Original article

Molecular characterization of *Theileria orientalis* from cattle in Ethiopia

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ABSTRACT

This study reports the first molecular characterization of *Theileria orientalis* in local breeds of cattle in Ethiopia. A conventional PCR utilizing major piroplasm surface protein (*MPSP*) gene and an established multiplexed tandem PCR (MT-PCR) were used to characterize *T. orientalis* and to assess the infection intensity, respectively. Of 232 blood samples tested, *T. orientalis* DNA was detected in only 2.2% of samples using conventional PCR; two genotypes *buffeli* (1.3%; 3/232) and type 5 (0.9%; 2/232) of *T. orientalis* were detected. Phylogenetic analysis revealed that the *buffeli MPSP* sequences from Ethiopia were closely related to those reported from Kenya, Sri Lanka and Myanmar, and type 5 sequences from Ethiopia grouped with those from Korea, Japan, Vietnam and Thailand. A higher number of samples (3.9%; 9/232) were test-positive by MT-PCR and four genotypes (*buffeli*, *chitose*, *ikeda* and type 5) of *T. orientalis* were detected. The average intensity of infections with genotypes *buffeli* (DNA copy numbers 11,056) and type 5 (7,508) were significantly higher ($P < 0.0001$) than the pathogenic genotype *ikeda* (61 DNA copies). This first insight into *T. orientalis* from cattle in Ethiopia using *MPSP* gene provides a basis for future studies of *T. orientalis* in various agroclimatic zones and of the impact of oriental theileriosis on cattle in this and other countries of Africa.

Keywords: *Theileria orientalis*; Cattle; PCR; MT-PCR; Ethiopia

Introduction

Theileriosis is caused by tick-borne, haemoprotozoan parasites of the genus *Theileria* spp. and is one of the most economically important diseases of bovines, particularly in tropical and sub-tropical regions of the world (Mehlhorn and Schein, 1984; Uilenberg, 1995; Ahmed et al., 2002; Makala et al., 2003; Aktas et al., 2006). The most important *Theileria* spp. infecting bovines are *T. parva* and *T. annulata*, which cause East Coast fever and tropical theileriosis, respectively (Salih et al., 2007; Yusufmia et al., 2010), whereas other taxa, such as *T. mutans*, *T. velifera* and those of the *T. orientalis* complex are believed to cause milder and/or non-pathogenic theileriosis (Uilenberg et al., 1977; Salih et al., 2007; Tomassone et al., 2012). However, recently, *T. orientalis* complex, transmitted mainly by *Haemaphysalis* spp. caused significant morbidity, economic losses and/or mortality in cattle in the Asia-Pacific region (Izzo et al., 2010; Aparna et al., 2011; Kamau et al., 2011; McFadden et al., 2011; Perera et al., 2013, 2014; Gebrekidan et al., 2015). Based on the sequence analysis of the major piroplasm surface protein (*MPSP*) gene, two (*chitose* and *ikedai*) of the 11 currently known genotypes (called *chitose* or *type 1*, *ikedai* or *type 2*, *buffeli* or *type 3*, *types 4 to 8*, and *N1 to N3*) of *T. orientalis* are considered to be pathogenic, leading to disease (oriental theileriosis) (reviewed by Sivakumar et al., 2014).

Although *T. orientalis* is known to be a cosmopolitan parasite of bovines (Uilenberg et al., 1985), the taxonomy of this complex is still controversial, and most of the studies describing various genotypes of *T. orientalis* have been carried out in the Asia-Pacific region, mainly utilizing the *MPSP* gene (reviewed by Sivakumar et al., 2014). To date, most of molecular studies focused on the characterization of the *T. orientalis* from the African continent have utilized the 18S rRNA gene using conventional PCR, followed by DNA sequencing (Allsopp et al., 1994; Collins et al., 2002; Darghouth, 2004; Salih et al., 2007; Altay et al., 2008; M'ghirbi et al., 2008; Chaisi et al., 2011, 2014). However, recently, Elsify et al. (2015)

analysed *T. orientalis* for the first time using the *MPSP* gene from Africa, and reported *iked*
and *chitose* genotypes from cattle and buffalo in Egypt.

Very little is known about *T. orientalis* in cattle from Ethiopia. The first suspected cases of
T. orientalis infection in cattle were reported in 1983; the parasite was detected using
conventional (microscopic and serological) methods (Becerra et al., 1983), which are known
to have limitations such as low specificity and sensitivity (cf. Perera et al., 2015a). Recently,
Gebrekidan et al. (2014) detected *T. orientalis* in asymptomatic cattle in northern Ethiopia by
conventional PCR, targeting the 18S rRNA gene. Since this study did not utilize the *MPSP*
gene, *T. orientalis* could not be classified genetically as “pathogenic” or “non-pathogenic”
genotypes (cf. Sivakumar et al., 2014). The present study detected, quantitated and
characterized *T. orientalis* in local cattle in Ethiopia using PCR-based tools.

Materials and methods

Blood samples from cattle

A total of 232 blood samples randomly collected from clinically healthy/asymptomatic
cattle were tested. Of these blood samples, 100 were available from a previous study carried
out between October and November 2010 in three localities, including Addis Zemen (12° 7'
11"N, 37° 46' 48"E), Humera (14° 16' 20"N, 36° 38' 24"E) and Sheraro (14° 24' 0"N, 37° 56'
0"E) in northern Ethiopia, which were tested for various piroplasms (Gebrekidan et al.,
2014). In addition, 132 more blood samples were collected in February 2013 from three more
villages in Sheraro in northern Ethiopia.

Blood samples were taken from the jugular vein and collected into EDTA-coated tubes.
The study was approved by the Ethiopian National Research Ethics Review Committee

(NRERC; permit no. 3.10/3398/04). Consent was obtained from cattle owners for the collection of blood samples by an experienced, practicing veterinarian. Different cattle breeds were sampled in different locations, including Fogera and Zebu local breeds from Addis Zemen, and Arado and Barka (Begait) breeds from both Humera and Sheraro.

Isolation of DNA from blood, conventional PCR and DNA sequencing

Genomic DNA was extracted from 300 µL of each blood sample using the illustra blood genomicPrep Mini Spin Kit (GE HealthCare, Buckinghamshire, UK), following the manufacturer's instructions and stored at -20 °C until further analysis.

Primers MPSP-F and MPSP-R were used for the amplification of a 776 bp region of the *MPSP* gene as described previously (Ota et al., 2009). PCRs were conducted in 50 µL volumes containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl (Promega), 3.5 mM MgCl₂, 6.25 µM of each deoxynucleotide triphosphate (dNTP), 100 pmol of each primer, and 1 U of *GoTaq* polymerase (Promega, USA). PCR cycling conditions were an initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, 58 °C for 30 s and 72 °C for 1 min, and 72 °C for 5 min. Negative and positive controls were included in each PCR. PCR products were examined by agarose (1.5%) gel electrophoresis.

PCR products were then treated with shrimp alkaline phosphatase and exonuclease I (Germentas Inc., USA) (Werle et al., 1994), and subjected to bi-directional, automated sequencing (BigDye[®] Terminator v.3.1, Applied Biosystems, CA, USA) using (separately) the same primers employed in PCR. The quality of nucleotide sequences was appraised using the program Geneious Pro 5.6.5 (Biomatters Ltd., Auckland, New Zealand), and polymorphic sites were designated using International Union of Pure and Applied Chemistry (IUPAC) codes. The *MPSP* sequences were identified by local alignment comparison (six reading frames) using amino acid sequences conceptually translated (using an online tool,

http://www.ebi.ac.uk/Tools/st/emboss_transeq/) from reference sequences of *T. orientalis* available from the GenBank database.

Quantitative PCR

In order to estimate the infection intensity of *T. orientalis* genotypes present in Ethiopian cattle, we used a semiquantitative multiplex tandem PCR (MT-PCR) assay. This assay was conducted in the Easy-Plex platform (AusDiagnostics Pty Ltd, Australia) as described previously by Perera et al. (2015a) using primers designed specifically to the piroplasm surface protein (*p23*) gene (for genotype *buffeli*), *MPSP* gene (*chitose* and *type 5*) and the first internal transcribed spacer (ITS-1) of nuclear ribosomal DNA (*ikedai*) (cat. no. 38170R; AusDiagnostics, Australia). Following primary and secondary amplifications, the peak high resolution melting (HRM) temperature of each amplicon was compared with pre-determined reference temperatures representing individual genotypes: *buffeli* (83.6 ± 1.5 °C), *chitose* (82.1 ± 1.5 °C), *ikedai* (87.4 ± 1.5 °C) and *type 5* (81.6 ± 1.5 °C) (Perera et al., 2015a). The relative intensity of infection of each of these four genotypes was expressed as a DNA copy number (Perera et al., 2015a). All amplicons had the peak melting temperatures within respective reference values. Amplicons from each genotype were subjected to single-strand conformation polymorphism (SSCP) analysis and targeted sequencing (Cufos et al., 2012; Perera et al., 2013).

Phylogenetic analyses

Nucleotide sequences were aligned using the MUSCLE V 3.8.31 program (Edgar, 2004) and adjusted manually employing the program Mesquite V 3.03 (Maddison and Maddison, 2008). Based on pairwise comparisons, sequence differences were calculated using the program MEGA 6.0. (Tamura et al., 2013).

A dataset representing the *MPSP* sequences detected in Ethiopia were compiled, together with key reference data from previous studies (Kawazu et al., 1992; Kubota et al., 1996; Kakuda et al., 1998; Kim et al., 1998; Kawazu et al., 1999; Gubbels et al., 2000; Zakimi et al., 2006; Altangerel et al., 2011a, b; Khukhuu et al., 2011; Sivakumar et al., 2013; Bawm et al., 2014; Elsify et al., 2015) available from the GenBank, with *T. annulata* (see Shiels et al., 1995) as the outgroup, and sequences were aligned (705 bp) and adjusted manually as described above. Phylogenetic analyses were performed using Bayesian Inference (BI) and Neighbor Joining (NJ) methods. The BI was conducted, using Monte Carlo Markov Chain (MCMC) analysis in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The likelihood parameters for BI were based on the Akaike Information Criterion (AIC) test in jModeltest v2.1.5 (Darriba et al., 2012). The Tamura 3-parameter model of evolution, with gamma distribution and a proportion of invariable sites (T92+ Γ +I), was utilized for the analysis of the sequence data. The estimates of the base frequencies, the substitution rate model matrix and the proportion of invariable sites were fixed. Posterior probabilities (pp) were calculated using 2,000,000 generations, employing four simultaneous tree-building chains, with every 100th tree being saved. A consensus tree (50% majority rule) was constructed based upon the remaining trees generated by BI. The NJ analyses were performed using the program MEGA 6.0, and the nodes were tested for robustness with 10,000 bootstrap replicates. The phylogenetic trees produced from the BI and NJ analyses were compared for concordance in their topologies.

Statistical analyses

Pearson's chi-square contingency table analysis, Fisher's Exact Test and descriptive statistics were used to analyse the prevalence of genotypes detected by PCR and MT-PCR. DNA copy numbers (a measure of the intensity of infection) for the four *T. orientalis*

genotypes were log-transformed (log₁₀) and analysed by one-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference test. The SPSS Statistics 22 package (IBM) was used for statistical analyses, and a *P*-value of < 0.05 was considered as statistically significant.

Results and discussion

Of 232 blood samples tested, 5 (2.2%) and 9 (3.9%) were test-positive for *T. orientalis* by conventional PCR and MT-PCR assays, respectively, and this difference in prevalence was not statistically significant (Table 1). All of the test-positive samples were from one locality, Addis Zemen (Table 1).

DNA sequencing of conventional PCR amplicons revealed *buffeli* (two variants; GenBank accession nos. KU170784 and KU170785) and *type 5* (two variants; KU170786 and KU170787) genotypes of *T. orientalis* with an overall prevalence of 1.3% (3/232) and 0.9% (2/232), respectively (Table 1), and mixed infections with these genotypes were not detectable. Using MT-PCR, four genotypes, including *buffeli*, *chitose*, *ikeda* and *type 5* were detected, and mixed infections were more prevalent (3%; 7/232) than single infections (0.9%; 2/232) (Table 1). Four samples were test-positive for *buffeli* + *ikeda* and one each for *buffeli* + *chitose*; *buffeli* + *type 5*; and *buffeli* + *ikeda* + *type 5* (Table 1). The estimated intensity of infection with the genotype *buffeli* was dominant with an average DNA copy number of 11,056, followed by *type 5* (7,508) and *ikeda* (61). The average intensity of infection with both *buffeli* and *type 5* were significantly higher than for *ikeda* ($P < 0.0001$), and that did not differ between *buffeli* and *type 5*.

The pairwise comparisons of both Ethiopian *buffeli* (KU170784 and KU170785) and *type 5* (KU170786 and KU170787) *MPSP* sequences revealed a nucleotide difference of 0.7%, and 2.1%, respectively (see Supplementary Tables 1 and 2). Phylogenetic analyses of *T.*

orientalis MPSP sequence data from Ethiopia with those reported previously using BI and NJ showed a concordance topology for the two methods. Ethiopian *buffeli* (KU170784 and KU170785) and *type 5* (KU170786 and KU170787) genotypes grouped, with strong statistical support, with sequences representing the *buffeli* and *type 5* genotypes of *T. orientalis*, respectively, to the exclusion of all other *T. orientalis* genotypes presently recognized (Fig. 1).

Overall, the *T. orientalis* infections in cattle were rare, with only two non-pathogenic genotypes *buffeli* and *type 5* being detected by conventional PCR, and four genotypes *buffeli*, *chitose*, *ikeda* and *type 5* by MT-PCR. Using the latter PCR, the average intensity of infection with *buffeli* was shown to be dominant, followed by *type 5*.

The apparent prevalence of *T. orientalis* was shown to be low (2.2%) using conventional PCR, which was considerably lower than estimated in several previous reports in asymptomatic cattle from various Asia-Pacific countries, including Japan (64.8%; Ota et al., 2009), Mongolia (41.7%; Altangerel et al., 2011a), Myanmar (36.2%; Bawm et al., 2014), Sri Lanka (53.5%; Sivakumar et al., 2013), Thailand (31.5%; Altangerel et al., 2011b) and Vietnam (13.8%; Khukhuu et al., 2011) using the same conventional PCR targeting the MPSP gene. However, it was higher than that (0.68%; 3/439) reported recently in cattle from Egypt (Elsify et al., 2015). MT-PCR also revealed a lower prevalence (3.9%) of *T. orientalis* infection in Ethiopia compared with those estimated in recent studies following outbreaks of oriental theileriosis in cattle from Australia (53.8 to 75.5%) and New Zealand (99.4%) using the same assay (Gebrekidan et al., 2015; Perera et al., 2015b,c). The variation in prevalence of *T. orientalis* among different countries could be due to numerous factors, including the presence and abundance of ticks (e.g., *Haemaphysalis* spp.) and/or other vectors, susceptibility of animal breeds, the presence of wildlife reservoirs and/or other ecological or climatic factors. The prevalence of *T. orientalis* reported in this study should be interpreted

carefully, as we sampled only 232 cattle from only two (i.e., Amhara and Tigray) out of the nine administrative regions of Ethiopia, and future nationwide molecular survey are warranted to better understand the extent and impact of *T. orientalis* infections on cattle health in this country.

We found that two non-pathogenic genotypes of *T. orientalis*, *buffeli* and *type 5*, were dominant (see Table 1), with their average infection intensities being 11,056 and 7,508, respectively. In contrast, the most pathogenic genotype *ikeda* was uncommon, and its average infection intensity was also very low (61). This contrasts the situation in countries of the Asia-Pacific, where pathogenic genotypes are common and their infection intensities are quite high, even in asymptomatic animals. The low intensity of infection of pathogenic genotypes of *T. orientalis* may explain why clinical oriental theileriosis is uncommon in Ethiopia. However, to date, only two suspected cases of oriental theileriosis have been reported in cattle from Ethiopia (Becerra et al., 1983). For instance, in two localities (Illubabor and Wollega) of western Ethiopia, Becerra et al. (1983) investigated diseased Horro cattle exhibiting pyrexia, nasal discharge, salivation, anaemia, emaciation and swelling of superficial lymph nodes, and detected *T. orientalis* infections by blood smear examination and serological testing (indirect fluorescent antibody technique). These authors also detected other haemoparasites, such as piroplasms (*T. velifera* and *B. bigemina*), *Anaplasma* (*A. marginale*) and *Trypanosoma* (*T. congolense*), but they did not conclude which pathogens were most likely associated with mortality in cattle (Becerra et al., 1983). Thus, a true clinical case of oriental theileriosis in cattle from Ethiopia still remains to be identified.

The phylogenetic analyses of *MPSP* sequence data for *T. orientalis* genotypes found in Ethiopia and all ($n = 11$) currently known genotypes of *T. orientalis* showed that the *buffeli* *MPSP* sequences from Ethiopia were closely related to those from Kenya (pairwise differences 0.5 to 0.6%), Myanmar (0.9 to 1.2%) and Sri Lanka (0.9 to 1.0%) (Supplementary

Fig. 1; Table 1). Similarly, genotype *type 5 MPSP* sequences determined in this study were similar to those from Japan (1.1 to 1.8%), Myanmar (1.4 to 2.3%), Sri Lanka (1.4 to 2.1%), Thailand (1.3 to 2.3%) and Vietnam (1.7 to 2.1%) (Supplementary Fig. 2; Table 2).

All of the blood samples test-positive for *T. orientalis* originated from one locality, Addis Zemen, which is a midland region with a relatively high average annual rainfall (800-1038 mm) and a low (14-21 °C) average annual temperature, unlike Humera and Sheraro, which are dry lowland and/or semi-arid regions with a prolonged dry season of 9-10 months with low average rainfalls (350-750 mm) and high (25-42 °C) average annual temperatures. Such differences in climate could lead to considerable variation in the distribution of tick vectors. The tick vectors for *T. orientalis* transmission are largely unknown in Africa. Recently, DNA of *T. orientalis* was detected in *Amblyomma* sp. and *Rhipicephalus* sp. collected from cattle in western Oromia and south-western Oromia, along the Didessa valley, Ethiopia (Hornok et al., 2014; Kumsa et al., 2014). However, in the Asia-Pacific region, where the clinical form of oriental theileriosis is common, *Haemaphysalis* spp. are considered the principal tick vectors for the transmission of *T. orientalis* (see Ota et al., 2009; Khukhuu et al., 2011; McFadden et al., 2011; Hammer et al., 2015). Future studies should focus on understanding the role of various ticks in the transmission of *T. orientalis* in different agroclimatic zones of Ethiopia.

In conclusion, this study provides the first genetic characterization of *T. orientalis* using *MPSP* gene from the Ethiopian local cattle. In addition, a quantitative PCR also allowed an estimation of infection intensities of the prevalent genotypes of *T. orientalis* in Ethiopia. Future studies should focus on testing more blood samples covering larger geographic regions from indigenous, crossbred and exotic cattle breeds from Ethiopia to ascertain whether indigenous breeds are more resistant for *T. orientalis* than the exotic breeds. Also, further studies are needed to identify the local tick vectors capable of transmitting the *T. orientalis* genotypes in the country.

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References

- Ahmed, J., Yin, H., Schnittger, L., Jongejan, F., 2002. Ticks and tick-borne diseases in Asia with special emphasis on China. *Parasitol. Res.* 88, 51–55.
- Aktas, M., Altay, K., Dumanli, N., 2006. A molecular survey of bovine *Theileria* parasites among apparently healthy cattle and with a note on the distribution of ticks in eastern Turkey. *Vet. Parasitol.* 138, 179–185.
- Allsopp, M.T., Cavalier-Smith, T., De Waal, D.T., Allsopp, B.A., 1994. Phylogeny and evolution of the piroplasms. *Parasitology* 108, 147–152.
- Altangerel, K., Battsetseg, B., Battur, B., Sivakumar, T., Batmagnai, E., Javkhlan, G., Tuvshintulga, B., Igarashi, I., Matsumoto, K., Inokuma, H., Yokoyama, N., 2011a. The first epidemiological survey of *Theileria orientalis* infection in Mongolian cattle. *Vet. Parasitol.* 182, 343–348.
- Altangerel, K., Sivakumar, T., Inpankaew, T., Jittapalapong, S., Terkawi, M.A., Ueno, A., Xuan, X., Igarashi, I., Yokoyama, N., 2011b. Molecular prevalence of different genotypes of *Theileria orientalis* detected from cattle and water buffaloes in Thailand. *J. Parasitol.* 97, 1075–1079.
- Altay, K., Aydin, M.F., Dumanli, N., Aktas, M., 2008. Molecular detection of *Theileria* and *Babesia* infections in cattle. *Vet. Parasitol.* 158, 295–301.
- Aparna, M., Ravindran, R., Vimalkumar, M.B., Lakshmanan, B., Rameshkumar, P., Kumar, K.G., Promod, K., Ajithkumar, S., Ravishankar, C., Devada, K., Subramanian, H., George, A.J., Ghosh, S., 2011. Molecular characterization of *Theileria orientalis* causing fatal infection in crossbred adult bovines of South India. *Parasitol. Int.* 60, 524–529.

309 Bawm, S., Shimizu, K., Hirota, J., Tosa, Y., Htun, L.L., Maw, N.N., Thein, M., Kato, H.,
310 Sakurai, T., Katakura, K., 2014. Molecular prevalence and genetic diversity of bovine
311 *Theileria orientalis* in Myanmar. *Parasitol. Int.* 63, 640–645.

312 Becerra, V.M., Eggen, A.A.S., De Rooy, R.C., Uilenberg, G., 1983. *Theileria orientalis* in
313 cattle in Ethiopia. *J. Res. Vet. Sci.* 34, 362–364.

314 Chaisi, M.E., Collins, N.E., Oosthuizen, M.C., 2014. Phylogeny of *Theileria buffeli*
315 genotypes identified in the South African buffalo (*Syncerus caffer*) population. *Vet.*
316 *Parasitol.* 204, 87–95.

317 Chaisi, M.E., Sibeko, K.P., Collins, N.E., Potgieter, F.T., Oosthuizen, M.C., 2011.
318 Identification of *Theileria parva* and *Theileria* sp. (buffalo) 18S rRNA gene sequence
319 variants in the African buffalo (*Syncerus caffer*) in southern Africa. *Vet. Parasitol.* 182,
320 150–162.

321 Collins, N.E., Allsopp, M.T., Allsopp, B.A., 2002. Molecular diagnosis of theileriosis and
322 heartwater in bovines in Africa. *Trans. R. Soc. Trop. Med. Hyg.* 96, S217–S224.

323 Cufos, N., Jabbar, A., de Carvalho, L.M., Gasser, R.B., 2012. Mutation scanning-based
324 analysis of *Theileria orientalis* populations in cattle following an outbreak.
325 *Electrophoresis* 33, 2036–2040.

326 Darghouth, M., 2004. Piroplasmids of livestock in Tunisia. *Arch. Inst. Pasteur Tunis.* 81, 21–
327 25.

328 Darriba, D., Taboada, G. L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new
329 heuristics and parallel computing. *Nat. Methods* 9, 772–772.

330 Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high
331 throughput. *Nucleic Acids Res.* 32, 1792–1797.

332 Elsify, A., Sivakumar, T., Nayel, M., Salama, A., Elkhtam, A., Rizk, M., Mosaab, O., Sultan,
333 K., Elsayed, S., Igarashi, I., 2015. An epidemiological survey of bovine *Babesia* and
334 *Theileria* parasites in cattle, buffaloes, and sheep in Egypt. *Parasitol. Int.* 64, 79–85.

335 Gebrekidan, H., Hailu, A., Kassahun, A., Rohoušová, I., Maia, C., Talmi-Frank, D., Warburg,
336 A., Baneth, G., 2014. *Theileria* infection in domestic ruminants in northern Ethiopia.
337 *Vet. Parasitol.* 200, 31–38.

338 Gebrekidan, H., Gasser, R.B., Perera, P.K., McGrath, S., McGrath, S., Stevenson, M.A.,
339 Jabbar, A., 2015. Investigating the first outbreak of oriental theileriosis in cattle in South
340 Australia using multiplexed tandem PCR (MT-PCR). *Ticks Tick Borne Dis.* 6, 574–578.

341 Gubbels, M.-J., Hong, Y., van der Weide, M., Qi, B., Nijman, I.J., Guangyuan, L., Jongejan,
342 F., 2000. Molecular characterisation of the *Theileria buffeli/orientalis* group. *Int. J.*
343 *Parasitol.* 30, 943–952.

344 Hammer, J. F., Emery, D., Bogema, D. R., Jenkins, C., 2015. Detection of *Theileria*
345 *orientalis* genotypes in *Haemaphysalis longicornis* ticks from southern
346 Australia. *Parasit. Vectors* 8, 229.

347 Hornok, S., Abichu, G., Meli, M.L., Tánčzos, B., Sulyok, K.M., Gyuranecz, M., Gönczi, E.,
348 Farkas, R., Hofmann-Lehmann, R., 2014. Influence of the biotope on the tick infestation
349 of cattle and on the tick-borne pathogen repertoire of cattle ticks in Ethiopia. *PloS One* 9,
350 e106452.

351 Izzo, M.M., Poe, I., Horadagoda, N., De Vos, A.J., House, J.K., 2010. Haemolytic anaemia in
352 cattle in NSW associated with *Theileria* infections. *Aust. Vet. J.* 88, 45–51.

353 Kakuda, T., Shiki, M., Kubota, S., Sugimoto, C., Brown, W.C., Kosum, C., Nopporn, S.,
354 Onuma, M., 1998. Phylogeny of benign *Theileria* species from cattle in Thailand, China

355 and the USA based on the major piroplasm surface protein and small subunit ribosomal
 356 RNA genes. *Int. J. Parasitol.* 28, 1261–1267.

357 Kamau, J., de Vos, A.J., Playford, M., Salim, B., Kinyanjui, P., Sugimoto, C., 2011.
 358 Emergence of new types of *Theileria orientalis* in Australian cattle and possible cause of
 359 theileriosis outbreaks. *Parasit. Vectors* 4, 22.

360 Kawazu, S., Kamio, T., Kakuda, T., Terada, Y., Sugimoto, C., Fujisaki, K., 1999.
 361 Phylogenetic relationships of the benign *Theileria* species in cattle and Asian buffalo
 362 based on the major piroplasm surface protein (p33/34) gene sequences. *Int. J. Parasitol.*
 363 29, 613–618.

364 Kawazu, S., Sugimoto, C., Kamio, T., Fujisaki, K., 1992. Analysis of the genes encoding
 365 immunodominant piroplasm surface proteins of *Theileria sergenti* and *Theileria buffeli*
 366 by nucleotide sequencing and polymerase chain reaction. *Mol. Biochem. Parasitol.* 56,
 367 169–175.

368 Khukhuu, A., Lan, D.T.B., Long, P.T., Ueno, A., Li, Y., Luo, Y., Macedo, A. C., Matsumoto,
 369 K., Inokuma, H., Kawazu, S., Igarashi, I., Xuan, X., Yokoyama, N., 2011. Molecular
 370 epidemiological survey of *Theileria orientalis* in Thua Thien Hue province, Vietnam. *J.*
 371 *Vet. Med. Sci.* 73, 701–705.

372 Kim, S.J., Tsuji, M., Kubota, S., Wei, Q., Lee, J.M., Ishihara, C., Onuma, M., 1998.
 373 Sequence analysis of the major piroplasm surface protein gene of benign bovine
 374 *Theileria* parasites in East Asia. *Int. J. Parasitol.* 28, 1219–1227.

375 Kubota, S., Sugimoto, C., Onuma, M., 1996. Population dynamics of *Theileria sergenti* in
 376 persistently infected cattle and vector ticks analysed by a polymerase chain reaction.
 377 *Parasitology* 112, 437–442.

378 Kumsa, B., Signorini, M., Teshale, S., Tessarin, C., Duguma, R., Ayana, D., Martini, M.,
379 Cassini, R., 2014. Molecular detection of piroplasms in ixodid ticks infesting cattle and
380 sheep in western Oromia, Ethiopia. *Trop. Anim. Health Prod.* 46, 27–31.

381 M'ghirbi, Y., Hurtado, A., Barandika, J.F., Khlif, K., Ketata, Z., Bouattour, A., 2008. A
382 molecular survey of *Theileria* and *Babesia* parasites in cattle, with a note on the
383 distribution of ticks in Tunisia. *Parasitol. Res.* 103,435–442.

384 Maddison, W.P., Maddison, D.R., 2008. Mesquite: a modular system for evolutionary
385 analysis. Version 2.5 <http://mesquiteproject.org>.

386 Makala, L.H, Mangani, P., Fujisaki, K., Nagasawa, H., 2003. The current status of major
387 tick- borne diseases in Zambia. *Vet. Res.* 34, 27–45.

388 McFadden, A.M., Rawdon, T.G., Meyer, J., Makin, J., Clough, R.R., Tham, K., Mullner, P.,
389 Geysen, D., 2011. An outbreak of haemolytic anaemia associated with infection of
390 *Theileria orientalis* in naive cattle. *N. Z. Vet. J.* 59, 79–85.

391 Mehlhorn, H., Schein, E., 1984. The piroplasms: life cycle and sexual stages. *Adv. Parasitol.*
392 23, 37–103.

393 Ota, N., Mizuno, D., Kuboki, N., Igarashi, I., Nakamura, Y., Yamashina, H., Hanzaike, T.,
394 Fujii, K., Onoe, S., Hata, H., Kondo, S., Matsui, S., Koga, M., Matsumoto, K., Inokuma,
395 H., Yokoyama, N., 2009. Epidemiological survey of *Theileria orientalis* infection in
396 grazing cattle in the eastern part of Hokkaido, Japan. *J. Vet. Med. Sci.* 71, 937–944.

397 Perera, P.K., Gasser, R.B., Anderson, G.A., Jeffers, M., Bell, C.M., Jabbar, A., 2013.
398 Epidemiological survey following oriental theileriosis outbreaks in Victoria, Australia,
399 on selected cattle farms. *Vet. Parasitol.* 197, 509–521.

400 Perera, P.K., Gasser, R.B., Firestone, S.M., Anderson, G.A., Malmo, J., Davis, G., Beggs,
 401 D.S., Jabbar, A., 2014. Oriental theileriosis in dairy cows causes a significant milk
 402 production loss. *Parasit. Vectors* 7, 73.

403 Perera, P.K., Gasser, R.B., Firestone, S.M., Smith, L., Roeber, F., Jabbar, A., 2015a. Semi-
 404 quantitative multiplexed tandem PCR for detection and differentiation of four *Theileria*
 405 *orientalis* genotypes in cattle. *J. Clin. Microbiol.* 53, 79–87.

406 Perera, P.K., Gasser, R.B., Read, E., Malmo, J., Nguyen, H., Nyein, S., Cheng, A., Jex, A.R.,
 407 Rawlin, G., Spithill, T.W., Jabbar, A., 2015b. Use of multiplexed tandem PCR to
 408 estimate the prevalence and intensity of *Theileria orientalis* infections in cattle. *Infect.*
 409 *Genet. Evol.* 32, 68–73.

410 Perera, P.K., Gasser, R.B., Pulford, D.J., Stevenson, M.A., Firestone, S.M., McFadden, A.M.,
 411 Jabbar, A., 2015c. Comparison of the performance of three PCR assays for the detection
 412 and differentiation of *Theileria orientalis* genotypes. *Parasit. Vectors* 8, 192.

413

414 Ronquist, F., Huelsenbeck, J.P., 2003. MRBAYES 3: Bayesian phylogenetic inference under
 415 mixed models. *Bioinformatics*, 19, 1572–1574.

416 Salih, D.A., El Hussein, A.M., Seitzer, U., Ahmed, J.S., 2007. Epidemiological studies on
 417 tick-borne diseases of cattle in central equatorial state, southern Sudan. *Parasitol. Res.*
 418 101, 1035–1044.

419 Shiels, B.R., d'Oliveira, C., McKellar, S., Ben-Miled, L., Kawazu, S., Hide, G., 1995.
 420 Selection of diversity at putative glycosylation sites in the immunodominant
 421 merozoite/piroplasm surface antigen of *Theileria* parasites. *Mol. Biochem. Parasitol.* 72,
 422 149–162.

423 Sivakumar, T., Yoshinari, T., Igarashi, I., Kothalawala, H., Abeyratne, S.A.E., Vimalakumar,
424 S.C., Meewewa, A.S., Kuleswarakumar, K., Chandrasiri, A.D.N., Yokoyama, N., 2013.
425 Genetic diversity within *Theileria orientalis* parasites detected in Sri Lankan cattle.
426 Ticks Tick Borne Dis. 4, 235–241.

427 Sivakumar, T., Hayashida, K., Sugimoto, C., Yokoyama, N., 2014. Evolution and genetic
428 diversity of *Theileria*. Infect. Genet. Evol. 27, 250–263.

429 Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA 6: molecular
430 evolutionary genetics analysis version 6.0. Mol. Boil. Evol. 30, 2725–2729.

431 Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic
432 trees. Bioinformatics, 17, 754–755.

433 Tomassone, L., Grego, E., Callà, G., Rodighiero, P., Pressi, G., Gebre, S., Zeleke, B.,
434 Meneghi, D., 2012. Ticks and tick-borne pathogens in livestock from nomadic herds in
435 the Somali Region, Ethiopia. Exp. Appl. Acarol. 56, 391–401.

436 Uilenberg, G., 1995. International collaborative research: significance of tick-borne
437 hemoparasitic diseases on world animal health. Vet. Parasitol. 57, 19–41.

438 Uilenberg, G., Mpangala, C., McGregor, W., Callow, L.L., 1977. Biological differences
439 between African *Theileria mutans* (Theiler 1906) and two benign species of *Theileria* of
440 cattle in Australia and Britain. Aust. Vet. J. 53, 271–273.

441 Uilenberg, G., Perie, N.M., Spanjer, A.A., Franssen, F.F., 1985. *Theileria orientalis*, a
442 cosmopolitan blood parasite of cattle: demonstration of the schizont stage. Res. Vet.
443 Sci. 38, 352–360.

444 Werle, E., Schneider, C., Renner, M., Völker, M., Fiehn, W., 1994. Convenient single-step,
 445 one tube purification of PCR products for direct sequencing. *Nucleic Acids Res.* 22,
 446 4354.

447 Yusufmia, S.B., Collinsa, N.E., Nkunaa, R., Trotsky, M., Van den Bosschea, P., Penzhorna,
 448 B. L., 2010. Occurrence of *Theileria parva* and other haemoprotozoa in cattle at the edge
 449 of Hluhluwe-iMfolozi Park, KwaZulu-Natal, south Africa. *J. S. Afr. Vet. Assoc.* 81, 45–
 450 49.

451 Zakimi, S., Kim, J.Y., Oshiro, M., Hayashida, K., Fujisaki, K., Sugimoto, C., 2006. Genetic
 452 diversity of benign *Theileria* parasites of cattle in the Okinawa prefecture. *J. Vet. Med.*
 453 *Sci.* 68, 1335–1338.

454 **Table 1**455 Apparent prevalence and infection intensity of each *Theileria orientalis* genotype detected in cattle from Ethiopia.

Study site	No. of sample	PCR method	Genotype(s)				
		Conventional PCR No. of test-positive (%)	<i>buffeli</i> % (proportion)	<i>chitose</i> % (proportion)	<i>ikedai</i> % (proportion)	<i>type 5</i> % (proportion)	% (proportion)
Addis Zemen	59	5 (8.5)	5.1 (3/59)	-	-	3.4 (2/59)	
Humera	20	-	-	-	-	-	
Sheraro	153	-	-	-	-	-	
Total	232	5 (2.2)	1.3 (3/232)	-	-	0.9 (2/232)	
		MT-PCR No. of test-positive (%)	<i>buffeli</i>	<i>buffeli+chitose</i>	<i>buffeli+ikedai</i>	<i>buffeli+type 5</i>	<i>buffeli+ikedai+type 5</i>
Addis Zemen	59	9 (15.3)	3.4 (2/59)	1.7 (1/59)	6.8 (4/59)	1.7 (1/59)	1.7 (1/59)
Humera	20	-	-	-	-	-	-
Sheraro	153	-	-	-	-	-	-
Total	232	9 (3.9)	0.9 (2/232)	0.4 (1/232)	1.7 (4/232)	0.4 (1/232)	0.4 (1/232)

Fig. 1. Genetic relationships of *Theileria orientalis* genotypes from Ethiopia (bold) with reference genotypes selected from previous studies. The relationships were inferred based on the phylogenetic analysis of major piroplasm surface protein sequences using Neighbor Joining (NJ) and Bayesian inference (BI) methods, and *Theileria annulata* was used as the outgroup. Nodal support values are indicated: posterior probability for BI (first/above), and bootstrap for NJ (second/below). The scale bar indicates distance.