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Short Communication

Genetic variation in three mitochondrial genes among cattle tick *Rhipicephalus microplus* originating from four provinces of China

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Abstract. The cattle tick, *Rhipicephalus microplus* (formerly *Boophilus microplus*), is the most important blood-feeding ectoparasite of cattle in tropical and subtropical regions of the world. In this study, we examined sequence variability in three mitochondrial (mt) DNA (*cox*1, *nad*1, *nad*4) among cattle tick *R. microplus* originating from four provinces of China. A portion of *cox*1 (p*cox*1), *nad*1 (p*nad*1) and *nad*4 (p*nad*4) genes were amplified by polymerase chain reaction (PCR) separately from adult *R. microplus* individuals and the amplicons were subjected to sequence from both directions. The sequence of mt *cox*1, *nad*1, *nad*4 genes was 817 bp, 350 bp, and 794 bp in size, respectively. The intraspecific sequence variations within *R. microplus* were 0-8.6% for *cox*1, 0-4.9% for *nad*1 and 0-10.3% for *nad*4. However, the interspecific sequence differences among the members of the *Rhipicephalus* [*R. sanguineus* (JX416325) and *R. turanicus* (NC035946)] were significantly higher, being 16.9–20.5%, 18–22.8%, 22.8–25.3% for p*cox*1, p*nad*1 and p*nad*4, respectively. In addition, genetic differences were 7.9–8.6% for *cox*1, 4.3–4.9% for *nad*1 and 10–10.3% for *nad*4 between the two detected lineages (*R. microplus* clade A and clade B). Phylogenetic analyses indicated that all the *Rhipicephalus* isolates from the present study represents *R. microplus*, supporting that *R. microplus* represents species complex. Our result provided an additional genetic evidence for the existence of species complex within *R. microplus* in China.

INTRODUCTION

Ticks are the most important ectoparasites of cattle and other animals, causing major economic losses to the livestock industry (de la Fuente *et al.*, 2008). Cattle tick *Rhipicephalus microplus* (formerly *Boophilus microplus*) is the most common tick of livestock, especially cattle. This tick not only causes dermatitis and blood loss by bite, but also is considered as a vector of many pathogenic microorganisms (Lu *et al.*, 2013; Giles *et al.*, 2014; Bhat *et al.*, 2017). Recently, it was estimated that annual loss associated with *R. microplus* was more than $2.5 billion around the word (Lew-Tabor *et al.*, 2014).

Accurate identification and differentiation of *R. microplus* and other closely-related *Rhipicephalus* species are very different based on their morphological features, hosts or geographical origins (Kamani *et al.*, 2017; Baron *et al.*, 2018). However, these criteria are sometimes insufficient for accurate identification and differentiation of many hard species, especially species complex (Coimbra-Dores *et al.*, 2018). Employing molecular tools, the internal transcribed spacers (ITS) of the nuclear ribosomal DNA (rDNA) region and mt *cox*1, 12S genes, 16S

genes have provided an additional tool for identification and differentiation of *R. microplus* (Brahma *et al*., 2014; Labruna *et al*., 2009). Previous studies have indicated that *R. microplus* were divided into five taxa: *R. annulatus*, *R. australis* and *R. microplus* clades A, B, C based on the molecular datasets (Low *et al*., 2015). In addition, Burger *et al.* have also reported that *R. microplus* from Southern China belongs to species complex (Clade A and B) (Burger *et al*., 2014). Very recently, Li *et al.* have also indicated that *R. mircoplus* tick samples from Southern China belong to *R. microplus* Clade A (Li *et al*., 2018a). Beyond that, phylogenetic analysis using the *cox*1 gene sequences revealed that *R. microplus* ticks from a county on the China-Myanmar border belong to clade C (Li *et al*., 2018b). However, *R. microplus* has never been reported in other provinces of China.

The objectives of the present study were to examine genetic variation in three mtDNA genes, namely cytochrome *c* oxidase subunits 1 (*cox*1) and NADH dehydrogenase subunits 1 and 4 (*nad*1 and *nad*4), among *R. microplus* isolates from cattle in China. Based on the *cox*1 sequences, phylogenetic relationships of *R. microplus* with other five *Rhipicephalus* species were also reconstructed. Our results would provide baseline information for further control of the cattle tick and tick-borne diseases in China.

MATERIALS AND METHODS

Parasites collection and DNA extraction

All adult cattle ticks of *R. microplus* (n=35) were collected from naturally infested cattle in four provinces (Henan, Hunan, Guizhou and Hainan) in China. All ticks were preliminary identified species according to morphological structure (Kang *et al*., 1985). These ticks were fixed in 70% (V/V) ethanol and stored at -20°C until used. Total genomic DNA was isolated from individual samples using sodium dodecyl sulphate/proteinase K treatment, followed by spin column purification (Wizard® SV Genomic DNA Purification System, Promega, Madison, Wisconsin, USA). The molecular identity of each specimen was then verified by PCR-based sequencing of regions in the internal transcribed spacers of nuclear ribosomal DNA (ITS rDNA) using an established method (Chitimia *et al*., 2009). Both regions ITS-1 and ITS-2 had 99% identity to previously published sequences for *R. microplus* from South Africa and China (GenBank accession nos. KY457506 and KC503274, respectively).

PCR amplification and sequencing

A portion of the mt *cox*1 gene (p*cox*1) was amplified using previous primers *cox*1F (5'-GGAACAATATATTTAATTTTTGG-3'), and *cox*1R (5'-ATCTATCCCTACTGTAAATATATG-3') (Chitimia *et al*., 2010). The primer sets for amplifying mt *nad*1 and *nad*4 were designed based on well-conserved mt sequences of *R. microplus* (KP143546), namely *nad*1F (5'-TGAGCGAATCCTCGATATGA-3'), *nad*1R (5'-CCGATGAGAATCAGGTTGG-3'), *nad*4F (5'-ATTGTTATAGGGGCTGATATATT-3'), and *nad*4R (AATATTAATAGCAAGCCGATTA-3'). PCR reactions (25 µL) were performed in 3.0 µL of $MgCl_2$ (25 mM), 0.25 µL of each primer (50 pmol/µL), 2.5 µL 10×rTaq buffer (100 mM Tris-HCl and 500 mM KCl), 2 µL of dNTP Mixture (2.5 mM each), 0.25 µL of rTaq (5 U/µL) DNA polymerase (TaKaRa Biotechnology, Dalian, China) and 2 µL of DNA sample in a thermocycler (Biometra, Göttingen, German). The cycling conditions were: 95°C for 5 min (initial denaturation), followed by 35 cycles of 95°C for 30s (denaturation), 54°C (for p*cox*1), 53°C (for p*nad*1) or 50°C (for p*nad*4) for 1 min (annealing), 72°C for 1 min (extension) and then 72°C for 5 min (nal extension). Negative control (without DNA template) was included in each amplification run. Each amplicon (5 µL) was examined by 1% (w/v) agarose gel electrophoresis to validate amplification efficiency. PCR products were sent to BGI-shenzhen (Shenzhen, China) for sequencing from both directions.

Sequences analysis and reconstruction of phylogenetic relationships

Sequences of the three mt genes were separately aligned using the software Clustal X 1.83 (Thompson *et a*l., 1997). The

haplotypes, nucleotide diversity (Pi) and haplotype diversity (Hd) of each gene were determined using the DnaSP 5.0 program (Librado & Rozas, 2009).

The p*cox*1 sequences of all tick samples in this study were used for phylogenetic analyses. Maximum likelihood (ML) was used for phylogenetic re-constructions. ML analyses were performed using PhyML 3.0 (Guindon *et al.*, 2010), and the GTR+I model with its parameter for the concatenated dataset was determined for the ML analysis using JModeltest (Posada 2008) based on the Akaike information criterion (AIC). Bootstrap support (BS) for ML trees was calculated using 100 bootstrap replicates. To study the phylogenetic relationships, 35 *R. microplus* samples from four provinces in China and other *Rhipicephalus* species were included in this study, using *Amblyomma americanum* (DQ168131) as the outgroup. Phylograms were drawn using the Tree View program version 1.65 (Page 1996).

RESULTS AND DISCUSSION

Amplicons of p*cox*1, p*nad*1 and p*nad*4 (about 850bp, 570bp, 860bp, respectively) were amplified individually and subjected to agarose gel electrophoresis. For each mtDNA region, no product was amplified from the no DNA samples or host DNA control (not shown). The sequences of p*cox*1, p*nad*1 and p*nad*4 were 817 bp, 350 bp, 794 bp in size, respectively. These sequences have been deposited in the GenBank under the accession numbers: for *cox*1 (MH788922-MH788956), for *nad*1 (MH794289-MH794323), for *nad*4 (MH794324-MH794358). The A+T contents of the sequences were 68.3–68.8% (p*cox*1), 81.4–83.7% (p*nad*1) and 78.2–79.1% (p*nad*4), respectively. The intra-specific sequence variations among different populations of *R. microplus* isolates were 0–8.6% for *cox*1, 0–4.9% for *nad*1 and 0–10.3% for *nad*4. However, the interspecific sequence differences among members of the *Rhipicephalus* were significantly higher, being 16.9–20.5%, 18–22.8%, 22.8–25.3% for p*cox*1, p*nad*1 and p*nad*4, respectively. In addition, genetic differences were 7.9–8.6% for *cox*1, 4.3–4.9% for *nad*1 and 10–10.3% for *nad*4 between the two detected lineages (*R. microplus* clade A and clade B). These studies have clearly indicated that *R. microplus* represented a species complex. These results were consistent with the previous studies (Roy *et al.*, 2018; Burger *et al.*, 2014).

Many studies have indicated that mt sequences are unique genetic markers to indicate geographical movements and population genetic structure of parasites (Lv *et al.*, 2014; Li *et al.*, 2017). In the present study, 72 polymorphic sites, 9 haplotypes, *Hd*=0.775 and *Pi*=0.01331 were determined in all sequences of p*cox*1. 17 polymorphic sites, 5 haplotypes, *Hd*=0.267 and *Pi*=0.00508 were determined in all sequences of p*nad*1. 81 polymorphic sites, 12 haplotypes, *Hd*=0.837 and *Pi*=0.01264 were determined in all sequences of p*nad*4 (Table 1).

mtDNA sequences are useful molecular markers for phylogenetic studies of many ectoparasites, including ticks (Song *et al.*, 2011; Latrofa *et al.*, 2013; Chitimia *et al.*, 2010). In the present study, all the *R. microplus* isolates grouped together, indicating that all studied isolates represent *R. microplus* Clade A and B (Fig. 1). Our result was consistant with previous studies

Table 1. Number and diversity of nucleotide variations in the sequences of cytochrome c oxidase subunits 1 gene (p*cox*1) and NADH dehydrogenase subunits 1 and 4 genes (p*nad*1 and p*nad*4) within 35 *R. microplus* samples

MtDNA region	Polymorphic sites	Haplotypes	Haplotype diversity (Hd)	Nucleotide diversity (Pi)
*cox*1	72	9	0.775	0.01331
*nad*1	17	5	0.267	0.00508
*nad*4	81	12	0.837	0.01264

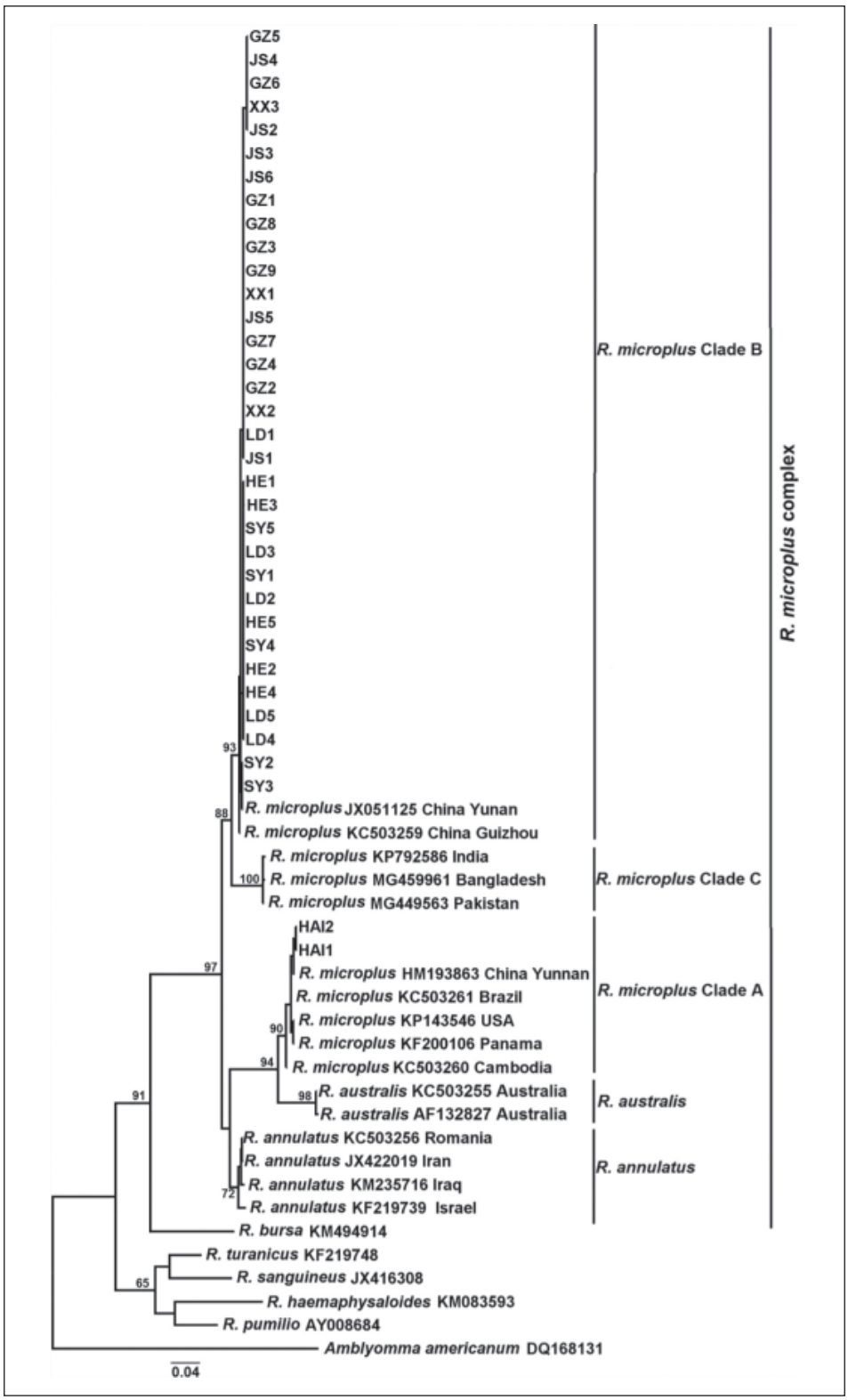

Figure 1. Phylogenetic relationship among *Rhipicephalus microplus* isolates in China with other *Rhipicephalus* species inferred by maximum likelihood analyses using *cox*1, with *Amblyomma americanum* (DQ168131) as out-group.

based on mt *cox*1 and 16S genes (Low *et al*., 2015). In this study, the *R. microplus* formed a monophyletic group with high statistical support (BS=97), and all the *R. microplus* isolates were segregated into five major clades (Fig. 1). Isolates from Hainan province clustered together in one clade (Clade A) with high statistical support (BS=90) (Fig. 1). However, isolates from the other three provinces clustered together in another clade (Clade B) without reflecting geographical origin, with weak statistical support (BS=93) (Fig. 1). Our results indicated that *R. microplus* consists of at least two closely related species in China.

Taken together, the findings supported the proposal that *R. microplus* consisted of at least two lineages in China. Sequence variations among *R. microplus* isolates from four different geographical localities in China were revealed by sequence analyses of mt *cox*1, *nad*1 and *nad*4 genes. These datasets of *R. microplus* provided an addition genetic marker for epidemiology, population genetics and biology of *R. microplus* in animals in China.

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REFERENCES

de la Fuente, J., Estrada-Pena, A., Venzal, J.M., Kocan, K.M. & Sonenshine, D.E. (2008). Overview: ticks as vectors of pathogens that cause disease in humans and animals. *Front Biosci-Landmrk* **13**: 6938-6946.

Lu, X., Lin, X.D., Wang, J.B., Qin, X.C., Tian, J.H., Guo, W.P., Fan, F.N., Shao, R., Xu, J. & Zhang, Y.Z. (2013). Molecular survey of hard ticks in endemic areas of tick-borne diseases in China. *Ticks and Tick Borne Diseases* **4**: 288-296.

Giles, J.R., Peterson, A.T., Busch, J.D., Olafson, P.U., Scoles, G.A., Davey, R.B., Pound, J.M., Kammlah, D.M., Lohmeyer, K.H. & Wagner, D.M. (2014). Invasive potential of cattle fever ticks in the southern United States. *Parasties & Vectors* **7**: 189.

Bhat, S.A., Singh, N.K., Singh, H. & Rath, S.S. (2017). Molecular prevalence of *Babesia bigemina* in *Rhipicephalus microplus* ticks infesting cross-bred cattle of Punjab, India. *Parasite Epidemiology and Control* **2**: 85-90.

Lew-Tabor, A.E., Bruyeres, A.G., Zhang, B. & Valle, M.R. (2014). *Rhipicephalus* (*Boophilus*) *microplus* tick *in vitro* feeding methods for functional (dsRNA) and vaccine candidate (antibody) screening. *Ticks and Tick Borne Diseases* **5**: 500-510.

Kamani, J., Apanaskevich, D.A., Gutiérrez, R., Nachum-Biala, Y., Baneth, G. & Harrus, S. (2007). Morphological and molecular identification of *Rhipicephalus* (*Boophilus*) *microplus* in Nigeria, West Africa: a threat to livestock health. *Experimental and Applied Acarology* **73**: 283-296.

Baron, S., van der Merwe, N.A. & Maritz-Olivier, C. (2018). The genetic relationship between *R. microplus* and *R. decoloratus* ticks in South Africa and their population structure. *Molecular Phylogenetics and Evolution* **129**: 60-69.

Coimbra-Dores, M.J., Maia-Silva, M., Marques, W., Oliveira, A.C., Rosa, F. & Dias, D. (2018). Phylogenetic insights on Mediterranean and Afrotropical *Rhipicephalus* species (Acari: Ixodida) based on mitochondrial DNA. *Experimental and Applied Acarology* **75**: 107-128.

Brahma, R.K., Dixit, V., Sangwan, A.K. & Doley, R. (2014). Identification and characterization of *Rhipicephalus* (*Boophilus*) *microplus* and *Haemaphysalis bispinosa* ticks (Acari: Ixodidae) of northeast India by ITS2 and 16S rDNA sequences and morphological analysis. *Experimental and Applied Acarology* **62**: 253-265.

Labruna, M.B., Naranjo, V., Mangold, A.J., Thompson, C., Estrada-Peña, A., Guglielmone, A.A., Jongejian, F. & de la Fuente, J. (2009). Allopatric speciation in ticks: genetic and reproductive divergence between geographic strains of *Rhipicephalus* (*Boophilus*) *microplus*. *BMC Evolutionary Biology* **25**: 9-46.

Low, V.L., Tay, S.T., Kho, K.L., Koh, F.X., Tan, T.K., Lim, Y.A., Ong, B.L., Panchadcharam, C., Norma-Rashid, Y. & Sofian-Azirun, M. (2015). Molecular characterisation of the tick *Rhipicephalus microplus* in Malaysia: new insights into the cryptic diversity and distinct genetic assemblages throughout the world. *Parasties & Vectors* **8**: 341.

Burger, T.D., Shao, R. & Barker, S.C. (2014). Phylogenetic analysis of mitochondrial genome sequences indicates that the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, contains a cryptic species. *Molecular Phylogenetics and Evolution* **76**: 241-253.

Li, J., Chen, Z.H., Jiang, L., Wu, C.Y., Liao, S.Q., Lin, X.H., Xiang, R., Lv, M.N., Qi, N.S., Zhang, J.F., Chen, Q.L. & Sun, M.F. (2018a). Characterization of cattle-origin ticks from Southern China. *Acta Tropica* **187**: 92-98.

Li, L.H., Zhang, Y., Wang, J.Z., Li, X.S., Yin, S.Q., Zhu, D., Xue, J.B. & Li, S.G. (2018b). High genetic diversity in hard ticks from a China-Myanmar border county. *Parasties & Vectors* **11**: 469.

Kang, Y.B. & Jang, D.H. (1985). Scanning electron microscopic observations on the surface structure of the tick *Boophilus microplus* (Canestrini, 1887) female specimens. *Kisaengchunghak Chapchi* **23**: 313-323.

Chitimia, L., Lin, R.Q., Cosoroaba, I., Braila, P., Song, H.Q. & Zhu, X.Q. (2009). Molecular characterization of hard ticks from Romania by sequences of the internal transcribed spacers of ribosomal DNA. *Parasitology Research* **105**: 1479-1482.

Chitimia, L., Lin, R.Q., Cosoroaba, I., Wu, X.Y., Song, H.Q., Yuan, Z.G. & Zhu, X.Q. (2010). Genetic characterization of ticks from southwestern Romania by sequences of mitochondrial *cox*1 and *nad*5 genes. *Experimental and Applied Acarology* **52**: 305-311.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997). The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876-4882.

Librado, P. & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451-1452.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol Reprod Med* **59**: 307-321.

Posada, D. (2008). jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* **25**: 1253-1256.

Page, R.D. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**: 357-358.

Roy, B.C., Estrada-Peña, A., Krücken, J., Rehman, A. & Nijhof, A.M. (2018). Morphological and phylogenetic analyses of *Rhipicephalus microplus* ticks from Bangladesh, Pakistan and Myanmar. *Ticks and Tick Borne Diseases* **9**: 1069-1079.

Lv, J., Wu, S., Zhang, Y., Zhang, T., Feng, C., Jia, G. & Lin, X. (2014). Development of a DNA barcoding system for the Ixodida (Acari: Ixodida). *Mitochondrial DNA* **25**: 142-149.

Li, Z.B., Cheng, T.Y., Xu, X.L., Song, L.L. & Liu, G.H. (2017). Genetic variation in mitochondrial genes of the tick *Haemaphysalis flava* collected from wild hedgehogs in China. *Experimental and Applied Acarology* **71**: 131-137.

Song, S., Shao, R., Atwell, R., Barker, S. & Vankan, D. (2011). Phylogenetic and phylogeographic relationships in *Ixodes holocyclus* and *Ixodes cornuatus* (Acari: Ixodidae) inferred from *cox*1 and ITS2 sequences. *International Journal of Experimental Pathology* **141**: 871-880.

Latrofa, M.S., Dantas-Torres, F., Annoscia, G., Cantacessi, C. & Otranto, D. (2013). Comparative analyses of mitochondrial and nuclear genetic markers for the molecular identification of *Rhipicephalus* spp. *Infection Genetics and Evolution* **20**: 422-427.