Genetic variation in *Rhipicephalus appendiculatus* (Acari: Ixodidae) from Zambia: correlating genetic and ecological variation with *Rhipicephalus appendiculatus* from eastern and southern Africa

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ABSTRACT: Based on their ecology, *Rhipicephalus appendiculatus* ticks from eastern and southern Africa have been divided into three groups. We investigated how two geographic genetically differentiated stocks of *R. appendiculatus* from the southern and the eastern provinces of Zambia, representing two ecological groups, i.e., southern African and transition groups, respectively, genetically compare to stocks from east Africa (Rwanda) and southern Africa (Zimbabwe and South Africa). The ITS2 and two mitochondrial genes segments, 12s rDNA and COI, were used in the investigations. The ITS2 tree did not show support for differentiation into any groups, while the two mitochondrial genes trees (12s rDNA and COI) showed two genetically differentiated groups: an east African genetic group which included specimens from Rwanda and the plateau area of the eastern province of Zambia, and a southern African genetic group represented by specimens from South Africa, Zimbabwe and specimens collected on the fringes of the eastern province plateau in the Nyimba district of Zambia. This suggests that the two geographically differentiated stocks of the southern and eastern provinces of Zambia might be part of two wider geographic genetically differentiated *R. appendiculatus* groups that extend beyond Zambia. Stocks of "transition" ecology (eastern province) belong to the east African genetic group and the differences in ecology within this genetic grouping may be due to genetic polymorphism, phenotypic plasticity, and other local factors. ***Journal of Vector Ecology* 32 (2): 168-175. 2007.**

Keyword Index: *Rhipicephalus appendiculatus*, ITS2, 12S rDNA, COI, geographic genetic differentiation.

INTRODUCTION

The African brown ear tick, *Rhipicephalus appendiculatus* Neumann, 1901, is the main field vector of the haemoprotozoan parasite *Theileria parva,* the causative agent of East Coast fever (ECF). East Coast fever is one of the most economically important cattle diseases in Zambia (Makala et al. 2003) and other eastern, central, and southern African countries due to the high mortality, morbidity, and other production losses associated with the disease (Mukhebi et al. 1992). The distribution of *R. appendiculatus* is not continuous but is limited by factors like the availability of suitable hosts, vegetation, and climate (Norval et al. 1992, Walker et al. 2000). In addition, climate influences the number of generations per year (Norval et al. 1992).

Based on the number of generations per year, the adult body size and the expression or non-expression of a behavioral diapause (the suppression of host-seeking activity in unfed adult ticks), *R. appendiculatus* have been divided into three groups (Madder et al. 1999, Madder et al. 2002, Speybroeck et al. 2004). The first group comprises *R. appendiculatus* stocks that cycle through two or more generations per year originating from parts of east Africa, e.g., Kenya, Rwanda, Burundi, Tanzania, and Uganda where the climate is characterized by short dry seasons and high ambient humidity throughout most of the year (Smith 1969a,b, Tatchell and Easton 1986, Kaiser et al. 1988). The ability of the east African stocks to cycle through more than one generation per year has been attributed to the absence of a long dry season which precludes the necessity for a behavioral diapause (Berkvens et al. 1995, Madder et al. 2002) in these stocks. Further, climate, as it is influenced by latitude, has an effect on the adult body size of some insects (James et al. 1995, Silver and Renshaw 1999) and this seems to be the case for *R. appendiculatus* (Speybroeck et al. 2004). The latter authors have described eastern African stocks to be of smaller adult body size than southern African stocks.

The southern African stocks (i.e., stocks from the southern province of Zambia through Zimbabwe to South Africa) constitute the second group (Madder et al.

2002, Speybroeck et al. 2004). The climate of southern Africa is marked by a well-defined rainy season that is followed by a dry season of almost equal duration. *Rhipicephalus appendiculatus* in this region cycles through one generation with peak adult activity coinciding with the rainy season (Short and Norval, 1981, Minshull and Norval 1982, Paine 1982, Pegram et al. 1986, Pegram and Banda 1990, Speybroeck et al. 2002). Southern African stocks exhibit either an obligatory or a very intense, photoperiod-induced (short day) behavioral diapause (Pegram and Banda 1990, Norval et al. 1991, Madder et al. 2002) that allows them to delay oviposition until conditions favorable for the survival of eggs and larvae, the most vulnerable stages of their life cycle, are present. Termination of behavioral diapause in these stocks is determined by aging (Southern province of Zambia), or in the case of Zimbabwe, increasing photoperiod (Madder et al. 2002). A comparatively larger adult body size in southern African stocks has been associated with the ability to survive harsh conditions (higher temperature, low ambient humidity, and delayed feeding) during diapause (Speybroeck et al. 2004).

The third group consists of *R. appendiculatus* stocks from the eastern province of Zambia, whose ecology has been described as a "transition" between eastern and southern African stocks (Chaka et al. 1999, Madder et al. 1999, Speybroeck et al. 2004) based on the following observations: (a) *R. appendiculatus* stocks from the eastern province of Zambia have an average adult body size that is between that of *R. appendiculatus* from eastern and southern Africa (Speybroeck et al. 2004), (b) their phenology is characterized by two adult activity periods per year (Berkvens et al. 1998, Chaka et al. 1999) in spite of a climate that is marked by well-defined alternate rainy and dry seasons of approximately equal duration, and (c) their expression of behavioral diapause is mixed, i.e., ticks may or may not enter behavioral diapause depending on the amounts of rain or length of the rainy season (Madder et al. 2002). Diapause in the eastern province stocks is terminated by aging (Madder et al. 2002). Recently Mtambo et al. (2007a,b) have shown that *R. appendiculatus* from eastern and southern provinces of Zambia are genetically differentiated.

The aims of the current work were two-fold: (1) to clarify the phylogenetic relationship of the two geographic genetically differentiated stocks of *R. appendiculatus* from the southern and eastern provinces that are ecologically classified as "southern African" and "transition," respectively, with those of eastern and southern Africa in general, and (2) to compare the resultant phylogenetic relationships with the three group ecological characterization of east African, transition (eastern province of Zambia), and southern African stocks.

MATERIALS AND METHODS

Rhipicephalus appendiculatus were collected from Zambia, Rwanda, Grande Comore, Zimbabwe and South Africa. Adults of *R. appendiculatus* from Zambia originating from the southern and eastern provinces were collected from cattle in 1997 and 2003, respectively. Southern province tick collections were collected from the plateau districts of Mazabuka (15°52' 0S, 27°46'0E) and Monze (16°16'60S, 27°28'60E), and the valley areas of Gwembe (16°49'0S, 27°46'60E), Livingstone (17°49'60S, 25°23'60E), and Sinazongwe (17°7'60S, 27°25'0E) districts. Ticks collected from the southern province were killed in 70% ethanol immediately after collection. They were preserved in 70% ethanol in sealed vacutainer tubes at ambient temperatures. In the eastern province, ticks were collected from the plateau districts of Chipata (13°37'60S, 32°38'60E) and Petauke (14°15'0S, 31°19'60E) and Nyimba (14°33'0S, 30°49'60E), a district on the fringes of the eastern province plateau and the Luangwa Valley. These ticks were preserved at -80° C. In Rwanda, ticks were collected from cattle in different agro-ecological regions of the country in the districts of Byumba, Kibuye, Gitarama, Gikongoro, Kigali, and Kibungo in 2004. These ticks were killed in 70% ethanol immediately upon capture. Later they were washed in tap water, air dried, and packed in Ziplock bags with silica gel and preserved at 4° C. Ticks from South Africa were collected from cattle in Rietvlei (29°10'60S, 30°16'60E) and Hluhuwe (28°1'60S, 32°16'60E) in the KwaZulu Natal midlands in 2000 and 2004, respectively. These specimens were killed and preserved in 70% ethanol. The ticks from Grande Comore were collected from cattle in May 2004 at Madjeouéni (11°49'18S, 43°16'41E) on the northeastern part of the island. They were killed and preserved in 70% ethanol. Ticks from Zimbabwe were collected from cattle in West Mashonaland in 2000. They were allowed to die naturally after collection, dried, and then stored in sealed conical tubes at ambient temperature. Different stocks were subjected to different preservation methods appropriate to the specific purposes for which they were collected other than this work.

DNA was isolated using the method of Boom et al. (1990, 1999). The primers, PCR conditions, and temperature profiles used for amplification of the ITS2 and the COI including the sequencing techniques have been described by Mtambo et al. (2006). Mitochondrial 12S rDNA was amplified with the primers SR-J-1499 (5'-TACTATGTTACGACTTAT-3') and SR-N-14594 (5'-AAACTAGGATTAGATACCC-3') (Simon et al. 1994). The PCR conditions and thermal cycler temperature profile for amplification of the 12S rDNA were identical to those used in the amplification of the COI (Mtambo et al. 2006). To distinguish between *R. appendiculatus* and the closely related *Rhipicephalus Zambeziensis* Walker, Norval and Corwin, 1981, a PCR-RFLP technique (Mtambo et al. 2007b) was used. Briefly, ITS2 amplicons of the specimens were digested with the restriction endonuclease *Bau*I in a 25µl volume containing 17 µL Mqw, 2.5 µl 10X buffer (Tango™), 0.5 µl Enzyme (0.1U; *Bau*I), and 5 µl amplicon. The mixture was incubated at 37° C for 150 min. Fragment profiles were resolved by electrophoresis through 2% (w/v) agarose gel at 100 volts for 30 min and visualized after staining for 30 min in ethidium bromide.

Multiple alignments were generated for 59 (ITS2), 59 (COI), and 58 (12S rDNA) gene sequences using default options in ClustalX 1.83 (Thompson et al. 1997). The alignments were imported into Genedoc ver.2.6.001 (Nicholas and Nicholas 1997), wherein they were visually examined and redundant sequences were removed. The remaining sequences were subjected to a BLAST to confirm the target gene segment. These sequences were then aligned together with sequences of *Rhipicephalus turanicus* Pomerantsev, 1936 (accession numbers **DQ849267** [ITS2]; **DQ849231** [12S rDNA]; **DQ859260** [COI]) as the outgroup species. These alignments are available in the EMBL-Align database under the accession numbers: **ALIGN_001066**=ITS2; **ALIGN_001067**=COI; **ALIGN_001065**=12S rDNA. The best models of evolution for the sequences of the three target genes were determined in Modeltest (Posada and Crandall 1998) using the Akaike Information Criterion. Modeltest chose the General Time Reversible with a proportion of invariable sites (GTR+I) as the model best fitting the COI data with the following model parameters: base frequencies; freqA = 0.2755, freqC =0.1832, freqG = 0.1376 and freqT = 0.4037; Rmat=1.7849, 3.4740, 3.7650, 0.1735, and 10.8297; Rates=equal and proportion of invariable sites (I) =0.6340. For the ITS2 data, the GTR model with no invariable sites was the most appropriate. The parameters of the model were: base frequencies; freqA = 0.1839, freqC = 0.3016, freqG = 0.3487 and freqT = 0.1658; Rmat =0.9242, 1.5422, 1.3804, 0.3855, and 3.3218; Rates=equal and Pinvar (I) =0. The model best fitting the 12S rDNA was the K81uf+I. This model had the following parameters: base frequencies; freqA= 0.3658, freqC= 0.1178, freqG= 0.0916 and freqT= 0.4248; Rmat= 1.0000, 2003660.7500, 1123197.3750, 1123197.3750, and 2003660.7500; Rates=equal and Pinvar (I) = 0.7203. Phylogenetic relationships among the ITS2 sequences and COI and 12S rDNA haplotypes were evaluated by both a distance (NJ: Neighbor-joining) and a maximum likelihood (ML) analysis in PAUP* (Swofford 2003). Starting trees were obtained through the random addition of input taxa with the heuristic search option and tree bisection-reconnection (TBR) branch swapping. In all the analyses, gaps were treated as missing data. The reliability of the groups on the trees was evaluated through bootstrap analysis with 1,000 iterations with TBR branch swapping.

RESULTS

Targeted genes failed to amplify in the dried *R. appendiculatus* material from Zimbabwe and those that amplified did not give interpretable sequences. Instead, we obtained ITS2 (Accession No. **U97704**) and COI (Accession No. **AF132833**) sequences for Zimbabwe from GenBank. These and the rest of the sequences used in this study are summarized in Table 1. Sequences for the ITS2 and the two mitochondrial (COI and 12S rDNA) fragments obtained for *R. appendiculatus* material from Rwanda and Grande Comore appear in GenBank under accession numbers **DQ901321**-**DQ901355** for the ITS2, **DQ901356**-**DQ901363** for COI, and **DQ901277**-**DQ901320** for 12S rDNA. Ticks from Zambia and South Africa appear in GenBank under the following accession numbers: ITS2= (**DQ849239**-**DQ849254** and **DQ849269**-**DQ849272**); 12S rDNA= (**DQ849203**-**DQ849218** and **DQ849233**-**DQ849236**); COI= (**DQ859261**-**DQ859266**). Specimens were submitted to the Royal Belgium Museum of Natural sciences under the General Inventory number **I.G. 30662.**

Table 1: Source and numbers of *R. appendiculatus* sequences for the three gene segments analyzed.

Country	Gene Segment	No. of Sequences
Rwanda	ITS2	15
	COI	15
	12S rDNA	15
Grande Comore	ITS2	2
	COI	2
	12S rDNA	2
Zambia	ITS2	35
	COI	35
	12S rDNA	35
Zimbabwe	ITS2	1
	COI	1
	12S rDNA	0
South Africa	ITS2	6
	COI	6
	12S rDNA	6

There were five different ITS2, eight COI, and five 12S rDNA sequences (Tables 2-4). The eastern province had five COI and 12S rDNA haplotypes compared to one in both segments in the southern province. The eastern province and Rwanda shared some COI and 12S rDNA haplotypes. In addition, COI and 12S rDNA haplotypes endemic to either Rwanda or the eastern province but sympatric to the shared haplotypes were observed.

Results of the analysis of the ITS2 data did not provide support for grouping *R. appendiculatus* from Rwanda, Zambia (eastern and southern provinces), and South Africa into any special groupings. The COI tree had two well-supported groups (Figure 1). One group, with BS values of 95% (ML) and 100% (NJ), was an unresolved polytomy of the eastern province plateau and Rwanda haplotypes, within which there was weak support (BS values 64% ML and 67% NJ) for the exclusion of eastern province plateau haplotype 8. The second group had BS values of 70% (ML) and 98% (NJ). This group comprised ticks from South Africa, Zimbabwe, Zambia (southern province and the southwest edges [Nyimba] on the eastern province plateau) and Grande Comore. The 12S rDNA tree (Figure 2) was resolved into two groups (BS values 78% [NJ] and 87% [ML]). One group consisted of eastern province (Nyimba), southern province of Zambia, South Africa, and Grande Comore stocks, while the second was made up of stocks

Table 2. Variation of the ITS2 sequences in *R. appendiculatus* from six eastern, central, and southern African countries.

		Variable Base Positions															
Sequence No.	Number of Sequences	34	135	346	364	400	408	445	509	510	606	755	788	850	1003	1006	Sequence Origin
Sequence 1	2	T	C	A	T	C	C	C	▪	▪	C	T	A	T	A	C	Zambia (east and south)
Sequence 2	26	–	–	–	–	–	–	T	▪	▪	–	–	–	–	–	–	Zambia (east and south), South Africa
Sequence 3	1	–	▪	T	▪	T	T	T	C	T	▪	T	A	▪	–	–	Zimbabwe (McIllwaine)
Sequence 4	25	–	C	A	T	C	C	T	▪	▪	C	G	T	T	–	–	Rwanda and Zambia (east)
Sequence 5	5	T	–	–	–	–	–	–	▪	▪	–	T	–	–	–	C	Grande Comore, Zambia (south), South Africa

Key: ▪ indel; – nucleotide identical to one immediately above, Zambia (south); southern province of Zambia, Zambia (east); eastern province of Zambia, Zambia (east & south); both eastern and southern provinces of Zambia.

Table 3. Variation of the COI sequences in *R. appendiculatus* from five eastern, central, and southern African countries.

		Variable Base Positions																					
Haplotype	No. of specimens	12	24	57	94	147	159	165	211	240	246	261	303	315	339	345	372	393	394	432	453	465	Sources
Haplotype 1	30	T	G	T	C	T	C	T	A	G	C	T	A	T	T	G	T	A	G	G	C	C	Zambia (south and east), Zimbabwe, South Africa
Haplotype 2	2	—	—	C	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Grande Comore
Haplotype 3	19	C	—	—	T	C	T	—	—	—	T	—	G	C	—	A	G	A	G	A	T	T	Zambia (east), Rwanda
Haplotype 4	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	A	—	—	—	Zambia (east)
Haplotype 5	1	—	—	—	—	—	—	—	C	—	—	—	—	—	—	—	—	—	G	—	—	—	Zambia (east)
Haplotype 6	1	—	—	—	—	—	—	—	A	—	—	—	—	—	—	—	—	G	—	—	—	—	Rwanda
Haplotype 7	1	—	—	—	—	—	—	C	—	A	—	—	—	—	—	—	—	A	—	—	—	—	Rwanda
Haplotype 8	4	—	—	—	—	—	—	T	—	G	—	—	—	—	—	—	—	—	—	G	—	—	Zambia (east)

Key: — nucleotide identical to one immediately above, Zambia (south); southern province of Zambia, Zambia (east); eastern province of Zambia, Zambia (east & south); both eastern and southern provinces of Zambia.

Table 4. Variation of the 12S rDNA sequences in of *R. appendiculatus* from five eastern, central, and southern African countries.

Haplotype	Number of Sequences	Variable Base Positions 69	81	88	164	220	309	315	Haplotype location
Haplotype 1	33	T	T	T	T	G	G	A	Grande Comore, Zambia (East &South), South Africa
Haplotype 2	4	C	C	C	▪	—	—	—	Zambia (East)
Haplotype 3	1	—	T	—	▪	—	—	—	Zambia (East)
Haplotype 4	2	—	—	—	▪	—	—	G	Rwanda and Zambia (East)
Haplotype 5	18	—	—	—	▪	—	—	A	Rwanda and Zambia (East)

Key: ▪ indel, — nucleotide same as one immediately above, Zambia (South); southern province of Zambia, Zambia (East); eastern province of Zambia, Zambia (east and south); both eastern and southern provinces of Zambia.

from Rwanda and eastern province plateau districts.

DISCUSSION

The non-resolution of *R. appendiculatus* from Rwanda, Zambia, Zimbabwe, and South Africa into any special groups by the current ITS2 data show that, in spite of their ecological differences, *R. appendiculatus* from Rwanda (east Africa), Zambia, Zimbabwe, and South Africa (southern Africa) are one and the same species. However, the mitochondrial (12S rDNA and COI) DNA data show that, *R. appendiculatus* from Rwanda, Zambia, Zimbabwe, and South Africa may be divided into two geographic genetically differentiated groups: (1) A group consisting of specimens from Rwanda and the plateau districts of the eastern province of Zambia which we here refer to as the "east African genetic group (eAGG)," and (2) a group comprising specimens from South Africa, Zimbabwe, Grande Comore, the southern province of Zambia, and the southwestern edges of the eastern province plateau (Nyimba) in Zambia, which we have referred to as the "southern African genetic group (sAGG)" (Figures 1 and 2). This geographic genetic differentiation into sAGG and eAGG is an indicator of possible temporal and/or spatial partial isolation of the two populations (Avise 2000).

That the stocks from the eastern province that are considered transitional and stocks from Rwanda form a coherent monophyletic eAGG is at variance with their ecological groupings. The difference in ecology within the eAGG may be due to genetic polymorphism and/or phenotypic plasticity. Eastern province transition stocks had five COI and 12S rDNA haplotypes, respectively, compared to a single haplotype for both segments in all the southern African stocks. It is possible that some haplotypes may be favored by the existing local conditions in one area more than in others, e.g., a shorter rainy season for the eastern province than for most places in Rwanda or east Africa in general may be favorable to some haplotypes and not others. Which haplotype corresponds to a particular phenotype or phenotypes (size, diapause, and phenology) under discussion cannot be ascertained with the current data. It is also possible that individuals of the same haplotype may have varied phenotypic expression of their genotype in different environmental conditions. Indeed, the most prevalent haplotypes, i.e., COI haplotype 3 and 12S rDNA haplotype 5, were common to both Rwanda and the eastern province. Similarly, the reported difference in body size (Speybroeck et al. 2004) in stocks arising from the same regions as those that constituted our eAGG may be attributed to genetic polymorphism, phenotypic plasticity, differences in latitude (Speybroeck et al. 2004), and/or any other unidentified local factors (Speybroeck et al. 2004). Madder et al. (1999), studying diapause induction in three stocks of ticks from Kiambu (Kenya), Mtenguleni (eastern province Zambia), and West Mashonaland (Zimbabwe), speculated the existence of an extensive genetic variation for diapause after observing as many responses as were the stocks. We, however, suggest that an extensive genetic variation for diapause might exist especially within the eAGG.

The composition of the sAGG agrees with the ecological similarities of the constituent stocks (Norval et al. 1991, Madder et al. 1999, Speybroeck et al. 2004) except for Grande Comore ticks for which no prior ecological information was obtained. However, the island of Grande Comore has a single rainy season (November to April) and a dry season of equal duration (May to October) with temperatures ranging from 20-28° C (Ben Daoud and Scheltens 1985). Such climatic conditions could be more favorable to the establishment of *R. appendiculatus* that express a behavioral diapause in order to synchronize oviposition with favorable environmental conditions (Speybroeck et al. 2004). However, the actual situation in Grande Comore can only be established by further surveys as local ecological conditions may modulate these climatic conditions. The failure of amplification of dried specimens from Zimbabwe may have been due to the nature of the killing and storage methods (Mtambo et al. 2006).

Within the limits of these data, we conclude that *R. appendiculatus* stocks from the southern and eastern provinces of Zambia are geographic genetically differentiated stocks that might be part of two wider geographic genetically differentiated populations of *R. appendiculatus* in which southern province stocks are part of the sAGG and

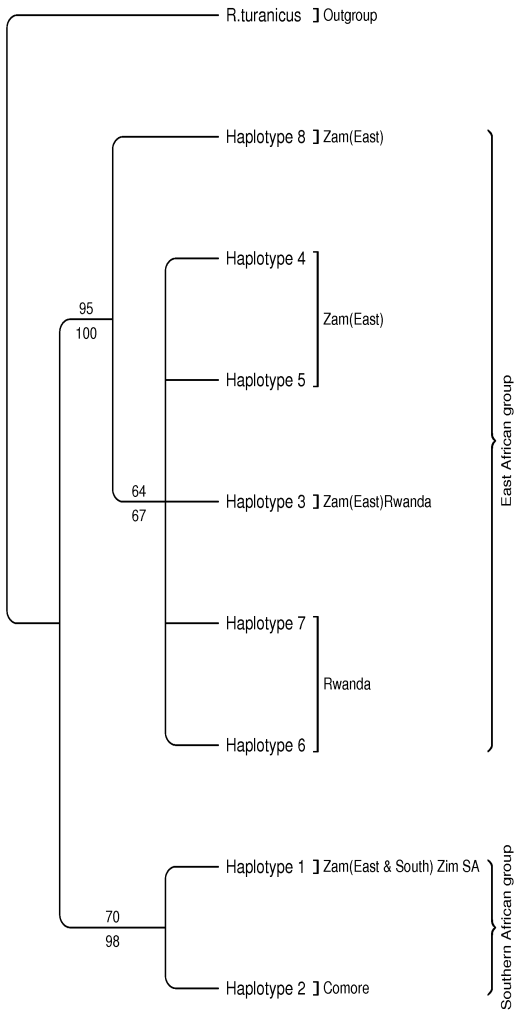

Figure 1. A cladogram of COI haplotypes with Bootstrap support values; Top = ML; Bottom = Neighbor Joining (NJ); Names after the braces refer to the area where haplotypes were found. SA=South Africa; Zam (south) = southern province of Zambia; Zam (east) = eastern province Zambia; Zam (east and south) = eastern and southern province of Zambia; Zim= Zimbabwe; Comore= Grand Comore.

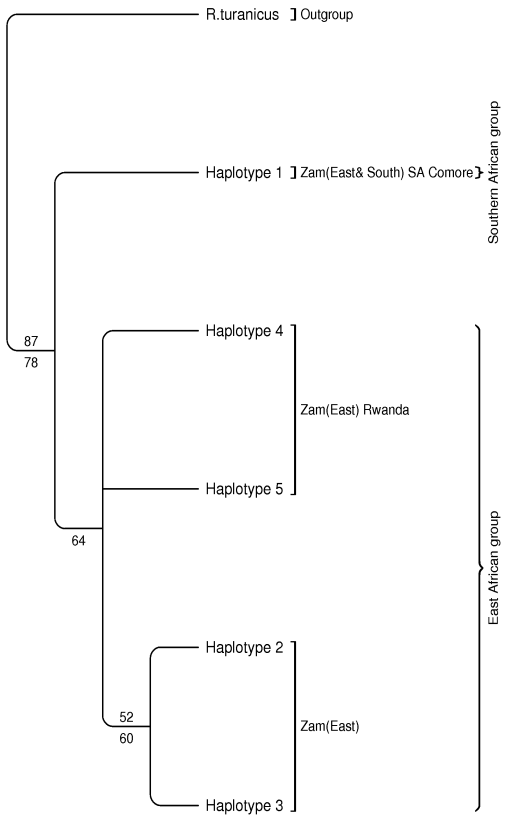

Figure 2. A cladogram of 12S rDNA sequences with Bootstrap support values; Top = ML, Bottom= Neighbor Joining. Names after the braces refer to the area where haplotypes were found. SA=South Africa; Zam (south) = southern province of Zambia; Zam (east) = eastern province Zambia; Zam (east and south) = eastern and southern province of Zambia; Comore= Grand Comore.

the eastern province stocks belong to the eAGG. It will be important to delimit the ranges of these two groups both within and outside Zambia since their respective roles in the epidemiology of ECF may be different (McLain et al. 1995, Avise 2000, Speybroeck et al. 2004).

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