Dr. Leonhard Schnittger (ORCID ID 0000-0003-3484-5370)

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Title: High genetic diversity and differentiation of the *Babesia ovis* population in Turkey

Running Title: *Babesia ovis* population diversity and structure in Turkey

Authors: Anabela Mira[1,2], Ahmet Hakan Unlu[3], Huseyin Bilgin Bilgic[4], Serkan Bakirci[4], Selin Hacilarlioglu[4], Tulin Karagenc[4], Tamara Carletti[1], William Weir[5], Brian Shiels[5], Varda Shkap[6], Munir Aktas[7], Monica Florin-Christensen[1,2], Leonhard Schnittger[1,2,*]

Affiliation:

[1]Instituto de Patobiología Veterinaria, CICVyA, INTA-Castelar, Hurlingham, Argentina

[2]Consejo Nacional de Ciencia y Tecnología (CONICET), Buenos Aires, Argentina

[3]Vocational School of Gevas, Van Yuzuncu Yil University, Van, Turkey

[4]Department of Parasitology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Aydin, Turkey

[5]Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary & Life Sciences, University of Glasgow, 464 Bearsden Road, Glasgow G61 1QH, UK

[6]Division of Parasitology, Kimron Veterinary Institute, PO Box 12, Bet Dagan 50250, Israel.

[7]Department of Parasitology, Faculty of Veterinary Medicine, Firat University, Elazig, Turkey

*corresponding author address:

Instituto de Patobiologia Veterinaria, CICVyA, INTA-Castelar, Los Reseros y Nicolas Repetto, s/n, 1686 Hurlingham, Prov. de Buenos Aires, Argentina

E-mail address: schnittger.leonhard@inta.gob.ar

SUMMARY

Babesia ovis is a tick-transmitted protozoan hemoparasite causing ovine babesiosis in sheep and goats leading to considerable economic loss in Turkey and neighboring countries. There are no vaccines available, therapeutic drugs leave toxic residues in meat and milk, and tick vector control entails environmental risks. A panel of eight mini- and microsatellite marker loci was developed and applied to study genetic diversity and substructuring of *B. ovis* from western, central, and eastern Turkey. A high genetic diversity (H_e = 0.799) was found for the sample of the overall *B. ovis* population (n = 107) analyzed. Principle component analysis (PCoA) revealed the existence of three parasite subpopulations: (i) a small subpopulation of isolates from Aydin, western Turkey, (ii) a second cluster predominantly generated by isolates from western Turkey, and (iii) a third cluster predominantly formed by isolates from central and eastern Turkey. Two *B. ovis* isolates from Israel included in the analysis clustered with isolates from central and eastern Turkey. This finding strongly suggests substructuring of a major Turkish population into western vs. central-eastern subpopulations, while the additional smaller *B. ovis* population found in Aydin could have been introduced, more recently, to Turkey. STRUCTURE analysis suggests a limited exchange of parasite strains between the western and the central-eastern regions and *vice versa*, possibly due to limited trading of sheep. Importantly, evidence for recombinant genotypes was obtained in regionally interchanged parasite isolates. Important climatic differences between the western and the central/eastern region, with average yearly temperatures of 21ºC versus 15ºC, correspond with the identified geographical substructuring. We hypothesize that the different climatic conditions may result in variation in the activity of subpopulations of *Rhipicephalus* spp. tick vectors, which, in turn, could selectively maintain and transmit different parasite populations. These findings may have important implications for vaccine development and the spread of drug resistance.

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51

52 **KEYWORDS**

53 *Babesia ovis;* ovine babesiosis; satellite marker; multilocus typing; genetic diversity; population

54 structure

55

1 INTRODUCTION

Ovine babesiosis is a tick-borne disease principally caused by *B. ovis* and is endemic in southern Europe, Africa, the Middle East and Asia (Mehlhorn and Schein, 1984; Ahmed et al., 2002; Schnittger et al., 2012). The disease has been reported to result in important economic losses in Turkey and Iran (Ranjbar-Bahadori et al., 2012; Esmaeilnejad et al., 2015; Sevinc et al., 2013, 2014). Clinical signs of the infection include fever, hemolytic anemia, hemoglobinuria, and icterus leading to high morbidity, with a significant number of fatalities observed in some areas (Sevinc et al., 2013; Hurtado et al., 2015). Currently, ovine babesiosis is predominantly controlled by chemotherapeutic treatment of animals during acute disease but, to a limited degree, chemical control of the vector tick is also applied. The drawback of the former approach is that it is expensive, requires early diagnosis of the disease, and drug residues may be introduced into the food chain, whereas the downside of the latter approach is that it poses environmental risks and acaricide resistance is observed.

The transmitting tick vectors of *B. ovis* are species of the genus *Rhipicephalus*, such as *R. bursa, R. turanicus, R. sanguineus,* and *R. annulatus,* which are abundant in western, central, and eastern Turkey, and also endemic in Iran (Sayin et al., 1997a,b,c; Aydin and Bakirci, 2007; Shayan et al., 2007). Accordingly, a recent comprehensive molecular epidemiological study demonstrated the highest *B. ovis*-infection rates in sheep and goats from the Kutahya and Afyon regions of western, and the Konya and Aksaray regions of eastern Turkey (Bilgic et al., 2017). Notwithstanding the observed high infection rate of and seroprevalence against *B. ovis* in Turkey, endemic instability prevails in most regions, thus an environment-friendly immunoprophylactic approach is required to control the disease (Mahoney and Ross, 1972, Ekici et al., 2012; Sevinc et al., 2014).

Although attenuated live vaccines have been shown to be effective against bovine babesiosis caused by the closely related species, *B. bovis,* and by *B. bigemina*, a similar vaccine is not yet available for ovine babesiosis (Shkap et al., 2007; Schnittger et al., 2012; Florin-Christensen et al., 2014; Sevinc et al., 2014). In a recent preliminary study, twelve successive blood passages in splenectomized lambs mitigated but could not eliminate the virulence of a pathogenic *B. ovis*

strain when tested in lambs (Sevinc et al., 2014). Analogous to the situation for *B. bovis* and *B. bigemina*, effective attenuation may be achieved through additional *in vivo* passage or, alternatively, through *in vitro* passage of cultured *B. ovis* strains (Hora et al., 2014; Florin-Christensen et al., 2014).

Population genetic analysis of *Babesia* allows the investigation of parasite genetic diversity and structure, and can estimate the extent of genetic exchange in the field. Estimated parameters are able to inform on the most promising approaches for vaccine development and application, and provide information on the risk of dissemination of drug resistance-encoding genes in the population. Population genetic studies are currently lacking for *B. ovis* but have been carried out for the closely related species *B. bovis* (Perez-Llaneza et al., 2010, Simuunza et al., 2011; Schnittger et al., 2012; Flores et al., 2013). An extremely high level of genetic differentiation and diversity of the *B. bovis* worldwide metapopulation has been estimated by comparing reference isolates originating from distant countries and continents (Perez Llaneza et al., 2010; Flores et al., 2013). Likewise, a study of the *B. bovis* populations in Turkey and Zambia suggests a high level of genetic diversity and frequent genetic exchange, under preconditions of a relatively low geographic distance between populations, a high transmission rate, and exchange of infected animals (Simuunza et al., 2011; Schnittger et al., 2012). In contrast, linkage disequilibrium is commonly observed when isolates from distant geographic regions are compared.

In this study, a panel of micro- and mini-satellite markers for multilocus typing based on the available *B. ovis* genome was developed and applied, as these types of markers have been shown to be highly suitable for population genetic analysis of related *Babesia* and *Theileria* parasites. Satellite markers were used to assess the genetic diversity, population structure, frequency of recombination, and multiplicity of infection of the *B. ovis* population of Turkey. The data obtained indicated separation of major populations of parasites within Turkey, which has implications for both vaccine development and the dissemination of genes that confer drug resistance.

2 Materials & Methods

2.1 Parasite sampling, genomic DNA isolation, and satellite marker development

A total of 107 blood samples of sheep from different areas in Turkey were analyzed (Figure 1). These originated from (i) the western [Aegean and Mediterranean region (n = 51), including the provinces of Afyon (n = 22), Aydin (n = 7), Burdur (n = 2), and Kütahya (n = 20)], (ii) the central [central Anatolian region (n = 34) region, including the provinces of Aksaray-Eskil (n = 7), Konya (n = 19), and Niğde (n = 8)], and (iii) the eastern region [(Eastern Anatolia and Southeastern Anatolia region (n = 22), including the provinces of Bingöl (n = 1), Elaziğ (n = 2), Erzincan (n = 4), Erzurum (n = 1), Iğdir (n = 2), Malatya (n = 2), Mardin (n = 2), Muş (n = 3), and Şirnak (n = 5)] (Figure 1). Genomic DNA was isolated using the DNAeasy blood and tissue kit, according to the recommendation of the manufacturer (Qiagen, Hilden, Germany). In addition, genomic DNA was also isolated from the *in vitro* cultured *B. ovis* strain, Israel and from the field isolate *B. ovis* strain, Itamar (Kimron Veterinary Institute, Israel). Isolated DNA was quantified using a Nanodrop spectrophotometer, adjusted to 500 pg/µl, and stored at −20 ºC until further use. Selected blood samples were shown to test positive in a PCR assay, using RLB primers generic for *Babesia* spp. infecting small ruminants (Schnittger et al., 2004; Horta et al., 2014). Micro- and mini-satellite markers were developed as described in Perez-Llaneza et al., (2010), and designed based on the as yet publicly unavailable *B. ovis* strain Israel genome sequence (University of Glasgow, Glasgow, UK).

2.2 PCR amplification of micro- and mini-satellite loci

PCR was performed using primers designed to amplify each of the eight selected marker repeat regions on the *B. ovis* isolates and strains (Supplementary Table 1). The reverse primer of each pair was labeled at the 5’ end with one of the standard fluorescence dyes: 6-FAM (Blue), NED (Yellow), PET (Red), or VIC (Green) (Applied Biosystems), enabling detection of product on an ABI Genetic Analyzer. PCR amplification was performed in a final reaction volume of 10 µl comprising of 500 pg of template genomic DNA, 0.025 U DreamTaq polymerase (ThermoFisher Scientific), 2 mM dNTPs, 10 µM of each primer, 2 mM $MgCl_2$, and DreamTaq

buffer (1x) (ThermoFisher Scientific). Nuclease-free water and *B. ovis* genomic DNA were used as a negative and positive controls, respectively. The thermocycler program consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles comprising denaturation at 95 °C for 30 s, annealing at 55 to 60 °C (see Supplementary Table 1) for 30 s, and extension at 72 °C for 20 s; and a final extension step at 72 °C for 7 min. Five microliters of each PCR reaction were analyzed on an ethidium bromide-containing 1.8% agarose gel to verify amplification success. Genotyping was performed at the Unidad de Genómica, Instituto de Biotecnología, CICVyA, INTA-Castelar. DNA fragment sizes were analyzed relative to ROX-labeled GS600 LIZ size standards (Applied Biosystems) using Peak Scanner software (ThermoFisher Scientific), which allowed resolution of 1 bp differences in amplicon size. The presence of multiple amplification products generated in a single PCR reaction of a sample demonstrated the presence of two or more *B. ovis* genotypes. Data output generated by capillary electrophoresis included the area under the peak as a quantitative measurement of allele amplification. The predominant allele of each marker locus of a given sample was identified by the largest peak area, and a multi-locus genotype (MLG) representing the most abundant *B. ovis* strain present in the isolate was generated. An excel file of MLG data was produced to assess genetic diversity and structure of the population. A second file comprised the allelic marker profile of each sample that was used to estimate the multiplicity of infection (MOI).

2.3 Data analysis

Population genetic analysis for the estimation of F-statistics was performed using Genepop version 4.2 (http://genepop.curtin.edu.au/index.html; Raymond and Rousset, 1995; Rousset, 2008). The Microsoft Excel plug-in software, GenAlEx6.5 (http://biology.anu.edu.au/GenAlEx/, Peakall and Smouse, 2012), was used to estimate the Analysis of MOlecular VAriance (AMOVA), to construct a similarity matrix, and perform principal component analysis (PCoA) of MLG data. Population structure was also assessed using Bayesian clustering analysis as implemented in STRUCTURE 2.3.4. (Pritchard et al., 2000). The EVANNO Method Delta K and STRUCTURE Harvester was used to derive the best

fit for the parameter value K (Earl and von Holdt, 2012). Assuming that the four populations from each sample location are genetically distinct and using the admixture model with correlated allele frequencies, 20 iterations were run for each K ranging from 1 to 10 selecting a 'burn in' of 100,000 and 200,000 MCMC simulations.

The null hypothesis of linkage equilibrium was tested using LIAN which computes the standardized index of association (I^S_A) and quantifies linkage equilibrium/disequilibrium as previously defined by Haubold and Hudson (2000) (http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl/query). The I^S_A measures the association between alleles at pairs of loci. I^S_A values close to 0 or negative are indicative of panmixia, while those significantly greater than 0 are indicative of a non-panmictic situation. Linkage equilibrium is characterized by the statistical independence of alleles across all loci and is investigated by initially determining the number of loci at which each pair of MLGs differs. From the distribution of mismatch values, a variance (V_D) is calculated which is compared to the variance expected for linkage equilibrium, termed V_e. The null hypothesis that $V_D = V_e$ is tested by both a Monte Carlo simulation and a parametric method in order to estimate a 95% confidence limit (95% CI), which are denoted L_{mc} and L_{para}, respectively. When V_D is found to be greater than L, the null hypothesis is rejected and linkage disequilibrium is accepted.

The mean number of all identified alleles for all eight loci was calculated to represent the MOI as an estimation of the number of genotypes within an isolate. The average MOI of each population was estimated by calculating the overall mean of the MOI of all representative samples.

3 RESULTS

3.1 Satellite marker diversity

In each isolate, the presence of numerous *B. ovis* genotypes (MLG) was evident through the observation of more than one allele at one or multiple marker loci. The foremost allele for each marker loci was scored to determine the prominent MLG in each sample. This MLG was used to assess satellite marker and genetic diversity as well as the genetic differentiation within the

196 population. Of the eight employed micro- and mini-satellite markers, five showed a very high
197 level of genetic diversity, ranging from 0.802 (Oms_8) to 0.899 (OMS_47), compared to the
198 remaining three that displayed a somewhat lower genetic diversity, ranging from 0.703
199 (Oms_11) to 0.755 (OMS_103) (Table 1). This high polymorphism indicated that markers can
200 be assumed to be informative for linkage disequilibrium analysis of the *B. ovis* population and
201 also suitable to describe population diversity. The number of alleles per marker identified in all
202 isolates studied was very high, ranging from 14 alleles for marker locus OMS_103 up to 28
203 alleles for markers OMS_47 and OMS_93. Across all eight markers, an average of 21.6
204 different alleles was determined. Predominant allele frequencies ranged from 20.6% for marker
205 OMS_47 to 51.4% for marker Oms_11. As an example, the allele frequency distribution of
206 three markers in the three studied geographic regions of Turkey is shown in Figure 2, and for
207 the remaining markers in Supplementary Figure 1.
208
209 **3.2 Population diversity and structure**
210 The genetic diversity (H_e) estimated for the three geographic regions of Turkey was found to be
211 moderate for the population of the western region (0.663), compared to a very high value
212 obtained for both the population of the central (0.770) and eastern regions (0.791), which were
213 found to be similar to that of the overall population (0.799) (Figure 3, Supplementary Table 2).
214 The number of different alleles in the western, central and eastern population (Na) was 10.8,
215 10.6, and 8.8, respectively, with a mean number of 19.5 different alleles in the overall
216 population. In contrast, the mean number of effective alleles (N_e) was determined as 4.1, 5.3,
217 and 6.0 and the mean number of private alleles was found to be 5.5, 4.5 and 2.4, with a high
218 mean number of 12.4 private alleles at each marker locus in the overall population. This
219 considerable number of private alleles for each marker can be directly observed in Figure 2 and
220 Supplementary Figure 1, and this information is detailed in Supplementary Table 2.
221 The predominant allele at each marker locus was identified and the MLG for each isolate
222 established. Altogether, 100 different MLG could be identified in the 107 isolates,
223 demonstrating a high level of genomic diversity in the overall *B. ovis* population of Turkey. On

the basis of the MLG data, a matrix of Nei's genetic distance was calculated to carry out a principal component analysis (PCoA). PCoA allows visualization of the genetic relationships between MLGs and the comparison of genotypes from the three geographic regions together with *B. ovis* reference isolates from Israel (Figure 4). Two main genetic clusters are observed, one cluster is predominantly composed of samples from western although also includes seven (corresponding to six data points in Figure 4) from central Turkey and two from eastern Turkey (cluster 1), while the second cluster is mainly composed of samples from central and eastern Turkey but also includes five isolates (corresponding to four data points in Figure 4) from western Turkey and the two Israel isolates (cluster 2). Cluster 1 and 2 touch to the right of the perpendicular axis. Importantly, a third smaller separate grouping (cluster 3) is formed by some samples from western Turkey.

With regard to sample origin, most samples from western Turkey clustered to the right of the perpendicular axis of the plot into cluster 1, with the exception of all isolates originating from the Aydin region (n = 7), situated at the Aegean coast, that formed a separate cluster in the lower left quadrant (cluster 3). In contrast, samples from central and eastern Turkey clustered mainly left of the perpendicular axis of the plot including also the two isolates from Israel to produce cluster 2. Seven samples that originate from Aksaray, central Turkey, clustered jointly with the majority of western Turkish isolates to the right of the perpendicular axis forming a subgroup within cluster 1.

The STRUCTURE analysis predicts three different *B. ovis* genotype clusters (K=3) as determined by the EVANNO delta K method that match the three clusters identified by PCoA. The three genotype clusters are represented in Figure 5 as red genotype 1 (western population including seven isolates from Aksaray, central Turkey, one from Mus and one from Sirnak, eastern Turkey, and four isolates of a mixed genotype from Kutahya, western Turkey corresponding to cluster 1), blue/green genotype 2 (central and eastern population including one isolate from Afyon, two from Burdur, two from Kutahya, western Turkey and both parasite strains from Israel, corresponding to cluster 2) and the green genotype 3 (seven isolates from Aydin, corresponding to cluster 3). AMOVA analysis showed that the molecular variation

252 between the three geographically defined populations of western Turkey (cluster 1),
253 eastern/central Turkey (cluster 2), and Aydin (cluster 3) was 24 %, whereas the variation within
254 populations accounted for the remaining 76 % of the observed variation.
255 In order to verify the existence of a separate Aydin population as predicted by the PCoA and
256 STRUCTURE analyses, samples were divided into the four geographic populations Aydin,
257 western Turkey* (excluding Aydin samples), central Turkey, and eastern Turkey; and the
258 pairwise genetic differentiation determined by estimation of the unbiased Nei´s genetic distance
259 (uD) and Wright´s fixation index (Table 2). An extremely high level of genetic differentiation
260 was estimated between the Aydin *vs*. the western population (uD = 2.464; Fst = 0.544) and was
261 also very high between samples from Aydin *vs*. the central (uD = 0.421; Fst = 0.241) and also
262 *vs*. the eastern population (uD = 0.380; Fst = 0.244), strengthening the evidence that Aydin
263 isolates should be considered as a discrete population. Furthermore, a high genetic
264 differentiation was observed between the population of western Turkey* *vs*. that of central (uD
265 = 0.889; Fst = 0.241) and eastern Turkey (uD = 0.830; Fst = 0.233). In contrast, the population
266 of central Turkey showed negligible genetic differentiation to that of eastern Turkey (uD =
267 0.049; Fst = 0.011), corroborating their joint grouping into cluster 2 and exhibiting a similar
268 blue/green genotype 2 in the PCoA and STRUCTURE analysis, respectively. Consequently, it
269 seems justified to regard *B. ovis* isolates from central and eastern Turkey as one single
270 population.
271
272 **3.3 Linkage analysis**
273 Using the assessed MLG dataset, the standard index of association (I^s_A) was calculated in order
274 to determine whether an association between alleles could be observed when all pairwise
275 combinations of loci were compared. After estimation of I^s_A the null hypothesis of linkage
276 equilibrium for MLG data was tested. Using the MLG data, the LD analysis was carried out for
277 all available data from Turkey, by pairwise combination of the data representing western,
278 central, and eastern regions, for each region separately, and also for individual/farms, whenever
279 a sufficient number of samples were available. This approach allows testing hierarchically

nested population subdivisions until eventually linkage equilibrium is observed. The observance of linkage equilibrium demonstrates a geographic area, which may correspond to a single farm, within which frequent recombination of MLGs is observed.

The combined population of Turkey resulted in an I^s_A of 0.1615 demonstrating the existence of LD (Table 3). Likewise, pairwise combination of the population representing western/central Turkey and western/eastern Turkey showed even higher values for LD of 0.1992 and 0.1916, respectively. In contrast, the pairwise combination of data representing central/eastern Turkey resulted in a substantially lower I^s_A of 0.0889, indicating a much less pronounced LD. Thus, the central and eastern populations of Turkey are best regarded as a single population as they are substantially closer to equilibrium than the central *vs.* the western or the eastern *vs*. the western population, which is consistent with the population differentiation between the western *vs.* the central/eastern population, evident on the PCoA and F_{ST} analysis. Linkage disequilibrium was also observed when testing each region, the western population showing a higher I^s_A of 0.2111 followed by the central and eastern populations with 0.1317 and 0.0919, respectively. Further geographic subdivision resulted in the observation of significant linkage equilibrium in samples originating from Konya and Aydin, but in the remaining subpopulations LD was still observed. Notwithstanding that in the case of Aydin (n = 7) the sample size was relatively low, this result indicates that shorter geographic distances facilitate genetic exchange between MLGs, resulting eventually, in the observation of LE in populations from local geographic or farm populations as has been reported for related piroplasmid species (Simuunza et al., 2011; Weir et al., 2011; Gomes et al., 2016).

3.4 Multiplicity of infection

In a single sample, the number of alleles at a given loci ranged from 1 to a maximum of 5 for marker Oms_8 and from 1 to a maximum of 15 for marker Oms_11 and OMS_62. Overall, the mean number of alleles per sample across all markers was found to range from 1.65 for Oms_8 up to 4.36 for OMS_62 (Supplementary Table 2). For each isolate, the detection of several alleles at one or more markers was indicative of multiple genotypes within all isolates

originating from the three studied regions. Correspondingly, the number of genotypes for each sample could be estimated by calculating the average number of alleles across all marker loci for a given sample. In turn, the MOIs determined for each isolate were used to estimate the average MOI in the populations of the western (3.31), central (2.48) and eastern region (2.62), as well as for the total *B. ovis* population of Turkey (2.91) (Table 4).

4 Discussion

Population genetic studies using micro- and mini-satellites have been shown to be invaluable for the assessment of parasite diversity, recombination frequency, and behavior of the parasite at the population level. Genome sequence analysis allows the identification of putatively neutral, highly diverse micro- and mini-satellites with relative ease and these markers have been shown to be a useful tool for population genetic studies.

Ovine babesiosis has an important economic impact in Turkey and population genetics should be applied to provide insights on the most promising approaches and strategies for parasite and disease control. Similar to other apicomplexan hemoparasites, *Babesia* spp. are haploid and the only diploid stage is the zygote, which is formed during sexual reproduction in the tick host. Accordingly, only a single allele can be detected in the vertebrate host and thus, detection of two or more alleles is evidence of dual or multiple infections. Consequently, heterozygosity cannot be directly observed, and is estimated based on the observed allele frequencies in the population.

As measured using expected heterozygosity, the overall genetic diversity of the *B. ovis* population in Turkey was found to be extremely high (H_e = 0.799) and similar to that observed for its phylogenetically closest relative, the cattle-infecting *B. bovis* (H_e=0.837) (Simuunza et al., 2011). Likewise, in a recent study comparing reference isolates from diverse countries and continents around the world, a comparably high genetic diversity was observed in the *B. bovis* metapopulation (H_e = 0.780, Flores et al., 2013). *Babesia ovis* belongs with the closely-related species *B. bovis* to the *Babesia* sensu stricto group of piroplasmids, characterized by transovarial transmission.

In contrast, population genetic studies of *Theileria lestoquardi* (classified within *Theileria* sensu stricto), the causative agent of malignant ovine theileriosis in sheep, have shown a considerably lower genetic diversity in Sudan (H_e=0.550 to 0.572) and Oman (H_e=0.528 to 0.610) as compared to its sister species *T. annulata* in Oman (H_e=0.833). Furthermore, the genetic diversity of *T. lestoquardi* was also found to be considerably lower to that determined in the present study on *B. ovis* (H_e=0.799) and to *B. bovis* (H_e=0.837) in Turkey (Simuunza et al., 2011; Al-Hamidhi et al., 2016; Ali et al., 2017; Awad et al., 2018). While each of these species was analysed using a different set of markers, these various markers were identified using broadly similar criteria and for this reason they are broadly comparable. It has been proposed that the generally lower diversity of *T. lestoquardi* compared to that of the closely related *T. annulata* is because present day *T. lestoquardi* and *T. annulata* evolved relatively recently from a common ancestor, following adaptation of *T. lestoquardi* to the small ruminant host, as supported by high 18S rRNA gene identity (Schnittger et al., 2000, 2003; Awad et al., 2018). Importantly, although *B. ovis* and *B. bovis* also represent sister species, it can be predicted that they have undergone a strikingly longer independent evolution, as evidenced by phylogenetic analysis (Schnittger et al., 2012).

A high diversity of the *B. ovis* population in Turkey (n=107) is further substantiated by the high mean number of different alleles identified at each marker loci (19.5), whereas lower mean numbers of 11.3, 10.8, and 9.7 different alleles were observed in the *B. bovis* population of Turkey (n = 40), *T. annulata* population in Portugal (n = 90), and in the *T. lestoquardi* population of Sudan (n = 36), respectively (Gomes et al., 2016; Ali et al., 2017). Noteworthy, a high mean number of 12.4 private alleles in the overall *B. ovis* population suggests that future population admixture and subsequent recombination have the potential to significantly further increase the *B. ovis* genetic diversity in Turkey.

The observation of a lower genetic diversity in the *B. ovis* population of western Turkey (H_e = 0.663), as compared to the central (H_e = 0.770) and eastern populations (H_e = 0.791) suggests the existence of a degree of genetic differentiation between these two populations. Indeed, PCoA demonstrated two larger discrete clusters of *B. ovis* isolates, one comprising

predominantly isolates originating from western Turkey (cluster 1) and another composed predominantly of isolates from the central and eastern regions (cluster 2). Strikingly, a third cluster (cluster 3) was observed, indicating that an additional separate *B. ovis* subpopulation exists within the parasite population of western Turkey. A number of assumptions may be drawn from this analysis. Firstly, cluster 1 includes *B. ovis* isolates originating from Aksaray, central Turkey, strongly suggesting that sheep infected with *B. ovis* originating from western Turkey have been introduced into the Aksaray region of central Turkey. This finding strongly suggests that some, though restricted, trading of sheep from western to eastern Turkey has taken place. Secondly, cluster 3 comprises exclusively *B. ovis* isolates from Aydin, a coastal region of western Turkey. Thus, Aydin isolates are distinctly different from the other western and from the eastern/central *B. ovis* populations, which may be due to strain selection by unique sheep breeds such as, *sakız*, *karya*, and *çine çaparı* kept in this region (pers. comm.). Thirdly, isolates from the *B. ovis* population of central and eastern Turkey cluster together suggesting that they represent a single parasite population. Fourthly, the two *B. ovis* isolates from Israel sit within cluster 2, demonstrating that they possess a MLG similar to those defined for central/eastern Turkey.

Significant linkage disequilibrium was observed in the overall population of Turkey, in pairwise combinations and within each of the regions of western, central, and eastern Turkey. A somewhat lower LD ($I^S_A = 0.0889$) was found for the pairwise combination of the population of central and eastern Turkey suggesting that there is a state of panmixia but recombination is not frequent enough to reach LE. Exclusively in two more confined regions, Konya and Aydin, linkage equilibrium could be observed. This finding also suggests that no genotypes have been introduced into these two regions, whereas, as shown by PCoA and STRUCTURE analysis, introduction of genotypes from other regions of Turkey to Afyon, Aksaray, and Kutahya can be implied and may be responsible for the observed LD due to a recent admixture of novel genotypes. In order to reach LE, recombination frequency must be high which is underpinned by conditions where transmission intensity is high, the vector tick is abundant, and a high level of sheep infestation is observed (Weir et al., 2011). Due to the restricted mobility of the tick

vector off the host, LE is most likely observed at the farm level or a more confined local geographic area under the premise that no novel genotypes are introduced. Thus, LD is generally observed in the overall population and in larger geographical regions, while LE is observed in the two smaller confined regions of Konya and Aydin. This observation suggests that any scenario where resistance to a drug has arisen in a particular region, dissemination of the resistance gene(s) would occur primarily in a local manner, unless resistant parasites were spread via trading of infected animals across regions.

A high multiplicity of infection (MOI) of 3.31 was found in sheep of western Turkey, as compared to that of central (2.48) and eastern Turkey (2.62). In comparison, a considerably lower MOI of 2.04 has been observed for *B. bovis*-infection of cattle of Turkey (Simuunza et al., 2011). This high MOI in combination with a high genetic diversity is indicative of a high infection pressure with ticks carrying distinct parasite genotypes. It may be concluded that pathogen transmission is considerably more intense in western and elevated in eastern Turkey, which coincides with a higher *B. ovis* infection of sheep as reported for these regions by Bilgic et al (2017).

Babesia ovis is characterized by transovarial transmission which allows direct vertical transfer of the parasite into tick eggs and successive larvae, nymph, and adult tick stages in the absence of an infected vertebrate host. Although, it has been recently reported that exclusively the adult tick effectively transmits *B. ovis* while larvae and nymph stages have none or inefficient vectorial competence (Erster et al., 2016), it may be assumed that compared to transstadial transmission as seen for *Theileria*, transovarial transmission might have the potential to exert higher infection intensity.

Importantly, two *B. ovis* isolates from Israel show a similar genotype to those from the central/western population of Turkey. A limited exchange of parasite genotypes seems to take place between the western and central/eastern populations, possibly due to mutual trading of sheep. This is supported by the observation that some *B. ovis* MLGs display combinations of western–central/eastern genotypes, suggesting recent recombination events. Interestingly, the substructuring of the western Turkish parasite population versus the eastern/central parasite

population corresponds with important climatic differences of the western region with an average yearly temperature of about 21ºC as compared to the central-eastern region with an average yearly temperature of about 15ºC. We hypothesize that the different climatic conditions result in activity differences of the respective *Rhipicephalus* spp. subpopulations, which, in turn, possess discrete parasite populations.

The significance of the observed genetic differentiation is that the development of an attenuated live vaccine based on a single strain may possibly not protect against challenges of strains from both populations. Importantly, the *in vitro B. ovis* strain Israel may be suitable for the development of a vaccine against the central/eastern parasite population but would be predicted to less likely protect against challenges from parasites of the population of western Turkey.

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Ethics approval

This study was approved by the Adnan Menderes University Animal Experiment Ethic Committee dated 28/08/2012 in accordance with decision number B.30.2.ADÜ.0.00.00.00/050.04/2012/047.

Conflict of interest

None declared

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563 **Table 1.** Allelic variation in the *Babesia ovis* population of Turkey

	Oms_8	Oms_10	Oms_11	OMS_47	OMS_58	OMS_62	OMS_93	OMS_103	Mean
Total number of alleles	17	18	15	28	16	20	28	14	21.6
Predominant allele frequency (size in bp)	0.393 (313)	0.346 (181)	0.514 (142)	0.206 (241)	0.421 (130)	0.290 (189)	0.290 (250)	0.393 (282)	0.747
Genetic diversity (H_e)	0.802	0.812	0.703	0.899	0.723	0.823	0.865	0.755	0.799

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566 **Table 2.** Pairwise comparison of Wright´s fixation index (F_{st}) and unbiased Nei´s genetic distance (uD)

	Wright´s fixation index (F_{st})			
Unbiased Nei's Genetic Distance (uD)	Aydin	Western Turk.*	Central Turk.	Eastern Turk.
Aydin	-	0.544	0.241	0.244
Western Turk.*	2.464	-	0.241	0.233
Central Turk.	0.421	0.889	-	0.011
Eastern Turk.	0.380	0.830	0.049	-

567 Western Turk.*, isolates from western Turkey exempt Aydin

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569

570 **Table 3.** Linkage disequilibrium analysis of *Babesia ovis* populations of Turkey

Region	n	H_e	I^S_A	V_D	L	p-value	Linkage
Turkey	107	0.799	0.1615	2.5879	1.2863	<0.01	LD
Western/central Turk.	85	0.784	0.1992	3.1198	1.4101	<0.01	LD
Central/eastern Turk.	56	0.812	0.0889	1.8018	1.2209	<0.01	LD
Western/eastern Turk.	73	0.770	0.1916	3.1558	1.4702	<0.01	LD
Western Turk.	51	0.677	0.2111	3.7629	1.7158	<0.01	LD
Central Turk.	34	0.793	0.1317	2.2986	1.4165	<0.01	LD
Eastern Turk.	22	0.829	0.0919	1.6608	1.2347	<0.01	LD
Konya	20	0.690	0.0331	1.8630	1.9571	0.11	LE
Afyon	22	0.503	0.0660	2.0908	1.7604	<0.01	LD
Kutahya	20	0.643	0.1483	2.6554	1.7877	<0.01	LD
Aydin	7	0.143	0.0196	0.9286	1.9286	0.12	LE
Aksaray/Nigde	15	0.801	0.2120	2.9557	1.5518	<0.01	LD

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573 **Table 4.** Multiplicity of *Babesia ovis* infection in different regions of Turkey

Region	n	Multiplicity of infection			
		Mean	SD	Min	Max
Turkey	107	2.91	0.93	1.25	6.13
Western Turk.	51	3.31	0.97	1.33	6.13
Central Turk.	34	2.48	0.75	1.25	5
Eastern Turk.	22	2.62	0.69	1.63	4

574 n: number of samples; SD: standard deviation

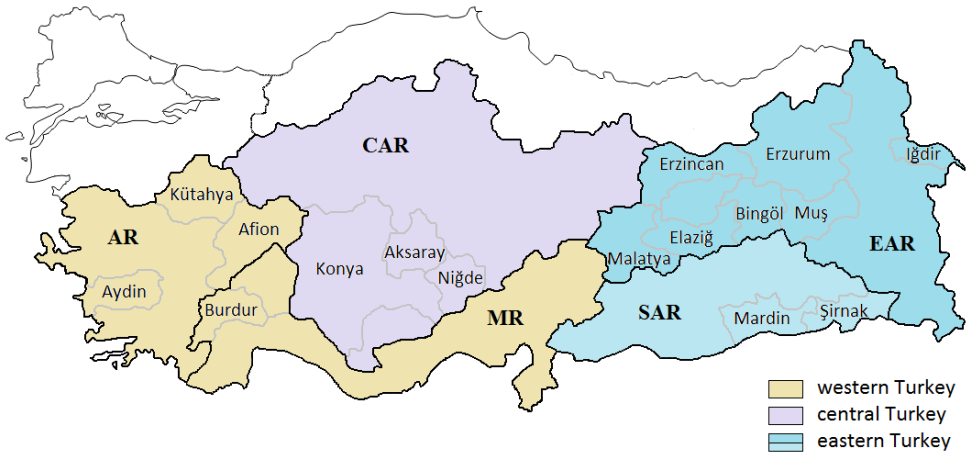

Figure 1. Map of Turkey showing the main sampling regions: western Turkey comprising of the Aegean (AR) and Mediterranean Region (MR), central Turkey represented by the Central Anatolian Region (CAR), and eastern Turkey consisting of the Eastern Anatolian Region (EAR) and the Southeastern Anatolia Region (SAR). Western Turkey has a medium temperature of 15.9 ºC and a medium humidity of 62.4 %; central Turkey has a medium temperature of 10.5 ºC and a medium humidity of 62.6 %; EAR and SAR of eastern Turkey have a medium temperature of 9.7 ºC and 16.5 ºC and a medium humidity of 60.9 % and 53.4 %, respectively.

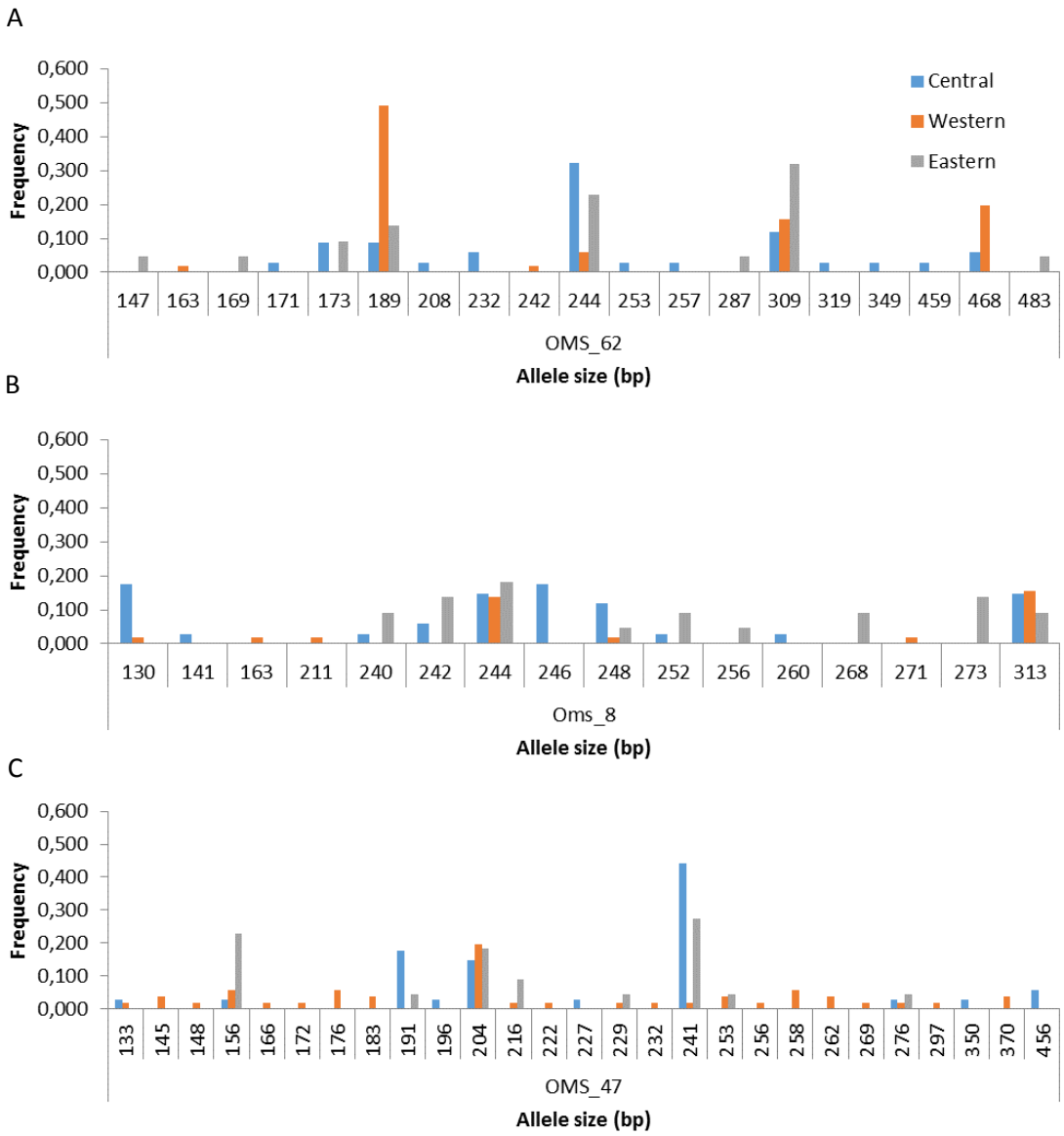

Figure 2. Allele frequency histograms of selected satellite markers of *Babesia ovis* field populations of the western, central, and eastern regions of Turkey. Satellite marker OMS_62 shows a high number of private alleles (n = 8) in the central population, and low to moderate number of private alleles in the central (n = 4) and western populations (n = 2) (A). A bell-like frequency distribution is observed from allele 211 to 260 bp for marker Oms_8 (B). Marker OMS_47 shows a high number of private alleles (n = 14) in the western parasite population (C).

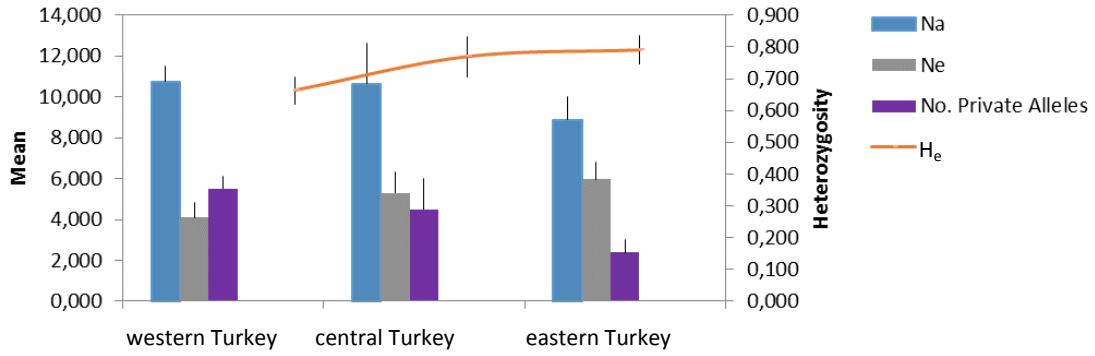

Figure 3 Genetic diversity (H_e), mean number of different (N_a), effective (N_e), and private alleles in the *B. ovis* populations of western, central and eastern Turkey.

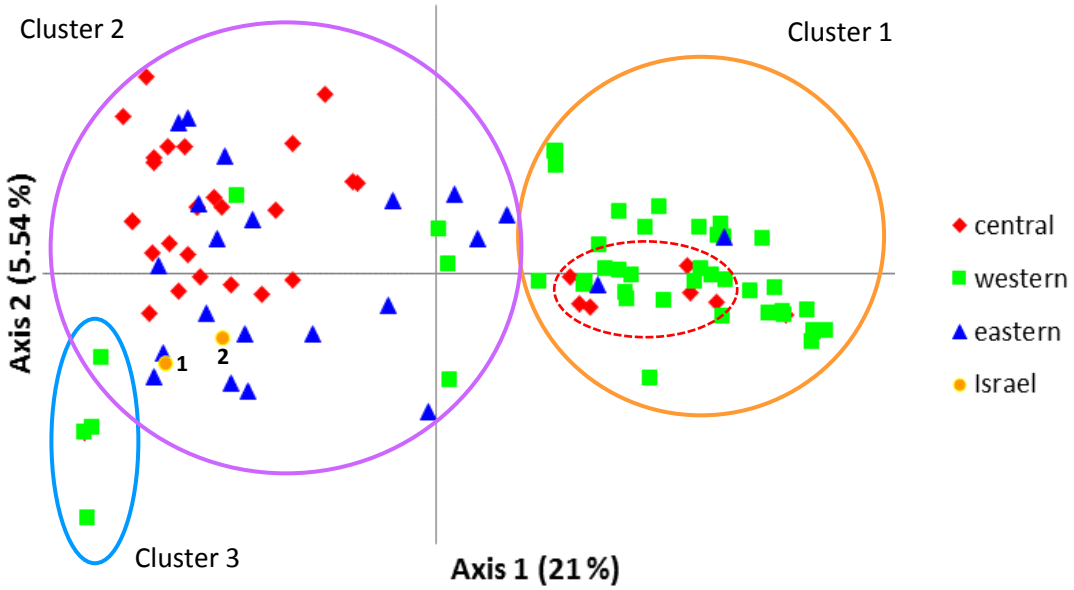

Figure 4. Principal Component Analysis (PCoA) of the multi-locus genotypes of *B. ovis* isolates of western, central, and eastern Turkey. The two principal axes are shown and the portion of variation in the dataset explained by each axis is indicated in parenthesis. The subgroup indicated with broken lines is formed by samples from Aksaray, eastern Turkey and falls within cluster 1, mainly composed by samples from western Turkey. 1: *B. ovis* strain Israel, 2: *B. ovis* strain Itamar, Israel.

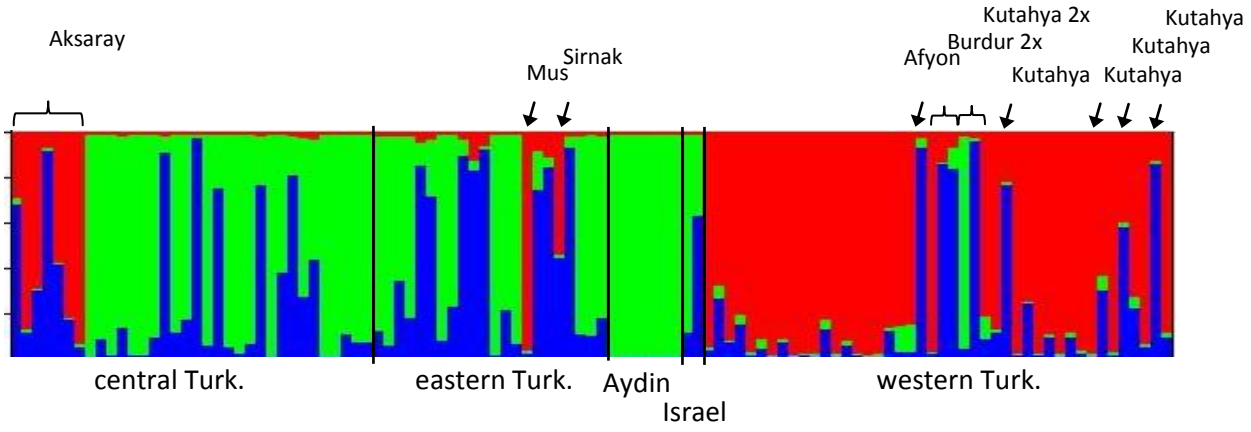

Figure 5. STRUCTURE and EVANNO Method Delta K analysis. The assumption of three genotype clusters (K = 3) was found to best fit data which are represented by green (Aydin, western Turkey), green/blue (predominantly central and eastern Turkey), and red stripes (predominantly western Turkey with the exclusion of Aydin) within the bar. The origin of population samples are given below. Genotypes that did not fit into the genotype background of the respective geographic population are given above.