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Theileria lestoquardi displays reduced genetic diversity relative to sympatric *Theileria annulata* in Oman

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Abstract

The Apicomplexan parasite *Theileria lestoquardi* and *T. annulataT. annulata* the causative agents of theileriosis in small and large ruminants, are widespread in Oman, in areas where cattle, sheep and goats co-graze. Genetic analysis can provide insight into the dynamics of the parasite and the evolutionary relationship between species. Here we identified ten genetic markers (micro- and mini-satellites) spread across the *T. lestoquardi* genome, and confirmed their species specificity. We then genotyped *T. lestoquardi* in different regions in Oman. The genetic structures of *T. lestoquardi* populations were then compared with previously published data, for comparable panels of markers, for sympatric *T. annulata* isolates. In addition, we examined two antigens genes in *T. annulata* (*Tams1* and *Ta9*) and their orthologues in *T. lestoquardi* (*Tlms1* and *Tl9*).

The genetic diversity and multiplicity of infection (MOI) were lower in *T. lestoquardi* (*He*=0.64–0.77) than *T. annulata* (*He*=0.83–0.85) in all populations. Very limited genetic differentiation was found among *T. lestoquardi* and *T. annulata* populations. In contrast, limited but significant linkage disequilibrium was observed within regional populations of each species. We identified eight *T. annulata* isolates in small ruminants; the diversity and MOI were lower among ovine/caprine compared to bovine. Sequence diversity of the antigen genes, *Tams1* and *Ta9* in *T. annulata* (π=0.0733 and π=0.155 respectively), was 10-fold and 3-fold higher than the orthologous *Tlms1* and *Tl9* in *T. lestoquardi* (π=0.006 and π=0.055, respectively).

Despite a comparably high prevalence, *T. lestoquardi* has lower genetic diversity compared to sympatric *T. annulata* populations. No evidence of differentiation among populations of either species. In comparison to *T.lestoquardi*, *T. annulata* has a larger effective population size. While, genetic exchange and recombination occurs in both parasite species, the extent of diversity, overall, is less for *T. lestoquardi*. It is, therefore, likely that *T. lestoquardi* evolved from an ancestor of present day *T. annulata* and that this occurred either once or on a limited number of occasions.

Keywords:

Theileria lestoquardi, *Theileria annulata*; population genetics, evolution, host species jump, Oman

1. Introduction

Theileria lestoquardi is a highly pathogenic ovine and caprine parasite and is considered to be the only *Theileria* species of economic significance in small ruminants (Leemans *et al.*,2001; Li *et al.*,2014). The parasite is transmitted by *Hyalomma anatolicum anatolicum*, which is common in South-eastern Europe, Northern Africa, Southern Russia and the Middle East. However, distribution of *T. lestoquardi* is limited compared to the range of its vector. Although *T. lestoquardi* has been shown to be antigenically closely related to *T. annulata* (Leemans *et al.*,1997), it has been reported as being incapable of infecting cattle (Leemans *et al.*,1999). Conversely, it is known that *T. annulata* can infect sheep; experiments in sheep indicate that *T. lestoquardi* infection protects against subsequent *T. annulata* infection (Leemans *et al.*,1999) and although prior infection with *T. annulata* does not prevent infection from *T. lestoquardi* sporozoites, it does protect against the major clinical effects. However, these experiments were carried out on limited numbers of animals with a very limited number of parasite genotypes, and the actual transmission dynamics in the field are unknown.

Theileria lestoquardi was first reported in sheep in Sudan and Egypt (Littlewood,1916), and later detected in sheep and goats in other countries of the Middle East such as Algeria (Lestoquard,1927), Turkey (Baumann,1939), Iraq (Khayyat *et al.*,1947), Iran (Hooshmand-Rad *et al.*,1976; Hawa,1981) as well as India (Raghvachari,1959) and Serbia (Dschunkovsky *et al.*,1924). A previous study in Oman demonstrated a high level of theileriosis-attributed mortality in a local sheep breed (Tageldin *et al.*,2005). This confirmed previous individual case reports and outbreak records of a pathogenic species of *Theileria* in sheep and goats in Oman (Annual Reports VRC2004-2006) (MOAF,2008). These reports indicated that in Oman, sheep, in general, were significantly more at risk of clinical theileriosis than cattle and goats, and this has been attributed to a higher tick infestation of sheep. However, the relative distribution of the major pathogenic species of *Theileria* (*T. lestoquardi* and *T. annulata*) is not yet known in the Sultanate of Oman. Thus, there is currently no information on the prevalence of *T. lestoquardi* in different regions in

Oman and nothing is known regarding the *T. lestoquardi* population structure. In contrast, a recent survey demonstrated that *T. annulata* is widely distributed across the country and is comprised of a highly genetically diverse, inter-breeding population (Al-Hamidhi *et al.*,2015).

Genetic analysis of parasite populations can provide important information about the epidemiology of disease and may facilitate the development of rational control approaches. Polymorphic genetic markers have been developed for some species of *Theileria*, e.g. *T. annulata* and *T. parva* (Oura *et al.*,2003; Weir *et al.*,2007), however, such tools are not yet available for the small ruminant *Theileria* species parasites, *T. lestoquardi* and *T. ovis*. Micro- and mini-satellites are considered as highly appropriate molecular markers for population genetics applications. Their high mutation rate and Mendelian mode of inheritance make them particularly useful for the study of both fine and broad-scale population genetic structure (Abdelkrim *et al.*,2009). Common applications include assessing genetic diversity, degree of population inbreeding, bottleneck effects, gene flow and migration rates, the assignment of population of origin and parental lineages (Goldstein *et al.*,1999). The present study included the development of micro- and mini-satellite genotyping for *T. lestoquardi* and their application to investigate the genetic diversity of parasite populations from four regions in Oman. The extent of diversity and population structure of *T. lestoquardi* was then compared to available published data on sympatric *T. annulata* populations for three of the four regions. We aimed to gain an understanding of whether local gene flow and genetic diversity differs between these two species in an area of similar prevalence and distribution of tick species. We also investigated the hypothesis that *T. lestoquardi* is a relatively recently evolved species that has diverged from the more ancient cattle parasite species, *T. annulata*, following a host species jump to small ruminants.

2. Materials and method

2.1. Parasite material and DNA preparation

Blood samples (n = 1,454) were collected from clinically healthy sheep and goats in four governorates of Oman: Batinah (n = 584), Dhira (n = 357), Sharqia (n = 369) and Dakhiliya (n = 144) (Figure 1). The climate across these regions is hot and dry throughout the year, with 3-4 months (Oct to Feb) of relatively moderate temperatures (below 30 ºC).

For comparison of diversity and population structure, genotyping data representing 97 *T. annulata* isolates from Batinah (n = 21), Dhira (n = 57) and Sharqia (n = 19) derived from cattle co-grazed on the same farms as the sheep/goats that provided *T. lestoquardi* isolates was utilised. These were previously genotyped with a set of *T. annulata* specific micro- and mini-satellites (Al-Hamidhi *et al.*,2015).

2.2. Identification of specific *T. lestoquardi* micro- and mini-satellite sequences

A draft sequence of the *T. lestoquardi* genome has been generated (Weir *et al*., unpublished). To identify micro- and mini-satellite loci specific for *T. lestoquardi*, sequence contigs were screened using the tandem repeat finder program (Benson,1999). A filtration pipeline was used to identify a subset of high-value loci, which could be tested using a panel of available stocks and isolates. Filtration included discarding repeat regions greater than 500 bp in length and those that possessed insufficient flanking sequence for primer design. The remaining sequences were ranked, based on the fidelity of the repeat within each region (> 70 % fidelity) and the number of repeats. A subset of 28 loci with conserved repeat motifs was then derived.

2.3. PCR amplification of specific micro- and mini-satellite loci

Primers were designed to unique sequence flanking each repeat and used to amplify DNA purified from a panel of stocks (*T. lestoquardi*, *T. annulata* and *T. ovis*) and field isolates to test marker specificity and polymorphism. In addition, to test for marker sensitivity, serial dilutions of *T. lestoquardi* DNA were generated and PCR performed with each primer set and sample.

PCR was carried out in a total reaction volume of 20 µl using conditions described previously (Al-Hamidhi *et al*., 2015). Thermocycler parameters were as follows: denaturation at 94 °C for 5 minutes, 32 cycles at 94 °C for 30 seconds, 42-55 °C for 30 seconds, and 65 °C for 30 seconds, followed by a final extension step of 5 minutes at 65 °C. Amplified products were observed on a 2

% ethidium bromide pre-stained agarose gel and their size determined with reference to either a 1 kb or 100 bp DNA ladder.

To identify length polymorphism down to the level of 1 base pair (bp), PCR products were denatured and then capillary electrophoresed in an ABI3130 xl Genetic Analyser (Applied Biosystems, UK). DNA fragment sizes were determined relative to ROX-labeled GS500 size-standards (Applied Biosystems) using GeneMapper software (Applied Biosystems). For all loci and DNA samples, fragment size (i.e. peak position) was determined to two decimal places. Analysis of the distribution of fragment sizes facilitated the creation of 'fixed bins' of variable size to score alleles. Since these loci represent genomic regions encoding hypothetical proteins, variation among allele sizes was assumed to be in steps of three base pairs or multiples thereof.

The single or predominant allele for each of the ten selected loci was utilised to compute allele frequencies. Each of the markers selected for further analysis was shown to represent a different single-copy locus based on genome data and PCR fragments amplified from *T. lestoquardi* (Lahr) DNA. Since *Theileria* parasites are haploid, the presence of one or more additional alleles at a particular locus was interpreted as a co-infection with one or more genetically distinct genotypes. An additional allele was scored if the peak was at least one-third the height of the predominant allele (highest peak) on the electropherogram traces, a method that has been widely used in previous studies (Anderson *et al.*,1999). In this way, the predominant allele at each locus was identified for each sample and the data combined to generate a multi-locus genotype (MLG), representing an estimate of the most abundant genotype in each sample, as described previously (Weir *et al.*,2007).

The MLG dataset was then used to measure population genetic indices such as heterozygosity, linkage disequilibrium and population differentiation. Since *Theileria* is haploid and heterozygosity cannot be observed directly, the estimated heterozygosity was calculated using the predominant allele dataset for each marker and averaged across all ten loci.

2.4. Sequence analysis of *Tams1*/*Tlms1* and *Ta9/Tl9* orthologues in *T. annulata* and *T. lestoquardi*

Theileria annulata and *T. lestoquardi* isolates were obtained from the same farms in Sharqia and Dhira, since a high level of *Theileria* infection had been detected in animals from each region. The PCR products for *Tams1*/*Tlms1* and *Ta9*/*TL9* genes were generated and cloned using the Topo sequencing vector. DNA from 5 purified colonies representing each isolate was sequenced by ABI3130 xl Genetic Analyser (Applied Biosystems, UK). The obtained nucleotide sequence was confirmed by via the NCBI BLAST web interface (http://www.ncbi.nlm.nih.gov/), and nucleotide sequences translated to amino acid sequences using MEGA4 software (Tamura *et al.*,2007). Nucleotide and translated amino acid sequences were aligned with the corresponding reference gene from the *T. annulata* and *T. lestoquardi* genome sequence using MEGA V. software. Sequence polymorphism and diversity was estimated using DnaSP version 5.0 (Librado *et al.*,2009) by calculating the total number of polymorphic sites (S); the average pair-wise nucleotide diversity (π), the average number of nucleotide differences (k) and haplotype diversity combinations for all divergent sequences. The HKY+G mutational model applied was chosen using jmodeltest (http://jmodeltest.org). The tree for nucleotide sequence of *Tams1*/*Tlms1* gene was constructed using a PhyML 3.0. software (Guindon S.,2010), and visualize using archaeopteryx software (https://sites.google.com/site/cmzmasek /home/software/archaeopteryx).

2.5. Data analysis

The Excel Microsatellite toolkit (Bowcock *et al.*,1994) was used for a similarity comparison of MLGs. Genetic diversity parameters were calculated for the entire population using GenAlex v6.5 (Peakall *et al.*,2012) by determining the number of alleles per locus (A) and the expected heterozygosity (Dschunkovsky *et al.*,1924). Allelic diversity was determined using the formula for ‘unbiased heterozygosity’, the equivalent of diploid expected, also named as haploid genetic diversity, $H_e = [n/(n-1)][1-\sum p^2]$ where *n* is the number of isolates and *p* the frequency of each different allele at a locus (Anon,1996). Expected heterozygosity ranges between 0 and 1, with values close to 1 reflecting high genetic diversity levels in a population.

To determine whether the *T. lestoquardi* and *T. annulata* populations in different regions comprised a single panmictic population with a high degree of genetic exchange, linkage disequilibrium (LD), i.e. the non-random association of alleles among loci was quantified using the standard index of association (I^S_A). Each region was analysed separately and then the samples were pooled and analysed as a single set. Both I^S_A and the variance data were calculated using the program LIAN, version 3.5 (Haubold *et al.*,2000). This software tests for independent assortment of alleles by determining the number of loci at which each pair of MLGs differs, and from the distribution of mismatch values, a variance V_D (the variance of the number of alleles shared between all pairs of haplotypes observed in the population) is calculated which is then compared with the variance expected for linkage equilibrium (LE), termed V_e. The null hypothesis that $V_D = V_e$ is tested by either a Monte Carlo simulation or a parametric method and the results provide 95 % confidence limits, which are denoted L_{MC} and L_{PARA}, respectively. If there is limited or no association between alleles at different loci, indicating panmixia, a value close to zero is obtained for the I^S_A, whereas if association is detected at a value significantly greater than 0, LD is indicated (Haubold *et al.*,2000). The variance of pair-wise difference (V_D) between the data and that predicted for panmixia (V_e) and L were calculated in order to test the hypothesis of panmixia. To test whether the populations in each region were genetically differentiated, the reduction in heterozygosity for sub-populations compared to the overall population, Wright's fixation index (F_{ST}) (Brown,1970) value was calculated.

As some of the loci are located in regions near or inside coding genes, we have conducted a F_{ST} outlier tests for detect loci that might have being under selective pressure. These tests were conducting using the algorithm included in F_{ST} concept (Beaumont MA *et al.*,1996), using the java based software user-friendly Mcheza (Antao *et al.*,2011).

Principal co-ordinate analysis (PCoA), a multivariate analysis also known as multidimensional scalling (MDS), was used to investigate the genetic relationships between the isolates MLGs. A F_{ST} based genetic distances matrix was used to calculate the PCoA, which the results can be plotted to visualize the genetic relationships between individuals and/or populations. This analysis was calculate using Genalex V6, excel plugin software (Peakall *et al.*,2012).

2.6. Multiplicity of infection

Multiplicity of infection was defined as the "presence of multiple genotypes per isolate" by the detection of more than one allele at a locus, when minor peaks were >33% the height of the predominant allele present. The mean number of alleles across ten selected loci in each sample was calculated and this index value was used to represent the multiplicity of infection within each sample. The overall mean for the index value for each sample was then calculated to provide the average multiplicity of infection for each region.

3. Results

3.1. Identification and evaluation of *T. lestoquardi* micro- and mini-satellites

A panel of twenty-eight repeat-containing single-copy loci were initially identified by screening the draft genome of *T. lestoquardi* with repeat finder (Benson,1999). These loci represented 13 micro-satellite (motif size 3 - 6 bp) and 15 mini-satellite (motif size 9 - 24 bp) markers. Of the 28, only ten loci had flanking sequence suitable for designing primers specific for *T. lestoquardi*; the other 18 were either flanked with sequence common to *T. annulata* and *T. lestoquardi* or the flanking sequences were too short to allow primer design. The ten selected loci consisted of four micro-satellites (TL_MS07, TL_MS13, TL_MS19 and TL_MS16) and six mini-satellites (TL_MS05, TL_MS281, TL_MS280, TL_MS18, TL_MS04 and TL_MS25). The characteristics of these loci are summarised in supplementary Table 1 and supplementary Table 2. Eight of the ten loci are located in exons; one is in an intron and another in an intergenic region. The genes associated with or flanking these loci are all annotated as hypothetical proteins with orthologues present in the *T. annulata* genome (Pain *et al.*,2005).

PCR of the selected ten loci generated amplicons of the predicted size with *T. lestoquardi* DNA, but no product was obtained with *T. annulata* and *T. ovis* template DNA, demonstrating that the selected markers were specific for *T. lestoquardi*. The selected marker primer sets were then used to genotype 36 DNA samples representing *T. lestoquardi* field isolates, after the presence of *T. lestoquardi* DNA was confirmed by PCR-RFLP of the 18S rRNA locus. Each DNA sample/marker combination produced an amplicon. Variation in amplicon size among isolates was observed for each marker, confirming these loci as being polymorphic and thus informative for population analysis. The differences in allele size for each marker ranged from 3 to 9 bp and agreed well with the motif size of each marker. A subset of DNA samples showed evidence of more than one allele at one or more loci, indicating the presence of multiple genotypes in a number of animals.

3.2. Prevalence and multiplicity of infection of *T. lestoquardi* relative to *T. annulata* across three regions in Oman

The ten micro- and mini-satellites were then used to analyse *T. lestoquardi* populations in four regions in Oman. The extent of diversity and population structure of *T. lestoquardi* were then compared to *T. annulata* using previously published mini- and micro-satellite data for 97 isolates from cattle, obtained from three of the four regions where cattle and small ruminants co-graze.

Of the 1,688 blood samples collected [1454 small ruminate and 234 bovine], a total of 190/1454 (13 %) and 97/234 (41 %) were positive for *T. lestoquardi* or *T. annulata* parasites, respectively, as detected by PCR/RFLPs and/or PCR/RLB (Al-Fahdi *et al*., 2015). The difference in prevalence of either species across the different regions was not significant (chi squared test, $P > 0.05$). However, the prevalence of *T. annulata* in cattle was significantly higher than that of *T. lestoquardi* in small ruminants.

Genotyping data generated from the ten micro- and mini-satellite markers for each of the two species showed significantly greater MOI among *T. annulata* cattle isolates (ranging between 2.9 and 3.2) than the *T. lestoquardi* small ruminant isolates (1.49 to 1.63) (t test, $P < 0.001$) (Table 1). Similarly, the *T. annulata* dataset had a significantly larger proportion of multiple infections

(52 %), with more than one allele at one or more loci, than the *T. lestoquardi* dataset (44 %) (Chi-squared test, 1 df: P = 0.0045).

3.3. Relative diversity of mini- and micro- satellite markers

All ten markers for *T. lestoquardi* were found to be polymorphic, with the number of alleles for each marker ranging from four, for TL_MS25, to 22 for TL_MS280. The average of number of alleles per marker was 12.6. Broadly similar allele frequencies were observed for each marker in each region, as the example of TL_07 in Figure 2 shows, while a limited number of private alleles specific to sub-populations from each region were observed (Table 2). Three markers revealed a lower level of diversity (H_e range 0.121-0.441), compared to higher levels observed for the remaining seven (H_e range 0.548-0.867) (Table 2). The average heterozygosity identified within each of the four geographical regions was found to be moderate, ranging from 0.637 within Sharqia to 0.575 in Batinah (Table 2).

The extent of gene diversity among *T. lestoquardi* isolates was compared to that of *T. annulata* cattle isolates obtained from the same sites. Genetic diversity was consistently higher for *T. annulata*, where the estimate of diversity within each region (H_e range 0.820 to 0.854) was similar to the average of combined diversity in all regions (H_e = 0.836) (Table 3), consistent with little or no differentiation between sub-populations.

3.4. Comparative analysis of sequence diversity of antigen genes

We assessed the extent of diversity of two antigen genes in *T. annulata*: the immunodominant merozoite/piroplasm surface antigen of *T. annulata* (*Tams1*) (Shiels *et al.*,1995) and *Ta9* which encodes peptides recognised by $CD8^+$ T cells from immune animals (MacHugh *et al.*,2011). The level of sequence diversity in Omani isolates was then compared to that of the orthologous genes in sympatric *T. lestoquardi* isolates (*Tlms1* and *Tl9*).

Partial sequence of *Tams1* and *Tlms1* were obtained from *T. lestoquardi* (38 isolates) and *T. annulata* (36 isolates) from the same region in Oman. For *Tams1*, 144 nucleotide site polymorphisms were found among aligned *T. annulata* sequences in comparison with the reference genome sequence strain (Ankara, C9), while only 19 polymorphisms were detected across the *T. lestoquardi* sequences (Table 4).

Nucleotide alignment of *Tlms1* revealed eight haplotypes with haplotype diversity of 0.649 among *T. lestoquardi* sequences. However, 20 haplotypes were identified for the *T. annulata* orthologue *Tams1*, with a haplotype diversity (Hd) of 0.968 (Table 4). The overall nucleotide diversity (π) for *T. annulata* (π = 0.0733) *Tams1* was 10-fold higher than that computed for the *Tlms1* sequences (π = 0.006) and the average number of pair-wise nucleotide differences (k) was 3.902 and 45.832 in *T. lestoquardi* and *T. annulata*, respectively (Table 4). Thus, these results demonstrate that nucleotide diversity of the major merozoite/piroplasm surface antigen gene is significantly higher in *T. annulata* than in its *T. lestoquardi* orthologue, based on analysis of a similar number of sympatric isolates. This difference in sequence diversity between alleles representing the two orthologues was illustrated by the generation of a phylogenetic tree Figure 3. Clearly, the branch lengths are longer within the *T. annulata* tree, indicating more diversity/distant relationship between sequences. In addition, the sequences for both species, as might be predicted, show clear separation, with the *T. lestoquardi* indicated as branching/evolving from a common ancestor of the *T. annulata* sequences.

For the *Tl9*/*Ta9* comparison, 9 and 23 distinct sequences were obtained from a similar number of *T. lestoquardi* and *T. annulata* isolates, respectively. Haplotype number and Hd was 7 and 0.9, for *T. lestoquardi* 10 and 0.978 for *T. annulata*, respectively (Table 4). However, nucleotide diversity (π) was 3-fold higher for *T. annulata* (π = 0.155) sequences compared to that of *T. lestoquardi* (π = 0.055). Thus, the results for *Ta9*/*Tls9* reflect those of *Tams1*/*Tlms1*, demonstrating that two antigen genes selected for analysis have higher diversity in *T. annulata* than in their *T. lestoquardi* orthologues (Table 4), and this consistent with the results of the micro- and mini-satellites.

3.5. Genetic diversity of *T. annulata* isolated from small ruminants in Oman

Eight *T. annulata* isolates collected from small ruminants were genotyped using the ten published *T. annulata* micro- and mini-satellites and compared to the *T. annulata* genotyping results from the bovine isolates (Al-Hamidhi *et al.*,2015). Similar to bovine-derived isolates, each of the small ruminant isolates was found to carry multiple genotypes, with several alleles identified at one or more loci. However, the mean MOI was lower compared to that obtained for bovine isolates (average of 2.9 in small ruminants compared to 3.27 in bovine), but this difference was not significant. Six private alleles were observed, for the small ruminant isolates, on four loci (one allele each for Ts12 and Ts9 and two alleles each for Ts6 and Ts8). Due to the small number of isolates from small ruminants, genetic differentiation between *T. annulata* genotypes derived from the different host species could not be estimated.

3.6. Linkage disequilibrium analysis

To assess whether *T. lestoquardi* parasites in the study regions undergo random mating with a high level of genetic exchange, the extent of LD at pairs of loci was measured using the standard index of association (I^S_A). Low, yet significant LD was found when each region was treated as a single population and a low overall I^S_A value of 0.0264 was obtained. A V_D value (2.28) greater than L (1.98) was calculated indicating LD (Table 5). However, when each regional population was treated separately Dhira and Batinah showed (I^S_A) close to zero with pair-wise variance (V_D) less than the critical L value, indicating that those two populations were in LE. The above inconsistencies was likely a reflection of variation in effective population size (*Ne*) and sub-population structure, Wahlund effect (Waples *et al.*,2011). This agrees with the small effective population size among *T. lestoquardi* in different regions, which ranged between 2.71 and 3.73 compared to a higher *Ne* seen among *T. annulata*, which ranged between 6.96 and 8.46 (Table 3). For *T. annulata*, a lower but significant LD score was found for two of the three populations, whereas linkage equilibrium was evident in the Batinah population (Table 5).

3.7. Detection of possible selection on mini- and microsatellites

The F_{ST} outlier test conducted to detect departures from neutrality found in four loci showing low F_{ST}/He, with significant statistical support to be classified as lower threshold outliers (Table 6). Loci showing low F_{ST} are often under balancing selection, as this process forces alleles to maintain heterozygosity and lower differentiation across populations under the same environments.

3.8. Population structuring

A low level of F_{ST} was detected between each pair of the four *T. lestoquardi* populations (Table 7), as well as between pair-wise combinations of the three *T. annulata* populations, indicating a lack of differentiation between regional populations. A low level of differentiation between regional parasite populations is supported by Principal Coordinate Analysis (PCoA) (Figure 4A and B). PCoA demonstrated no evidence of regional structuring for either species, with haplotypes distributed throughout the main cluster independent of geographic origin.

4. Discussion

Small ruminant theileriosis is a major problem in Oman, as it is a leading cause of morbidity and mortality and is associated with significant economic loss. To establish innovative control measures and assess their effectiveness, information on the extent of genetic diversity and population structure of *T. lestoquardi* is desirable. It is also of interest to investigate how Apicomplexan parasites may evolve by adapting to novel host species, and to determine whether such events occur at low or high frequency. The *T. annulata*/*T. lestoquardi* relationship provides a good model for this, as biological and molecular phylogenetic data suggest that *T. lestoquardi* has most likely evolved from an ancestral *T. annulata* infection of small ruminants (Leemans, *et al.,* 1998; Katzer *et al.,* 1998; Schttinger *et al.,* 2000) generating a parasite species that manifests acute pathology in susceptible hosts. In this study we investigated these questions by developing and validating a set of ten micro- and mini-satellites markers specific for *T. lestoquardi* and used them in a comparative analysis of *T. lestoquardi* and *T. annulata* parasites in four regions of Oman.

Although micro- and mini-satellites representing *T. annulata* (Weir *et al.*,2007) and *T. parva* (Oura *et al.*,2003) have previously been identified and characterised, this study is the first to report similar markers for estimating genetic diversity within and between isolates of *T. lestoquardi*. The present study describes the development of a panel of ten *T. lestoquardi*-specific markers, which are distributed over the four chromosomes. The ten loci showed considerable diversity within the studied populations with seven having an excess of high H_e. Together these markers represent a useful tool for analysing *T. lestoquardi* populations in the field, as they negate any issues of co-infection with related *Theileria* species and can provide an estimate of the level of genetic diversity and divergence within and between populations.

The markers revealed a high level of genetic diversity, a limited degree of linkage disequilibrium and an absence of differentiation across different *T. lestoquardi* populations in Oman. However, the extent of diversity among *T. lestoquardi* isolates was much lower than observed within *T. annulata* isolates in three regions where the two species co-exist. The mean H_e index for *T. lestoquardi* isolates in each site ranged from 0.575 to 0.637, lower than that observed among *T. annulata* in Oman (*He* ranged between 0.819 and 0.854) (Al-Hamidhi *et al.*,2015) and other endemic countries (Weir *et al.*,2011), as well as that reported for *T. parva* in Zambia (Muleya *et al.*,2012). The higher level of genetic diversity in the *T. annulata* population may be the result of genetic recombination over an extended period of time compared to *T. lestoquardi*, which may have emerged more recently. Whether the higher MOI of *T. annulata* in the cattle population is simply a reflection of increased diversity in this parasite population is difficult to gauge. However, given the high level of identity at the 18S rRNA locus between the *T. lestoquardi* and *T. annulata* (Schnittger *et al.*,2000), it is most probable that *T. lestoquardi* has evolved from an ancestral cattle-infective parasite related to present day *T. annulata* and that parasite speciation occurred as the parasite adapted to the small ruminant host. A similar conclusion on host switching and parasite speciation has been made, following analysis of mitochondrial genome sequences, for primate malaria parasites among several species that live in sympatry (Escalante *et al.*,1998).

The above hypothesis is also consistent with the greater diversity of two antigen genes in *T. annulata* compared to that of their orthologues in *T. lestoquardi*. Indeed, construction of phylogenetic trees from sequence data from each antigen gene clearly shows separation of sequences representing either species with no indication that any *T. lestoquardi* sequence showed a closer relationship to *T. annulata* than the rest of the dataset (Figure 3). Taken together the data indicate that the jump from the ancestral species that allowed adaptation to small ruminants is not a frequent event, and may have only happened on a limited number of occasions, involving a low number of genotypes. Thus, much of the pre-existing diversity in the cattle population would not have been carried over into the *T. lestoquardi* population. Whether speciation events linked to host adaptation of vector-borne Apicomplexan parasites are generally infrequent is unknown. However, a study of the evolution of *Plasmodium falciparum* concluded that the jump of the ancestral parasite from gorillas may have resulted from a single cross-species transmission event (Liu *et al.*,2010). These studies may indicate that, while evolution of new pathogenic Apicomplexan species after transmission to a novel host has occurred on a number of occasions (Arisue *et al.*,2015), the probability of this happening on a frequent basis is not high.

We identified eight *T. annulata* isolates in small ruminants (seven ovine and one caprine), and found that the average number of alleles and MOI were slightly lower (2.9 vs 3.27) in the ovine/caprine isolates than in bovine isolates of *T. annulata*. This preliminary data suggests *T. annulata* is less well adapted to sheep than *T. lestoquardi* and that establishing *T. annulata* infection is more difficult in ovine cells than bovine. Exactly how competent small ruminants are in the transmission of *T. annulata* in the field is unknown; however the weight of evidence to date does not suggest they play a major role in the epidemiology of tropical theileriosis in comparison to cattle. With the common ancestor of *T. annulata* and *T. lestoquardi* presumed to be a cattle parasite, essentially similar to modern day *T. annulata*, adaptive changes promoting establishment and transmission capability in small ruminants would likely have developed as *T. lestoquardi* established as a species. However, whether the most recent common ancestor shared *T. annulata*'s inability to produce piroplasms in small ruminants (Li *et al.*,2014) is impossible to say and the degree of each host-species adaptation in the intervening time is unknown.

The high diversity of antigen genes *Tams1* and *Ta9* (Table 4) in *T. annulata* has been proposed to confer a selective advantage to parasite genotypes by facilitating evasion from a protective immune response (Wang *et al.*,2014). This and genetic diversity, in general, could promote a more widespread distribution and survival of this species, even in the face of various control strategies. However, it should be noted that although less divergent, a stable transmissible endemic population of *T. lestoquardi* exists in a number of countries of the Middle East and Africa.

The high genetic diversity of bovine *T. annulata* populations in Oman compared to that detected among sympatric *T. lestoquardi* is consistent with the multiplicity of infection data (Table 1). MOI is a prerequisite for cross-mating and recombination among different parasite genotypes in the tick vector midgut, and thus the generation of novel recombinant genotypes. The proportions of animals harbouring multiple infections were similar for each species; however the mean MOI values differed considerably, being two-fold higher for *T. annulata*. This cannot be attributed to variation in density of infection between the two species, as PCR detection can favor the most abundant genotypes existing at high parasitaemia compared to those at low levels, as all samples were collected from animals not showing clinical signs. MOI could result from inoculation of multiple clones from one infected tick or multiple ticks infected with distinct parasite genotypes feeding on a single bovine (superinfection). The former is expected to happen more readily in *T. annulata* due to the high level of diversity displayed by infected bovine isolates. Whatever the cause of MOI, the higher multiplicity of *T. annulata* genotypes could sustain a high rate of cross-mating and recombination in the tick vector, which in turn would result in increased genetic diversity in the bovine host, as demonstrated for the human malaria parasite *P. falciparum* (Babiker *et al.*,1994; Conway *et al.*,1999). In addition, 98% of the adult ticks collected from examined cattle and sheep were *H.anatolicum*: thus, it appears unlikely that transmission by different tick vectors could account for the differences in diversity of the two *Theileria* species.

The significant LD seen among some populations of both species contrasts with the expected high levels of out-crossing; however the LD is essentially mild and is only a slight departure from panmixia. LD can be influenced by demography and/or selection events. Diverse factors, other than the extent of inbreeding including the recombination rate, the local parasite effective population size and population differentiation (Hill and Babiker, 1995; Hill *et al.*,1995). Similar to other vector-transmitted parasites, *T. lestoquardi* genotypes are not randomly distributed, but rather the population is fragmented, with individual host animals supporting a sub-population of genotypes. Similarly, effect can also be achieved by undergoing selection. Individual carrying a genotype that positively affects its fitness, will be selected and increase in frequency in the population, at the expenses of the less "fitted" genotypes which will be erased, reducing the number of possible genotype combination available in a given population. The moderate LD values observed in the *T. lestoquardi*, can be explained by undergoing balancing selection. In this type of selection, several genotypes bring similar advantages to the individuals and therefore the frequency of those genotypes tends to be even. In other words, balancing selection promotes diversity (heterozygosity) rather than positive selection that promotes fixation (homozygosity). However, co-uptake of sexual stages of closely related genotypes by the feeding tick may result in non-random mating and consequently LD. Assuming random pairing of male and female gametes, the frequency of cross-mating equals the probability these gametes are sampled from different genotypes carried in a single animal. The probability of inbreeding can be related to the numbers of genotypes detected per infection, assuming that all blood form parasites are represented in the gametocyte population (Hill *et al.*,1995). It has been shown that a small number of genetically related parasites in the vertebrate host can generate significant linkage disequilibrium (Anderson *et al.*,2000). Thus, the observed LD and lower level of genetic diversity in *T. lestoquardi* relative to *T. annulata*, does not necessarily indicate the absence of a broadly panmictic population structure or reduced levels of genetic recombination.

Very low levels of genetic differentiation were detected between *T. lestoquardi* populations in the four sites in Oman with most pair-wise F_{ST} values being less than 0.04. This is consistent with the analysis of sympatric *T. annulata* populations which also show a low level of population differentiation (Al-Hamidhi *et al.*,2015). The results suggest a rate of genetic exchange and gene flow between parasites in different parts of the country, sufficient to allow the population to remain homogenous and to overcome genetic drift through geographical and genetic isolation. It is likely that homogenisation of the population is underpinned by movement of infected/infested animals from one area to another. It would be of interest to examine *T. lestoquardi* populations in neighboring countries in the region, where theileriosis is also a major problem, to determine how closely related these populations are. This could determine whether control measures, based on vaccine or drug therapy should be implemented separately or if a regional policy can and should be adopted.

In conclusion, the present study compared genotypic and population diversity between sympatric *T. lestoquardi* and *T. annulata* in Oman. *Theileria annulata* populations were shown to be consistently more diverse and hosts displayed a greater MOI. These results provide an insight into the evolution of *T. lestoquardi*, reinforcing the hypothesis that it has diverged from ancestral *T. annulata* and evolved following adaption to small ruminant hosts, potentially via a single cross-species adaptive event. Further work investigating the molecular basis that promoted host adaptation and speciation of *T. lestoquardi* is warranted, together with investigation of whether a reduced level of antigenic diversity impacts on transmission efficiency of *T. lestoquardi* relative to *T. annulata* in the field.

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Figure 1: Locations of sample collection sites in Oman for *T. lestoquardi* and *T. annulata*.

Figure 2: The frequency of *T. lestoquardi* alleles in the four governorates of Oman for the representative marker TL07. The size of each allele (in bp) is given on the x- axis

Figure 3: ML phylogenetic tree showing relationships between *Tams1* and *Tlms1* alleles**.** Evolutionary distances were computed using the Maximum Composite Likelihood method and are

in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. TA (*T. annulata*), TL (*T. lestoquardi*), and TP (*T. parva*).

Figure 4: A) Principal Coordinates Analysis of *T. lestoquardi* from four regions in Oman. B) Principal component analysis of *T. annulata* from three regions in Oman. The amount of variation explained by each axis is shown as a percentage of the overall variation.

Table 1: Prevalence and multiplicity of infection of small ruminant *T. lestoquardi* and bovine *T. annulata* populations in three regions in Oman

Species	Region	No. of animals samples	No. of infected animals (%)	No. of infected animals with multiple genotypes (%)	Mean MOI (SD)
T. annulata					
	Batinah	78	21 (26.9)	21 (100)	3.3 (1.0)
	Dhira	120	57 (47.5)	57 (100)	2.9 (0.8)
	Sharqia	36	19 (48.7)	19 (100)	3.3 (0.7)
T. lestoquardi					
	Batinah	584	57 (9.8)	53 (93)	1.63 (0.33)
	Dhira	357	52 (14.6)	52 (100)	1.65 (0.30)
	Dakhiliya	144	25 (17.4)	24 (96)	1.64 (0.30)
	Sharqia	369	56 (15.2)	53 (95)	1.49 (0.26)

Table 2: Allelic diversity and heterozygosity at ten micro- and mini-satellite loci from 190 *T. lestoquardi* isolates in Oman

		TL_MS05		TL_MS18		TL_MS281		TL_MS04		TL_MS07		TL_MS13		TL_MS16		TL_MS19		TL_MS280		TL_MS25		
Region	**n**	H_e	**Private alleles**	H_e	**Private alleles**	H_e	**Private alleles**	H_e	**Private alleles**	H_e	**Private alleles**	H_e	**Private alleles**	H_e	**Private alleles**	H_e	**Private alleles**	H_e	**Private alleles**	H_e	**Private alleles**	**Average** H_e
Batinah	57	0.823	1	0.531	2	0.805	0	0.395	0	0.736	1	0.741	1	0.342	0	0.595	1	0.679	3	0.102	0	0.575
Dakhiliya	25	0.830	0	0.550	1	0.720	0	0.527	0	0.690	0	0.730	0	0.477	0	0.717	2	0.570	1	0.227	1	0.604
Dhair	52	0.850	2	0.521	0	0.824	3	0.115	0	0.705	0	0.847	0	0.419	0	0.632	0	0.816	4	0.111	0	0.584
Sharqia	56	0.882	2	0.581	2	0.858	5	0.410	2	0.655	0	0.815	5	0.510	5	0.733	4	0.816	4	0.105	1	0.637
Overall		0.867		0.548		0.839		0.359		0.705		0.796		0.441		0.665		0.760		0.121		0.610

He: gene diversity; heterozygosity

Table 3: Estimates of genetic diversity of *T. lestoquardi* and *T. annulata* populations in three regions in Oman

Species	Region	n	H_e	*Ne*
T. lestoquardi	Batinah	57	0.575	2.904
	Dhira	52	0.584	3.394
	Sharqia	56	0.637	3.736
	Dakhiliya	25	0.604	2.714
T. annulata				
	Batinah	21	0.854	6.967
	Dhira	57	0.820	8.460
	Sharqia	19	0.833	6.153

Table 4: Estimates of genetic diversity of antigen genes among *T. lestoquardi* and *T. annulata* isolates

Antigen gene	*Tl9*	*Ta9*	*Tlms1*	*Tams1*
Parasite species	*T. lestoquardi*	*T. annulata*	*T. lestoquardi*	*T. annulata*
Polymorphic sites (S)	33	134	19	144
Average number of nucleotide differences (k)	18.600	52.270	3.902	45.832
Nucleotide diversity (π)	0.055	0.155	0.006	0.073
Haplotype diversity (Hd)	0.900	0.978	0.649	0.968

Table 5: Linkage equilibrium among *T. lestoquardi* and *T. annulata* populations in Oman

Species	**Region**	I^S_A	V_D	L_{MC}	L_{PARA}	**Linkage**
T. annulata	Batinah	0.0028	1.1174	1.3	1.277	LE
	Dhira	0.0219	1.5537	1.4497	1.4378	LD
	Sharqia	0.0337	1.5627	1.4568	1.4342	LD
	Total	**0.0169**	**1.3841**	**1.2896**	**1.2835**	**LD**
T. lestoquardi	Batinah	0.0102	2.1347	2.2212	2.2073	LE
	Dhira	0.0018	1.7015	1.872	1.8559	LE
	Sharqia	0.0752	2.9698	2.0328	2.0174	LD
	Dakhiliya	0.0462	3.0053	2.6308	2.5871	LD
	Total	**0.0264**	**2.284**	**1.9792**	**1.975**	**LD**

Table 6: Outlier loci outputs Heterozigozity and F_{ST} obtained by using Mcheza (DFDIST algorithm). Statistical significance was obtained as Simulated F_{ST} <sample F_{ST}

Locus	*He*	F_{ST}
TL_MS05	0.139	-0.026**
TL_MS18	0.191	-0.020**
TL_MS281	0.139	-0.021**
TL_MS04	0.202	0.032
TL_MS07	0.379	0.111
TL_MS13	0.252	-0.016
TL_MS16	0.188	0.016
TL_MS19	0.188	-0.021**
TL_MS280	0.188	0.013
TL_MS25	0.187	0.104

** Significant at P<0.01

Table 7: Pair-wise F_{ST} value between *T. lestoquardi* and *T. annulata* populations in Oman

		Batinah	Dhira	Dakhiliya
T. annulata	Batinah	0.0		
	Dhira	0.0257		
	Sharqia	0.0201	0.0266	ND
T. lestoquardi	Batinah	0.0		
	Dhira	0.0256		
	Dakhiliya	-0.0013	0.0457	
	Sharqia	0.0227	0.0281	0.0232

Figure 1


United Arab Emirates
Batinah
Dhira
Dakhiliya
Sharqia
Saudi Arabia
Dofar
Yemen
OMAN
OUTLINE WITH ADMINISTRATIVES

Figure 2

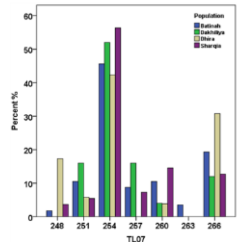

Population
Batinah
Dakhiliya
Dhira
Sharqia
Percent %
0
10
20
30
40
50
60
248
251
254
257
260
263
266
TL07

Figure 3

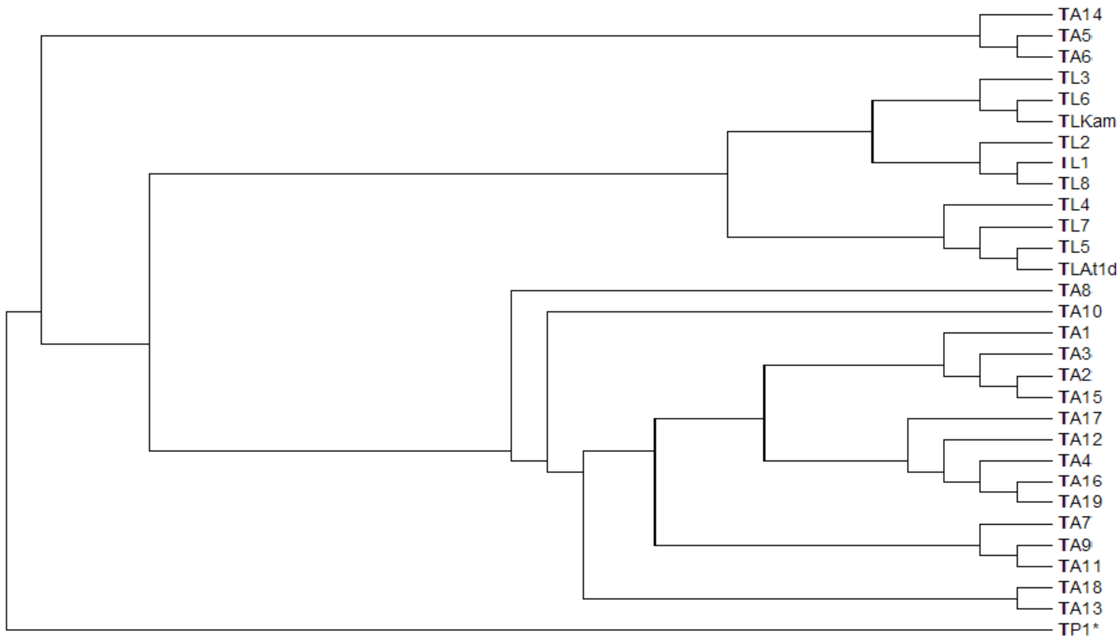
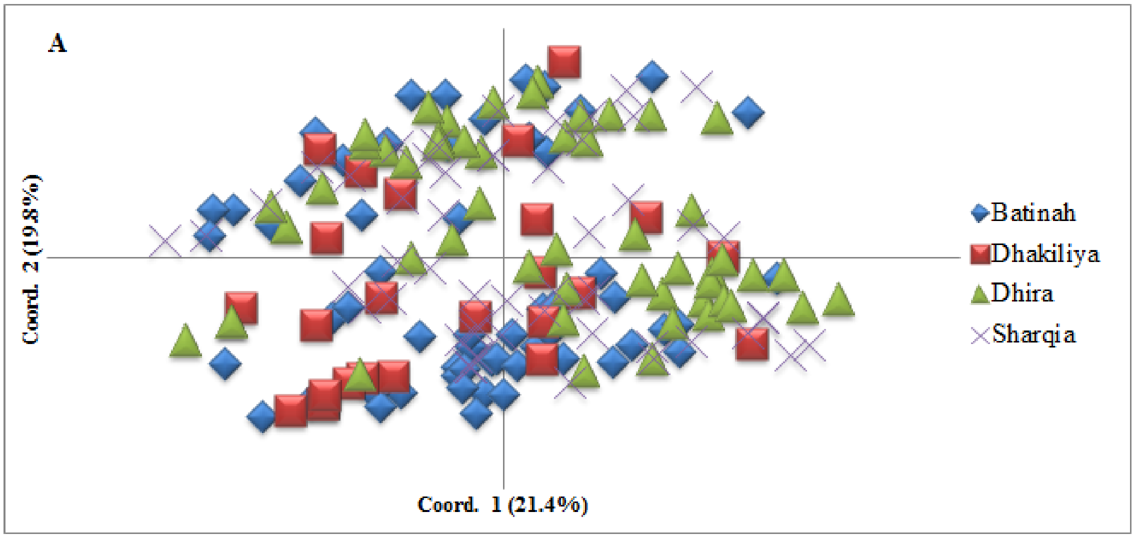
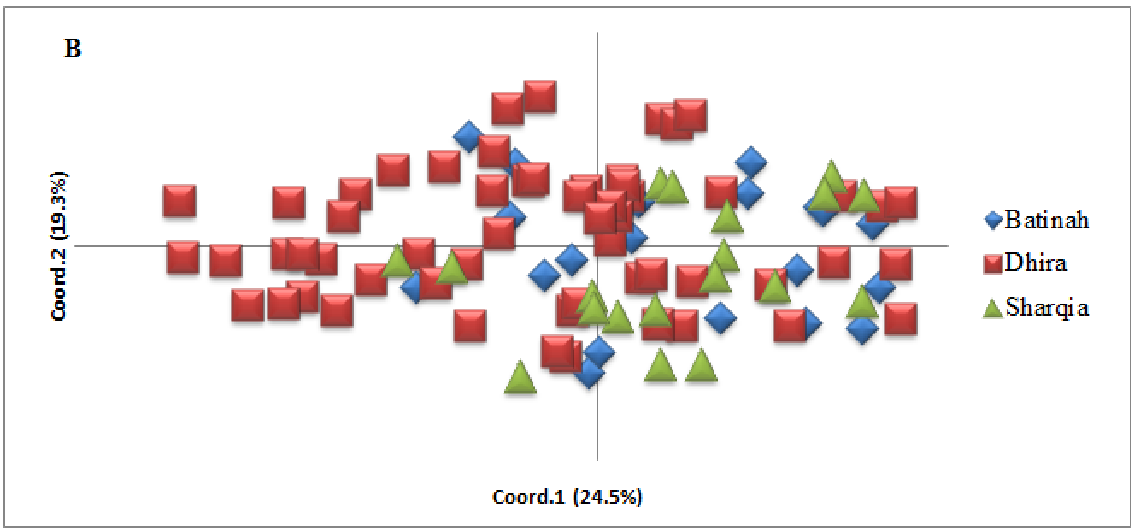
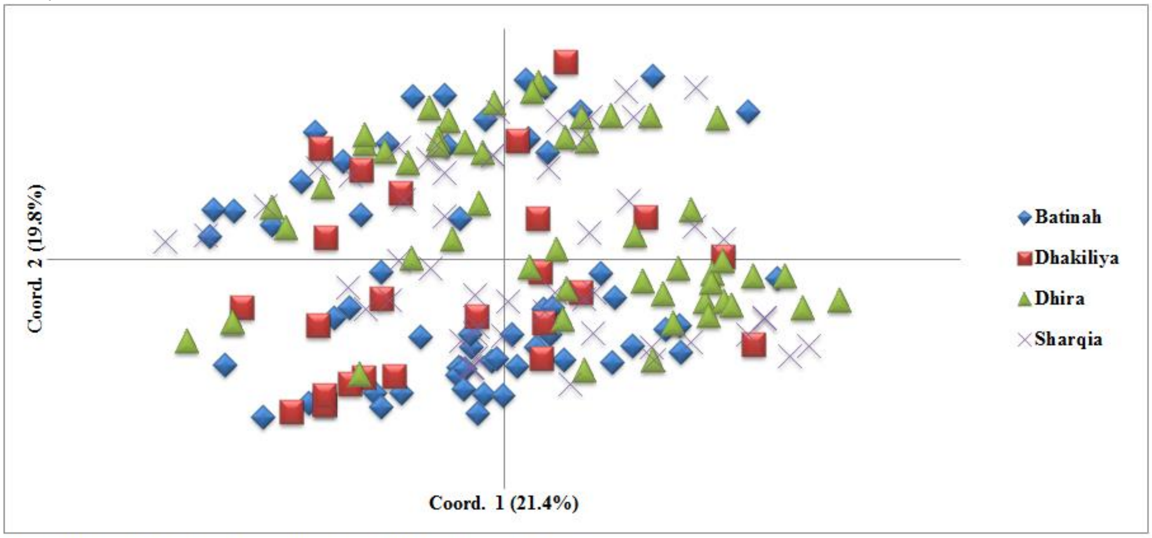

TA14
TA5
TA6
TL3
TL6
TLKam
TL2
TL1
TL8
TL4
TL7
TL5
TLAt1d
TA8
TA10
TA1
TA3
TA2
TA15
TA17
TA12
TA4
TA16
TA19
TA7
TA9
TA11
TA18
TA13
TP1*

Figure 4


A
Coord. 2 (19.8%)
Coord. 1 (21.4%)
Batinah
Dhakiliya
Dhira
Sharqia


B
Coord.2 (19.3%)
Coord.1 (24.5%)
Batinah
Dhira
Sharqia

Graphical abstract



Principal Coordinates Analysis of *T. lestoquardi* from four regions in Oman

Highlights

1. This study identified, and confirmed the species specificity, of ten micro- and mini-satellites spread across the *T.lestoquardi* genome and used them for analysis of genetics of four *T. lestoquardi* populations in Oman.

2. In addition, we examined two antigens genes in *T.annulata* (*Tams1* and *Ta9*) and their orthologues in *T. lestoquardi* (*Tlms1* and *Tl9*).

3. We compared the above data with previously published data, for comparable panels of markers, for sympatric *T.annulata*.

4. Despite a comparably high prevalence, *T.lestoquardi* has lower genetic diversity and multiplicity of infection (MOI) compared to sympatric *T.annulata* populations.

5. Similar to microsatellites, sequence diversity of the antigen genes, *Tams1* and *Ta9* in *T.annulata*, was higher than the orthologous *Tlms1* and *Tl9* in *T.lestoquardi*.

6. It is, therefore, likely that *T.lestoquardi* evolved from an ancestor of present day *T.annulata*.