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Genomewide association of male reproductive traits in Aksaray Malakli dogs

Muhammed Enes İnanç[1] | Koray Tekin[2] | Mustafa Yenal Akkurt[3] | Kemal Tuna Olgac[2] | Burak Yılmaz[2] | Beste Çil[2] | Mehmet Kızılaslan[4] | Umut Taşdemir[5] | Pürhan Barbaros Tuncer[6] | Serhat Büyükleblebici[6] | Ongun Uysal[2] | Bengi Çınar Kul[3]

[1]Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Mehmet Akif Ersoy University, Burdur, Turkey

[2]Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Ankara University, Ankara, Turkey

[3]Faculty of Veterinary Medicine, Department of Genetics, Ankara University, Ankara, Turkey

[4]International Center for Livestock Research and Training, Ankara, Turkey

[5]Technical Sciences Vocational School, Aksaray University, Aksaray, Turkey

[6]Technical Sciences Vocational School, Mersin University, Mersin, Turkey

Correspondence
Muhammed Enes İnanç, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Mehmet Akif Ersoy University, Burdur, Turkey.
Email: enesinanc@hotmail.com

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Contents

Male reproductive parameters are often used for the functional examination and evaluation of predicted genetic values for future aspects. However, these traits are relatively reliable until the measurable effects are expressed on desired traits. Therefore, we aimed to associate the single nucleotide polymorphism (SNP) genotype of the investigated characteristics and reproductive loci. A total of 46 male dogs are divided into three age groups (I ≤ 3 years, n = 19; II 4–6 years; n = 19, and III ≥7 years, n = 8). The testis, scrotum and body weight, libido sexualis and ejaculation time for each fraction were monitored as functional traits, while the pH, fractional semen volume, motility, concentration, and abnormal and dead spermatozoa rate were recorded as spermatological traits. The Affymetrix Canine 127 K SNP genotyping array v2 (Affymetrix Inc., California, USA) was used for SNP genotyping. In the primary results, the scrotal circumference was found to be higher in group II compared to other groups ($p < 0.05$) and the lowest total abnormal spermatozoa rate was found in group I ($p < 0.05$). The normal spermatozoa rate was found to be significantly above the threshold in relation to the SNP in chromosome 17. In conclusion, this study represents an exciting first step towards SNP association with dog semen spermatological parameters. Future studies might be undertaken to evaluate this SNP region for gene-knockout and expression analysis and for fine mapping to validate and/or discover the exact position of the effect region.

KEYWORDS

GWAS analysis, Malakli dog, single nucleotide polymorphism, spermatological parameters

1 | INTRODUCTION

Despite extraordinary phenotypic variations, all dog breeds are derived from a single species (*Canis familiaris*) that produces a fertile offspring when bred within (Mellersh et al., 1997). The fact that 2.1 million single nucleotide polymorphs (SNPs) were identified in dog genome and the availability of linkage disequilibrium (LD) information (nonrandom associations between alleles at different loci) have attracted research attention towards canine genomic studies (Modiano et al., 2005). Now, with commercially available whole-genome SNP chips, it is possible to identify the position and effect size of quantitative trait loci (QTL) underlying the phenotypic distribution of interest with high precision and accuracy. At present, genomewide association study (GWAS) is statistically the most powerful and precise method available for QTL detection (Risch & Merikangas, 1996). Through this technology, it is possible to detect idiopathic male infertility (Tanaka et al., 2007) and select candidate animals for bulls on the basis of genomic breeding values (Hayes, Bowman,

Chamberlain, & Goddard, 2009). This value is calculated as the sum of dense genetic or haplotype markers across the entire genome for potential QTL contribution to the desired traits. However, the QTL effect must be estimated in a large reference population with the desired phenotypic information.

Basically, the aim of association studies was to reveal the correlation between different allelic versions of the loci and phenotypic variations. Using SNP markers for investigating the genomic regions underlying the phenotypic distributions is a preferred approach as they are more informative and reliable. SNPs are biallelic (two allelic) markers and are spread throughout the genome (İnanç & Daşkın, 2015). Due to their prevalence and ability to cover and represent the entire genome, SNP markers in the existence of LD are much more suitable than other markers (Fan, Du, Gorbach, & Rothschild, 2010; Ke et al., 2011). SNPs are also becoming increasingly important in the development of selection methods for increased animal production and for the identification of genetic mechanisms underlying yield-related quantitative traits in species, races and domestic pets (Modiano et al., 2005).

Several studies state that some male reproductive traits are heritable. For instance, some authors reported that scrotal circumference (Burrow, 2001; Cammack, Thomas, & Enns, 2009; Meyer, Hammond, Parnell, Mackinnon, & Sivarajasingham, 1990), normal/abnormal spermatozoa rate and sperm motility, in general, have a heritability range of 0.04–0.47 in North American and European Bos Taurus breeds (Corbet et al., 2013; Gredler, Fuerst, Fuerst-Waltl, Schwarzenbacher, & Sölkner, 2007; Kealy, MacNeil, Tess, Geary, & Bellows, 2006; Yilmaz, Davis, & Simmen, 2004). There is some information available on the relationship between andrological and spermatological examination and ageing in humans, but very few reports are available on dogs. Some authors reported that age has a negative relationship with the rate of normal spermatozoa in dogs (Rijsselaere, Maes, Hoflack, De Kruif, & Van Soom, 2007; Rota, Tesi, Petta, Sabatini, & Vannozzi, 2016; Stone, Alex, Werlin, & Marrs, 2013). However, the alteration of andrological and spermatological parameters with age in Aksaray Malakli dogs needs more studies for a better understanding of the involved factors. Most of the available literature on shepherd dogs in Turkey are concerned with Akbaş and Karabaş (Kangal) breeds; only a very few studies can be found on Malakli dogs. The Federation Cynologique International (FCI) is involved in the registration of dogs with morphological biochemical and genetic parameters (Anonymous, 2016). Despite the Aksaray Malakli dogs not being recognized as a breed by the FCI, it is stated that the Kangal, Akbaş and Kars breeds, which are other shepherd dog breeds in Turkey, are genetically distinct from the Aksaray Malakli dogs and may be identified as separate breeds (Atasoy, Erdoğan, Yüceer, Özarslan, & Kocakaya, 2011; Atasoy et al., 2014). These dogs are local breeds in Turkey and are being crossbred with other breeds owing to the risk of extinction.

In line with the above-mentioned literature, there is no information available on the genomewide association and spermatological and andrological characteristics of Aksaray Malakli dogs. Therefore, we aimed to determine the some reproductive characteristics of Aksaray Malakli dogs, which are indigenous to Turkey, and to analyse the andrological and spermatological results according to their characteristics and age groups. Furthermore, using animal genotyping with the Affymetrix 127K Canine array v2, a genomewide association study of spermatological traits was carried out, with the primary aim of identifying the loci affecting sperm characteristics.

2 | MATERIALS AND METHODS

2.1 | Animals

The reproductive traits of Aksaray Malakli dogs were analysed using three age groups: Group I (n = 19), ≤3 years of age, Group II (n = 19), 4–6 years of age, and Group III (n = 8), ≥7 years of age; the three groups of dogs were bred in public ownership. Animal experiments were conducted according to ethical principles, and this study was approved by the animal ethics committee of Ankara University, Turkey.

2.2 | Morphological measurement of external genital organs

Before the experimental design, all the dogs were examined for their medical history, reproductive status and pathological conditions. The body weights (kg) of the dogs were measured with metal stage-mounted spring scales. For andrological evaluation, all the dogs were subjected to examination in the standing position to measure their testicular height (mm) (from the beginning of the caput epididymis to its end), depth (mm) (the portion from the lateral to the medial margin), width (mm) (from the widest diameter in median region) and scrotal thickness using electronic callipers. The scrotal circumference (cm) was measured with a tape (measure placed all around the widest part of the scrotum).

2.3 | Semen collection and functional examination

Ejaculates were collected by digital manipulation, and the fractional (first, second and third) volumes (mL) were determined using graded conical tubes; the fraction times were determined using a stopwatch. Libido sexualis, the mounting behaviour and ejaculation time (first, second, third fractions and total in seconds) were recorded. Libido sexualis was determined according to Table 1 (the first secretion immediately before bulbus manipulation was assigned a value of 5 and the visible first fraction in 5 min or longer is assigned a value in the range of 0–5).

2.4 | Semen evaluation parameters

Motility was evaluated using a phase-contrast microscope (100x), with a warm stage maintained at 37°C. Sperm concentration was determined according to a hemocytometric method (Bearden & Fuquay, 2000). Abnormal spermatozoa rate was examined according to the procedure

TABLE 1 Evaluation parameters of Libido Sexualis

Libido Sexualis	
Stimulation of secretion	5
Duration of bulbus manipulation: 0–45 s	4
Duration of bulbus manipulation: 45 s–1.5 min	3
Duration of bulbus manipulation: 1.5–5 min	2
Duration of bulbus manipulation: >5 min	1

prescribed by Schafer and Holzmann (2000), and the sperm viability rate was defined using an eosin staining method (Akalın et al., 2015).

2.5 | Statistical analysis

All the obtained variables were examined by the Shapiro–Wilks test, which is a parametric test, for normality. The assumptions and variables were evaluated using the Levene test for homogeneity before materiality tests. Significant differences between the groups were analysed by ANOVA with normal dividing variables; further, a Kruskal–Wallis test was used for dividing abnormal variables. To determine the differences between various groups, a Tukey test was conducted. In addition, statistical analyses were carried out with a minimum margin of error of 5%. The measured values are presented in the form of "arithmetic mean ($\bar{X}$) ± standard deviation (*SD*)." SPSS® for Windows 14.1 (Licence No: 9869264) package was used for data analysis.

2.5.1 | SNP array genotyping and quality control

Genomic DNA was extracted from the blood samples taken from the *Vena cephalica antebrachia* of the animals using a phenol–chloroform extraction method (Sambrook, Fritsch, & Maniatis, 1989). All the DNA samples were diluted to 50 ng/μl and genotyped with the Affymetrix Canine 127 K SNP genotyping array v2 (Affymetrix Inc., California, USA). The arrays were scanned using a Gene Chip Scanner according to the manufacturer's instructions, and the obtained data were stored as a "CEL" file. The genotypes were evaluated using quality control (QC) procedures, which are crucial to avoid false associations and increase statistical power, which eventually leads to the reduction in Type II error. To this end, a total of 127.132 SNPs were evaluated with the "check.marker" function of the GenABEL package (Aulchenko, Ripke, Isaacs, & Van Duijn, 2007) in a R statistical environment according to the following criteria: (I) call rate ≥95%, (II) minor allele frequency (MAF) ≥0.05% (Laurie et al., 2010) and (III) deviation from the Hardy–Weinberg equilibrium (HWE) $p \geq 0.001$ (Foulkes, 2009). The sex chromosomes were excluded. At last, 51.750 SNPs on 38 autosomal chromosomes that passed the QC filters were retained for downstream analysis.

2.5.2 | Genomewide association analysis

GWAS was performed for all the traits presented in Table 6 using the *mmscore* function in the GenABEL package (Aulchenko et al., 2007) with a regression-based score test method (Chen & Abecasis, 2007). A generalized linear mixed-model approach is used to avoid possible false-positive associations originating from sampling. In this context, the model included the genomic relationship matrix (Astle and Balding, 2009) supplying the phenotype parameters least affected by inbreeding and environmental conditions. Furthermore, all the phenotypic values were analysed for rank-based normalization using the *rntransform* function in the GenABEL package, owing to the presence of a non-normal distribution resulting from a small sample size and age heterogeneity. We fitted the following model:

$$\boldsymbol{y} = \boldsymbol{\mu} + \boldsymbol{X\beta} + \boldsymbol{Zu} + \boldsymbol{e}$$

Here, $\boldsymbol{y}$ is the vector of the phenotypic observations of interest, μ is the mean of the population corresponding to the analysed trait in that specific run, β is the vector of fixed effects (SNP effects and other factors known to have an effect on the observations), and μ is the vector of random background genetic effects for each animal and it is assumed to be drawn from a multivariate normal distribution $(0, \sigma_u^2\boldsymbol{G})$, where $\boldsymbol{G}$ is the genomic relationship matrix obtained from the SNP genotypes of the animals. Meanwhile, $\boldsymbol{e}$ is the vector of random residual effects drawn from a multivariate normal distribution $(0, \sigma_e^2\boldsymbol{I})$, where $\boldsymbol{I}$ is the identity matrix. At last, $\boldsymbol{X}$ and $\boldsymbol{Z}$ are designed matrices mapping the fixed effects and random background effects on the observations, respectively. Thus, SNP effects were estimated once in every iteration.

Bonferroni adjustment (Pearson & Manolio, 2008) was used to correct the inflated false positives resulting from multiple comparisons. The thresholds for genomewide and chromosome-wide significances (*p*-values) were estimated by dividing the traditional significance value (0.05) by the number of independent markers from the genome (51.750) and each chromosome. For ease of display, Manhattan plots were constructed using the qqman function (Turner, 2014) to visualize the −log 10 values of individual *p*-values of the SNPs (Pearson & Manolio, 2008). Q-Q plots were utilized to represent the deviation in the observed *p*-values from the null hypothesis of "no association."

3 | RESULTS

The main outcomes of the study should be evaluated in two sections—male reproductive parameters and GWAS analysis. Primarily, among all the reproductive parameters, the scrotal

circumference (Table 2) was higher in group II compared to group I ($p < 0.05$); there were no statistically significant differences between the three groups with respect to testis height, testis diameter, scrotal thickness and body weight ($p > 0.05$). This clearly shows that scrotal circumference measurement is not always correlated with the body weight or sperm output (in terms of testicular volume).

Ejaculation time with regard to sperm fraction and duration was shorter in group I ($p < 0.05$) (Table 3). Age led to no significant differences between the groups with respect to semen volume, pH, motility, concentration and dead spermatozoa rate ($p > 0.05$) (Table 4), but there were differences in the total abnormal spermatozoa rate ($p < 0.05$). The number of total sperm abnormalities showed a significant correlation with age, especially in age group III (>7 years); in this group, a large number of head and tail abnormalities were observed (Table 5). Moreover, the significance of normal spermatozoa rate between different groups was confirmed by association analysis based on chromosomal wide significance. In Table 6, descriptive statistics [$(\bar{X}) \pm (SD)$] corresponding to the spermatological parameters are shown; these values were derived from GWAS analysis. The genomewide significant threshold was calculated as $p = 9.66 \times 10^{-7}$. Only one SNP (rs9109232) in the intron region of the sideroflexin 5 (SFXN5) gene on chromosome 17 showed chromosome-wide significance ($p = 2.4 \times 10^{-5}$) for normal spermatozoa rate, while none of the other SNPs passed on the genomewide significance ($p < 0.05$). The results shown in Figure 1 correspond to the Manhattan plot of GWAS analysis of normal spermatozoa rate (%).

A Q-Q plot of the *p*-values estimated for SNP marker effects in the GWAS analysis of normal spermatozoa rate (%) and their expected values under the null hypothesis of "no association" is presented in Figure 2. Only a small number deviated from the line and only one of them are significantly deviated (chromosome-wide) from the assumption of "no association."

4 | DISCUSSION

The evaluation and examination of male animals become more precise with the introduction of genomic selection. These transmitting genomic traits are already being considered as tools for candidate bull selection in artificial insemination centres. However, the establishment of genomewide maps for each species creates new opportunities for the early selection of animals according to their predicted spermatological characteristics and for addressing problems, specifically on animal genomes (Goddard & Hayes, 2007). In this regard, we aim to find out whether the reproductive traits Aksaray Malakli dogs exhibit a significant relationship with specific genes.

With regard to phenotypic reproductive traits, an increase in testicular size with age was reported by some authors (Akçay & Tekin, 2002; Bhanmeechao, Srisuwatanasagul, Prapaiwan, & Ponglowhapan, 2018; England et al., 2017). Some researchers stated that the quality of semen decreases as the age of the animals increases (Eskenazi et al., 2003; Rijsselaere et al., 2007). In addition, Kidd, Eskenazi, and Wyrobek (2001) reported an inverse relationship between sperm motility and age in humans.

Even though there were no significant differences between the groups regarding libido sexualis, live weight, and first and third fraction durations ($p > 0.05$), age-dependent differences were observed between the groups in terms of the second fractional duration ($p < 0.05$). Akçay and Tekin (2002) reported that the duration of ejaculation reduced due to ageing. On the contrary, in this study, we found that the ejaculation duration increased with ageing. The shortest ejaculation duration was found in group I (≤3 years) ((42 ± 3.8) s) for the second fraction. Furthermore, Cupps (1991) stated that the duration of sperm delivery may change depending on the live weight.

During spermatological evaluation, the semen volume, concentration, motility, dead spermatozoa rate, and pH were observed to be within the normal range; this result agrees with many other studies (Günay, Polat, Güneş, Soylu, & Kil, 2003; Güngor & Bucak, 2016),

TABLE 2 Morphological evaluation of genital organs in Aksaray Malakli dog

Groups	Age	Scrotal thickness (mm)	Scrotal circumference (cm)	Testis diameter (mm)		Testis heights (mm)		Live weight (kg)
				Right	Left	Right	Left	
I	≤3	4.38 ± 1.3	15.4 ± 2.2[a]	36.4 ± 5.7	35.9 ± 10.3	64.0 ± 8.5	62.07 ± 15.3	75.2 ± 2.9
II	4–6	4.57 ± 4.1	17.7 ± 1.9[b]	39.5 ± 5.6	37.8 ± 4.8	68.3 ± 7.1	64.8 ± 8.1	73.4 ± 2.5
III	≥7	3.46 ± 1.0	16.9 ± 3.4[a,b]	39.8 ± 7.3	38.2 ± 7.1	68.3 ± 6.4	61.4 ± 8.6	69.2 ± 3.8

Different superscript letters ([a, b]) within the same column demonstrate significant differences ($p < 0.05$).

TABLE 3 Functional examination of Aksaray Malakli dog

Groups	Age	Libido Sexualis	Fraction duration (s)		
			1.	2.	3.
I	≤3	4.7 ± 0.42	271 ± 1.7	42 ± 3.8[a]	515 ± 14.6
II	4–6	4.63 ± 0.68	285 ± 3.5	55 ± 2.5[b]	462 ± 16.1
III	≥7	4.37 ± 0.91	279 ± 5.34	56 ± 2.9[b]	479 ± 59.5

Different superscript letters ([a, b]) within the same column demonstrate significant differences ($p < 0.05$).

TABLE 4 Semen evaluation of Aksaray Malakli dog

Groups	Age	Volume (ml) Fraction 1.	Volume (ml) Fraction 2.	Volume (ml) Fraction 3.	pH Fraction 1.	pH Fraction 2.	pH Fraction 3.	Motility (%)	Concentration (ml/10^6)	Dead (%)
I	≤3	2.7 ± 1.7	2.1 ± 1.1	6.0 ± 4.7	6.0 ± 0.2	5.8 ± 0.2	5.8 ± 0.2	85.6 ± 5.38	328.0 ± 100.9	16.41 ± 7.71
II	4–6	2.7 ± 1.7	2.4 ± 1.2	5.2 ± 3.7	5.8 ± 0.1	5.7 ± 0.1	5.8 ± 0.1	80.5 ± 10.5	315.0 ± 94.7	12.59 ± 7.41
III	≥7	3.2 ± 2.3	2.7 ± 1.3	3.6 ± 2.4	5.7 ± 0.2	5.8 ± 0.2	5.8 ± 0.1	77.4 ± 15.3	312.8 ± 46.8	16.92 ± 12.93

TABLE 5 Semen evaluation of Aksaray Malakli dog

Groups	Age	Abnormal spermatozoa rate (%) Acrosome	Head	Neck	Tail	Cytoplasmic droplet	Total abnormal
I	≤3	0.54 ± 0.1	1.5 ± 0.2	3.5 ± 0.3	7.1 ± 0.3	2.3 ± 1.1	12.42 ± 5.52[a]
II	4–6	0.81 ± 0.1	2.7 ± 2.4	1.6 ± 0.5	11.2 ± 1.3	7.7 ± 0.6	28.57 ± 20.2[b]
III	≥7	1.25 ± 0.1	3.7 ± 1.6	3.2 ± 2.1	13.1 ± 1.9	4.7 ± 2.0	25.42 ± 19.33[b]

Different superscript letters ([a, b]) within the same column demonstrate significant differences ($p < 0.05$).

TABLE 6 Descriptive statistics [($\bar{X}$) ± (SD)] for spermatological parameters used in GWAS analysis

Spermatological parameters	$\bar{X} \pm SD$
Semen volume (ml) (2. Fraction)	2.2 ± 1.04
pH (2. Fraction)	5.7 ± 0.2
Concentration (x10^6)	320.7 ± 88.5
Dead spermatozoa rate (%)	14.4 ± 8.6
Normal spermatozoa rate (%)	80.1 ± 15.5
Abnormal acrosome rate (%)	0.7 ± 0.2
Abnormal head rate (%)	3.5 ± 1.3
Abnormal neck rate (%)	1.6 ± 0.3
Abnormal tail rate (%)	9.2 ± 3.4
Total abnormal spermatozoa rate (%)	20.51 ± 17.5
Total motility (%)	82.4 ± 10.3

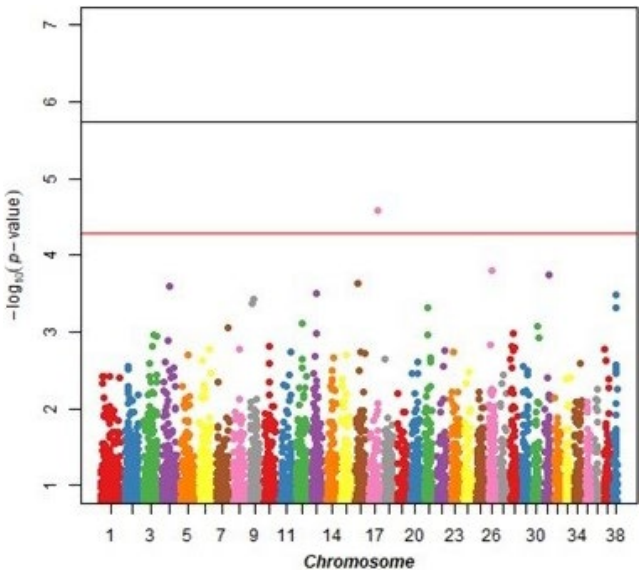

FIGURE 1 Manhattan plot of GWAS for normal spermatozoa rate. One SNP showed chromosomal wide significance. (*X*-axis shows chromosomal positions. *Y*-axis shows −log10 (*p* values) obtained from animal mixed model-based score test. Black and red horizontal lines represent the genomewide and chromosome-wide thresholds, respectively)

but not with the observations made by Tekin, İzgür, and Özyurt (1987). The results lying between the highest and lowest total abnormal spermatozoa ratios obtained from different races were found to be within the normal range (Baran, Ak, & İleri, 2000). Based on our results, it is thought that the primer (disruptions of spermatozoa production in tubulus seminiferus contortus) and seconder (transportation of spermatozoa to the epididymis, storage in the epididymis, and ejaculation and genital infections) abnormalities in the reproductive tract caused an increase in the abnormal spermatozoa rate with ageing.

Spermatological parameters can be defined characteristically as quantitative traits, which are influenced by the genotype (many loci with a small effect on the phenotype of interest) and environmental factors. These quantitative traits are also known to have a phenotype distribution of a persistent, unimodal (normal) type (Khatib, 2015). It has been argued that the results obtained from studies with fewer than 1,000 samples of case–control subjects are not ideal for identifying a specific effect with genomic QTL mapping (Hong & Park, 2012; Korte & Farlow, 2013). However, there are studies in which fewer samples are used in the presence of SNPs; major effects were observed in these studies (Forman, De Risio, & Mellersh, 2013; Hayward et al., 2016). Bianchi et al. (2015) conducted GWAS analysis to elucidate the genetic basis underlying hypothyroidism in dogs and found that the 167-kb haplotype is associated with the disease in the studied dog

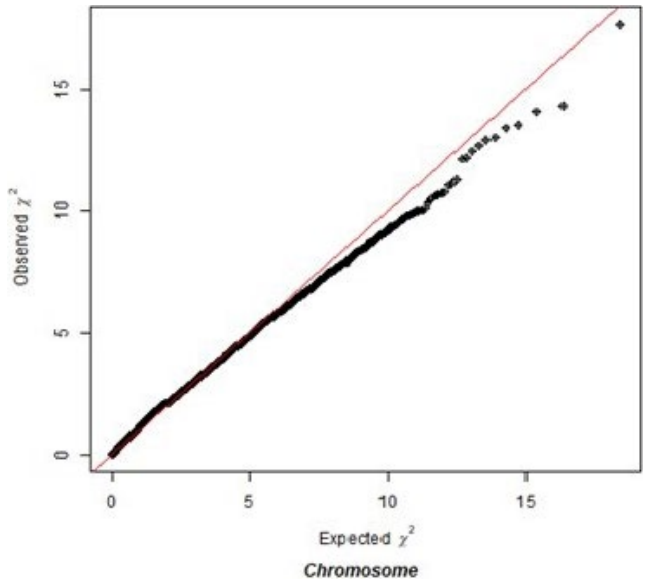

FIGURE 2 The Q-Q plot graphic for GWAS of normal spermatozoa rate (%)

population. The researchers identified three candidate genes in this region but regarding the biological function of these genes, they reported that there is no direct effect of these genes on hypothyroidism. However, they may be involved in pathways that play a role in the development of this disease. Pfahler and Distl (2012) conducted a study to identify quantitative trait loci in relation to hip and elbow dysplasia in Bernese dogs. It was determined that SNPs spread over chromosome 4 and chromosome 3 are associated with these traits. A similar study conducted in humans identified 20 SNPs associated with azoospermia and severe oligospermia (Aston Kenneth & Carrell, 2009). However, there are no reports in the literature on the genomewide association analysis of the andrological and spermatological characters of dog semen. In this study, only one SNP (rs9109232) in the intron region of sideroflexin 5 (SFXN5) gene on chromosome 17 showed a chromosome-wide significance for normal spermatozoa rate and none of the other SNPs passed the genomewide significance threshold at a significance level of 0.05. SFXN5 is responsible for potential iron transport and citrate transmembrane transport activity as a molecular function; further, it is responsible for iron-ion homeostasis. However, Gibbons, Watson, Coffey, Brady, and Fitzpatrick (2000) showed that SFXN5 is highly expressed in urothelial, testis and prostate malignancies. When the genomic region of the discovered SNP was examined, there was no well-defined function in the development of spermatozoa. This may suggest that there may be an effect of this SNP on different pathways or the presence of a large number of small-effect genes on this character.

It is strongly suggested that the andrological and spermatological parameters should be evaluated according to breed, environment and age conditions (Balic, Milinkovic-Tur, Smardzija, & Vince, 2012). Besides, this study represents an exciting first step in SNP association analysis/QTL discovery of dog semen spermatological parameters using genomewide SNP markers. It should be remembered that genomewide association of this SNP does not necessarily mean that the SNP undergoes causal mutation with itself; it is quite possible that the causal mutation, or in other words, the QTL segregating under normal spermatozoa rate's phenotypic distribution, might be in strong linkage disequilibrium (LD) with the discovered chromosome-wide significant SNP. Future studies might be undertaken to evaluate this SNP region for gene-knockout and expression analysis as well as fine mapping to validate and/or discover the exact position of the effect region. Furthermore, if a biological relationship is to be built, this SNP can be used as a marker for selection, aiming at higher normal spermatozoa rate in this particular breed. At last, the SNP might be validated by implementing GWAS studies in different breeds with additional samples to increase the statistical power.

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CONFLICT OF INTEREST

There are no conflict of interests to declare.

AUTHOR CONTRIBUTIONS

Muhammed Enes İnanç contributed to coordination and design of the study and drafted the manuscript. Koray Tekin collected semen samples in dog and controlled whole manuscript. Mustafa Yenal Akkurt collected blood samples in dog. Kemal Tuna Olgac extended and performed the examination of the semen. Burak Yılmaz evaluated the abnormal spermatozoa rate. Beste Çil controlled the English grammar of the manuscripts. Mehmet Kızılaslan performed GWAS analysis. Umut Taşdemir examined the andrological and spermatological parameters. Pürhan Barbaros Tuncer examined the andrological parameters. Serhat Büyükleblebici examined the fresh spermatological parameters. Ongun Uysal designed the study. Bengi Çınar Kul performed GWAS analysis.

ORCID

Muhammed Enes İnanç http://orcid.org/0000-0001-6954-6309

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