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

Xinzhu Zhou[1], Celine L. St. Pierre[2], Natalia M. Gonzales[3], Riyan Cheng[4], Apurva Chitre[4], Greta Sokoloff[5], Abraham A. Palmer[4,6*]

[1] Biomedical Sciences Graduate Program, University of California San Diego, La Jolla, CA, USA;

[2] Department of Genetics, Washington University School of Medicine, St. Louis, MO, USA

[3] Department of Human Genetics, University of Chicago, Chicago, IL, USA

[4] Department of Psychiatry, University of California San Diego, La Jolla, CA, USA

[5] Department of Psychological & Brain Sciences, University of Iowa, Iowa City, IO, USA

[6] Institute for Genomic Medicine, University of California San Diego, La Jolla, CA, USA

[*]Corresponding author

Email: aapalmer@ucsd.edu (AAP)

Author contributions

XZ imputed genotypes, performed SNP and individual QC, and conducted GWAS in F_{34} and F_{39-43} AILs under supervision of AAP. AAP also provided computational resources for the analyses in this paper. CS prepared GBS libraries for sequencing, as well as organizing portions of the F_{39-43} phenotypes. NMG de-multiplexed GBS sequencing results and performed alignment and variant calling. RC helped with kinship relatedness matrix calculated from AIL pedigree. AC provided technical support for running programs and scripts. GS collected F_{39-43} phenotypes, respectively. XZ co-wrote the manuscript with AAP, who designed the study and oversaw data collection.

Data Availability

All relevant data are within the paper and its Supporting Information files. The GeneNetwork accession numbers for genotypes and phenotypes of F_{34}, F_{39-43}, and mega-analysis cohort of 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.

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.

In the present study, we used genotyping-by-sequencing (**GBS**), which is a reduced-representation sequencing method [40–42], to obtain a much denser set of SNPs in the F_{34} and to genotype mice from the F_{39}-F_{43} generations for the first time. With this denser set of SNPs, we attempted to identify novel loci in the F_{34}s that were not detected using the sparse SNPs. We also performed a GWAS on using the mice from the F_{39}-F_{43} AILs. Because F_{39}-F_{43} AILs are descendants of the F_{34}, they are uniquely suited to be a replication population for GWAS in the F_{34} generation. Moreover, F_{39}-F_{43} may provide improved resolution and allow for the discovery of novel loci not detected in the F_{34} generation. We also performed a mega-analysis of F_{34} mice and F_{39}-F_{43} mice to identify loci that were not identified in either individual dataset. Finally, we explored whether imputation from the array SNPs could have provided the additional coverage we obtained using the denser GBS genotypes. This study investigates the effect of SNP density on association analyses, addresses rarely explored questions about replication in outbred rodent populations, and identifies novel loci associated with a range of physiological and behavioral traits in mice.

Results

We used 214 males and 214 females from generation F_{34} (Aap:LG,SM-G34) and 305 males and 295 females from generations $F_{39\text{-}43}$. We performed association studies on 79 traits in F_{34} AIL and 49 traits in $F_{39\text{-}43}$ AIL (S1 Table). F_{34} mice had been previously genotyped on a custom SNP array [35,36]. The average minor allele frequency (**MAF**) of those 4,593 array SNPs was 0.388 (Fig 1). To obtain a denser set of SNP markers, we used genotyping-by-sequencing in F_{34} and $F_{39\text{-}43}$ AIL mice. Since data about the $F_{39\text{-}43}$ AIL mice had been collected over the span of approximately two years, we carefully considered the possibility of sample contamination and sample mislabeling [43]. We removed samples based on four major features:

heterozygosity distribution, number of reads aligned to sex chromosomes, discrepancies between pedigree and genetic kinship relatedness, and coat color genotype to phenotype mismatch (see Methods; S1 and S2 Figs). The final SNP sets included 60,392 GBS-derived SNPs in 428 F_{34} AIL mice, 59,790 GBS-derived SNPs in 600 $F_{39\text{-}43}$ AIL mice, and 58,461 GBS-derived SNPs that existed in both F_{34} and $F_{39\text{-}43}$ AIL mice (S2 Table). The MAF for the GBS SNPs was 0.382 in F_{34}, 0.358 in $F_{39\text{-}43}$, and 0.370 in F_{34} and $F_{39\text{-}43}$ (Fig 1). There were 66 SNPs called from our GBS data that were also present on the genotyping array. The genotype concordance rate, which reflects the sum of errors from both sets of genotypes, was 95.4% (S3 Fig). We found that LD decay rates using F_{34} array, F_{34} GBS, $F_{39\text{-}43}$ GBS, and F_{34} and $F_{39\text{-}43}$ GBS genotypes were generally similar to one another, though levels of LD using the GBS genotypes appear to be slightly reduced in the later generations of AILs (S4 Fig).

GBS genotypes produced more significant associations than array genotypes in F_{34}

We used a linear mixed model (**LMM**) as implemented in GEMMA [44] to perform GWAS. We used the leave-one-chromosome-out (**LOCO**) approach to address the problem of proximal contamination, as previously described [39,45–47]. We performed GWAS using both the sparse array SNPs and the dense GBS SNPs to determine whether additional SNPs would produce additional genome-wide significant associations. Autosomal and X chromosome SNPs were included in all GWAS. We obtained adjusted significance threshold for each SNP set using MultiTrans and SLIDES [48,49].To select independently associated loci (“lead loci”), we used a LD-based clumping method implemented in PLINK to group SNPs that passed the adjusted genome-wide significance thresholds over a large genomic region flanking the index SNP [50]. Applying the most stringent clumping parameters (r^2 = 0.1 and sliding window size = 12,150kb, S3 Table), we identified 110 significant lead loci in 49 out of 79 F_{34} phenotypes using the GBS

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.

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).

180

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

190 replicated at nominal levels of significance ($p<0.05$) in $F_{39\text{-}43}$; similarly, the only body weight
191 locus identified in $F_{39\text{-}43}$ 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 $F_{39\text{-}43}$ (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).

			Discovery set				Replication set			
Phenotype	**rG(*s.e.*)(*p<0.05)**	**SNP**	**P**	**af**	**beta**	**se**	**P**	**af**	**beta**	**se**
			F34 GBS				**F3943 GBS replicate**			
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).

204

205

Table 2. Lead SNPs in F_{34} and $F_{39\text{-}43}$ (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 $F_{39\text{-}43}$ 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, $F_{39\text{-}43}$ GBS, combined set

206

207

208

209

210

211

212

Discussion

Outbred model organism populations have become important resources for genetics studies. We used F_{34} and $F_{39\text{-}43}$ generations of a LG/J x SM/J AIL to investigate how SNP density influences GWAS results, heritability, and genetic correlations. Using the ~60K SNPs in the F_{34} GBS set, we found 110 loci, of which 36 were unique to the GBS genotypes. Notably, F_{34} array genotypes were able to detect the majority of loci that were identified in F_{34} GBS SNP set, indicating that many but not all loci were successfully 'tagged' by the sparse array SNPs. Although the genetic correlations between traits measured in F_{34} and $F_{39\text{-}43}$ were high, replication was incomplete, with the locomotor traits showing especially poor replication. Finally, imputation to reference panels increased the number of SNPs available for analysis but did not meaningfully enhance the number of loci we discovered because it did not improve our ability to capture recombination events.

Previous work from our lab used the array genotypes described in this study to map QTLs for various behavioral and physiological traits in 688 F_{34} AILs. With F_{34} GBS SNPs, which were only available for 428 of the initial 688 AIL mice, we were able to replicate many of our previously published loci. For instance, using the array SNPs in the F_{34} generation in conjunction with the LMM software QTLRel, Cheng et al. [35] found a significant locus on chromosome 8 for locomotor day 2 activity that contained only one gene: *Csmd1* (CUB and sushi multiple domains 1). Gonzales et al. [39] replicated this finding in $F_{50\text{-}56}$ AIL and identified a *cis*-eQTL mapped to the same region. *Csmd1* mutant mice showed increased locomotor activity compared to wild-type and heterozygous mice, indicating that *Csmd1* is likely a causal gene for locomotor and related traits [39]. We replicated this locus in our own analysis of the F_{34} array and GBS SNP, using GEMMA rather than QTLRel (S7 Fig). We also replicated a locus on chromosome 17 for distance traveled in the periphery in the open field test (Fig 4; [36,39]), three loci on chromosomes 4, 6, and 14 for body weight (Supplementary Fig 7; [54]), one locus on

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 $F_{39\text{-}43}$ 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 $F_{39\text{-}43}$ 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 $F_{39\text{-}43}$ data were collected were not ideal, likely contributing to
253 their lower heritability and the limited replication. Unlike the F_{34} dataset, the $F_{39\text{-}43}$ 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 $F_{39\text{-}43}$ 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 $F_{39\text{-}43}$ 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.

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 $F_{39\text{-}43}$ 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, $F_{39\text{-}43}$ 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 $F_{39\text{-}43}$ AIL mice
285 allowed us to identify novel loci for locomotor activity, open field test, fear conditioning, light dark

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].
289

290 # Materials and Methods

291 ## Animals

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.

304

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

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

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 $F_{39\text{-}43}$ 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

315 **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

320 **F_{34} and $F_{39\text{-}43}$ GBS Genotypes**

321 F_{34} and $F_{39\text{-}43}$ 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 $p < 1.0\times10^{-6}$ 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

QC of individuals

We have found that large genetic studies are often hampered by cross-contamination between samples and sample mix-ups. We used four features of the data to identify problematic samples: heterozygosity distribution, proportion of reads aligned to sex chromosomes, pedigree/kinship, and coat color. We first examined heterozygosity across autosomes and removed animals where the proportion of heterozygosity that was more than 3 standard deviations from the mean (S1 Fig). Next, we sought to identify animals in which the recorded sex did not agree with the sequencing data. We compared the ratio of reads mapped to the X and Y chromosomes. The 95% CI for this ratio was 196.84 to 214.3 in females and 2.13 to 2.18 in males. Twenty-two F_{34} and $F_{39\text{-}43}$ animals were removed because their sex (as determined by reads ratio) did not agree with their recorded sex; we assumed this discrepancy was due to sample mix-ups. To further identify mislabeled samples, we calculated kinship coefficients based on the full AIL pedigree using QTLRel. We then calculated a genetic relatedness matrix (**GRM**) using IBDLD, which estimates identity by descent using genotype data. The comparison between pedigree kinship relatedness and genetic kinship relatedness identified 7 pairs of animals that showed obvious disagreement between kinship coefficients and the GRM. Lastly, we excluded 14 $F_{39\text{-}43}$ animals that showed discordance between their recorded coat color and their genotypes at markers flanking *Tyr*, which causes albinism in mice. The numbers of animals filtered at each step are listed in S2 Table. Some animals were detected by more than one QC step, substantiating our belief that these samples were erroneous.

At the end of SNP and sample filtering, we had 59,561 autosomal and 831 X chromosome SNPs in F_{34}, 58,966 autosomal and 824 X chromosome SNPs in $F_{39\text{-}43}$, and 57,635 autosomal and 826 X chromosome SNPs in the combined F_{34} and $F_{39\text{-}43}$ set (S2 Table). GBS genotype quality was estimated by examining concordance between the 66 SNPs that were present in both the array and GBS genotyping results.

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 $F_{39\text{-}43}$ 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.

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 $F_{39\text{-}43}$ 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

390 **Identifying suspicious SNPs**

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

399 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

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

Genetic correlation and heritability estimates between F_{34} and $F_{39\text{-}43}$ phenotypes

Locomotor activity, body weight, and coat color had been measured in both F_{34} and $F_{39\text{-}43}$ populations. We calculated both SNP heritability and genetic correlations between F_{34} and $F_{39\text{-}43}$ animals using GCTA bivariate GREML analysis [69]. Because $F_{39\text{-}43}$ day 1 locomotor activity data were not normally distributed, we quantile normalized locomotor activity data when estimating SNP heritabilities and genetic correlations.

LocusZoom Plots

LocusZoom plots were generated using the standalone implementation of LocusZoom [65], using LD scores calculated from PLINK v.1.9 --ld option and mm10 gene annotation file downloaded from USCS genome browser.

Ethics Statement

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC protocol: S15226) Euthanasia was accomplished using CO_2 asphyxiation followed by cervical dislocation

.

Acknowledgements

We would like to recognize Jackie Lim and Kaitlin Samocha for collecting F_{34} AIL phenotype data and Ryan Walters for collecting $F_{39\text{-}43}$ AIL phenotype data. We wish to acknowledge Alex Gileta for input on a draft of this manuscript.

447

448

449

450

451

452 # References

453 1. Parker CC, Gopalakrishnan S, Carbonetto P, Gonzales NM, Leung E, Park YJ, et al. Genome-wide
454 association study of behavioral, physiological and gene expression traits in outbred CFW mice. Nat
455 Genet. 2016;48(8):919.

456 2. Gatti DM, Svenson KL, Shabalin A, Wu L-Y, Valdar W, Simecek P, et al. Quantitative trait locus
457 mapping methods for diversity outbred mice. G3 Genes Genomes Genet. 2014;4(9):1623–1633.

458 3. Chesler EJ. Out of the bottleneck: the Diversity Outcross and Collaborative Cross mouse
459 populations in behavioral genetics research. Mamm Genome. 2014;25(1–2):3–11.

460 4. Churchill GA, Gatti DM, Munger SC, Svenson KL. The diversity outbred mouse population. Mamm
461 Genome. 2012;23(9–10):713–718.

462 5. Consortium CC. The genome architecture of the Collaborative Cross mouse genetic reference
463 population. Genetics. 2012;190(2):389–401.

464 6. Talbot CJ, Nicod A, Cherny SS, Fulker DW, Collins AC, Flint J. High-resolution mapping of
465 quantitative trait loci in outbred mice. Nat Genet. 1999;21(3):305.

466 7. Demarest K, Koyner J, McCaughran J, Cipp L, Hitzemann R. Further characterization and high-
467 resolution mapping of quantitative trait loci for ethanol-induced locomotor activity. Behav Genet.
468 2001;31(1):79–91.

469 8. Valdar W, Solberg LC, Gauguier D, Burnett S, Klenerman P, Cookson WO, et al. Genome-wide
470 genetic association of complex traits in heterogeneous stock mice. Nat Genet. 2006;38(8):879.

471 9. Ghazalpour A, Doss S, Kang H, Farber C, Wen P-Z, Brozell A, et al. High-resolution mapping of gene
472 expression using association in an outbred mouse stock. PLoS Genet. 2008;4(8):e1000149.

473 10. Svenson KL, Gatti DM, Valdar W, Welsh CE, Cheng R, Chesler EJ, et al. High-resolution genetic
474 mapping using the Mouse Diversity outbred population. Genetics. 2012;190(2):437–447.

475 11. Yalcin B, Willis-Owen SA, Fullerton J, Meesaq A, Deacon RM, Rawlins JNP, et al. Genetic dissection
476 of a behavioral quantitative trait locus shows that Rgs2 modulates anxiety in mice. Nat Genet.
477 2004;36(11):1197.

478 12. Nicod J, Davies RW, Cai N, Hassett C, Goodstadt L, Cosgrove C, et al. Genome-wide association of
479 multiple complex traits in outbred mice by ultra-low-coverage sequencing. Nat Genet.
480 2016;48(8):912.

481 13. Carbonetto P, Cheng R, Gyekis JP, Parker CC, Blizard DA, Palmer AA, et al. Discovery and
482 refinement of muscle weight QTLs in B6$\times$ D2 advanced intercross mice. Physiol Genomics.
483 2014;46(16):571–582.

484 14. Coyner J, McGuire JL, Parker CC, Ursano RJ, Palmer AA, Johnson LR. Mice selectively bred for High
485 and Low fear behavior show differences in the number of pMAPK (p44/42 ERK) expressing neurons
486 in lateral amygdala following Pavlovian fear conditioning. Neurobiol Learn Mem. 2014;112:195–
487 203.

488 15. Parker CC, Cheng R, Sokoloff G, Palmer AA. Genome-wide association for methamphetamine
489 sensitivity in an advanced intercross mouse line. Genes Brain Behav. 2012;11(1):52–61.

490 16. Samocha KE, Lim JE, Cheng R, Sokoloff G, Palmer AA. Fine mapping of QTL for prepulse inhibition in
491 LG/J and SM/J mice using F2 and advanced intercross lines. Genes Brain Behav. 2010;9(7):759–
492 767.

493 17. Hernandez Cordero AI, Carbonetto P, Riboni Verri G, Gregory JS, Vandenbergh DJ, P Gyekis J, et al.
494 Replication and discovery of musculoskeletal QTLs in LG/J and SM/J advanced intercross lines.
495 Physiol Rep. 2018;6(4).

496 18. Baud A, Guryev V, Hummel O, Johannesson M, Hermsen R, Stridh P, et al. Genomes and phenomes
497 of a population of outbred rats and its progenitors. Sci Data. 2014;1:140011.

498 19. Besnier F, Wahlberg P, Rönnegard L, Ek W, Andersson L, Siegel PB, et al. Fine mapping and
499 replication of QTL in outbred chicken advanced intercross lines. Genet Sel Evol. 2011;43(1):3.

500 20. Johnsson M, Henriksen R, Höglund A, Fogelholm J, Jensen P, Wright D. Genetical genomics of
501 growth in a chicken model. BMC Genomics. 2018;19(1):72.

502 21. Guryev V, Koudijs MJ, Berezikov E, Johnson SL, Plasterk RH, Van Eeden FJ, et al. Genetic variation in
503 the zebrafish. Genome Res. 2006;16(4]:491–497.

504 22. Patowary A, Purkanti R, Singh M, Chauhan R, Singh AR, Swarnkar M, et al. A sequence-based
505 variation map of zebrafish. Zebrafish. 2013;10(1]:15–20.

506 23. Mackay TF, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, et al. The Drosophila
507 melanogaster genetic reference panel. Nature. 2012;482(7384):173.

508 24. Vonesch SC, Lamparter D, Mackay TF, Bergmann S, Hafen E. Genome-wide analysis reveals novel
509 regulators of growth in Drosophila melanogaster. PLoS Genet. 2016;12(1):e1005616.

510 25. King EG, Macdonald SJ, Long AD. Properties and power of the Drosophila Synthetic Population
511 Resource for the routine dissection of complex traits. Genetics. 2012;genetics–112.

512 26. Kislukhin G, King EG, Walters KN, Macdonald SJ, Long AD. The genetic architecture of
513 methotrexate toxicity is similar in Drosophila melanogaster and humans. G3 Genes Genomes
514 Genet. 2013;g3–113.

515 27. Marriage TN, King EG, Long AD, Macdonald SJ. Fine-mapping nicotine resistance loci in Drosophila
516 using a multiparent advanced generation inter-cross population. Genetics. 2014;198(1):45–57.

517 28. Doitsidou M, Jarriault S, Poole RJ. Next-generation sequencing-based approaches for mutation
518 mapping and identification in Caenorhabditis elegans. Genetics. 2016;204(2):451–474.

519 29. Diouf IA, Derivot L, Bitton F, Pascual L, Causse M. Water Deficit and Salinity Stress Reveal Many
520 Specific QTL for Plant Growth and Fruit Quality Traits in Tomato. Front Plant Sci. 2018;9:279.

521 30. Cockram J, Mackay I. Genetic Mapping Populations for Conducting High-Resolution Trait Mapping
522 in Plants. 2018;

523 31. Rishmawi L, Bühler J, Jaegle B, Hülskamp M, Koornneef M. Quantitative trait loci controlling leaf
524 venation in Arabidopsis. Plant Cell Environ. 2017;40(8):1429–1441.

525 32. Parker CC, Palmer AA. Dark matter: are mice the solution to missing heritability? Front Genet.
526 2011;2:32.

527 33. Darvasi A, Soller M. Advanced intercross lines, an experimental population for fine genetic
528 mapping. Genetics. 1995;141(3):1199–1207.

529 34. Gonzales NM, Palmer AA. Fine-mapping QTLs in advanced intercross lines and other outbred
530 populations. Mamm Genome. 2014;25(7–8):271–292.

531 35. Cheng R, Lim JE, Samocha KE, Sokoloff G, Abney M, Skol AD, et al. Genome-wide association
532 studies and the problem of relatedness among advanced intercross lines and other highly
533 recombinant populations. Genetics. 2010;

534 36. Parker CC, Carbonetto P, Sokoloff G, Park YJ, Abney M, Palmer AA. High-resolution genetic
535 mapping of complex traits from a combined analysis of F2 and advanced intercross mice. Genetics.
536 2014;198(1):103–116.

537 37. Lionikas A, Cheng R, Lim JE, Palmer AA, Blizard DA. Fine-mapping of muscle weight QTL in LG/J and
538 SM/J intercrosses. Physiol Genomics. 2010;42(1):33–38.

539 38. Carroll AM, Cheng R, Collie-Duguid ESR, Meharg C, Scholz ME, Fiering S, et al. Fine-mapping of
540 genes determining extrafusal fiber properties in murine soleus muscle. Physiol Genomics.
541 2017;49(3):141–150.

542 39. Gonzales NM, Seo J, Hernandez-Cordero AI, Pierre CLS, Gregory JS, Distler MG, et al. Genome wide
543 association study of behavioral, physiological and gene expression traits in a multigenerational
544 mouse intercross. bioRxiv. 2017 Dec 8;230920.

545 40. Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, et al. A robust, simple
546 genotyping-by-sequencing (GBS) approach for high diversity species. PloS One. 2011;6(5):e19379.

547 41. Davey JW, Hohenlohe PA, Etter PD, Boone JQ, Catchen JM, Blaxter ML. Genome-wide genetic
548 marker discovery and genotyping using next-generation sequencing. Nat Rev Genet.
549 2011;12(7):499.

550 42. Fitzpatrick CJ, Gopalakrishnan S, Cogan ES, Yager LM, Meyer PJ, Lovic V, et al. Variation in the form
551 of Pavlovian conditioned approach behavior among outbred male Sprague-Dawley rats from
552 different vendors and colonies: sign-tracking vs. goal-tracking. PloS One. 2013;8(10):e75042.

553 43. Toker L, Feng M, Pavlidis P. Whose sample is it anyway? Widespread misannotation of samples in
554 transcriptomics studies. F1000Research (Internet). 2016 Sep 30 (cited 2018 Jul 11);5. Available
555 from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5034794/

556 44. Zhou X, Stephens M. Genome-wide efficient mixed-model analysis for association studies. Nat
557 Genet. 2012;44(7):821.

558 45. Listgarten J, Lippert C, Kadie CM, Davidson RI, Eskin E, Heckerman D. Improved linear mixed
559 models for genome-wide association studies. Nat Methods. 2012;9(6]:525.

560 46. Cheng R, Parker CC, Abney M, Palmer AA. Practical considerations regarding the use of genotype
561 and pedigree data to model relatedness in the context of genome-wide association studies. G3
562 Genes Genomes Genet. 2013;g3–113.

563 47. Yang J, Zaitlen NA, Goddard ME, Visscher PM, Price AL. Advantages and pitfalls in the application of
564 mixed-model association methods. Nat Genet. 2014;46(2):100.

565 48. Joo JWJ, Hormozdiari F, Han B, Eskin E. Multiple testing correction in linear mixed models. Genome
566 Biol. 2016;17(1):62.

567 49. Han B, Kang HM, Eskin E. Rapid and accurate multiple testing correction and power estimation for
568 millions of correlated markers. PLoS Genet. 2009;5(4):e1000456.

569 50. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for
570 whole-genome association and population-based linkage analyses. Am J Hum Genet.
571 2007;81(3):559–575.

572 51. Nikolskiy I, Conrad DF, Chun S, Fay JC, Cheverud JM, Lawson HA. Using whole-genome sequences
573 of the LG/J and SM/J inbred mouse strains to prioritize quantitative trait genes and nucleotides.
574 BMC Genomics. 2015;16(1):415.

575 52. Browning SR, Browning BL. Rapid and accurate haplotype phasing and missing-data inference for
576 whole-genome association studies by use of localized haplotype clustering. Am J Hum Genet.
577 2007;81(5):1084–1097.

578 53. Yang J, Zeng J, Goddard ME, Wray NR, Visscher PM. Concepts, estimation and interpretation of
579 SNP-based heritability. Nat Genet. 2017 Sep;49(9):1304–10.

580 54. Parker CC, Cheng R, Sokoloff G, Lim JE, Skol AD, Abney M, et al. Fine-mapping alleles for body
581 weight in LG/J × SM/J F<Subscript>2</Subscript> and F<Subscript>34</Subscript> advanced
582 intercross lines. Mamm Genome. 2011 Oct 1;22(9–10):563.

583 55. Bartnikas TB, Parker CC, Cheng R, Campagna DR, Lim JE, Palmer AA, et al. QTLs for murine red
584 blood cell parameters in LG/J and SM/J F2 and advanced intercross lines. Mamm Genome. 2012
585 Jun;23(5–6):356–66.

586 56. Cheng R, Abney M, Palmer AA, Skol AD. QTLRel: an R package for genome-wide association studies
587 in which relatedness is a concern. BMC Genet. 2011 Jul 27;12:66.

588 57. Logan RW, Robledo RF, Recla JM, Philip VM, Bubier JA, Jay JJ, et al. High-precision genetic mapping
589 of behavioral traits in the diversity outbred mouse population. Genes Brain Behav.
590 2013;12(4):424–437.

591 58. Cervino AC, Li G, Edwards S, Zhu J, Laurie C, Tokiwa G, et al. Integrating QTL and high-density SNP
592 analyses in mice to identify Insig2 as a susceptibility gene for plasma cholesterol levels. Genomics.
593 2005;86(5):505–517.

594 59. Conneally PM, Edwards JH, Kidd KK, Lalouel J-M, Morton NE, Ott J, et al. Report of the committee
595 on methods of linkage analysis and reporting. Cytogenet Genome Res. 1985;40(1–4):356–9.

596 60. Lander ES, Botstein D. Mapping mendelian factors underlying quantitative traits using RFLP linkage
597 maps. Genetics. 1989;121(1):185–199.

598 61. Mangin B, Goffinet B, Rebai A. Constructing confidence intervals for QTL location. Genetics.
599 1994;138(4):1301–1308.

600 62. Dupuis J, Siegmund D. Statistical methods for mapping quantitative trait loci from a dense set of
601 markers. Genetics. 1999;151(1):373–386.

602 63. Manichaikul A, Dupuis J, Sen S, Broman KW. Poor performance of bootstrap confidence intervals
603 for the location of a quantitative trait locus. Genetics. 2006;

604 64. Sequencing RG, Baud A, Hermsen R, Guryev V, Stridh P, Graham D, et al. Combined sequence-
605 based and genetic mapping analysis of complex traits in outbred rats. Nat Genet. 2013;45(7):767.

606 65. Pruim RJ, Welch RP, Sanna S, Teslovich TM, Chines PS, Gliedt TP, et al. LocusZoom: regional
607 visualization of genome-wide association scan results. Bioinformatics. 2010;26(18):2336–2337.

608 66. Gardenghi S, Marongiu MF, Ramos P, Guy E, Breda L, Chadburn A, et al. Ineffective erythropoiesis
609 in β-thalassemia is characterized by increased iron absorption mediated by down-regulation of
610 hepcidin and up-regulation of ferroportin. Blood. 2007;109(11):5027–5035.

611 67. Graziano JH, Grady RW, Cerami A. The identification of 2, 3-dihydroxybenzoic acid as a potentially
612 useful iron-chelating drug. J Pharmacol Exp Ther. 1974;190(3):570–575.

613 68. Korneliussen TS, Albrechtsen A, Nielsen R. ANGSD: analysis of next generation sequencing data.
614 BMC Bioinformatics. 2014;15(1):356.

69. Yang J, Lee SH, Goddard ME, Visscher PM. GCTA: a tool for genome-wide complex trait analysis. Am J Hum Genet. 2011 Jan 7;88(1):76–82.

Main figure legends

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

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

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

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

Supporting information

S1 Fig. Autosomal heterozygosity distribution in F_{34}, $F_{39\text{-}43}$ 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 $F_{39\text{-}43}$ 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, $F_{39\text{-}43}$ GBS, and F_{34} and $F_{39\text{-}43}$ 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, $F_{39\text{-}43}$ 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.

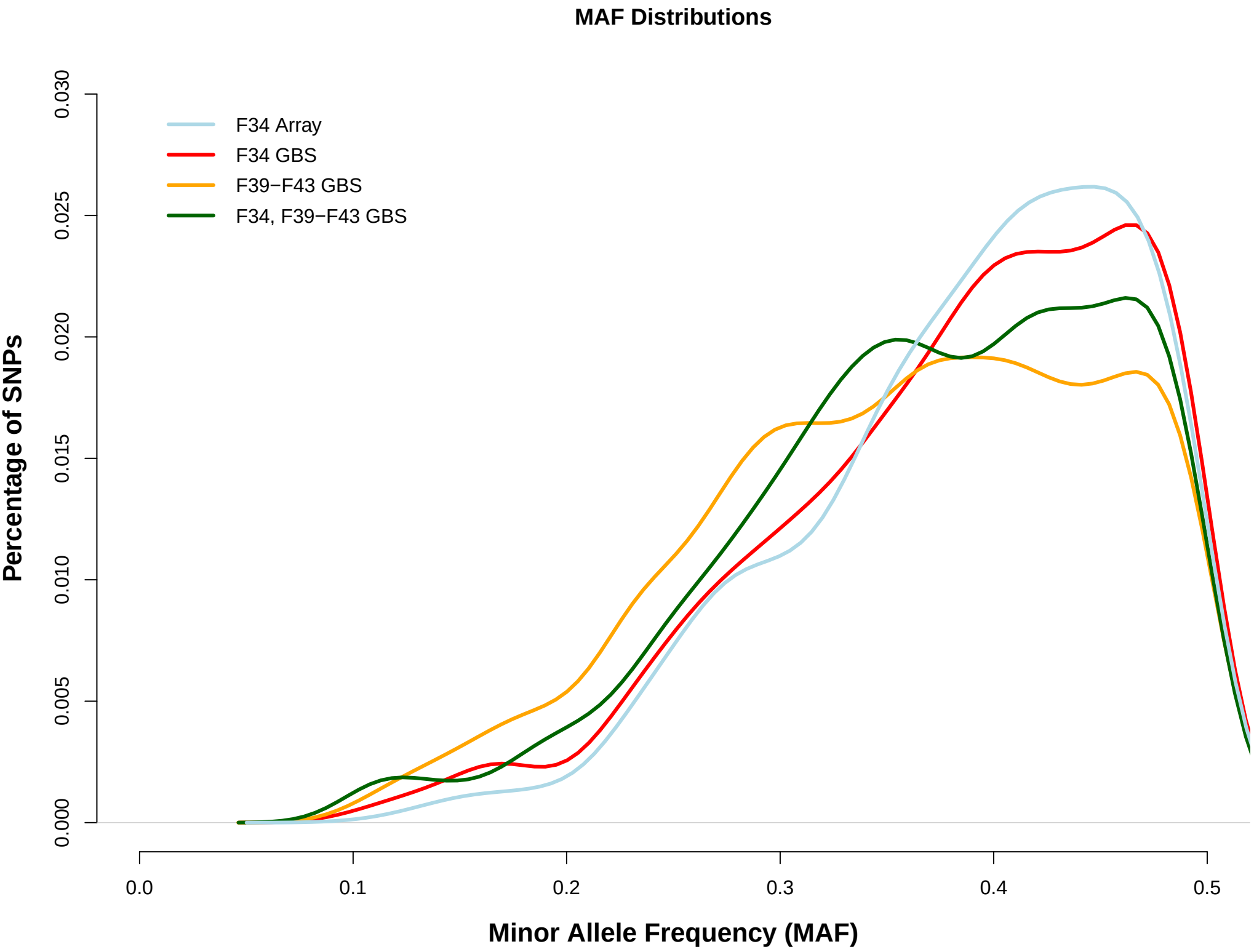
S5 Table. F_{34} GBS and array SNP heritability estimates.

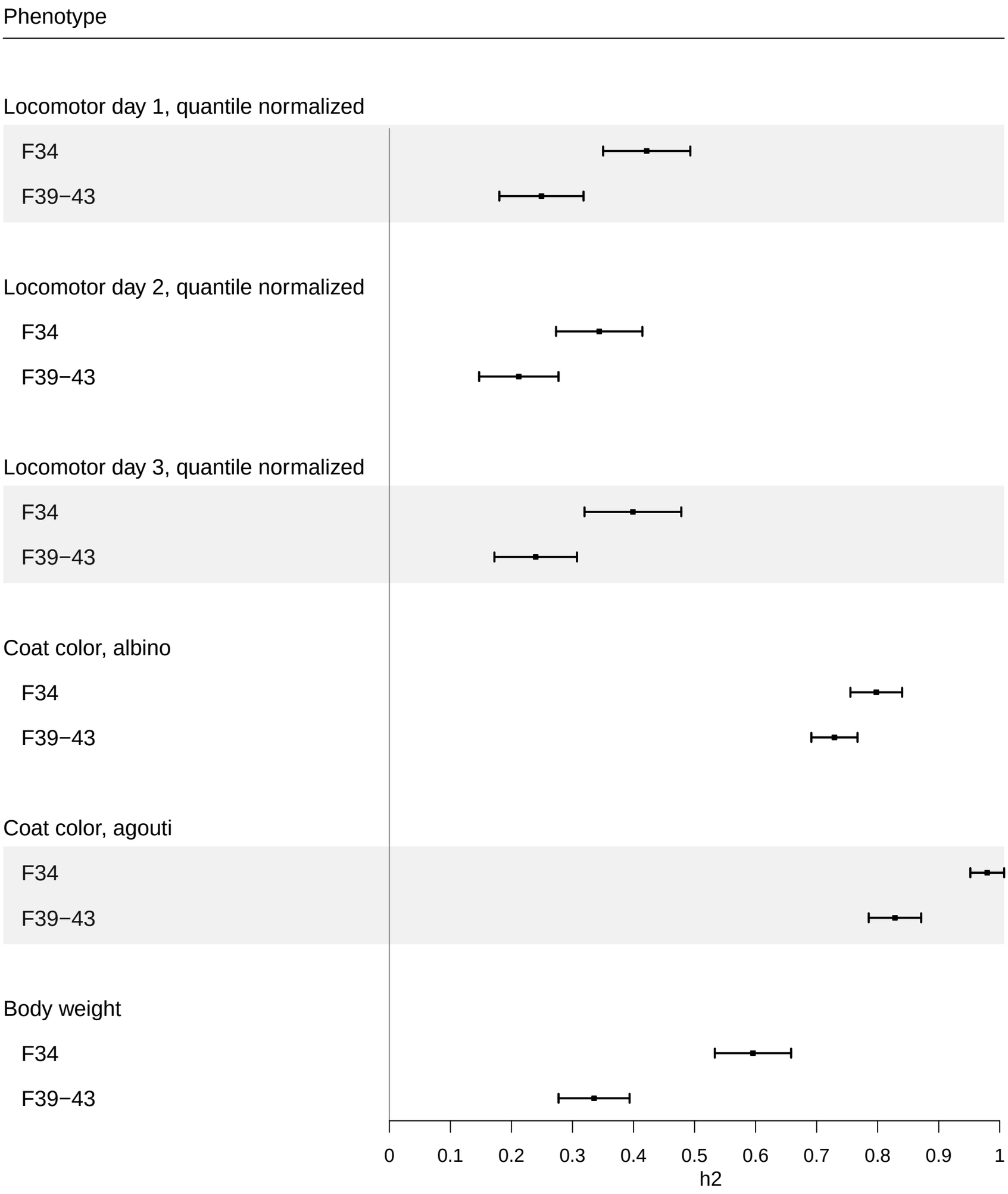
S6 Table. F_{34} and $F_{39\text{-}43}$ genetic correlations in locomotor activity, coat color, and body weight.

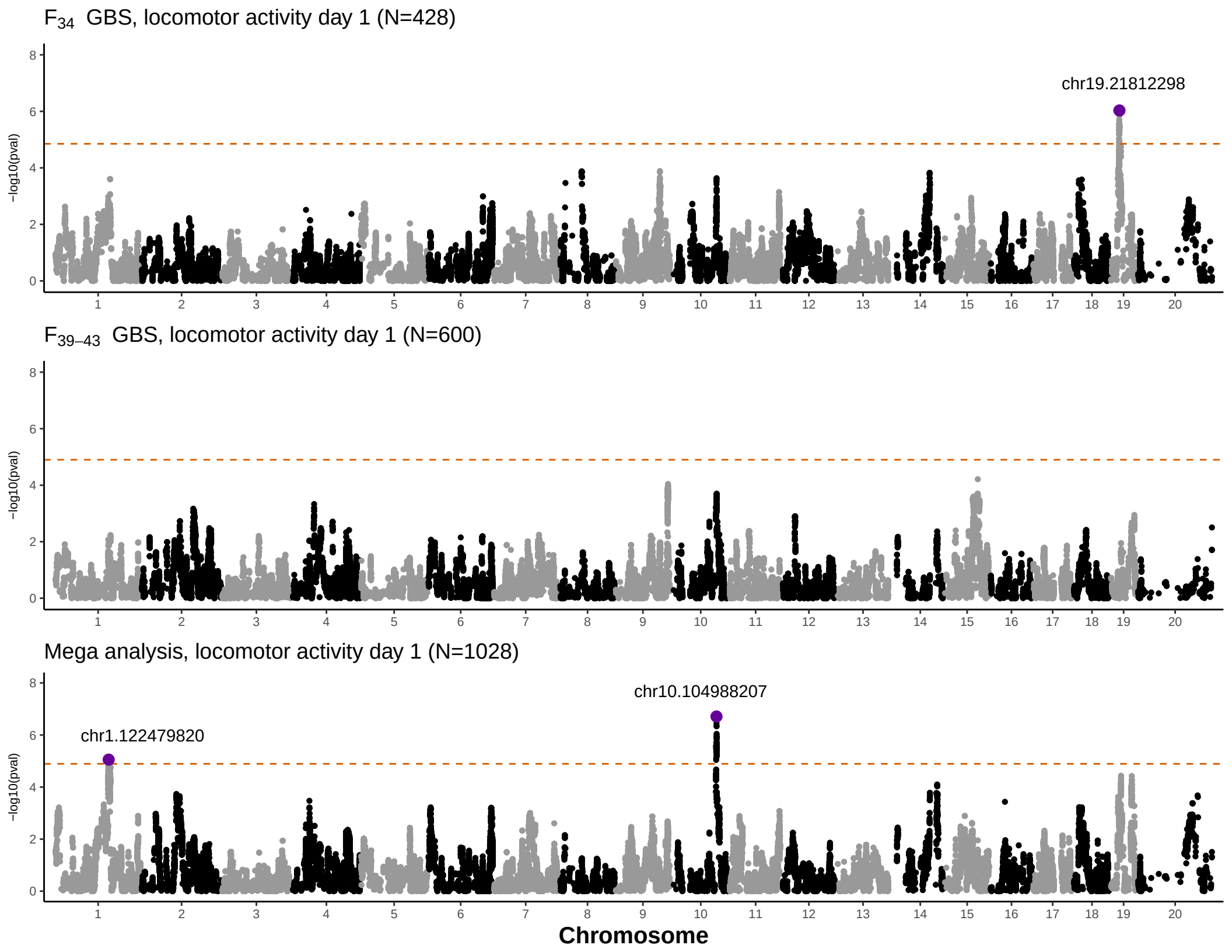
S7 Table. SNP-heritability comparison between F_{34} and $F_{39\text{-}43}$ GBS.

694
695 **S8 Table. Lead QTL in $F_{39\text{-}43}$ 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 $F_{39\text{-}43}$ (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 $F_{39\text{-}43}$ 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.**

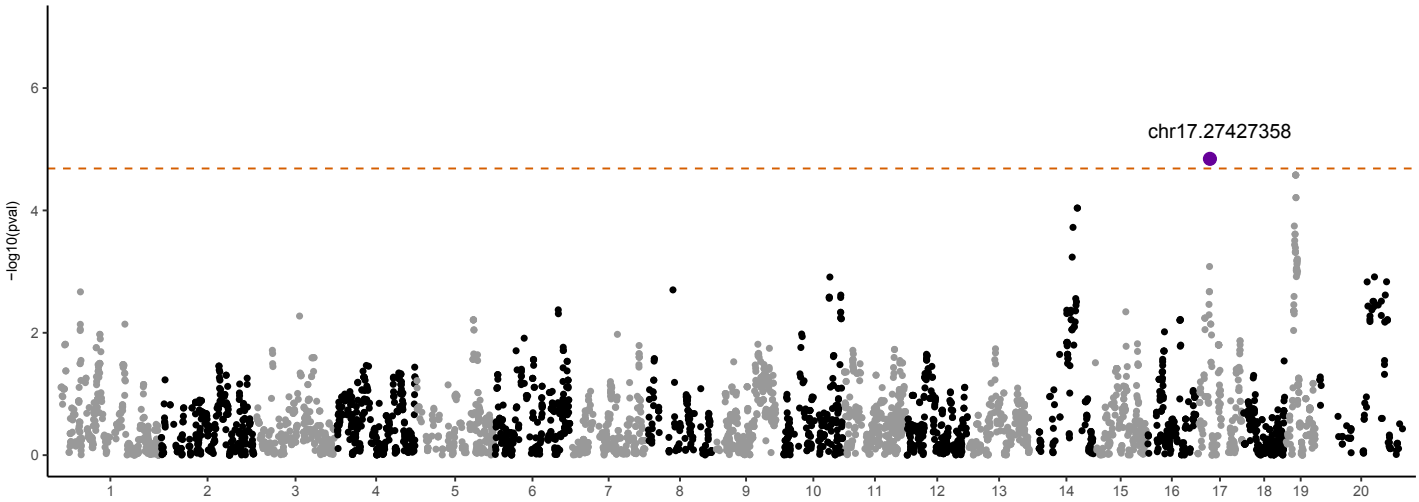

MAF Distributions
F34 Array
F34 GBS
F39−F43 GBS
F34, F39−F43 GBS
Percentage of SNPs
0.000
0.005
0.010
0.015
0.020
0.025
0.030
0.0
0.1
0.2
0.3
0.4
0.5
Minor Allele Frequency (MAF)


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

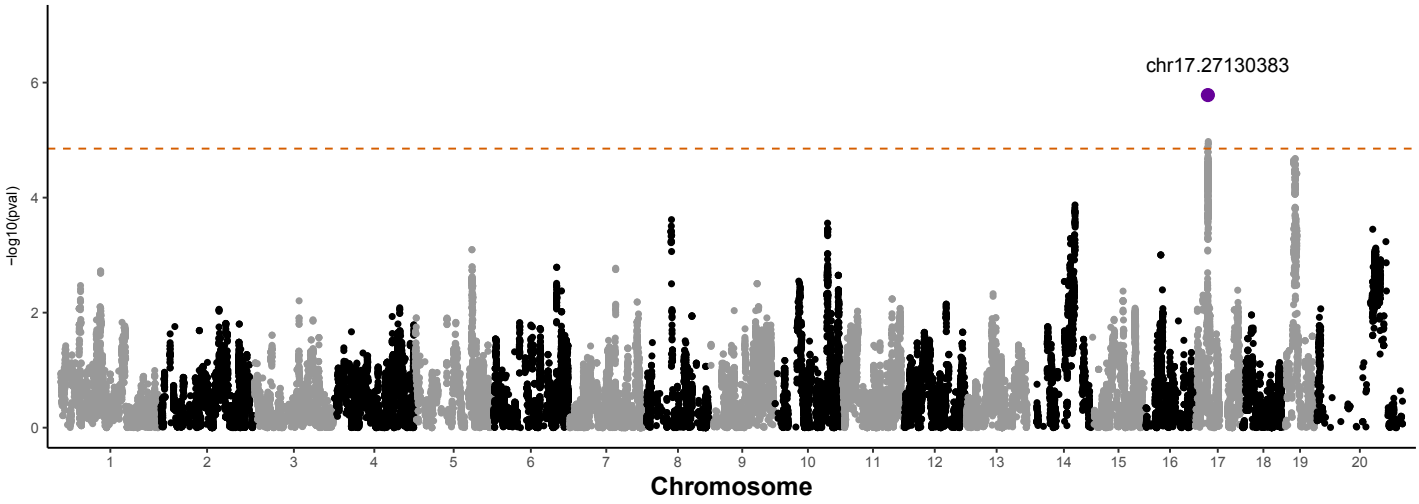

F34 GBS, locomotor activity day 1 (N=428)
chr19.21812298
−log10(pval)
F39–43 GBS, locomotor activity day 1 (N=600)
−log10(pval)
Mega analysis, locomotor activity day 1 (N=1028)
chr10.104988207
chr1.122479820
−log10(pval)
Chromosome

a

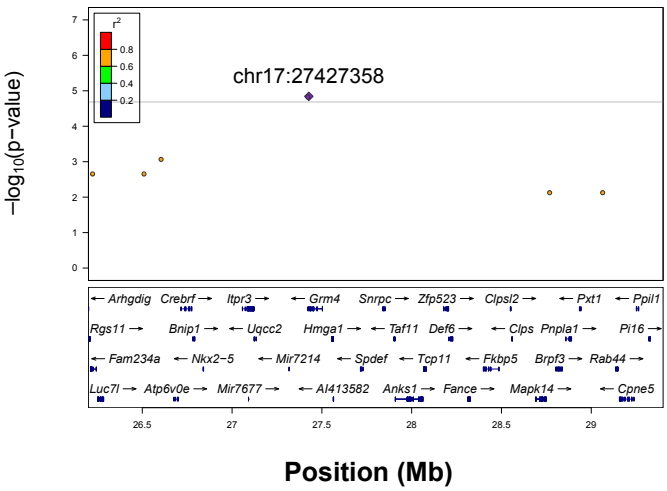
F_{34} array, open filed, distance traveled in periphery



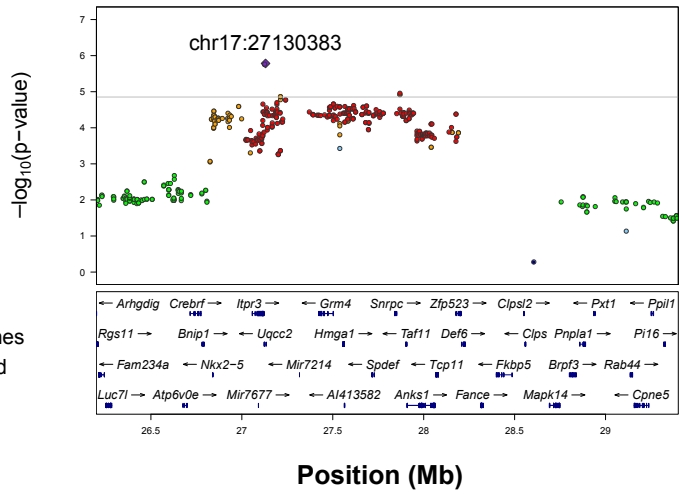
F_{34} GBS, open filed, distance traveled in periphery



b



46 genes omitted



46 genes omitted