Genetic dissection of behavioral traits related to successful training in drug-detection dogs

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Abstract

Drug detection dogs play integral roles in society; however, the interplay between their behaviors and genetic characteristics remains uninvestigated. To profile the genetic traits associated with various behaviors related to the successful training of drug detection dogs, we collected and analyzed more than 230,000 genetic variants from 326 dogs belonging to the German Shepherd or Labrador Retriever breeds. Behavioral breed differences were observed in 'friendliness to humans' and 'tolerance to dogs'. A strong positive correlation was observed in the genomic heritability of behavioral traits between breeds (Pearson's $r = 0.964$, $P = 0.008$), indicating a similar degree of genetic influence on the behavioral traits between breeds. A genome-wide association study identified 18 single nucleotide polymorphisms potentially associated with drug detection abilities and three behavioral traits (interest in the dummy, tolerance to dogs, and friendliness to humans) related to drug detection abilities. Among them, 61 protein coding genes, including those associated with anxiety-related or exploration behavior in mice, such as *Atat1* and *Pfn2*, were located surrounding the candidate polymorphisms. These findings highlight genetic characteristics associated with behavioral traits that are important for the successful training of drug detection dogs, which might support improved breeding and training of these dogs.

Introduction

Dogs are one of the oldest domesticated animals worldwide and play important roles as companions and working dogs. To ensure their suitability for such roles, directional selection of behavioral traits with genetic alterations has been performed[1]. Particularly for working dogs, behavioral traits such as herding have been selected during the domestication process[2].

Drug detection dogs are working dogs widely used for tasks aiming to finding objects with drug odors, and are used for interdicting the smuggling of prohibited drugs in transport (e.g., airport, port) in Japan. Compared to other working dogs, including herding dogs, guide dogs, and rescue dogs, they should have an acute ability to detect chemical odors. However, due to the limited number of studies on the use of drug detection dogs[3], limited information is available regarding the association between drug detection behavioral traits and genetic characteristics. Such scientific evidence could prove useful for the efficient selection of appropriate dogs for drug detection, as well as for strengthening drug detection abilities through selective breeding.

Over the past decades, the genetic basis for behavioral traits in dogs have been widely assessed using several genetic markers based on the knowledge of human behavior (i.e., the candidate gene approach). For example, the dopamine receptor D4 gene (*DRD4*) and serotonin transporter 1A gene (*5HTT*) have been investigated to explore the relationship between genetic polymorphisms and behavioral traits in dogs[4–7].

A more comprehensive analysis using genome-wide single nucleotide polymorphisms (SNPs) has also been used to uncover genetic regions associated with behavioral traits related to dog breed stereotypes[8]. Genome-wide association studies (GWAS) with large numbers of genetic variants and large sample sizes

have successfully uncovered associations between various genetic variants and individual differences in behavioral traits (i.e., personality) in dogs[9–12]. For odor detection dogs, genetic polymorphisms in olfactory receptor genes and oxytocin receptor genes have been shown to be associated with performance in odor detection tasks and training success[13, 14]. To date, however, no GWAS studies have been conducted on odor detection dogs.

In this study, we adopted these techniques to evaluate the genetic characteristics associated with behaviors related to successful training as drug detection dogs in two breeds, namely German Shepherds (GSs) and Labrador Retrievers (LRs). First, we characterized seven behavioral traits related to the qualification of drug detection using inter-breed comparisons. Second, we estimated the genomic heritability for each trait to assess the size of the genetic effect. Finally, we used GWAS to detect the candidate genetic regions associated with the behavior and qualification of drug detection dogs, and discussed the candidate SNPs and genes derived from a biological database.

Materials And Methods

Animals and Training

All dogs used in this study were either purebred German Shepherds (GSs, *n* = 127) or purebred Labrador Retrievers (LRs, *n* = 497). These two breeds are the most common dog breeds used as drug detection dogs by Japan Customs.

Dogs were trained to be drug detection dogs at the Canine Training Center, Tokyo Customs, between 2002 and 2019. They were provided by dog breeders (i.e., general dog population in Japan) at about 1 year of age. They did not receive any specific training for detection dogs until then. At the training center, dogs were kept in kennels and the animal management staff provided daily care in accordance with the relevant regulations in Japan. We could not obtain the precise pedigree data for confidentiality reasons.

The training method was based on positive reinforcement with social rewards (i.e., food rewards were not used). Canine trainers played tug-of-war with the dogs using a rolled towel with a target scent (i.e., reinforcer) so that dogs were motivated to search for an object with the scent. The training period lasted for about 4 months, and was divided into 3 phases (i.e., familiarization, basic training, and advanced training). The Familiarization phase was aimed at habituating the dogs to the training methods and to the novel environment of the training facility. Basic and advanced training phases were aimed at training the dogs to detect strong- and soft-scent drugs, respectively. At the end of each training phase, dog experts determined which dogs were suitable for scent work. Dogs that succeeded in all training sessions and phases were employed for drug detection at each field (e.g., airport, port) and were categorized as ‘qualified,’ whereas those that did not, were categorized as ‘unqualified.’

Finally, 121 GSs and 205 LRs were subjected to genetic analysis. The qualification ratios were 64/121 (52.9%) in GSs and 73/205 (35.6%) in LRs. Previous studies have suggested that the rate of successful training of working dogs reached 30% to 50%[15,16], which is consistent with the qualification rate in the

present study. Note that these ratios refer to the qualification rate of dogs for which detailed genetic analysis was possible in this study. Japan Customs reported that the qualification rate is about 30 % for both breeds. The outline of the training protocol has been described in further detail in previous studies[5,14]. This study was approved by the Ethics Committee of the Wildlife Research Center, Kyoto University (WRC 2010EC001) and performed in accordance with the relevant guidelines and regulations. This study used a non-invasive method based on behavioral observation, except for blood sampling. All methods using dogs in this study were reported in accordance with ARRIVE guidelines (https://www.nature.com/srep/journal-policies/editorial-policies#experimental-subjects).

Assessment of behavioral traits

The dogs' behavioral traits were assessed after 2 weeks of training in the Canine Training Center. We obtained scores of seven behavioral traits regarding suitability for scent work as follows: activity, boldness, concentration, friendliness to humans, independence, and interest in the target (dummy) (Table 1). Using a 5-point scale (i.e. 1 to 5) with a score of 5 indicating 'very high,' dog experts working at the training facility rated the extent to which a behavioral trait was applicable to each dog. The detailed procedure of behavioral assessment has been described in previous studies[5,14].

Behavioral differences based on sex, qualification status, and breed were evaluated using the Wilcoxon rank-sum test implemented in R software (version 3.6)[17]. Multiple comparisons (seven traits based on sex, qualification, and breeds) were conducted, and the Bonferroni correction was applied. The significance level of the corrected *P* value was 0.0014 (0.05/35).

Genotyping

Genomic DNA of all dogs was extracted from drawn blood, which was obtained via syringe, using a DNeasy Blood and Tissue Kit (Qiagen, CA, USA), according to manufacturer's instructions. The Canine 230K Consortium BeadChip Array (Illumina, CA, USA) for genome-wide SNP genotyping was then employed following the standard protocols provided by the manufacturer. Prior to genomic analyses, quality control for samples and markers was performed using PLINK v1.90[18] and v2.00a2LM with the following settings: missingness per dog < 0.05; miner allele frequency > 0.01; missingness per marker < 0.05; remove high linkage disequilibrium SNPs by –indep-pairwise option including 50 kb windows, 10 SNPs step, and r^2 threshold 0.1; sex-check by confirming genetic sex and interview sheet; remove genetically identical dogs based on kinship value exceeding 0.354; exclude mitochondrial DNA, sex chromosomes, and unlocalized SNPs.

Population genetic analysis

We determined the inbreeding coefficient and performed principal component analysis (PCA) to evaluate the population genetic structure in target populations. The inbreeding coefficient considering runs of homozygosity (F_{ROH}) was calculated using PLINK v1.90 and detectRUNS v.0.9.6 R package (options: maxOppRun = 0, maxMissRun = 0, minSNP = 2, minLengthBps = 100, and maxGap = 500,000), and was

based on the methods of sliding windows[19] and consecutive runs[20]. The difference in F_{ROH} between breeds were tested using the Welch Two Sample t-test implemented in R. Statistical significance was set at 0.05. PCA was performed using the --pca option implemented in PLINK v1.90.

Genomic heritability estimation

The genomic heritability for each trait and qualification was estimated using Genome-wide Complex Trait Analysis (GCTA) software v1.91.7beta[21]. The log-likelihood ratio test was used to estimate genetic variance, residual variance, phenotypic variance, and standard errors. Genomic heritability was estimated based on genome-wide SNP data as a ratio of genetic variance to phenotypic variance. SNP heritability (h^2_{SNP}) was tested using the Genomic Restricted Maximum Likelihood (GREML) method implemented in GCTA software.

Further, the Pearson's product-moment correlation coefficient was used to reveal the relationships of heritability estimates between breeds. The significance level was set as 0.05 for each heritability analysis.

Genome-wide association study

In the GWAS, a linear mixed model was fitted using GEMMA software[22]. We tested the associations between SNP genotypes and behavioral scores for seven traits (1 to 5) or binary for qualification (qualified or unqualified). To handle the effect of population structure on the GWAS, we used a centered relatedness matrix option (-gk 1) in GEMMA as a random effect. P-values were calculated using the Wald test. The genomic inflation factor (λ) was calculated using the R package GenABEL v. 1.8. to estimate the effect of the population genetic structure on GWAS results. The λ values were estimated using a regression model. To address the multiple testing problem, we used a test measuring the proportion of false positives incurred (the false discovery rate, FDR). The FDR was calculated using p.adjust function in R software (version 3.6). The genome-wide significance and suggestive level thresholds for adjusted p-values were set at 0.05 and 0.10, respectively.

Candidate SNP and gene analysis

A subsequent analysis based on linkage disequilibrium was performed to identify the potentially effective genes for each trait. We denoted the candidate loci associated with traits as follows. The candidate SNPs exceeding significance and suggestive level thresholds were subjected to uncover the candidate genes. All gene data sets were obtained from Ensembl genome browser (release 104, CanFam 3.1). We denoted the length between adjacent SNPs as within 200 kb. When the SNP did not share the high-LD SNPs then the region located + /− 200 kb of the SNP was denoted as the same locus for target[12]. The bedtools v2.27.1 were used to extract the Ensembl gene IDs from the gene feature format (GTF) file downloaded from the Ensembl genome browser (release 104, CanFam 3.1).

To determine the functional classes of genes associated with the analyzed traits, we used the gene ontology (GO) enrichment analysis powered by PANTHER[23]. The target genes were selected using the

above linkage disequilibrium-based analysis. PANTHER Overrepresentation Test (Released 20210224) was used for GO enrichment analysis. We used “Biological process” for annotated analysis with PANTHER 16.0. for domestic dog data sets (*Canis lupus familiaris*) as the analysis type. Ensembl gene IDs were used for all annotation data.

As *in vivo* evidence in mice facilitates the search of genes associated with traits, we used Mouse Genomics Informatics (MGI) database (v 6.17) to investigate the relationships between genes and phenotypes (http://www.informatics.jax.org/). The phenotype annotations related to “behavior/neurological” categoly were targeted to identify the relationship.

Results

Breed and sex differences with respect to the seven behavioral traits

The differences between breed, sex, and qualification status were evaluated for seven behavioral traits (Fig. 1 and Table 2). LRs had higher scores for ‘friendliness to humans’ and ‘tolerance to dogs’ compared to those for GSs (Fig. 1). No significant effect was observed between sexes in either dog breed.

In GSs, we found that the qualified group significantly differed from the unqualified group in four behavioral traits (activity, concentration, boldness, and interest in the dummy). Meanwhile in LRs, all traits except ‘Friendliness to humans’ differed between the two qualification groups.

Population genetic structure

In total, 124,675 SNPs in GSs (n = 121) and 154,340 SNPs in LRs (n = 205) were obtained after quality control measures were applied. Over eighty and sixty thousand SNPs were removed due to minor allele thresholds. The genetic data were used for all subsequent genetic analyses.

The mean and standard error of F_{ROH} were 0.549 ± 0.003 (GSs) and 0.553 ± 0.002 (LRs) and thus, no difference was observed between GSs and LRs (t = -1.118, df = 238.06, p-value = 0.264) (Fig. 2A). The PCA revealed that a population structure was found in GSs (Fig. 2B) whereas no clear population structure was found in LRs by PCA (Fig. 2C). No obvious difference was observed between qualified groups.

Genomic heritability of seven behavioral traits and qualification

The heritability of the seven behavioral traits and qualification was estimated based on the genomic data (Table 3). We found heritability estimates for six traits in both breeds with statistical significance (range 0.309 – 0.999 [GSs], 0.403 – 0.759 [LRs]). In both breeds, ‘friendliness to humans’ had the highest heritability among the seven behavioral traits. ‘friendliness to humans’ in GSD was almost the maximum value (i.e. 1), likely due to the low sample size. The heritability on qualification in both breeds pertaining to the traits ‘tolerance to dogs’ in GSs and ‘boldness’ in LRs was not significant. Further, the Pearson’s

product-moment correlation coefficient for heritability estimates for five behavioral traits with statistical significance was high between breeds (Pearson's $r = 0.964$, $P = 0.008$).

Genome-wide association study analysis

Seven behavioral traits and qualifications were subjected to GWAS to genetically separate GSs and LRs. The genomic inflation factors (λ) ranged from 1.028 (activity in LRs) to 1.108 (tolerance to dogs in GSs) (SupplementaryTable S1). The fact that λ was found to be larger than 1.1 only in qualification in GSs indicated that the other analyses were well corrected to the effect of population structure.

No genome-wide significant SNP was found in all analysis. However, we found one SNP (chr9_50771276, genomic position: 50,771,276 on chromosome 9) associated with 'tolerance to dogs' with nearly genome-wide significance in GSs (Adjusted *P* value = 0.0504, Fig. 3A and Table 4). In addition, at a suggestive level, we identified 5 SNPs associated with 'interest in the dummy' in GSs (Fig. 3B), and 3 and 9 SNPs in LRs were candidates for 'friendliness to humans' and qualification, respectively (Fig. 3C, 3D, and Table 4).

Candidate SNP and gene analysis

We found 9 regions as candidates for association with the traits used by our criteria (SupplementaryTable S2). Sixty-one protein-coding genes were located on these regions. No significant term was found in the GO gene enrichment analysis for the 61 ready to analyze genes.

Additional database search using MGI detected 12 genes (Supplementary Table S3) among 61 candidate genes, which had phenotype annotations related to behavior/neurological systems. Considering the relatedness between behavioral traits in this study, anxiety and exploratory and social behavior could be the candidates associated with the traits targeted in this study. We found such traits in seven genes including *Slc35c2*, *Atat1*, *Ddr1*, *Dhx16*, *Pnpla1*, *Pfn2*, and *Wwtr1*.

Discussion

Due to limited studies on the use of drug detection dogs[3], the behavioral and genetic aspects of these animals are under-researched. In the present study, we used phenotypic and genomic approaches to characterize the behaviors and genetic basis of behaviors related to the successful training of drug detection dogs in two breeds. In particular, for LRs, we found 9 SNPs, and 6 genes were potentially associated with training success in drug detection dogs. Although details of the genetic influence and its function on the qualification for drug detection dogs are currently unclear, such genes regulate dogs' individual differences in responsiveness to intensive training of exploratory work.

Behavioral differences between dog breeds are widely known and well-documented[24]. Our behavioral analysis contributed to this body of knowledge by elucidating breed differences with respect to 'friendliness to humans' and 'tolerance to dogs' traits between GSs and LRs. However, we found the

highest SNP heritability in ‘friendliness to humans’ in both breeds and the correlation of SNP heritability between breeds. These findings indicate that behavioral traits could be influenced by genetic factors, and that the degree might tend to be similar among dog breeds.

GWAS have successfully identified SNPs associated or potentially associated with various behavioral traits in dogs[12]. Compared to a previous study, the sample size of this study was smaller ($n < 200$ in both breeds). There are three possibilities for successful identification of the candidate regions (i.e. (1) the number of times phenotyping was performed, (2) the number of humans phenotyping, and (3) environmental variance). The first is regarding the measuring times of phenotyping. The Canine Behavior Assessment and Research Questionnaire (C-BARQ) is widely used for behavioral GWAS in dogs, as reported in a previous study reported[11, 12, 25]. The C-BARQ is usually collected only once for answering the questions. However, in the present study, we used a different method based on behavioral evaluation over 2 weeks. Second, only one person (usually the owner) answers the questions in the C-BARQ; however, in our method, each behavioral trait was scored by several animal management staff. Although further studies are needed to compare the results between C-BARQ and our methods, our methods should be relatively objective compared to C-BARQ thanks to the above two reasons. Third, environmental factors generally affect dog behavior[26]; however, the dogs examined in this study shared the same breeding facility and the same trainers, resulting in lower environmental variance compared to that in common households, which were used in previous reports. Taken together, better measures for behavioral traits and low environmental variance should provide better results; our strategies are powerful for elucidating genetic influences on behavior. However, increasing the number of phenotyped dogs should improve the detection rate, and further analysis should use increased sample sizes as discussed above.

Estimating heritability represents one of the primary methods employed to evaluate genetic effects on traits. Traditionally, estimates of heritability were calculated based on pedigree[27]; however, more recently, significant improvements in genomic technologies have accelerated the estimation of heritability based on genomic data (genomic heritability)[11, 12]. Both pedigree-based and genomic heritability estimates are influenced by scoring traits as well as environmental effects. Our results uncovered higher heritability estimates with statistical significance in behavioral traits, as reported by previous studies (0.00–0.16[12], and 0.00–0.23[11]). This difference should be obtained by measures on traits and low environmental variance as discussed above, as well as by the genetic relationships within the dog population, as reported in the human population[28].

We found significant and higher heritability in both breeds for ‘friendliness to humans,’ which could be associated with strong selection by humans. A meta-analysis identified that traits believed to be under strong selection maintained high heritability[29]. As friendliness to humans is be one of the fundamental factors for dog domestication[30], high heritability for ‘friendliness to humans’ may reflect the outcome of selective pressure by domestication.

Our results thus elucidate the genetic effect on behavioral traits related to successful training in drug detection dogs. which might support improved breeding and training protocols for working dogs. Further analysis using more comprehensive data sets (i.e. $n > 500$) with better behavioral phenotyping and low environmental variance is needed to confirm our results, and to compare the C-BARQ results.

Declarations

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Author contributions

M. I-M., Y.M. and A. K. designed research, M. I-M., Y.M., A. K., and G. I. performed research, contributed with the analytical tools and analyzed data, and all authors wrote the paper.

Competing interests:

The authors declare no competing interests.

Data Availability statement

All SNP and behavioral data can be found in the Dryad database (https://datadryad.org/stash/share/vVhbcaRDt98fHq1Avzkg0sVRdtlQ7jf-zd5FNnq318Y).

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Tables

Tables are available in the Supplemental Files section.

Figures

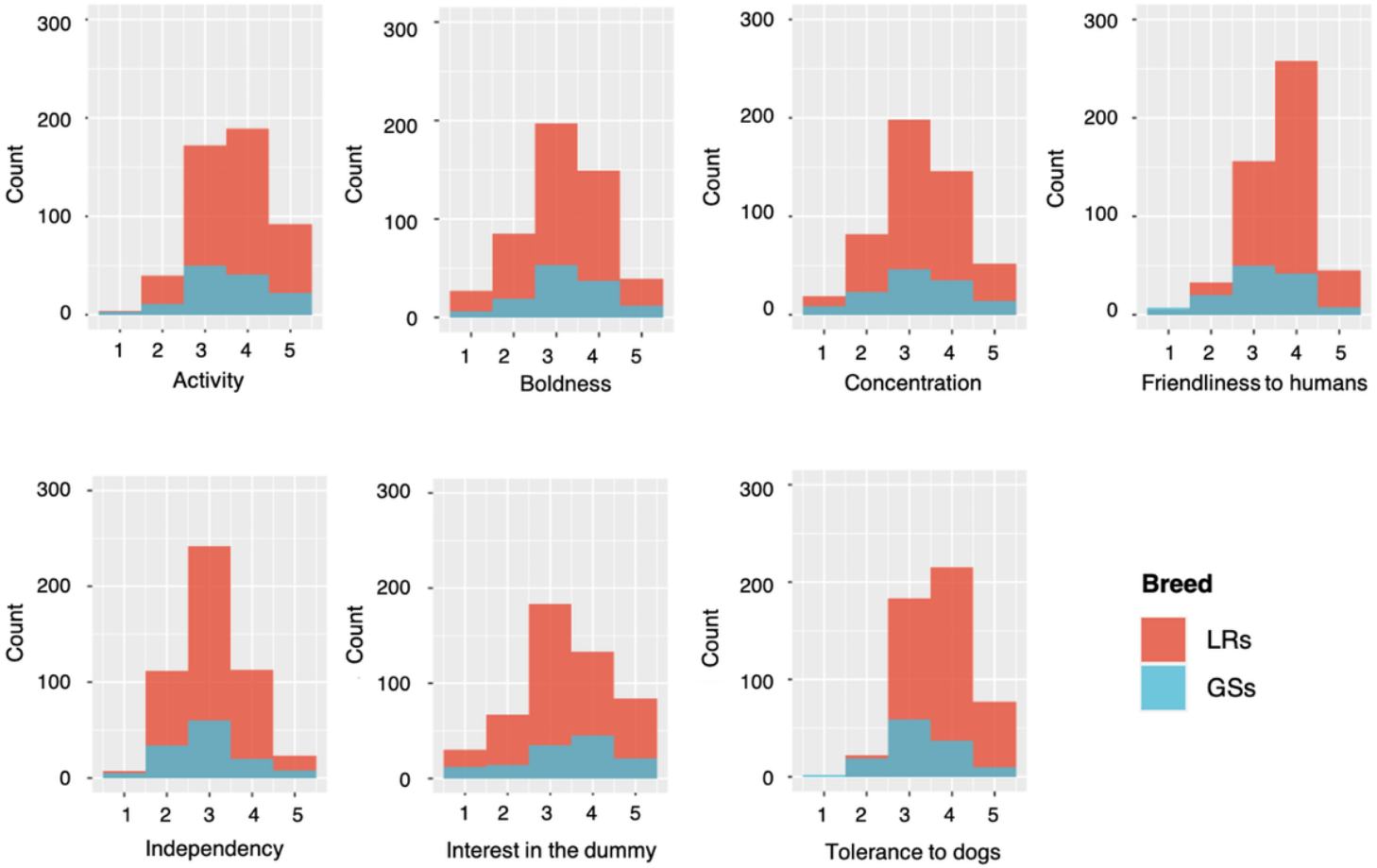

Figure 1

Histogram of the seven behavioral traits related to qualification (the success of training) of drug detection dogs. Four cells for each trait were indicated for the breeds (left: Labrador Retrievers (LRs), right: German Shepherds (GSs)) and for qualification (top: qualified, bottom: unqualified).

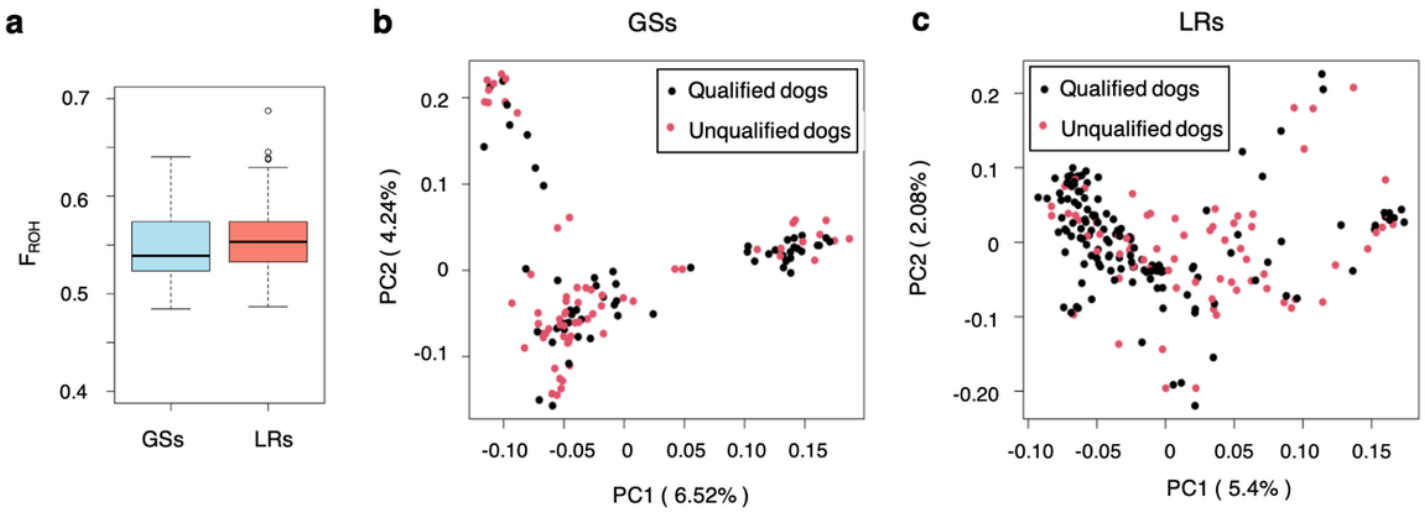

Figure 2

Population genetic structure in GSs and LRs. (**a**) Runs of homozygosity-based inbreeding coefficients (F_{ROH}) for both breeds. (**b**) Principal component analysis (PCA) in GSs. (**c**) Principal component analysis (PCA) in LRs.

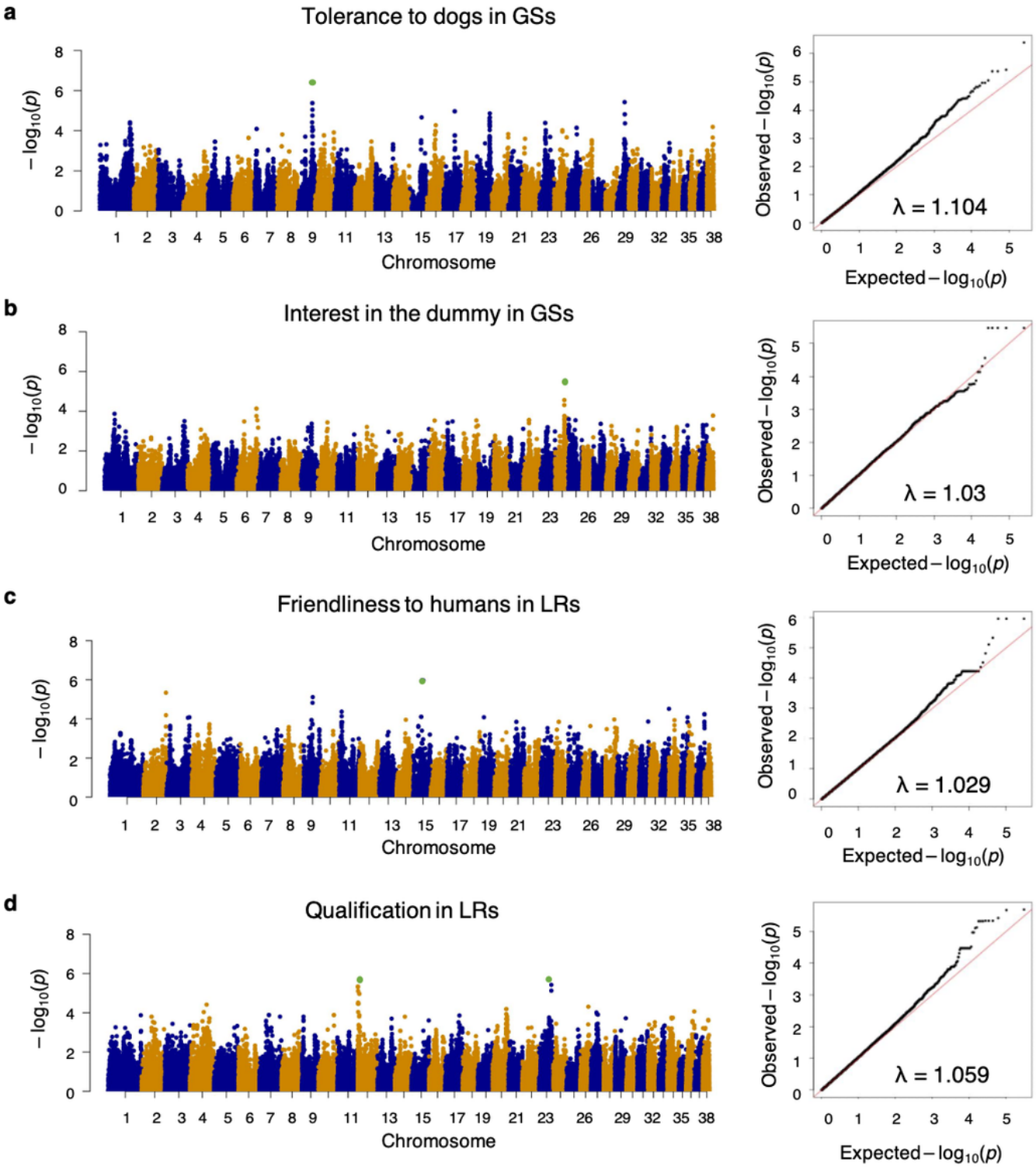

Figure 3

Genome-wide association study results in German Shepherds (GSs) and Labrador Retrievers (LRs). (Left) Manhattan plot for ‘tolerance to dogs’ in GSs (A-B) and for Qualification in LRs (C-D), which detected a single nucleotide polymorphism (SNP) exceeding the genome-wide suggestive level (green dot, adjusted *P* value < 0.10). (Right) Quantile-quantile plot for each manhattan plot.

Supplementary Files

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