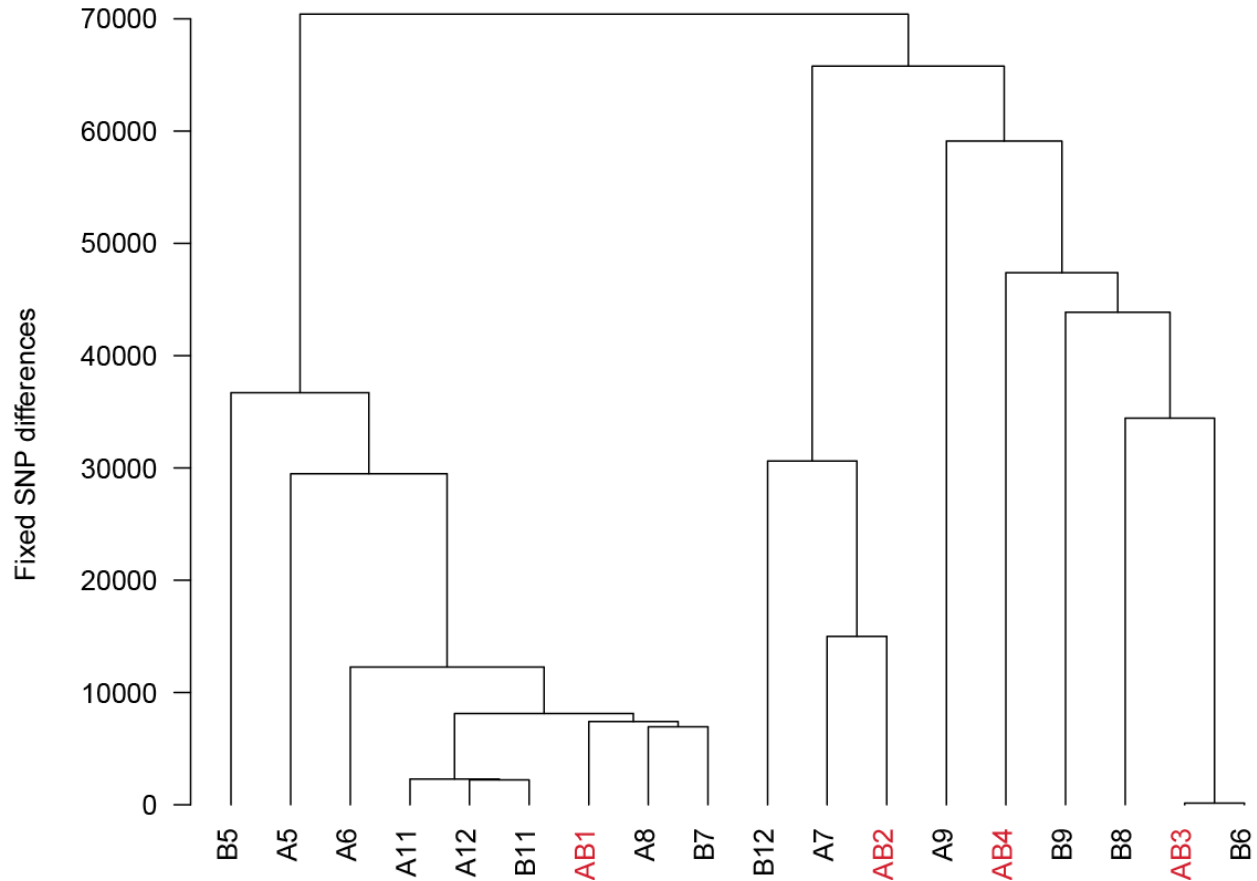
Supplementary Information



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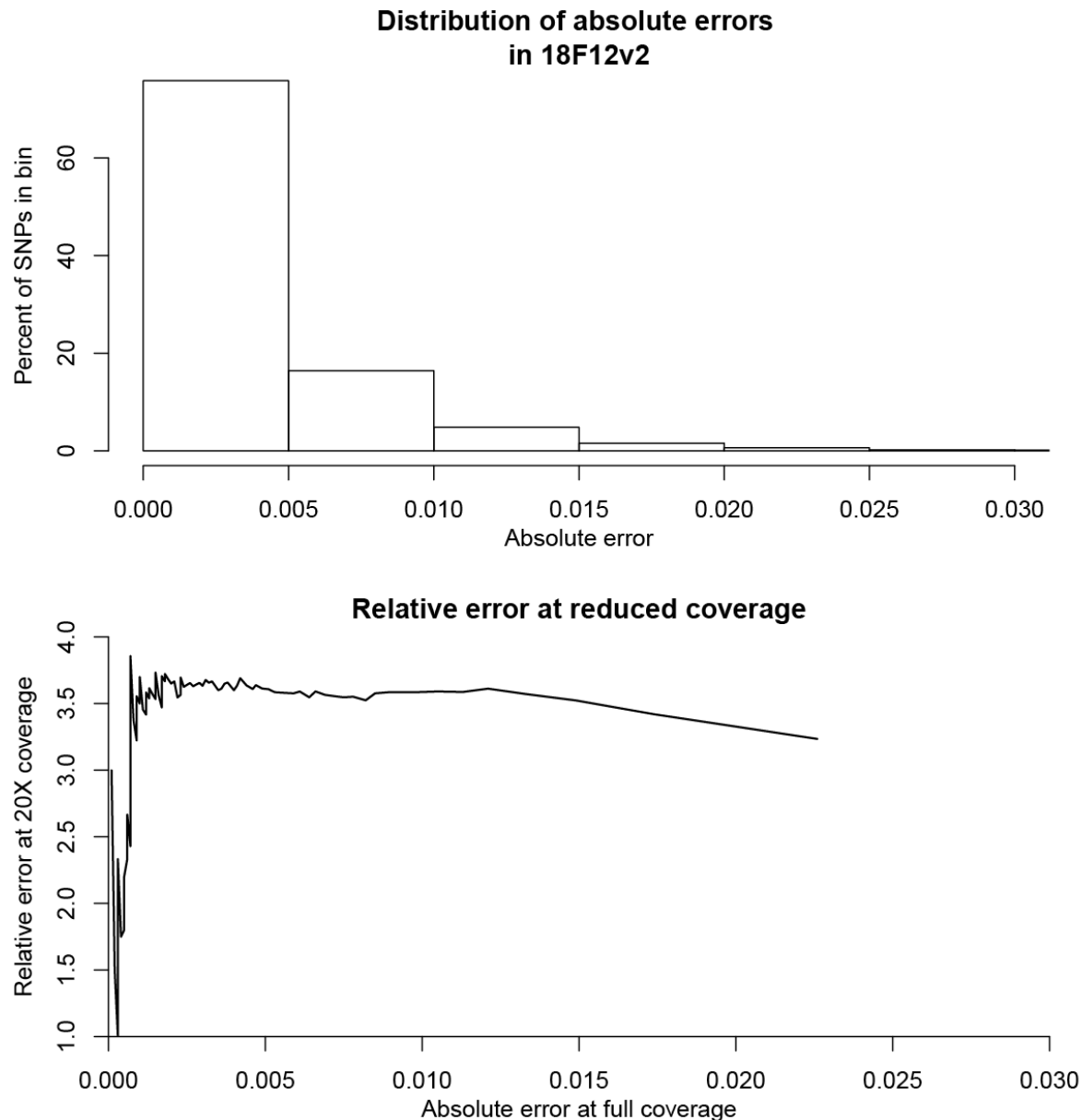
Figure S1. Dot plots showing the numerous structural variants present at this particular site on chromosome IV. Each dot plot was constructed by plotting the sequence alignments of regions from each founder strain (y-axis) against the S288C reference strain (x-axis).

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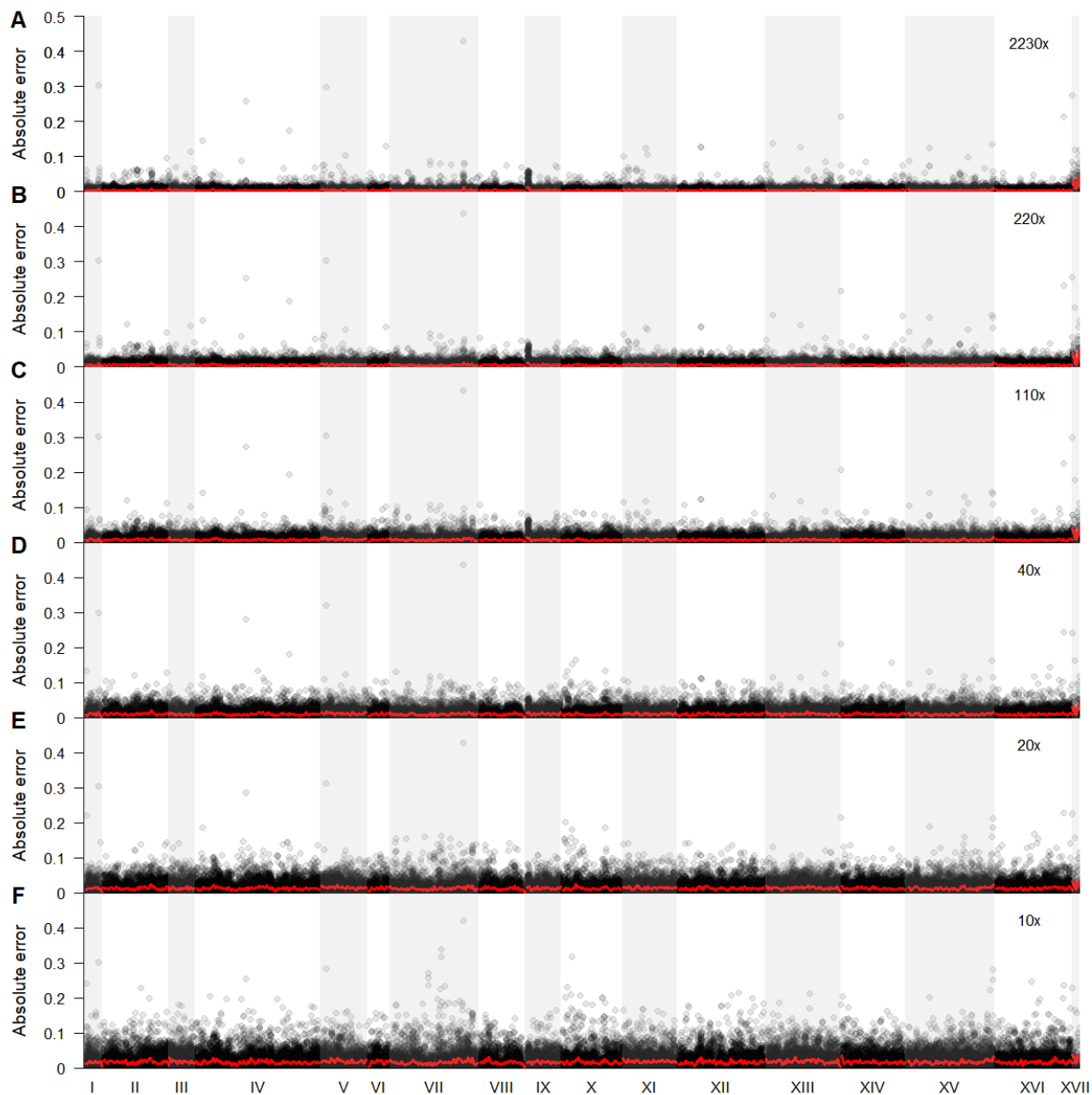


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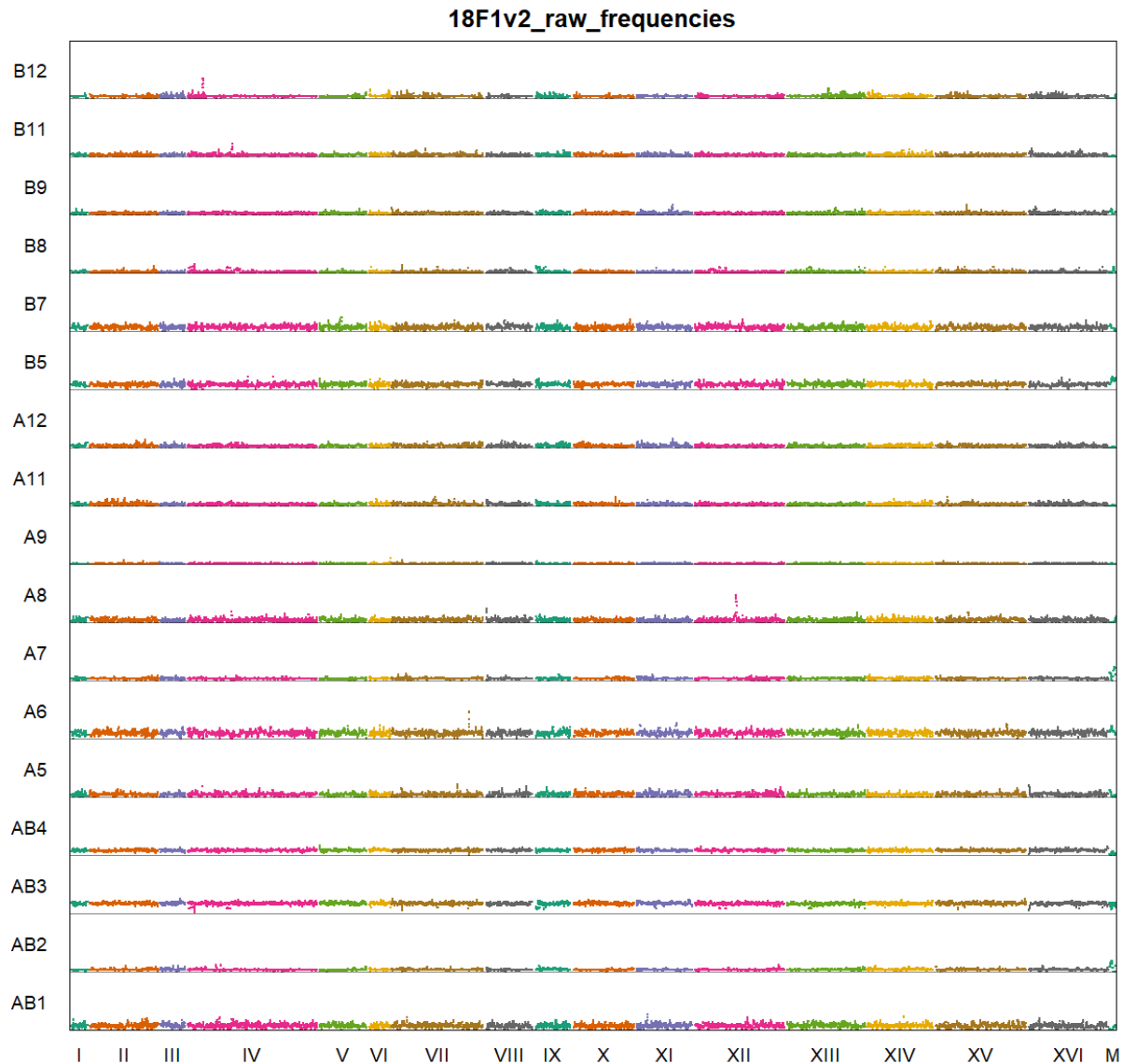
Figure S2. Dendrogram showing the degree of relatedness between the 18 founder strains used in this study. This was constructed in *R* using the *hclust* function on a distance matrix representing the number of differences between each pair of founders. Strains colored in red are the founders from the four-way cross described in Cubillos *et al.* and used in (Cubillos *et al.* 2013, 2017).



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1165 **Figure S3.** As a measure of the accuracy of the haplotype calling algorithm, we
1166 calculated the absolute error between haplotype calls at sites harboring private SNPs
1167 and the SNP frequencies in 18F12v2 estimated from 2230X coverage (A). The vast
1168 majority of sites tested in this manner showed an error rate of less than one percent.
1169 We also computed the relative error following downsampling the 18F12v2 data to
1170 approximately 20x coverage as a function of the absolute error at full coverage (B). At
1171 20X coverage haplotype frequencies are estimated much more accurately than SNP
1172 frequencies could be directly estimated at 20X coverage based on binomial sampling.
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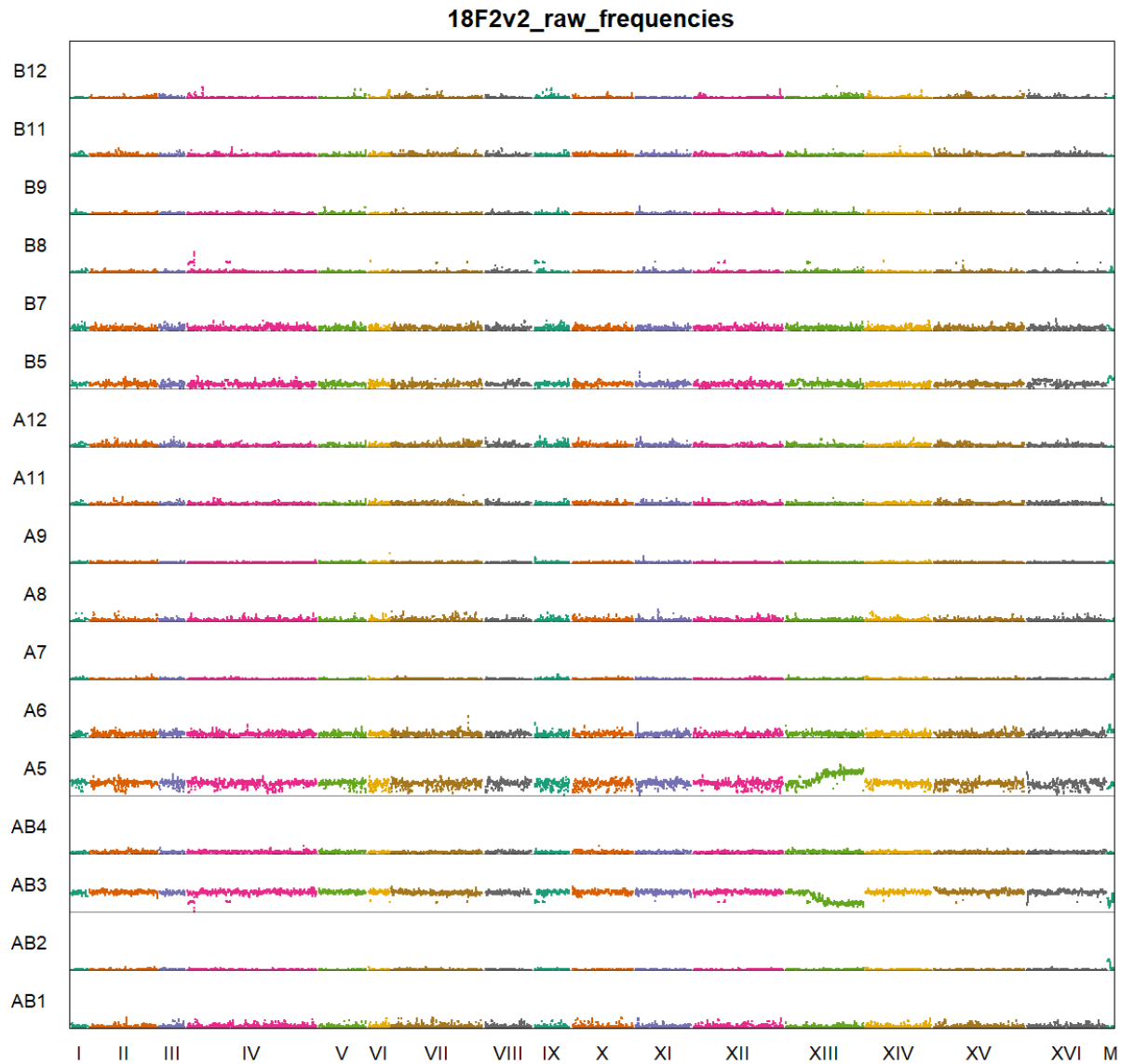


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1178 **Figure S4.** Shown is the absolute error rate between haplotype and SNP frequencies at
1179 sites with private SNPs for 18F12v2. Panels A-F represent 100%, 10%, 5%, 2%, 1%,
1180 and 0.5% of the full dataset, which approximately corresponds to the coverages shown
1181 in the upper right of each panel. The red line represents a kernel regression run using
1182 the `ksmooth()` function in R with kernel set at 'normal' and bandwidth set at 20000,
1183 approximating the local expected error rate. Despite clear outliers (that occur
1184 irrespective of coverage), haplotype frequency estimates are accurate on average.
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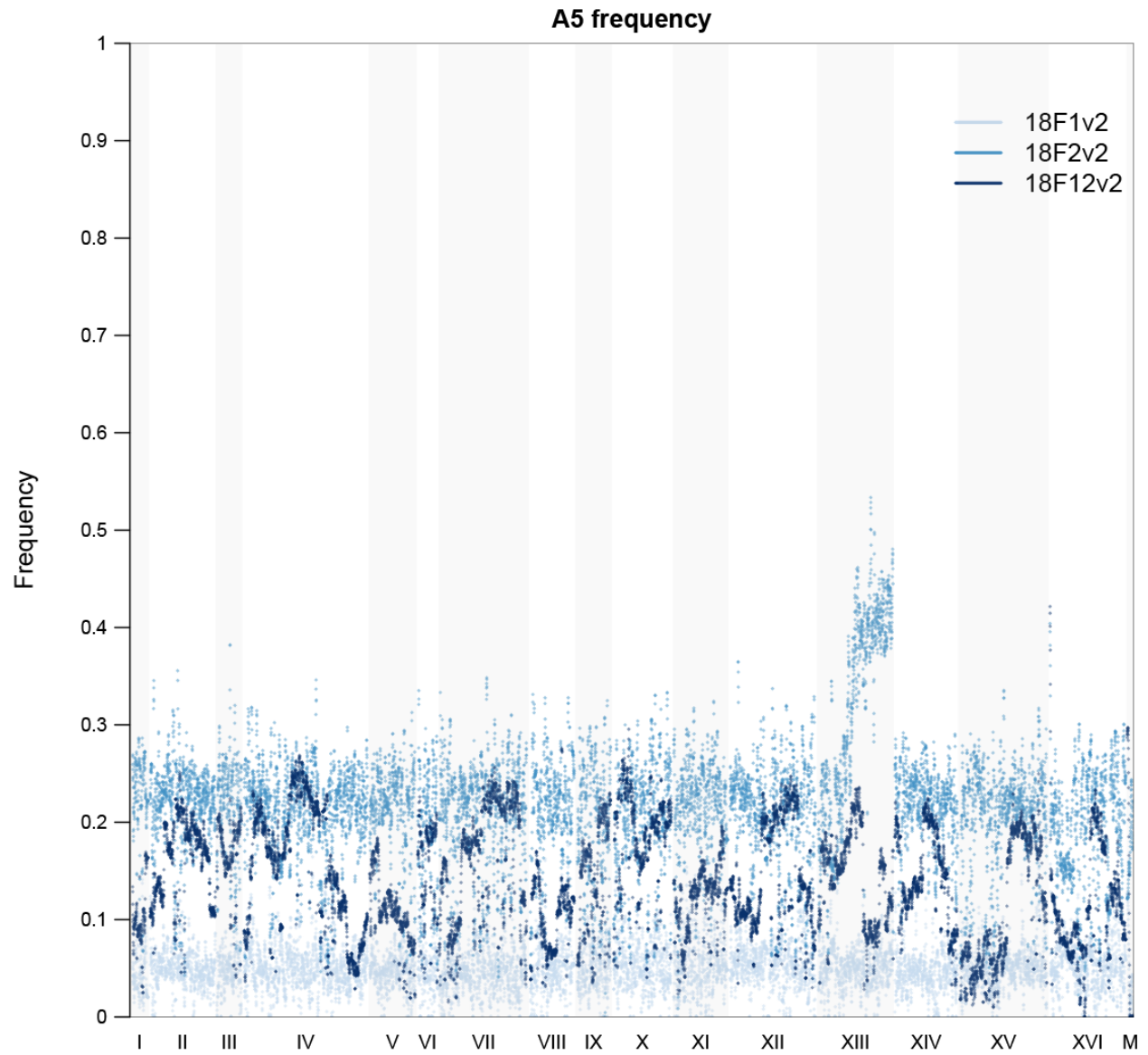
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Figure S5. Haplotype frequencies genome-wide for 18F1v2. This sample was taken after the first meiotic generation of version two of the outcrossed population and shows a more balanced distribution of founder haplotypes than the final generation. Haplotype calls for founder B6 were merged with founder AB3, while founders A12 and B11 were merged with founder A11 due their high sequence similarity.



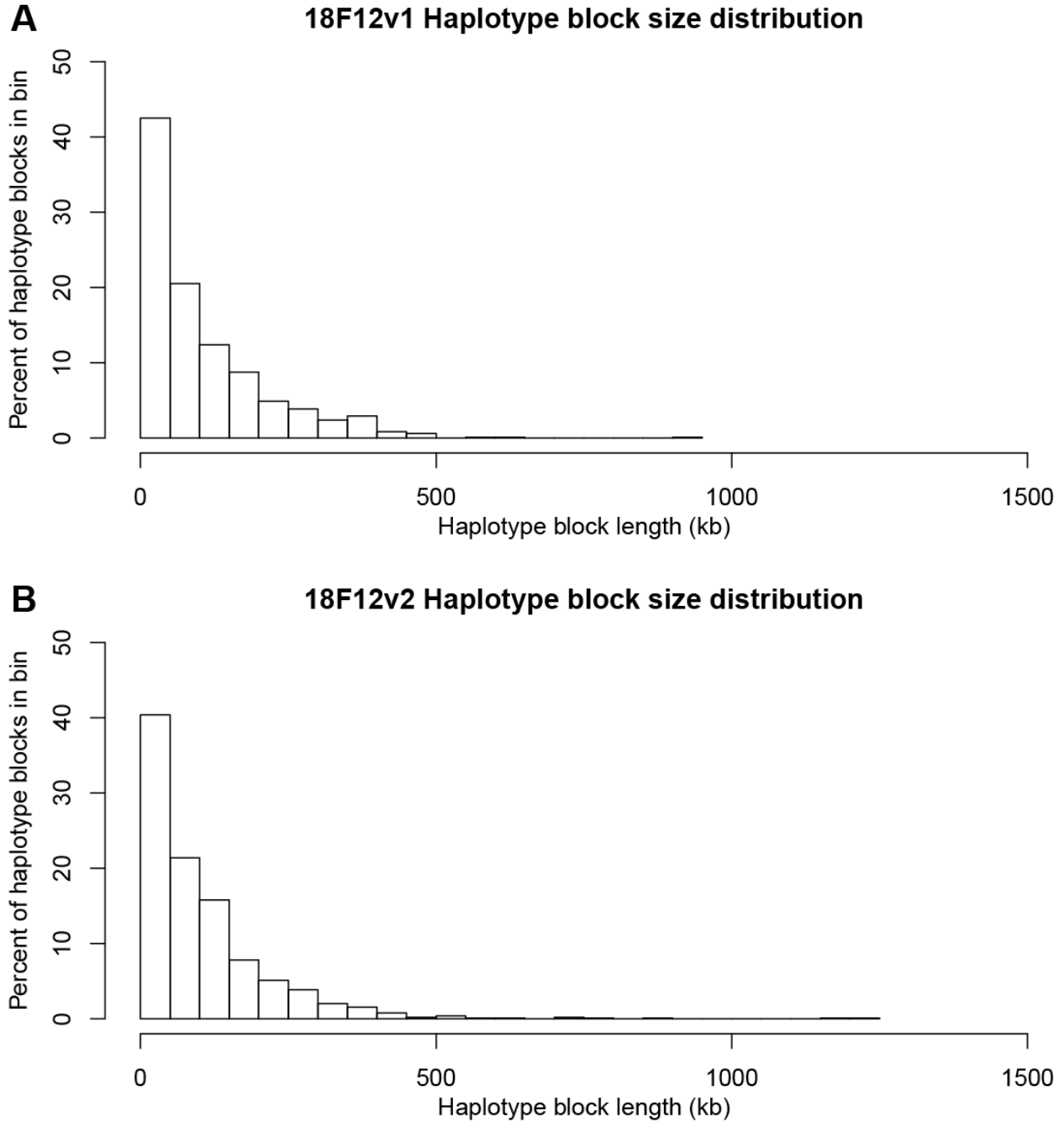
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Figure S6. Haplotype frequencies genome-wide for 18F2v2. This sample was taken after the second meiotic generation of version two of the outcrossed population and shows founders AB3 and A5 increasing significantly in frequency as compared to the remaining founders. As above, haplotype calls for founder B6 were merged with founder AB3, while founders A12 and B11 were merged with founder A11.



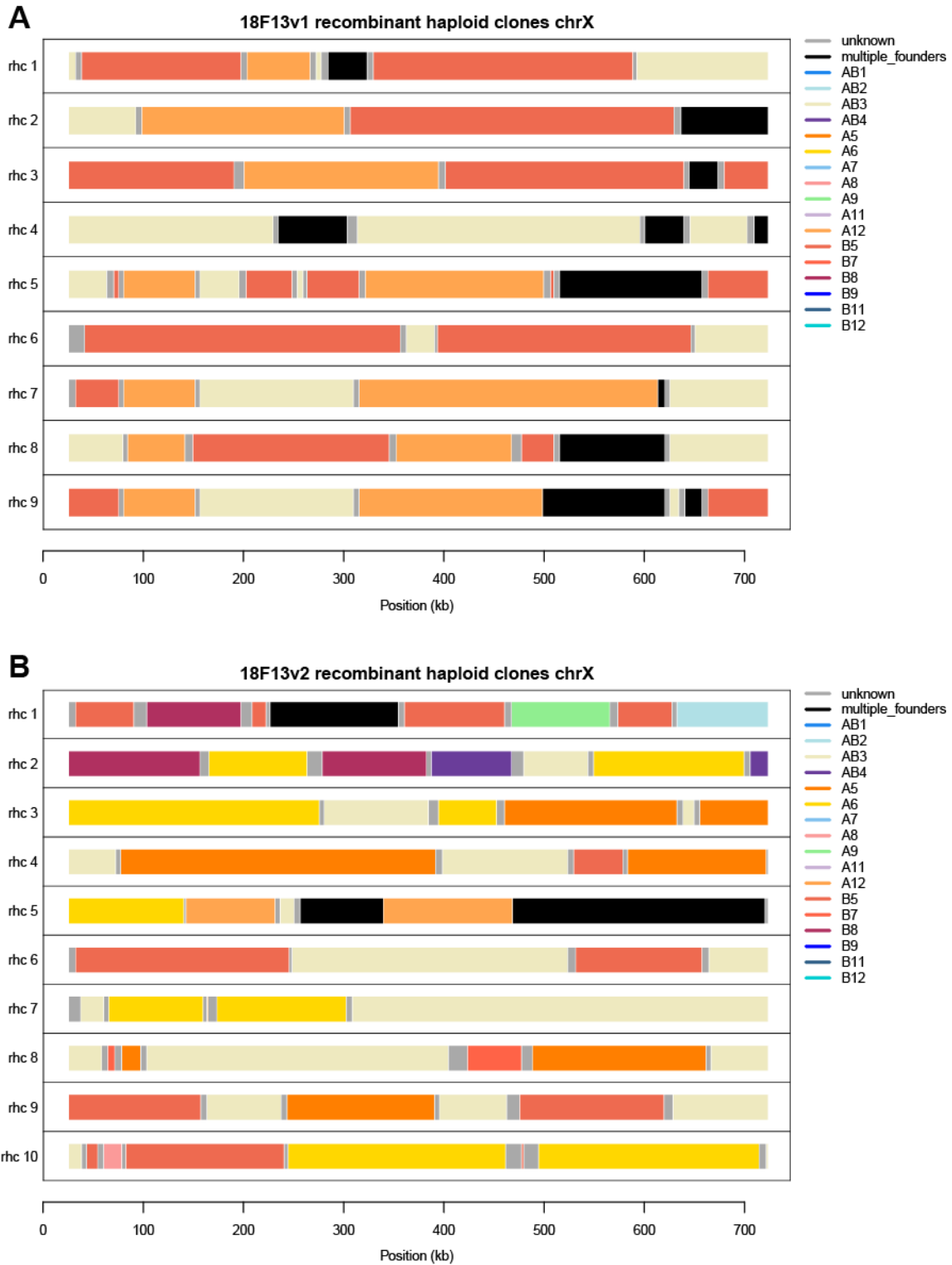
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Figure S7. Haplotype frequency genome-wide for the A5 founding strain across the first (18F1v2), second (18F2v2), and twelfth (18F12v2) meiotic generations of the version 2 outcrossed population.



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Figure S8. Distribution of haplotype block lengths in recombinant haploid clones derived from 18F12v1 (**A**) and 18F12v2 (**B**). All inferred haplotype blocks, including those called as ties between multiple founding strains, were included in this analysis.



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Figure S9. Haplotype diversity at chromosome XII present in 18F13v1 recombinant haploid clones (A) and 18F13v2 recombinant haploid clones (B).

1220 **Table S1.** List of primers used in this study.

Primer	Sequence (5'-3')	Template	Purpose
pBS_fwd	gcggccgcCCCGGTACCCAGCTTTTG	pBluescript II KS	Assemble <i>hoΔ</i> construct
pBS_rev	CGGGGGATCCACTAGTTC	pBluescript II KS	Assemble <i>hoΔ</i> construct
HO-US_fwd	tagaactagtgatcccccgTATTCTGATGGCTAACGGTG	Founder B1 gDNA	Assemble <i>hoΔ</i> construct
HO-US_rev	gcttcagctgCCTTTAGAGCGCTCCATTTTC	Founder B1 gDNA	Assemble <i>hoΔ</i> construct
pAG61_fwd	gctctaaggCAGCTGAAGCTTCGTACG	pAG61	Assemble <i>hoΔ</i> construct
pAG61_rev	aaattttatgATAGGCCACTAGTGGATC	pAG61	Assemble <i>hoΔ</i> construct
HO-DS_fwd	agtggcctatCATAAAAATTTCTTGCTTGGC	Founder B1 gDNA	Assemble <i>hoΔ</i> construct
HO-DS_rev	aacaaaagctgggtaccggggcgccgcTGC GTTGTACCA CAACTC	Founder B1 gDNA	Assemble <i>hoΔ</i> construct
HO-big-flank-F	AAGCGTTCTAAACGCACTATTCA	all <i>hoΔ</i> founder transformants	<i>hoΔ</i> check
HO-big-flank-R	ATGGCGTATTTCTACTCCAGCAT	all <i>hoΔ</i> founder transformants	<i>hoΔ</i> check
YCR043C-US_MX4_F	TAGAACTAAATGCAAAGAATATTTGTGCCAAGAT AAGGCCAAGAAATTTTGGGGCAACAGTAGGCAG TAAAAGCGCTCTTAACGTA CTTTGGCAAAGGccag ctgaagctctgtacgc	pAG25 (<i>natMX4</i>) or pAG32 (<i>hphMX4</i>)	<i>ycr043c::natMX4</i> or <i>ycr043c::hphMX4</i> deletion
YCR043C-DS_MX4_R	CTCTATGTAGACATATACATATTATTTTCATTGCTTT TTGTAATAAACGAGGAAAATGGTGGGATAAAAAA ATGCATTGTTATTCCTTCGAAACCAGCAGTgcatag gccactagtggatctg	pAG25 (<i>natMX4</i>) or pAG32 (<i>hphMX4</i>)	<i>ycr043c::natMX4</i> or <i>ycr043c::hphMX4</i> deletion
YCR043C-cds-F	agctccactgatgcttcctta	all <i>YCR043C</i> deletion transformants	<i>YCR043C</i> deletion check
YCR043C-cds-R	cacctctccatcttaagtcg	all <i>YCR043C</i> deletion transformants	<i>YCR043C</i> deletion check
YCR043C-flank-F	GGCGGAATGTTGGCTTGGAC	all <i>YCR043C</i> deletion transformants	<i>YCR043C</i> deletion check/ create larger deletion cassette
YCR043C-flank-R	GCGCTACCGTAATGCAGTG	all <i>YCR043C</i> deletion transformants	<i>YCR043C</i> deletion check/ create larger deletion cassette
YCR043C-seq-check-RC	CACTTACCCCTCTGTCAATAGTT	all <i>YCR043C</i> deletion transformants	<i>YCR043C</i> deletion check via Sanger Sequencing
barcode-check-F	gattcggaatctccgagcagaag	all founder strains	barcode check
barcode-check-R	ttcagaacaactctggcgca	all founder strains	barcode check

1221 *Uppercase letters that follow lowercase letters represent the priming sequence, while
1222 the lowercase letters represent regions of overlap homology.
1223 **Lowercase letters that follow uppercase letters represent the priming sequence, while
1224 the uppercase letters represent regions of flanking homology.
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1267 **Table S2.** Sporulation efficiency of all crosses*

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B11	B12
A1	0	4	1	3	3	4	3	4	4	1	4
A2	2	1	4	1	4	4	4	3	2	1	3
A3	5	3	3	ND**	4	3	4	0	2	4	4
A4	4	4	3	4	3	2	4	4	3	3	4
A5	3	2	3	1	1	2	4	2	0	0	1
A6	3	4	4	3	3	4	4	4	3	2	4
A7	3	4	5	4	4	5	5	5	4	4	5
A8	3	3	3	4	3	2	4	0	4	0	4
A9	5	4	3	3	4	2	4	1	2	4	4
A11	4	4	4	4	4	5	4	3	4	1	4
A12	4	4	4	3	3	3	4	4	4	0	4

1268 *A score of 0 means no asci were visible in the field of view while a score of 5 means
1269 approximately 90% or more of the objects in the field of view were asci.

1270 **No data

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1300 **Table S3.** Specific sporulation conditions used in this study.

Population	Outcrossing Cycle	Sporulation time (in days)	Sporulation volume (in mL)	Sporulation vessel	Additional notes
18F1v1	1	5	200	1L erlenmeyer	
18F2v1	2	5	200	1L erlenmeyer	
18F3v1	3	5	200	1L erlenmeyer	
18F4v1	4	5	200	1L erlenmeyer	
18F5v1	5	5	200	1L erlenmeyer	
18F6v1	6	5	200	1L erlenmeyer	
18F7v1	7	5	200	1L erlenmeyer	
18F8v1	8	5	200	1L erlenmeyer	
18F9v1	9	5	200	1L erlenmeyer	
18F10v1	10	5	200	1L erlenmeyer	
18F11v1	11	5	200	1L erlenmeyer	
18F12v1	12	5	200	1L erlenmeyer	
18F1v2	1	6	4	24DWP	All crosses sporulated separately
18F2v2	2	4	200	1L erlenmeyer	
18F3v2	3	3	200	1L erlenmeyer	
18F4v2	4	3	50	250mL erlenmeyer	
18F5v2	5	4	50	250mL erlenmeyer	
18F6v2	6	4	50	250mL erlenmeyer	
18F7v2	7	3	50	250mL erlenmeyer	
18F8v2	8	4	50	250mL erlenmeyer	
18F9v2	9	4	50	250mL erlenmeyer	
18F10v2	10	3	50	250mL erlenmeyer	
18F11v2	11	4	50	250mL erlenmeyer	
18F12v2	12	5	50	250mL erlenmeyer	

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1303 **Table S4. All pairwise SNP differences between founder strains**

	AB1	AB2	AB3	AB4	A5	A6	A7	A8	A9
AB1	0	68178	60061	66679	27765	12131	63233	7410	70076
AB2	68178	0	48848	55578	64142	67101	15008	68575	63395
AB3	60061	48848	0	40134	53034	58437	48360	60383	52287
AB4	66679	55578	40134	0	54606	64986	50777	67027	59111
A5	27765	64142	53034	54606	0	29485	59901	27980	66127
A6	12131	67101	58437	64986	29485	0	62410	12281	69433
A7	63233	15008	48360	50777	59901	62410	0	12281	63205
A8	7410	68575	60383	67027	27980	12281	63629	0	70409
A9	70076	63395	52287	59111	66127	69433	63205	70409	0
A11	8140	68612	60416	67088	28220	12261	63669	7898	70395
A12	7988	68533	60365	67049	27943	12154	63594	7794	70361
B5	28183	63545	53668	55176	36714	30476	59484	28362	66654
B6	60023	48876	157*	40176	53023	58407	48373	60347	52315
B7	7320	68536	60350	66977	27893	12207	63582	6964	70372
B8	58073	52026	34405	44614	54177	56026	51878	58356	53822
B9	54032	56216	43784	47394	49980	53422	54434	54244	57068
B11	8083	68613	60397	67067	28111	12214	63672	7807	70382
B12	45831	25006	52332	59231	49973	46772	30619	46234	65780

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	A11	A12	B5	B6	B7	B8	B9	B11	B12
AB1	8140	7988	28183	60023	7320	58073	54032	8083	45831
AB2	68612	68533	63545	48876	68536	52026	56216	68613	25006
AB3	60416	60365	53668	157*	60350	34405	43784	60397	52332
AB4	67088	67049	55176	40176	66977	44614	47394	67067	59231
A5	28220	27943	36714	53023	27893	54177	49980	28111	49973
A6	12261	12154	30476	58407	12207	56026	53422	12214	46772
A7	63669	63594	59484	48373	63582	51878	54434	63672	30619
A8	7898	7794	28362	60347	6964	58356	54244	7807	46234
A9	70395	70361	66654	52315	70372	53822	57068	70382	65780
A11	0	2283*	28464	60394	7989	58473	54447	2294*	46503
A12	2283*	0	28328	60343	7885	58408	54405	2217*	46396
B5	28464	28328	0	53655	28475	54111	52160	28451	49324
B6	60394	60343	53655	0	60315	34439	43783	60375	52328
B7	7989	7885	28475	60315	0	58340	54268	8006	46164
B8	58473	58408	54111	34439	58340	0	43869	58454	54419
B9	54447	54405	52160	43783	54268	43869	0	54438	53416
B11	2294*	2217*	28451	60375	8006	58454	54438	0	46520
B12	46503	46396	49324	52328	46164	54419	53416	46520	0

1305 *Cells bolded and filled in with blue represent founders that were collapsed due to high
 1306 sequence similarity (AB3/B6; A11/A12/B11). The table was split for readability.

1307

1308 **Table S5.** Mean haplotype frequencies genome-wide through one, two, and twelve
1309 rounds of outcrossing in 18F12v2.

Founder	18F1v2_frequency	18F2v2_frequency	18F12v2_frequency
AB1	8.0%	3.2%	1.2%
AB2	4.4%	1.3%	0.5%
AB3	17.6%	33.2%	41.4%
AB4	9.0%	3.3%	3.3%
A5	5.0%	21.8%	14.0%
A6	10.5%	6.4%	14.3%
A7	4.2%	1.2%	0.4%
A8	4.3%	2.2%	0.7%
A9	1.1%	1.4%	0.5%
A11	3.5%	2.8%	1.2%
A12	3.6%	3.4%	1.7%
B5	9.1%	8.4%	11.3%
B7	7.2%	4.6%	1.0%
B8	2.3%	1.4%	5.7%
B9	3.2%	1.3%	0.8%
B11	3.1%	2.6%	1.3%
B12	3.8%	1.4%	0.6%

1310

1311

1312 **Note S1.** Strains A9, B1, and B9 all grew slowly after O/N incubation, so all 5mL from
1313 each culture was transferred to 5mL of YPD and incubated an additional 3h at 30°C at
1314 250 RPM. During this time, the rest of the haploid strains remained at RT. For mating,
1315 200uL of culture from these slow-growing strains was mixed with 100uL of culture from
1316 the rest of the haploid strains..

1317 **Note S2.** Before taking OD630 measurements, the following crosses were accidentally
1318 combined: A5xB6 with A5xB7, A6xB2 with A6xB3, A6xB8 with A6xB4, and A11xB7
1319 with A11xB6. To correct for this, twice of much of the combined cultures were added to
1320 the final spore pool after normalizing cell density.

1321 **Note S3.** Founder strains A5, A7-A9, A11, A12 and B5-B12 (excluding B10) were
1322 sequenced using PE100 reads, while founders A1-A4 and A6 were sequenced using
1323 PE150 reads.

1324 **Note S4.** In one of the recombinant haploid clones derived from 18F12v2 (rhc 10), the
1325 entirety of chromosome I was called as unknown. Closer examination showed that this
1326 chromosome is duplicated and heterozygous for two founding strains, suggesting a
1327 partial diploidization event.

1328