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Genetic diversity, piroplasms and trypanosomes in *Rhipicephalus microplus* and *Hyalomma anatolicum* collected from cattle in northern Pakistan

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Abstract
The two most important tick species in Pakistan are *Rhipicephalus microplus* and *Hyalomma anatolicum*. When associated with cattle, these have one or three host life cycles, respectively, with potential implications for their population genetics and for their vector role in the transmission of pathogens. To compare the two tick species in this context with molecular-phylogenetic methods, during the present study 123 ticks were collected from cattle in northern Pakistan. Two mitochondrial markers of 36 ticks were molecularly analyzed. All 11 *R. microplus* specimens had identical cox1 haplotypes, whereas the 25 *H. anatolicum* specimens had nine cox1 haplotypes. The latter belonged to two distinct phylogenetic lineages with high support. However, in the 16S rRNA gene these differences were less evident. Among the 113 ticks molecularly analyzed for tick-borne protozoa, the sequence of *Babesia occultans* was successfully amplified from two specimens of *H. anatolicum*. *Theileria annulata* was present in both *R. microplus* (10.4%) and *H. anatolicum* (27.3%), with significantly higher prevalence rate in the latter species. Only one tick, a *H. anatolicum* female, was positive in the PCR detecting *Trypanosoma* spp. Sequencing revealed the presence of a new genotype, with the closest phylogenetic relationship to stercorarian trypanosomes (in particular, to a tick-associated *Trypanosoma* sp. from Japan). In conclusion, the above differences between *R. microplus* and *H. anatolicum* may be partly related to their life cycles involving one host or three hosts, respectively. Among the others, host switching (reducing chances of inbreeding) and shorter periods spent on-host (reducing gene flow between cattle herds) are supposed to be important drivers of cox1 gene diversification in case of *H. anatolicum* as a three host tick species. These results highlight the importance of studying differences in intraspecific genetic diversity and piroplasm burdens between one host and three host ticks in the local scale. In addition, a *Trypanosoma* sp. molecularly identified in *H. anatolicum* is reported here for the first time from South Asia, deserving further evaluation concerning its host and vector species.

Keywords Cox1 · 16S rRNA · One host tick · Three host tick · *Theileria annulata* · *Babesia occultans*

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Introduction

Hard ticks (Acari: Ixodidae) are blood-sucking ectoparasites, which have outstanding medical and veterinary importance (Jongejan and Uilenberg 2004). The reason for this is that tick infestation of humans and animals may entail various harmful consequences, including blood loss, as well as pathogenic effects of toxic substances and microorganisms inoculated by the tick during its blood-sucking. Among these consequences, the transmission of tick-borne pathogens by hard ticks accounts for the highest economical losses, especially in tropical and subtropical regions of the world (Jongejan and Uilenberg 2004).

Pakistan is situated in the temperate zone and has (according to Köppen classification) warm climate in most of its area. In addition, Pakistan is among the ten countries of the world, which have been most severely affected by global warming (Eckstein et al. 2018). Although during the past few years several ecological and molecular studies targeted ticks and tick-borne pathogens in Pakistan, not all taxonomic and eco-epidemiological aspects have been addressed. For instance, the survey of tick distribution across different ecological zones revealed that the two predominant tick species infesting ruminants in Pakistan are *Hyalomma anatolicum* and *Rhipicephalus microplus* (Rehman et al. 2017). However, when these tick species were analyzed with molecular tools from this country, their specimens were compared either in a broad geographical context (12 samples of *R. microplus* from three regions of Pakistan: Roy et al. 2018) or with a method (amplifying the second internal transcribed spacer, ITS2) that is not able to resolve intraspecific differences (15 samples of *H. anatolicum* randomly selected from different farms of Pakistan were identical: Rehman et al. 2017). Thus, to our knowledge, the local genetic diversity of these important tick species was not assessed or compared.

Similarly, despite being a currently unfolding field of research, molecular data on tick-borne pathogens are still restricted to a few studies from Pakistan. In particular, while the presence of at least three piroplasms has relatively long been known in Pakistan (*Theileria annulata*, *Babesia bigemina* and *Babesia bovis*: Jabbar et al. 2015), there are some limitations to the availability of their sequences in GenBank [e.g. considering the most widely used genetic marker of piroplasms (18S rRNA gene), relevant sequences are only published for *T. annulata*, and only from one study in Pakistan: Khan et al. 2013]. In addition, tick-infecting trypanosomes have also been reported from the above tick species (from *H. anatolicum* in Africa: Latif et al. 2004; from *R. microplus* in South America: Martins et al. 2008), but similar data have not been published from the region of Pakistan.

With this in mind, ticks were collected from cattle in one locality in northern Pakistan. After morphological identification of tick species, their DNA extracts were used for molecular and phylogenetic comparison of intraspecific heterogeneity of two mitochondrial markers. Of these, the cytochrome *c* oxidase subunit I (cox1) gene was already shown to resolve close (even infra-specific) taxonomic relationships and population genetics of *R. microplus* (Burger et al. 2014), but to the best of our knowledge, it was not used in the same context for the other target species, *H. anatolicum* in the region of Pakistan (Southern Asia). Taken together, this also provided an opportunity to compare the local population structure of a one host and a three host tick species. Finally, conventional PCR was used for screening a larger number of tick DNA extracts for the presence of piroplasms and trypanosomes, followed by sequencing of positive samples.

Materials and methods

Ethics statement

The ticks used in this study were removed from cattle during veterinary care. The study design was approved by the Ethical Committee of Department of Zoology (Abdul Wali Khan University, Mardan). Consents from farmers and veterinarians were also obtained.

Sample collection

Ticks were collected in 2018, in 20 small cattle herds at one locality (coordinates: 34.7865°N, 71.5249°E) of Bajaur Agency in northwestern Pakistan, bordering Afghanistan. Ticks were collected from a total of 50 randomly chosen cattle (2 to 4 in a herd), which were found tick-infested. Up to six ticks were removed from each animal with tweezers and placed into 96% ethanol. Thus, in this study 123 ticks were included (Table 1). Ten adult ticks (four males, two females of *H. anatolicum* and four females of *R. microplus*) were used for morphological identification and molecular confirmation of their species following DNA extraction from two (third and fourth) legs (Table S1: samples labelled as "PAK"). These were used as voucher specimens during morphological identification of the remaining 113 ticks, in case of which DNA was extracted individually, from their whole body for pathogen screening (Table S1: samples labelled as "PACA"). For morphological identification, a VHX-5000 digital microscope (Keyence, Osaka, Japan) was used (Fig. S1), taking into account descriptions of *H. anatolicum* (Vatansever 2017) and *R. microplus* (Walker et al. 2003). Molecular phylogenetic analyses of tick mitochondrial markers were performed with the DNA extracts of 36 ticks (all samples labelled as "PAK" and those which were PCR positive for piroplasms: Table S1).

DNA extraction

The DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as reported in Hornok et al. (2016), except that ticks were disinfected on their surface with 10% sodium hypochlorite solution.

Table 1 Results of molecular screening for piroplasms according to the species and developmental stage or sex of ticks analyzed in this study

Detected piroplasm	Number of relevant specimens/all (percentage)			
	Rhipicephalus microplus		*Hyalomma anatolicum*	
	Female	Nymph	Male	Female
Theileria annulata	***7/67 (10.4)***	0/2	***2/17 (11.8)***	***10/27 (37)***
Babesia occultans	0/67	0/2	***1/17 (5.9)***	***1/27 (3.7)***
None	60/67 (89.6)	2/2 (100)	14/17 (82.3)	16/27 (59.3)

Data of positive samples are highlighted with italic and bold numbers

PCR methods

For molecular taxonomic analyses, the cytochrome *c* oxidase subunit I (cox1) gene was chosen as the first target, on account of its suitability as a DNA-barcode sequence for tick species identification (Lv et al. 2014). The PCR was modified from Folmer et al. (1994) and amplifies an approx. 710 bp long fragment of the gene. The primers LCO1490 (forward: 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (reverse: 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) were used. To confirm the results obtained with the cox1 gene, another PCR was used to amplify an approx. 460 bp fragment of the 16S rRNA gene of Ixodidae (Black and Piesman 1994), with the primers 16S+1 (forward: 5′-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3′) and 16S-1 (reverse: 5′-CCG GTC TGA ACT CAG ATC AAG T-3′) as described (Hornok et al. 2017).

For the detection of piroplasms, the primers BJ1 (forward: 5′-GTC TTG TAA TTG GAA TGA TGG-3′) and BN2 (reverse: 5′-TAG TTT ATG GTT AGG ACT ACG-3′) were used. The method was modified from Casati et al. (2006) as reported in Hornok et al. (2016).

DNA samples were also screened for trypanosomes and related kinetoplastids, with a conventional PCR that amplifies an approx. 900 bp long fragment of the 18S (SSU) rRNA gene. The primers 609F (forward: 5′-CAC CCG CGG TAA TTC CAG C-3′) (da Silva et al. 2004) and 706R (reverse: 5′-CTG AGA CTG TAA CCT CAA-3′) (Ramírez et al. 2012) were used, as reported (Szőke et al. 2017).

Sequencing, phylogenetic and statistical analyses

Purification and sequencing of the PCR products were done by Biomi, Inc. (Gödöllő, Hungary). Obtained sequences were manually edited, then aligned and compared to reference GenBank sequences by nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov). Representative sequences were submitted to the GenBank (accession numbers MK462194–MK462203 for the cox1 and MK495911–MK495917 for the 16S rRNA gene sequences of ticks; MK421148 and MK421149 for the 18S rRNA gene of *T. annulata* and *Babesia occultans*, respectively; MK495918 for *Trypanosoma* sp; see also Table S1). In the phylogenetic analyses reference sequences with high coverage (i.e. 99–100% of the region amplified here) were retrieved from GenBank and trimmed to the same length. This dataset was resampled 1000 times to generate bootstrap values. The MEGA model selection method was applied to choose the appropriate model for phylogenetic analyses. Phylogenetic analyses were conducted initially with the neighbour-joining method (P-distance model) (data not shown), followed by the maximum likelihood method (Tamura–Nei and K2 model for the cox1 gene of ticks and 18S rRNA gene of trypanosomes, respectively) by using MEGA version 6.0. Prevalence rates were compared by Fisher's exact test ($\alpha = 0.05$).

Results

Molecular investigation of tick mitochondrial markers

In this part of the study 36 ticks were analyzed. All 11 *R. microplus* specimens had identical cox1 haplotypes (100% identical with MG459963 in GenBank), whereas the 25 *H. anatolicum* specimens had 9 cox1 haplotypes (Table S1). The latter differed from each other in up to four nucleotides (meaning 645–649/649 = 99.4–100% identity). The majority of these substitutions were synonymous in the protein, but one isolate

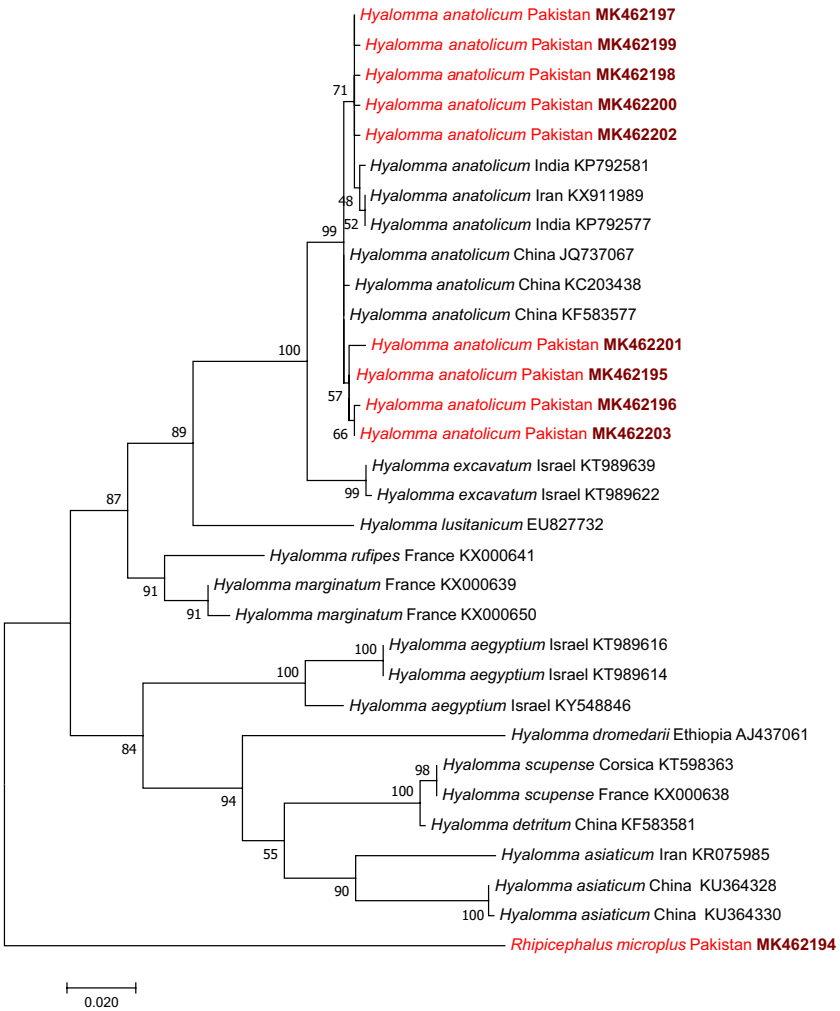

Fig. 1 Maximum likelihood tree of *Hyalomma* cox1 sequences, shown according to the country of origin before GenBank accession numbers. Sequences from this study are highlighted with red color, including that of *Rhipicephalus microplus* used as outgroup. The scale-bar indicates the number of substitutions per site. (Color figure online)

(PACA-117) had a mutation indicating different amino acid sequence (valine instead of alanine at position 100). The cox1 sequence heterogeneity in *H. anatolicum* was confirmed by phylogenetic analysis: isolates of this tick species belonged to two distinct genetic lineages, the separation of which was highly (bootstrap: 99%) supported (Fig. 1).

Regarding the shorter sequences of the more conserved 16S rRNA gene, differences between intraspecific genetic variation were less evident: *R. microplus* had three, whereas *H. anatolicum* had four haplotypes, differing in up to three nucleotides (meaning 410–413/413 = 99.3–100% identity).

DNA of protozoa in ticks

In the category of piroplasms, two species have been identified (Table 1). *Babesia occultans* was only detected in *H. anatolicum* (in a male and a female of the same haplotype: Table S1). This piroplasm from Pakistan had 100% identical sequence with other, geographically diverse isolates (e.g., from Italy: KC157568, from China, Xinjiang: AY726557, from South Africa: EU376017).

On the other hand, *T. annulata* was present in both *R. microplus* (10.4%) and *H. anatolicum* (27.3%) with significantly higher prevalence rate in the latter species (when only females are compared: P = 0.0058; when both sexes of *H. anatolicum* are taken into account: P = 0.037). The sequence of *T. annulata* identified here from Pakistan was 100% identical with another, formerly reported conspecific sequence from this country (JQ743630), as well as with others from various countries (e.g., from Italy: KX375830, from China: KU554731).

Only one tick, a *H. anatolicum* female was positive in the PCR detecting *Trypanosoma* spp. Sequencing revealed the presence of a new genotype, with the highest (423/451 bp = 93.8%) identity with a *Trypanosoma* sp. from a tick species (*Haemaphysalis hystricis*) in Japan (AB281091). Although the successfully sequenced part of the 18S rRNA gene had incomplete (approx. 76%) coverage with the majority of *Trypanosoma* sp. sequences in GenBank, phylogenetic analysis indicated that this novel genotype clustered in the same group with the tick-associated trypanosome reported from Japan (AB281091) and with *T. pestanai* from badgers and their fleas in Western Europe (AJ009159) (Fig. 2).

Discussion

To our knowledge, this is the first study to demonstrate cox1 gene heterogeneity of *H. anatolicum* from Pakistan and Southern Asia, and (in contrast to this) cox1 gene homogeneity of sympatric *R. microplus* populations. The latter finding is also confirmed with the 100% identity of our cox1 sequence with that of *R. microplus* formerly reported from Pakistan (MG459963, belonging to clade "C" in Roy et al. 2018). On the other hand, previously *H. anatolicum* has been reported with two identical cox1 sequences from Pakistan (KU130580–KU130581 in Sands et al. 2017). However, here it was shown that in northern Pakistan *H. anatolicum* is represented by two cox1 mitochondrial phylogenetic lineages, the separation of which is highly supported. Nevertheless, because of the limited sample size analyzed here, it will be necessary to extend the scope of these results before drawing any final conclusions.

Both tick species, *R. microplus* and *H. anatolicum* have long been indigenous in the study region (*H. anatolicum* being a Palearctic species: Guglielmone et al. 2014; and *R.*

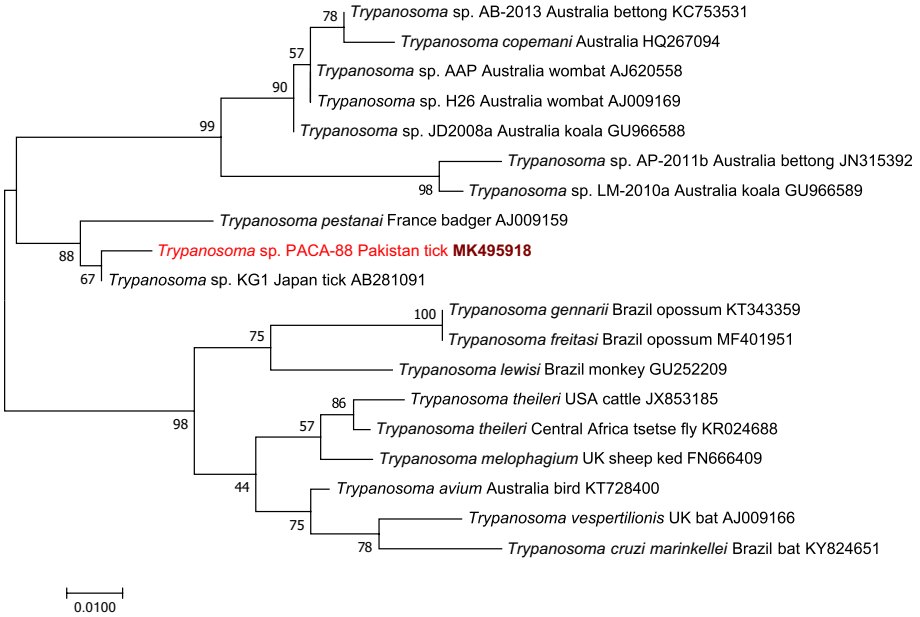

Fig. 2 Maximum likelihood tree of *Trypanosoma* 18S rRNA gene sequences, shown according to the country of origin and generic name of isolation source before GenBank accession numbers. The sequence from this study is highlighted with red color. The scale-bar indicates the number of substitutions per site. (Color figure online)

microplus, now widespread even in neotropics, most likely originated from South Asia: Barré and Uilenberg 2010). This could therefore counterbalance major differences in their genetic diversity resulting from more or less recent introduction into the region. Similarly, natural gene flow over larger geographic distances is not expected to play an important role in the population genetics of *R. microplus* and *H. anatolicum*, because birds are regarded as only exceptional hosts of both species (Guglielmone et al. 2014).

As recently suggested for *R. microplus*, very low levels of genetic differentiation *between* tick populations (in different localities or cattle herds) could result from frequent movement of livestock from one area to another (Sungirai et al. 2018). In this context the period of time spent by the tick on its cattle host is an important factor. Therefore, contrasting mitochondrial cox1 gene diversity (homogeneity vs. heterogeneity) between local populations of *R. microplus* and *H. anatolicum* as demonstrated here, maybe in part related to their different host usage. *H. anatolicum* is a three host tick species when feeding on cattle, which implies that in each of its stages the duration of on-host blood sucking does not exceed approx. 7–10 days (Vatansever 2017). This could increase the chances of forming relatively isolated local populations, which may differ from each other in their haplotype. By contrast, *R. microplus* is a one host tick species, which may spend up to 149 days on its cattle host after having attached as larvae (Hoogstraal 1956). This entails higher chances of (also longer distance) movement of *R. microplus* compared to *H. anatolicum*, implying a tendency of gene flow and low genetic differentiation within the region of local cattle movements.

Adding to this, the relative absence of genetic differentiation at one locality, as also observed here, may partly result from high chances of inbreeding in the case of *R. microplus* (Koffi et al. 2006). As recently postulated by Sungirai et al. (2018), low genetic diversification in this tick species can be partly attributed to its biology at the infra-population level (as a one host tick species), because development occurs simultaneously within large brotherhoods of individuals, which go on to seek hosts as a group, develop to adults simultaneously, and (frequently) mate with each other (Koffi et al. 2006). This results in inbreeding and increased homozygosity (Dharmarajan et al. 2011), as also suggested by the present results. Complying with this, a similar phenomenon (low, only 0.73% *within* population divergence of *R. microplus*) was also reported from South America (Csordas et al. 2016).

The difference in genetic diversity between the two tick species (*R. microplus* vs. *H. anatolicum*) was less evident in the 16S rRNA gene, which has usually lower "resolution" to reflect intraspecific diversity compared to the barcoding gene, cox1 (Lv et al. 2014). As an example, the genetic distances between *R. microplus* and *R. annulatus* for the cox1 and the 16S rRNA genes were 6.0% and 1.2%, respectively (Lv et al. 2014). In addition, the amplified fragment and thus the sequences of the latter marker were considerably shorter than in case of cox1, contributing to low efficacy in revealing infra-population differences of *H. anatolicum*.

The different pattern of host switching characteristic of *R. microplus* and *H. anatolicum* (a one and a three host tick species of cattle, respectively) is also relevant to differences in their role as vectors of piroplasms. Here engorged ticks were analyzed, therefore it cannot be determined if a PCR-positive tick acquired piroplasms with the blood of its host or had been infected prior to its blood meal. Nevertheless, taking into account that the three host tick species, *H. anatolicum* transmits *T. annulata* (the causative agent of tropical theileriosis in cattle) transstadially (Jongejan and Uilenberg 1994), this may have contributed to the higher number of *H. anatolicum* carrying this piroplasm, when compared to *R. microplus*.

Babesia occultans is a piroplasm having mild to more severe pathogenicity in cattle (Decaro et al. 2013). It has hitherto been reported from various parts of the Palearctic, from Italy to South Africa and China (Decaro et al. 2013; Gray and de Vos 1981; Song et al. 2018). This study provides the first sequence of *B. occultans* from Southern Asia. This piroplasm is transmitted transovarially by *Hyalomma marginatum* and related species (Aktas et al. 2014). The latter potentially includes *H. anatolicum*, in which tick species this piroplasm was formerly detected in Pakistan by reverse line blot hybridization (Rehman et al. 2019) and by PCR and sequencing here. Nevertheless, both studies analyzed engorged ticks, therefore these results do not preclude that *B. occultans* was obtained by the ticks from the host with the blood meal.

Recently, novel tick-infecting trypanosomes have been described from South America (Marotta et al. 2018) and from Africa (Latif et al. 2004), as well as from Eastern Asia (Thekisoe et al. 2007). Here an apparently novel *Trypanosoma* sp. (tentatively denoted here as "PACA-88": Table S1) was molecularly detected in *H. anatolicum* from Southern Asia. The presence of this *Trypanosoma* sp. in an engorged tick does not necessarily imply that it is also tick-associated (i.e., it may have also originated from the blood meal of the tick). Nevertheless, sequence and phylogenetic analyses reflected the close relationship of the new genotype with *Trypanosoma* sp. KG1 from the tick species *Ha. hystricis* in Japan (Thekisoe et al. 2007). The latter is a stercorarian trypanosome (Thekisoe et al. 2007), where numerous flea-associated species belong, as exemplified by *T. pestanai* (also closely related to *Trypanosoma* sp. PACA-88 as shown here). Further studies are needed to characterize this species morphologically, as well as concerning its host and vector species.

In conclusion, the above differences between *R. microplus* and *H. anatolicum* are at least partly related to their life cycles involving one host or three hosts, respectively. Among the others, host switching (reducing chances of inbreeding) and shorter periods spent on-host (reducing gene flow between cattle herds) are supposed to be important drivers of cox1 gene diversification in case of *H. anatolicum* as a three host tick species. These results highlight the importance of studying differences in intraspecific genetic diversity and piroplasm burdens between one host and three host ticks in the local scale. In addition, a *Trypanosoma* sp. potentially associated with *H. anatolicum* is reported here for the first time from South Asia, deserving further evaluation.

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