

Further evidence for geographic differentiation in *R. appendiculatus* (Acari: Ixodidae) from Eastern and Southern provinces of Zambia

Jupiter Mtambo · Maxime Madder · Wim Van Bortel · George Chaka · Dirk Berkvens · Thierry Backeljau

Received: 16 August 2006 / Accepted: 10 January 2007 / Published online: 6 March 2007
© Springer Science+Business Media B.V. 2007

Abstract Studies in the biology, ecology and behaviour of *R. appendiculatus* in Zambia have shown considerable variation within and between populations often associated with their geographical origin. We studied variation in the mitochondrial COI (mtCOI) gene of adult *R. appendiculatus* ticks originating from the Eastern and Southern provinces of Zambia. *Rhipicephalus appendiculatus* ticks from the two provinces were placed into two groups on the mtCOI sequence data tree. One group comprised all haplotypes of specimens from the Eastern province plateau districts of Chipata and Petauke. The second group consisted of a single haplotype of specimens from the Southern province districts and Nyimba, an Eastern province district on the

Nucleotide sequence data reported in this paper are available in the GenBank™ under the Accession Numbers DQ859259–DQ859266

J. Mtambo

Department of Veterinary and Livestock Development, P.O. Box 670050, Mazabuka, Zambia

J. Mtambo (✉) · M. Madder · D. Berkvens

Department of Animal Health, Institute of Tropical Medicine, Nationalestraat 155,
2000 Antwerp, Belgium
e-mail: jmtambo@itg.be

W. Van Bortel

Department of Parasitology – Entomology Section, Institute of Tropical Medicine,
Nationalestraat 155, 2000 Antwerp, Belgium

G. Chaka

Department of Veterinary and Livestock Development, P.O. Box 510155, Chipata, Zambia

T. Backeljau

Department of Invertebrates – Malacology Section, Royal Belgian Institute of Natural Sciences,
Vautierstraat 29, 1000 Brussels, Belgium

T. Backeljau

Department of Biology, Evolutionary Biology Group, University of Antwerp,
Groenenborgerlaan 171, 2020 Antwerp, Belgium

fringes of the valley. This variation provides additional evidence to the earlier observations in the 12S rDNA and ITS2 data for the geographic subdivision of *R. appendiculatus* from Southern province and Eastern province plateau. The geographic subdivision further corresponds with differences in body size and diapause between *R. appendiculatus* from these geographic areas. The possible implications of these findings on the epidemiology of East Coast fever (ECF) the disease for which *R. appendiculatus* is one of the vectors are discussed.

Keywords *Rhipicephalus appendiculatus* · COI · Geographic differentiation · Zambia

Introduction

The Ixodid tick *Rhipicephalus appendiculatus* Neumann, 1901, one of the main field vectors of East Coast fever (ECF), a cattle disease, is widely distributed in eastern, southern and central African countries (Norval et al. 1992) including Zambia (Pegram et al. 1986; Speybroeck et al. 2002). Studies in the biology, ecology and behaviour of *R. appendiculatus* in Zambia have shown considerable geographic variation within and between populations (Berkvens et al. 1995; Chaka et al. 1999; Madder et al. 2002; Speybroeck et al. 2002, 2004). In a recent study, 12S rDNA and ITS2 sequence data of *R. appendiculatus* and *R. zambeziensis* from Eastern and Southern provinces of Zambia, showed that *R. appendiculatus* from the Eastern province constitutes a bootstrap-supported monophyletic group within an unresolved assemblage of *R. appendiculatus* specimens from the Southern province (JM in prep). Such intraspecific geographic genetic variation may have important taxonomical and epidemiological consequences (McLain et al. 1995; Avise 2000). The current work therefore aims at (1) providing further evidence for the geographic genetic differentiation between *R. appendiculatus* from the Eastern and Southern provinces of Zambia; and (2) correlating the observed molecular differentiation with literature data on ecological differences between *R. appendiculatus* from the two provinces (Table 1). To this end we sequenced a 600 bp fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene and considered possible epidemiological implications of this geographic patterning in *R. appendiculatus*.

Table 1. Published differences between Eastern province and Southern province *R. appendiculatus* stocks

Feature	East <i>R. appendiculatus</i> stock	South <i>R. appendiculatus</i> stock	References
Diapause induction	Photoperiod i.e. shortday	Obligatory	Madder et al. (2002)
Diapause intensity	Less intense	Greater intensity	Madder et al. (2002)
Body size	Mixed i.e. large and small	Large	Chaka et al. (1999); Speybroeck et al. (2004)
Phenology	Bimodal	Unimodal	Berkvens et al. (1998); Chaka et al. (1999)
<i>Theileria parva</i> (katete)	Lower infection rates	Higher infection rate	Tempia (1997)

Materials and methods

Sampling

Ticks were sampled from the Eastern (10°18–15°06'S; 29°56–33°42'E) and Southern (15°14–17°42'S; 25°01–28°40'E) provinces of Zambia (Fig. 1) between 1997 and 2003. Ecologically, Zambia is divided into three agro-ecological zones (Fig. 1). Both the Eastern and Southern provinces fall into agro-ecological zones 1 and 2a. Zone 1 is characterised by the hottest (40°C) and driest (600 mm) annual conditions. Rainfall is generally less than 800 mm per annum. Zone 2a receives 800–1200 mm of rain annually and is therefore a medium rainfall zone. The climate and vegetation of the Eastern province have been described by Berkvens et al. (1998). Briefly, the rainy season starts in November and ends in April. The hot dry season is from August to the beginning of the rainy season. There is an intervening cold dry season between May and July. The vegetation is broadly divided into four types viz., (1) Munga woodland: fairly open woodland found richer soils and with a richer grass cover; (2) Miombo woodland: fairly closed woodland on poorer soils with sparse grass cover. These vegetation types (1) and (2) are mainly features of the plateau. (3) Mopane woodland: found in low-lying valleys; (4) Grassland also known as Dambos: these are open grasslands with very few or no trees; their drainage is poor and therefore they tend to get water logged in the rainy season. Grasslands serve as communal grazing areas. The ticks from Eastern province were collected in January of 2003 from the plateau districts of Chipata (Kamulaza, Wafa) and Petauke (Kalindawalo, Nyamphande) and Nyimba (Beni, Chipembi) a district on the fringes of the valley (Fig. 2). These specimens were killed and preserved at –80°C. The climate and vegetation of the Southern province were described in Mulumba et al. (2000) and Speybroeck et al. (2002). The climate is characterized by a warm rainy season (November to April) which is followed by a cool dry season (May–August) and a hot dry season (September–October). The vegetation on the plateau is mainly Munga and Miombo woodlands. Mopane and scrub woodlands are found in the valley areas and areas of the Kalahari sands. Ticks from the Southern province were collected from the valley districts of Sinazongwe (Sinazeze), Gwembe (Syabwengo), Livingstone (Simonga) and the plateau districts of Mazabuka (Nkokola, Nega Nega) and Monze (Nteme) (Fig. 3). All the specimens were killed in 70% ethanol upon collection (1997) and stored in the same at ambient temperature.

Identification of ticks and DNA extraction

DNA extraction was based on the method of Boom et al. (1990, 1999) as described in Mtambo et al. (2006). All the 60 specimens of *R. appendiculatus* used in this study were identified by the use of a PCR-RFLP based on the digestion of the ITS2 with *Bau* I (JM unpublished). This allows distinguishing between *R. appendiculatus* and the closely related *R. zambeziensis*.

PCR amplification, DNA sequencing and alignment

Amplification and sequencing of the COI were set up as described in Mtambo et al. (2006). A total of 60 amplicons were sequenced. Sequences were assembled with ClustalX 1.83 (Thompson et al. 1997), visually inspected and manually edited in GeneDoc version 2.6.001 (Nicholas and Nicholas 1997). Multiple alignments were

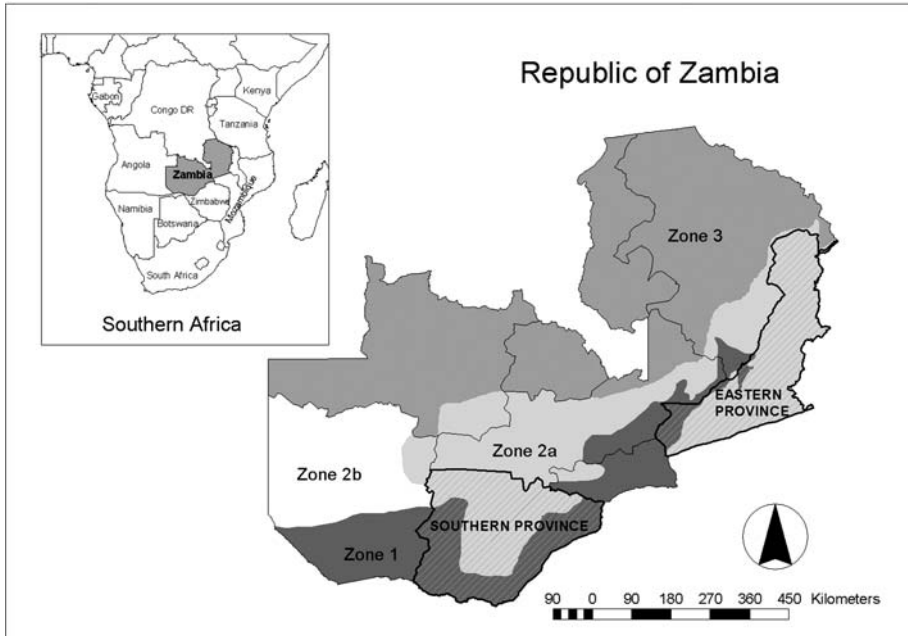


Fig. 1 Location map of Zambia with agro-ecological zones

executed in ClustalX 1.83 with its default settings and the alignments were visually inspected in GeneDoc.

Phylogenetic analysis

Using the program Modeltest (Posada and Crandall 1998) the best fit model of evolution for the COI sequences was determined as the GTR + I (General time reversible with proportion of Invariable sites). The parameters of the model were base frequencies; $\text{freqA} = 0.2755$, $\text{freqC} = 0.1832$, $\text{freqG} = 0.1376$ and $\text{freqT} = 0.4037$; $R_{\text{mat}} = 1.785$, 3.474, 3.765, 0.174, 10,830 and 1.000; Rates = equal and proportion of invariable sites (I) = 0.6340. Sequences of *R. evertsi evertsi* and *R. turanicus* were included in the alignment as outgroups. (The alignment file is available by anonymous FTP from <ftp.ebi.ac.uk> in directory pub/databases/embl/align under the accession numbers ALIGN_001061). *Rhipicephalus evertsi evertsi* and *R. turanicus* were collected from Kazungula and Simonga, respectively in the Livingstone district. These species were morphologically identified with the identification keys of Walker et al. (2000). Their sequences were subjected to a BLAST to confirm their morphological identification. A distance tree was generated with Neighbour Joining (NJ) in PAUP* version 4.0b10 (Swofford 2003) with the F84 as the distance measure using the parameters of the GTR + I model. The model was next used in a Maximum Likelihood (ML) analysis. The stabilities of the NJ and ML trees were evaluated via bootstrap analysis with 1000 iterations. Finally the same parameters were used in a Bayesian phylogenetic inference in MrBayes (Ronquist and Huelsenbeck 2003). The starting tree for each of the two simultaneous runs was random. We allowed for one cold chain and three heated chains (default settings). Stationarity was achieved after 600,000 cycles.

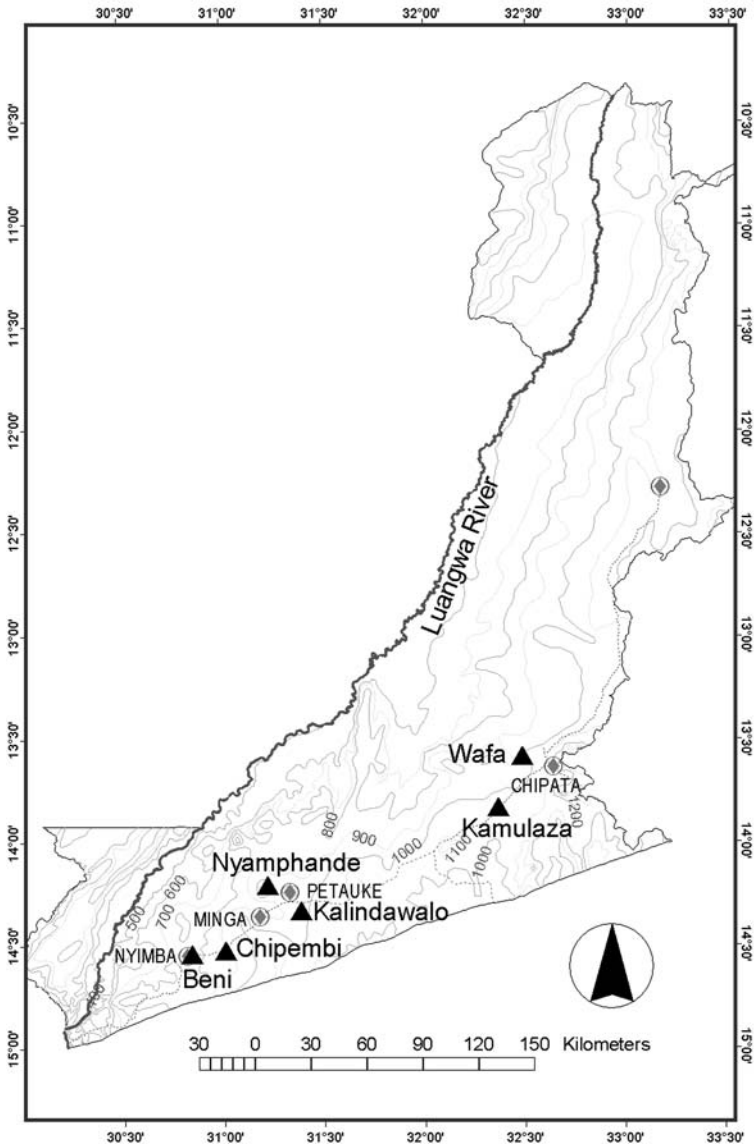


Fig. 2 Map of the Eastern province showing areas of tick collection

Results

PCR amplification and sequence alignment statistics

The COI gene fragment comprised 478 aligned bp with 14 polymorphic sites, 12 of which were parsimony informative and 2 were singletons. There was only a single haplotype in the Southern province, while there were five haplotypes in the Eastern

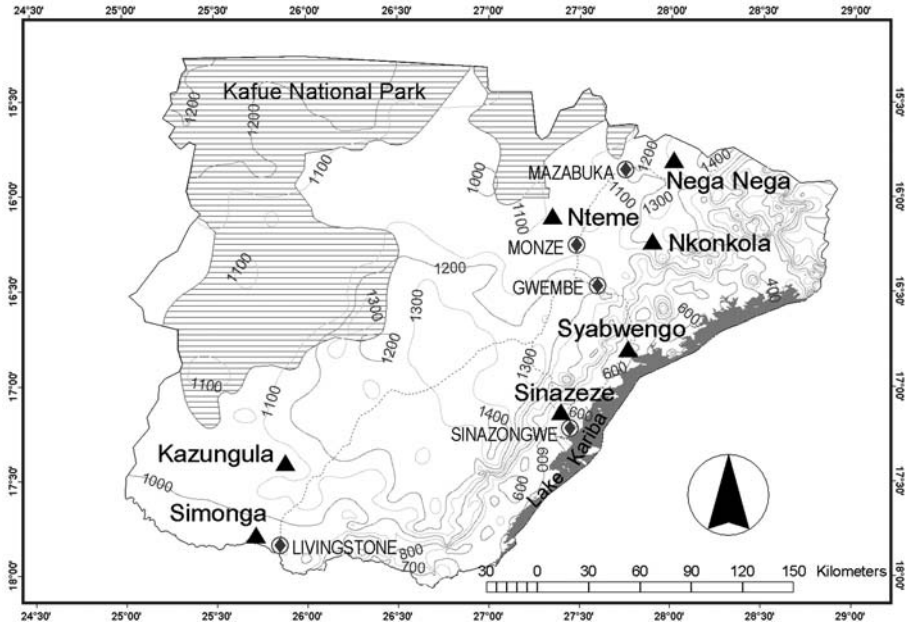


Fig. 3 Map of the Southern province showing areas of tick collection

Table 2 Polymorphism in the mitochondrial COI gene fragment in *R. appendiculatus* from Eastern and Southern provinces of Zambia

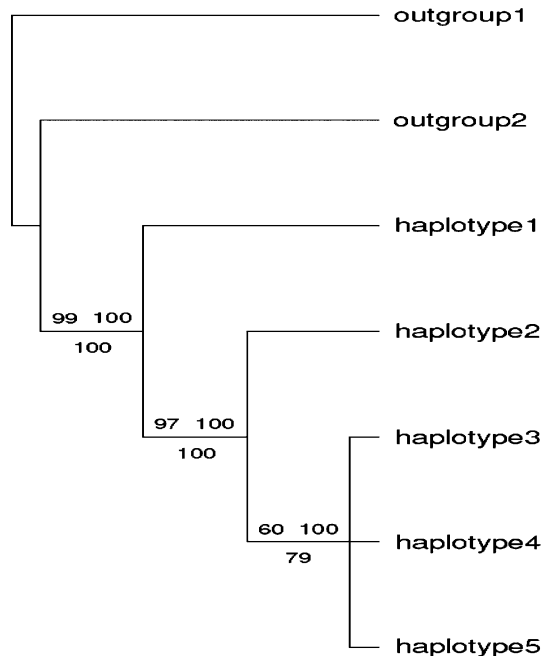
Haplotype	No. of specimens	Variable base positions														Source	
		12	94	147	159	212	246	303	315	345	372	394	432	453	465	Southern	Eastern
Haplotype 1	40	T	C	T	C	A	C	A	T	G	T	G	G	C	C	All districts	Nyimba
Haplotype 2	8	C	T	C	T	-	T	G	C	A	G	-	-	T	T		Chipata, Petauke
Haplotype 3	1	-	-	-	-	-	-	-	-	-	-	-	A	A	-	-	Chipata
Haplotype 4	1	-	-	-	-	C	-	-	-	-	-	G	-	-	-	-	Chipata
Haplotype 5	10	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	Chipata, Petauke

province (Table 2). Four of the five Eastern province haplotypes (i.e. haplotypes 2–5) only occurred in the plateau localities of Chipata and Petauke. The fifth (haplotype 1) was identical to the single Southern province haplotype and was only found in Nyimba, a district on the fringes of the valley.

Phylogenetic analysis

The ML, NJ and Bayesian trees consistently revealed that the four haplotypes of the plateau populations from the Eastern province represent a strongly supported monophyletic group (Bootstrap support 97(ML) and 100 (NJ) and clade credibility support 100 (Bayesian inference) to the exclusion of the single haplotype of the Southern province and Nyimba district (Fig. 4). Three of the four Eastern Province

Fig. 4 COI Maximum Likelihood (ML) cladogram for *R. appendiculatus* from Eastern and Southern provinces. Bootstrap and Clade credibility Values: Upper left = ML; Upper right = Bayesian; Bottom = NJ. Taxon labels: outgroup1 = *R. evertsi evertsi*; outgroup2 = *R. turanicus*; Haplotype location: Haplotype 1 = Southern province and Nyimba district; Haplotypes 2–5 = Eastern province plateau districts



plateau haplotypes (3–5) formed an unresolved subclade which received partial bootstrap support (NJ 79% and Bayesian inference 100%).

Discussion

The COI tree (Fig. 4) agrees with previous 12S rDNA sequence analyses results (MJ in prep) by revealing that the plateau haplotypes of the Eastern province constitute a monophyletic group to the exclusion of the single Southern province and Nyimba district haplotype. The COI data further agreed with 12S rDNA data by having equal number of haplotype diversity for the two provinces i.e. one for the Southern province and five for the Eastern province. In the COI data, as in the 12S rDNA, the one haplotype out of the five Eastern province haplotypes that was identical to the single haplotype of the Southern province was from Nyimba district. The COI tree was similarly in agreement with the previous ITS2 data which showed support for the Eastern province plateau sequences as a monophyletic group within an assemblage of *R. appendiculatus* sequences from the Southern province and Nyimba district.

The solid support for the separation of *R. appendiculatus* from Southern and Eastern provinces into two groups on the gene tree considered here agrees with published differences (Table 1). *Rhipicephalus appendiculatus* from the two provinces further differ by having different adult phenologies (Berkvens et al. 1998; Chaka 2001; Speybroeck et al. 2002) which are related to the differences in diapause induction and intensity (Madder et al. 2002; Speybroeck et al. 2002). In addition, Tempia (1997) reported higher infection rates for *Theileria parva* (Katete), strain from the Eastern province in adults of *R. appendiculatus* from the Southern province than

R. appendiculatus from the Eastern province plateau. These differences manifest themselves in significant differences in the epidemiology of ECF between the Southern and Eastern provinces (Speybroeck 2004; Billiouw 2005).

While both Southern and Eastern provinces fall within the same two agro-ecological zones (Fig. 1), there are two major climatic differences between them i.e. (1) Southern province experiences lower mean temperatures in the cold dry season and (2) generally receives lower total annual rainfall than Eastern province (Speybroeck et al. 2002). However, significantly higher differences in both total annual rainfall and temperatures exist between valley and plateau locations within both provinces. We therefore propose that haplotype 1 and the Eastern province plateau haplotypes represent geographical rather than ecologically differentiated haplotypes. The existence of the two geographic subdivisions may be an indication of partial isolation of the two geographic stocks resulting in insufficient demographic connection between them (Avisé 2000).

The presence of more than one haplotype in the Eastern province may be testimony to the more than one life history strategy developed by this species in this region (Madder 1999). Indeed, Madder (1999) suggested that the population in the eastern province, might consist of individuals with genetically variable critical photoperiod thresholds for diapause induction. However, which haplotype or haplotypes coincide with the reported phenotype groups remains to be decided. This, however, is not to suggest that the COI is involved in diapause control. Using the tool for determination of the physiological age of *R. appendiculatus* (Chaka et al. 2001) it should be possible to find if there is any correspondence between these haplotypes and the reported phenotypes. On the contrary, only a single COI haplotype was found in the Southern province. One factor, which though singly may not explain this phenomenon completely, is that the dispersal rate of ticks in the province is quite high (own unpublished observation). This is due to the traditional cattle keeping system in which cattle are grazed on communal areas and allowed to move from foci of disease outbreaks to disease free areas. Social-economic activities like the payment of stock “on the hoof” as dowry for marriage, sharing and hiring out oxen for ploughing, using oxen as transport to health centres and trekking to selling or trucking points also contribute to this high movement.

It is interesting that the Nyimba district (Eastern province) haplotype is identical to the Southern province haplotype. This is in spite of the district’s geographical proximity to the Eastern province plateau and the absence of a topographical barrier between Nyimba (on the fringes of the valley) and Petauke on the plateau (Berkvens et al. 1998) that would otherwise prevent movement of hosts (livestock and wild animals) and therefore dispersal of the ticks. Despite that, Berkvens et al. (1998) did predict a limit in the westward spread of *R. appendiculatus* on the eastern plateau to around Minga an outpost 35 km west of Petauke (55 km East of Nyimba) because of unfavourable conditions i.e. higher minimum temperatures and lower relative humidity for *R. appendiculatus*. That the Eastern province plateau haplotypes were not found in Nyimba is in line with that prediction but a contradiction for haplotype 1. However, Speybroeck et al. (2004) suggested that *R. appendiculatus* stocks that do not go through a diapause cannot survive in areas with unfavourable conditions where a diapause is necessary. We, however, further add that *R. appendiculatus* stocks that do not go through an obligatory diapause may not survive harsh conditions. Conversely, *R. appendiculatus* stocks that go through an obligatory diapause are likely to survive a wide range of conditions within the acceptable limits of

this species survival (Speybroeck et al. 2004). Therefore, we hypothesize that in Nyimba (an area with unfavourable conditions) *R. appendiculatus* may require an obligatory diapause to survive, just like the Southern province ticks with an identical haplotype. If this assumption is correct, we suggest that haplotype 1 has more chances of advancing eastwards on the Eastern province plateau, than any of the four Eastern plateau haplotypes advancing westwards into the valley.

The reported difference in the epidemiology of ECF in Eastern and Southern Zambia (Billiouw 2005; Speybroeck et al. 2004) is therefore due in a large part to the difference in the vector ticks. The spread and establishment of either group of ticks into another group's area of endemicity has a potential to change the epidemiology of ECF for that area. This would be due to the differences in infection rates for *Theileria parva* strain or strains present in the new area of establishment (Tempia 1997) and the differences in ticks' life history traits. There is therefore need to both confirm whether haplotype 1 ticks from Nyimba are an extension of the southern province ticks and therefore undergo an obligate diapause. Further the biology of the progeny of this haplotype with the Eastern province plateau haplotypes should be explored. This may help reveal further future epidemiological possibilities.

Acknowledgements This work was funded by the Belgian Technical Co-operation through the "Assistance to the Veterinary Services of Zambia (ASVEZA) project in the Southern Province. We thank the farmers of the two provinces for their co-operation. Special thanks to Mr Geff Mukwalantila without whose commitment collections from Petauke and Nyimba would have not been possible.

References

- Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge and London
- Berkvens DL, Pegram RG, Brandt JRA (1995) A study of the diapausing behaviour of *Rhipicephalus appendiculatus* and *R. zambeziensis* under quasi-natural conditions in Zambia. *Med Vet Entomol* 9:307–315
- Berkvens DL, Geysen DM, Chaka G, Madder M, Brandt JRA (1998) A survey of the Ixodid ticks parasitizing cattle in the Eastern Province of Zambia. *Med Vet Entomol* 12:234–240
- Billiouw M (2005) The epidemiology of bovine theileriosis in the eastern province of Zambia. PhD thesis, Ghent University, Ghent, Belgium
- Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, Van Der Noordaa J (1990) Rapid and simple method of purification of nucleic acids. *J Clin Microbiol* 28:495–503
- Boom R, Sol C, Beld M, Weel J, Goudsmit J, Wertheim-van Dillen PME (1999) Improved silica-guanidiniumthiocyanate DNA isolation procedure based on selective binding of bovine alpha-casein to silica particles. *J Clin Microbiol* 37:615–619
- Chaka G, Billiouw M, Geysen DM, Berkvens DL (1999) Spatial and temporal variation in *Rhipicephalus appendiculatus* size in eastern Zambia. *Trop Med Int Health* 4:A43–A48
- Chaka G, Madder M, Speybroeck N, Tempia S, Tona K, Berkvens D (2001) Determination of physiological age of *Rhipicephalus appendiculatus* (Acari: Ixodidae). *Syst Appl Acarol* 10:1–16
- Chaka G (2001) Eco-physiology of *Rhipicephalus appendiculatus* (Acari: Ixodidae) in eastern Zambia. PhD Thesis, Institute of Tropical Medicine, Antwerp (ITMA), Antwerp, Belgium, pp 89–105
- Madder M (1999) Behavioural Diapause in *Rhipicephalus appendiculatus* Neumann (Acari: Ixodidae), PhD Thesis, Ghent University, pp 109–122
- Madder M, Speybroeck N, Brandt J, Tirry L, Hodek I, Berkvens D (2002) Geographic variation in diapause response of adult *Rhipicephalus appendiculatus* ticks. *Exp Appl Acarol* 27:209–221
- McLain DK, Wesson DM, Olivier JH Jr, Collins FH (1995) Variation in ribosomal DNA internal transcribed spacer 1 among Eastern populations of *Ixodes scapularis* (Acari: Ixodidae). *J Med Entomol* 32:353–360

- Mtambo J, Van Bortel W, Madder M, Roelants P, Backeljau T (2006) Comparison of preservation methods of *Rhipicephalus appendiculatus* (Acari: Ixodidae) for reliable DNA amplification by PCR. *Exp Appl Acarol* 38:189–199
- Mulumba M, Speybroeck N, Billiow M, Berkvens DL, Geysen DM, Brandt JRA (2000) Transmission of theileriosis in the traditional sector in the southern province of Zambia during 1995–1996. *Trop Animal Health Prod* 38:303–314
- Nicholas KB, Nicholas HB Jr (1997) GeneDoc: a tool for editing and annotating multiple sequence alignments. Distributed by author. <http://www.psc.edu/biomed/genedoc/gddl.htm>
- Norval RAI, Perry BD, Young AS (1992) The epidemiology of theileriosis in Africa. Academic Press, London, pp 99
- Ronquist F, Huelsenbeck JP (2003) MRBAYES: Bayesian phylogenies under mixed models. *Bioinformatics* 19:1572–1574
- Pegram RG, Perry BD, Musisi FL, Mwanaumo B (1986) Ecology and phenology of ticks in Zambia: seasonal dynamics in cattle. *Exp Appl Acarol* 2:25–45
- Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–818
- Speybroeck N, Madder M, Van Den Bossche P, Mtambo J, Berkvens N, Chaka G, Mulumba M, Brandt J, Tirry L, Berkvens D (2002) Distribution and phenology of ixodid ticks in Southern Zambia. *Med Vet Entomol* 16:430–441
- Speybroeck N, Madder M, Thulke HH, Mtambo J, Tirry L, Chaka G, Marcotty T, Berkvens D (2004) Variation in body size in the tick complex *Rhipicephalus appendiculatus*/*Rhipicephalus zambezensis*. *J vector Ecol* 29:347–354
- Swofford DL (2003) PAUP* phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland, MA
- Tempia S (1997) Comparison of the vectorial capacity of *Rhipicephalus appendiculatus* (Acari: Ixodidae) nymphs and adults originating from eastern and southern provinces of Zambia infected by *Theileria parva* Katete stock. M.Sc. Thesis 65, Institute of Tropical Medicine, Antwerp (ITMA), Belgium
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876–4882
- Walker JB, Keirans JE, Horak IG (2000) The Genus *Rhipicephalus* (Acari: Ixodidae): a guide to the brown ticks of the world. Cambridge University Press, Cambridge