1 **Genome-wide association study, replication, and mega-analysis using a dense marker**
2 **panel in a multi-generational mouse advanced intercross line**

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Data Availability

30 All relevant data are within the paper and its Supporting Information files. The GeneNetwork
31 accession numbers for genotypes and phenotypes of F_{34}, F_{39-43}, and mega-analysis cohort of
32 AIL are currently pending but will be secured prior to final acceptance/publication.

Abstract

Genome-wide association studies (**GWAS**) in multigenerational outbred populations offer improved mapping precision compared to traditional populations such as F_2s. Advanced intercross lines (**AILs**) are the simplest possible multigenerational intercross; AILs are produced by crossing two inbred strains and then breeding unrelated offspring for additional generations. New recombinations accumulate with each successive generation; these new recombinations provide increased mapping resolution. We used genotyping-by-sequencing (**GBS**) to re-genotype a cohort of sparsely genotyped F_{34} LG/J x SM/J AIL mice that were the subject of several prior publications as well as to obtain genotypes for new cohort of AIL mice from the F_{39-43} generations. The denser set of GBS markers allowed us to identify 110 significant loci, 36 of which were novel, for 79 behavioral and physiological traits in the 428 F_{34} mice. Genetic correlations between F_{34} and F_{39-43} were high, though F_{39-43} AILs showed systematically lower SNP-heritability estimates. We explored replication of loci identified in either F_{34} or F_{39-43} in the other cohort for the traits measured in both F_{34} and F_{39-43}: locomotor activity, body weight, and coat color. While coat color loci were robustly replicated, we observed only partial replication of associations for locomotor activity and body weight. We then performed a mega-analysis of locomotor activity, body weight by combining F_{34} and F_{39-43} mice (N=1028), which identified four novel loci. Finally, we showed that imputation using the old, sparse marker set and the newer dense marker set had little impact on our results, emphasizing the need of the denser GBS genotypes. The present study provides empirical insights into replication, the utility of denser genotyping and identifies new candidate loci that can be explored in future studies.

Author summary

Using a sufficiently dense marker set is essential for genome-wide association studies. The required SNP density is a function of population history. When considering populations derived from a cross between two or more inbred strains, more recombinations will require a correspondingly greater number of markers. We performed several genome-wide association studies using different marker sets - sparse SNPs from a previously publish dataset, dense SNPs obtained using genotyping-by-sequencing, and imputed versions of both the sparse and dense SNPs - to compare their association results in 34th and 39-43rd generations of an LG/J x SM/J advanced intercross line (**AIL**). We found that the dense SNP set substantially increased the number of significant results, while the imputed sets did not. To achieve maximum power, we performed a mega-analysis using all the mice and the dense SNP set; this analysis identified several novel loci. Finally, we explored the replicability of associated loci discovered in either cohort by examining the same SNP in the other cohort. We have made these data publicly available on www.genenetwork.org, providing the community with a unique reference dataset.

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Introduction

The use of multi-parental crosses and commercially available outbred populations for genome wide association studies (**GWAS**) in model organisms such as mice [1–17], rats [18], chickens [19,20], zebrafish [21,22], fruit flies [23–27], *C. elegans* [28] and various plant species [29–31] has become increasingly popular over the last decade. These mapping populations can further be categorized as multi-parental crosses, which are created by interbreeding two or more inbred strains, and commercially available outbred populations, in which the founders are of unknown provenance. An F_2 cross between two inbred strains is the prototypical mapping population; however, F_2s provide poor mapping resolution [32]. To improve mapping resolution, Darvasi and Soller [33] proposed the creation of advanced intercross lines (**AILs**), which are produced by intercrossing F_2 mice for additional generations. AILs accumulate additional crossovers with every successive generation, leading to a population with shorter linkage disequilibrium (**LD**) blocks, which improves mapping precision, albeit at the expense of power [32,34].

The longest running mouse AIL was generated by crossing LG/J and SM/J inbred strains, which were selectively bred for large and small body size. We obtained this AIL in 2006 at generation 33 from Dr. James Cheverud (Jmc: LG,SM-G_{33}, Washington University in St. Louis). Since then, we have collected genotype and phenotype information from multiple generations, including F_{34} [16,35–38], F_{39}-F_{43} and $F_{50\text{-}56}$ [39]. Our previous publications using the F_{34} generation used a custom Illumina Infinium genotyping microarray to obtain genotypes for 4,593 SNPs [35,36]. Although not previously published, we also collected phenotype information from the F_{39}-F_{43} generations, including body weight, fear conditioning, locomotor activity in response to methamphetamine, and the light dark test for anxiety.

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93   In the present study, we used genotyping-by-sequencing (**GBS**), which is a reduced-

94   representation sequencing method [40–42], to obtain a much denser set of SNPs in the F_{34} and

95   to genotype mice from the F_{39}-F_{43} generations for the first time. With this denser set of SNPs,

96   we attempted to identify novel loci in the F_{34}s that were not detected using the sparse SNPs. We

97   also performed a GWAS on using the mice from the F_{39}-F_{43} AILs. Because F_{39}-F_{43} AILs are

98   descendants of the F_{34}, they are uniquely suited to be a replication population for GWAS in the

99   F_{34} generation. Moreover, F_{39}-F_{43} may provide improved resolution and allow for the discovery

100 of novel loci not detected in the F_{34} generation. We also performed a mega-analysis of F_{34} mice

101 and F_{39}-F_{43} mice to identify loci that were not identified in either individual dataset. Finally, we

102 explored whether imputation from the array SNPs could have provided the additional coverage

103 we obtained using the denser GBS genotypes. This study investigates the effect of SNP density

104 on association analyses, addresses rarely explored questions about replication in outbred

105 rodent populations, and identifies novel loci associated with a range of physiological and

106 behavioral traits in mice.

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108 # Results

109 We used 214 males and 214 females from generation F_{34} (Aap:LG,SM-G34) and 305

110 males and 295 females from generations F_{39-43}. We performed association studies on 79 traits in

111 F_{34} AIL and 49 traits in F_{39-43} AIL (S1 Table). F_{34} mice had been previously genotyped on a

112 custom SNP array [35,36]. The average minor allele frequency (**MAF**) of those 4,593 array

113 SNPs was 0.388 (Fig 1). To obtain a denser set of SNP markers, we used genotyping-by-

114 sequencing in F_{34} and F_{39-43} AIL mice. Since data about the F_{39-43} AIL mice had been collected

115 over the span of approximately two years, we carefully considered the possibility of sample

116 contamination and sample mislabeling [43]. We removed samples based on four major features:

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117 heterozygosity distribution, number of reads aligned to sex chromosomes, discrepancies

118 between pedigree and genetic kinship relatedness, and coat color genotype to phenotype

119 mismatch (see Methods; S1 and S2 Figs). The final SNP sets included 60,392 GBS-derived

120 SNPs in 428 F_{34} AIL mice, 59,790 GBS-derived SNPs in 600 F_{39-43} AIL mice, and 58,461 GBS-

121 derived SNPs that existed in both F_{34} and F_{39-43} AIL mice (S2 Table). The MAF for the GBS

122 SNPs was 0.382 in F_{34}, 0.358 in F_{39-43}, and 0.370 in F_{34} and F_{39-43} (Fig 1). There were 66 SNPs

123 called from our GBS data that were also present on the genotyping array. The genotype

124 concordance rate, which reflects the sum of errors from both sets of genotypes, was 95.4% (S3

125 Fig). We found that LD decay rates using F_{34} array, F_{34} GBS, F_{39-43} GBS, and F_{34} and F_{39-43} GBS

126 genotypes were generally similar to one another, though levels of LD using the GBS genotypes

127 appear to be slightly reduced in the later generations of AILs (S4 Fig).

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129 **GBS genotypes produced more significant associations than array genotypes in**

130 **F_{34}**

131 We used a linear mixed model (**LMM**) as implemented in GEMMA [44] to perform

132 GWAS. We used the leave-one-chromosome-out (**LOCO**) approach to address the problem of

133 proximal contamination, as previously described [39,45–47]. We performed GWAS using both

134 the sparse array SNPs and the dense GBS SNPs to determine whether additional SNPs would

135 produce additional genome-wide significant associations. Autosomal and X chromosome SNPs

136 were included in all GWAS. We obtained adjusted significance threshold for each SNP set using

137 MultiTrans and SLIDES [48,49].To select independently associated loci ("lead loci"), we used a

138 LD-based clumping method implemented in PLINK to group SNPs that passed the adjusted

139 genome-wide significance thresholds over a large genomic region flanking the index SNP [50].

140 Applying the most stringent clumping parameters (r^2 = 0.1 and sliding window size = 12,150kb,

141 S3 Table), we identified 110 significant lead loci in 49 out of 79 F_{34} phenotypes using the GBS

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142 SNPs. In contrast, we identified 83 significant lead loci in 45 out of 79 F_{34} phenotypes using the

143 sparse array SNPs. Among F_{34} lead loci, 36 were uniquely identified in the GBS GWAS,

144 whereas 11 were unique to the array GWAS. GBS SNPs consistently yielded more significant

145 lead loci compared to array SNPs regardless of the clumping parameter values (S3 Table),

146 indicating that a dense marker panel was able to detect more association signals compared to a

147 sparse marker panel. Significant lead loci in F_{34} GBS and array are summarized in Table S4.

148 We considered whether the disparity between the numbers of loci identified by the two

149 SNP sets could be resolved by imputation. We used LG/J and SM/J whole genome sequencing

150 data as reference panels [51] and performed imputation on array and GBS SNPs using Beagle

151 v4.1 [52]. After QC filtering, we obtained 4.3M SNPs imputed from the array SNPs and 4.1M

152 SNPs imputed from the GBS SNPs. More imputed GBS SNPs were filtered out because GBS

153 SNPs were called from genotype probabilities, thus introducing uncertainty in imputed SNPs.

154 We found that imputed array genotypes and imputed GBS genotypes did not meaningfully

155 increase the number of new loci discoveries (Fig S5), indicating that the greater number of

156 genome-wide significant associations obtained using the GBS SNPs could not be overcome by

157 imputation.

158 Under a polygenic model where a large number of additive common variants contribute

159 to a complex trait, heritability estimates could be higher when more SNPs are considered [53].

160 Given that there were more GBS SNPs than array SNPs, we used autosomal SNPs to examine

161 whether GBS SNPs would generate higher SNP heritability estimates compared to the sparse

162 array SNPs. Heritability estimates were similar for the two SNP sets, with the exception of

163 agouti coat color, which showed marginally greater heritability for the GBS SNPs (S6 Fig; S5

164 Table). Our results show that while the denser GBS SNP set was able to identify more genome-

165 wide significant loci, greater SNP density did not improve the polygenic signal.

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166 **Genetic correlation and SNP heritability of traits in F_{34} and $F_{39\text{-}43}$**

167   One goal of collecting phenotypic information in the $F_{39\text{-}43}$ generations was to evaluate

168 whether or not the genetic signal driving the phenotypes are consistent across cohorts. To this

169 end, we used two approaches to evaluate replication between F_{34} and F_{39}-F_{43}. First, we used

170 autosomal SNPs to calculate genetic correlations between the F_{34} and $F_{39\text{-}43}$ generations for

171 body weight, coat color, and locomotor activity phenotypes (S6 Table). Locomotor activity on

172 days 1 and 2, albino and agouti coat color, and body weight were highly genetically correlated

173 (r_Gs >0.7). In contrast, locomotor activity on day 3 showed a significant but weaker genetic

174 correlation (r_G=0.577), perhaps reflecting variability in the quality of the methamphetamine

175 injection, which was not given on days 1 and 2. Overall, these results suggest that genetic

176 influences on these traits were broadly similar in the two cohorts. Despite the relatively robust

177 genetic correlations, SNP heritability was consistently lower in the $F_{39\text{-}43}$ compared to the F_{34},

178 possibly a result of increased experimental variance introduced by our extended phenotype

179 collection period (Fig 2; S7 Table).

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181 **Replication of loci detected in F_{34} using $F_{39\text{-}43}$**

182   Using the dense GBS SNP set, we identified 27 genome-wide significant loci in 21 out of

183 49 $F_{39\text{-}43}$ phenotypes. A subset of those traits, including coat color, body weight, and locomotor

184 activity, were also phenotyped in the F_{34} AILs (S8 Table). To assess replication, we determined

185 whether loci that were significant in one cohort (either F_{34} or $F_{39\text{-}43}$) would also be significant in

186 the other. We termed the cohort in which a locus was initially discovered as its "discovery set"

187 and the cohort we attempted replication in as the "replication set" (Table 1). Coat color

188 phenotypes (both albino and agouti) are Mendelian traits and thus served as positive control. As

189 expected, all body weight loci replicated. All three body weight loci identified in the F_{34} were

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190   replicated at nominal levels of significance (p<0.05) in F_{39-43}; similarly, the only body weight

191   locus identified in F_{39-43} was replicated in F_{34}. However, none of the five locomotor activity

192   associated loci were replicated in the reciprocal (replication) cohorts. We also used the "sign

193   test" to determine whether the directions of the effect (beta) of the loci were in the same

194   direction between the discovery and replication cohorts. We found that 11 of 13 loci passed this

195   much less stringent test of replication. The two loci that did not pass the sign test were the two

196   locomotor loci "discovered" in F_{39-43} (Table 1).

Table 1. Replication of significant SNPs between F$_{34}$ and F$_{39-43}$ AIL association analyses. "Discovery set" indicates the AIL generation that significant SNPs were identified. "Replication set" shows the association p-value, β estimates, etc. of the "discovery set" significant SNPs in the replication AIL generation. SNPs that replicated (p<0.05, same sign for the beta) between F$_{34}$ and F$_{39-43}$ are highlighted in bold italics. Genetic correlations for phenotypes measured in both F$_{34}$ and F$_{39-43}$ are listed (see also Supplementary Table 6).

Phenotype	rG(*s.e.*)(*p<0.05)	SNP	Discovery set				Replication set			
			P F34 GBS	af	beta	se	P F3943 GBS replicate	af	beta	se
Body weight		*chr4.66414508*	*8.06E-08*	0.419	-2.50E-01	4.56E-02	*1.56E-02*	0.406	-1.05E-01	4.33E-02
	0.711(0.25)*	*chr6.81405109*	*5.89E-06*	0.497	2.06E-01	4.51E-02	*2.36E-02*	0.518	9.48E-02	4.18E-02
		chr14.79312393	*7.53E-06*	0.514	-2.01E-01	4.45E-02	*1.44E-02*	0.566	-1.04E-01	4.26E-02
Coat color, albino	0.967(0.04)*	*chr7.87642045*	*5.00E-106*	0.432	-5.81E-01	1.91E-02	*2.85E-163*	0.387	-6.07E-01	1.55E-02
Coat color, agouti	0.971(0.04)*	*chr2.154464466*	*9.43E-191*	0.129	9.39E-01	1.25E-02	*5.70E-93*	0.207	7.20E-01	2.57E-02
Locomotor test day 1, total distance travelled in 30min	0.968(0.24)*	chr19.21812298	9.28E-07	0.461	-6.90E+02	1.39E+02	7.72E-01	0.52	-1.94E-02	6.74E-02
Locomotor test day2, total distance travelled in 30min	0.988(0.19)*	chr7.45084416	1.12E-05	0.246	6.77E+02	1.53E+02	3.40E-01	0.217	7.38E-02	7.77E-02
		chr8.17410225	4.96E-06	0.171	7.91E+02	1.71E+02	4.03E-01	0.192	7.04E-02	8.43E-02
			F3943 GBS				F34 GBS replicate			
Body weight	0.711(0.25)*	*chr14.82586326*	*2.63E-06*	0.658	-2.09E-01	4.43E-02	*2.87E-05*	0.575	-1.89E-01	4.50E-02
Coat color, albino	0.967(0.04)*	*chr7.87255156*	*5.91E-167*	0.389	-6.24E-01	1.57E-02	*7.80E-97*	0.444	-5.75E-01	2.07E-02
Coat color, agouti	0.971(0.04)*	*chr2.155091628*	*1.78E-115*	0.218	7.42E-01	2.17E-02	*1.51E-185*	0.135	8.98E-01	1.33E-02
Locomotor test day 2, total distance travelled in 30min	0.988(0.19)*	chr15.67235072	3.21E-06	0.47	3.14E-01	6.63E-02	1.69E-01	0.522	-1.72E+02	1.25E+02
Locomotor test day 3, total distance travelled in 30min	0.577(0.22)*	chr7.113250866	5.88E-06	0.389	3.29E-01	7.20E-02	8.27E-01	0.483	-1.21E+02	5.45E+02

199 Mega-analysis

200   Due to the high genetic correlations between cohorts (S6 Table), we suspected that a

201 mega-analysis using the combined sample set would allow for the identification of additional

202 loci. This analysis identified four novel genome-wide significant associations that were not

203 discovered in either individual cohort (Fig 3; Table 2; S9 Table).

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Table 2. Lead SNPs in F_{34} and F_{39-43} (N=1028) mega-analysis in locomotor activity (day 1 – 3) and body weight. Significant SNPs are clumped using parameters r_2=0.1, 12150kb. Rows in bold indicate SNPs that were discovered in the mega-analysis but not in individual F_{34} or F_{39-43} AILs.

Phenotype	SNP	P	af	Beta	se	Discovery set
Locomotor test day 1, total distance travelled in 30min	**chr1.122479820**	**8.84E-06**	**0.292**	**-2.03E-01**	**4.56E-02**	**Combined set**
Locomotor test day 1, total distance travelled in 30min	**chr10.104988207**	**1.94E-07**	**0.373**	**2.16E-01**	**4.13E-02**	**Combined set**
Locomotor test day 2, total distance travelled in 30min	**chr1.40907532**	**2.65E-07**	**0.664**	**2.27E-01**	**4.38E-02**	**Combined set**
Locomotor test day 2, total distance travelled in 30min	**chr1.123997667**	**3.53E-08**	**0.289**	**-2.53E-01**	**4.56E-02**	**Combined set**
Body weight	**chr2.157502044**	**1.10E-05**	**0.42**	**-1.35E-01**	**3.06E-02**	**Combined set**
Body weight	chr4.66866758	1.64E-09	0.403	-1.84E-01	3.01E-02	F_{34} array, F_{34} GBS, combined set
Body weight	chr6.81267890	8.61E-07	0.507	1.40E-01	2.83E-02	F_{34} GBS, combined set
Body weight	chr14.82672838	2.06E-10	0.623	-1.90E-01	2.96E-02	F_{34} array, F_{34} GBS, F_{39-43} GBS, combined set

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Discussion

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214   Outbred model organism populations have become important resources for genetics

215   studies. We used F_{34} and F_{39-43} generations of a LG/J x SM/J AIL to investigate how SNP

216   density influences GWAS results, heritability, and genetic correlations. Using the ~60K SNPs in

217   the F_{34} GBS set, we found 110 loci, of which 36 were unique to the GBS genotypes. Notably,

218   F_{34} array genotypes were able to detect the majority of loci that were identified in F_{34} GBS SNP

219   set, indicating that many but not all loci were successfully 'tagged' by the sparse array SNPs.

220   Although the genetic correlations between traits measured in F_{34} and F_{39-43} were high,

221   replication was incomplete, with the locomotor traits showing especially poor replication. Finally,

222   imputation to reference panels increased the number of SNPs available for analysis but did not

223   meaningfully enhance the number of loci we discovered because it did not improve our ability to

224   capture recombination events.

225   Previous work from our lab used the array genotypes described in this study to map

226   QTLs for various behavioral and physiological traits in 688 F_{34} AILs. With F_{34} GBS SNPs, which

227   were only available for 428 of the initial 688 AIL mice, we were able to replicate many of our

228   previously published loci. For instance, using the array SNPs in the F_{34} generation in

229   conjunction with the LMM software QTLRel, Cheng et al. [35] found a significant locus on

230   chromosome 8 for locomotor day 2 activity that contained only one gene: *Csmd1* (CUB and

231   sushi multiple domains 1). Gonzales et al. [39] replicated this finding in F_{50-56} AIL and identified a

232   *cis*-eQTL mapped to the same region. *Csmd1* mutant mice showed increased locomotor activity

233   compared to wild-type and heterozygous mice, indicating that *Csmd1* is likely a causal gene for

234   locomotor and related traits [39]. We replicated this locus in our own analysis of the F_{34} array

235   and GBS SNP, using GEMMA rather than QTLRel (S7 Fig). We also replicated a locus on

236   chromosome 17 for distance traveled in the periphery in the open field test (Fig 4; [36,39]), three

237   loci on chromosomes 4, 6, and 14 for body weight (Supplementary Fig 7; [54]), one locus on

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238   chromosome 7 for mean corpuscular hemoglobin concentrations (MCHC, complete blood count;

239   S7 Fig; [55]), and numerous loci on chromosome 4, 6, 7, 8, and 11 for muscle weights

240   (Supplementary Fig 7; [37]). We noticed that while using the same set of F_{34} array genotypes,

241   some previously published loci were not detected in our GWAS. The most likely explanation is

242   that we had only 428 of the 688 mice used in the previous publications in this study.

243   Methodological differences between prior studies and the current study, such as the use of

244   QTLRel rather than GEMMA and the choice of pedigree rather than genotypes in relatedness

245   calculation, may also lead to lack of complete replication [56].

246   F_{39-43} AILs replicated some, but not all, significant loci identified in F_{34}, despite generally

247   high genetic correlations between the two cohorts. Significant loci for coat color, which are

248   monogenic and viewed as positive controls, were consistent between the two AIL cohorts. Loci

249   for body weight were fully replicated between F_{34} and F_{39-43} using the $p<0.05$ cutoff for the

250   replication cohort, whereas loci for locomotor activity were not. Nevertheless, the beta estimates

251   for all but two loci shared the same sign, which provides modest evidence for replication. The

252   circumstances under which the F_{39-43} data were collected were not ideal, likely contributing to

253   their lower heritability and the limited replication. Unlike the F_{34} dataset, the F_{39-43} used multiple

254   technicians to conduct the behavioral tests and occurred over a prolonged period of time in

255   which numerous environmental factors may have changed.

256   We performed a mega-analysis using F_{34} and F_{39-43} AIL mice. The combined dataset

257   increased our power and allowed us to identify four novel genome-wide significant associations

258   that were not detected in either the F_{34} or the F_{39-43} cohorts. For example, the mega-analysis

259   identified a locus for body weight on chromosome 2 (Fig S7). Parker et al. [54] identified the

260   same locus using an integrated analysis of LG/J x SM/J F_2 and F_{34} AILs.

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261   Many GWAS use a 1.0~2.0 LOD support interval to approximate the size of the

262   association region (see [57,58]). The LOD support interval, proposed by Conneally et al. [59]

263   and Lander & Botstein [60], is a simple confidence interval method involving converting the p-

264   value of the peak locus into a LOD score, subtracting "drop size" from the peak locus LOD

265   score, and finding the two physical positions to the left and to the right of the peak locus location

266   that correspond to the subtracted LOD score. Although Mangin et al. [61] showed via simulation

267   that the boundaries of LOD support intervals depend on effect size, others observed that a 1.0 ~

268   2.0 LOD support interval accurately captures ~95% coverage of the true location of the loci

269   when using a dense set of markers [60,62,63]. In the present study, we considered using LOD

270   support intervals but found that the sparse array SNPs produced misleadingly large support

271   intervals. Various methods have been proposed for calculating confidence intervals in

272   analogous situations (e.g. [12,64]). Rather than adopting a formal method, we compared

273   LocusZoom plots of the same locus region between array SNPs and the GBS SNPs (S7 Fig;

274   [65]). For example, the benefit of the denser SNP coverage is easily observed in the locus on

275   chromosome 7 for "complete blood count, repeat measure of retic parameters, cell hemoglobin

276   concentration mean" (Fig S7). Thus, there are advantages of dense SNP sets that go beyond

277   the ability to discover additional loci (Table 1).

278   Our study has notable limitations. First, not all F_{34} and F_{39-43} animals that were

279   phenotyped were later genotyped by GBS due to missing tissue samples, which in turn lowered

280   our sample size and reduced the power of association analyses. Second, F_{39-43} traits have been

281   collected by different technicians over the span of several years, which introduced noise and

282   diminished trait heritability (Fig 2).

283   The present study explored the role of marker density and imputation in GWAS.

284   Furthermore, the combination of denser marker coverage and the addition of 600 F_{39-43} AIL mice

285   allowed us to identify novel loci for locomotor activity, open field test, fear conditioning, light dark

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286   test for anxiety, complete blood count, iron content in liver and spleen, and muscle weight. Our

287   findings add to the large body of phenotypic/genotypic data available for the LG/SM AIL, which

288   can be found on GeneNetwork [http://www.genenetwork.org].

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Materials and Methods

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Animals

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292   All mice used in this study were members of the advanced intercross line (**AIL**) between

293   LG/J and SM/J that was originally created by Dr. James Cheverud (Washington University, St.

294   Louis, MO). The AIL line has been maintained in the Palmer laboratory since generation F_{33}.

295   Age and exact number of animals tested in each phenotype are described in S1 Table. Several

296   previous publications [16,35–38,54,55] have reported on association analyses of the F_{34} mice

297   (N=428). No prior publications have described the $F_{39\text{-}43}$ generations (N=600). The sample size

298   of F_{34} mice reported in this study (N=428) is smaller than that in previous publications of F_{34}

299   (N=688) because we only sequenced a subset of F_{34} animals using GBS. With the exception of

300   coat color and locomotor activities, we quantile normalized all phenotypes. Coat color traits

301   were coded in binary numbers (albino: 1 = white, 0 = non-white; agouti: 1 = tan, 0 = black,

302   NA=white). Locomotor activity traits were not quantile transformed in order to follow the

303   guideline described in Cheng et al. [35] for direct comparison.

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F_{34}, $F_{39\text{-}43}$ Phenotypes

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306   We have previously described the phenotyping of F_{34} animals for locomotor activity [35],

307   fear conditioning [36], open field [36], coat color, body weight [54], complete blood counts [55],

308   heart and tibia measurements [37], muscle weight [37]. Iron content in liver and spleen, which

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309 have not been previously reported in these mice, was measured by atomic absorption

310 spectrophotometry, as described in Gardenghi et al. [66] and Graziano, Grady and Cerami [67].

311 Although the phenotyping of F_{39-43} animals has not been previously reported, we used method

312 that were identical to those previously reported for locomotor activity [35], open field [36], coat

313 color, body weight [54], and light/dark test for anxiety [15].

314

F_{34} AIL Array Genotypes

316 F_{34} animals had been genotyped on a custom SNP array on the Illumina Infinium

317 platform [35,36], which yielded a set of 4,593 SNPs on autosomes and X chromosome that we

318 refer to as 'array SNPs'.

319

F_{34} and F_{39-43} GBS Genotypes

321 F_{34} and F_{39-43} animals were genotyped using genotyping-by-sequencing (**GBS**), which is

322 a reduced-representation genome sequencing method [1,39]. We used the same protocol for

323 GBS library preparation that was described in Gonzales et al [39]. We called GBS genotype

324 probabilities using ANGSD [68]. GBS identified 1,667,920 autosomal and 43,015 X-

325 chromosome SNPs. To fill in missing genotypes at SNPs where some but not all mice had calls,

326 we ran within-sample imputation using Beagle v4.1, which generated hard call genotypes as

327 well as genotype probabilities [52]. After imputation, only SNPs that had dosage $r^2 > 0.9$ were

328 retained. We removed SNPs with minor allele frequency < 0.1 and SNPs with $p < 1.0 \times 10^{-6}$ in the

329 Chi-square test of Hardy–Weinberg Equilibrium (**HWE**) (S2 Table). All phenotype and GBS

330 genotype data are deposited in GeneNetwork (http://www.genenetwork.org).

331

17

332 **QC of individuals**

333   We have found that large genetic studies are often hampered by cross-contamination

334 between samples and sample mix-ups. We used four features of the data to identify problematic

335 samples: heterozygosity distribution, proportion of reads aligned to sex chromosomes,

336 pedigree/kinship, and coat color. We first examined heterozygosity across autosomes and

337 removed animals where the proportion of heterozygosity that was more than 3 standard

338 deviations from the mean (S1 Fig). Next, we sought to identify animals in which the recorded

339 sex did not agree with the sequencing data. We compared the ratio of reads mapped to the X

340 and Y chromosomes. The 95% CI for this ratio was 196.84 to 214.3 in females and 2.13 to 2.18

341 in males. Twenty-two F_{34} and F_{39-43} animals were removed because their sex (as determined by

342 reads ratio) did not agree with their recorded sex; we assumed this discrepancy was due to

343 sample mix-ups. To further identify mislabeled samples, we calculated kinship coefficients

344 based on the full AIL pedigree using QTLRel. We then calculated a genetic relatedness matrix

345 (**GRM**) using IBDLD, which estimates identity by descent using genotype data. The comparison

346 between pedigree kinship relatedness and genetic kinship relatedness identified 7 pairs of

347 animals that showed obvious disagreement between kinship coefficients and the GRM. Lastly,

348 we excluded 14 F_{39-43} animals that showed discordance between their recorded coat color and

349 their genotypes at markers flanking *Tyr*, which causes albinism in mice. The numbers of animals

350 filtered at each step are listed in S2 Table. Some animals were detected by more than one QC

351 step, substantiating our belief that these samples were erroneous.

352   At the end of SNP and sample filtering, we had 59,561 autosomal and 831 X

353 chromosome SNPs in F_{34}, 58,966 autosomal and 824 X chromosome SNPs in F_{39-43}, and

354 57,635 autosomal and 826 X chromosome SNPs in the combined F_{34} and F_{39-43} set (S2 Table).

355 GBS genotype quality was estimated by examining concordance between the 66 SNPs that

356 were present in both the array and GBS genotyping results.

18

357   LD decay

358   Average LD (r^2) was calculated using allele frequency matched SNPs (MAF difference <

359   0.05) within 100,000bp distance, as described in Parker et al. [1].

360

361   Imputation to LG/J and SM/J reference panels

362   F_{34} array genotypes (N=428) and F_{34} GBS genotypes (N=428) were imputed to LG/J and

363   SM/J whole genome sequence data [51] using BEAGLE. For F_{34} array imputation, we used a

364   large window size (100,000 SNPs and 45,000 SNPs overlap). Imputation to reference panels

365   yielded 4.3 million SNPs for F_{34} array and F_{34} GBS imputed sets. Imputed SNPs with DR^2 > 0.9,

366   MAF > 0.1, HWE p value > 1.0×10^{-6} were retained, resulting in 4.1M imputed F_{34} GBS SNPs

367   and 4.3M imputed F_{34} array SNPs.

368

369   Genome-wide association analysis (GWAS)

370   We used the linear mixed model, as implemented in GEMMA [44], to perform a GWAS

371   that accounted for the complex familial relationships among the AIL mice [35,39]. We used the

372   leave-one-chromosome-out (**LOCO**) approach to calculate genetic relatedness matrix, which

373   effectively circumvented the problem of proximal contamination [46]. Separate GWAS were

374   performed using F_{34} array genotypes, F_{34} GBS genotypes, and F_{39-43} GBS genotypes. Apart

375   from coat color (binary trait) and locomotor activity, raw phenotypes were quantile normalized

376   prior to analysis. Locomotor activity was not quantile normalized because the trait was

377   reasonably normally distributed already and because we wanted our analysis to match those

378   performed in Cheng et al [35]. Because F_{34} AIL had already been studied using array genotypes

379   [35] and mapped using QTLRel [56], we used the same covariates as described in Cheng et al.

19

380 [35] in order to examine whether our array and GBS GWAS would replicate their findings. We

381 included sex and body weight as covariates for locomotor activity traits (see covariates used in

382 [35])and sex, age, and coat color as covariates for fear conditioning and open field test in F_{34}

383 AILs (see covariates used in [36]). We used sex and age as covariates for all other phenotypes.

384 Covariates for each analysis are shown in S1 Table. Finally, we performed mega-analysis of F_{34}

385 and F_{39-43} animals (N=1,028) for body weight, coat color, and locomotor activity, since these

386 traits were measured in the same way in both cohorts. For the mega-analyses, locomotor

387 activity was quantile normalized after the combination of the two datasets to ensure that data

388 were normally distributed across generations.

389

Identifying suspicious SNPs

391 Some significant SNPs in F_{34} GWAS and in the mega-analysis of F_{34} and F_{39-43} were

392 suspicious because nearby SNPs, which would have been expected to be in high LD (a very

393 strong assumption in an AIL), did not have high -log10 values. We only examined SNPs that

394 obtained significant p-values; these examinations reveled that these SNPs had suspicious ratios

395 of heterozygotes to homozygotes calls and had corresponding HWE p-values that were close to

396 our 1.0×10^{-6} threshold (S10 and S11 Tables). To avoid counting these as novel loci, we

397 removed those SNPs prior to summarizing our results as they likely reflected genotyping errors.

398

Selecting independent significant SNPs

400 To identify independent "lead loci" among significant GWAS SNPs that surpass the

401 significance threshold, we used the LD-based clumping method in PLINK v1.9. We empirically

402 chose clumping parameters (r^2 = 0.1 and sliding window size = 12,150kb) that gave us a

20

403   conservative set of independent SNPs (S3 Table). For the coat color phenotypes, we found that

404   multiple SNPs remained significant even after LD-based clumping, presumably due to the

405   extremely significant associations at these Mendelian loci. In these cases, we used a stepwise

406   model selection procedure in GCTA [69] and performed association analyses conditioning on

407   the most significant SNPs.

408

409   **Significance thresholds**

410   We used MultiTrans and SLIDE to set significance thresholds for the GWAS [48,49].

411   MultiTrans and SLIDE are methods that assume multivariate normal distribution of the

412   phenotypes, which in LMM models, contain a covariance structure due to various degrees of

413   relatedness among individuals. We were curious to see whether MultiTrans/SLIDE produces

414   significance thresholds drastically different from the threshold we obtained from a standard

415   permutation test ('naïve permutation' as per Cheng et al. [46]). We performed 1,000

416   permutations using the F_{34} GBS genotypes and the phenotypic data from locomotor activity

417   (days 1, 2, and 3). We found that the 95th percentile values for these permutations were 4.65,

418   4.79, and 4.85, respectively, which were very similar to 4.85, the threshold obtained from

419   MultiTrans using the same data. Thus, the thresholds presented here were obtained from

420   MultiTrans but are similar (if anything slightly more conservative) than thresholds we would have

421   obtained had we used permutation. Because the effective number of tests depends on the

422   number of SNPs and the specific animals used in GWAS, we obtained a unique adjusted

423   significance threshold for each SNP set in each animal cohort (S12 Table).

424

425

21

426 **Genetic correlation and heritability estimates between F$_{34}$ and F$_{39-43}$ phenotypes**

427   Locomotor activity, body weight, and coat color had been measured in both F$_{34}$ and F$_{39-}$

428 $_{43}$ populations. We calculated both SNP heritability and genetic correlations between F$_{34}$ and

429 F$_{39-43}$ animals using GCTA bivariate GREML analysis [69]. Because F$_{39-43}$ day 1 locomotor

430 activity data were not normally distributed, we quantile normalized locomotor activity data when

431 estimating SNP heritabilities and genetic correlations.

432

433 **LocusZoom Plots**

434 LocusZoom plots were generated using the standalone implementation of LocusZoom [65],

435 using LD scores calculated from PLINK v.1.9 --ld option and mm10 gene annotation file

436 downloaded from USCS genome browser.

437

438 # Ethics Statement

439 All procedures were approved by the Institutional Animal Care and Use Committee (IACUC

440 protocol: S15226) Euthanasia was accomplished using CO_2 asphyxiation followed by cervical

441 dislocation

442 .

443 # Acknowledgements

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622 **Main figure legends**

623

624 **Fig 1. Minor allele frequency distributions for F_{34} array, F_{34} GBS, F_{39}-F_{43} GBS, and F_{34} and**
625 **F_{39}-F_{43} GBS SNP sets.** MAF distributions are highly comparable between AIL generations.

626 **Fig 2. Chip-heritability estimates in F_{34} and $F_{39\text{-}43}$ AILs.** All heritability estimates are highly
627 significant (p < 1.0E-05; see S7 Table).

628 **Fig 3. Manhattan plots comparing F_{34} GBS, $F_{39\text{-}43}$ GBS, and mega-analysis on locomotor**
629 **day 1 test using 57,170 shared SNPs in all AIL generations.** Mega-analysis identified a locus
630 on chromosome 10 (chr10.104988207) that was not detected in the F_{34} or F_{39}-F_{43} alone,
631 suggesting that mega-analysis enhanced power to detect some loci.

632 **Fig 4. Significant loci on chromosome 17 for open field, distance traveled in periphery in**
633 **F_{34} AIL.** (**a**) Genome-wide Manhattan plots highlighting the significant loci on chromosome 17
634 associated with open field, distance traveled in periphery in F_{34} array SNP set (chr17:27427358)
635 and F_{34} GBS SNP set (chr17:27130383). (**b**) As exemplified in this pair of LocusZoom plots,
636 GBS SNPs defined the boundaries of the loci much more precisely than array SNPs. GBS
637 SNPs that are in high LD (r_2 > 0.8, red dots) with lead SNP chr17:27130383 resides between 27
638 ~ 28.2 Mb. In contrast, too few SNPs are present in the array plot to draw any definitive
639 conclusion about the boundaries or LD pattern in this region. This locus has also been observed
640 in GWAS for a variety of other traits, including locomotor activity, startle and prepulse inhibition
641 [39] and open field test for anxiety [36]. LocusZoom plots for all loci identified in this paper are in
642 Fig S7.

643

644 **Supporting information**

28

645

S1 Fig. Autosomal heterozygosity distribution in F_{34}, F_{39-43} AILs. Animals with excessive or insufficient heterozygosity (3 *s.d.* away from mean) were removed from further analysis. As controls, we have sequenced two F_2s of LG and SM, four LG mice and four SM mice (see annotated data points with 1 and 0 heterozygosity).

S2 Fig. Kinship coefficients in F_{34} and F_{39-43} AILs calculated from pedigree against genetic relatedness matrix calculated using IBDLD [49]. Each circle represents a pair of animals, which their genetic kinship relatedness on the x-axis and pedigree kinship relatedness on the y-axis. Color signifies relatedness based on AIIL pedigree. Blue circles represent identical twins, red full siblings, yellow parent-offspring pairs, grey other relationships. Seven animal pairs that deviate from the pedigree relationship clusters were excluded (see black arrows).

S3 Fig. Heatmap showing F_{34} array and F_{34} GBS genotype concordance in percentages, using 66 shared SNPs. "A" codes for the LG/J allele, and "B" codes for the SM/J allele. "AA" genotype concordance between array and GBS is 24.54%, "AB" 43.23%, "BB" 27.60%.

S4 Fig. LD decay in F_{34} array, F_{34} GBS, F_{39-43} GBS, and F_{34} and F_{39-43} GBS SNP sets.

S5 Fig. Manhattan plots comparing 4,593 F_{34} array, 60.3K F_{34} GBS, 4.3M imputed F_{34} array, and 4.1M imputed F_{34} GBS (N=428) SNPs on day 2 locomotor activity. Adjusted significance thresholds for imputed array and GBS SNPs were estimated using LD pruned SNPs (r2=0.1, window size=20kb; PLINK v1.9). Notice that even though the imputed sets have more SNPs (the two right panels), they are frequently blocks of many SNPs with almost identical position and LD=1, therefore making it hard to visualize the additional SNPs.

S6 Fig. SNP heritability using F_{34} GBS and F_{34} array SNPs (slope=1).

S7 Fig. LocusZoom for F_{34} array, F_{34} GBS, F_{39-43} GBS, and mega-analysis QTLs.

S1 Table. List of phenotypes used in GWAS.

S2 Table. SNP and individual QC filter table. Numbers of animals and SNPs remained after each step of filtering are shown per GBS SNP set.

S3 Table. Effect of PLINK v1.9 clump-based pruning parameters on number of independent SNPs remained. At all r_2 values examined, a sliding window size of 12150kb was the first smallest window that yield the most stringent number of clumped SNPs in both array and GBS GWAS.

S4 Table. Lead QTL in F_{34} GBS and F_{34} array GWAS studies across phenotypes. Significant SNPs are clumped using parameters r_2=0.1, 12150kb.

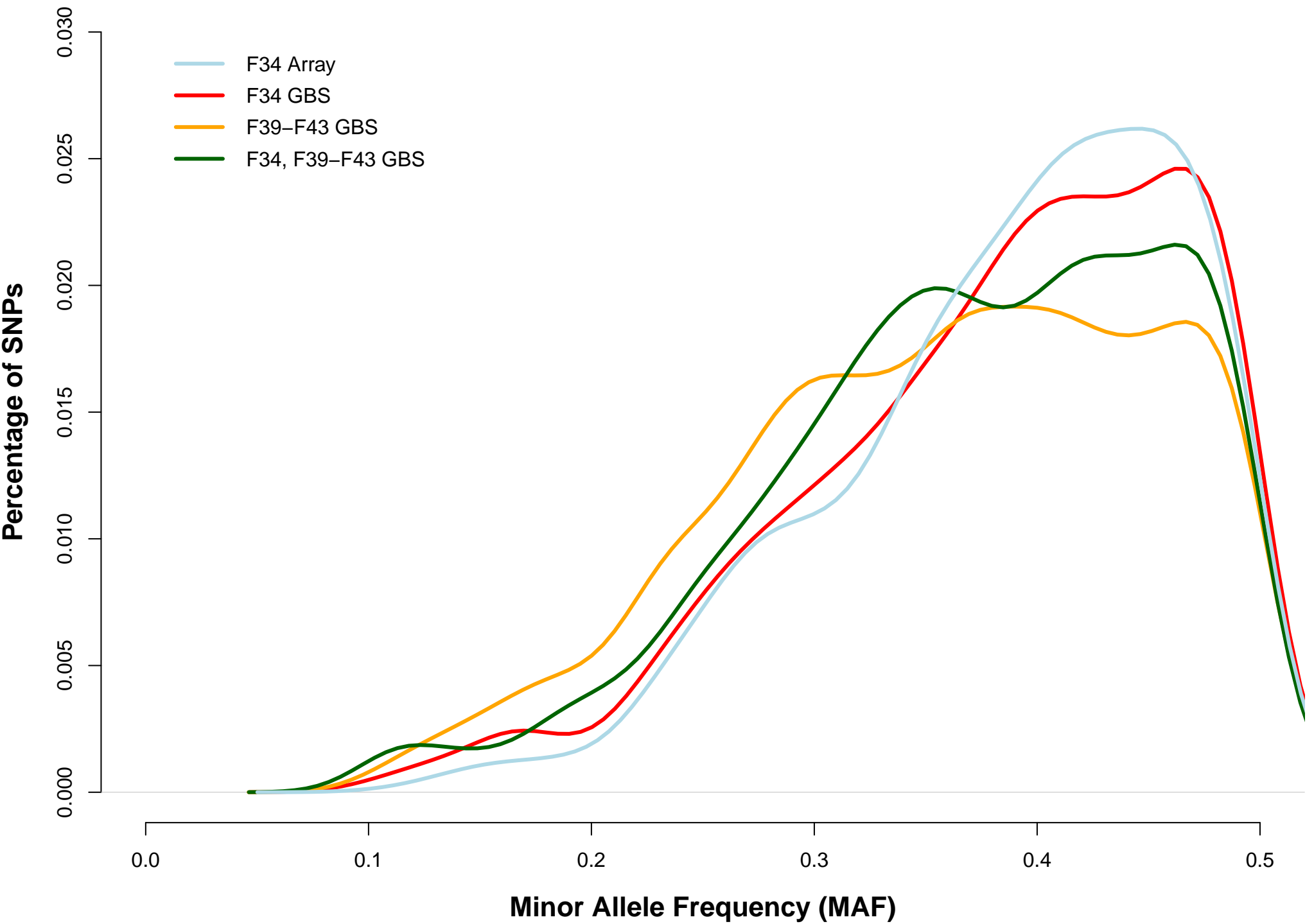**S5 Table. F_{34} GBS and array SNP heritability estimates.**

S6 Table. F_{34} and F_{39-43} genetic correlations in locomotor activity, coat color, and body weight.

S7 Table. SNP-heritability comparison between F_{34} and F_{39-43} GBS.

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694

695 **S8 Table. Lead QTL in F$_{39-43}$ N=600 GBS GWAS studies across phenotypes.** Significant
696 SNPs are clumped using parameters r_2=0.1, 12150kb.

697

698 **S9 Table. Lead QTL in F$_{34}$ and F$_{39-43}$ (N=1028) mega-analysis across phenotypes.**
699 Significant SNPs are clumped using parameters r_2=0.1, 12150kb.

700

701 **S10 Table. SNPs in F$_{34}$ GBS set with HWE p-values close to 1.0×10-6 cutoff threshold.**
702 These SNPs are removed from QTL summary tables.

703

704 **S11 Table. SNPs in F$_{34}$ and F$_{39-43}$ mega-analysis GBS set with HWE p values close to**
705 **1.0×10-6 cutoff threshold.** These SNPs are removed from QTL summary tables.

706

707 **S12 Table. Adjusted significance threshold for each SNP set and GWAS cohort.**

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MAF Distributions

Percentage of SNPs

Minor Allele Frequency (MAF)

F34 Array
F34 GBS
F39–F43 GBS
F34, F39–F43 GBS

Phenotype

Locomotor day 1, quantile normalized

- F34
- F39−43

Locomotor day 2, quantile normalized

- F34
- F39−43

Locomotor day 3, quantile normalized

- F34
- F39−43

Coat color, albino

- F34
- F39−43
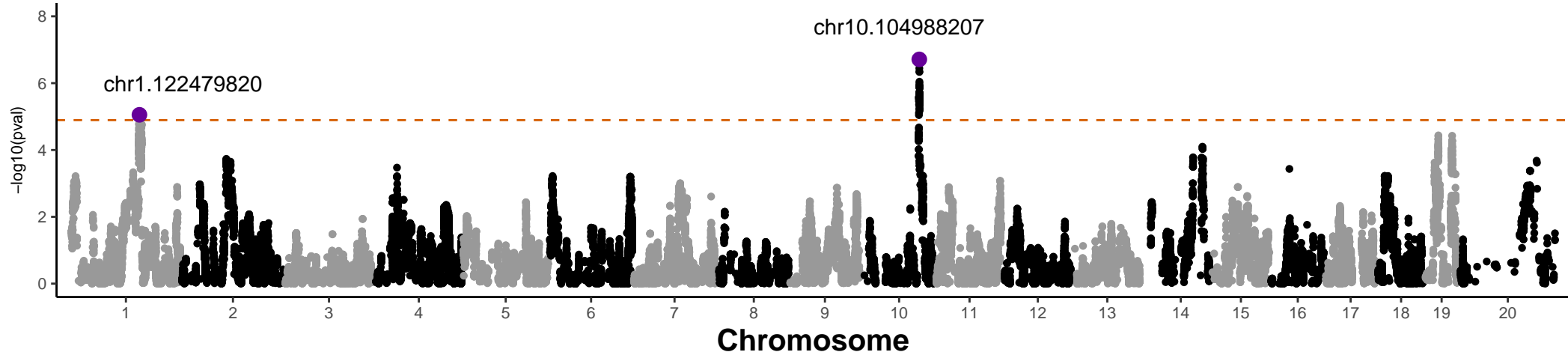
Coat color, agouti

- F34
- F39−43

Body weight

- F34
- F39−43

0   0.1   0.2   0.3   0.4   0.5   0.6   0.7   0.8   0.9   1

h2
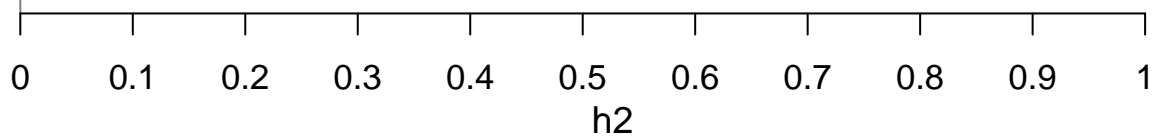
F$_{34}$ GBS, locomotor activity day 1 (N=428)

chr19.21812298
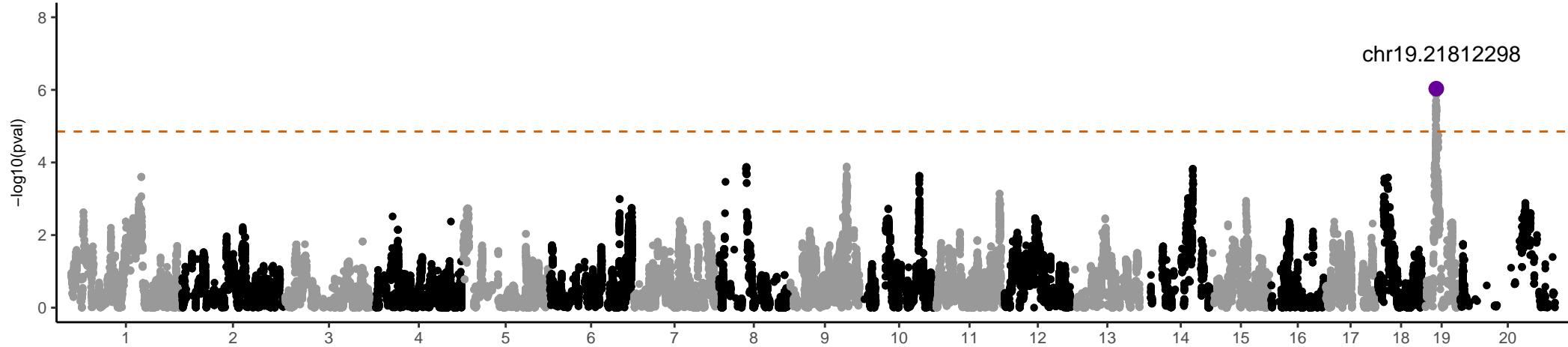
F$_{39-43}$ GBS, locomotor activity day 1 (N=600)
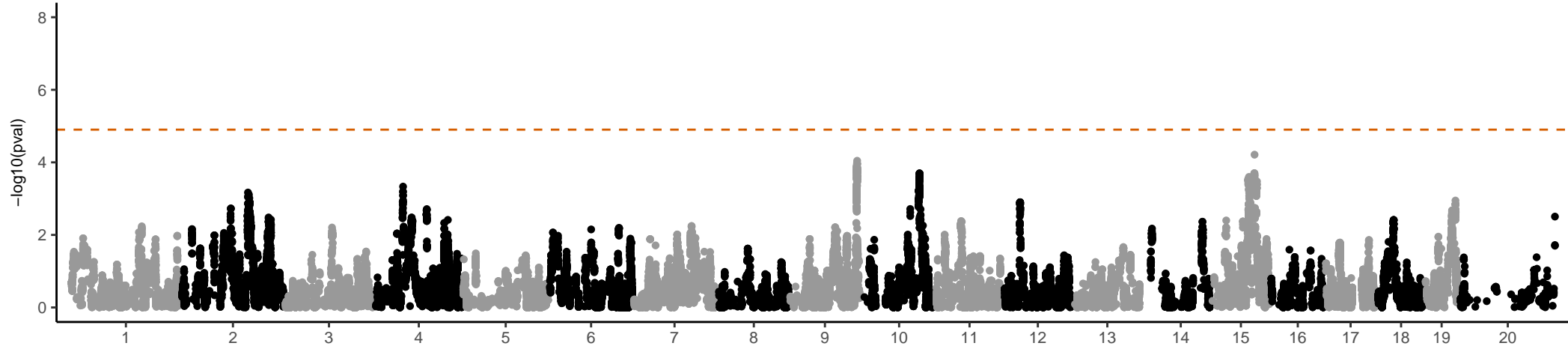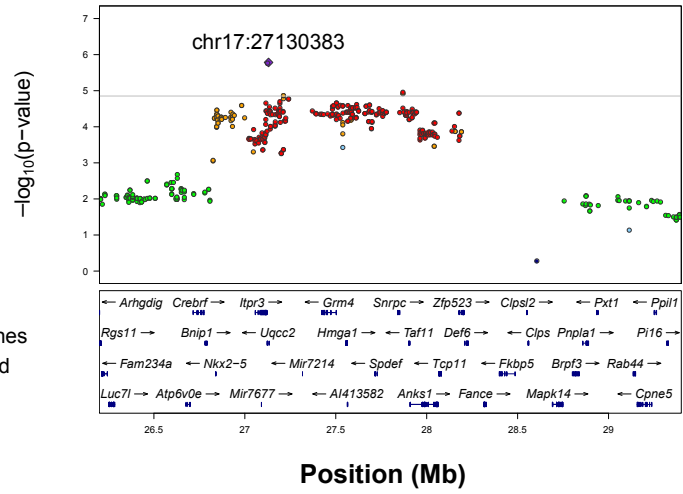
Mega analysis, locomotor activity day 1 (N=1028)

chr10.104988207

chr1.122479820

Chromosome

a

F$_{34}$ array, open filed, distance traveled in periphery
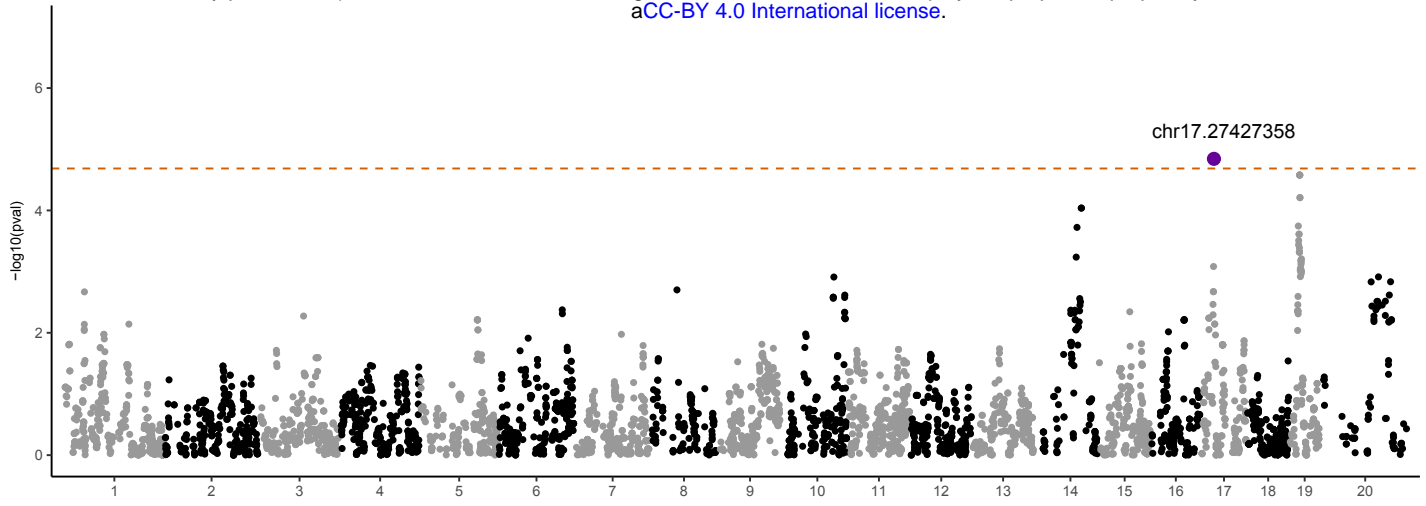
F$_{34}$ GBS, open filed, distance traveled in periphery

b