Genome Variation in Tick Infestation and Cryptic Divergence in Tunisian Indigenous Sheep

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Abstract

Background: Ticks are obligate haematophagous ectoparasites considered second to mosquitoes as vectors and reservoirs of multiple pathogens of global concern. Individual variation in tick infestation has been reported in indigenous sheep, but the genes regulating the trait are poorly understood.

Results: Here, we report 397 genome-wide signatures of selection overlapping 991 genes from the analysis, using four methods (ROH, LR-GWAS, XP-EHH, FST), of 600K SNP genotype data from 170 Tunisian sheep exhibiting high and low resistance to ticks. We considered 45 signatures detected by consensus results of at least two methods as high-confidence selection sweep regions. These spanned 104 genes which included immune system function genes, solute carriers and chemokine receptor. One region spanned STX5, that has been associated with tick resistance in cattle, implicating it as a prime candidate in sheep. We also observed RAB6B and TF in a high confidence candidate region that has been associated with growth traits suggesting natural selection is enhancing growth and developmental stability under tick challenge. The analysis also reveals fine-scale genome structure suggesting the existence of cryptic divergence in the Tunisian sheep.

Conclusion: Our findings provide genomic reference that could enhance our understanding of the genetic architecture of tick resistance and cryptic divergence in indigenous sheep.

Background

Ticks are among the most ancient acari and possibly the earliest to evolve blood-feeding capacity through the development of immune-active proteins and lipids that induce vasodilatory, anti-haemostatic and immunomodulatory activities that guarantee successful engorgement and acquisition of a blood meal [1–5]. Compared to other arthropod vectors, ticks transmit a greater variety of pathogenic microorganisms including protozoa, bacteria and viruses, implicated in severe infections in humans, wild and domestic animals [6, 7]. Globally, ticks and tick-borne infections (T-TBI) are a major health impediment to efficient livestock production and reproductive performance. These translates to reduced farm incomes, increased consumer and producer costs, and disruption of local and global markets, and trade through the imposition of quarantines and market bans.

Prophylactic use of acaricides is the commonly employed strategy to control and eradicate ticks [8, 9]. The (over)use of acaricides has however imposed selection pressure that has resulted in the development of acaricide-resistant tick strains, persistent environmental contamination, infection of farmers and treated animals, retention of chemical residues in livestock products, and increased costs of existing and of developing and manufacturing of new more potent, acaricides [9–13]. Anti-tick vaccines are a promising option, but they do not confer protection against multiple tick species [7, 14, 15] which is a common occurrence in most regions, especially the (sub)tropics [16–18]. These factors are driving the search for alternative sustainable tick control strategies such as the use of tick-resistant animal genotypes. The host’s natural resistance to ticks can be exploited as a long-term sustainable control strategy. It offers a low-cost, permanent solution that demands no extra resources and costs to generate a unit of product [19]. Moreover, the inherent resistance targets all tick species.

Genetic variation in resistance to parasites has been demonstrated in many livestock species with investigations on individual variation in resistance to T-TBI being the subject of intense research in cattle (see review by [20]) compared to other livestock species. Most of the studies have shown that an effective immunological response (resistance/tolerance) to T-TBI is genetically determined with a heritability of between 0.05 and 0.42 (see review by [20]). For haemopathogens, the immunological response has been associated with the ability to resist the development of anemia following infection [21, 22], variation in the expression of extracellular matrix metalloproteinase [23], differences in chemokines, toll-like and chemokine receptors [24, 25], variation in genes that limit the supply of blood-meal to ticks and enhance both innate and adaptive immune responses [26]. Several genes have been proposed as candidates for tick resistance. Lumican (LUM) was identified as a potential biomarker for tick resistance in cattle [26]. [27] reported two SNPs in the ELTD1 gene that are associated with tick burdens in both dairy and beef cattle, and a haplotype of nine tag SNPs and two others that are associated with tick counts in dairy cattle. The authors reported also haplotypes spanning the ITGA11 gene that were associated with tick burdens. Class I antigens of the bovine major histocompatibility complex have been reported to be associated with tick loads [28, 29]. Alleles at the bovine lymphocyte antigen (BOLA-DRB3) have also been linked with resistance to Rhipicephalus (Boophilus) microplus infestation [30–32].

Domestic sheep (Ovis aries) are the second most abundant ruminant livestock after cattle [33] and are an important component of livestock enterprises in tick-endemic areas. Ticks transmit to sheep several pathogens, including viruses (tick-borne encephalitis virus, Thogoto virus, Louping-ill virus, Crimean-Congo haemorrhagic fever virus), bacteria (Mycoplasma ovis, Anaplasma ovis, Borelia burgdorferi s.l., Francisella tularensis, Dermatophilus congolensis, Coxiella burnetti) and protozoa (Babesia spp. and Theileria spp.) [34–40]. Some of these pathogens cause important zoonotic diseases such as Crimean Congo Haemorrhagic fever, Q fever and Human granulocytic anaplasmosis [41] that result in dramatic human and animal health impacts including significant socio-cultural and economic losses. Despite reports on individual variations in tick burdens in sheep including both prevalence and infestation intensity [42–45], little is known regarding the genetic basis of this phenotype.

Taking advantage of observed individual natural variation in tick infestation in two indigenous sheep populations (Barbarine (B; fat-tailed) and Queue Fine de l'Ouest (Q; thin-tailed)) from Tunisia [40], we investigated the likely genetic basis of the variation by analysing genome-wide 600K SNP genotype data that was generated using 170 individuals showing high- and low-infestation/resistance (HR/LR) to ticks. Our results suggest that the resistance could be the outcome of multigene associations with RAB6B, TF, SLC02A1, and STXB6 the likely potential candidates and STX5 as the possible prime candidate driving the genetic variation in tick infestation and thus resistance in sheep.
Results

Population genetic structure

Genetic structure and relationship were investigated with principal component analysis (PCA) and ADMIXTURE tool. The first and second PCs of the PCA explained respectively, 7.63% and 6.04% of the total genetic variation (Figure 1). They separate the study individuals into four genetic groups, herein named G1, G2, G3 and G4 (Figure 1a). These four groups do not correspond to the resistance status to ticks (HR/LR) (Figure 1b), the sampling region (northeast, northwest, southeast) (Figure 1c) and breed (B/Q) (Figure 1d).

Following ADMIXTURE analysis, the lowest CV error was at K = 4 (Figure 1e) suggesting the presence of at least four genetic clusters in the dataset. These clusters correspond to the four genetic groups revealed by PCA and do not correspond to the resistance status to ticks, sampling region and breed. Therefore, the results from the two algorithms corroborate each other and for consistency, we also named the four genetic clusters as G1, G2, G3 and G4 (Figure 1f). The G1 cluster occurs in a few individuals of G2, G3 and G4, suggesting the introgression of G1 into these three clusters.

Genome variation

Descriptive statistics, that provide insights on the levels of genetic diversity and estimated for the overall dataset, the two breeds (B/Q), the two cohorts (HR/LR) and the four genetic groups (G1, G2, G3, G4), are shown in Table 1. The overall estimates of genetic diversity represented by observed (H_{O}) and expected (H_{E}) heterozygosity averaged 0.3305 ± 0.0305 and 0.3470 ± 0.00006, respectively. The individual values for each statistic are in general higher than 0.3286 ± 0.0344. The mean length of ROH was 3.406 ± 0.4072, ranging between 3.032 ± 0.5213 Mb (G1) and 3.728 ± 1.004 Mb (G4). The mean value of F_{ROH} was 0.0549 ± 0.0842 and ranged between 0.0239 ± 0.0527 (G1) and 0.1612 ± 0.1029 (G3). The average value of F was 0.0476 ± 0.0881 with a range of -0.0866 ± 0.1339 (G4) and 0.0525 ± 0.0991 (HR). The LR cohort shows higher values of H_{O} and H_{E} compared to the HR cohort. At the opposite, the LR cohort shows lower values of ROH size, F_{ROH} and F. For the four genetic groups, G4 had the highest values of H_{O}, H_{E} and mean length of ROH while the lowest values were observed in G1, G2 and G1, respectively. The F_{ROH} was highest in G3 and lowest in G1. Except G1, the other three genetic groups show negative values of F.

We assessed the decay in linkage disequilibrium (LD; Figure 2a) and the trends in effective population size (N_{E}; Figure 2b) for the overall dataset, the two cohorts (HR/LR) and the four genetic groups (G1-G4). The overall pattern of decay in LD as a function of genomic distance is the same across all the datasets analysed. It generally reveals higher LD at shorter distances which decays rapidly and plateaus off at around 0.2Mb. The G2, G3 and G4 groups show persistently higher r^2 values (r^2 > 0.15) and thus higher LD compared to the overall dataset, HR, LR and G1 (r^2 < 0.15). The trends in N_{E} over generation time show different profiles for the data classes analysed. The overall dataset and G1 group show the highest N_{E} which increases gradually reaching the maxima around 350 generations ago after which it declines rapidly to the present time. The HR and LR cohorts show the next lowest estimates of N_{E} which increase gradually up to around 350 generations ago followed by a rapid decline to the present. The G2, G3 and G4, have the lowest N_{E} across all generations which declines gradually to the present time.

Genome-wide selection signature analysis

We assessed selection signatures using runs of homozygosity (ROH), Logistic regression- genome-wide association analysis (LR-GWAS), cross-population extended haplotype homozygosity (XP-EHH) and genetic differentiation (F_{ST}) to gain insights on the regions of the genome correlating with resistance to ticks (HR verses LR). We also used the XP-EHH and F_{ST} algorithms to investigate selection signatures that may explain the divergence between the four genetic groups, G1-G4, revealed by PCA and ADMIXTURE algorithms.

The ROH approach identified 110 and 105 ROH regions that spanned 280 and 281 annotated genes in the HR and LR cohorts (Figure 3a, 3b; Supplementary Table S1, S2), respectively. The LR-GWAS identified 242 candidate regions spanning 561 genes (Figure 4a), and F_{ST} identified 79 candidate regions, showing genetic differentiation between the HR and LR cohorts (Figure 4b), that spanned 243 genes. The XP-EHH identified 76 candidate regions under positive selection spanning 187 genes (Figure 4c). These 397 candidate regions overlapped 991 genes (Supplementary Table S3, S4, S5) of which 104 annotated genes were found in candidate regions that were commonly identified by at least two approaches.

We used the ROH analysis as an approach for identifying cohort-specific selection signatures. We therefore compared the ROH results of HR and LR (Figure 4a, 4b). This identified 30 ROH regions, spanning 57 annotated and 20 unannotated (prefix “LOC”) genes that were specific to the HR cohort (Supplementary Table S6). Considering these HR-specific ROH regions and the regions identified by LR-GWAS, XP-EHH and F_{ST}, there were 45 candidate regions that were simultaneously identified by at least two methods across 17 autosomes and spanning 160 genes of which, 56 remain unannotated (Table 2). Among the putative candidate genes identified in the 45 candidate regions were immune system function genes (CD242EP1, CD164, CD180, CD181, MYO), solute carriers (SLCO2A1, SLC26A3, SLC24A3, SLC22A8) and a chemokine receptor (CCIN). Of these genes, SLCO2A1 (OAR1), MYO (OAR8), SLC24A3 (OAR13), CD180 (OAR16) and CDH18 (OAR16) occurred closest to the most significant SNP identified by LR-GWAS in the respective candidate regions. Four genes (RAB6B (OAR1), TF (OAR1), SLCO2A1 (OAR1), STXBP6 (OAR18) and STX5 (OAR21)) that occurred in our candidate regions are of particular interest with regard to the objectives of this study. They have direct or indirect association with body weight traits in various livestock species.
and resistance to ticks in cattle. Worth noting is that STX5 occurred in an ROH region that was specific to the HR cohort (Table 3a) and in the most significant region that differentiated HR from LR (Figure 4b).

Functional enrichment analysis was tested in the pool of 104 putative genes, excluding 56 that remain uncharacterized, that were present in the 45 candidate regions that overlapped between at least one HR-specific ROH region and the candidate regions that were identified by LR-GWAS, FST and XP-EHH (Table 2). The analysis resulted in nine KEGG Pathways and four GO terms that were significantly enriched (Supplementary Table S7). The two top-most significant terms were Lysozyme (osas04142) and microRNAs in cancer (osas05206). These functions are represented by clusters of genes (such as GGA1, CD164, and BCL2L11) having roles in innate immunity and disease-related inflammation [46, 47].

To determine genomic regions associated with divergence between G1, G2, G3, and G4, we contrasted these groups with each other using XP-EHH and FST. The candidate regions that were identified by the two methods following comparative analysis involving each group are shown in Supplementary Table S8. The selection regions detected by XP-EHH were generally broader (Figure 5) compared to those identified by FST (Figure 6) which are narrower and perhaps more accurate. This may be because XP-EHH is LD-based, and LD is expected to extend over longer distances in regions under recent selection [48]. To narrow down the regions that overlapped between the two methods, we considered the ones identified by FST as the reference candidate regions. In total, 10, 18, 18 and 26 regions that overlapped in a comparative analysis involving at least one group-pair were identified for G1, G2, G3 and G4, respectively (Supplementary Table S8). These regions spanned 16, 44, 25 and 86 genes, respectively. The most significant region for G1, G2, G3 and G4 were located on OAR13, 7, 16 and 27, respectively. These regions spanned respectively 4, 2, 1 and 5 genes.

We performed functional enrichment analysis with the pool of 11, 38, 23 and 76 genes, excluding the uncharacterized ones, that were identified in each of the four genetic groups (G1, G2, G3, G4). The functional term clusters that were significantly enriched for the cluster of genes for each genetic group are shown in Supplementary Table S9.

Discussion

Ticks and TBI impact negatively ruminant livestock production worldwide. Therefore, the control and ultimate elimination of T-TBI should be prioritized to minimize their impacts not only on animal health and production but also on long-term human and environmental wellbeing. Large variations in susceptibility to T-TBI points to genome-wide variability underpinning inter-animal variation in both tick infestation and the pathogens they transmit. Here, we generated ovine 600K SNP genotype data, from 170 animals of two breeds of Tunisian indigenous sheep graze natural communal pastures with no history of specific anti-tick prevention measures. The sheep comprised of individuals showing high and low resistance (HR/LR) to tick infestation and piroplasm infection under natural conditions [40]. We analysed the data with four approaches, ROH, LR-GWAS, XP-EHH and FST, to investigate signatures of selection underpinning variation in tick infestation and therefore resistance. As noted recently by [49], we also acknowledge that using naturally infected animals runs the risk of resistant animals being a cocktail of truly highly resistant animals and those that were never exposed to infestations. These factors may dilute the certainty that animals showing high or low resistance to different pathogens, including viruses, bacteria and parasites, could in fact be functionally dissimilar [49].

Compared to the HR cohort, LR showed a comparatively higher level of genetic diversity and lower levels of inbreeding, although the differences were not significant (P > 0.05). The differences in the levels of inbreeding in the two cohorts, as determined from FRoh and FST, were also not significant. The variability in the four genetic groups revealed by PCA and ADMIXTURE was of the same magnitude as that of the two cohorts. Comparable levels of genetic variability have been reported in Egyptian [50], Algerian [51], Tunisian [52] and Russian [53] breeds of sheep.

The PCA and ADMIXTURE provided corroborating evidence suggesting absence of genetic stratification that is consistent with the a priori classification of the study individuals based on their susceptibility/resistance status to tick infestation, geographic sampling regions and breeds. The lack of genetic differentiation based on susceptibility/resistance to tick infestation is not surprising. A similar lack of genetic stratification that corresponds to a priori classification of sheep based on prolificacy [54] or resistance to gastro-intestinal nematodes [49, 55] has been reported. [56] also observed a lack of clear differentiation between sub-populations of dual-purpose black and white and German Holstein cattle. Our result suggests that the animals that were identified to be of high- and low-resistance to ticks are not highly divergent for this phenotype. This can be attributed to various reasons including, weak selection pressure driving differences in tick susceptibility/resistance and thus any favourable alleles are likely to be very rare, low level of tick burden that does not result in detectable genomic differentiation, and absence of farmer-driven preferential use of tick resistant animals for breeding. One or more of these reasons may explain the lack of genetic differentiation between the two cohorts. The lack of genetic differentiation corresponding to breeds and sampling regions was not unexpected. Past and recent human-mediated translocations and dispersal of the two breeds across Tunisia has brought the two breeds in close geographic proximity [57] and is resulting in cross-mating that is homogenizing their genomes. A similar finding was reported by [52]. The fact that the genomes of the B and Q breeds could not be differentiated by ADMIXTURE in each cluster provides evidence at the genome level that supports past and on-going uncontrolled mating of the fat-tailed Barbarine with thin-tailed breeds to reduce the fat content, especially of the tail, in the Barbarine carcass [58].

Linkage disequilibrium was estimated for all marker pairs using the metric r² plotted as a function of increasing genomic distance. The overall pattern of decay in LD is the same for all the classes of datasets analysed. It reveals higher LD at shorter distances which declines rapidly and plateaus off at around 200 Kb. This differs from what has been observed in commercial breeds whose LD plateaus off at around 150 Kb [59] and is most likely the result of differences in breeding history. Specifically, the study populations comprise a broad genetic base while that of commercial breeds has been narrowed down by bottlenecks associated with breed formation and stringent artificial selection for traits of economic importance. Both the HR and LR
cohorts showed identical patterns of LD decay over increasing genomic physical distances, and in the trend in $N_e$ over the past 1,000 generations. Although the two cohorts showed differences in the prevalence of tick infestation [40], this result suggests that they share aspects of their past and recent population demographic, genetic and breeding history. This may be the case as their genomes show no genetic differentiation that is commensurate with their a priori classification based on tick susceptibility/resistance. We also assessed the demographic profiles of the four genetic groups that underly the genome architecture of the study individuals as revealed by PCA and ADMIXTURE. The G2, G3 and G4 genetic groups had the highest $r^2$ values ($r^2 > 0.15$) and thus highest LD over short genomic distances and a decay curve with persistently elevated values compared to G1, HR and LR cohorts. A similar pattern was observed in the primitive Soay sheep [59] and was attributed to its small effective population size due to its genetic isolation in Scotland's St. Kilda Island. Indeed, G2, G3 and G4 show the lowest $N_e$ which declines gradually over all generations. The possible reason(s) for the observed decline in $N_e$ in these three groups remains unknown and is worth further investigation. In contrast, LD was lower ($r^2 < 0.15$) in HR, LR and G1 across all genomic distance intervals and they also showed the highest $N_e$ sizes over all generations. A similar pattern and magnitude of LD was reported in the Iranian Qezel sheep and was attributed to high genetic diversity in the breed [59]. This explanation may not suffice to explain our results as there was no significant differences in the level of genetic diversity across the different classes of datasets that we analysed. Despite being unexplained, we postulate that the low LD and high $N_e$ in HR, LR and G1, and similar magnitude of genetic diversity is unlikely to reflect any biological phenomenon.

In line with what we observe in our study, the analysis of SNP arrays shows that LD decays faster and at much shorter genomic distances in sheep [59, 60]. While LD estimates are not comparable between studies due to differences in sample sizes, number and type of markers used, it is evident that sheep are characterized by lower LD than other domestic animals. For instance, the extent of LD in various cattle breeds [61] is greater than that found in our study. [62] reported higher $r^2$ values of between 0.19 and 0.26 in four pig breeds. This observation may be the result of less intensive selection in sheep for traits of economic importance and that the species is derived from a larger initial gene pool compared to other domestic animals [63]. Indeed, multiple male and female lineages and introgression from wild relatives have been implicated to constitute the modern-day diversity of domestic sheep [64–66].

Since millennia, ticks have parasitized animals for a blood-meal to the extent of developing, through co-evolution, a sophisticated armoury that guarantees their biological success. Here, to explore genomic signatures associated with individual variation in tick infestation and thus host resistance to ticks, we segregated the study individuals into two extreme groups comprising animals showing high- and low-resistance (HR/LR) to tick infestations. The HR group comprised individuals showing neither tick infestation nor piroplasm infection, and the LR group included animals that were infested by ticks and infected by piroplasms. The differences in tick infestation/resistance phenotype between the two groups was significant [40] and their comparison was used to maximize the likelihood of identifying biologically meaningful and statistically significant results. We therefore performed a comparative analysis with ROH, LR-GWAS, XP-EHH and $F_{ST}$ approaches to detect selection signatures and SNPs that are likely associated with variation and thus resistance to tick infestation.

The four analytical approaches revealed 45 candidate regions, that overlapped between at least two approaches, which spanned 104 annotated genes. We observed a selection signature on OAR21 that was identified by LR-GWAS and $F_{ST}$ which spanned nine genes including $STX5$ (Syntactin-5). The ROH analysis showed that this region was specific to the HR cohort and it was the most significant in differentiating the HR from the LR cohort in the $F_{ST}$ analysis. In a study of Belmont Red cattle, $STX5$ was amongst 11 out of 14 genes that showed a significant increase in its expression levels in the skin of animals that were highly resistant (HR) to ticks at time zero post infestation compared to animals of low resistance (LR) [67]. The expression level was more pronounced in 3-hour skin samples, suggesting a response to tick attachment and this could contribute to host innate immunity and to a higher resistance to ticks. $STX5$ regulates the endoplasmic reticulum channel-release properties of polycystin-2, a member of the transient receptor potential cation channel family that can function as an intracellular calcium ($Ca^{2+}$) release channel [68]. Based on the increased expression levels of the 11 out of 14 genes they tested, most of which are $Ca^{2+}$ dependent genes, [67] suggested that the high mRNA transcription levels of $Ca^{2+}$ signaling genes in skin of HR animals accounts for their greater resistance to ticks. Thus, previous tick exposure may prime animals that exhibit the HR phenotype to resist further infestations via increased expression of $Ca^{2+}$ signaling genes. Based on these results, we suggest that the $STX5$ could be the prime candidate gene driving tick resistance in sheep.

Environmental changes can exert either positive or negative effects on thermoregulation mechanisms that could influence tick burdens [69]. It has been observed that tick burdens in cattle might be correlated with traits that influence thermal comfort [70]. For instance, traits such as skin thickness, hair density and skin secretions (quantity and quality) could influence tick resistance in domestic livestock and might also influence thermal comfort since they affect the ability of the animal to dissipate heat [71]. Observations in Colombian cattle showed that high temperature humidity index (THI) values were associated with lower tick burdens and a higher tick infestation would be expected when animals experience higher thermal discomfort [70]. These findings are of relevance in our study because one of our candidate regions found on OAR18 that was revealed by LR-GWAS and XP-EHH, spanned $STXB6$ (Syntxin Binding Protein 6) and another candidate region on OAR1, that was revealed by LR-GWAS, XP-EHH and $F_{ST}$, spanned $SLCO2A1$ (OATP2A1). Both genes were closest to the most significant SNP as revealed by LR-GWAS. $STXB6$ was one of the genes found to be upregulated in the testes of roosters exposed to acute heat stress [72]. Transcripts of $STXB6$ were also found to be among eight other genes that were correlated with the modified Rodnan skin thickness score and forced vital capacity in humans suffering from scleroderma and systemic sclerosis [73], a condition that is characterized by thickening and hardening of the skin. One of the physical barriers that can confer resistance to tick infestation(s) include skin thickness, with animals with thin skins having significantly lower tick burdens [67, 74, 75]. Furthermore, except for birds and canids, thin skins allow animals to dissipate more heat through sweating and evaporative cooling when ambient temperatures are above the thermoneutral zones [69], and at the same
time, it decreases tick attachment rate to the host's skin. SLC02A1, a prostaglandin transporter, maintains an increased interstitial concentration of PGE2, a major chemical mediator of febrile response, in the hypothalamus which plays a key role in thermoregulation [76]. The function of these genes is most likely complemented by the GNE, the gene closest to the top-most SNP as identified by LR-GWAS on a candidate region on OAR2, that likely plays a role in adaptation to climate-mediated selective pressures in sheep [77, 78]. Taken together, and the fact that Tunisia is a relatively hot country with annual average temperature of 29 °C (range 5.7 °C-37.1 °C; www.climate-data.org), this information leads us to hypothesize that the STXBP6 could have a potential pleiotropic effect on skin thickness and thermoregulation in sheep that enhances tick and heat stress tolerance. SLC02A1 on the other hand, can enhance tick resistance by regulating fever during infusionation with ticks and infections by TBI as well as in thermoregulation. However, these hypotheses need to be validated with more data on appropriate phenotypes such as skin characteristics.

Birth weight is the earliest available body weight trait with considerable impact on lamb survivability and growth performance traits [79]. Our analysis revealed a candidate region on OAR1 that was identified by LR-GWAS, XP-EHH and FST that spanned among others the RAB6B and TF genes. This region was observed, following genome-wide association analysis, to be significantly associated with birth weight in sheep and could be considered potential candidates for the trait [80, 81]. Growth is essentially associated with bone development and it was found that STXBP6 had potential pleiotropic effect on bone tissue and fecundity traits in chickens [82] and was found to be in a region under selection in broilers [83] and layers [84] suggesting that STXBP6 may influence body weight. In several cases, T-TBI can destabilize host growth and development. To counter against this destabilization, we suggest that natural selection may be acting on the regions spanning RAB6B, TF and STXBP6 to ensure growth and development stability early in life as an adaptive strategy that ensures survival in the context of high T-TBI challenge.

The differentiation of the study individuals into four genetic groups independent of tick infestation status, geographic sampling and breed was surprising. It was also surprising that the G1 group was characterized by a different pattern of LD decay and the trend in N\textsubscript{e} compared to G2, G3 and G4, implicating a different genetic and demographic history and/or profile for G1. To gain further insights into the genetic differentiation between these four genetic groups, we performed selection signature analysis with XP-EHH and F\textsubscript{ST} approaches. These two analyses revealed several candidate regions spanning a number of putative genes showing differentiation between the four genetic groups. However, based on the functions of the genes, we are unable to suggest the exact genetic mechanisms that are driving the differentiation of the four genetic groups given that the identified candidate genes are associated with multiple functions some of which overlap. The lack of phenotypic data, such as production, reproduction, adaptation and physical traits, that describe the individuals of the four genetic groups makes it difficult to interpret our findings. We therefore suggest that there could be some underlying but yet unknown factors that are driving the genome divergence of the study populations. To determine these factors, more data as well as sampling of other breeds found in Tunisia and neighbouring countries will be required.

Conclusions

In this study, we investigated the possible genetic basis of resistance to tick infestation in two populations of Tunisian indigenous sheep. We therefore examined selection signatures in an a priori defined two groups of animals presenting contrasting phenotypes of high- and low-resistance to ticks, based on tick counts and piroplasm infections. The two cohorts were characterized by similar levels of genetic variation and a fine scale genomic structure that could not be explained by tick resistance status, geographic sampling region and breeds, suggesting that other unknown factors could be driving the genomic divergence. Four methods of detecting selection signatures identified regions of the genome that were most likely associated with differences in tick infestation and thus resistance with our results suggesting that STX5 could be a prime candidate gene for tick resistance in sheep. We further hypothesize that STXBP6 and SLC02A1 could be potential candidate genes for tick resistance in indigenous sheep which should be investigated further. The occurrence of RAB6B and TF in a candidate region that was significantly associated with body weight traits indicates that natural selection could be enhancing growth and development stability as an adaptive strategy to tick infestation. Although the sample size used in our study is relatively small, the high density of marker loci used increases the confidence of our findings. Nevertheless, [85] found that a rather limited value of n = 15 diploid individuals, equivalent to 30 gametes, is sufficient to reach reasonable power when used with high density markers. This research confirms the importance of studying indigenous livestock genotypes within their production environments to gather information on genomic footprints of adaptive significance. Further studies on understanding the genetics of tick resistance can benefit from the inclusion of variables such as skin and coat characteristics in order to analyze their influence on tick infestation.

Methods

Study animals, sampling and genotyping

The blood samples used in this study were collected by veterinary surgeons, during routine animal health surveillance in farmers herds/flocks, using standard veterinary procedures in full respect of animal welfare and minimizing stress as per the guidelines for the care and use of animals of the Tunisian National Council of Veterinary Surgeons (TNCVS). The blood sampling was also done with the verbal permission, and in the presence, of animal owners. Therefore, permission from the Ethics Committee of the TNCVS was not required.

Two breeds, Barbarine (B) and Queue Fine de l'Ouest (Q), of Tunisian indigenous sheep were sampled. The animals graze predominantly on natural communal pastures year-round except during summer when they are grazed on crop residues. Prophylactic treatment is rare, but vaccinations against Brucellosis, Bluetongue, Foot and Mouth disease and Sheep-pox are done annually by the National Veterinary Services. The animals sampled for this study were from the northeast, northwest and southeast regions of Tunisia and were sampled as part of a larger cross-sectional study on T-TBI that was
carried out between 2018 and 2020 [40]. The northeast and southeast regions are the homelands of the B breed although it has been dispersed throughout the country. It forms the foundation of the "Tunis" and "Barbaresca" breeds found respectively, in the USA and Italy [86]. The B is also found in Libya and eastern Algeria [87]. The Q breed predominates in the northwest region although they have been dispersed to the central, eastern and western plateaus of Tunisia. The breed is also found in mixed sheep-cropping systems in eastern Morocco and is genetically close to the Algerian Ouled Jellal breed [88].

For the cross-sectional study, 439 mature ewes were tagged and monitored through eight rounds of sampling. From each animal, whole blood (5 mls) was collected from each animal in EDTA coated vacutainers via jugular venipuncture and ticks were also collected from both ears, identified, counted and preserved in 70% ethanol. From the 439 ewes, 170 animals (B = 108; Q = 62) were purposely selected for this study based on tick scores and piroplasm infections. Genomic DNA was extracted from the blood samples with the Rapid Blood Genomic DNA Extraction Kit (Bio Basic, Markham, Canada). Tick scores, which were used to determine tick infestations, were visually estimated based on a scale from 0 (no ticks) to 5 (> 150 ticks) [89] while piroplasms were detected with the polymerase chain reaction technique [40].

The 170 samples were assigned to two cohorts based on extreme values of tick load and piroplasm infections viz i) high tick-resistant (HR) - animals with no tick infestation(s) and/or no piroplasm infection(s) and ii) low tick-resistant (LR) - animals that are highly infested with ticks and/or infected by piroplasms (Table 1). The DNA from the 170 samples were genotyped with the Illumina 600K SNP BeadChip (Illumina Inc., San Diego, CA, USA) at Neogen GeneSeek, Lincoln NE, USA. The BeadChip comprises 606,006 probes targeting genome-wide SNPs, of which 577,401 are autosomal, 27,314 are on the X chromosome and 1,291 remain unassigned [90].

The raw genotypes were assessed for quality with PLINK1.9 [91] and then pruned with the following criteria: (i) one individual from a pair of highly related animals was discarded if they had an identity-by-state (IBS) score greater than 0.99, (ii) SNPs with minor allele frequencies (MAF) of no less than 0.01 were retained, (iii) individuals and SNPs with call rates lower than 90% and 95%, respectively were discarded and (iv) all unmapped SNPs were discarded. This generated a dataset of 537,214 SNPs comprising 74 HR (B = 43; Q = 31) and 96 LR (B = 65; Q = 31) individuals. This dataset was subjected to LD pruning with the parameters 50 5 0.5 which represent window size, step size and r² threshold, respectively resulting in 338,180 SNPs that were used for genetic structure and phylogenetic analysis.

Assessment of population genetic structure

Although the study individuals were classified a priori into two extreme cohorts, HR and LR, we first assessed population genetic structure and divergence to determine whether the underlying genome architecture corresponds to the two cohorts. We therefore performed principal component analysis (PCA) with PLINK v1.9 and the first two PC’s were plotted to visualize genetic relationships. We also inferred fine-scale genome structure and shared genome ancestry with the unsupervised mode of ADMIXTURE tool v1.30 [92], independent of any background information on the number and frequency of alleles in the ancestral gene pool. The ADMIXTURE tool was run with a K range of 1–8 clusters and we performed five replicate runs for each K to generate cross-validation (CV) errors. The lowest CV error was used to infer the optimal number of distinct genetic clusters in the dataset.

Assessment of genetic diversity and demographic dynamics

Expected (Hₑ) and observed (Hₒ) heterozygosity, patterns of LD decay across the genome and effective population size (Nₑ) across generation time were investigated for each breed, the HR and LR cohorts and the genetic groups revealed by PCA and ADMIXTURE algorithms. The Hₑ and Hₒ were calculated with PLINK v1.9. The extent of LD between pairwise SNPs was evaluated with the r² statistic calculated with PLINK v1.9. The Nₑ over generation time was estimated with the equation Nₑ = (1/4c) (1/r² – 1) (Sved 1971), where Nₑ is the Nₑ t generations ago (t = 1/2c); r² is the LD between pairwise SNPs; and c is the genetic distance in Morgans between pairs of SNPs.

Two coefficients of inbreeding were calculated with PLINK v1.9; the SNP based “F” and the runs of homozygosity (ROH) based “F_ROH”. The latter was computed as the ratio of the total length of ROH to the length of autosomes (2.45 Gb) [93]. The ROHs were identified for each animal with the following parameters: (i) the minimum number of SNPs in a sliding window was set to 50; (ii) the minimum ROH length was set to 1 Mb to eliminate the impact of strong LD; (iii) each ROH spanned a minimum of 80 consecutive SNPs; (iv) one heterozygous and five missing calls per window were allowed to avoid false negative results that can arise due to genotyping errors or missing genotypes; (v) the minimum SNP density was set to one SNP/100 kb, and (vi) the maximum gap between consecutive SNPs was set to 1 Mb.

Detection of selection signatures and association analysis

To detect selection signatures spanning regions associated with genomic targets for resistance to ticks, we first investigated the distribution of ROH across the genomes of the HR and LR cohorts. For each cohort, the frequency of a SNP within an ROH region was determined and a Manhattan plot visualising all the tested SNPs against their autosomal positions was generated. The most frequent SNPs occurring above the 50% cut-off threshold of the empirical distribution were taken as the most significant loci underlying ROH stretches that were possibly under selection. To identify the ROH stretches that are likely associated with tick resistance, we contrasted the HR and LR ROH regions and identified the ones that were specific to the HR cohort.

We used the population differentiation statistic, F_ST, to investigate regions of the genome showing divergence between the HR and LR cohorts. The unbiased pairwise F_ST [94] was computed for each SNP with the R package “HIERFSTAT” [95] using a window size of 100 Kb and a window-step size of
10 Kb. Windows with less than five SNPs were excluded from the analysis. The \(F_{ST}\) values were standardized by Z transformation following [96]. The windows that occurred within the top 0.001% of the \(F_{ST}\) values in each chromosome were considered the putative candidates under divergent selection.

We used the software developed by [97] to estimate the unstandardized XP-EHH statistics for all SNPs, after quality control, following the comparative analysis between the HR and LR cohorts. The unstandardized XP-EHH statistics were standardized using their means and variances. We estimated the p-values of the SNPs using the standard normal distribution following findings of previous studies [98–100]. We determined the candidate regions under positive selection by clustering the significant core SNPs (p-value < 0.05) within a distance of less than 200 kb from the top-most SNP.

We performed the logistic regression (LR) GWAS with PLINK v1.9 to explore further, the possible genomic regions and SNPs associated with variation in tick resistance. The HR and LR cohorts were used as the test and control groups, respectively. To obtain the 99% confidence intervals for the estimated parameters, the “-ci 0.99” and “-covar” options were invoked, and Fisher’s exact test was used to generate the p-values considering age, sampling region and breed as covariates. The generated p-values were Bonferroni corrected to minimize false positive results among the SNPs that were likely under selection. The corrected p-values were standardized and the -Log10 (p-value) of 4.25 (the top 0.001) was set as the cut-off threshold to identify the candidate regions and associated SNPs. The estimations were summarized in 100 Kb window sizes and the genes and the top-most SNP found within the candidate regions were identified. The Manhattan plots showing the genome-wide distribution of the SNPs were generated with the R package “qqman” v3.5.1.

To determine the genomic regions associated with the divergence of the genetic groups revealed by PCA and ADMIXTURE algorithms, we used the XP-EHH and \(F_{ST}\) approaches. The former detects signatures arising from recent selection or those that are approaching fixation, and the latter identifies regions that show allele frequency differentiation which may be the result of long-term selection. The analyses were performed by contrasting one group with the others for example, G1 vs G2, G3, G4 etc. The approaches used to perform XP-EHH and \(F_{ST}\) while contrasting the HR and LR cohorts were also used in this analysis.

**Functional annotation of candidate regions**

The candidate regions revealed by ROH were analysed and the ones that were specific to the HR cohort identified. These HR-specific ROH regions and the regions identified by LR-GWAS, XP-EHH and \(F_{ST}\) were analysed, and those that overlapped between at least two methods were identified and merged using Bedtools v 2.28.0 [101]. The same analysis was performed to determine the regions that overlapped between the ones identified by XP-EHH and \(F_{ST}\) from the comparative analysis involving at least two genetic groups that were identified by the clustering algorithms. The genes spanned by the overlapping candidate regions were retrieved using the Biomart/Ensembl (http://www.ensembl.org/biomart) tool based on the Ovine v3.1 reference genome assembly. These set of genes were assessed for biological enrichment gene ontology (GO) and KEGG Pathway (www.kegg.jp/kegg/kegg1.html) terms compared to the full list of \(O. aries\) autosomal protein-coding genes with the functional annotation tool in DAVID v6.8 [102]. We further determined gene functions using the NCBI database (http://www.ncbi.nlm.nih.gov/gene/) and review of literature.

**Abbreviations**

B = Barbarine; Q = Queue Fine de l’Ouest; HR = High resistant; LR = Low resistant; PCA = Principal component analysis; T-TBI = Ticks and tick-borne infections; ROH = runs of homozygosity; LR-GWAS = Logistic regression genome-wide association analysis; XP-EHH = cross-population extended haplotype homozygosity; \(F_{ST}\) = genetic differentiation statistic; OAR = Ovine chromosome

**Declarations**

**Ethics approval and consent to participate**

The animals used in this study belonged to farmers who provided their verbal informed consent to their sampling which was performed as a routine veterinary monitoring activity (blood sampling and tick collection) by licensed veterinarians following the guidelines for the care and use of animals of the Tunisian National Council of Veterinary Surgeons; no ethical approvals were required.

**Consent for publication**

Not applicable

**Availability of data and materials**

The data used in this study is available from the communicating author on reasonable request upon signing a material transfer agreement.

**Competing interests**

The authors declare no competing interests in all aspects of the study.
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Author’s contributions

GM, RM, HA, RB and MJM conceived the study. KKM, RH and SL carried out the field work including sampling, DNA extraction and PCR under supervision of GM and RM. AAM analysed the data under supervision from MJM and both wrote the manuscript. All authors read and approved the final version of the manuscript.

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Tables

| Table 1 |
|-----------------------------|
| Genetic diversity indices generated for the breeds, cohorts and genetic groups of Tunisian Sheep used in this study |
| Breed                  | N  | $H_O$ (Mean ± Sd) | $H_E$ (Mean ± Sd) | ROH (Mb) (Mean ± Sd) | $F_{ROH}$ (Mean ± Sd) | $F$ (Mean ± Sd) |
|------------------------|----|-------------------|-------------------|----------------------|----------------------|-----------------|
| Barbarine (B)          | 104| 0.3313 ± 0.0299   | 0.3470 ± 0.00003  | 3.332 ± 0.4434       | 0.0515 ± 0.0826      | 0.0452 ± 0.0862 |
| Queue Fine de l’Ouest (Q) | 60 | 0.3291 ± 0.0322   | 0.3468 ± 0.00010  | 3.520 ± 0.5242       | 0.0625 ± 0.0881      | 0.0511 ± 0.0929 |
| **Cohort**             |    |                   |                   |                      |                      |                 |
| LR                     | 77 | 0.3321 ± 0.0289   | 0.3477 ± 0.00007  | 3.377 ± 0.4750       | 0.0507 ± 0.0803      | 0.0447 ± 0.0832 |
| HR                     | 77 | 0.3286 ± 0.0344   | 0.3468 ± 0.00006  | 3.439 ± 0.4117       | 0.0618 ± 0.0940      | 0.0525 ± 0.0991 |
| **Genetic group**      |    |                   |                   |                      |                      |                 |
| G1                     | 115| 0.3416 ± 0.0192   | 0.3487 ± 0.00007  | 3.032 ± 0.5213       | 0.0239 ± 0.0527      | 0.0204 ± 0.0551 |
| G2                     | 18 | 0.3424 ± 0.0326   | 0.3331 ± 0.00003  | 3.667 ± 0.5966       | 0.1335 ± 0.0798      | -0.0278 ± 0.0979 |
| G3                     | 20 | 0.3606 ± 0.0464   | 0.3397 ± 0.00007  | 3.435 ± 0.4571       | 0.1612 ± 0.1029      | -0.0616 ± 0.1367 |
| G4                     | 12 | 0.3798 ± 0.0468   | 0.3495 ± 0.00002  | 3.728 ± 1.004        | 0.0850 ± 0.1105      | -0.0866 ± 0.1339 |
| **Overall**            | 170| 0.3305 ± 0.0305   | 0.3470 ± 0.00006  | 3.406 ± 0.4072       | 0.0549 ± 0.0842      | 0.0476 ± 0.0881 |

Note: N: Sample size; $H_O$: observed heterozygosity; $H_E$: expected heterozygosity; ROH: Runs of homozygosity; $F_{ROH}$: ROH based inbreeding; F: inbreeding coefficient; LR: Low-resistant; HR: High-resistant

Table 2

Candidate regions, SNPs with topmost score in LR-GWAS, and associated genes found in candidate regions that were identified by at least two methods of selection signature analysis between the high- (HR) and low-resistance (LR) sheep cohorts.
| Reg | OAR | Start     | Stop      | Size (Mb) | Method   | Top SNP (LR-GWAS) | No. of genes Genes (Top gene*) | P value* |
|-----|-----|-----------|-----------|-----------|----------|-------------------|--------------------------------|---------|
| 1   | 1   | 122490001 | 122680000 | 0.190     | ×        | oar3_OAR1_122581225 | 4 | 3.10E-06 |
| 2   | 1   | 226950001 | 227160000 | 0.210     | ×        | oar3_OAR1_227042621 | 5 | 2.55E-06 |
| 3   | 1   | 253380001 | 253660000 | 0.280     | ×        | oar3_OAR1_253478525 | 4 | 2.70E-06 |
| 4   | 2   | 519100001 | 521400000 | 0.230     | ×        | oar3_OAR2_520439393 | 7 | 2.72E-06 |
| 5   | 2   | 531100001 | 532500000 | 0.140     | ×        | oar3_OAR2_53171448 | 4 | 1.79E-06 |
| 6   | 2   | 629700001 | 631600000 | 0.190     | ×        | oar3_OAR2_63061644 | 3 | 2.68E-06 |
| 7   | 2   | 845500001 | 847400000 | 0.190     | ×        | oar3_OAR2_84646297 | 4 | 3.47E-06 |
| 8   | 2   | 858100001 | 861400000 | 0.330     | ×        | oar3_OAR2_86046574 | 2 | 3.18E-06 |
| 9   | 2   | 201150001 | 201380000 | 0.230     | ×        | oar3_OAR3_201281356 | 5 | 2.72E-06 |
| 10  | 3   | 183020001 | 183260000 | 0.240     | ×        | oar3_OAR3_183165082 | 2 | 2.76E-06 |
| 11  | 3   | 193980001 | 194170000 | 0.190     | ×        | oar3_OAR3_194077322 | 1 | 2.65E-06 |
| 12  | 3   | 213290001 | 213550000 | 0.260     | ×        | oar3_OAR3_213385724 | 11 | 2.70E-06 |
| 13  | 3   | 224020001 | 224277781 | 0.258     | ×        | oar3_OAR3_224118862 | 11 | 3.35E-06 |
| 14  | 4   | 487600001 | 490000000 | 0.240     | ×        | oar3_OAR4_48859309 | 5 | 2.82E-06 |
| 15  | 5   | 441100001 | 443000000 | 0.190     | ×        | oar3_OAR5_44206370 | 3 | 2.68E-06 |
| 16  | 5   | 105810001 | 106000000 | 0.190     | ×        | oar3_OAR5_105908550 | 1 | 2.56E-06 |
| 17  | 6   | 55800001 | 57700000 | 0.190     | ×        | oar3_OAR6_5672674 | 2 | 2.60E-06 |
| 18  | 8   | 25200001 | 27100000 | 0.190     | ×        | oar3_OAR8_2618920 | 2 | 2.55E-06 |
| Chromosome | Position | Gene(s)                                                                 | p-value |
|------------|----------|-------------------------------------------------------------------------|---------|
| 19         | 8        | AK9, ZBTB24, MICAL1, SMPD2, PPIL6, POLE4, CD164                          | 2.72E-06|
| 20         | 8        | NBSL1                                                                  | 2.59E-06|
| 21         | 9        | TRIB, LOC105611791, NSMCE2                                             | 2.63E-06|
| 22         | 9        | PCMTD1, ST18, NHSL1, TRIB, LOC105611791, NSMCE2                         | 2.71E-06|
| 23         | 9        | POLE4, CD164                                                           | 2.70E-06|
| 24         | 11       | OAR8_27875909, 7                                                       | 2.60E-06|
| 25         | 12       | KIF26B, LOC105611791, NSMCE2                                           | 2.58E-06|
| 26         | 12       | TNN, LOC105611791, NSMCE2, NHSL1, TRIB, LOC105611791, NSMCE2            | 2.30E-06|
| 27         | 13       | NHSL1                                                                  | 1.46E-06|
| 28         | 15       | MRE11A, ANKRD49, AASDHPPT, KBTBD3, MSANTD4                             | 2.73E-06|
| 29         | 15       | MMP27, MMP20, MMP7                                                     | 3.01E-06|
| 30         | 15       | NPAT, ATM, C15H11orf65                                                 | 2.68E-06|
| 31         | 15       | LOC10562151                                                           | 1.71E-06|
| 32         | 16       | LOC10562434, SPDL1, LOC10560244, DOCK2                                 | 2.93E-06|
| 33         | 16       | LOC105602490, CD180, LOC105602489, MAST4                               | 2.55E-06|
| 34         | 16       | LOC10562500, LOC10562501                                              | 3.40E-06|
| 35         | 16       | CDH18, TRNAW-CCA                                                       | 2.60E-06|
| 36         | 16       | EFTUD1, MEX3B                                                          | 2.65E-06|
| 37         | 18       | STXBP6                                                                 | 2.59E-06|
| 38         | 18       | LOC105603171                                                          | 3.14E-06|
| 39         | 18       | LOC105601927, 7                                                       | 2.89E-06|
*Significant markers following LR-GWAS (Bonferroni correction P < 0.001); ** Top genes closest to the topmost SNP are indicated in bold.

**Figures**

**Figure 1**

Population genetic structure analysis of the two cohorts (HR, LR) of autochthonous Tunisian sheep (a, b, c and d) PCA cluster analysis showing PC1 and PC2; (e) Cross-validation plot for admixture analysis; (c) Admixture analysis plot showing the genetic backgrounds present in the study cohorts for 2≤K≤5
Figure 2

Trends in LD decay (a) and NE (b) across 1000 generations in non-infected and infected cohorts (HR = high resistance cohort; LR = low resistance cohort) and different genetic backgrounds (G1, G2, G3 and G4) of study Tunisian sheep.

Figure 3

**a**

**ROH: HR**

ACOXL, BCL2L11, DENND5B, G18S, G1H2, G1A3, ZMYM2, EFTUD1, MEX6B, PAG6, STX3, WDR74

**b**

**ROH: LR**

Chromosomes

SNP occurrences [%]
Manhattan plots showing genome-wide distribution frequency of SNPs in stretches of ROH regions. The dashed lines indicate the 25% threshold for each cohort. HR = High resistance; LR = Low resistance cohort

Figure 4

Manhattan plots showing the genome-wide distribution of SNPs following (a) LR-GWAS (b) FST and (c) XP-EHH analysis using the non-infected and infected cohorts of autochthonous Tunisian sheep. HR = High resistance; LR = Low resistance cohort.
Figure 5
Manhattan plots showing the genome-wide distribution of SNPs following FST pairwise contrasting the genomic pools (G1, G2, G3 and G4) of study Tunisian sheep.

Figure 6
Manhattan plots showing the genome-wide distribution of SNPs following XP-EHH pairwise contrasting the genomic pools (G1, G2, G3 and G4) of study Tunisian sheep.

Supplementary Files
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