GENETIC VARIATION IN TWO POPULATIONS OF THE BROWN EAR TICK, *RHIPICEPHALUS APPENDICULATUS*, IN KENYA

BY

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To: Dr. E. O. Osir

With compliments from Dr. F. N. Baliraine.

"The fear of the Lord is the beginning of wisdom...Righteousness exalts a nation, but sin is a reproach to any people...Blessed is the nation whose God is the Lord..."
(Psa. 111:10; Prov. 14:34; Psa. 33:12).

God Bless You!

DECLARATION

I, **Frederick Ndhoga Baliraine**, hereby declare that this thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This thesis is dedicated to four people. Each has moulded my personality, emotions, and intellect in his own unique, loving manner. First and foremost is the Lord Jesus Christ, to whom I owe my very self and from whom comes my inspiration and direction in everything. Second is my Dad, Mr. Yonasani Kintu Mutabule (who went to glory on Oct. 13, 1998, just as I was writing up this thesis) and third, my Mom, Mrs. Wamukisa Efulansi Muwanse-Mutabule. Fourth is my senior brother, Mr. James Dhi'Kusooka Asinze. I present this thesis with joy, love, and insufficient thanks in honour of these people and above all the Almighty God, who is going to see me to greater heights.

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ABBREVIATIONS

ABBREVIATIONS	FULL NAME/MEANING
2-DE	Two-dimensional electrophoresis
APS	Ammonium persulphate
ß	Beta
BCA	Bicinchoninic acid
bp	Base pairs
BSA	Bovine serum albumin
Conc.	Concentrated
CTAB	Cetyl-trimethyl ammonium bromide
dH_2O	De-ionized water
DNA	De-oxyribonucleic acid
ECF	East Coast fever
EDTA	Ethylenediamine tetraacetic acid
e.g.	For example
EMBL	European molecular biology laboratory
h	Hour
IAT	Infection and treatment
ICIPE	International Centre of Insect Physiology and Ecology
i.d	Internal diameter
i.e.	That is

IEF	Isoelectric focusing
kDa	Kilo daltons
M	Molar (Moles/litre)
mer	base pairs
mg	Milligram(s)
min	Minute(s)
mM	Millimolar (Millimoles/litre)
M_r	Relative molecular mass
NEPHGE	Non-equilibrium pH gradient electrophoresis
ng	Nanogram(s)
nm	Nanometre(s)
NP-40	Non-ionic detergent P-40 (Octylphenoxypolyethoxyethanol)
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pH	-Log hydrogen ion concentration
pI	Isoelectric point
PMSF	Phenylmethylsulfonylfluoride
RAPD	Random amplified polymorphic DNA
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate

Tris	Tris-(hydroxymethyl-aminomethane)
TBE	Tris borate EDTA
TEMED	N,N,N'N'-tramethylethylenediamine
µ	Micro (10^{-6})
µg	Microgram(s)
µl	Microlitre(s)
U.O.N	University of Nairobi
UPGMA	Unweighted pair-group method with averaging
V	Volts
v/v	Volume per unit volume
w/v	Weight per unit volume

SUMMARY

Several studies have shown that *Rhipicephalus appendiculatus* from geographically isolated areas differ in their susceptibilities to *Theileria parva*, the haemoprotozoan causative agent of East Coast Fever. In Kenya, *R. appendiculatus* from Muguga have been reported to be less susceptible to *T. parva* infection than those from Rusinga Islands. As the same tick species from different areas shows variation in vector competence, it was considered to be of epidemiological interest to determine whether these tick populations are genetically different. Most population genetic studies have been done by comparing protein electrophoretic patterns, and more recently by DNA analysis. However, all available techniques have limitations. It is therefore not advisable to rely on only one technique.

In this study, two-dimensional protein electrophoresis and random amplified polymorphic DNA Polymerase chain reaction were used to compare two *R. appendiculatus* populations from Muguga and Rusinga, in Kenya. Most proteins were common to both populations. However, some proteins were population-specific. Two proteins (M_r ~14.4-19,000), one protein (M_r ~26,000) and two proteins (M_r ~47-50,000), were specific to the Muguga population. On the other hand, three proteins (M_r ~14,000), one protein (M_r ~22,000), one protein (M_r ~29,000), one protein (M_r ~41,000), and three proteins (M_r ~45-48,000), were specific to the Rusinga population. Homologous proteins are probably species-specific.

In the RAPD technique, both pooled and individual tick DNA samples were analysed. Five different primers were used. In the pooled DNA, each primer gave some population-specific bands. However, individual DNA samples showed considerable band

variation among individuals within each population. The genetic distance between the two populations averaged 0.047. This implies that these populations are closely related, sharing about 95% of their genes.

The presence of population-specific proteins and DNA fragments, together with a genetic distance greater than zero suggests that genetic differences exist between the two populations. The observed differences may not be directly linked to a particular phenotype. Nevertheless, it is possible that the differences in the vectorial capacities of these populations may be accounted for by genetic factors. However, further studies will be required to confirm this.

CHAPTER 1

INTRODUCTION

1.1 The tick problem

Ticks are ectoparasites that transmit various fatal or debilitating diseases to humans and livestock (Dipeolu, 1989; Nuttall, 1998). They transmit a larger number and variety of diseases to livestock than any other arthropod (Obenchain and Galun, 1982). About 80% of the estimated world cattle population of 1.29 billion are at risk from ticks and tick-borne diseases (de Castro, 1997). Ticks also cause enormous economic losses by direct damage to their hosts. Tick bite wounds are usually very painful, of long duration, and predispose cattle to other diseases (Sonenshine, 1991). In addition, tick bites can cause severe toxic conditions and adverse immune responses (Sonenshine, 1991). Ticks also cause emaciation, reduction in growth rate and milk production, prolonged calving intervals and death of cattle by decreasing their blood supply (Dipeolu *et al.*, 1992; Solomon and Kaaya, 1998). Ticks also reduce the recreational value of many parks, campsites, and hiking paths. The global economic loss attributed to ticks and tick-borne diseases is in billions of dollars annually (de Castro, 1997). Africa annually incurs a greater loss in livestock production attributed to ticks than all other continents put together (Dipeolu *et al.*, 1992). In the developing world, the losses from ticks and tick-borne diseases are not merely economic. In many areas, malnourished people are deprived of animal protein and fat needed to enhance their resistance to debilitating infectious diseases (FAO, 1984; de Castro, 1997).

In Africa, the human population is growing rapidly and is projected to triple by the year 2025 (ILRI, 1995). This calls for increased animal protein production to meet increased demand. Unfortunately, most of Africa has reached her maximum livestock-carrying

capacity, given the traditional systems of management. The solution is to increase sustainable productivity rather than the number of animals. Thus, the focus should be on enhancing individual animal productivity. In view of this, African governments are encouraging farmers to import the highly productive exotic breeds and their crossbreeds from Europe, and to genetically improve the indigenous cattle (ILRI, 1995). Unfortunately, farmers are often unable to viably crossbreed their relatively small and less productive indigenous animals with more productive cattle. This is because the latter are highly susceptible to tick-borne diseases. About 90% of the estimated 200 million cattle in Africa are infested with ticks (Dipeolu *et al.*, 1992). *Rhipicephalus appendiculatus* is the most important tick in Africa, because it transmits *Theileria parva*, which kills most cattle in this continent (de Vos, 1981). This tick is predominant in the savannah zone (Dipeolu *et al.*, 1992). The savannah zone can be referred to as the potential "food basket of Africa" because of its fertility. This is where most of Africa's crop and livestock production occurs (Dipeolu *et al.*, 1992). Ticks therefore impede viable livestock production in Africa. If Africa's livestock sector is to develop up to the stage that will assure self-sufficiency of animal protein supply, a viable solution must be sought for the tick problem.

So far, three main approaches have been employed to handle the problem: (1) killing the ticks using acaricides, (2) anti-*Theileria* chemotherapy, and (3) anti-*Theileria* vaccination. However, acaricide resistance is now a problem (Willadsen, 1997). Chemotherapy, besides being expensive, may only be of help at the start of the clinical disease (Nyindo, 1992). The vaccines are yet to be improved from the current "crude" ones to the use of novel molecular vaccines (Musoke *et al.*, 1993). This requires accurate identification and keeping of tick colonies with defined infection rates; to avail the parasites for the identification of candidate molecules for vaccine target. Studies on the population

genetics of *R. appendiculatus* may eventually lead to the discovery of molecular markers and genes for *T. parva* refractoriness and susceptibility in ticks. This may then enable accurate identification of tick colonies, and development of genetic methods of controlling tick-borne diseases.

This project does not intend to go as far as identifying specific molecular markers or genes for *T. parva* susceptibility or refractoriness. It is just an attempt to unearth some genetic information on *R. appendiculatus* population in Kenya. This is to be done by comparative 2-DE of proteins and RAPD-PCR of DNAs of a tick population from Muguga and another population from Rusinga Islands. This is to be followed by a calculation of the genetic distance between the two populations, and relating the obtained information with the observed differences in the *T. parva* susceptibilities of these two tick populations. It is hoped that the results of this research will make some contribution to the body of information required for the designing of novel tick and tick-borne disease control strategies.

CHAPTER 2

LITERATURE REVIEW

2.1 Population genetics: an overview

Population genetics is the study of genetic variation in natural populations and the factors that influence this variation (Tabachnick and Black, 1996). It is a vital area of study in arthropod vector biology. Understanding genetic variation in vector species such as *R. appendiculatus* may provide a foundation for understanding the role of the vector in disease epidemiology. This is because the factors that shape genetic variation in vector species will also influence variation in vector capacity and vector competent traits (Tabachnick and Black, 1996).

Population genetics essentially deals with genotypic variation, but only phenotypic variation can be observed. Some of this variation has direct correspondence to genotype. For example, an allelic substitution at a structural gene locus causes a change in the amino acid sequence of the protein coded for by the affected gene (Tait, 1985). For reasons of experimental convenience, most population genetics work has concentrated on such phenotypes. However, some characters of interest, such as vector competence, could be quantitative and having a complex relation to genotype. Consequently, the study of such variation should be a two step process, involving (1) the description of the phenotypic variation, and (2) a genetic analysis of that variation (Suzuki *et al.*, 1986; Hartl and Clark, 1989).

2.1.1 The sources of genetic variation

For a given population, there are three main sources of genetic variation: mutation, recombination, and immigration of genes from other populations (Tabachnick and Black, 1996; Clark and Russell, 1997).

Although mutations are a source of variation, mutation rates are so low that they cannot account for rapid evolution of populations and species. The rate of gene-frequency change from the mutation process is very low because spontaneous mutation rates are low. For example, for the average animal mitochondrial DNA genome, only one site substitution is expected every 3000 years (Szalanski *et al.*, 1996). Thus, the process of mutation does not itself drive evolution.

The creation of genetic variance by recombination can be a great deal faster than that due to mutation. This is because a single homologous pair of chromosomes that is heterozygous at n loci can produce $n(n\text{-}1)/2$ new unique gametic types per generation of recombination (Suzuki *et al.*, 1986). If the heterozygous loci are well spread on the chromosomes, these new gametic types will be frequent, and a considerable variance will be generated (Suzuki *et al.*, 1986; Watson *et al.*, 1987).

A further source of variation is migration or introduction of genes into a population from other populations with different gene frequencies. This will cause a change in gene frequency (Nei, 1972). Unlike the mutation rate, the migration rate can be large, so the change in frequency may be substantial (Suzuki *et al.*, 1986; Tabachnick and Black, 1996).

2.2 Genetic variation in ticks

Several studies have revealed some genetic variation even among related species. For example, significant allelic differences have been found in populations of the sheep tick, *Ixodes ricinus*, in Ireland (Sonenshine, 1991). In contrast, very low genetic variability has been noted in the Australian reptile tick, *Aponomma hydrosauri* (Sonenshine, 1991). Oliver (1983) suggests that the diversity of hosts and rapid dispersal by transport of sheep stock allows greater potential gene flow in *I. ricinus* than the dispersal achieved by the slow moving reptile hosts of *A. hydrosauri*. Thus, ecological differences, host range and dispersal parameters play important roles in genetic variability between populations.

An example of how genetic divergence between closely related species may be assessed may be seen in the study of two *Amblyomma* species: *A. cajennense* and *A. immitator*. Morphologically, these two species are almost indistinguishable. However, protein electrophoresis revealed considerable genetic variation between them, with a number of species-diagnostic proteins (Hilburn *et al.*, 1989). Similar studies were conducted to determine genetic similarity between populations of *Boophilus microplus* and between the two closely related species *B. microplus* and *B. annulatus* (Sattler *et al.*, 1986). Comparisons of genetic identity of *B. microplus* populations from diverse regions indicated a very high degree of genetic similarity. The index of genetic identity (*I*) was 0.984 ± 0.012, suggesting that they shared the same undifferentiated genetic pool. In contrast, comparisons between the two species indicated much less similarity, at a level characteristic of distinct but sibling species ($I = 0.716 \pm 0.013$) (Sattler *et al.*, 1986). Hilburn and Sattler (1986) reported electrophoretically detectable protein variations in various natural populations of the lone star tick, *Amblyomma americanum.*

2.3 The tick, *Rhipicephalus appendiculatus*

2.3.1 Taxonomy, distribution, and hosts

Rhipicephalus appendiculatus (Acari: Ixodidae) is a reddish or blackish-brown tick (Newson, 1978; de Vos, 1981; Nyindo, 1992). It is economically the most important tick species in Africa (de Vos, 1981).

The tick is mainly found in the eastern, central, and southern parts of Africa, south of the equator. It does not occur in West Africa (Newson, 1978). On the Kenyan coast, it is found from sea level up to 2100 m inland (Newson, 1978). It is absent from deserts and areas lacking shrub cover. It is most common in areas of tall grass rather than short grass, and in areas of moderate or higher rainfall, which is often defined as more than 750 mm per annum. In areas where the rainfall exceeds 1000 mm, heavy infestations could be expected. The distribution of *R. appendiculatus* is the result of a complex interaction between climate, vegetation and cattle (Newson, 1978; Randolph, 1994).

Rhipicephalus appendiculatus is primarily a parasite of cattle but also occurs on sheep and goats, and to a limited extent on wild animals. Up to 2000 adults have been found on a single animal, but infestations are usually considerably lower (Newson, 1978).

2.3.2 Economic importance

Rhipicephalus appendiculatus transmits *Theileria parva*, the most economically important tick-borne protozoan parasite of cattle in Africa (Chiera *et al.*, 1989; Nyindo, 1992; Mbassa *et al.*, 1998). *Theileria parva* causes theileriosis, commonly known as East Coast Fever. The distribution, abundance, and seasonal population dynamics of *R. appendiculatus* directly determine the epidemiology of ECF (Randolph, 1994). East Coast Fever debilitates and kills infected susceptible livestock in 11 countries of Eastern, Central

and Southern Africa: Burundi, Democratic Republic of the Congo, Kenya, Malawi, Mozambique, Rwanda, Sudan, Tanzania, Uganda, Zambia and Zimbabwe (Brocklesby *et al.*, 1961; ILRAD, 1990, 1993/4; Radostits *et al.*, 1994). About 40% of the cattle raised in this region are at risk from the disease, which kills over a million cattle annually. In 1989 alone, ECF caused a loss of US $168 million in Africa (Norval *et al.*, 1992). This was due to mortalities, loss of production in sick and recovering cattle, and cost of control measures (Norval *et al.*, 1992; Mbassa *et al.*, 1998). The disease most severely affects the highly productive exotic breeds and their crossbreeds, and the genetically improved indigenous cattle encountering *T. parva* for the first time. These animals will develop severe disease from infection and die if untreated.

Among indigenous cattle where the disease is endemically stable, up to 50% of the calves exposed to *T. parva* die. In areas where the disease is endemically unstable, such as in herds encountering the parasite for the first time, 80-100% of animals of all age groups may die (ILRAD, 1990, 1991). Thus, the economic losses sustained from *R. appendiculatus* and ECF include not only the loss of local stocks, but also the expensive imported breeding animals. The contribution these animals would have made to greater productivity and genetic improvement of national herds is therefore also lost. East Coast Fever is therefore a major constraint to the development and expansion of the livestock industry in Kenya and other afflicted areas.

2.3.3 Life cycle

The life cycle of ticks goes through four successive stages: eggs, larvae, nymphs and adults. Depending on the number of host changes and moults during their metamorphosis, ticks can be divided into four life cycle groups: one-host, two-host, three-

host and multi-host (Nyindo, 1992). *Rhipicephalus appendiculatus* belongs to the three-host group (Musoke *et al.*, 1992). The larvae and nymphs may feed on rodents, reptiles or birds in the wild. The adult stage seeks large mammals such as cattle, buffalo or sheep. In the adults, mating influences feeding. Females need a small blood meal before mating (Nyindo, 1992). A large blood meal is taken soon after mating. The engorged, mated female ticks drop off the host and lay eggs, which hatch into larvae after about 2 weeks. The larvae climb the vegetation to await passing hosts. The larvae feed on a new host and when engorged, fall to the ground and moult into nymphs. These in turn attach to fresh hosts, engorge, and moult into adults. Adults are often found attached on cattle ears. In heavy infestations, the ticks occur elsewhere on the head, spreading to the neck, body and peri-anal region (Newson, 1978; Nyindo, 1992). It is probably because of its colour and predilection site that *R. appendiculatus* is commonly referred to as the *brown ear tick.*

2.4 Control of ECF

2.4.1 Vector control

At present, the main method for controlling of ECF aims at breaking the transmission cycle of *T. parva* between cattle and ticks. This is achieved through application of acaricides (Radostits *et al.*, 1994; Mbogo *et al.*, 1996), adherence to legislation on cattle movements and quarantine, and good livestock and pasture management (Thomson *et al.*, 1978; Beesley, 1982; Dipeolu *et al*, 1992; Malonza *et al.*, 1992; Radostits *et al.*, 1994).

However, acaricides are becoming increasingly expensive and they pollute the environment (Willadsen, 1997). In Africa, about 92% of cattle have no access to acaricide treatments, due to the high costs of the chemicals, and of construction and management of

dip tanks and spray races (ICIPE, 1992). In addition, water is in chronic short supply in rural Africa (ILRAD, 1993/94). Besides, development of resistance to acaricides by *R. appendiculatus* and other African ticks is now an established fact (Dipeolu *et al.*, 1992).

In areas heavily infested with ticks, cattle herds are walked to acaricide dips or spray races as often as twice a week for treatment. This creates foci for disease spread. This practice also helps erode the land, pollute the environment with toxic residues, and possibly quickens the development of acaricide resistance. Moreover, since cattle regularly treated with acaricides are not exposed to *T. parva*, they develop no immunity to ECF. In effect, they are vulnerable to the parasites if acaricide treatment is interrupted or stopped altogether. Furthermore, some acaricides may not kill the ticks before they have had time to attach and transmit infection (Mwase, *et al.*, 1991). Generally, the control of three-host ticks has always proved difficult due to the range of alternative wild hosts available (Mwase *et al.*, 1991). This strategy for tick control has therefore not been very successful.

2.4.2 Chemotherapy

Curative drugs are relatively new tools for ECF control. To be effective, the disease must be diagnosed accurately and early so that treatment can be given at the start of clinical disease. Unfortunately, early diagnosis of ECF is difficult. This is because the disease becomes clinically apparent only when it has reached an advanced stage (Nyindo, 1992; Mbassa *et al.*, 1998). Moreover, besides being expensive, the drugs have withdrawal periods, which the rural farmers will hardly observe. Chemotherapy therefore poses a potential public health problem in developing countries.

Alternative and improved methods of controlling ECF are therefore urgently needed. No single method is likely to avail a permanent solution. An integrated approach,

including the induction of resistance in animals to tick infestation and the development of vaccine s against the parasites, is more logical (Dipeolu *et al.*, 1992; Nyindo *et al.*, 1996; Willadsen, 1997).

2.4.3 Vaccination

Although most cattle die from ECF, the few that recover become permanently protected against re-infection by the same parasite strain (Musoke *et al.*, 1982, 1992, 1993; Radostits *et al.*, 1994). However, some cross-immunity has been noted between antigenically different strains (Radostits *et al.*, 1994; Mbogo *et al.*, 1996). In endemic areas, immunity is established early and this provides life-long protection if re-infection continues (Radostits *et al.*, 1994).

Theiler (1911), showed that it was possible to protect cattle from infections with *T. parva* by inoculating animals with lymph node or splenic material derived from infected cattle. He conducted 25 experiments using 224 cattle and made several important observations. Among the observations was that the cattle had a 50% survival rate upon field challenge inoculations by ticks.

Theiler's observations acted as an eye opener for vaccination against ECF. It was later observed that oxytetracycline, when administered during the incubation period, could alter the course of ECF. Some animals thus treated did not die of the disease and were later protected from challenge (Brocklesby and Bailey, 1965; Young *et al.*, 1988).

For a more positive control programme, vaccination has obvious advantages. Vaccines offer sustained protection, and cross-species action is less likely to occur than with drugs; since they are intrinsically specific (Willadsen, 1997). Vaccines are cheaper than many chemical agents, and resistance to them is unlikely to occur (Willadsen, 1997).

In addition, unlike drugs and acaricides, vaccines are free of residues (Willadsen, 1997). The need for vaccines should also be seen in the light of the cost of developing a new drug, estimated at an average cost of US $230 million per compound (de Castro, 1997). The importance of immunization therefore needs not be over emphasized.

So far, the only field-effective immunization method against ECF is immunotherapy or "infection-and-treatment" method (Radley, 1981; Radostits, 1994; Mbogo *et al.*, 1996). A promising recombinant sporozoite vaccine is being developed, but this has so far only shown partial protection (Musoke *et al.*, 1992, 1993).

In IAT, cryopreserved suspensions of *T. parva* sporozoites from homogenized infected *R. appendiculatus* ticks are injected into cattle. The resultant infection is controlled by the administration of long acting oxytetracycline, given at the same time so that immunity is established (Radostits *et al.*, 1994). Either one stock of parasite or a cocktail of different stocks may be used. Vaccination is usually successful provided the local parasite strains are included (Radley, 1981; Taracha *et al.*, 1988; Nyindo, 1992; Radostits *et al.*, 1994). Large-scale field trials of this vaccine have been carried out throughout East and Central Africa, and a similar procedure using Parvaquone instead of Tetracycline has given even better results (Radostits *et al*, 1994; Mbassa *et al.*, 1998).

However, for the IAT vaccine to be produced, it is necessary to maintain highly infected ticks to provide the sporozoites. This is because sporozoites are difficult to grow *in vitro* (Nyindo, 1992). Highly infected ticks are also required to provide the sporozoites for isolation and studying of antigenic molecules of *T. parva* that could be used to develop novel recombinant vaccines. These are envisaged to be cheaper and probably more satisfactory than the live vaccines currently in use (ILRAD, 1993/4; de Castro, 1997; Prichard, 1997; Willadsen, 1997). Lowly infected ticks that mimic field infection rates are

also needed. These may be useful in assessing the levels of protection afforded cattle by experimental vaccines.

Cattle also acquire resistance to infestation with hard ticks (Brown, 1985; Chiera *et al.*, 1985, 1989; Rubaire-Akiki, 1990; Nuttall, 1998). Host resistance against ticks drastically reduces tick numbers (Chiera *et al.*, 1989), and has been used in Australia as part of the integrated management of *Boophilus microplus* (Sutherst *et al.*, 1979). Acquired resistance to ticks could also possibly interfere with the transmission of disease agents. This phenomenon has been reported for *Theileria annulata* in *Hyalomma anatolicum*-resistant cattle (Rubaire-Akiki, 1990). The induction of tick resistance in cattle by judicious exposure to *T. parva*-refractory ticks could also be a viable approach to ECF control (Nyindo *et al.*, 1996).

Since the rates with which *T. parva* parasites infect their tick vectors as well as the competence of the ticks to transmit the parasites vary greatly (Norval *et al.*, 1989, 1991; Kubasu,1992; ILRAD, 1993/4), studies on tick population genetics in relation to vector competence are necessary. Such studies may unearth genetic information, which may eventually be used in the development of molecular markers for *T. parva* resistance or susceptibility. This may aid in the selection and production of tick colonies with defined infection rates for the above purposes.

2.5 Genetics of susceptibility and vector competence

The discovery that certain blood-sucking arthropods may transmit pathogens led to the realization that each pathogen is transmitted by a limited number of arthropods, and that each arthropod has the ability to transmit only certain pathogens. This gave the first indication that vector genetics was involved in determining vector competence (Gooding,

1996).

2.5.1 Interpopulation variation in vector-pathogen relationships

The frequency of genes for susceptibility or refractoriness within a population is influenced by the usual evolutionary forces such as mutation, selection, founder effects, and genetic drift (Gooding, 1996). Interpopulation differences in the levels of susceptibility to pathogens can therefore be expected. This has already been demonstrated in various vector species.

Büscher and Otim (1986) reported that the distribution of *Theileria* infection in *R. appendiculatus* populations is over dispersed or aggregated. They found most of the ticks to have few or no *Theileria* parasites, but a few had many parasites. This suggested the existence of *Theileria*-susceptible and unsusceptible *R. appendiculatus* populations (Büscher and Otim, 1986). Norval *et al.*, (1989) found stocks of *R. appendiculatus* from geographically different areas to differ in their susceptibility to infection with *T. parva*. The highest infection rates were obtained in ticks from Ol Pejeta Ranch, Kenya. Intermediate infection rates were obtained in the Muguga stock and generally low infection rates in a stock from Lake Mcllwaine, Zimbabwe (Norval *et al.*, 1989). Kubasu (1990, 1992), using isoenzyme analysis, studied the biological diversity of *R. appendiculatus* in relation to transmission of *T. parva* in Kenya. He observed that ticks from Kilifi, South Nyanza and Uashin Gishu were more efficient transmitters of the *T. parva* stocks investigated. On the other hand, ticks from Kiambu were least efficient in transmitting the parasites. Basing on enzyme profile results, he concluded that *R. appendiculatus* populations from different geographical areas of Kenya are different. In another study, four geographically isolated *R. appendiculatus* populations in Kenya (Baragoi, Malaral, Embu and Lanet) were compared

by RAPD-PCR. The tick DNA samples were amplified using twenty 10-mer primers of random sequences and the products analysed by agarose gel electrophoresis. Concurrently, nymphal ticks from the same locations were allowed to feed on cattle that had been infected with *T. parva* Marikebuni and *T. parva* Lanet. The results showed wide variations in the mean infection rates of the ticks infected with the two parasite strains. Resistance to infection differed according to location, with ticks from Embu and Lanet being more susceptible than those from Baragoi and Malaral (Kiara and Osir, unpublished). The RAPD-PCR analysis also showed distinct differences between the tick populations (ICIPE, 1996/97).

Variation in vectorial capacity has also been noted in other arthropod species. For example, tsetse fly species have been found to differ greatly in their ability to transmit trypanosomes. *Glossina pallidipes* and *Glossina morsitans centralis* appear to have similar vector competence for *Trypanosoma vivax* (Moloo *et al.*, 1992). However, they differ greatly with regard to susceptibility to *T. congolense* and *T. brucei*; in which case *G. pallidipes* has a lower vector competence than *G. m. centralis* (Moloo *et al.*, 1992). It is possible that the differences in infection rates reflect differences in their gut environments (Moloo and Kutuza, 1988). This could be a result of genetic differences. Already, susceptibility to trypanosome infection in *G. morsitans morsitans* has been shown to be a maternally inherited trait (Maudlin *et. al*, 1986; Welburn and Maudlin, 1991). A similar phenomenon has been reported in mosquitoes. For example, *Aedes aegypti* has a heritable trypsin-like proteinases activity in its midgut, responsible for the destruction of ingested Ookinates of *Plasmodium* (Gass and Yeats, 1979; Yeats and Steiger, 1981). A similar heritable property, which prevents the survival of *Leishmania donovani*, has been suggested in *Phlebotomus papatasi* (Brovsky and Schlein, 1987).

Recently, scientists at EMBL embarked on a project to study the molecular genetics of interaction between the vector mosquito, *Anopheles gambiae*, and the malaria parasite (Kafatos *et al.*, 1994). Already, a high resolution genetic map of *Anopheles gambiae* based upon microsatellite markers and complemented with RAPD markers has been produced at EMBL (Kafatos *et al.*, 1994; Collins *et al.*, 1997). This map is being used to analyze genetic refractoriness to *Plasmodium*. Refractoriness has been found to be largely dominant, with intermediate phenotypes which can be measured quantitatively (Kafatos *et al.*, 1994). Genes that condition vector competence for filarial and malaria parasites in *Aedes aegypti* have also been mapped (Mutebi *et al.*, 1997).

2.5.2 Risk assessment based upon vector susceptibility to pathogens

Given enough information on the genetics of vector susceptibility to pathogens, it may be possible to assess the risk of a vector-borne disease in any locality. For example, tsetse fly susceptibility to trypanosome infection is vital in assessing the risk which man and his animals face in different tsetse infested areas (Maudlin, 1985).

In North America, malaria is largely unheard of. Many physicians may therefore be ill-prepared to diagnose the disease. However, the widespread occurrence of potential malaria vectors such as *Anopheles quadrimaculatus* sensu lato (Cornel *et al.*, 1996) serve as a reminder that the occasional introduction of malaria carriers can create avenues for malaria outbreaks.

During the second World War, a malaria-risk assessment enabled successful selection of military camps in Italy, based upon the distribution of populations of *Anopheles maculipennis* that bite humans and those that do not (Curtis, 1984). An ECF-risk assessment should also be possible, given a reliable knowledge of the geographical

distribution of *T. parva*-susceptible and *T. parva*-refractory ticks.

However, the assessment of local risk factors using the classical procedures for determining vector competence is problematic. Besides being time consuming, classical procedures are also expensive (Gooding, 1996). They are therefore unlikely to be of help in the absence of an obvious imminent threat. New molecular techniques should provide methods for rapid assessment of risk factors. For example, it is now possible to quickly assess the risk of trypanosome transmission posed by any tsetse fly population. This is done by using immunological procedures to determine the prevalence of maternally inherited factors that control tsetse susceptibility to trypanosomes (Maudlin and Dukes, 1985). Similarly, DNA probes have been developed to distinguish between females of *Anopheles gambiae* and *Anopheles arabiensis* (Gale and Crampton, 1988).

2.5.3 Potential uses of refractory and susceptible ticks

By using standard selection procedures, colonies of ticks that are highly susceptible or highly resistant to *T. parva* can be established. Such colonies are a potential source of material for investigating the mechanisms by which ticks and *Theileria* interact. A thorough understanding of such mechanisms is a must for the successful application of genetic techniques to control theileriosis.

Ticks that are highly susceptible to *T. parva* may be useful in producing the vertebrate-infective form of the pathogen for use in developing and testing vaccines and prophylactic agents (Musoke *et al.*, 1982, 1992, 1993; Mbogo *et al.*, 1996; Mbassa *et al*, 1998). Highly refractory colonies may be useful for the introduction of deleterious genes into natural populations, or as replacements for a population having high vector competence (Beard *et al.*, 1992, 1993; Crampton *et al.*, 1994).

Although not yet tried in ticks, the feasibility of population replacement has been demonstrated in other arthropod species. Graves and Curtis (1982) reduced the susceptibility of caged populations of *Anopheles gambiae* to *Plasmodium yoelii nigeriensis* by releasing males of a poorly susceptible strain. After the end of the release programme, the susceptibility of the population remained half its original level. Van der Kaay *et al.* (1982) obtained similar results with a caged population of *Anopheles atroparvus* that was susceptible to *Plasmodium berghei berghei*, by introduction of males from a line that had low susceptibility to this malaria parasite. A number of mosquito genes that can be manipulated to inhibit vector competence, and DNA transfer techniques to effect this have been studied (Spielman, 1994; Curtis and Sinkins, 1998). Similar techniques could be adapted to manipulate *T. parva* vector competence in *R. appendiculatus*. However, before any genetic control programme is implemented, a thorough knowledge of the genetics of the target populations is required (Hanson *et al.*, 1993; Gooding, 1996).

2.6 Biochemical genetics

Biochemical genetics, particularly involving studies of electrophoretic variation in proteins, have been widely applied to vectors and have revealed that a significant amount of variation exists in most vector populations. These techniques have demonstrated, or confirmed, the existence of sibling species and genetic structuring within several species of vectors (Gooding, 1996; Tabachnick and Black, 1996).

2.6.1 Rationale for using biochemical methods to measure genetic variability

Morphological characteristics have traditionally been used in the identification and classification of organisms. There are, however, instances where organisms having different behavioural characteristics are morphologically indistinguishable. This poses epidemiological and taxonomic problems. For example, behavioural and intra-species genetic variation has been demonstrated in morphologically identical species and sub-species of all sub-genera of genus *Trypanosoma* (Young and Godfrey, 1983; Myler, 1993). In view of this, modern scientists have sought to utilize biochemical (e.g. protein and DNA) properties, which are stable and intrinsic to the organisms (Post, 1985; Overmeyer *et al.*, 1996; Besansky *et al.*, 1997). At present, most data have been obtained from protein electrophoresis, and direct measurement of DNA variability (Cornel *et al.*, 1996; Gooding, 1996).

2.6.2 The measurement of genetic variation

2.6.2.1 Protein polymorphism as a biochemical measure of genetic variation

The study of protein variation is an indirect means of examining gene diversity (Tait, 1985). Most of the variations observed result from amino acid alterations within the polypeptides under study, which in turn reflect nucleotide alterations within the genes coding for the polypeptides (Tait, 1985; Tabachnick and Black, 1996). Thus, studies of genetic polymorphism are now being carried down to the level of the polypeptides coded by the structural genes themselves. A nonredundant codon change in a structural gene will result in amino acid substitution in the polypeptide produced at translation. For example, a

codon change of GGA to GAA will give a respective change in the translation product from glycine, which is neutral and uncharged, to glutamic acid, which is acidic and carries a strong negative charge (Clark and Russell, 1997). If a specific protein could be purified and sequenced from separate individuals, it would be possible to detect genetic variation in a population at this level. In practice, this is tedious for large organisms and impossible for small ones unless a large mass of protein can be produced from a homozygous line (Suzuki *et al.*, 1986).

There is, however, a practical substitute for sequencing that makes use of the change in the physical properties of a protein when an amino acid is substituted. The amino acids: arginine, aspartic, glutamic acid, histidine, and lysine have ionizable side chains that give a protein a characteristic net charge, depending on the pH of the surrounding medium (Stryer, 1981; Watson *et al.*, 1987). Amino acid substitutions may either directly replace these charged amino acids or affect their degrees of ionization. Substitutions can also cause changes in the three-dimensional structures of the amino acids (Suzuki *et al.*, 1986). In all these cases, the net charge on the polypeptide will be altered (Wilson and Walker, 1994). To detect the change in net charge, protein can be subjected to the method of gel electrophoresis (Hames and Rickwood, 1990).

2.6.2.2 Gel electrophoresis in the measurement of genetic variation

Gel electrophoresis is based on the principle that any charged ion or group will conduct electricity and migrate when placed in an electric field (Dunbar, 1987; Hames and Rickwood, 1990). Proteins carry a net charge at any pH other than their isoelectric point. They will therefore migrate, and their rate of migration will depend upon their charge density (the ratio of charge to mass). The higher the charge density, the faster the molecules

will migrate (Hames and Rickwood, 1990). The application of an electric field to a protein mixture in solution will therefore result in different proteins migrating at different rates towards one of the electrodes. Thus, once electrophoresis is completed, the proteins will be fractionated by charge or size (Tabachnick and Black, 1996). However, unlike proteins, which can be neutral, positively or negatively charged depending on their overall amino-acid composition, all nucleic acids are negatively charged. Molecules of nucleic acids have exactly one negative charge per nucleotide so that the charge and size factors cancel out. In effect, all molecules of DNA will move at the same speed towards the positive electrode as long as they are free in solution (Clark and Russell, 1997). Gel electrophoresis will therefore help fractionate DNA according to size (Clark and Russell, 1997).

2.6.2.2.1Movement of molecules in support media during electrophoresis

A variety of matrices can be used as supporting material for separation of molecules during electrophoresis. These include paper, cellulose acetate, starch, agarose, and polyacrylamide (Dunbar, 1987). These supporting materials affect the mobility of charged molecules in many ways. For example, polyacrylamide will reduce molecular movement due to "molecular sieving" (Dunbar, 1987; Harris and Angal, 1989). Polyacrylamide is very popular because it is transparent, inert, and stable over a wide range of pH, temperatures, ionic strengths (Dunbar, 1987; Hames and Rickwood, 1990). Furthermore, its pore size is easily adjustable and resolution improved by either using a gel of increasing acrylamide concentration (gradient gel) or by using a stacking gel on top of the resolving gel, or both (Dunbar, 1987; Maizels *et al.*, 1991). A stacking gel helps increase resolution by first concentrating the proteins before they enter the resolving gel (Dunbar, 1987).

Gradient gels are better than gels of uniform concentration (straight gels). With a

gradient gel, a wide range of protein sizes can be optimally resolved on the same gel, and the bands are sharper. In addition, the gel separations are more reproducible, and the gels are easier to handle than straight gels (Dunbar, 1987; Harris and Angal, 1989; Maizels *et al.*, 1991).

2.6.2.2.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The anionic detergent, sodiumdodecyl(laurel)sulphate, $NaDodSO_4$, ($H_3(CH_2)_{10}CH_2OSO_3Na$) is now routinely used in PAGE. It binds to the hydrophobic regions of proteins and separates them into component subunits. In addition, SDS gives a large negative charge to the denatured, randomly coiled polypeptides; making them easy to separate during electrophoresis. Provided the size difference is large enough, PAGE can separate proteins of different sizes, but identical charge densities. This is because its molecular sieving effect will slow down the migration of larger proteins relative to that of the smaller proteins (Dunbar, 1987). This forms the basis of protein separation according to differences in mass.

2.6.2.2.3 Two-dimensional polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis is an extremely useful analytical tool for the separation of proteins from complex mixtures, and for estimating genetic variation (O'Farrel, 1975; Hames and Rickwood, 1990). O'Farrell (1975), Dunbar (1987) and Hames and Rickwood (1990) have given a sound theoretical basis for analysing the results from this relatively inexpensive technique, which also differs fundamentally from all other methods of genetic analysis in allowing the study of loci that are not segregating (Suzuki *et al.*, 1986).

This method involves the separation of proteins in the first dimension according to their charge (pI) and in the second dimension according to their relative mobility in SDS-PAGE. It was made popular for use in protein separation by O'Farrell (1975). Since the two dimensions separate proteins on the basis of independent properties, even complex protein mixtures can be excellently resolved (Dunbar, 1987; Maizels *et al.*, 1991). About 30% of all the base substitutions will give a charge change in a protein (O'Farrel, 1975; Tait, 1985). Mutations can produce amino acid substitutions, which result in charge changes, and consequently change the pI values of the proteins. These changes produce significant changes in band positions in IEF, and consequently on the SDS-PAGE gels. Since the mobility of proteins in SDS-PAGE is primarily a function of their molecular mass, it is also possible to obtain an indication of the molecular mass of the separated protein (Dunbar, 1987).

The limitation however is that the first dimension is not reliable with basic proteins (Piperno *et al.*, 1977; Maizels *et al.*, 1991). An alternative method, the non-equilibrium pH gradient electrophoresis, can resolve basic proteins (Maizels *et al.*, 1991). However, NEPHGE cannot separate proteins at their exact pI (Dunbar, 1987; Maizels *et al.*, 1991). Urea is usually included as a denaturant in the IEF gels and also in the sample solubilization buffer. At alkaline pH however, urea progressively breaks down to form cyanate ions, which react with protein amino groups to form stable carbamylated derivatives (Harris and Angal, 1989). These derivatives will have altered charge properties. This can result in anomalous electrophoretic behaviour and lead to artifactural separation profiles (Harris and Angal, 1989). Samples could also be run under native conditions (without urea). However, this is limited to protein samples which are soluble and which will not precipitate or aggregate during electrophoresis (Harris and Angal, 1989). In general, the resolution

obtained by the IEF is superior to that of the NEPHGE method (Maizels *et al.*, 1991). Many species have been sampled by the 2-DE method, including protozoa, bacteria, fungi, higher plants, vertebrates, and invertebrates with consistent results (O'Farrel, 1975, 1978; Tait, 1981, 1985; Ochieng *et al.*, 1993). For example, Tait (1981), used 2-DE as a means of measuring genetic variation in *Plasmodium falciparum*. The method revealed the occurrence of area specific variants and that *P. falciparum* is divided into discrete non-interbreeding populations. Racine and Langley (1980), examined whole kidney proteins of a wild population of the house mouse, *Mus musculus*, using starch gel electrophoresis and 2-DE. Both methods revealed protein heterozygosity.

However, the protein electrophoretic technique has the disadvantage of detecting variation only in structural genes (Suzuki *et al.*, 1986). If most of the evolution of shape, physiology, and behaviour rests on changes in regulatory elements, then the observed variation in structural genes would be misleading.

2.6.2.3DNA polymorphism as a biochemical measure of genetic variation

The limit in sensitivity inherent in examining variation in gene products rather than the genes themselves can be overcome by DNA analysis. The DNA-based techniques also measure the same parameter as that measured by protein variation, namely variation in the nucleotide sequence of different genes (Tait, 1985). With DNA analysis, a greater and more direct detection of genetic diversity is achieved (Black *et al.*, 1992; Stothard, 1997). Closely related species can be accurately distinguished from each other using their DNAs. This is because each organism is a "living fossil"; containing its own evolutionary record in the sequence of nucleotides of its DNA. A sequence unique to a particular organism will precisely identify it. Furthermore, the sensitivity and utility of DNA analysis has been

recently enhanced by the advent of the PCR, which can enable the analysis of even minute quantities of DNA (Linacre, 1995; Ellis, 1998).

2.6.2.4 Principles and application of PCR in biochemical genetics

The polymerase chain reaction is a process of DNA amplification with aid of thermostable DNA polymerase, resulting in the production of large amounts of the target DNA (Linacre, 1995). In the process of DNA replication, a single strand is copied into the double stranded molecule. This is achieved by the synthesis of a complementary strand by the enzyme DNA polymerase, provided that a short priming section of double stranded DNA is first present. The synthesis of the second strand occurs in the 5' to 3' direction (Wilson and Walker, 1994).

For DNA amplification to occur *in vitro*, all the components of *in vivo* DNA replication need to be assembled (Linacre, 1995). Short oligonucleotide primers are designed such that they will anneal to a target nucleotide sequence. The sample containing the target sequence is denatured, the primers are annealed and then extended enzymatically to produce a copy of each strand using a DNA polymerase. The same cycle is repeated at least 30 times so that copies are made from both the original target and copy templates. The result is exponential amplification of the target sequence. The PCR has greatly facilitated the analysis of nucleotide sequence variation, and has made possible a new level of phylogenetic investigation (Stothard, 1997). It has been used as a rapid means of screening for variation among a large number of individuals in population studies, including studies of extinct species like the mammoth and the quagga (Paabo *et al.*, 1989; Clark and Russell, 1997).

2.6.2.3.1 RAPD-PCR analysis: Use in taxonomy and population genetic studies

The RAPD-PCR is a novel and sensitive method for revealing polymorphisms randomly distributed throughout any given genome (Welsh and McClelland, 1990, 1991; Gwakisa *et al.*, 1994). It is based on the observation that a single short oligonucleotide of a random nucleotide sequence, when mixed with any DNA and *Taq* DNA polymerase then subjected to the PCR, will prime the amplification of several segments of the DNA (Williams *et al.*, 1990). However, the term *random* may be somewhat misleading in that the only random component is the initial choice of a primer for the PCR. The amplification itself is not random. It occurs only in regions of the genome where a particular DNA sequence and its reverse component are found (Haymer, 1994). The polymorphisms are detected as DNA fragments, that are present in one but absent in another individual or strain (Black, 1993; Tibayrenc *et al.*, 1993). Although short oligonucleotides (about 10-mer) of arbitrary nucleotide sequence are often used as primers, non-random, longer primers have also been used in certain cases (Welsh *et al.*, 1991). The RAPD patterns so obtained have been used in the derivation of molecular taxonomy and in the study of population genetics of diverse organisms (Welsh *et al.*, 1991; Black, 1993; Tibayrenc *et al.*, 1993). In phylogenetic studies, RAPD markers have been used to demonstrate genetic relationships between species or strains of various organisms in relation to their geographical distribution, pathogenicity, host specificity and other behavioural characters (Ballinger-Crabtree *et al.*, 1992; Tibayrenc *et al.*, 1993; Stothard, 1997). Tibayrenc *et al* (1993) analyzed genetic polymorphisms in several *Trypanosoma, Leishmania* and *Plasmodium* species using the RAPD-PCR method. The RAPD results were in agreement with those obtained by multilocus enzyme electrophoresis, and the phylogenetic

relationships among the parasites were consistent with previous knowledge. This parity between two sets of results confirmed that RAPD markers are reliable genetic markers (Tibayrenc *et al.*, 1993).

However, the validity of the RAPD-PCR in broad practice remains to be established. Questions arise as to the consistency of the results, the genetic significance of the amplified DNA sequences, and the possibility of artifactual outcomes (Riedy *et al.*, 1992). Williams *et al.* (1990) and Apostol *et al.* (1996) have highlighted some practical problems with RAPD analysis. These include: (1) the appearance of many markers on the same gel. This creates some uncertainty in assigning markers to specific loci in the absence of preliminary pedigree analysis; (2) the possibility for products of different loci to have similar molecular weights, and therefore comigrate; and hence be indistinguishable, and (3) the dominance of RAPD markers. Over 90% of alleles segregate as dominant markers (Williams *et al.*, 1990). The RAPD-PCR amplifies DNA from individuals that are either homozygous or heterozygous for an amplifiable allele. No fragment is produced in homozygous recessive individuals because amplification is disrupted in both alleles (Apostol *et al.*, 1996). Thus, if one of the alleles at a RAPD site is unamplifiable, then marker/marker homozygotes cannot be distinguished from marker/null heterozygotes. Nevertheless, the estimation of allele frequencies necessary for population genetic analysis is not prevented, provided there is a single amplifiable allele per locus (Lynch and Milligan, 1994).

2.7 Importance of the study

The present study was prompted by the observation that ticks from different geographical areas differ in their *T. parva* vector competence. For example, Kubasu (1992) carried out infection-rate studies on *R. appendiculatus* populations in Kenya. He infected ticks from South Nyanza (Suba) and Kiambu districts with *T. parva* Muguga and *T. parva* Kilae strains of *Theileria.* Ticks from Kiambu had mean infection rates of 6.4% and 2.6% with *T. parva* Muguga and *T. parva* Kilae respectively. Ticks from Suba had mean infection rates of 13.7% and 6.7% *T. parva* Muguga and *T. parva* Kilae respectively. He concluded that ticks from Suba are more efficient transmitters of *T. parva* than ticks from Kiambu. Similar recent studies on ticks from Muguga and Rusinga Islands in those respective districts using a different strain of *Theileria* gave higher infection rates, but agree with the above conclusion. The mean infection rates of ticks from Muguga and Rusinga with *T. parva* Marikebuni were 34% and 40.2% respectively (Kiara and Osir, unpublished observations). Due to these variations in *T. parva* susceptibility, it was considered of epidemiological interest to determine whether these populations differ genetically.

Understanding the genetic diversity that occurs in vectors is particularly important to epidemiologists and disease control workers. Populations of a species may look alike but exhibit marked differences in such important factors as host preferences, vectorial capacity and susceptibility to toxicants and natural enemies. Information on the genetic biodiversity of ticks could be extrapolated to give a better understanding of the relationships between pathogens and their tick vectors, ticks and their hosts, and between ticks and their natural enemies. This is vital for the designing of appropriate and effective disease control strategies. For example, instead of trying to eradicate vectors, vector biologists now hope to produce transgenic strains that are incapable of transmitting disease or incapable of

reproducing (Ikenshoji *et al.*, 1990; Aldhous, 1993; Crampton *et al.*, 1994; Osir and Gould, 1994; Collins *et al.*, 1997; O'Brochta and Atkinson, 1997; Curtis and Sinkins, 1998). In addition, there is need to produce ticks with defined infection rates for vaccine research and development (ILRAD, 1993/4). Even the available IAT strategy has proved difficult to standardise (Mbogo *et al.*, 1996; Mbassa *et al.*, 1998). This is in part due to the wide variation in the infection rates of ticks and the intensity of this infection. The use of ticks of a uniformly infected nature would assist in this process. For then a stabilate of known virulence can be made. For all this to be achieved, prior knowledge of the population genetics of the ticks is vital.

Genetic information may also enable prediction of the possible responses of natural populations to control measures (Tabachnick and Black, 1996). Lack of genetic variation in the tick populations would imply that the same management tactics could work over different regions (Sattler *et al.*, 1986). High variability between populations may imply that research results from one region cannot be extrapolated to other areas. Consequently, different strategies would have to be devised for managing the vector in different regions. It is for these reasons that this project proposed to examine the genetic variability among *R. appendiculatus* populations in Kenya.

3 Objectives of the study

The overall objective of this study was to compare the genetic variation between two distinct populations of the brown ear tick, *Rhipicephalus appendiculatus*, in Kenya, and to possibly relate this information to the observed differences in their susceptibility to *Theileria parva*, the causative agent of East Coast fever. The specific objectives were:

(1). Analyze whole *R. appendiculatus* protein samples by 2-DE.

(2). Analyze *R. appendiculatus* DNA samples by RAPD-PCR.

(3). Determine the genetic distance between the tick populations analyzed.

CHAPTER 3

MATERIALS AND METHODS

3.1 Work station

The experiments were carried out in the Molecular Biology and Biotechnology laboratories at ICIPE.

3.2 Reagents

All chemicals used in the experiments were of analytical grade. They were obtained from: BDH, BRL, Koch-Light, LKB, May & Baker, MERCK, Operon, SERVA, SIGMA, and Stratagene (Appendix I). All water used was distilled and de-ionized.

3.3 Ticks

Rhipicephalus appendiculatus ticks were obtained from Muguga, and Rusinga Island, which are geographically and ecologically isolated areas in Kiambu and Suba districts in Kenya, respectively. The geographical conditions of these areas have been described elsewhere (Kubasu, 1990). The ticks were kept in the Animal Rearing and Quarantine Unit at ICIPE and fed on rabbit ears (Irvin and Brocklesby, 1970). The rabbits were tick-naive male adult New Zealand whites (*Oryctolagus cuniculus*), free of mites. Each rabbit was used only once in the tick feeding procedure. Some of the ticks were cultured through one generation and cohorts used for protein extraction, and for infection-rate studies (Kiara and Osir, unpublished results).

3.4 Protein extraction

Protein was extracted according to Kiely and Riddiford (1985 a, b). Unfed adult male ticks were pulverized under liquid nitrogen in 1.5 ml Eppendorf tubes, using Teflon pestles. The resultant powder was homogenized at 4°C in 800 µl of 6.25 mM Tris pH 6.8 buffer, containing 1% (v/v) NP-40 and 1 mM PMSF (Sigma Chemical Co., St. Louis, MO). The protein content of the extracts was determined using the BCA assay (Pierce, Rockford, IL). Bovine serum albumin (Fraction V; Calbiochem, San Diego, CA) was used as the protein standard. Samples were stored at -80°C.

3.5 Two-dimensional gel electrophoresis

3.5.1 Sample preparation

The proteins were diluted with IEF sample solubilization buffer (9.5 M urea, 2% (v/v) NP-40, 2% (v/v) Ampholines (pH 5-8, LKB) and 5% (v/v) ß-mercaptoethanol). The following proportions were used: 10 µl protein sample + 10 µl sample buffer + 10 mg crystalline urea. After mixing, the samples were centrifuged at 12000 rpm for 10 seconds in a table top centrifuge (Eppendorf GmBH, model 5415 C) before loading them on the first dimension gels.

3.5.2 Procedures for the first dimension.

3.5.2.1 Preparation of the tubes

Soft glass tubes (140 x 2.5 mm i.d) were used. The tubes were soaked in chromic acid for at least 24 hours, thoroughly washed in water, rinsed with 70% (v/v) ethanol and dried (Dunbar, 1987). Each tube was then sealed at the bottom with four layers of Parafilm

and marked at 10.5 cm using a felt pen.

3.5.2.2 Preparation and running of the first dimension

The first dimension gel electrophoresis was done according to O'Farrel *et al.* (1975). In order to ensure reproducibility of the gel patterns, the same parameters were maintained between samples that were being compared. To prepare 10 ml of gel mixture, 5.5 g of urea was added to a 125 ml side arm flask, followed with 1.33 ml of 30% (w/v) acrylamide (Appendix II), 2 ml of 10% (v/v) NP-40, 1.96 ml H_2O, and 0.5 ml of pH 5-8 Ampholines. Complete disolution of the urea was achieved by brief warming of the mixture at 37°C in a water bath. The solution was degassed for 5 min with regular stirring. Immediately after adding 10 µl of 10% (w/v) APS and 7 µl of TEMED, the solution was loaded into the gel tubes. Using a Pasteur pipette, each tube was filled to the 10.5 cm mark. The gels were overlaid with 20 µl of H_2O. After 1 hour, the water was replaced with 20 µl of IEF sample buffer, and left to polymerize at room temperature for one more hour. The Parafilm was carefully cut off to avoid damaging the bottom of the gels. The overlay was then replaced with another 20 µl of IEF buffer. Freshly made and degassed cathode buffer (20 mM NaOH) was then added to fill the tubes. The lower reservoir of the vertical tube gel electrophoresis system (BRL, Life Technologies, Inc.) was filled with freshly made anode buffer (10 mM H_3PO_4). Any air bubbles were removed from the bottom of the gels by gently tapping the tubes. The upper reservoir was then filled with the cathode buffer and the gels pre-run according to the following schedule for ten gels: (a) 200 V for 15 min; (b) 300 V for 30 min; (c) 400 V for 60 min. The power supply was turned off and the upper reservoir emptied. The IEF buffer and NaOH were removed from the surfaces of the gels, and 100 µg of protein was loaded per gel. Each sample was overlaid with 10 µl of sample

overlay solution (made by adding 20 µl of dH_2O to 100 µl of IEF sample buffer). The tubes were then topped up with cathode buffer and the chamber was refilled with the same. The gels were ran at 400 V for 1 h; the cathode buffer was then quickly replaced with fresh buffer and the run continued for 15 h, and then at 800 V for 1 h. The gels were either immediately equilibrated and loaded onto the second dimension, or kept frozen in the tubes at -20°C, for running within 5 days.

In order to remove the gels from the tubes, they were "rimmed" by inserting a thin needle between the gel and the glass, and gently running it around while injecting in some water.

3.5.3 Equilibration

Each gel was extruded into a 1.5 ml Eppendorf tube containing 1 ml of equilibration buffer (10% (v/v) glycerol, 2.3% (w/v) SDS, 5% (v/v) ß-mercaptoethanol, 62 mM Tris, pH 6.8). The gels were equilibrated for 30 min at room temperature, and then loaded onto the second dimension, or frozen at -80°C for later use.

3.5.4 Procedures for the second dimension

3.5.4.1 Preparation of slab gels

The notched plate system was used (Studier, 1973). Each gel was formed between two uniform glass plates, each about 3 mm thick. One plate was a rectangular glass of 19.5 x 19.0 cm and the other plate was the same width, but with a length of 16.2 cm and a notch 0.4 cm deep and 17.0 cm wide. Perspex spacers, 1.0 mm thick, 1.3 cm wide and 18 cm long (22.5 cm for the bottom spacer) were used. The gel plates were carefully cleaned with detergent, followed by several rinses in water. A final wash with acetone was done to

eliminate any remaining grease that could affect polymerization. The two glass plates and three spacers were mounted together. One spacer was placed along the bottom, while the other two ran down the vertical sides of the plates. Care was taken to ensure that the vertical spacers were in close contact with the one at the bottom. To ensure a complete seal, the spacers were slightly greased before the assembly. The assembled plates were held together with strong clips, which were positioned to press over the spacers.

The discontinuous SDS gel system was used (Laemmli, 1970). Resolution was done in 8-20% (w/v) acrylamide gradient gels, stacked with 3% (w/v) acrylamide gels. To make the resolving gel, the following proportions were mixed:

		8%	20%
30% monomer	:	5.3 ml	13.3 ml
Separating buffer	:	5.1 ml	5.1 ml
H_2O	:	9.3 ml	1.3 ml
10% SDS	:	0.1 ml	0.1 ml
10% APS	:	0.044 ml	0.1 ml
TEMED	:	0.0067 ml	0.0067 ml

The gradients were cast using a gradient marker (BRL). The solution was degassed before adding TEMED and freshly made APS. After adding these components, the solution was carefully mixed and poured into the assembled vertical plates up to 2 cm from the top of the notched plate. The resolution gel was then carefully overlaid with either acetone or water. The gels were left to polymerize for at least 30 min. The overlay was removed and the gel-top rinsed with water and dried with blotting paper before pouring the stacking gel solution (1.02 ml of 30% (w/v) acrylamide, 2.5 ml of stacking gel buffer, 6.1 ml of H_2O, 0.1 ml of 10% (w/v) SDS, 0.05 ml of 10% (w/v) APS and 0.01 ml of TEMED). The

stacking gel was also degassed before adding APS and TEMED. It was then poured as far as the top of the notched plate and overlaid as described for the resolution gel.

3.5.4.2 Running of the IEF gel on the slab gel

Before the IEF gel was applied on top of the slab gel, the stacking gel was rinsed with water and dried with blotting paper. All the clamps were removed together with the bottom spacer and the plates laid flat or slightly inclined to facilitate the application of the first dimension gel. The latter was removed from the equilibrating solution and carefully laid on top of the stacking gel, avoiding any stretching. Then 2-3 ml of molten 0.5% agarose solution (0.025 M Tris pH 6.8, 0.192 M glycine, 0.1% (w/v) SDS, 0.5% (w/v) agarose) was used to keep the IEF gel in place. Using a Pasteur pipette, the gel was completely covered with the agarose solution. Any trapped air bubbles were eliminated and the agarose left to set before the slab gel was clamped to the electrophoresis chamber (BRL). Running buffer (25 mM Tris pH 8.3, 192 mM glycine, 0.1% (w/v) SDS) was then added to the upper and lower tanks. Using running buffer in a 20 ml syringe with a bent needle, air bubbles were eliminated from the bottom of the gels to allow good contact with the buffer. Electrodes were connected, the voltage set at 300 V and the gels ran at a constant current of 30 mA for 7 h.

3.6 Silver staining

After SDS-PAGE, the gels were stained for protein using silver (Wray *et al.*, 1990). They were first fixed in 50% (v/v) methanol-10% (v/v) acetic acid for 2 min. Each gel was then transferred to a staining dish containing 50% (v/v) methanol, and washed for 20 min. Next, it was washed in dH_2O for 5 min and again washed in 50% (v/v) methanol

for 20 min. The methanol was discarded, and the gel washed in 1% (v/v) aqueous glutaraldehyde for 30 min. At this stage, the gel was either stored in dH_2O overnight or washed with 5 changes of dH_2O at 5 min intervals. It was further fixed in 50% (v/v) methanol for 20 min before staining. About 4 min before staining, the staining solution for each gel was prepared as follows: Solution A: 0.8g $AgNO_3$ in 2.5 ml dH_2O and Solution B: 1.0 ml of 2 M NaOH in 20 ml dH_2O plus 1.6 ml of conc. ammonia solution. While stirring, Solution A was slowly added to Solution B, and dH_2O added to make 100 ml. The gel was then stained with this silver solution for 15 min. After discarding the solution, the gel was washed with 3 changes of dH_2O at 5 min intervals. Meanwhile, the colour developer, to be used within 5 min, was made by adding 150 µl of 37-41% (v/v) formaldehyde solution to 2.5 ml of 1% (w/v) citric acid, and making upto 250 ml with dH_2O. The gel was then covered with the developer and shaken until a satisfactory staining was observed. Colour development was stopped using 5% (v/v) acetic acid. Gels were then either photographed immediately, or stored in 5% (v/v) acetic acid, sealed in plastic bags. The individual proteins were resolved as discrete spots, and were quantified by direct counting. In order to easily locate the same spot on two different separations, the surrounding spots were used for orientation (O'Farrel *et al.*, 1977).

3.7 Tick DNA preparation and analysis

3.7.1 DNA extraction

Autoclaved or sterilized solutions, tubes, pipette tips were used, and gloves worn to prevent contamination from nucleases and extraneous nucleic acids. The DNA was extracted from the legs of individual *R. appendiculatus* ticks (Doyle and Doyle, 1987, 1990; Stevens and Wall, 1995). After briefly rinsing the ticks in absolute ethanol, all the

eight legs of each tick were detached and placed in a 1.5 ml Eppendorf tube. Using sterile disposable plastic grinders, the tick legs were rapidly pulverised in liquid nitrogen to powder. To this, 100 µl of CTAB extraction buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.7 M NaCl, 1.0% (w/v) CTAB, 0.1% (v/v) ß-Mercaptoethanol) pre-warmed to 60°C was added and the tube inverted several times to disperse the lamp. This was incubated at 60°C for 15 min in a water bath, with occasional gentle mixing by inversion. Next, 100 µl of chloroform was added and the tube gently inverted until an emulsion formed. This was followed by centrifugation at 13000 rpm at 25°C for 10 min in a table top centrifuge (Eppendorf GmBH, model 5415C). Using sterile, disposable Pasteur pipettes, the aqueous phase was placed in a fresh Eppendorf tube followed again by addition of chloroform, mixing, and centrifugation as described above. The now clear aqueous phase was transferred to a fresh tube. To this, 0.6 volumes (60 µl) of isopropanol were added, followed by mixing by inversion to precipitate the DNA. This was incubated at 25°C for 10 min, followed by centrifugation at 13000 rpm at 25°C for 10 min. All liquid was then carefully removed without disturbing the pellet. Next, 500 µl of 70%e ethanol was added, followed by inversion for several times to mix. The sample was centrifuged again for 5 min and the liquid carefully removed without disturbing the pellet. The latter was then vacuum dried for 15 min. The DNA pellet was resuspended in 50 µl of TE buffer and incubated at 60°C prior to storage at -20°C. The quantity of the DNA was assayed by measuring the absorbance of a 1:1000 dilution at 260 nm in a Beckman model DU 640 spectrophotometer.

3.7.2 RAPD-PCR amplification

3.7.2.1Primers

A total of 35 random decamer primers (Operon Technologies, Alamada, CA, USA) were screened. Only primers that gave polymorphic bands were selected for subsequent use. These were primer OPA-05 (5'-AGGGGTCTTG-3'), OPA-10 (5'-GTGATCGCAG-3'), OPA-11 (5'-CAATCGCCGT-3'), OPA-16 (5'-AGCCAGCGAA-3') and OPA-20 (5'-GTTGCGATCC-3').

3.7.2.2 PCR amplification

All reaction tubes, pipette tips, and water (dH_2O) were sterilized to destroy any possible contaminating surface DNA (Gawell and Bartlett, 1993) and all manipulations done in a Laminar flow hood. The RAPD-PCRs were set up in a final volume of 25 µl containing 20 ng genomic DNA, 5 picomoles of single decamer primer, 2.5 µl of 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.4, 1 mg gelatin/ml) (Promega, Madison, USA), 2 µl of 25 mM $MgCl_2$ (2 mM $MgCl_2$), 1 µl of 5 mM dNTP (0.2 mM dNTP), 0.4 µl (2 units) *Taq* DNA polymerase, and 8.5 µl sterile water, in a 0.5 ml Microfuge tube. After thorough mixing, the contents were centrifuged at 13000 rpm at 25°C for 4 sec and one drop of mineral oil added to each reaction tube to prevent evaporation of the samples. The amplifications were done in a PTC-100™ programmable thermal controller (MJ Research, Inc., Watertown, USA).

The following programme was used:

1. Initial denaturation - 94°C for 4 min
2. Denaturation - 94°C for 1 min
3. Primer annealing - 45°C for 1 min
4. Primer extension - 72°C for 2 min
5. Repetition of steps 2-4 - 39 times
6. Final primer extension - 72°C for 10 min
7. Incubation - 4°C (Innis *et al.*, 1990).

The PCR products were kept at 4°C. The consistency of amplified products was verified by running at least two reactions of the same template DNA. The RAPD-PCR products were separated at 4.7 V/cm for 2 h in 2% agarose gels in 0.5 TBE pH 8.3 buffer. The DNA fragments were visualized after staining in 5 µg/ml ethidium bromide and photographed on a 310 nm UV translluminator (Sambrook *et al.*, 1989). A negative control containing all the reaction components except template DNA was included.

3.7.3 Analysis of RAPD-PCR markers as alleles

Fifty four tick DNA samples from each population were subjected to RAPD-PCR using each of the above five primers. In total, 32 bands that were amplified by the 5 primers were scored. This is far less than the total number of amplified bands. Only those bands that were well amplified and showed clear presence or absence of polymorphisms among individuals were included in the data analysis. Since RAPD-PCR is not a quantitative technique (Shufran and Whalon, 1995), the intensity of the bands was not measured. Instead, scoring was done only for presence or absence of well-amplified bands less than 2.6 kb. Pools were made by mixing equal quantities of DNA from 54 individuals of the

same population.

The following assumptions were used in data analysis (Clark and Lanigan, 1993; Lynch and Milligan, 1994; and Apostol *et al.*, 1996):

(i) There is random mating within the populations

(ii) The populations were analysed at a time when the genotype frequencies were in Hardy-Weinberg equilibrium.

(iii) Each locus is a two-allele system, with only one of the alleles per locus being amplifiable by PCR. Thus, it is assumed that RAPD products segregate as dominant alleles in a Mendelian fashion. If each locus is biallelic, then every observable band represents a unique locus.

(iv) Alleles in both homozygous recessive and dominant individuals are identical in state (i.e. that they arose from identical mutations) among and within individuals.

(v) The amplification of a product depends strictly on the exact match between the oligonucleotide primer and a site on the DNA template.

(vi) The interpretation of banding patterns on gels can be accomplished in a completely unambiguous manner. It is thus assumed that marker alleles from different loci do not comigrate to the same position on the gel.

3.7.3.1 Genetic fingerprinting.

Genetic fingerprinting involves comparison of genetic markers among individuals and requires pair-wise comparison of measures (Weir, 1990; Black, 1993). The similarity of individuals was measured by scoring both the shared presence and shared absence of a band. The presence of a band was entered into the computer as "1", the absence of a band

as "0", and missing data as "•" (Appendix III). The proportion of matches (M) was estimated using the following formula (Black, 1993):

$$M = N_{AB}/N_T$$

Where N_{AB} is the total number of matches (both bands present or absent) in individuals A and B, and N_T is the total number of bands scored in the overall study. An M value of 1 indicates that two individuals have identical patterns; a value of 0 indicates that two individuals share no band in common. M values were used to construct a symmetrical distance matrix, which was collapsed to construct a dendogram using UPGMA with a FORTRAN program RAPDPLOT (Black, 1993).

CHAPTER 4

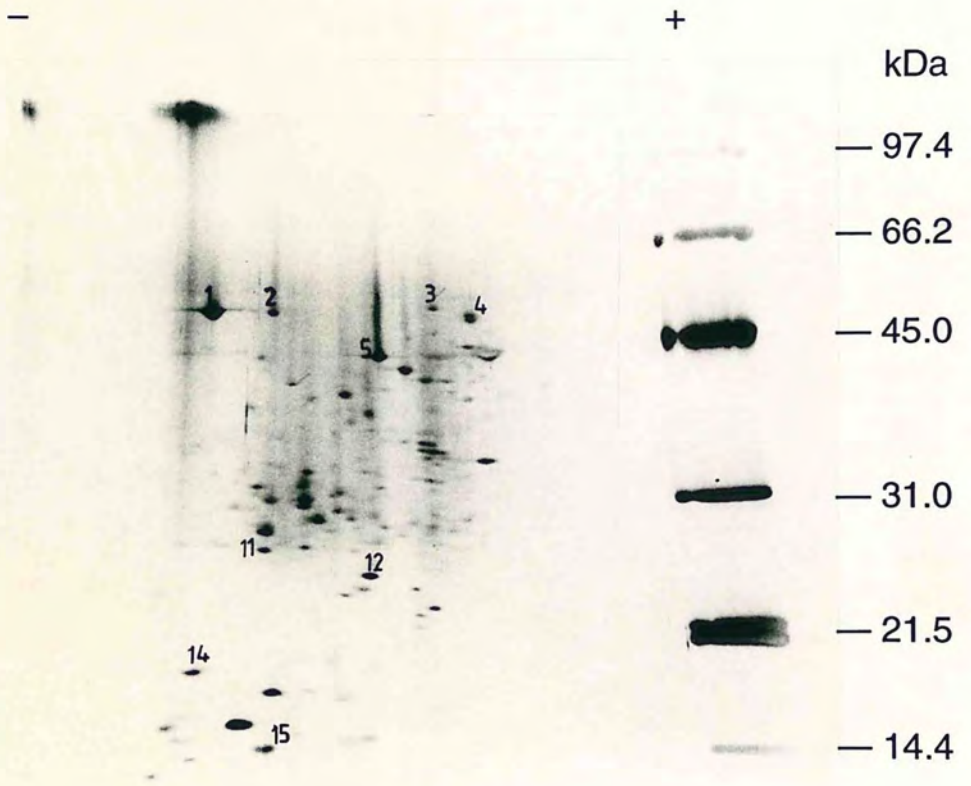
RESULTS AND DISCUSSION

4.1 2-DE results and discussion

Initial attempts were made to extract and analyze the tick cuticular proteins. The cuticular proteins were extractable in a buffer containing 7 M Urea, 5 mM Tris pH 8.6, 1 % (v/v) NP-40, 1 mM PMSF and also in another buffer containing 9 M Urea, 4% (v/v) NP-40, 2% (v/v) Ampholines pH 9-11 and 2% (v/v) ß-mercarptoethanol (Dunbar, 1987). The protein concentrations were high: 16.2 mg/ml and 32.3 mg/ml, respectively. Extraction of the cuticle proteins using a buffer containing 6.25 mM Tris, pH 6.8, 1% (v/v) NP-40 and 2% (v/v) ß-mercarptoethanol gave only 0.38 mg/ml. However, the cuticular proteins precipitated at the top of the IEF gel and could therefore not be resolved by IEF. On the other hand, when whole ticks were homogenized in the latter buffer, concentrations of at least 6.7 mg/ml were obtained, and no precipitation was observed during IEF. The protein separations by this technique were so reproducible that it was possible to match each spot on one separation with a spot on a different separation.

The dark spots (Plates 1, 2) represent the silver stained whole-tick proteins. Protein spots of interest were numbered 1 to 18 (Plates 1, 2). The un-numbered protein spots were either common to both populations or weakly-stained or overcrowded together. Only four of the shared proteins: 1, 3, 5 (M_r~45-66,000) and 12 (M_r~26,000) were numbered. Most of the proteins were common to both populations. However, proteins 2, 4 (M_r ~47-50,000), 11 (M_r ~26,000), 14 and 15 (M_r ~14.4-19,000) (Plate 1) were specific to the Muguga population, whereas proteins 6, 7, 8, (M_r ~45-48,000), 9 (M_r ~41,000), 10 (M_r ~29,000), 13 (M_r ~22,000) and 16, 17, 18 (M_r ~14,000) (Plate 2) were specific to the

Rusinga population. Weakly stained proteins (whether shared or populations-specific) were ignored.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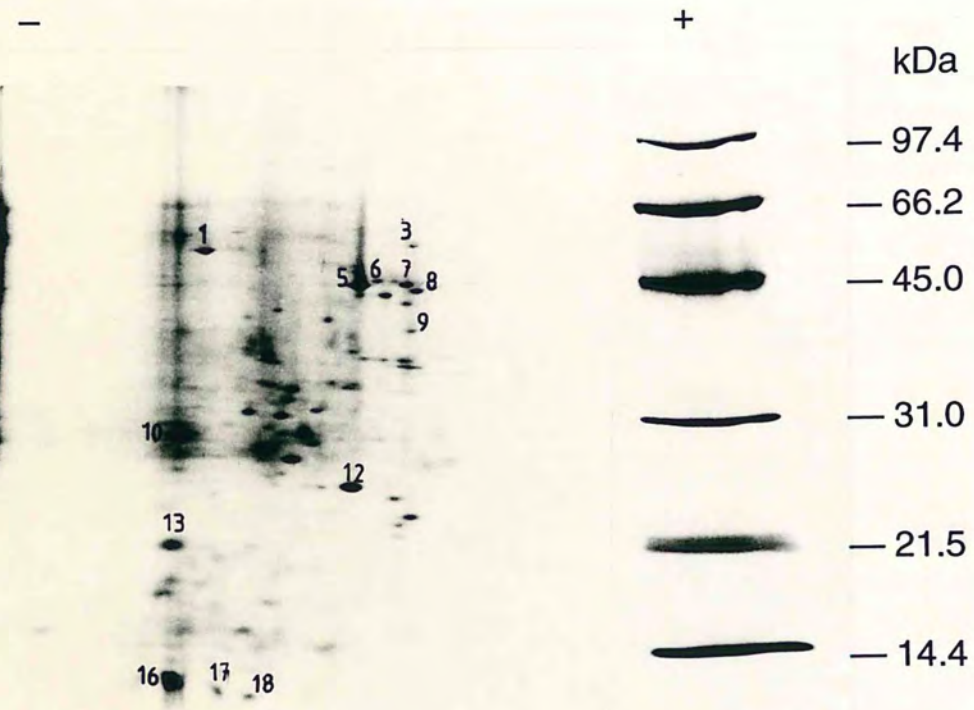
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Over the years, most population genetic studies have been done by comparing protein electrophoretic patterns, and more recently by DNA analysis (Cornel *et al.*, 1996; Gooding, 1996). However, it is not advisable to rely on only one technique, since all the available techniques have limitations (Tait, 1985; Black, 1993). It is because of this that both whole tick protein analysis by 2-DE and genomic DNA analysis by RAPD-PCR have been carried out in the present study.

For protein analysis, 2-DE has been used, because no uni-dimensional technique can adequately analyse proteins from ticks, since ticks have a complex biological system (O'Farrel, 1975; Sonenshine, 1991). Both IEF and the discontinuous SDS gel system of Laemmli (1970) have been used because of the high resolution of each system and because proteins are separated according to different properties. This gel system can be used in combination with genetics because even a single charge change can produce a detectable change in the position of a protein spot (O'Farrel, 1975).

Proteins such as 1, 3, 5 and 12 which were found in both populations are probably species specific. The presence of proteins 2, 4, 9, 11, 14 and 15 only in the Muguga population and proteins 6, 7, 8, 10, 13, 16, 17 and 18 only in the Rusinga ticks suggests that there may be genetic differences between the two populations. This is because the presence of a protein is sure evidence of a structural gene, and the protein electrophoretic technique detects changes in proteins coded for by the structural genes (Suzuki *et al.*, 1986; Horodyski and Riddiford, 1989). The difference in the banding patterns of the proteins on the gels may be a result of some changes in the structural genes, which in turn lead to changes in the amino acid compositions of the polypeptides. This results in mass and charge differences in the proteins. In effect, the individual proteins have different pIs and charge densities, and migrate at different rates. Since the resolution of charge differences depends

on molecular weight and pI, and since 2-DE separates proteins according to these parameters, the degree of resolution of even single charge differences can be predicted from the position on the gel (O'Farrel, 1975; Dunbar, 1987).

It is possible that some of the observed population-specific proteins may be responsible for the observed variations in vector competence (Kubasu, 1990, 1992; Kiara and Osir, unpublished). However, these protein data alone are not enough for a firm conclusion in this respect. This is in view of the fact that the genetic code is redundant, in that a number of different codons can determine the same amino acid (Adams *et al.*, 1992; Clark and Russell, 1997). Many nucleotide changes will therefore go undetected (Tait, 1985). By inference, some nucleotide changes (which could reduce or enhance vector competence) will not be detected by the protein electrophoretic method. Also, since the method detects charge changes, it is possible that some amino acid changes (and hence nucleotide substitutions) can escape detection. This is if no charge change in the protein occurs, as is the case where a neutral amino acid is replaced by another neutral amino acid (Tait, 1985; Knudson *et al.*, 1996). Thus, if the differences in vector competence are due to changes such as a substitution of one neutral amino acid with another neutral amino acid, or due to changes in regulatory elements, then the observed protein variation could be misleading in this respect. The differences could as well be due to genes (and hence proteins) other than those responsible for vector competence. This is in view of at least two possibilities. Firstly, the first dimension does not generally resolve proteins with a pI greater than 7, even when basic Ampholines are used (Piperno *et al.*, 1977; Maizels *et al.*, 1991). In addition, some proteins have pI values beyond the range of commercially available Ampholines (O'Farrel, 1975). Such proteins will either be lost or remain on top of the IEF gel (O'Farrel, 1975; Sinclair and Rickwood, 1981; Andersen *et al.*, 1986). For example,

Andersen *et al.* (1986) characterized *Locusta migratoria* cuticular proteins using 2-DE. Most of the proteins had pI values close to 11. The presence of highly basic proteins made isoelectric focusing of the locust cuticular proteins difficult. Due to cathodic drift, the proteins tended to migrate out of the gels before complete equilibration could be achieved (Andersen *et al.*, 1986). Similarly, if the gene products responsible for vector competence have a very basic pI, they may not be available for detection.

Secondly, since the technique is biased towards loci with more abundant gene products, loci with minuscule gene products may not be detected. Thus, if the latter loci are the ones carrying the genes for vector competence, then their gene products may escape detection. However, silver staining can detect as low as 0.02 ng protein/mm^2 (Celis and Bravo, 1984). Considering the sensitivity of this technique and the observed number of population specific proteins, it is still possible that some of those gene products may be responsible for *T. parva* susceptibility or refractoriness in the ticks.

In general, however, protein electrophoretic methods are relatively insensitive means of detecting genetic diversity, since only about 30% of amino acid substitutions will be detected (O'Farrell, 1975; Tait, 1985). Moreover, this estimate overlooks the fact that "silent" substitutions are not detected either. If these are also considered, then the sensitivity of detecting variation in nucleotide sequence of the gene under study is even lower. These considerations raise two important points. Firstly, there is need for alternative and more sensitive methods for detecting genetic diversity. Secondly, given the limited sensitivity of protein electrophoresis to detect genetic diversity, extreme caution must be exerted in concluding that two organisms or populations are identical or different based on protein electrophoretic criteria alone.

4.2 RAPD-PCR results and discussion

Amplification of tick DNA with the primers resulted in a series of discrete fragments (Plates 3-8). Polymorphisms were detected both within and between the two tick populations. This was both in the pooled and individual DNA samples. In the pooled samples (Plate 3), primer OPA-05 gave two bands (400-500 bp) and three bands (700-900 bp), specific to Muguga, and two bands (~1400 bp), specific to Rusinga. For primer OPA-10 one band (~400 bp) was specific to Rusinga. For primer OPA-11, two bands (~480 and 700 bp) were specific to Rusinga. For primer OPA-16 one band (~250 bp) was specific to Muguga, and one band (~1500 bp) was specific to Rusinga. For primer OPA-20, a total of five bands were specific to the Muguga population: 1 band (~250 bp), 1 band (~450 bp), 2 bands (~550-600 bp) and 1 band (~2070 bp). In the pooled DNA, the two populations were readily distinguishable by their RAPD patterns (Plate 3, arrows point at population-specific bands), although some primers (e.g. OPA-05) gave more population-specific fragments within patterns than the others (e.g. OPA-11).

However, individual DNAs showed considerable variation among individuals within each population (Plates 4-8). With primer OPA-05 (Plate 4), the Mw of bands in the Muguga population (lanes 3-20) ranged from ~150 bp (lanes 3, 5, 9) to ~2000 bp (lanes 6, 9, 10, 11, 12, 13, 14, 15, 16, 18). The number of amplified bands per template ranged from 3 (~200-500 bp, lane 24) to 11 (~200-2000 bp, lanes 9, 10, 12). For Rusinga (lanes 22-39) the band Mw range was ~180 bp (lanes 25, 30, 33, 37) to 2642 bp (lane 24). The number of amplified bands ranged from 1 (~1000 bp, lane 26) to 12 (~400-2000 bp, lane 27; ~180-1400 bp, lane 37). Some templates did not amplify (lanes 8, 23). Some bands were easy to score (e.g. mark a), but others were ambiguous (e.g. mark b).

For primer OPA-10 (Plate 5), the Mw of bands in Muguga (lanes 3-20) ranged

from ~200 bp (lane 13) to ~1300 bp (lanes 4, 5, 6, 7, 8, 9, 14, 16, 19). The number of amplified bands per template ranged from 1 (~250 bp, lane 17) to 13 (~300-1300 bp, lane 8). In Rusinga (lanes 22-39), the band Mw range was from ~180 bp (lane 25) to ~1500 bp (lane 23). The number of amplified bands ranged from 3 (~300-700 bp, lane 24; ~200-700 bp, lane 27) to 12 (~280-1300 bp, lane 37).

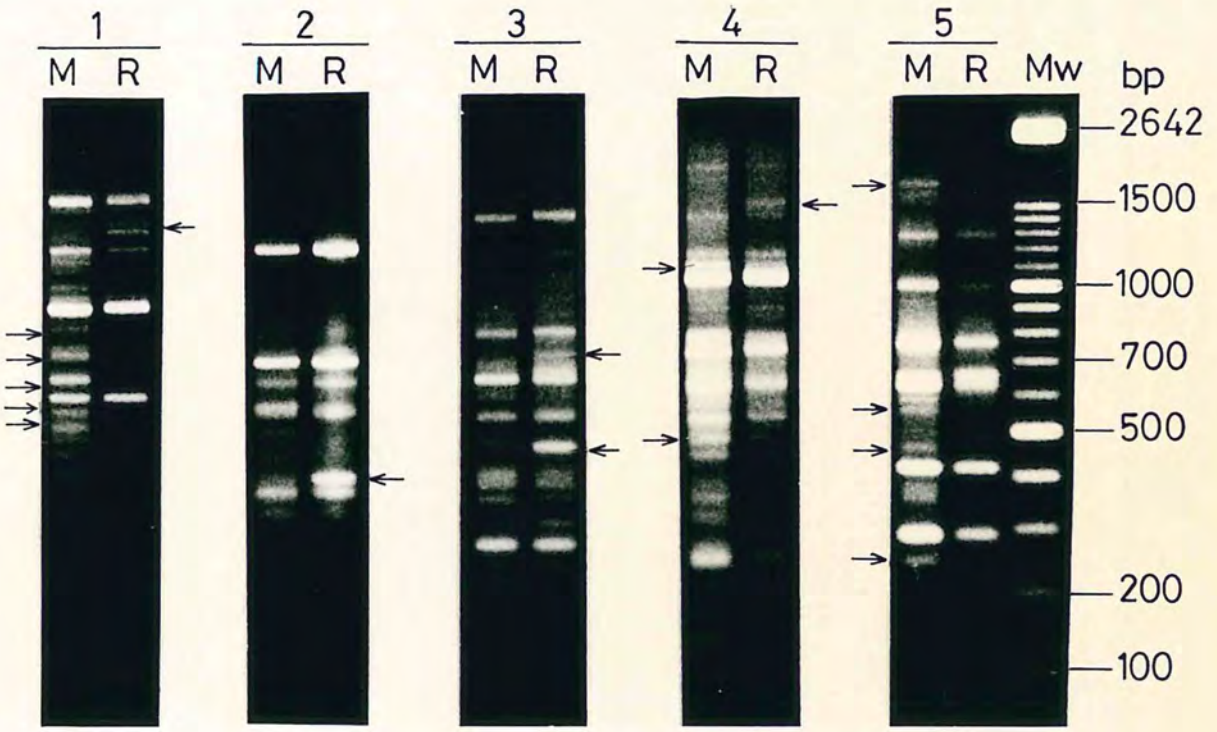
For primer OPA-11 (Plate 6), the Mw of bands in Muguga (lanes 3-20) ranged from ~100 bp (lanes 3, 5, 9) to ~900 bp (lane 18). The number of amplified bands ranged from 1 (~250 bp, lane 6) to 13 (~200-900 bp, lane 18). In Rusinga (lanes 22-39), the band Mw ranged from ~200 bp (lanes 34, 38, 39) to ~1000 bp (lanes 38, 39). The number of amplified bands ranged from 2 (~200-100 bp, lane 38) to 11 (~200-800 bp, lane 34).

For primer OPA-16 (Plate 7), the Mw of bands in Muguga (lanes 3-20) ranged from ~150 bp (lanes 3, 4, 5, 7, 16) to ~2600 bp (lane 10). The number of amplified bands ranged from 2 (~350-2200 bp, lane 6) to 13 (~250-2600 bp, lane 10). For Rusinga (lanes 22-39), the Mw of the bands ranged from ~140 bp (lane 38) to ~2600 bp (lanes 24, 31, 32, 35). The number of amplified bands ranged from 2 (~140-500 bp, lane 38) to 18 (~250-2600 bp, lane 32).

For primer OPA-20 (Plate 8), the Mw of bands in Muguga (lanes 3-20) ranged from ~250 bp (lanes 12, 19, 20) to ~1300 bp (lane 20). The number of amplified bands ranged from 3 (~250-950 bp, lane 19) to 9 bands (~250-850 bp, lane 12). For Rusinga (lanes 22-39), the Mw of the bands ranged from ~180 bp (lane 38) to ~2200 bp (lane 35). The number of amplified bands ranged from 1 (~1600 bp, lane 26) to about 13 (~250-1500 bp, lane 32; ~150-2200 bp, lane 35). Some bands were ambiguous (e.g. lane 22, 500-900 bp). Some templates did not amplify (e.g. lanes 6, 8, 23).

In both the pooled and individual DNA samples, the bands were found to vary in

intensity. Faint bands were found to be sporadically amplified. The presence of some faint bands complicated the scoring procedure and necessitated repeating amplification of some samples. Only consistently amplified bands were included in the RAPD data set (Graham *et al.*, 1994). These were identified by performing replicate reactions in a single run on DNA extract from each individual and then repeating this over several consecutive runs. Variations in band intensity were ignored. The genetic distance between the two populations averaged 0.047 (Fig. 1).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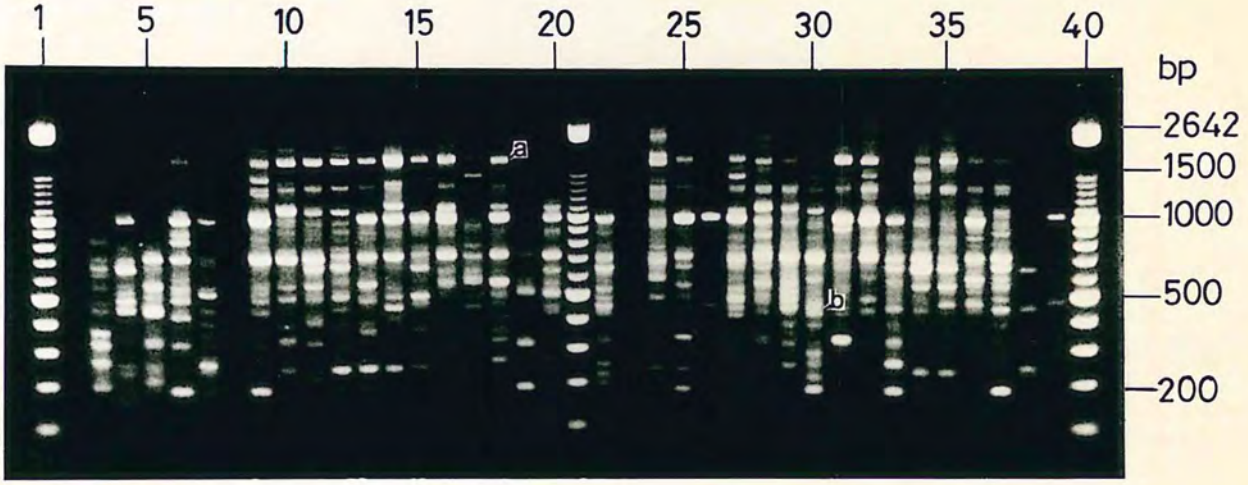
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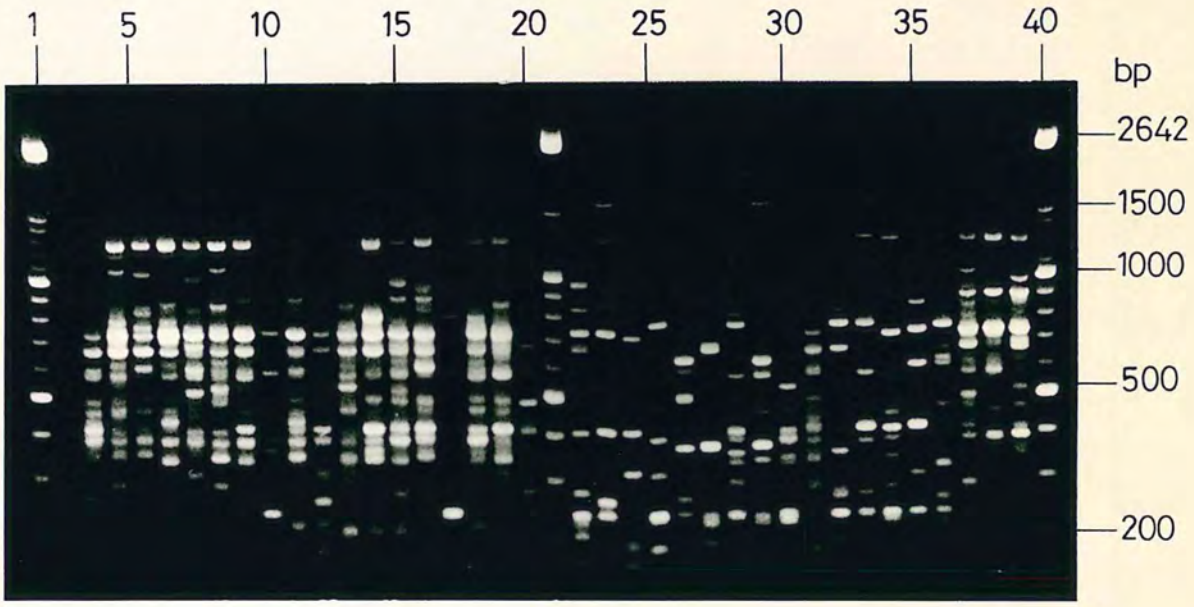
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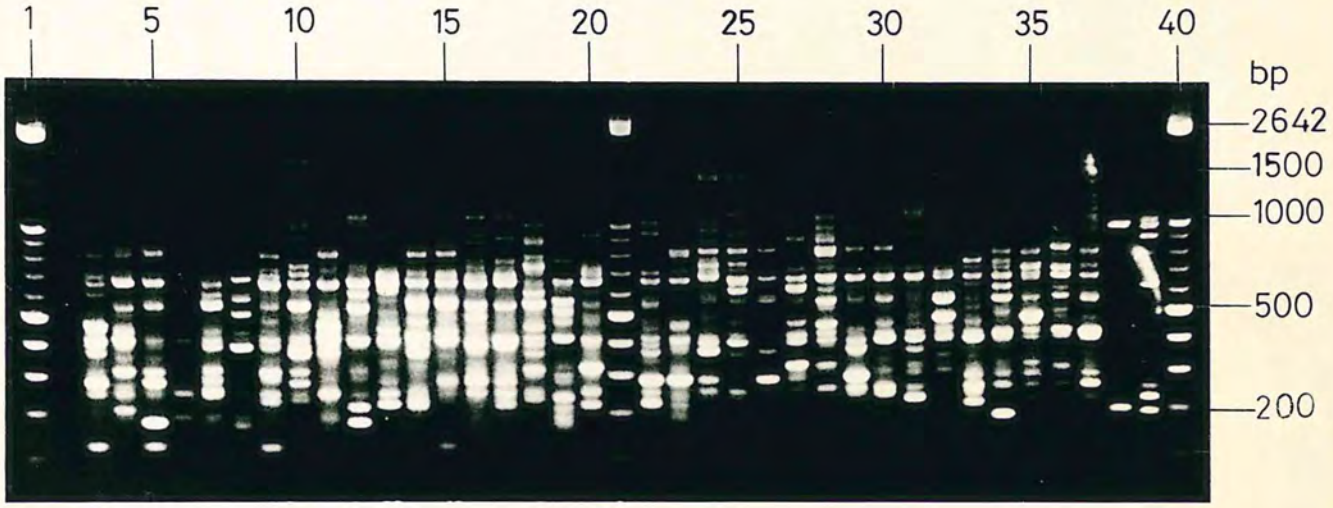
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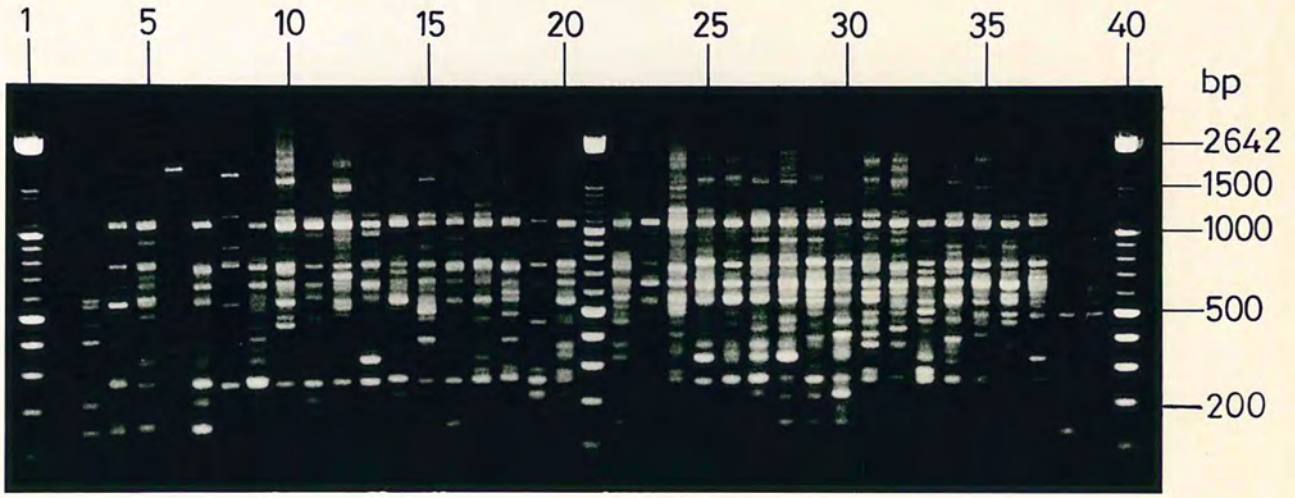
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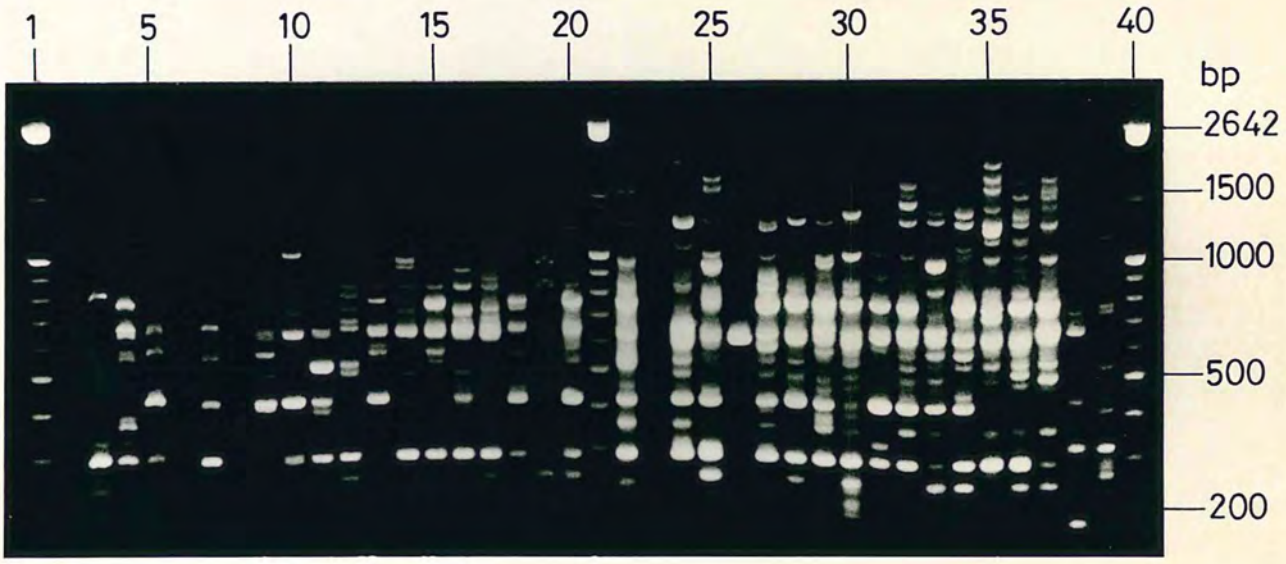
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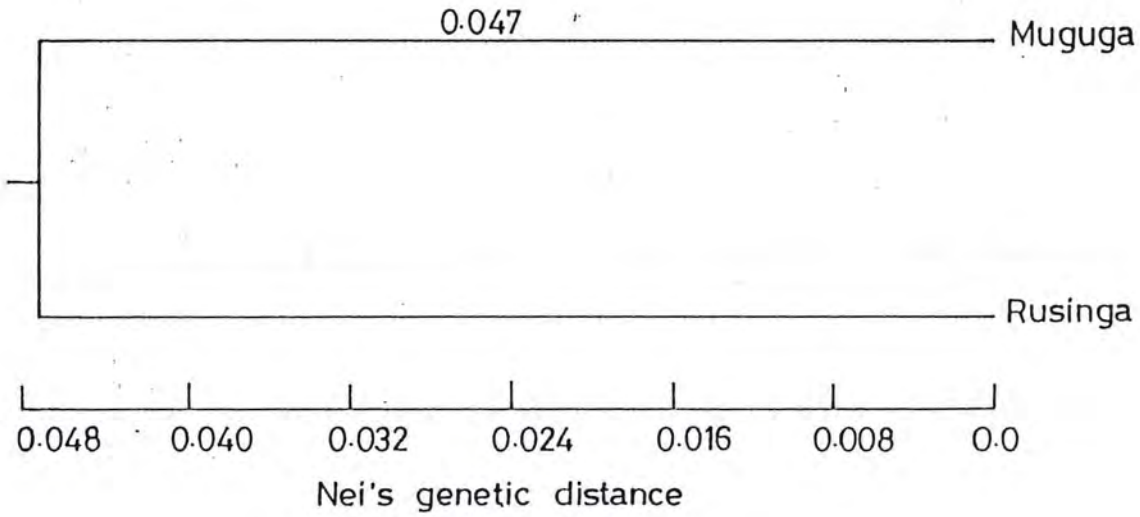
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0·040
0·032
0·024
0·016
0·008
0·0
Nei's genetic distance

Pools of genomic DNA from each population were used to screen the primers. This was based on previous studies. For example, pooled genomic DNA has been used in the identification of markers of loci controlling disease resistance in plants (Michelmore *et al.*, 1991), and in differentiation of bovine species (Kemp and Teale, 1994). Wilkerson *et al.* (1993) used pooled samples of genomic DNA from cryptic mosquito species to investigate the ability of 57 primers to produce RAPD patterns. Gwakisa *et al.* (1994) also used pooled DNA to characterize Zebu cattle breeds in Tanzania by the RAPD-PCR technique. The rationale is that the RAPD fragments unique to each pool will have a higher probability of being linked to the gene controlling the trait of interest, and will mask possible intra-population variability (Milchelmore *et al.*, 1991).

The observed DNA polymorphisms are a result of either chromosomal changes in the amplified regions or base changes that alter primer binding (Milchelmore *et al.*, 1991). Some polymorphisms were easy to score (e.g. Plate 4, mark a); but others appeared ambiguous and hence not useful as genetic markers (e.g. Plate 4, mark b). Ambiguous polymorphisms may result from poor discrimination by a primer between alternative priming sites of slightly different nucleotide sequences (Williams *et al.*, 1990). The presence of population-specific DNA bands (Plate 3, arrows) suggests that genetic differences exist between these two tick populations.

In this study, 54 samples were examined per population. This is because some population genetic studies have shown that a random sample of at least 50 is necessary to reliably estimate genetic diversity (Kiang *et al.*, 1992). If samples are too small, inaccurate estimates of genetic variability might be obtained. Nei (1978) pointed out the magnitude of the systematic bias introduced by a small sample size when ordinary methods of estimating average heterozygosity and genetic distance are used. Nevertheless, he also showed that the

number of individuals can be very small, if a large number of loci are studied. Indeed, as few as 25 samples or less have been reliably used in some genetic biodiversity studies (Kiang *et al.*, 1992; Gwakisa *et al.*, 1994).

As seen in figure 1, a distance of 0.047 was found between the Muguga and Rusinga populations. The genetic distance (*D*) is defined as the accumulated number of gene differences per locus (Nei, 1972). It provides an estimate of the mean number of mutations or substitutions separating the genes from two populations. The measurement of *D* is based on the identity of genes (*I*) between two populations. The identity of genes is a measure of the proportion of genes that are common in both populations under investigation. If there are no common alleles between the two populations $I = 0$, but when the two populations have the same alleles in identical frequencies, then $I = 1$ (Nei, 1972). Mathematically, *D* is defined as: $D = -\ln I$ (Nei, 1972; Tabachnick and Black, 1996). Thus, *D* is inversely proportional to *I*. If the *I* between populations is high, *D* will be small. Conversely, if *I* is low, *D* will be big. Thus, the *D* of 0.047 between the populations in the present study is rather small. This implies that the identity of genes between these populations is quite high.

As mentioned, over 90% of the RAPD markers are dominant (Williams *et al.*, 1990), and the RAPD technique is unlikely to detect homozygous-recessive markers (Lynch and Milligan, 1994; Apostol *et al.*, 1996). If RAPD-PCR detects 90% of the amino acid substitutions, it means that the average number of gene differences between the two populations is only about 0.05 per locus. Thus, these two populations share about 95% of common genes. Related individuals are expected to have more similar genotypes than non relatives. Thus, the fraction of loci for which two individuals or populations have identical phenotypes must increase with the degree of relationship. The small genetic distance

between the Muguga and Rusinga tick populations suggests that these two populations are closely related, but not identical — since D is greater than zero. Thus, some genetic differences do exist between the two populations.

Although the mobility of ticks is inherently low, that of their animal hosts is high. It is probably this latter factor that is responsible for the lack of significant genetic divergence over wide geographic areas (Hilburn and Sattler, 1986; Sattler *et al.*, 1986). The observed low level of genetic divergence suggests that there will probably be no genetic barriers to biological and other tick control operations between these populations (Sattler *et al.*, 1986).

CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

Many laboratory studies on arthropods are carried out on the assumption that colony material is similar to material from natural populations (Gooding, 1996). In this study however, only field-collected ticks were used for the DNA work, and their F_1 cohorts used for protein extraction (DeFoliart *et al.*, 1987). This is because long-colonized species may have reduced genetic variability due to factors like founder effects, genetic drift, and selection (DeFoliart *et al.*, 1987; Gooding, 1990).

Male *R. appendiculatus* only were used in this study. This avoided contamination of adult DNA with DNA from eggs. In addition, the possibility of contamination from ingested protein was also avoided since, unlike females, males do not require a proteinaceous meal prior to reproduction. By using only legs, the possibility of contaminating sample DNA with DNA from gut parasites or symbioants was also avoided (Stevens and Wall, 1995). The use of males alone could have limited the degree of variation observed. However, preliminary RAPD studies on pooled DNAs of other *R. appendiculatus* populations (Baragoi, Embu, Lanet and Maralal) have shown population-diagnostic bands in both sexes (Osir, unpublished observations). In addition, work on other arthropod species indicates that any study on either sex has the potential to cover the significantly major proportion of all genetic variation (Foster *et al.*, 1980; Stevens and Wall, 1995).

The RAPD-PCR method has previously been shown to detect high levels of DNA

variation where little or no protein variability was detected (Black *et al.*, 1992; Begun and Aquadro, 1993; Black, 1993; Edwards and Hoy, 1993; Peakall *et al.*, 1995). The enhanced ability to detect variation with RAPDs may lie in the physical requirements for the reaction to amplify DNA. For a region to be amplified, primers must anneal no further than 3 kb apart (Black, 1993; Haymer, 1994). Thus, regions of the genome that contain repetitive sequences or inverted terminal repeats may contain a greater number of sites amenable to RAPD amplification than do coding-sequence regions. The reason RAPDs can detect more variation within and among individuals therefore seems to be the sheer number of markers available, not withstanding the partial loss of resolution attendant upon using dominant markers (Gwakisa *et al.*, 1994; Peakall *et al.*, 1995; Tabachnick and Black, 1996). Re-arrangement, translocation and amplification of repetitive sequence DNA is a fundamental process of genome evolution (Rogers, 1985). Repetitive regions are targeted in genetic fingerprinting research because of their inherent variability (Jeffreys *et al.*, 1985a, b; Haymer, 1994). Perhaps it is the variability of these repetitive sequences that is the source of the variation detected by the RAPD-PCR procedure. However, Williams *et al.* (1990), working with *Glycine soja* and *G. max*, cloned 11 RAPD-PCR amplified fragments and used them as probes of total digested genomic DNA in a Southern analysis. Only 5 of these fragments were found to be repetitive. This indicates that not all amplified regions are repetitive. Thus, some of the amplified regions could be single-copy sequences (Kambhampati *et al.*, 1992; Haymer, 1994).

Single base changes can cause a complete change in the set of amplified DNA segments (Williams *et al.*, 1990). By inference, a single base change in the genome may also prevent amplification by introducing a mismatch at just one end of a DNA segment (Williams *et al.*, 1990). This could explain the presence of polymorphic bands and the

failure of amplification of some templates with certain primers (e.g. Plate 4, lanes 8, 23; Plate 8, lanes 6, 8, 23).

The studies discussed here illustrate the application of protein and DNA electrophoresis to distinguish between groups of morphologically very similar organisms and examining the population structure of arthropod vectors. They show the potential of using biochemical methods to gain insight into the population genetics of vectors and the epidemiology of diseases in situations where morphological and other criteria are of little use. The results seem to agree with the proposal that tick populations from different geographical areas are biologically different (Kubasu, 1990, 1992). As already mentioned, proteins are gene products, and differences have been observed both in the proteins and DNA of the two tick populations examined. This strongly suggests that these two tick populations are genetically different. These observations also raise the possibility that the reported differences in vector competence may be accounted for by genetic differences. It has been reported that the susceptibility of blood-sucking arthropods to pathogens is controlled by genes, or by maternally inherited factors (Gooding, 1992). Genetically-induced inter-population differences in the levels of susceptibility to pathogens has been demonstrated in other vector species. For example, in the mosquitoes, genetic variation has been shown to affect their ability to transmit filarial and malarial parasites (Kafatos *et al.*, 1994; Mutebi *et al.*, 1997). Collins *et al.* (1986, 1997) have documented heritable *Plasmodium* resistance in *Anopheles gambiae*. Many workers have documented the immune mechanisms in various arthropods against pathogens, and all seem to have a genetic basis (Ratcliffe *et al.*, 1985; Maudlin, 1985; Maudlin *et al.*, 1986, 1991; Hay, 1993; Horton and Ratcliffe, 1993; Paskewitz and Christensen, 1996).

The observed protein and DNA polymorphisms in this study are no surprise since any isolated populations of a species are expected to gradually diverge genetically (Ferguson, 1980; Post *et al.*, 1992; Gawell and Bartlett, 1993). Protein electrophoresis and DNA sequence analyses have revealed remarkable variation in natural populations of many species (Tait, 1985; Aquadro, 1992). Geographically isolated populations will drift in their genetic constitutions for a number of reasons. One of the reasons is the inevitable inbreeding, because of spatial proximity. Consider a population founded by a small number of individuals who mate at random to produce the next generation but no further immigration into the population ever occurs again. Excessive contribution to the gene pool by a few individuals will reduce the genetic variability in subsequent generations (Szalanski *et al.*, 1996). Thus, with time, everyone will be related to everyone else. This is because a back-trace of their family trees will reveal them to have common ancestors here and there in their pedigrees. Such a population is then inbred, in a sense that there is some probability of a gene being homozygous by descent. As generations go by, this probability will gradually increase until it finally reaches unity, so that the population is totally homozygous (Suzuki *et al.*, 1986). If this population was founded from a larger, heterozygous population and the two populations remain completely isolated from each other, each will become homozygous for one of the alleles at each gene locus. In effect, this form of inbreeding will result in genetic differentiation between the populations (Tabachnick and Black, 1996; Kraaijeveld *et al.*, 1998). In light of this, inbreeding can be seen as a process that converts genetic variation within a population into differences between populations by making each population homozygous for a randomly chosen allele.

It is also possible for changes in gene frequencies to result from sheer random chance. Unlike inbreeding or positive associative mating which only affects a single locus,

this phenomenon of genetic drift can affect the entire genome, including those traits that are important to vectorial capacity and competence (Tabachnick and Black, 1996). Arthropod vectors of disease differ from many other organisms because they are often directly subject to human efforts to control populations. Vector control plays a major role in changing gene frequencies through genetic drift (Tabachnick and Black, 1996). Furthermore, if a limited number of individuals move together by chance from a large population and begin or "found" a new population, by chance alone, the new population can differ from the starting large population. This chance effect caused by colonization is what is termed the "founder effect" (Tabachnick and Black, 1996).

In addition, the probability that the same mutations will occur in the two populations in the same sequence is extremely low. Each incorporated mutation changes the genetic background of the population and thus affects the selective value of all subsequent mutations. Furthermore, recombination will produce different genotypes in the two gene pools. Thus, since the same gene may have different selective values in different genotypes, it will lead to gradual change in gene frequencies (Giesel, 1972; Gould and Jonhson, 1972). The selection pressures to which two geographically isolated populations are exposed cannot be the same, since no two places can have an identical micro-environment. Every completely isolated population exists in a biotic environment that is different from any other. This difference adds another powerful selection pressure. Competition, predation, and other ecological interactions are apt to be entirely different in the new environment. These local conditions exert selection pressures reinforcing a steady change in gene contents (Mayr, 1964, 1970; Travis, 1990). In this case, genetic variation plays a key role in two related areas. First, enhanced additive genetic variation allows further response to selection and enables a population to increase its overall level of

adaptation to a specific selection pressure. Second, genetic variation enables a population to respond to selection dramatically and quickly. This enhances the likelihood for rapid differentiation among conspecific populations (Travis, 1990). Thus, if two populations are isolated for long, they may even acquire sufficient differences to be regarded as separate species (Mayr, 1964; Ferguson, 1980).

Nevertheless, although the results of this study indicate that there are genetic differences between the Muguga and Rusinga ticks, caution should still be exercised in relating this to the observed differences in *T. parva* vectorial capacity between the two populations (Kubasu, 1992; Kiara and Osir, unpublished). More work will be required to first rule out the possibility of the observed markers being linked to traits other than vector competence. This is because even individuals with the same gene may not necessarily always express the corresponding phenotype; owing to differences in penetrance and expressivity. Penetrance is defined as the percentage of individuals with a given genotype who actually exhibit the phenotype associated with that genotype (Gove, 1971). Failure of expression of the phenotype normally associated with a particular genotype could be due to presence of modifiers, epistatic genes, or suppressors in the rest of the genome, or because of a modifying effect of the environment (Watson *et al.*, 1987; Clark and Russell, 1997). Expressivity is a description of the degree or extent to which a given genotype is expressed phenotypically in an individual (Gove, 1971). Again, lack of full expression may be due to the rest of the genome or to environmental factors. For example, in a laboratory study of the factors affecting the infection rates of *T. parva*, Norval *et al.* (1989) found the air temperature to be prime. Lowest infection rates were obtained when the animals were kept warm during nymphal tick feeding, and highest infection rates when the animals were kept in a room cooled to ±15°C (Norval *et al.*, 1989). Work on other species has shown that a

single genotype can produce different phenotypes, and that a single phenotype can be produced by various genotypes, depending on the environment (Suzuki *et al.*, 1986). Seasonal variation in vector competence has also been reported (Reisen *et al.*, 1996). Thus, the impact of a gene at phenotype level depends not only on its dominance relations but also on the condition of the rest of the genome and on the condition of the environment. It is therefore possible for genetically identical organisms to differ phenotypically, and *vice versa*. Even when the genotype and environment are precisely fixed, random variations in phenotype could still be expected. Such uncontrollable variation in phenotype could result from random events in development; a phenomenon called *developmental noise* (Suzuki *et al.*, 1986). Thus, for a given genotype, developing in any given sequence of environments, there remains some uncertainty in the exact phenotype that will result. Geographically isolated populations like Muguga and Rusinga are apt to be exposed to different *developmental noise.* Thus, the differences observed in the present study may even not be directly linked to a particular phenotype (e.g. vector competence). Conclusions could be easily made if one were dealing with discrete variants, as in Mendel's experiments, or in modern Molecular Genetics involving variants of bacteria that show distinctive characters such as drug resistance (Clark and Russell, 1997). The expression of such genes is very clear cut and reliable. However, such simple direct relationships are rare. As mentioned, the relationships of genotype to phenotype are almost always an interaction of many factors (Watson *et al.*, 1987; Cole *et al.*, 1994; Yeh *et al.*, 1995; Tabachnick and Black, 1996; Yan *et al.*, 1998). This is probably why it may be difficult to find discrete phenotypic classes in nature, such as an *R. appendiculatus* population that is 100% susceptible or 100% refractory to *T. parva,* although on average, some populations will be more vector competent than others.

If the genetic basis of *T. parva* transmissibility is to be well defined, further studies need be done in order to establish the genetic linkages of loci for susceptibility. As already seen, Vector Biologists are now advocating for natural population replacement with transgenic, vector-incompetent strains. Transgenic ticks carrying genes for *T. parva* refractoriness may be used in future to replace the natural *R. appendiculatus* populations. In this case, it will be necessary to first gain a deeper understanding of the genetics of susceptibility and refractoriness as well as the chromosomal map location(s) for the gene(s). A genetic map is a prerequisite for localization, positional cloning and characterization of endogenous genes controlling refractoriness (Zheng *et al.*, 1996). Such a map should also be necessary for broadly based genetic studies of Rhipicephaline biology, which must underlie any reasonable theileriosis control strategy that takes account of the tick vector.

Extensive infection-rate and transmissibility studies should therefore be carried out on various tick populations. Ticks showing refractoriness or susceptibility to *T. parva* should be pooled and their DNA analyzed to identify markers that distinguish them. Since each DNA fingerprint may be representing or linked to a separate allele, any shared fingerprint may be contemplated as a product of the same allele (Gwakisa *et al.*, 1994). Accordingly, it is expected that markers that will be polymorphic between pools will be genetically linked to the loci determining the trait used to construct the pools (Michelmore *et al.*, 1991). Methods are already in place for rapidly identifying markers linked to any specific gene or genomic region (Michelmore *et al.*, 1991; Zheng *et al.*, 1996; Clark and Russell, 1997). In particular, RAPD markers have been found to provide a quick method for generating genetic maps and analyzing populations (Michelmore *et al.*, 1991). In future studies, polymorphic bands should be purified from the gels and cloned using standard techniques (Maniatis *et al.*, 1982). The cloned bands should be labelled and used directly as

probes in hybridization assays (Post, 1985; Keller and Manak, 1993). Probes could be used to rapidly screen the whole genome to locate several loci simultaneously (Michelmore *et al.*, 1991). The cloned bands should also be sequenced using standard DNA sequencing methods (Maniatis *et al.*, 1982). Specific primers could then be synthesized basing on these sequences, and used in PCR-based assays to routinely differentiate between different field *R. appendiculatus* populations. The need to develop reliable markers for susceptibility to infection should also be seen in view of the IAT vaccination method, whose standardization requires that ticks that give uniform levels of infection are used. It should also be possible to select highly *T. parva*-susceptible lines to avail sporozoites to aid in novel vaccines development (Musoke *et al.*, 1982, 1992, 1993). Markers for the *T. parva*-refractory trait could be used in developing a super-refractory line of ticks that could be used to "naturally" immunize the animals (Brown, 1985; Chiera *et al.*, 1989: Nyindo *et al.*, 1996).

Finally, owing to the limit in sensitivity inherent in examining gene products rather than genes themselves, a clear relationship between the variation observed with protein electrophoresis and genetic diversity needs to be established before the protein technique can be routinely used. This may involve isolation, purification and sequencing of the polymorphic proteins, and comparison with the sequences of isolated specific DNA bands. The RAPD-PCR and other techniques of DNA analysis offer greater and more direct detection of genetic variation and hence offer many advantages over protein electrophoresis (Post, 1985; Tait, 1985; Overmeyer *et al.*, 1996). Nonetheless, caution should always be exercised, especially when one is relying on only one technique. This is because DNA-analysis techniques also have limitations, and do not necessarily offer a

cure-all (Williams *et al.*, 1990; Black, 1993; Ellis, 1998). However, this study demonstrates that despite some limitations, both protein electrophoresis and RAPD analysis are of value for studies of intraspecific genetic variation, and are capable of elucidating relationships between even closely related populations of *R. appendiculatus*. In particular, analysis of RAPD data with novel numerical methods allows investigation of results beyond the purely descriptive level presented in many RAPD studies.

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APPENDIX

I. Reagents used

1. Acetic acid glacial 100% (MERCK, Germany)*
2. Acrylamide (SERVA, Feinbiochemica GmBH & Co.)
3. Ammonia solution 32% extra pure (MERCK)
4. Ammonium persulphate (APS) (SERVA)
5. Ampholytes (pH 5-8, LKB, Bromma)
6. Bis-Acrylamide (SERVA)
7. Citric acid (May & Baker ltd., England)
8. Ethanol (MERCK)
9. Formaldehyde solution (MERCK)
10. Glutaraldehyde 25% (SERVA)
11. Glycerol (SERVA)
12. Glycine (SERVA)
13. H_2O, deionized double-distilled water
14. Methanol (MERCK)
15. Molecular weight marker XIV, 100 bp ladder (Boehringer Mannheim GmBH, Germany)
16. ß-Mercaptoethanol (SERVA)
17. NaOH (Manigate Agencies Ltd., Kenya)
18. Nonidet P-40 (non-ionic detergent) (SIGMA chemical Co., USA)
19. Ortho-Phosphoric acid 89% (MERCK)
20. Primers (Operon Technologies, USA)
21. SDS (SERVA)
22. Silver nitrate ($AgNO_3$) (KOCH-LIGHT Ltd., England)
23. *Taq* DNA Polymerase enzyme (Stratagene Cloning Systems, USA)
24. TEMED (BRL, Life Technologies, Inc., USA)
25. Tris (SERVA)
26. Urea (BDH, Poole, England)

*Manufacturers in parentheses

II. Stock Solutions and Buffers for the First Dimension (IEF)

1. Protein extraction medium (6.25 mM Tris pH 6.8, 1% NP-40, 1 mM PMSF)

To prepare 1 ml of the extraction buffer, add the following proportions to a 1.5 m ml Eppendorf tube on ice:

6.25 mM Tris, pH 6.8	896.7 µl
10% NP-40	100 µl
300 mM PMSF	3.3 µl

The buffer should be vortexed and used immediately.

2. Sample solubilization buffer (9.5 M urea, 2% NP-40, 2% Ampholytes, 5% ß-mercaptoethanol):

Urea (MW 60.1)	2.95g
10% NP-40	2 ml
Ampholines pH range 5-8	0.25 ml
*ß-Mercaptoethanol	0.5 ml

Make up to 5 ml with H_2O and freeze at -80°C in 0.5 ml aliquots. Use aliquot once and discard the remainder.

*Add just before use.

3. IEF gel solution (10 ml)

Urea	5.5 g
30% Acrylamide	1.33 ml
10% NP-40	2.0 ml
Ampholines (pH 5-8)	0.5 ml
H_2O	1.7 ml
10% APS	10 µl
TEMED	7.0 µl

The APS and TEMED should be added last, after warming at 37°C in a water bath (to ensure complete disolution of urea) and degassing.

4. 30% Acrylamide stock solution (Monomer) (28.38% w/v acrylamide, 1.62% w/v N,N'- Methylene-bisacrylamide):

Acrylamide	28.38 g
Bis-acrylamide	1.62 g

Make up to 100 ml with H_2O and filter through a 0.22 µM filter (Millipore Corporation, Bedford). All acrylamide solutions are light sensitive and should be stored in dark bottles at 4°C. Acrylamide is a potent neurotoxin (Celis and Bravo, 1984), so extra caution should be exercised. Use hand gloves and face mask. Keep for no more than 3 months.

5. 10% NP-40 Solution:

NP-40	5 ml
H_2O	45 ml

Keep at 4°C

6. Anode electrode solution (Anode buffer):

10mM Phosphoric acid.
This should be made fresh from a 1M stock.

7. Cathode electrode solution (Cathode buffer):

20 mM Sodium hydroxide
This should be made fresh and extensively degassed before use.

8. Equilibration buffer (0.125 M Tris, 2% SDS, 10% glycerol, 0.01% Bromophenol blue, 0.5% ß-Mercaptoethanol):

Tris base	3g in 150 ml, adjust pH to 6.8 using conc. HCl
SDS	4 g
Glycerol	20 ml
Bromophenol blue	0.2 g

Make upto 200 ml with H_2O and filter through 0.22 µM. Freeze at -80°C in 10 to 15 ml aliquots. Add 0.5% ß-Mercaptoethanol just before use.

III. RAPD band scoring sheet for Muguga and Rusinga *R. appendiculatus* populations.

Number of individuals per population: 54
Number of scored bands: 32

	A05-01	A05-02	A05-03	A05-04	A05-05	A10-01	A10-02	A10-03	A10-04	A10-05	A10-06	A10-07	A11-01	A11-02	A11-03	A11-04	A11-05	A11-06	A11-07	A11-08	A16-01	A16-02	A16-03	A16-04	A16-05	A16-06	A20-01	A20-02	A20-03	A20-04	A20-05	A20-06
Muguga 01	0	1	1	0	1	1	0	0	0	0	1	1	1	0	1	0	1	0	1	0	1	0	0	1	0	1	1	0	0	0	1	1
Muguga 02	1	1	1	1	0	0	0	0	1	0	0	0	1	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1
Muguga 03	0	1	1	0	0	1	1	1	0	1	0	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	0	0	1	1
Muguga 04	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
Muguga 05	0	0	0	0	1	0	0	0	1	0	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	1	1	1	1
Muguga 06	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 07	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 08	1	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	1	1	0	1
Muguga 09	1	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Muguga 10	1	0	1	1	0	0	0	0	0	1	0	0	1	0	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Muguga 11	0	0	1	0	0	0	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	0	0	1	0	0	0	0	1
Muguga 12	0	1	0	0	0	1	1	1	1	1	1	0	1	0	1	1	1	0	1	0	1	0	0	0	1	0	1	1	1	0	1	1
Muguga 13	0	1	0	1	1	1	1	1	0	1	1	0	0	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 14	0	0	1	0	1	0	0	0	0	0	0	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 15	0	0	1	0	1	1	1	0	1	0	1	1	0	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 16	1	1	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	1	0	1	0	1	0	1
Muguga 17	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Muguga 18	1	1	1	1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	1	0	1	0	1	0
Muguga 19	0	0	1	1	0	1	0	0	0	1	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1
Muguga 20	0	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 21	0	0	1	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
Muguga 22	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Muguga 23	0	1	0	0	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	0	0	0
Muguga 24	.	.	.	.	.	0	1	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1	1	0	1	1	1	0
Muguga 25	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 26	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 27	1	1	1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1
Muguga 28	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 29	1	1	1	1	1	1	1	1	1	0	0	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 31	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 32	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 33	1	0	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 34	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 35	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Muguga 36	1	1	1	0	1	1	1	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 37	1	1	1	0	0	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 38	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 39	1	1	0	0	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 40	1	1	1	1	1	1	1	0	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 41	0	1	0	1	0	1	1	1	1	1	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 42	0	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 43	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 44	0	1	1	1	1	1	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 45	1	1	0	1	0	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 46	1	1	1	0	1	1	1	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 47	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 48	1	1	1	0	1	1	0	0	1	1	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 49	0	1	0	1	0	1	1	1	1	1	0	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
Muguga 50	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 51	1	1	1	0	1	0	1	0	0	0	0	1	0	0	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
Muguga 52	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 53	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Muguga 54	1	0	1	0	0	0	1	1	1	0	1	0	0	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	1	0	0	0

	A05-01	A05-02	A05-03	A05-04	A05-05	A10-01	A10-02	A10-03	A10-04	A10-05	A10-06	A10-07	A11-01	A11-02	A11-03	A11-04	A11-05	A11-06	A11-07	A11-08	A16-01	A16-02	A16-03	A16-04	A16-05	A16-06	A20-01	A20-02	A20-03	A20-04	A20-05	A20-06
Rusinga 01	1	1	1	1	1	1	1	0	1	0	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	1	0	1
Rusinga 02	1	0	1	1	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
Rusinga 03	0	0	1	0	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1	1	1	0	0	1	1	1
Rusinga 04	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 05	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 06	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 07	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 08	1	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 09	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	.	.	.	.	.	.
Rusinga 10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 11	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 12	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
Rusinga 13	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 14	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 15	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 17	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 18	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 20	.	.	.	.	.	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	.	.	.	.
Rusinga 21	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 22	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 23	0	1	1	0	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Rusinga 24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 25	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 26	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 27	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 28	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 29	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 30	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 31	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 32	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 33	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 34	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 35	0	0	0	1	1	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	1
Rusinga 36	0	1	0	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	1	1	0	1	1
Rusinga 37	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 38	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 39	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 40	0	0	0	0	1	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 41	0	1	1	1	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 42	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1	1	1	1	0	0	0	0	1	1
Rusinga 43	0	0	0	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 44	0	0	0	1	0	1	1	0	1	0	1	0	0	0	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 45	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 46	1	1	1	1	0	0	0	0	1	0	0	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0
Rusinga 47	0	0	1	1	1	1	0	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 48	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 49	0	0	0	0	1	0	1	1	0	1	1	0	1	0	1	1	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1
Rusinga 50	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 51	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 52	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 53	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rusinga 54	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Foot note: "1" — Band present; "0" — Band absent; "." — Missing data (No amplification)